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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY OF PORPHYRINS BY *IN SITU* FLUORESCENCE MEASUREMENTS

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SUMMARY

After separating porphyrins as their methyl esters by silica gel thin-layer chromatography, in the analytical range, a complementary fluorometric method was developed for quantitative *in situ* fluorometry of porphyrin thin-layer chromatograms. Commercial silica gel layers with an aluminium foil base are used. The fluorescence intensity of the porphyrin zones in the chromatogram was continuously registered on a Camag-Turner TLC-Scanner (primary filter Kodak-Wratten 47-B, secondary filter Kodak-Wratten 25; photomultiplier HTV-136). Using subsequent spectrophotometric analyses of the same material, factors were calculated for the quantitative evaluation of the fluorescence peaks of two or more porphyrins in each chromatogram, thus permitting the determination of the percentage distribution of the porphyrins in the separated mixture. The sensitivity of the method is such that amounts of less than 1 ng of porphyrin methyl ester/mm² of silica gel surface (layer thickness 0.2 mm) can be detected. The reproducibility depends mainly on the use of the same silica gel material in each analytical series, the quality of the chromatographic separation, the careful adjustment of the primary light, and the complete evaporation of the solvents from the layer. Under identical conditions the coefficient of variability ranges from 1 to 5% in groups of five similar chromatograms. The method of fluorescence scanning of porphyrins in thin-layer chromatograms is best suited for serial determinations, and has found application in the clinical chemistry of hepatic porphyrias and in studies on microbial tetrapyrrol biosynthesis.

INTRODUCTION

As a supplement to the separation of analytical amounts of porphyrins as methyl esters by thin-layer chromatography (TLC), we have tried to develop a method which would permit the rapid estimation of the porphyrin content of the individual spots. The procedure is mainly intended to provide rapid information concerning the relative distribution of the components of a porphyrin mixture, comparable to the evaluation of electrophoretically separated proteins. By taking advantage of the natural fluorescence of the porphyrins and by using a commercially available fluorometer for scanning the thin-layer chromatograms, the following requirements were

fulfilled: high sensitivity (provided by filters), detection of all biologically occurring porphyrins with eight to two carboxylic acid side chains with the same combination of filters, ease of application, reliability, and the possibility of calculating the actual amounts on the basis of spectrophotometric analyses using known amounts of the porphyrins and conversion factors relating the fluorescence measurements. It was found that such a method can be very useful in the biochemical analysis of porphyrins[1].

METHODS

Fluorescence scanning equipment

Porphyrins in silica gel thin-layer chromatograms were analysed on a Camag-Turner TLC-Scanner (Camag, Muttenz, Switzerland). The Turner filter fluorometer was equipped with the red sensitive photomultiplier HTV-R136. A Hitachi recorder QPD-33, 10 mV, also obtained from Camag, was connected to the Camag-Turner TLC-Scanner.

Fluorescence scanning conditions

The light source consisted of a low-pressure mercury arc lamp, Turner 110-853. A Kodak Wratten 47-B filter (Turner 110-813) was used as the primary filter and a Kodak Wratten 25 filter (Turner 110-820) as the secondary. The setting of the diaphragm aperture at the primary filter was usually 30 ×. The width of the excitation slit on the scanner scale was usually between 1.6 and 2.4. The scanner velocity was 2 or 1 cm/min. Scale expansion was effected by insertion of neutral density filters (Wratten 96, Turner 110-823).

Thin-layer chromatography of porphyrin methyl esters

TLC is carried out, as described elsewhere[2], on silica gel coated aluminium sheets having a layer thickness of 0.2 mm, purchased from Riedel-de Haën (BRD-3016 Seelze-Hannover) or Schleicher and Schüll (BRD-3354 Dassel/Kreis Einbeck). Absorption measurements on the porphyrin methyl esters used as reference substances were performed in chloroform solution[2]. The porphyrins eluted from the silica gel film of the chromatograms were also dissolved in chloroform for measurement, but in some cases they were transformed into their zinc chelates[3].

