THIN-LAYER CHROMATOGRAPHY OF METHYL ESTERS OF PORPHYRINS, CHLORINS AND RELATED COMPOUNDS WITH EASTMAN "CHROMAGRAM"*

T. C. CHU AND EDITH JU-HWA CHU

Department of Chemistry, Immaculate Heart College, Los Angeles, Calif. (U.S.A.)

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The separation of porphyrins by thin-layer chromatography (TLC) has been reported by other investigators[1-3]. The "Chromagram" made by Eastman Kodak Company offers a ready-to-use coated sheet, and also simplifies documentation of chromatograms. Direct application of DEMOLE's benzene–ethyl acetate–ethanol system[1] to "Chromagram" resulted in driving all the reported porphyrin esters almost to the solvent front, while most of the porphyrins made no movement with BALEK AND SZUTKA's benzene–acetone mixture[2]. JENSEN's solvent[3] is applicable to free porphyrins. The present communication describes a simple and rapid method for the separation of porphyrins, chlorins, chlorophylls and some chloroform-soluble metallo-complexes.

MATERIALS AND METHODS

The "Chromagram" is a sheet of polyethylene terephthalate coated with a layer of about 100 μ silica gel with polyvinyl alcohol as a binder and lead–manganese-activated calcium silicate as a fluorescent indicator. It is quite flexible and only slightly thicker than Whatman No. 1 filter paper. It comes in boxes of 20 sheets each, 20 × 20 cm in size. Any opened boxes may be stored in a desiccated chamber for months without activation.

A rectangular museum jar, 10.5 × 5 × 15 cm high, with glass edging and a ground glass cover was used as the developing chamber, and a metal trough, 9 × 2.5 × 1.5 cm high, was used as the solvent container. Other equipment is similar to what is needed for paper chromatography (PC).

Porphyrins, chlorins and other compounds used in this experiment are listed in Table I.

(A) Decane–chloroform (DC) system

This solvent system, consisting of 1 ml of *n*-decane and 9 ml of chloroform (U.S.P.), was used for the separation of methyl esters of porphyrins and related compounds in general. The developing chamber was lined with Whatman No. 1 paper, 27.5 × 12.5 cm, uniformly moistened with a mixture of 1.2 ml of decane and 2.8 ml of chloroform. The small metallic trough was lowered to the bottom of the

chamber without touching the lining. The solvent mixture was then introduced with a long-stem funnel, through a corner of the covered chamber into the trough. The chamber was left undisturbed for 1 h to attain saturation. In the meantime, the "Chromagram" was cut with a paper cutter into sheets of 6.5 × 12 cm. The sheet was spotted with chloroform solutions of samples along a base line drawn with a soft pencil 1 cm from one of the shorter edges. Because the "Chromagram" can hold more sample per unit area than the filter paper, it is easy to confine a spot to within 1 mm or so in diameter. Any irregular coatings along the two longer edges, resulting from cuttings, etc., were scraped off with a razor blade for a smooth solvent front. Using a pair of forceps, the spotted sheet was inserted through the half slide-opened cover of the saturated chamber into the solvent trough, with its back leaning against the chamber. The chromatogram was allowed to develop at room temperature for 30 min. By then, the solvent front was about 8 cm from the base line. Marking the solvent front, drying the chromatogram, and locating the spots under U.V. light were similar to those applied to PC. The developed "Chromagram" sheets marked with data were filed in a regular index box. Some earlier chromatograms, wrapped in a cellophane film, were found to be still fluorescent.

(B) Kerosene–2,4-pentanedione–methyl benzoate (KPM) system

This consists of 6 ml of kerosene, 3.5 ml of 2,4-pentanedione and 0.5 ml of methyl benzoate. All the solvents except kerosene were of the reagent grade. As described in (A), the paper lining of the chamber was wetted with a mixture of 3.6 ml of kerosene and 0.4 ml of 2,4-pentanedione. The spotted sheet was introduced after the atmosphere of the chamber had been equilibrated for 1 h. The chromatogram was allowed to develop for 40 min.

(C) Water–acetonitrile–p-dioxan (WAD) system

This was used for a reversed-phase partition chromatography. The blank "Chromagram" was first dipped into a light petroleum (b.p. 60–110°) solution of Dow Corning silicone No. 550 fluid (12.5:100, w/v). After draining and drying at 110° for 1 min, the sheet was placed on a piece of paper and the silicone was wiped off the back. The edges that showed any stains of silicone were trimmed off. Samples were then applied to the sheet as usual. In contrast to the other two solvent systems, the step of saturating the chamber was omitted. The spotted sheet was inserted immediately after the solvent mixture (2 ml of water, 7 ml of acetonitrile and 1 ml of p-dioxan) was introduced into the trough in the unlined but covered developing chamber. The time for development was 40 min.

