The quantitative separation of porphyrins and protohaemin as methyl esters by thin-layer chromatography

Other investigators have reported on the qualitative differentiation of porphyrins by thin-layer chromatography (TLC)[1,2]. Among porphyrin derivatives, tetraphenylporphines[3] have also been quantitatively separated by TLC. The present investigation shows that the porphyrins in biological material (urine, bacteria and culture filtrates) can be quantitatively separated on silicic acid after transformation to their methyl esters (ME). This procedure can also be used for the isolation and determination of the protohaemin di-ME. The latter is formed during the esterification of porphyrins.

Materials and methods

Chromatoplates (20 × 20 cm) were layered with Silica Gel H (10–40 μ) according to STAHL (E. Merck, Darmstadt); they were 0.5 mm in thickness, activated at 105° for 60 min, and developed by the usual ascending technique for 10–12 cm in 20–25 min in the following solvent systems:

(*A*) *Solvent system for porphyrin ME.* Benzene–ethyl acetate–methanol (BEM) (85:13:2, v/v). The porphyrin ME are separated according to the number of carboxyl groups[1] and can be visualised by their red fluorescence in U.V. light (355 nm).

(*B*) *Solvent system for haemin ME.* Chloroform–methanol (CM) (94:6, v/v). The haemin ME is detected by comparison with the test substance (a brownish-black spot) or when in very small amounts (< 1 μg) with *o*-tolidine reagent[4].

The porphyrin ME, prepared from culture fluid[5], bacteria[6], or urine[7] with methanol–H_2SO_4 (5 vol. %), after dissolving in chloroform, were applied in a linear pattern on the chromatoplates. The development of the chromatogram, with the substances isolated, in the solvent system BEM (saturated atmosphere) is shown in Fig. 1. Each red fluorescent silica gel zone is removed by means of a layer-suction and

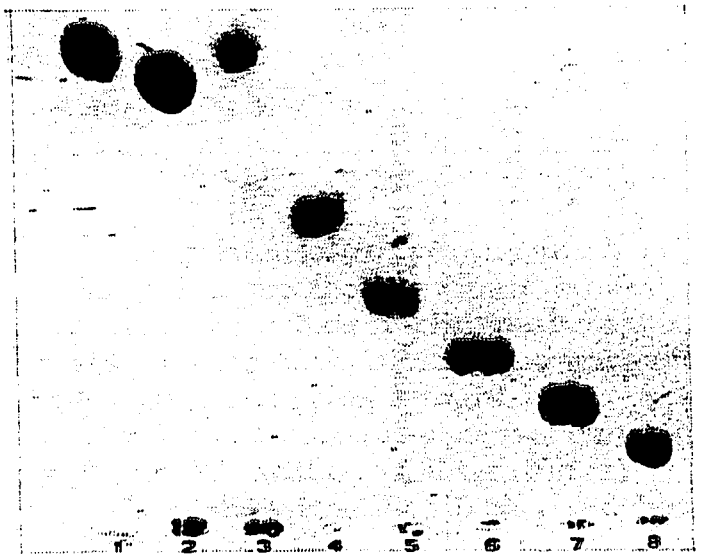

Fig. 1. Thin-layer chromatogram (Silica Gel H). Solvent system: benzene–ethyl acetate–methanol (85:13:2, v/v); porphyrins indicated by photography under U.V. light. Porphyrin ME: 1 = Meso IX; 2 = deutero IX; 3 = proto IX; 4 = copro III; 5 = pentacarboxylic; 6 = hexacarboxylic; 7 = heptacarboxylic; 8 = uro III; 4–8 are isolated from porphyric urine.