RESULTS AND DISCUSSION

The spectrophotometric properties of the porphyrin methyl esters are summarised in Table I. Since the excitation and emission maxima of the porphyrin methyl esters analysed in chloroform solution are separated by 200 nm, the filter fluorometer can provide suitable conditions for measurement. The excitation maxima of the porphyrin esters lie directly within the maximum transmission range of the Kodak-Wratten 47-B primary filter[1]. The properties of this filter and differences in molar fluorescence combine to produce different intensities in the fluorescence emission of each individual porphyrin. On the basis of the ratios of spectrophotometric to fluorometric data, correction factors were calculated, from which the values of the fluorescence measurements for each porphyrin in the separated mixture can be converted to correspond to the values obtained by spectrophotometric analysis[1]. The factors

TABLE I

RELEVANT SPECTRAL PROPERTIES OF PORPHYRIN METHYL ESTERS IN CHLOROFORM

Porphyrin	*Absorption Soret (nm)*	*Fluorescence excitation (nm)*	*Fluorescence emission (nm)*
Proto	408	410	632
Copro	400	401	623
Uro	405	405	627

vary according to the number of components in the porphyrin mixture. Under the conditions used here for fluorescence scanning, the factors for proto- and coproporphyrin methyl esters are less than 1, whereas that of uroporphyrin methyl ester is consistently greater than 1. As an example, for a porphyrin mixture consisting of six components separated in a silica gel chromatogram the following factors should be used: for protoporphyrin 0.5, coproporphyrin 0.7, pentacarboxylic porphyrin 1.2, and for hexa- and heptacarboxylic porphyrins and uroporphyrin 1.3 (ref. 1). The coefficients of variability, expressed as the standard deviations of five chromatograms, were found to be between 1 and 6% (ref. 1). Data from an experiment run parallel to one of these analyses, but conducted at another time and by another person from our laboratory for the purpose of testing the reproducibility, are listed in Table II. The registered peak area was taken as the measure of fluorescence of the porphyrin methyl esters. As shown in Fig. 1, a linear relationship over a wide range was found between peak area and concentration of uro-, copro-, and protoporphyrin. Quenching of fluorescence occurred in the presence of uroporphyrin when the amount exceeded 1 μg per cm^2 of silica gel film with a thickness of 0.20–0.25 mm.

TABLE II

DATA SHOWING THE REPRODUCIBILITY OF ANALYSES OF THIN-LAYER CHROMATOGRAMS OF PORPHYRINS BY FLUORESCENCE SCANNING

Porphyrin	*Mean (% of total porphyrins)*	*Standard deviation of individual determinations*	*Standard deviation of the mean*	*Coefficient of variability*	*Number of measurements*
Proto	29.4	0.56	0.16	1.1	12
Copro	21.0	0.43	0.12	2.0	12
Uro	49.7	0.69	0.20	1.4	12

In order to obtain good reproducibility in a series of analyses (such as in Table II), as well as in estimations from one day to another, the fluorometer must be warmed up for at least 2 h before scanning commences (Fig. 2). Although no differences in registration of the relative amounts of the porphyrins in any individual chromatograms were found when scanning was performed after a shorter warmup period, the base line and the peak area decreased steadily; this had to be compensated for later. Errors traceable to the apparatus were greatest in the first hour after it was turned on (coefficient of variability $>5\%$). Another source of error is the persistence of solvent in the silica gel layer after development; this causes the porphyrins to fluoresce more

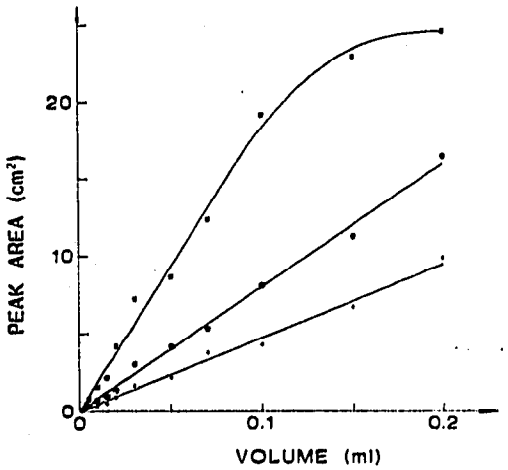

Fig. 1. Relationship between peak area and porphyrin concentration in a chromatogram. Uro-, copro-, and protoporphyrin methyl ester in chloroform solution were applied to a silica gel card by means of Camag self-filling micropipettes and separated in a benzene–ethyl acetate–methanol (85:13.5:1.5) solvent system. ■, Uro (10.1 nmoles/ml); ●, copro (6.1 nmoles/ml); ▲, proto (9.1 nmoles/ml). Scanning conditions: diaphragm aperture, 30×; excitation slit width, 5 (scanner scale); velocity, 2 cm/min.

