Rapid thin-layer chromatography of porphyrins and related compounds, and its application to the study of porphyrias*

Rapid methods of thin-layer chromatography (TLC) have been worked out in which porphyrins, chlorins, chlorophyll and related compounds are separated. The procedures are more rapid than our earlier methods[1]. A glass fiber reinforced silica gel sheet, made by Gelman Co.**, is used as the separating medium, and kerosene–chloroform and aqueous lutidine are used as the respective solvents for methyl esters of porphyrins and coproporphyrin (copro) isomers. A linear relationship between R_F values of the porphyrins and the number of ester groups can be obtained in 5 min and the separation of copro I, II and III (or IV) is achieved in 1 h.

Based upon the findings that different types of porphyria have different porphyrin patterns[2], the method is applied to differentiate major types of porphyria by a simple test tube chromatography of the porphyrins isolated from urine, or other porphyric materials, from such patients. Estimation of copro I and III isomers in such samples can be done in 30 min, which is much faster than the time required by paper chromatography (PC) (overnight)[3,4], or by JENSEN's TLC (2 h)[5].

Experimental

The Gelman sheets, ITLC-Type SG, are produced by combining silica gel with a slurry of microfilaments of glass fiber. With a thickness of about 0.3 mm, this glass fiber media is strong enough to withstand the chromatographic process.

Separation of methyl esters of porphyrins and related compounds. A rectangular museum jar, 10.5 × 5 × 15 cm high, with a ground glass cover was used as the developing chamber, and a mixture of 3 ml of kerosene and 7 ml of chloroform (U.S.P.) was used as the solvent. A 6 × 12 cm sheet cut out from the original 8 × 8 in. size, was spotted with chloroform solutions of samples along a pencilled base line 12 mm from one of the shorter edges. Because of the less compact nature of the Gelman sheet, the spotted area may easily spread over 2 mm in diameter. Therefore, repeated applications of dilute solutions should be avoided. The spotted sheet was inserted immediately after the solvent mixture was introduced with a long stem funnel into the chamber. In 5 min, the ascending solvent reached about 7 cm above the base line of the leaning chromatogram. The developed sheet was taken out, dried, and examined as usual.

For a single porphyric sample, a 2.5 × 15 cm test tube and a small watch glass were used. Two milliliters of the solvent mixture were introduced into the tube without touching the wall. A Gelman strip, 1.8 × 12 cm, spotted with an unknown and a proper marker, was then inserted. Methyl esters of crude porphyrins such as those originating from porphyria patients, were spotted with copro and uroporphyrin (uro) esters as references. The developing time was also 5 min.

Separation of copro isomers. A tall museum jar, 8 × 4.5 × 20 cm high, was used for this purpose. A mixture of 7 ml of 2,6-lutidine and 2 ml of water was the solvent. A necessary ammonia atmosphere was provided by 2 supported tubes of concentrated ammonium hydroxide. The supporting frame was made from a piece of No. 16 nichrome wire about 50 cm long. It was bent into a U-shape of 7–8 cm base. The 2

* This work was supported by a research grant AM-01000 from U.S. Public Health Service.
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ends were made into rings, each large enough to support a 12 × 75 mm test tube around its rim. The positions of the rings were adjusted in the jar, so that when the frame was leaning against the wall, the 2 tubes were hanging vertically at the upper corners of the jar. To help stabilize the tube, a piece of glass rod of appropriate diameter, and 5–5.5 cm long, was slid down into each tube. Concentrated ammonia was added with a pipet, to about 1 cm below the rim of the tube. Because of the glass rod, only about 2 ml of ammonia per tube is needed. The solvent mixture, previously cooled to room temperature, was then added through a long funnel into the chamber. It was left undisturbed for 30 min to attain equilibrium. A 6 × 18 cm sheet was spotted with freshly prepared ammoniacal solutions of copro isomers along the 12 mm high base line. The spreading effect of the sheet is much more pronounced with ammonia. Sample spots should be applied in equal volumes of approximately equal concentrations for better quantitative results. Using a pair of forceps, the spotted sheet was inserted quickly but steadily into the chamber, with its back leaning against the wall opposite the ammonia tubes. The solvent ascended about 14 cm in 1 h. In routine analysis of the naturally occurring copro I and III isomers, the developing time was reduced to 30 min.

TABLE I

R_F VALUES (× 100) OF PORPHYRINS AND RELATED COMPOUNDS

Methyl esters (Solvent: kerosene–chloroform, 3:7 ml)

°C	*Porphyrins*								
	Proto	*Meso*	*Deutero*	*Hemato*	*Copro*	*Penta-carbo-xylic*	*Hexa-carbo-xylic*	*Hepta-carbo-xylic*	*Uro*
20	67	67	67	3	50	40	28	18	9
23	68	68	68	3	50	40	29	19	10

	Chlorins and chlorophyll			
	Pyropheophorbide-a	*Mesochlorin-e_6*	*Uch**	*Chlorophyll-a*
20	60	62	4	73

	Metalloporphyrins			
	Cu-deutero	*Cu-copro*	*Cu-uro*	*Zn-uro*
20	72	56	3	3

Free coproporphyrins (Solvent: 2,6-lutidine–water, 7:2 ml; NH_3 atmosphere)

	I	*II*	*III*	*IV*
23	39	57	48	48

* Uch, a urinary chlorin isolated from a congenital porphyria patient[6].