J. Chromatog., 30 (1967) 265–269

TABLE I

R_F VALUES (× 100) OF METHYL ESTERS OF PORPHYRINS AND PROTOHAEMIN

*Solvent system**	*Temperature (°C)*	*Porphyrins*								
		Meso	*Proto*	*Deutero*	*Copro*	*Pentacar-boxylic*	*Hexacar-boxylic*	*Heptacar-boxylic*	*Uro*	*Haemin*
BEM	20	75	75	70	50	36	26	19	11	3
BEMB	20	85	85	80	66	52	39	36	34	7
CM	20	Porphyrin ME migrated with the front								45

* See text for details of the solvent systems.

elution apparatus[8]. Proto-, copro- and penta-carboxylporphyrin ME are eluted with chloroform, hexa-, hepta- and uroporphyrin ME with chloroform–methanol (9:1, v/v) under nitrogen. The photometric analysis of proto-, copro- and uroporphyrin ME in chloroform is carried out on the basis of the millimolar extinction coefficient of FALK[9].

Protohaemin di-ME, extracted after esterification from bacterial preparations[5], remains in the BEM system at the starting point. It is eluted with chloroform–methanol (4:1, v/v) from the silica gel and is purified by the CM system.

In the benzene–ethyl acetate–methanol–butanol (BEMB) system (82:14:3:1, v/v), a slight migration of the haemin ME occurs and separation of the whole porphyrin mixture is achieved. This system was mainly used for the isolation of proto-, copro- and uroporphyrin ME[7] and haemin ME[5]. It is, however, not as suitable for the isolation of the penta-, hexa- and hepta-carboxylporphyrins as the BEM system. The R_F values of the systems are summarized in Table I. Meso- and protoporphyrin ME cannot be separated in these systems.

Results

The recovery rate of the porphyrin ME, with the exception of the relatively unstable protoporphyrin IX di-ME, is over 90 %. The limits of error in the individual analyses permit the use of the method for analytical purposes (Table II).

TABLE II

THE RECOVERY OF PROTO-, COPRO-, AND UROPORPHYRIN ME AND PROTOHAEMIN ME FROM SILICA GEL BY PREPARATIVE TLC

Methyl esters	*μg applied*	*ME recovered* $\bar{x} \pm 2s$, $n = 4$	*% recovery*
Protoporphyrin	2.04	1.45 ± 0.36	70.8
	12.34	9.71 ± 1.05	78.7
Coproporphyrin	1.38	1.25 ± 0.66	90.7
	2.51	2.34 ± 0.35	93.3
	3.84	3.81 ± 0.15	99.1
Uroporphyrin	1.58	1.56 ± 0.14	98.7
	3.18	3.08 ± 0.09	97.0
Protohaemin	20.34	19.94 ± 1.16	98.1

$\bar{x}$ = Arithmetic mean; s = standard deviation; n = number of tests.

The greater loss of porphyrin IX in comparison with the other porphyrin ME is probably due, for the most part, to destruction by an artifact (benzene?); because even after immediate rechromatography of the protoporphyrin IX di-ME weakly fluorescent spots are visible in the start and migration zone. The pure preparation and the test substance (Koch-Light-Laboratories) behaved identically. Benzene, however, is preferable compared to other solvents as it gives a sharp separation of the individual porphyrin ME without tailing (Fig. 1).

Pure ME of penta-, hexa- and hepta-carboxylporphyrins were isolated from porphyric urine (Fig. 1), by means of rechromatography in the solvent system BEM, and were characterized by their absorption spectra (Table III). Infrared spectra of penta-, hexa- and hepta-carboxylporphyrin ME showed that these biologically occurring porphyrins are a mixture of the isomers of the I and III series[10].

TABLE III

SPECTRAL DATA* OF PENTA-, HEXA-, AND HEPTACARBOXYLIC PORPHYRIN METHYL ESTERS, SEPARATED BY TLC

Solvents: ((1)) chloroform, ((2)) dioxan, ((3)) ether.