Both (B) and (C) are solvent systems for the separation of dicarboxylic porphyrins. The choice should be made according to the nature of samples. The KPM system (B) does not distinguish between proto- and deuteroporphyrin esters, while the WAD system (C) does not differentiate proto- and mesoporphyrins.

RESULTS AND DISCUSSION

Examples of separation of methyl esters of various porphyrins by thin-layer "Chromagram" are shown in Fig. 1. The R_F values of these samples and related compounds are given in Table I.

TABLE I

R_F VALUES ($\times$ 100) OF METHYL ESTERS OF PORPHYRINS, CHLORINS AND RELATED COMPOUNDS IN DIFFERENT SOLVENT SYSTEMS

Porphyrins

Solvent system*	°C	Proto	Meso	Deutero	Hemato	Copro	Pentacar-boxylic	Hexacar-boxylic	Heptacar-boxylic	Uro
(A) DC	19	77	77	77	1	59	48	37	27	18
	23	72	72	72	1	56	44	33	25	17

Chlorins and chlorophylls

Solvent system*	°C	Pyropheo-phorbide-a	Mesochlorin-e6	Uch**	Chlorophyll-a	Chlorophyll-b
(A) DC	23	60	63	10	77	10

Metallo-porphyrins and chlorins

Solvent system*	°C	Cu-deutero	Cu-copro	Cu-uro	Zn-uro	Cu-Uch**
(A) DC	23	75	67	13	4	5

Porphyrins

Solvent system*	°C	Proto	Meso	Deutero	Hemato
(B) KPM	23	53	61	52	10
(C) WAD	23	52	52	65	82

* See Fig. 1 for definition of the solvents.
** Uch, a urinary chlorin isolated from a congenital porphyria patient[5].

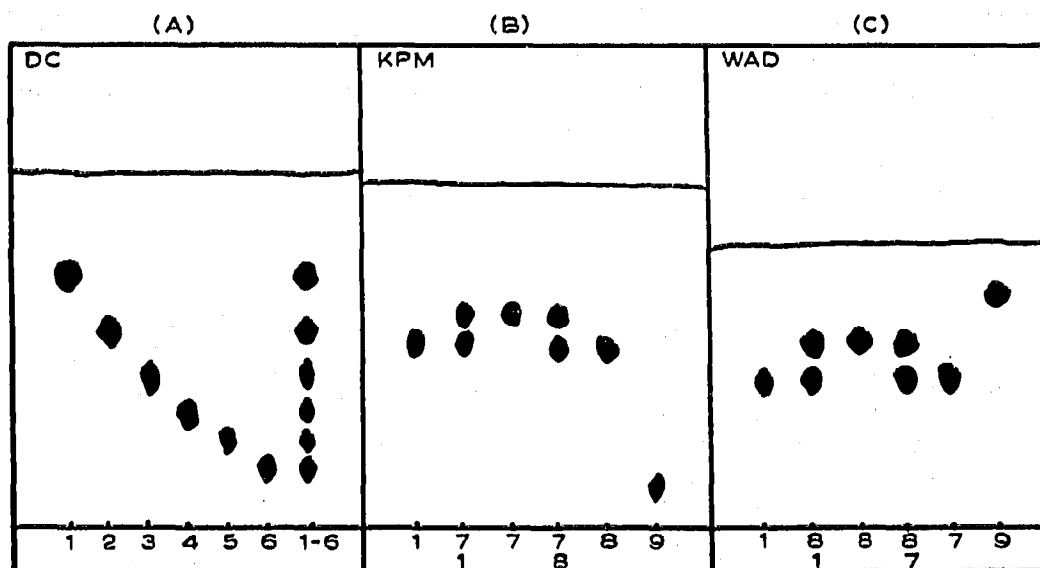Fig. 1. Thin-layer chromatograms of methyl esters of porphyrins. (A) DC, n-decane–chloroform (1:9, v/v), with solvent front marked after 30 min development at 23°; (B) KPM, kerosene–2,4-pentanedione–methyl benzoate (6:3.5:0.5); development time 40 min; (C) WAD, water–aceto-nitrile–p-dioxan (2:7:1), with silicone as stationary phase; time 40 min. (1) Protoporphyrin ester, (2) copro, (3), (4) and (5) penta-, hexa- and heptacarboxylic porphyrins respectively, separated from porphyria patient[4], (6) uro, (7) meso, (8) deutero, (9) hemato and (1–6) etc. artificial mixtures of specified porphyrins.

Although it is generally assumed that R_F values in TLC are influenced by more factors, such as nature of the adsorbent, thickness of the layer, etc., than those in PC, with improved techniques TLC has increasingly been giving useful R_F values. As shown in Fig. 2, some quantitative relationships between the chemical structure and the chromatographic behavior of the porphyrins are apparent. The R_M values, calculated from the experimental R_F values, are directly proportional to the number of the ester groups of the porphyrins. Based on this fact, the number of carboxyl groups of an unknown porphyrin is easily determined without the conversion of the pure ester to the free porphyrin as had been done in PC[6].

