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ISOLATION OF PORPHYRINS FROM PORPHYRIA URINE BY PREPARATIVE THIN-LAYER CHROMATOGRAPHY*

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SUMMARY

A preparative method of thin-layer chromatography for the isolation of porphyrins from bovine or human porphyric urine, is presented. The isolated uroporphyrin, 7-, 6-, and 5-carboxylic porphyrins and coproporphyrin from bovine urine were crystallized and identified as their methyl esters. Adaptation of this method for analytical work is described and examples are given of data from human and bovine porphyria samples.

Many methods[1-11] have been described for the identification and isolation of urinary porphyrins but in the main these have been used for small quantities and have not been preparative in type. Normal natural sources rarely provide sufficient amounts of porphyrins for preparation in any considerable quantity; in most cases previous methods have been concerned with isolation, purification, identification and quantitative determination in the micro- or, at most, milligram range. A few semi-preparative methods have been described for separation and crystallization of uroporphyrins, coproporphyrins and protoporphyrins from natural sources. The earlier techniques for the ether-soluble porphyrins involved extraction and fractionation with varying concentrations of hydrochloric acid[1]. These are tedious and highly difficult for large quantities and they do not permit efficient separation.

It is well known that the urine in human or bovine erythropoietic porphyria[9,12,13] has a remarkably high content of various porphyrins, especially uro-, hence is a rich source for an appropriate method of preparative isolation. Over many years a variety of methods have been used in this laboratory but none have proved adequate for this purpose. Recently extensive study has been made of high-voltage electrophoresis[13] and of solvent partition[1], with moderate success. These have now been compared with a preparative scale analytical method of thin-layer chromatography (TLC) which

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has been found to be simpler and more efficient, hence preferable to any method previously used by us or of which we are aware.

MATERIALS AND METHODS

Preparation of crude porphyrin mixture

Urine was collected from a number of bovine porphyrics[12] over a period of three months. After adjusting the pH to 5 with glacial acetic acid, each collection of approximately 25 gallons was mixed with 10 lb. of talc in a 30-gallon plastic drum. The foaming which occurred was controlled by addition of acetone. Several times over a two-day period the talc was stirred vigorously and finally allowed to settle. Most of the supernatant urine was decanted and the talc recharged with a fresh batch of acidified urine. The supernatant urine was again decanted and the remainder was filtered on a large Buchner funnel. After washing with distilled water, the porphyrin was eluted from the talc with dilute ammonium hydroxide and acidified to pH 3.2 with concentrated HCl. After standing overnight at 4° the precipitated porphyrins were collected on a sintered glass funnel and esterified with methanol–sulfuric acid (20:1, v/v). The esters were extracted into chloroform and worked up in the usual way[1]. Direct elution from the talc with methanol–sulfuric acid (20:1, v/v) was carried out with smaller volumes of urine from human cases of porphyria, including three of congenital erythropoietic (EP) type, one of hepatic porphyria cutanea tarda (PCT), and one of hepatic acute intermittent type (AIP).

After concentration to a small volume, the crude bovine prophyrin extract was

TABLE I

ANALYTICAL THIN-LAYER CHROMATOGRAPHY; COMPOSITION OF TOTAL URINE PORPHYRIN IN BOVINE AND HUMAN PORPHYRIA

a = Fluorometer; b = densitometer. For other abbreviations, see text.

	Bovine				*Human*				
	Erythropoietic				*Erythropoietic*			*PCT*	*AIP*
					M.M.	*M.H.*	*D.H.*	*R.V.*	*C.S.*
Amount of crude porphyrin applied	100 mg	1 mg	43 μg		10 μg	10 μg	10 μg	10 μg	10 μg
			a	b					
Amount recovered from TLC	63 mg	0.83 mg	26 μg	—	6.7 μg	8.5 μg	7.0 μg	7.8 μg	7.5 μg
Proportion of recovered porphyrins (%):									
Copro-(4-COOH)	31.6	28.9	20.4	27	40.8	22.8	25.3	1.3	38.9
5-COOH	8.1	7.8	6.9	3	4.3	5.3	4.4	2.3	5.3
6-COOH	0.6	0.5	0.3	—	0.9	0.7	0.6	3.1	1.8
7-COOH	1.4	1.2	1.2	1	1.9	3.0	1.8	22.7	1.8
Uro- (8-COOH)	50.0	53.2	63.1	50	40.3	59.3	55.0	53.0	41.6
Origin (unesterified) (see text)	8.3	8.4	8.1	19	11.8	8.9	12.9	17.6	10.6

