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SEPARATION AND SPECTRODENSITOMETRIC QUANTITATION OF POR-PHYRIN ESTERS ON THIN-LAYER CHROMATOGRAMS

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SUMMARY

Porphyrin esters separated on silica gel thin-layer chromatography have been quantitated spectrodensitometrically directly on thin-layer plates. The method can be applied to quantitation of porphyrins in natural samples.

INTRODUCTION

Naturally occurring porphyrins in human excreta, bile and tissues, have been determined by solvent fractionation methods [1–5], followed by spectrophotometry[1,3,4] or fluorimetry [1–3]. These methods are time-consuming, and are imprecise in separation of various porphyrins. The thin-layer chromatographic (TLC) methods permit accurate separation depending on carboxyl number[5–13]. Each porphyrin thus separated can be eluted and determined in solution, but this is again time-consuming, and attended by significant and unavoidable losses [13–16]. Preliminary studies have been described for a direct determination of the separated porphyrins on the TLC plates[17–20], as described for bile pigments[21].

Recently, chromatographic methods have been employed extensively for separation of porphyrins. Intermediate porphyrins discovered by the use of TLC techniques have proven of value in clinical diagnosis, *e.g.* the "isocopro" series and seven carboxylic porphyrins[10,22]. Several investigators have used the so-called "profile" or "constellation" of porphyrin excretion for diagnosis of porphyria[23,24]. In the present paper, a simple direct method of TLC–spectrodensitometry (TLC–SD) is described; an application of this method of TLC–SD to the porphyrins of urine, feces and tissues is discussed.

EXPERIMENTAL

Materials

Silica gel G was obtained from E. Merck (Darmstadt, G.F.R.) for preparation

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of TLC plates[9]. Glass plates, 20 × 20 cm, were purchased from Brinkmann (West-bury, N.Y., U.S.A.). Care must be taken to keep the plates free of scratches. Also, ready-made silica gel plates (LQ25, Quantum, Fairfield, N.J., U.S.A.) were used in order to simplify the spotting technique.

. Chloroform, methanol, ethyl acetate, benzene, ethanol, sulfuric acid and ammonia were all analytical grade reagents; water was deionized and distilled. Spectrodensitometry was carried out with the Schoeffel double-beam unit[25] equipped with a monochromator and a disc integrator.

Protoporphyrin IX was prepared according to Grinstein[26]. Porphyrin esters with three to eight carboxyl groups were isolated from urine of porphyric bovines as described previously[9]. In addition, synthetic type I coproporphyrin and uroporphyrin methyl esters were used. Isocoproporphyrin, hydroxyisocoproporphyrin and de-ethylisocoproporphyrin were kindly provided by Prof. Elder (University of Wales, Cardiff, Wales).

Methods

The silica gel plates (0.25 mm thick layer) were scored into bands 10 mm in width, with the Schoeffel scoring device[25]; the commercially (Quantum) available scored plates were also used. Every second band was spotted, the unspotted band serving as a reference.

The previously described chromatography solvent systems were used[8,11], with great care being taken to achieve reproducibility of R_F values. This was best effected with the system benzene–ethyl acetate–methanol (85:13:2, v/v/v) with short develop-ment time (ca. 20 min) and 100 mm distance from origin to front. Benzene and meth-anol could be substituted by toluene and ethanol[11]; the plates were then developed with toluene–ethyl acetate–ethanol (80:15:3, v/v/v)[11]. The silica gel plates were acti-vated and stored in an air-conditioned laboratory at 21 ± 1°, relative humidity 43–61 %.

Special care was taken in spotting with a syringe-type transfer micro-pipet, and in developing the plates. Aproximately 0.3 μl were delivered at a time, followed by drying with a stream of nitrogen. Spot sizes were controlled, and did not exceed 30 mm². Spot area measurements were made with the Desaga–Brinkmann standard labelling template.

