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THE SEPARATION OF FREE DICARBOXYLIC ACID PORPHYRINS USING THIN-LAYER AND PAPER CHROMATOGRAPHY

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SUMMARY

Mixtures of protoporphyrin, deuteroporphyrin, haematoporphyrin, pemptoporphyrin and mesoporphyrin can be separated as free acids on talc thin-layer chromatography plates by developing them with a mixture of ethanol–2,6-lutidine–water (30:3:67) in tanks containing an atmosphere saturated with ammonia vapour. Monoacrylic monopropionic deuteroporphyrin ("S/411" porphyrin) can be separated from coproporphyrin using this system. The same mixture of two-carboxyl porphyrins can also be separated on Whatman No. 1 chromatography paper by developing with pyridine–0.2 *M* sodium borate buffer pH 8.6 (1:9) by either ascending or descending techniques.

INTRODUCTION

During current investigations of porphyrin extracts from various tissues[1] it became apparent that chromatographic separation and identification of component porphyrins without prior esterification was desirable because of the known labile nature of some two-carboxyl porphyrins to esterification procedures[2].

Existing chromatographic methods for separating free (unesterified) porphyrins depend on the number of carboxyl groups. The lutidine methods of NICHOLAS AND RIMINGTON[3] and ERIKSEN[4], using paper, separate free porphyrins in this manner. Uroporphyrin (eight carboxyl groups) remains near the base line and protoporphyrin (two carboxyl groups) runs near to the solvent front. Porphyrins possessing seven to three carboxyl groups occupy intermediate positions on the chromatogram. Separation of coproporphyrin (four carboxyl groups) I and III isomers is also possible by these methods.

Other systems using paper (PC) and talc thin-layer chromatography (TLC)[5,6] separate free porphyrins in the reverse order, with uroporphyrin running near to the solvent front and two-carboxyl porphyrins remaining together near the base line.

A column method employing Sephadex dextran gels and borate buffers has been described by RIMINGTON AND BELCHER[7], which separates free porphyrins depending on differences of adsorptive properties rather than the number of carboxyl groups

thus making possible the separation of some two-carboxyl porphyrins from each other.

Techniques using porphyrin methyl esters on paper or thin layers usually do not separate two-carboxyl porphyrins. However, a convenient TLC method described by HENDERSON AND MORTON[8] will separate some two-carboxyl porphyrin esters. Also a two-dimensional method[9] employing two solvent systems and reversed phase will separate at least six dicarboxylic porphyrin esters. A quick method of separating haematoporphyrin dimethyl ester from other dicarboxylic porphyrin esters is described also by CHU *et al.*[10], which is useful for investigating porphyrins suspected of containing hydroxyl substituents by observing increased R_F values after acetylation[11].

The methods described below enable identification and preparative separation of haematoporphyrin (haemato), deuteroporphyrin (deutero), mesoporphyrin (meso), pemptoporphyrin (pempto) and protoporphyrin (proto), in addition to coproporphyrin (copro) and monoacrylic monopropionic deuteroporphyrin ("S/411"), all without prior esterification.

MATERIALS AND METHODS

All solvents used were of "Analar" quality except for 2,6-lutidine, which was supplied as laboratory reagent grade. Talc (fine powder purified by acids) was obtained from B.D.H. Ltd.

Pempto ester was kindly supplied by Professor A. H. JACKSON, University College, Cardiff and "S/411" ester, isolated from calf meconium, was a gift from Dr. D. NICHOLSON, Kings College Hospital Medical School, London. The esters were converted to the free acids by treating with 25% w/v HCl at room temperature in the dark for 14 and 72 h, respectively, with subsequent recovery into ether for application to chromatograms. The prolonged hydrolysis period required for the "S/411" ester is due to the known stability to normal hydrolysis procedures of the acrylic ester substituent[1].

Proto was obtained from the dimethyl ester by hydrolysing with 25% w/v HCl for 5 h at room temperature and recovering the free porphyrin by precipitation at the isoelectric point (approx. pH 3.9). Proto and other porphyrins used were obtained from the laboratory stock.