subjected to preliminary purification by three precipitations, each time in 50 volumes of petroleum ether (30–60°). The petroleum ether filtrate was yellow (see below under RESULTS), and had an intense green fluorescence with a strong absorption band at 400 mμ. The latter undoubtedly contributes to erroneously high porphyrin values in quantitative spectrophotometric determinations on the initial chloroform solution, which depend on the Soret band absorption. The amount of total porphyrin in the chloroform extract was determined spectrophotometrically using an uroporphyrin standard. This amount includes any absorption due to impurity as mentioned above and in the following. The various amounts applied for TLC (see Table I) were determined in the same way except for the 100 mg sample, which was weighed.

Silica Gel G plates (20 × 20 cm) were prepared according to directions in the Desaga–Brinkmann manual and using their applicator for making varying thicknesses (0.25–1.0 mm). Activation of plates is unnecessary and air drying overnight or for 24 h is sufficient. The amount of absorbed water on the plate has some influence on the quality of separation.

For preparative TLC the isolated porphyrin esters in chloroform solution were applied with the Rodder Streaker* on the plates (5–15 mg per 20 × 20 cm plate). Larger amounts, up to 30 mg per plate, have been separated satisfactorily on 40 × 20 cm plates. As noted below, the use of multiple plates permits processing of 1 g of crude porphyrin in one day. For analytical work the porphyrin esters were applied with a capillary tube or micropipet (see Table I).

The plates were developed in tanks (25 × 25 × 12 cm), using petroleum ether–chloroform (1:5, v/v) with 10% ammonia atmosphere (in a 25 ml cylinder). Previous saturation of tanks for 15–30 min was helpful. Other solvent systems found satisfactory were benzene–chloroform (1:5, v/v) with 10% ammonia atmosphere, and decane–chloroform (1:18, v/v).