The absorption spectra and $\lambda_{max.}$ of the various porphyrins were determined by repeated optical density measurements of the same spot on the TLC plate by manu-al change of wavelength at 10 nm intervals (see Table I and Fig. 1). The porphyrin ab-sorption spectra and their maxima on silica gel G TLC plates differ from those in solution. From the determined $\lambda_{max.}$, the wavelength closest to $\lambda_{max.}$ was selected for all of the porphyrins from two (proto-) to eight (uro-) carboxyls, and that wavelength, 390 nm (Table I), was then used in the preparation of the standard curves. Preceding each series of measurements, the instrument was checked with a set of five red filters, as previously described[21]. Coefficients of variation were determined[21,27] for the filter combination, and thereafter employed.

Before standard curves could be prepared, it was necessary to determine the error connected with spotting and chromatography. This was achieved by repeated spotting of 3 μl of the same solutions of mixtures of uro-, copro- and protoporphyrin, followed by TLC-SD. When integrator readings varied no more than ±5%, the method was assumed to be acceptable for standard curve preparation. Background

TABLE I

ABSORPTION MAXIMA FOR METHYL ESTERS

Number of carboxyls	Absorption maxima for methyl esters	$R_F \times 100$ values	
		Solvent 1[*]	Solvent 2[**]
8	400 nm	20	12
7	395 nm	29	19
6	390 nm	36	27
5	390 nm	46	35
4	390 nm	55	43
3	390 nm	67	50
2	390 nm	79	62

   [*] Solvent 1: benzene–ethyl acetate–methanol (85:13:2, v/v/v)[8].
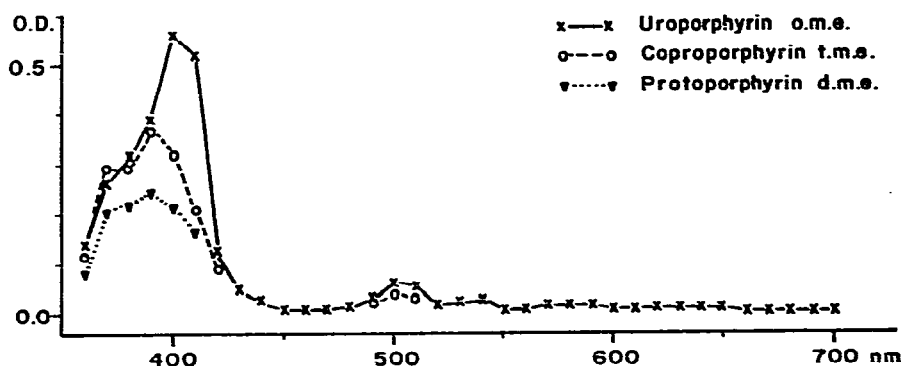   [**] Solvent 2: toluene–ethyl acetate–ethanol (80:15:3, v/v/v)[11].

Fig. 1. Absorption spectra of three porphyrin methyl esters on silica gel G after development with toluene–ethyl acetate–ethanol (85:15:3, v/v/v)[11]. Absorbance was measured at 10-nm intervals from 360 to 700 nm with a Schoeffel spectrodensitometer[25]. o.m.e. = octamethyl ester; t.m.e. = tetramethyl ester; d.m.e. = dimethyl ester.

scintillations were measured by running a clear TLC plate in the spectrodensitometer, thus establishing control values for silica gel G[21].

The general method for preparation of standard curves was as follows. The solutions of known concentrations were spotted on a plate with several spots containing various amounts, ranging from 0.01 to 2.0 μg. The plates were developed and the optical density was recorded in the spectrodensitometer at 390 nm with peaks integrated; a curve was prepared with the amount of porphyrin on the abscissa and the integrator readings on the ordinate. These curves permitted definition of the upper and lower limits of accuracy of densitometry (maximal sensitivity within the area in which Lambert–Beer law was obeyed) (see Fig. 2 and Table II). For comparison, two standard curves were recorded for uroporphyrin at 390 nm and 400 nm (the λ_{max} of uroporphyrin on silica gel G (Fig. 3).