intensely, as shown in Fig. 3. The scale for moisture in the layer is purely arbitrary and therefore without dimensions, as direct measurement was not possible; from the graph it looks as if the solvent moisture content of the silica gel layer is linearly proportional to the intensification of porphyrin fluorescence. This increase in porphyrin fluorescence due to solvent is inversely related to the number of methyl ester groups in the porphyrin molecule. Drying for 1 h in air at room temperature or for 15–20 min in a cold air current, freed the chromatograms of solvent to the extent that enhancement of fluorescence could no longer be observed. The section of the porphyrin band on the thin layer used for measurement is immaterial so long as only the relative amounts of the components of a porphyrin mixture are of interest. As Fig. 4 shows for a chromatogram with three porphyrins, scans made from the middle to the edge of the bands produced identical results concerning the relative porphyrin concentra-

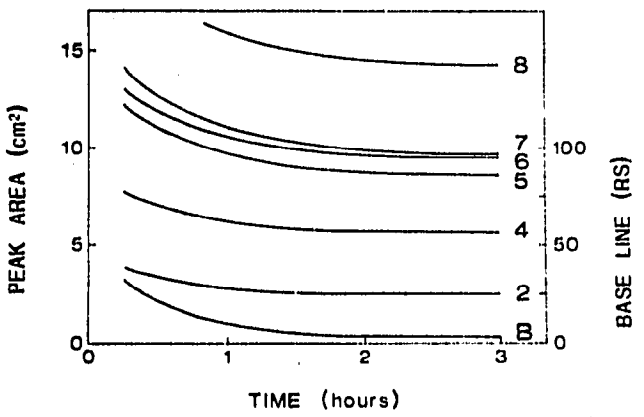

Fig. 2. Decrease in the peak areas of the prophyrins scanned (8–2 refer to the number of carboxyl groups in each molecule) and of the base line (B) after the fluorometer had warmed up. RS = recorder scale. Coefficients of variability of the percentage of each porphyrin in the mixture when scanning the whole chromatograms at six different times between 0 and 3 h: 8, 1.8%; 7, 4.6%; 6, 5.7%; 5, 3.7%; 4, 4.0%; 2, 6.7%.

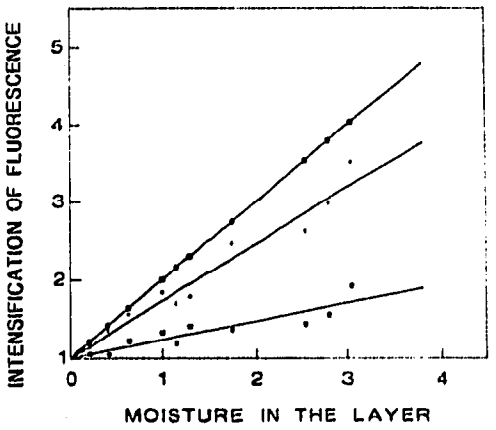

Fig. 3. Relationship between the output fluorescence of uro- (■), copro- (▲), and protoporphyrin (●) and the moisture in the layer due to the benzene–ethyl acetate–methanol solvent after running the chromatogram.

tions. The decrease in fluorescence is due to the fact that the aliquots were applied by hand to the chromatograms with fine, hand-drawn glass capillaries; the middle of the application zone received a greater amount of material than did the edges, for finger pressure on the rubber bulb of the capillary automatically increases toward the middle of the band.