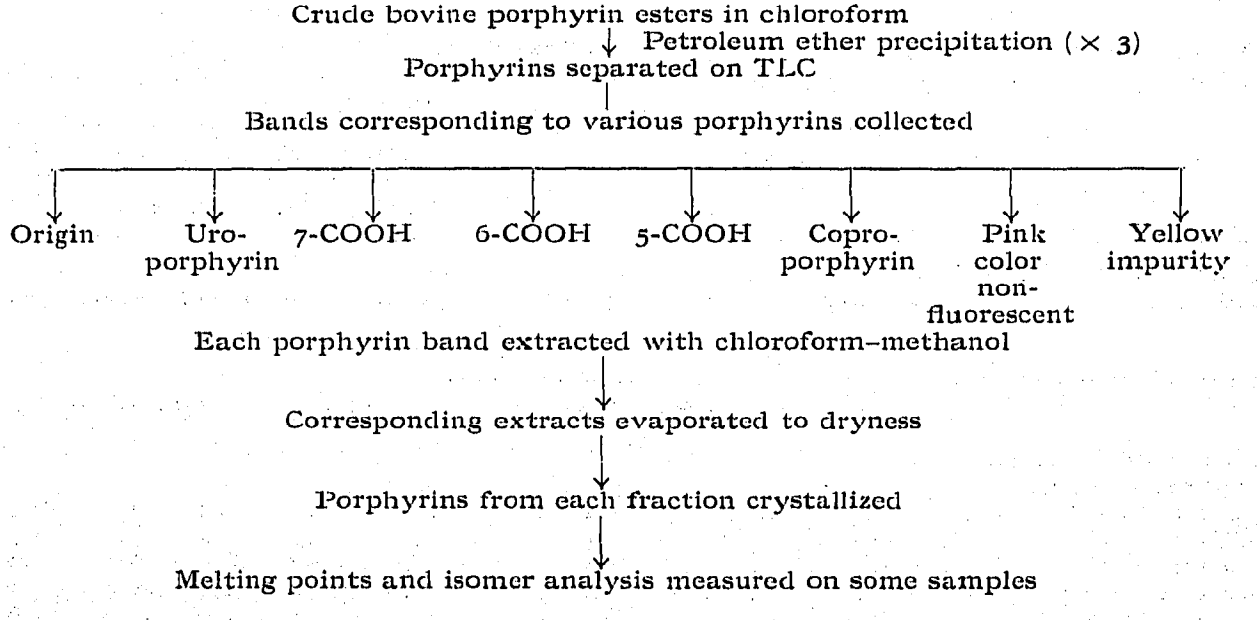

Fig. 1. Schematic pathway for the separation and isolation of bovine porphyrins by TLC on Silica Gel G in petroleum ether–chloroform (1:5) in ammonia atmosphere (10%).

* Rodder Instruments, Los Altos, Calif.

Development times varied with plate thickness. For analytical separation on a 0.25 mm plate 1 h was sufficient to have the solvent front run 11 cm. On 0.25–0.5 mm plates with 5–15 mg amounts of porphyrin 2–4 h were required. Thicker plates (1 mm) needed even more time and the zones were not as distinct because the plates were not as efficiently saturated with the ammonia atmosphere, which facilitates the separation.

After development the preparative plates were dried and the separate porphyrin bands were collected from many plates with the Brinkmann Vacuum Zone Collector. Each zone was extracted on a medium sintered glass filter, using methanol–chloroform (1:4). The eluates were taken to dryness on a Buchler flash evaporator, reconstituted in chloroform and the porphyrin esters crystallized from methanol (Fig. 1). Six plates were developed at a time, thus 1 g of crude porphyrin ester can be separated into its components in four days. By using 40 × 20 cm plates this can be reduced to one day.

Solvent partition was employed when the main objective was to obtain gram quantities of semi-purified uro- or coproporphyrin quickly. This procedure involves pouring the concentrated chloroform solution of porphyrin ester into 25 volumes of ethyl ether, producing an insoluble (mainly uro-) and a soluble (mainly copro-) fraction. These may then be purified further by TLC, if desired.

Previously described methods of paper chromatography[14,15] for isomer analysis were used. The copro- isomer ratios were determined in the Turner door fluorometer*[16]. Melting point determinations were made with a Fisher-Johns micro-melting point apparatus. Uncorrected temperature values are given.

