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THIN-LAYER CHROMATOGRAPHY OF FREE AND ESTERIFIED PORPHYRINS ON TALC

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SUMMARY

Free porphyrins and porphyrin esters are clearly and rapidly separated according to the number of carboxyl groups on 70 × 70 mm plates with 0.30 mm thick layers of talc prepared from methanol suspensions. Porphyrins were run with 0.5 *N* HCl–acetone (3:7) and porphyrin esters with glacial acetic acid–pyridine–acetone (1:1:1). Clear separations were achieved in 10–15 min even in the presence of impurities. Of both, porphyrins and porphyrin esters, the R_F value increased with the number of carboxyl groups. The methods are suitable for separating higher carboxyls, but less suitable for porphyrins with less than 4 carboxyl groups. As the opposite is the case with previously described techniques, the methods described here constitute a useful supplement to existing methods. A quick and convenient method of preparing plates from silica gel, kieselguhr, alumina and talc from methanol suspensions is also described.

INTRODUCTION

In experimental and clinical work with porphyrins methods for clear identification and rapid separation are of considerable advantage. Several methods for thin-layer chromatography (TLC) of porphyrins and their methyl esters have been proposed[1–10], but all these methods are relatively time-consuming (½ to 2 h) and also require presaturation of the chamber with solvents. Further, these methods require preliminary purification of the porphyrins as well as a porphyrin load within rather narrow limits to give clear separations. Moreover, all the published TLC methods for the separation of free or esterified porphyrins report the higher the R_F the lower the number of COOH-groups. Methods giving a clear and rapid separation of 4- to 8-carboxyl porphyrins have not yet been described although such separations are important in clinical analysis.

Experience in large scale porphyrin preparation[11–15] has shown that coproporphyrin (CP) adheres more strongly to talc than uroporphyrin (UP); also, UP is eluted from the talc more easily with HCl–acetone. It seemed reasonable, therefore, that a TLC technique could be developed based on these observations. As talc does not appear to be a conventional TLC medium, methods for preparation of talc plates

had to be developed. Previous observations[16] showed that a smooth talc surface could not be achieved from aqueous suspensions, but was possible using alcohol suspensions. The plates, therefore, were prepared from suspensions of talc in methanol. Preliminary observations with this method[17] were promising, but included no photographic documentation. A later report (in Danish)[18] included some photographs. Continued experiments with free porphyrins on talc thin-layer plates have confirmed that they give good separations without previous purification of the solutions. The plates work satisfactorily with both a light and heavy porphyrin load, are not dependent on the saturation of the chamber with solvent vapor, and can give separations within 15 min.

EXPERIMENTAL

Materials

Porphyrins and esters were prepared from human and bovine excreta by the methods developed in our laboratory[15]. The talc used was of the quality described in Pharmacopoeia Nordica, Vol. II (ed. Danica) (pp. 594–595, A. Busck, Copenhagen, 1963); before use it was passed through a 0.2 mm sieve (*cf. ibid.* Vol. III, pp. 6–7).

Plates and applicator

Long plates (200 mm) were prepared with a DeSaga apparatus, smaller plates with a special brass applicator (Figs. 1a and b).

Preparation of plates

Talc suspensions were prepared from 50 g of talc, 50 g of methanol and 2 g of gypsum, thoroughly mixed in a shaker. The mixture was immediately poured into