The amount of fluorescent light reaching the photomultiplier can be regulated by altering the size of the aperture (Fig. 5), as well as with neutral density filters

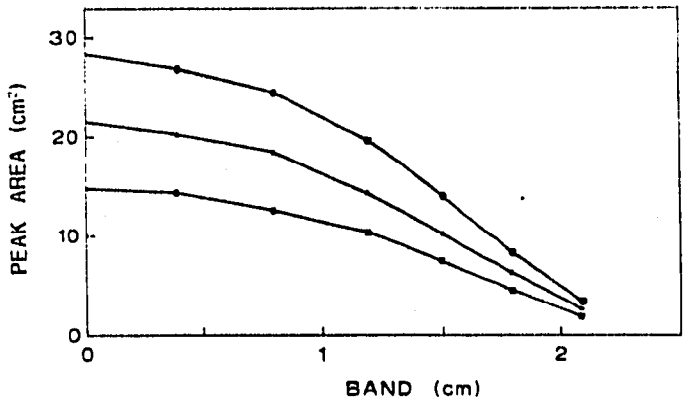

Fig. 4. Analyses of bands of uro- (■), copro- (▲), and protoporphyrin (●) from the middle (0) to the edge (2 cm) of the bands. A mixture of reference compounds was applied to a silica gel card in a band 4 cm wide, and separated as described in the legend to Fig. 1.

(Fig. 6) which can be used to provide optimal resolution of the bands and fullest utilisation of the recorder scale. All scans given in Figs. 7–9 were made with the aperture at the primary filter diaphragm completely open, whereas the width of the excitation slit and velocity of the scanner were varied according to the concentration of the porphyrins in the chromatogram, the shape of the bands or spots, the nature of the separation, the number of components to be registered, and the R_F range of the entire chromatogram, as explained in the legends. For microchromatograms with less than 10 ng of total porphyrins and for bands which occur close together a relatively

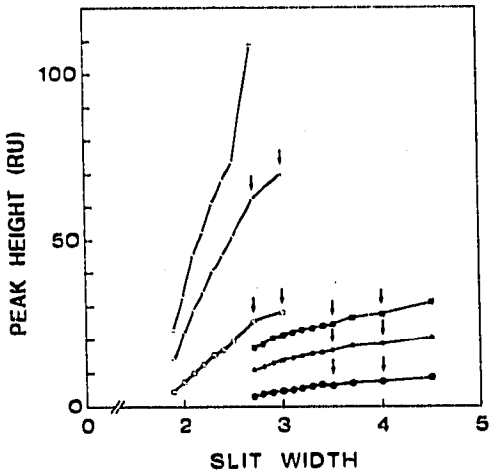

Fig. 5. Dependence of peak height on the width to the excitation slit, with the diaphragm aperture set at 10× (black symbols) and at 30× (open symbols). ■—□, Uro-; ▲—△, copro-; ●—○, protoporphyrin. The synchronised, simultaneous deviations of the curves from a straight line (section between the arrows) are due to the fact that the individual steps of the slit opening cannot be adjusted exactly.

large excitation slit width and low speed were preferable[1]. The same was found to apply in the case of isomer analyses (Fig. 8) and determinations of porphyrins of microbes in the nanogram range (Fig. 9). On the other hand, for the direct fluorometric measurement of larger porphyrin concentration on the chromatograms, neutral density filters can be used to expand the scale. Their effect on the fluorometric measurements is readily apparent and results from the decreased intrinsic fluorescence

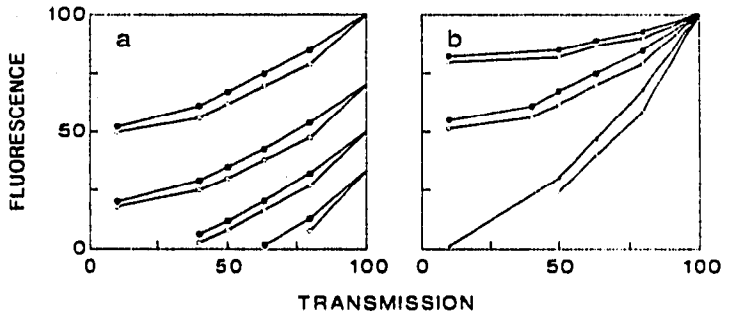

Fig. 6. Effect of neutral density filters (transmission "80", "63", "50", "40", and "10"%) on the fluorescence of a blank silica gel card measured at a constant excitation slit width in a and at various slit widths in b (■—□, 2.0; ●—○, 2.4; ▲—△, 3.0). Neutral density filters were inserted in addition to the primary (open symbols) and to the secondary filter (black symbols).