RESULTS

The preparative separation of the total porphyrin esters from bovine porphyria

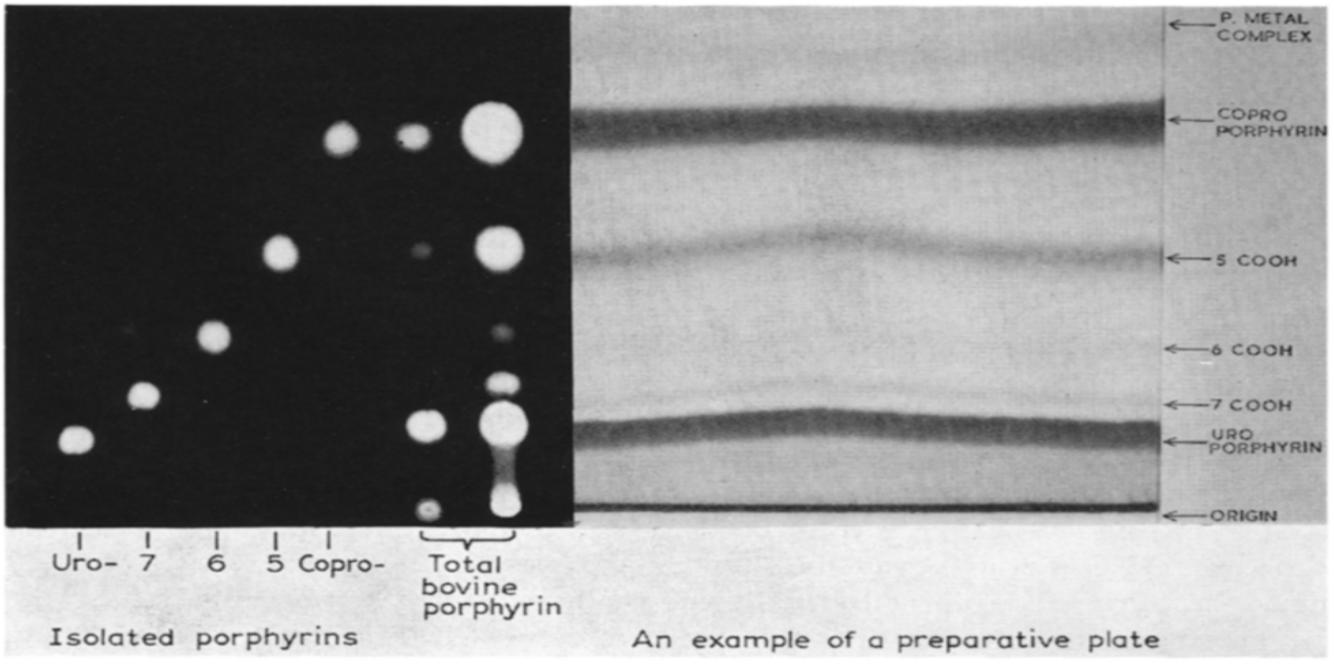

Fig. 2. Plates of Silica Gel G, developed with petroleum ether–chloroform (1:5) in ammonia atmosphere (10%).

* G. K. Turner Associates, Palo Alto, Calif.

urine is shown in Fig. 2. The isolated porphyrins were rerun analytically and proved to be homogenous (Fig. 2). The total bovine porphyrins were run on the same plate to demonstrate the separation achieved. On the primary plate a small amount of the yellow impurity ran to the front. This was identical to the pigment in the above described filtrate from petroleum ether precipitation which on crystallization gave a m.p. of 140–146°. This substance is as yet unidentified. The next band was a pink non-fluorescent one, a coproporphyrin metal complex, followed by the porphyrin bands (Figs. 1 and 2). The brown zone which remained at the origin was dissolved in methanol–sulfuric acid (20:1), extracted into chloroform and rechromatographed, all as described in the foregoing. A small amount of uro- and trace amounts of copro- to 7-COOH porphyrin* were found but the majority remained at the origin as unidentified brown pigment.

TABLE II

PREPARATIVE THIN-LAYER CHROMATOGRAPHY OF BOVINE PORPHYRINS

Porphyrin type	*Absorption maxima of porphyrin esters in $CHCl_3$*[a]					*Melting points (uncorr.) (°C)*
Copro-	622	568	533	499	400	239–245
5-COOH	623	569	536	500	401	218–223
6-COOH	624	568	538	503	403	230–240
7-COOH	625	570	537	501	404	238–243
Uro-	626	572	536	502	406	285–288

[a] Hitachi, Perkin-Elmer Model 139 spectrophotometer.

The absorption maxima and the melting points from the separated porphyrins are presented in Table II. The melting points shown are affected by minor proportions of type III isomer by the CORNFORD–BENSON[14] and ERIKSEN[15] methods. This was determined to be 15% and 14%, respectively, for uro- and copro-, and 18% for 7-COOH (after decarboxylation to copro-[17]).

Quantitative determination of porphyrin in each zone separated on TLC was made in two ways: (1) removal of each zone from the plate with a spatula, extraction from the silica gel with methanol–chloroform (1:4), followed by ordinary fluorometry[18]; (2) directly on the plate using the Photovolt densitometer. (See Table I for percentages of each porphyrin from the original sample.) Preliminary comparisons of direct densitometric determination on the plate indicates that the values compare favorably with fluorometric measurement of the eluted porphyrins. As yet the uro- and 7-COOH porphyrins have been determined in terms of an uroporphyrin standard, the copro-, 5- and 6-COOH porphyrins with a coproporphyrin standard (Table I). The present TLC method could be applied analytically and preparatively to any urinary porphyrins (adaptable to fecal), *e.g.*, the five cases of human porphyrias were investigated analytically and the representative values given in Table I are in agreement with those reported[9].