Several samples of recrystallized copro-, proto- and uroporphyrin methyl esters, dissolved in chloroform, were spotted on silica gel G plates. The chromatograms were developed and run in the Schoeffel spectrodensitometer. The values fell
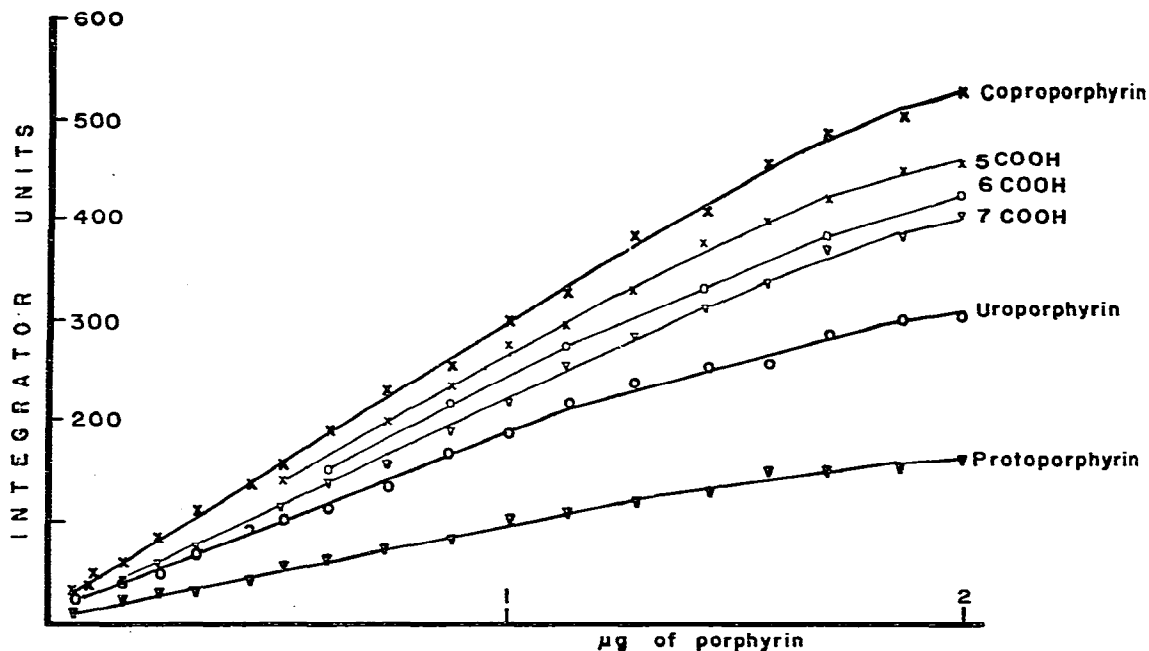
Fig. 2. Calibration curves for seven porphyrin methyl esters at 390 nm on silica gel G developed with benzene–ethyl acetate–methanol (85:13:2, v/v/v)[8].

TABLE II

STANDARD ERRORS AND RANGES FOR TLC–SD QUANTITATION OF PORPHYRIN ESTERS DEVELOPED WITH SOLVENT 1 (SEE TABLE I)

Number of carboxyls	Standard error of estimate (%)	Range (µg)	Ratio integrat. counts 1 µg of porphyrin
8	±7%	0.04–1.0	185
7	±8%	0.04–1.1	220
6	±7%	0.05–1.0	245
5	±5%	0.03–1.1	265
4	±6%	0.01–1.1	295
3	±7%	0.01–1.1	195
2	±10%	0.1 –1.3	90

within the ranges given in Table II. The upper limit of porphyrin esters applied was 2 µg, established in the same way as for bile pigments[21].

These methods have been applied to samples of normal and porphyric urine and faeces. The preparation of the samples for analysis is outlined in Fig. 4.