Talc plates (20 × 20 cm) were prepared by thoroughly shaking a mixture of 40 g of talc and 70 ml methanol and spreading over five glass plates to give a thickness of 0.25 mm using the "Shandon" thin-layer apparatus. The plates were allowed to dry at room temperature for 20 min before use. Chromatograms were prepared by applying porphyrins dissolved in 2 *N* ammonia and spread in bands 1–2 cm long and about 2 cm from the bottom of the plate. The optimum range of application to maintain consistently good separations was 0.1–20 μg per band. The chromatograms were developed in glass tanks (solvent ascending) with an atmosphere previously saturated for at least 1 h with ammonia vapour by placing two small beakers containing concentrated (s.g. 0.880) ammonium hydroxide at the bottom of the tanks and also a filter paper lining soaked in concentrated ammonia–water (1:1). The plates were placed in 200 ml of developing mixture containing 30% ethanol, 3% 2,6-lutidine and 67% water and allowed to develop in the tank for at least 2 h at a temperature range of 20–25°. For convenience, plates could be left to develop overnight giving an improved

separation of the two-carboxyl porphyrins. After drying the plate with a hair drier or allowing to dry at room temperature, the bands were eluted after observing their red fluorescence under UV light, by extracting the talc scrapings with 3 ml of 20% acetic acid–ether. The dissolved porphyrin was extracted from the ether into 1 ml of 1.5 *N* HCl and the Soret peaks determined using an Optica CF4R recording spectrophotometer.

Sheets of Whatman No. 1 chromatography paper (20 × 23 cm) were spotted 2 cm from the shortest edge with the previously described porphyrin solutions, Individual spots were not allowed to spread more than 0.5 cm diameter and if repeated application of sample to a spot was required a hair drier was used to dry the spot before further additions were made. The useful limits of loading were found to be 0.5–7 μg per spot. The papers were rolled into a cylinder and the edges stapled together to enable the chromatograms to stand freely in the tanks. Cylindrical glass tanks (13 × 28 cm) were allowed to equilibrate for 30 min before use with 20 ml of 0.880 ammonia placed at the bottom. The tanks were also lined with filter paper soaked in concentrated ammonia. The paper was conveniently soaked in water to enable the tanks to be lined, and concentrated ammonia was sprayed on to the paper *in situ* using a pasteur pipette.

Twenty millilitres of developing mixture containing pyridine and 0.2 *M* sodium borate buffer pH 8.6 (1:9) were placed in a suitably sized Petri dish placed at the bottom of the tanks and the chromatograms were developed with the mixture for at least 4 h at 20–25°. The chromatograms could also be developed overnight. After drying at 40° for 15 min the positions of the spots were marked after observing their red fluorescence under UV light.

This method was readily adapted for descending chromatography by preparing tanks as for the ascending technique and using larger volumes of developing solvent in the trough. A suitably sized tank to hold Whatman No. 1 paper 57 × 19 cm was a glass "Shandon" 300 Chromotank (55 × 20 × 35 cm). The papers were spotted on a base-line drawn 12 cm from the short edge. Development took at least 16 h at 20–25° and chromatograms could be left overnight allowing the solvent front to run off the end of the paper. Descending chromatograms could be more heavily loaded with upper limits of approx. 12 μg per spot.

RESULTS

Thin-layer chromatography

A mixture of two-carboxyl porphyrins consisting of haemato, deutero, meso,

TABLE I

TLC SEPARATION OF TWO-CARBOXYL PORPHYRINS SHOWING RELATIONSHIP OF R_F VALUE TO HCl NUMBER

Porphyrin	*R_F value*	*HCl No.*
Haemato	0.35	0.10
Deutero	0.32	0.30
Meso	0.25	0.50
Proto	0.19	2.50
Pempto	0.28	—