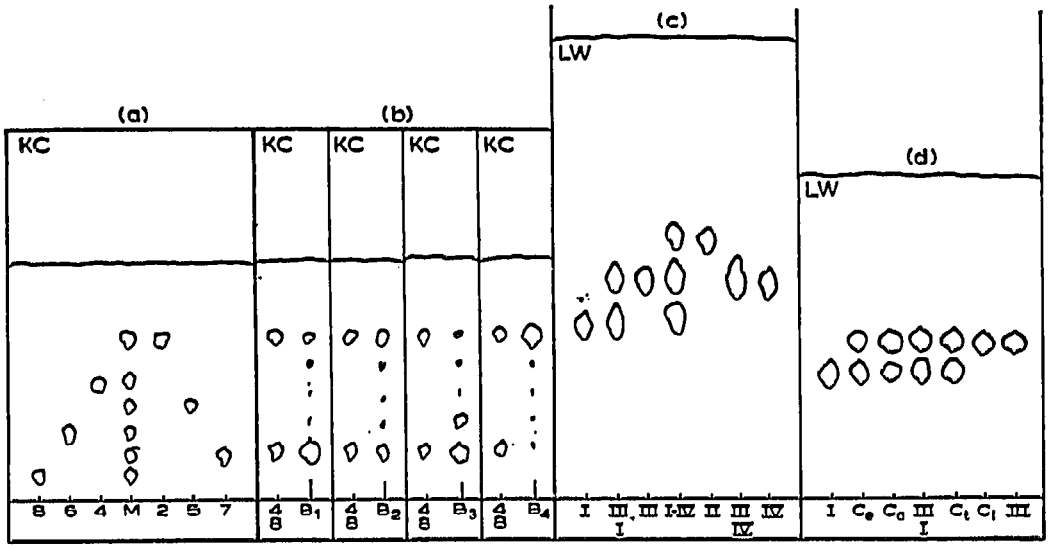

Fig. 1. Thin-layer chromatograms of porphyrins. (a) KC, kerosene–chloroform (3:7, v/v), with solvent front marked after 5 min development at 23°. 2 = Dimethyl ester of protoporphyrin; 4 = copro ester; 5, 6 and 7 = penta-, hexa- and heptacarboxylic porphyrin esters, respectively; 8 = uro ester; and M = artificial mixture of the esters. (b) Porphyrin patterns of major types of porphyria. B_1 = Total porphyrin esters prepared from urine sample of an erythropoietic porphyria patient; B_2 = from acute intermittent porphyria; B_3 = cutanea tarda; and B_4 = lead poisoning case. (c) LW, lutidine–water (7:2, v/v), with solvent front after 1 h. I, II, III, and IV = Free copro isomers of respective types; I–IV = mixture of the four; and I, III, etc. = mixtures of specified isomers. (d) Solvent front after 30 min. C_e = Copro from ertythropoietic porphyria patient; C_a = acute intermittent; C_t = cutanea tarda; and C_l = lead poisoning.

Results and discussion

The R_F values of porphyrins, chlorins, chlorophyll and metalloporphyrins are listed in Table I. The chromatograms showing separations of porphyrin esters and isomers are given in Fig. 1. From Table I, the R_F values of the methyl esters, revealing a linear function with the number of ester groups of the porphyrins, are quite constant at room temperature. The difference in the relative positions of porphyrins in Fig. 1a and b is due to the difference in the two experimental set-ups. Different batches of the Gelman sheets have also shown some differences in R_F values. Aside from some non-uniformity occasionally encountered, the sheet behaves almost like filter paper without the worry of its being deactivated on storage.

Fig. 1b shows the results obtained from urine samples from different porphyria patients. The uro is predominant in the erythropoietic or congenital porphyria (B_1), while the copro dominates in the lead poisoning porphyrinuria (B4). In the acute intermittent type of porphyria, the copro and uro are eliminated in comparable amounts (B_2), and in porphyria cutanea tarda, the excretion of the heptacarboxylic porphyrin is greatly enhanced (B_3).

Fig. 1c has reproduced the paper chromatographic result of FALK[3], with a better separation of the copros. Besides the much shortened developing time, the R_F values are reproducible even with the 30 min short runs. As in PC, the lutidine system does not separate copro III and IV.

Fig. 1d shows some typical isomeric compositions of copro isolated from various types of porphyria patients. The copro in the erythropoietic porphyria is mainly of

the type I, while that in acute and lead poisoning cases is mainly of the type III. In cutanea tarda, the copro has a wider range of the percentage composition, with an average of about 65 % III.

The minimum detectable amount of fluorescent porphyrin esters is 0.002 μg, and 0.1 μg is a convenient quantity to work with. In the case of copro isomers, 0.05–0.1 μg is only barely detectable, because of the spreading spots, and the easy fading of fluorescence on drying.

Acknowledgement

The authors are indebted to Dr. S. F. MACDONALD of National Research Council of Canada for the generous gift of his synthetic coproporphyrin isomers.

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Received November 8th, 1966

J. Chromatog., 28 (1967) 475–478

Thin-layer chromatography of isatins and corresponding isatin-N-Mannich bases

Recently we have synthesized a series of isatin-N-Mannich bases for pharmacological screening. These results are reported elsewhere[1]. During the course of this investigation it became necessary to develop a suitable method for the separation of isatins from their corresponding Mannich bases. Thin-layer chromatography was chosen because of its rapidity and simplicity. In this communication the results of thin-layer chromatography of these compounds are reported.

Materials

Commercially available isatin, 5-bromoisatin and 5-methylisatin were used.

Isatin-N-Mannich bases were prepared by condensing equimolar proportions of isatin, formaldehyde and appropriate secondary amine[1].

Solvent system

Benzene–ethyl acetate–diethylamine (75:20:5, v/v).

Visualization

Most of the spots were readily visible as such but the optimum visualization was achieved by the use of an ultraviolet lamp.