Number of COOH-groups of ME	*Solvent*	*Absorption spectra wavelength maxima in nm*				
		Soret	*IV*	*III*	*II*	*I*
5	1	400.5	498	532	566	619
	1**		500	535	569	623
	1***		500.9	535.0	566.2	621.8
	2	400	496	528	565	619
	2**	400	499	532	569	623
	2***	400				
	3	397.5	496	527	566	621
6	1	402.5	498	533	567	620
	1**		500	535	570	624
	1***		504.2	535.9	568.3	623.7
	2	400	497	530	566	620
	2**	402	499	533	569	624
	2***	402				
	3	399	497	528	567	622
7	1	404	499	534	568	621
	1**		500	537	571	625
	1***		504.8	535.8	568.6	625
	2	402.5	498	531	567	621
	2**	403	499	534	570	625
	2***	403.3				
	3	400	498	529	568	623

* Recording spectrophotometer Carry M 15.
** Values are from CHU AND CHU[11].
*** Values are from LOCKWOOD AND DAWIES[12].

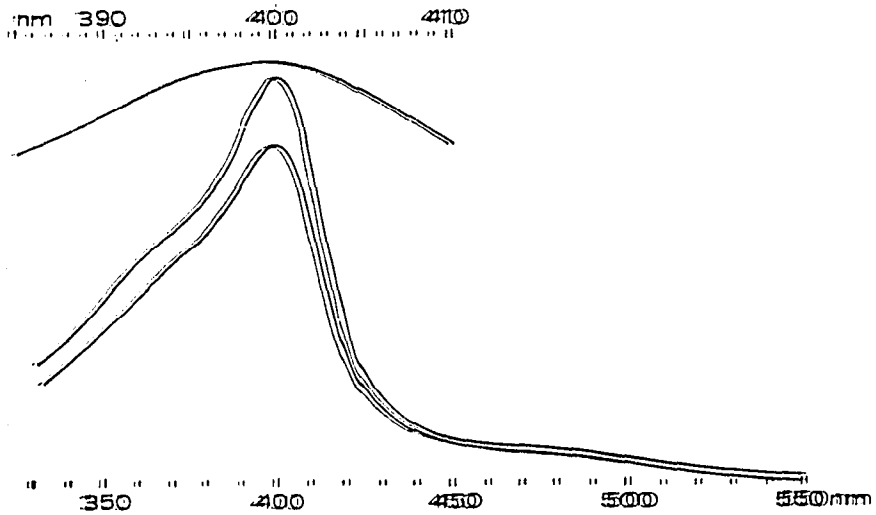

Fig. 2. The absorption maximum of protohaemin ME in chloroform. 1 = Haemin ME, prepared from *Achromobacter metalcaligenes* ($(E_{400} = 0.995)$); 2 = test haemin ME ($(E_{400} = 0.78)$). Recording spectrophotometer Carry M 15.

The absorption maximum of protohaemin ME was found at 400 nm in chloroform (Fig. 2). Under other conditions the absorption maximum was found at 401–403 nm[5]. With the relatively flat maximum of the absorption curve (see Fig. 2), such a difference is not significant for the quantification of haemin; this was tested by comparing the height of the absorption at these maxima with various weights.

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Calcium sulfate as an adsorbent for thin-layer chromatography

Calcium sulfate in the form of plaster of Paris ($CaSO_4 \cdot 1/2H_2O$) is commonly used as a binder in thin-layer chromatography (TLC). As such, it is present in small amounts (5–13 %) in commercially available adsorbents such as Silica Gel G, Aluminum Oxide G, and Cellulose Powder G.

As early as 1949, BROCKMAN[1], in work on column chromatography, reported that $CaSO_4 \cdot 2H_2O$ can be converted to an excellent adsorbent by heating it at temperatures between 150° and 200°. In 1962, KAUFMANN AND KHOE[2] used $CaSO_4 \cdot 1/2H_2O$ as an adsorbent for the thin-layer chromatographic separation of fatty acids and synthetic and natural triglycerides. More recently, ALFONSO[3] described the use of self-supporting strips of plaster of Paris ($CaSO_4 \cdot 1/2H_2O$), 1 to 5 mm thick, in preparative chromatography. Aside from these few references, the use of calcium sulfate by itself as a chromatographic adsorbent has been almost completely ignored. This may have been due to the difficulty in obtaining a plaster of Paris of suitable purity and uniformity.