RESULTS AND DISCUSSION

The prepared calibration curves (Fig. 2) have been used for quantitation of various porphyrin mixtures. However, it would be impractical to vary the wavelength used in determining the components of a mixture of porphyrins. As seen in Table I,
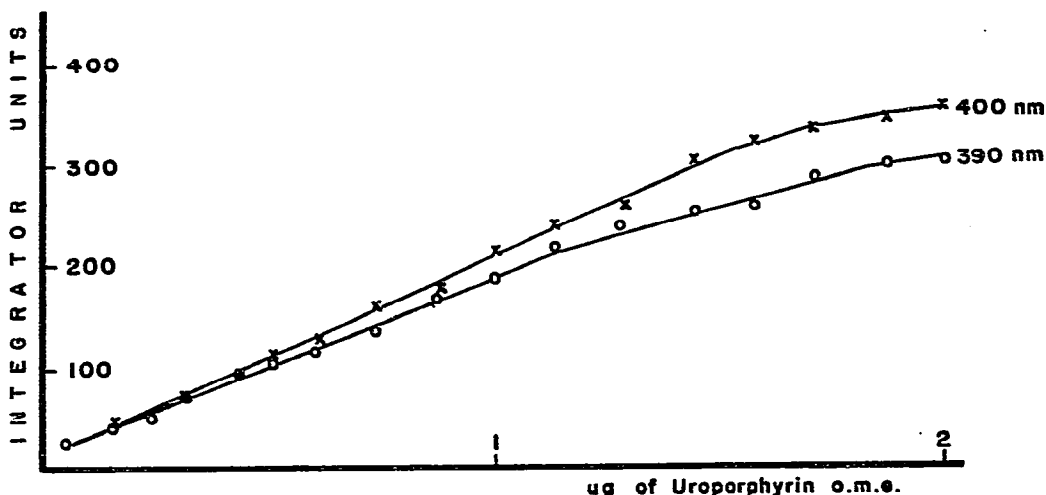
Fig. 3. Calibration curves for uroporphyrin I octamethyl ester (o.m.e.) at 390 and 400 nm on silica gel G after development with benzene–ethyl acetate–methanol (85:13:2, v/v/v)[8].
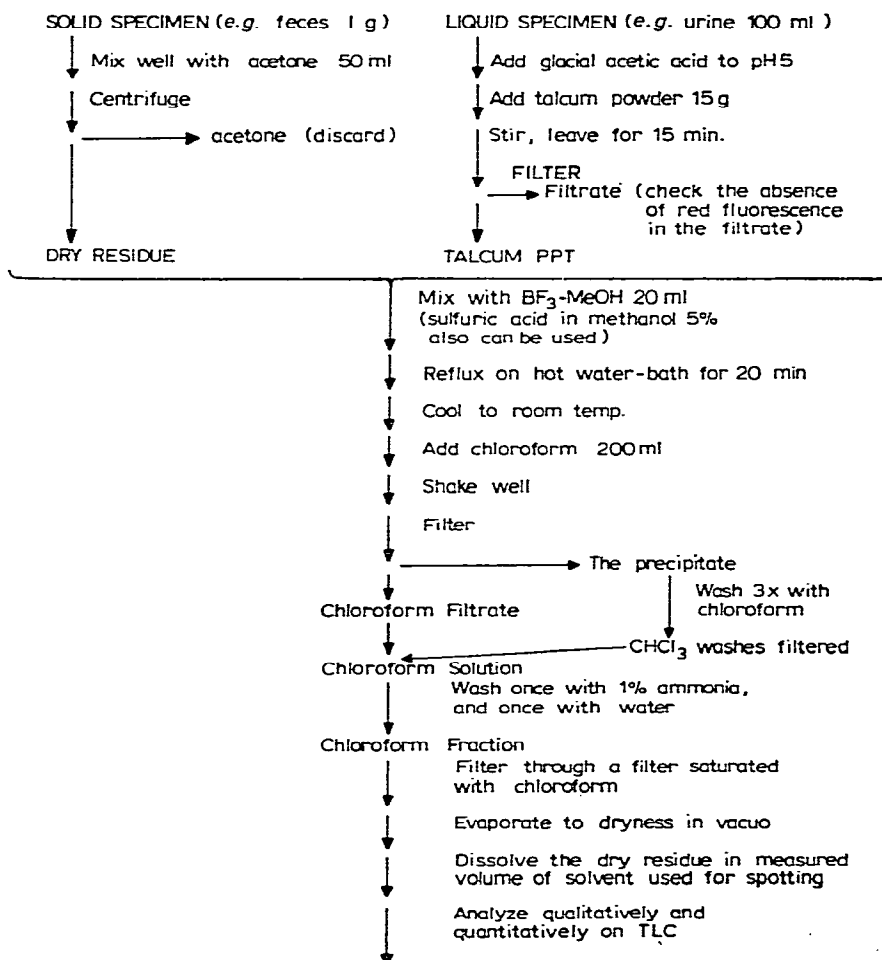
SOLID SPECIMEN (*e.g.* feces 1 g)