According to MARTIN's theory[7], the term $\log(1/R_F - 1)$ or R_M is a linear function of the number of equal groups, while the number of the other groups remains constant. The alignment of the R_M values of the penta-, hexa- and hepta-carboxylic porphyrins with proto-, copro- and uroporphyrins has shown the similarity in structure of these porphyrins, whereas hematoporphyrin containing two hydrophilic hydroxyl groups on the side chains is apparently an exception. It is also evident that the structural isomers of porphyrins are not resolved by the solvent system.

The effect of temperature on R_F values, especially those obtained from low-boiling solvent systems, is noticeable. Two sets of R_F values of porphyrins are given in Table I. The value becomes smaller at higher room temperature. However, the linear relationships mentioned above still hold nicely as shown in Fig. 2.

In the reversed-phase (WAD) system, a number of solvent mixtures has been tried, to control the atmosphere in the chamber, but none has been found to accomplish any better separation than the uncontrolled one. Any non-uniformity of the coating (silica gel or silicone), differences in the temperature and the atmosphere all

contributed to an error of more than ±0.02 in the R_F, which is the average value of the first system (DC) at a given temperature. In this respect, the KPM system registered the smallest error of ±0.01. Considering a similar separation by two-dimensional PC[8] with a development time of 4 h, TLC is a rapid method.
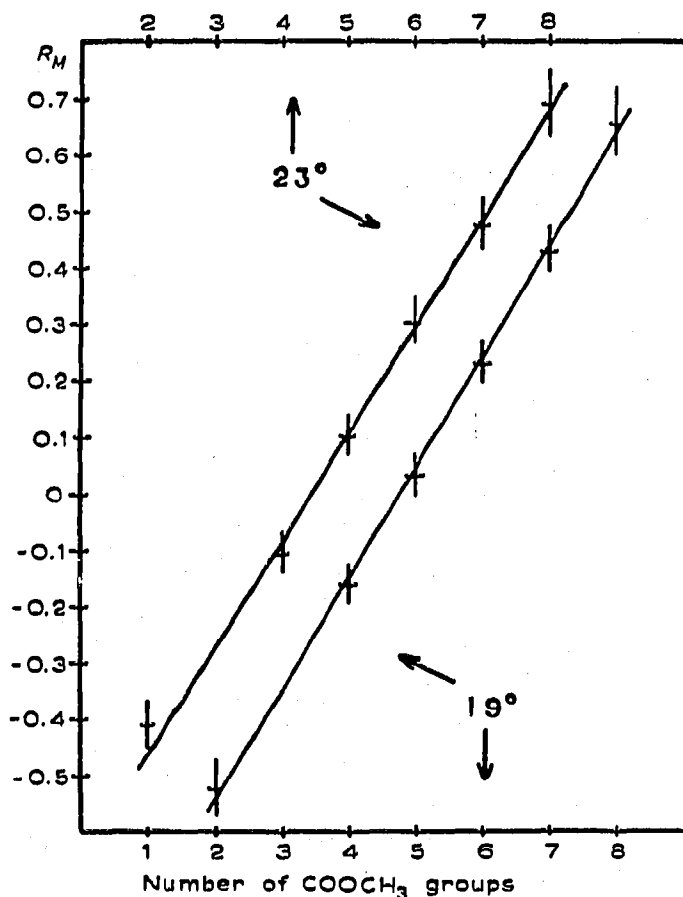

Fig. 2. Linear relation between R_M and the number of ester groups of porphyrins at room temperatures.

Highly fluorescent porphyrins such as copro- and uroporphyrins can be detected down to 0.002 μg under U.V. light, while weakly fluorescent or non-fluorescent ones such as zinc or copper complexes of uroporphyrin are visible at about 0.1 μg. For a ready identification, 0.2–0.5 μg is usually an adequate amount for the DC system and 0.2–0.3 μg for the KPM and WAD systems.

The "Chromagram" as a whole is quite uniformly coated. Only a few sheets in our first box showed some visible "grains" of coating. It is rigid enough to withstand the entire chromatographic process. "Chromagram" is attacked by dilute acids or alkalis, but is essentially unaffected by most other solvents.

SUMMARY

Three thin-layer chromatographic systems using Eastman Kodak "Chromagram" sheets are described. The decane–chloroform system is applicable to methyl esters of porphyrins, chlorins, chlorophylls and related compounds. Other systems

with kerosene–2,4-pentanedione–methyl benzoate and water–acetonitrile–p-dioxan are applicable to dicarboxylic porphyrins.

A linear relationship between the R_M values and the number of carboxyl groups of porphyrins is disclosed and discussed.

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