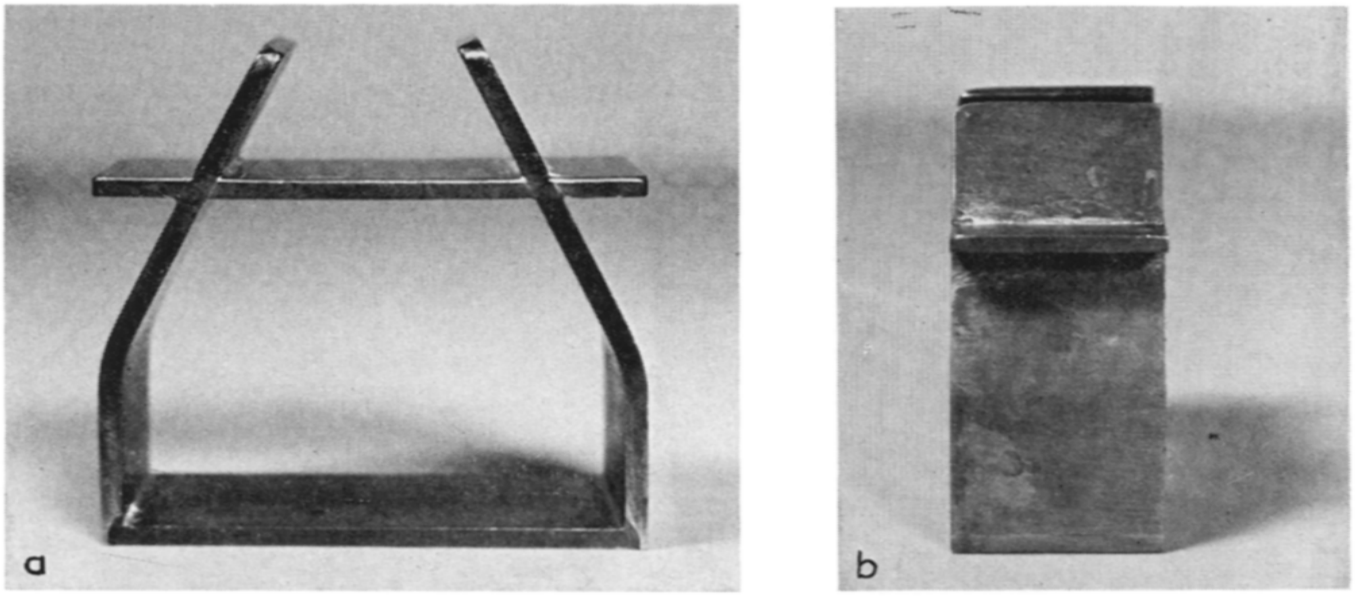

Fig. 1. Applicator for 0.30 mm thin-layer plates. The instrument is seen from the bottom (a) and from the side (b). Height 30 mm, length 80 mm, breadth 80 mm at the back and 25 mm at the front. The applicator is placed bottom downwards on the chromatographic plates and is filled with the suspension of the chromatographic medium, after which it is pushed slowly over the plates. The suspension pours out through the 0.30 mm slit formed between the plates and the back side of the applicator which is elevated exactly 0.30 mm—the difference between the level of the back and the sides of the applicator is clearly seen in (a).

the applicator placed in position on a row of clean plates of plain window glass lying on a smooth firm support. Immediately after pouring the suspension into the applicator, it was moved slowly forward over the plates to spread an 0.30 mm thick talc layer. After drying for ½ h at room temperature the plates were ready for use. They can be stored for several weeks at room temperature if required.

Application of the porphyrins or esters on the plates

Porphyrins were applied as solutions in HCl, ammonia or HCl–acetone; porphyrin esters as chloroform solutions. Urines rich in porphyrins can be applied directly; urine extracted with ether–amyl alcohol–glacial acetic acid (1:1:1) gives better results, but HCl extracts of these solvent preparations are preferable. 5–10 μl of a solution containing 0.1–20 μg of free or esterified porphyrin is applied. The spot must be dried before the run; suitable spots may be obtained by repeated application and drying with an electric fan until a spot with well-marked red fluorescence is obtained. For grossly impure solutions red fluorescence may be unobtainable because of impurities quenching porphyrin fluorescence; however, fluorescing spots are obtained after chromatography because the quenching substances and the porphyrins are separated. Application has to be performed cautiously to avoid "holes" in the talc surface which cause irregularly-shaped spots after the run.

Marker solutions

A CP and UP marker is run on each plate. Commercial preparations can be used. A fraction of a mg of ester is dissolved in chloroform in a small beaker or on a watch glass to give a porphyrin ester marker. To obtain a free porphyrin marker the ester is dissolved in 2–4 drops of analytical grade conc. HCl, and 1–2 drops of distilled water are added to give an HCl concentration of about 7 *N*. After 48 hours' hydrolysis at room temperature in a jar saturated with 7 *N* HCl, hydrolysis is complete.

Development of chromatograms

Free porphyrins are run with 0.5 *N* HCl–acetone (3:7) and esters with glacial acetic acid–pyridine–acetone (1:1:1). With 70 × 70 mm plates the run is completed in 15 min for free porphyrins and in 10–15 min for esters. The shape of the spots depends on the amount of porphyrin present, the heavier the load the more elongated the spots.

Esters can also be separated with chloroform–methanol (1:1) or ethanol–ethyl acetate (3:2), but these systems are considerably more liable to streaking than the glacial acetic acid–pyridine–acetone system which gives good separations even with heavy loads. The latter system may, however, give certain deformation of the front due to a wave-like line of impurities; this may deform the spots of UP, which run close to the front.