Mix well with acetone 50 ml

Centrifuge

→ acetone (discard)

DRY RESIDUE

LIQUID SPECIMEN (*e.g.* urine 100 ml)

Add glacial acetic acid to pH 5

Add talcum powder 15 g

Stir, leave for 15 min.

FILTER
→ Filtrate (check the absence of red fluorescence in the filtrate)

TALCUM PPT

Mix with BF$_3$-MeOH 20 ml
(sulfuric acid in methanol 5% also can be used)

Reflux on hot water-bath for 20 min

Cool to room temp.

Add chloroform 200 ml

Shake well

Filter

→ The precipitate
Wash 3x with chloroform

Chloroform Filtrate

←CHCl$_3$ washes filtered

Chloroform Solution
Wash once with 1% ammonia, and once with water

Chloroform Fraction
Filter through a filter saturated with chloroform

Evaporate to dryness in vacuo

Dissolve the dry residue in measured volume of solvent used for spotting

Analyze qualitatively and quantitatively on TLC

Fig. 4. Scheme for the isolation of porphyrins from natural samples.

the λ_{max} of the porphyrins with from two to eight carboxyls on silica gel G ranges from 390 to 400 nm; however, the 390 nm wavelength has been used for all porphyrins, since the loss in sensitivity is negligible (Fig. 3). The absorption spectra in Fig. 1 are characteristic of dry porphyrin esters dispersed on silica gel G, and they do not depend on the developing solvent[13,28]. Similar observations have been made for fluorescence spectra of porphyrins in solution and on solid supports[28].

Integrator readings in micrograms of porphyrin as used for the standard curves, are different for each porphyrin. This is due to differences in the molar extinction coefficient, ε, and to the different R_F values of each porphyrin. Although the differences in molar extinction coefficient are small for porphyrins with from four to eight carboxyls, when plotted with micrograms against scanned area, varying molecular weights account, in part, for the differences in integrator ratio (Table II). A change from a curve relating micrograms to peak area with a separate curve for each porphyrin to a curve relating micromoles to area results in convergence of the curves for porphyrins with three to eight carboxyls.

With the greater ratio (Table II) and greater R_F values (Table I), sensitivity of the densitometric method increases. The standard curves can be extrapolated to zero; the lowest detectable amount is considered to be an integrator value corresponding to twice the standard error of the estimate or twice zero line variation of integrator reading, whichever is greater.

Although the other absorption bands (Fig. 1) could also be used for this purpose, they are less intense than the 390 nm band, and the sensitivity diminishes at least ten-fold.

New porphyrins are frequently found in natural samples [22], and new chromatographic systems are developed[22,23] for their separation. The method described above is applicable for quantitation of any porphyrin on a great variety of TLC systems. It requires preparation of a standard curve for a new system. For many porphyrins, the differences between standard curves will be negligible; for others, a specific curve has to be prepared.

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