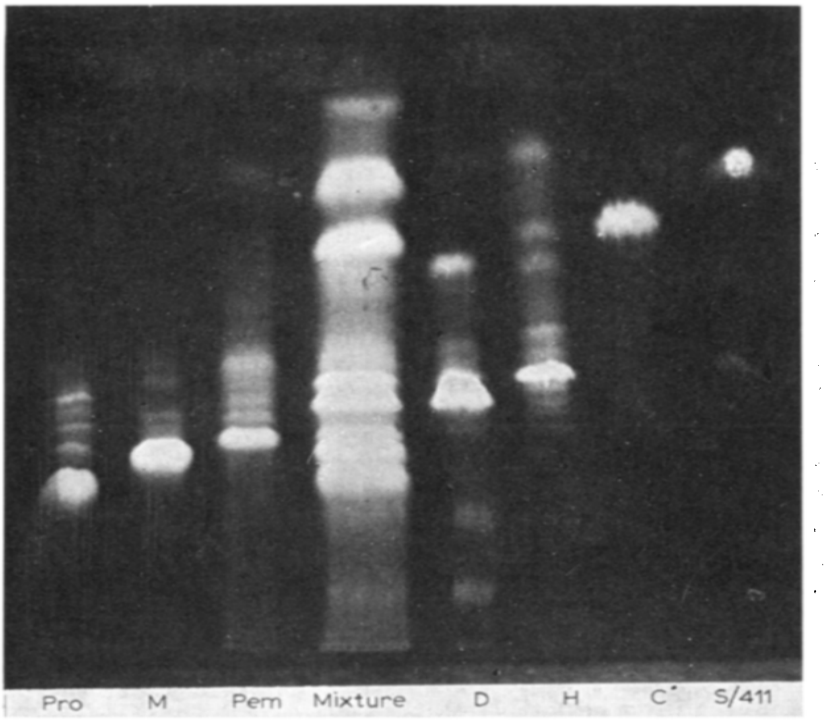

Fig. 1. Photograph taken under UV light of a typical TLC separation of porphyrins, Pro = proto, M = meso, Pem = pempto, D = deutero, H = haemato, C = copro.

proto, and pempto separated according to their HCl numbers[2], with the possible exception of pempto, whose HCl number is not published. Table I shows that the most water-soluble (low HCl No.) porphyrins have the highest R_F values.

"S/411" porphyrin separates well from copro (R_F values 0.7 and 0.58, respectively) and since both porphyrins are tetracarboxylic and occur naturally in bile and certain tissues, a convenient method for separation and identification is important. Fig. 1 shows a typical separation with a 2-h development time. The faint bands that accompany the major components are due either to trace impurities or to artefacts. The results shown in Table II were obtained by determination of the Soret maxima after elution of marker and the appropriate component porphyrin separated from the

TABLE II

COMPARISON OF SORET MAXIMA OF PORPHYRIN MARKERS AND APPROPRIATE COMPONENTS OF MIXTURE AFTER TLC SEPARATION

Porphyrin	*Soret (mμ) of marker*	*Soret (mμ) of porphyrin from mixture*
Haemato	403	403
Deutero	400	400
Meso	401–402	402
Proto	409	409
Pempto	404	404
S/411	410	411
Copro	403	403

TABLE III

COMPARISON OF R_F VALUES BY THREE METHODS OF TWO-CARBOXYL PORPHYRIN SEPARATION

Porphyrin	R_F *(TLC)*	R_F *(Paper, ascending)*	R_H[a] *(Paper, descending)*
Haemato	0.35	0.53	1.0
Deutero	0.32	0.33	0.62
Meso	0.25	0.21	0.36
Pempto	0.28	0.21	0.36
Proto	0.19	0.09	0.10

[a] R_H = position of spots relative to haemato.

mixture. The comparative values are sufficiently close to indicate good separation of bands with very little mutual contamination.

Paper chromatography

Table III shows the results of the separation of a mixture of porphyrins. It will be seen from the R_F values that the ascending and descending paper techniques give better separation of spots than the TLC technique. Consequently, the paper techniques are usually preferred for the identification of porphyrins. Unfortunately copro does not separate from the "S/411" porphyrin, and meso from pempto using the paper techniques.

Copro and "S/411" (R_F 0.87) run well above the two-carboxyl porphyrins when using the ascending method and do not interfere with their separation. Uroporphyrin runs with the solvent front.

DISCUSSION

The presence of ammonia in the tank atmosphere was essential for both TLC and PC. Mobilities and resolution were greatly depressed when ammonia was omitted[4]. Originally the TLC plates were prepared using Silica Gel G but because of unavoidable and extensive streaking of bands this medium was abandoned.

The use of borate buffer in the developing solvent prevented streaking of spots on paper runs. The effectiveness is probably due to the formation of a complex between the borate ion and the hydroxyl groups of the cellulose thus producing a de-adsorption effect. The pH of the buffer was found not to be critical. The usefulness of borate buffers in preventing streaking has been utilised for Sephadex column separation of free porphyrins[7].

Although the TLC method separates a greater number of porphyrins (particularly copro and "S/411") the two-carboxyl porphyrins tend to "crowd" together, thus occasionally making individual identification difficult. The PC methods give spacing of the two-carboxyl porphyrins, making identification comparatively easy. Therefore, a combination of TLC and PC will give an excellent and comprehensive method of investigating mixtures of porphyrins eluted from two- or four-carboxyl porphyrin bands produced on 2,6-lutidine–water silica gel TLC plates[12] or directly from extracts of tissues.

Proto, pempto, deutero and copro were readily detected in faecal extracts using the combination of TLC and PC. The "S/411" porphyrin was easily detected in ether extracts of dog bile contaminated with bile pigments. These did not interfere with the separation because they occupied positions between the two-carboxyl bands as small discrete areas. Other impurities ran diffusely near the solvent front.

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