Detection of the spots

Spots of free and esterified porphyrins are detected by their red fluorescence in U.V. light in a dark room. A convenient lamp which can stand on the working bench has been described[20]. The spots are still clearly visible after storage for 1½ year in a dark place at room temperature.

Photography of the fluorescent spots

The plates are irradiated with two lamps of the type described[20], each 25–30 cm from the plate, in a dark room. For color slides Agfa film C.T. 18, diaphragm 8, exposure for 1 min with color filter B + W, 46 E, KR 12, 2 × (Filterfabrik Johannes Weber, Wiesbaden) gave good results. For color negatives Koda color film, diaphragm 8, exposure for 1 min with filter as above was employed. For black-and-white exposures we used Kodak + X film, diaphragm 8, exposure for 5 min with Rotfilter B + W 091, Rotdunkel, 46 E 8 ×.

TABLE I

R_F VALUES OF FREE PORPHYRINS AND PORPHYRIN ESTERS ON 0.30 mm TALC PLATES

Number of carboxyl groups	*Free porphyrins 0.5 N HCl–acetone (3:7)*	*Porphyrin esters*		
		Glacial acetic acid–pyridine–acetone (1:1:1)	*Chloroform–methanol (1:1)*	*Ethanol–ethyl acetate (3:2)*
8 (Uroporphyrin)	0.60–0.80	About 0.90	About 0.60	About 0.80
4 (Coproporphyrin)	0.30–0.50	About 0.55	About 0.20	About 0.30
2 (Protoporphyrin and others)	Below 0.10	About 0.10	About 0.10	About 0.10

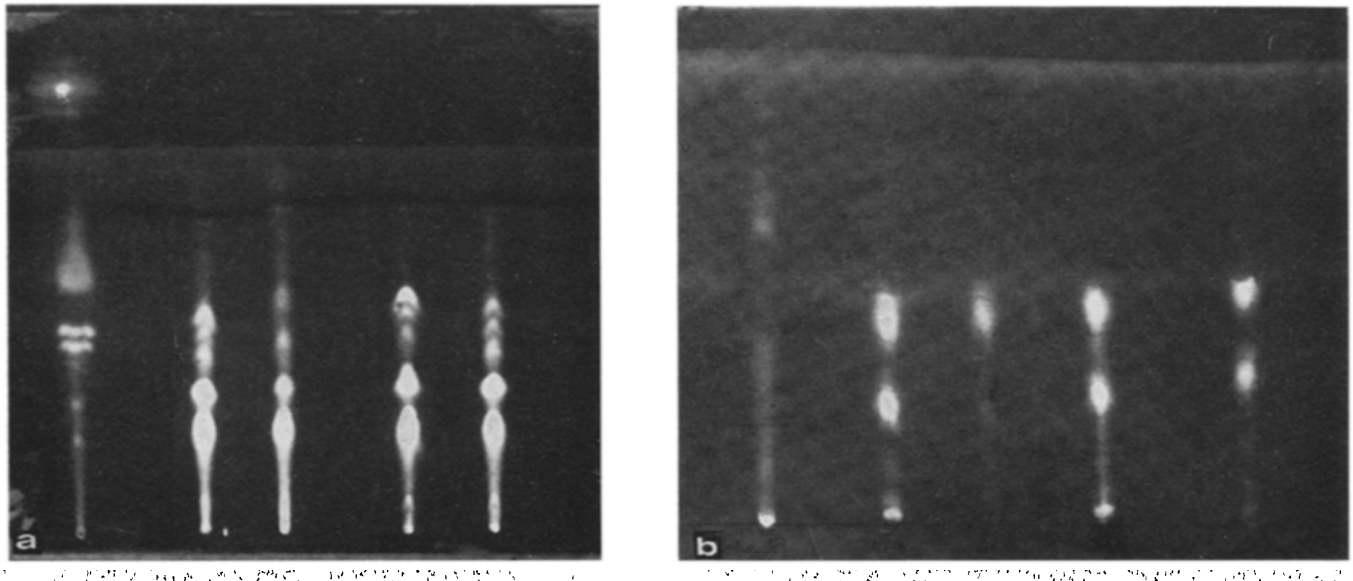

Fig. 2. Chromatograms of free porphyrins on talc run with acetone–HCl (a). The chromatogram on the left is a human acute intermittent porphyria urine applied to the starting spot without previous purification. Flattened spots of uro- and 7-carboxyl porphyrins are seen with traces of 5- and 4-carboxyl porphyrins. The elongated spot over the two flat spots exhibited greenish fluorescence, and consisted of impurities separated from the porphyrins during chromatography. The spot over the liquid front is urine applied, like the starting spot, for comparison. The other 4 chromatograms are from purified porphyrin mixtures obtained during preparative work with bovine porphyria urine. Spots of varying strength of porphyrins with 4, 5, 6, 7 and 8 carboxyl groups are visible. (b) shows paper chromatograms from the same 5 samples as in (a) in the same sequence from left to right and with a water–lutidine system.