* uro- = uroporphyrin; 7-COOH, 6-COOH, 5-COOH = hepta-, hexa- and pentacarboxylic; copro- = coproporphyrin.

DISCUSSION

The present TLC method provides a simple rapid separation of porphyrin esters. It has been used for preparative isolation of porphyrins from porphyrin rich urine, either human or bovine (EP). The latter often contains upwards of 1 g of porphyrin in 24 h, mainly uro-, while the urine of the comparable human disease generally contains about 50–100 mg. Both are, in porportion, excellent sources of smaller amounts of copro-, 5-, 7- and 6-COOH porphyrins, in approximately that order of amount. Used simply as an analytical method, the total porphyrin mixture in the range of 1–50 μg is readily separated on the TLC plate and each spot can be determined quantitatively with an appropriate densitometer. As yet our experience with the densitometric method is limited but it appears to be quite satisfactory. In the absence of a densitometer the individual spots are readily eluted as in the preparative method for quantitative determination in a sensitive photofluorometer.

Preliminary solvent partition is useful to obtain quickly a 90–95% pure uroporphyrin (with a small amount of 7-COOH) and 80% pure coproporphyrin (with small amounts of 5- and 6-COOH porphyrins). These may be further purified by TLC.

High-voltage electrophoresis was also tried for preparative separation of porphyrins but has been found far inferior because only small quantities can be isolated over long periods, as compared with TLC; there is too much overlapping of bands and considerable loss of porphyrin.

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REFERENCES

1 J. E. FALK, *Porphyrins and Metalloporphyrins*, B.B.A. Library, Vol. 2, Elsevier, Amsterdam, London, New York, 1964.
2 E. W. BAKER, M. LACKMAN AND A. H. CORWIN, *Anal. Biochem.*, 8 (1964) 503.
3 T. C. CHU AND E. J.-H. CHU, *Clin. Chem.*, 12 (1966) 647.
4 T. C. CHU AND E. J.-H. CHU, *J. Chromatog.*, 21 (1966) 46.
5 P. KOSKELO AND I. TOIVONEN, *Scand. J. Clin. Lab. Invest.*, 18 (1966) 543.
6 T. C. CHU AND E. J.-H. CHU, *J. Chromatog.*, 28 (1967) 475.
7 C. R. SCOTT, R. F. LABBE AND J. NUTTER, *Clin. Chem.*, 13 (1967) 493.
8 T. K. WITH, *Clin. Biochem.*, 1 (1967) 30.
9 T. C. CHU AND E. J. -H. CHU, *Clin. Chem.*, 13 (1967) 371.
10 Y. GROSSER, G. D. SWEENEY AND L. EALES, *S. African Med. J.*, 41 (1967) 460.
11 M. DOSS AND W. MANNHEIM, *Z. Klin. Chem. Klin. Biochem.*, 5 (1967) 260.
12 C. J. WATSON, V. PERMAN, F. A. SPURRELL, H. H. HOYT AND S. SCHWARTZ, *A.M.A. Arch. Internal Med.*, 103 (1959) 436.
13 L. HEILMEYER, *Disturbances in Heme Synthesis*, Charles C. Thomas, Springfield, Ill., 1966.
14 P. A. CORNFORD AND A. BENSON, *J. Chromatog.*, 10 (1963) 141.
15 L. ERIKSEN, *Scand. J. Clin. Lab. Invest.*, 10 (1958) 319.
16 I. T. KAY, *Proc. Natl. Acad. Sci. US*, 48 (1962) 901.
17 P. R. EDMONDSON AND S. SCHWARTZ, *J. Biol. Chem.*, 205 (1953) 605.
18 S. SCHWARTZ, M. H. BERG, I. BOSSENMAIER AND H. DINSMORE in D. GLICK (Editor), *Methods of Biochemical Analysis*, Vol. 8, Interscience, New York, 1960.