RESULTS AND DISCUSSION

The R_F values obtained are presented in Table I. They varied somewhat with the batch of talc used, the time the plates had been stored before use, the porphyrin load applied to the starting spot and the impurities present. Examples of chromatograms are given in Figs. 2a and b.

Originally we[17,18] employed 200 mm long plates made with the DeSaga apparatus. Later we found it more convenient to use 70 × 70 mm plates. To get a satisfactory separation with these smaller plates we introduced the solvent system 0.5 *N* HCl–acetone (3:7). For the 200 × 200 mm plates 0.5 *N* HCl–acetone (2:3) is more suitable.

The smaller plates give a considerably more rapid separation than the 200 mm plates. Addition of a small amount of gypsum to the talc suspension made the plates strong enough to resist damage from the air current from an electric hair-dryer blowing perpendicularly 2 cm above the surface.

We also prepared plates of silica gel, alumina and kieselguhr from methanol suspensions, both with the DeSaga apparatus and our applicator (Fig. 1). These plates were used for separation of free porphyrins according to SCOTT *et al.*[5] and for esters according to Doss[6] with results similar to those described by these authors. These plates from methanol suspensions could be used without previous activation after drying for 30 min at room temperature.

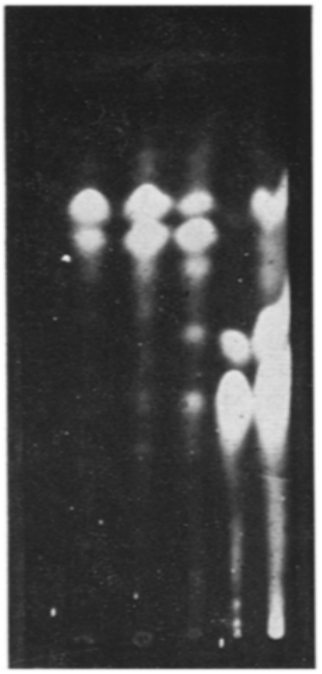

Fig. 3. Chromatograms of porphyrin esters with the glacial acetic acid–pyridine–acetone (1:1:1) system. The first three chromatograms from the left are from different Waldenström esters prepared from human porphyria urine. Clearly separated spots of uro- and 7-carboxyl porphyrin are seen as well as weak spots corresponding to 6, 5 and 4 carboxyls. No. 2 from the right is a purified coproporphyrin I ester from bovine porphyria urine showing a mixture of 4- and 5-carboxyl porphyrin. The chromatogram on the right is a raw ester from bovine porphyria urine containing both CP-, 5-carboxyl- and UP-esters. The elongated spots correspond to heavy loads of porphyrin ester. The separation is most clear in the 4–6 carboxyl region.

This TLC technique can also be used for the rapid preparation of small amounts of chromatographically pure porphyrins by scraping the spots off the plates, and eluting with 1 *N* HCl–acetone (1 : 9) for free porphyrins and with chloroform–methanol (9:1) for the esters. These elutions, however, are usually not quantitative as some porphyrin adheres to the talc.

The method for free porphyrins can be used for urines rich in porphyrins with direct application of the urine on the plate (Fig. 2), but better results are obtained with extracts (*cf.* above and ref. 19).

We have successfully identified free porphyrins using the above method and the lutidine paper chromatographic method[21]. The lutidine method was, however, much more time consuming and considerably more sensitive to interference by impurities. Thus urine rich in porphyrins can be applied without previous purification and gives a good separation on talc although no separation is obtained with lutidine paper chromatography (*cf.* Fig. 2).

Separation of esters on talc with chloroform–methanol (1:1) and ethanol–ethyl acetate (3:2) was less satisfactory than with glacial acetic acid–pyridine–acetone (1:1:1). The two former solvent systems gave considerable streaking if more than 0.1 μg of ester was applied to the plate; the latter (Fig. 3) could separate at least 10 μg of porphyrin esters without streaking and gave good results with crude ester extracts. Separation was obtained in about 10 min; thus this method is valuable as control in preparatory work (*cf.* ref. 14).

Various porphyrin preparations were subjected to chromatography, both as esters and after hydrolysis, and identical results were obtained in runs as free and esterified porphyrins.

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