of the silica gel layer. Fig. 6a shows the reduction in fluorescence measured and which is caused by neutral density filters of varying strength used with a constant width of the excitation slit in the scanner window. The effect can easily be reproduced by regulation of the fluorescence transmission from the zero mark on the fluorometer to various levels on the scale without the gray filter (T = 100). On the other hand, increasing the width of the slit augmented the decrease in fluorescence, and with it the scale expansion, whereby the relative reduction of fluorescence remained constant in relation to the different optical densities of the neutral density filters (Fig. 6b).

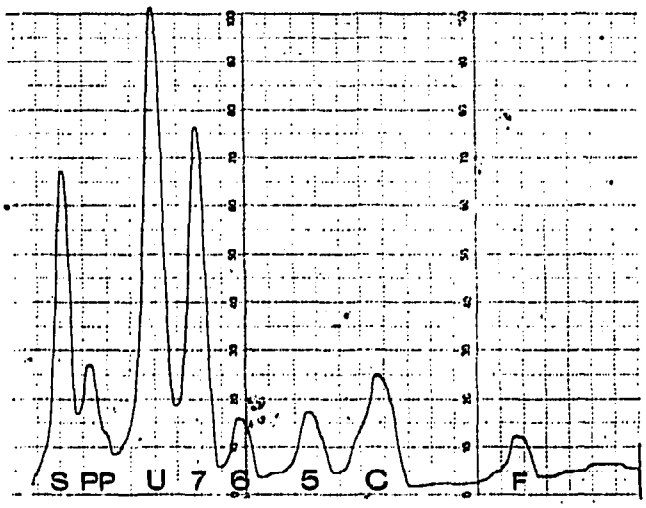

Fig. 7. Fluorescence record of a silica gel chromatogram of urinary porphyrins from a patient with porphyria cutanea tarda. U = Uroporphyrin; C = coproporphyrin; 7, 6, and 5 = hepta-, hexa-, and pentacarboxylic porphyrins. PP are red-fluorescent substances below U in the chromatogram; they probably belong to a new group of porphyrin peptides[6]. S represents the starting zone (orange fluorescence), and F the front zone (blue fluorescence) of the chromatogram. The excitation slit width is 2 and the scanner velocity 2 cm/min.

Fig. 6 also indicates that in the arrangement used here for determination of porphyrins the effect was the same regardless of whether the neutral density filters are inserted in front of the primary or the secondary filters. Factors corresponding to the various neutral density filters for the conversion of the measured peak area to that which would be obtained without use of a filter facilitate evaluation of porphyrin chromatograms in which one or more bands are recorded using the same or various neutral density filters[1].

This fluorescence scanning method has mainly been used in our laboratory for the determination of the relative distribution of porphyrins in serialised comparative studies. The excretion patterns of urinary porphyrins in human porphyrias[4], of which an example is shown in Fig. 7, as well as the porphyrin composition of bacteria[5] (Fig. 9), can be elegantly and comprehensively demonstrated by this method. The

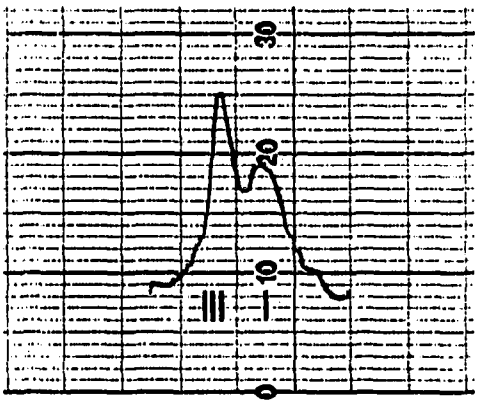

Fig. 8. Fluorometric scan of coproporphyrin isomers III and I, separated on a precoated silica gel plate from a urine sample of a 30-year-old man who had undergone hepatectomy and subsequent homologous liver transplantation six months previously[8]. The excitation slit width is 2.8 and the velocity of the scanner 1 cm/min.

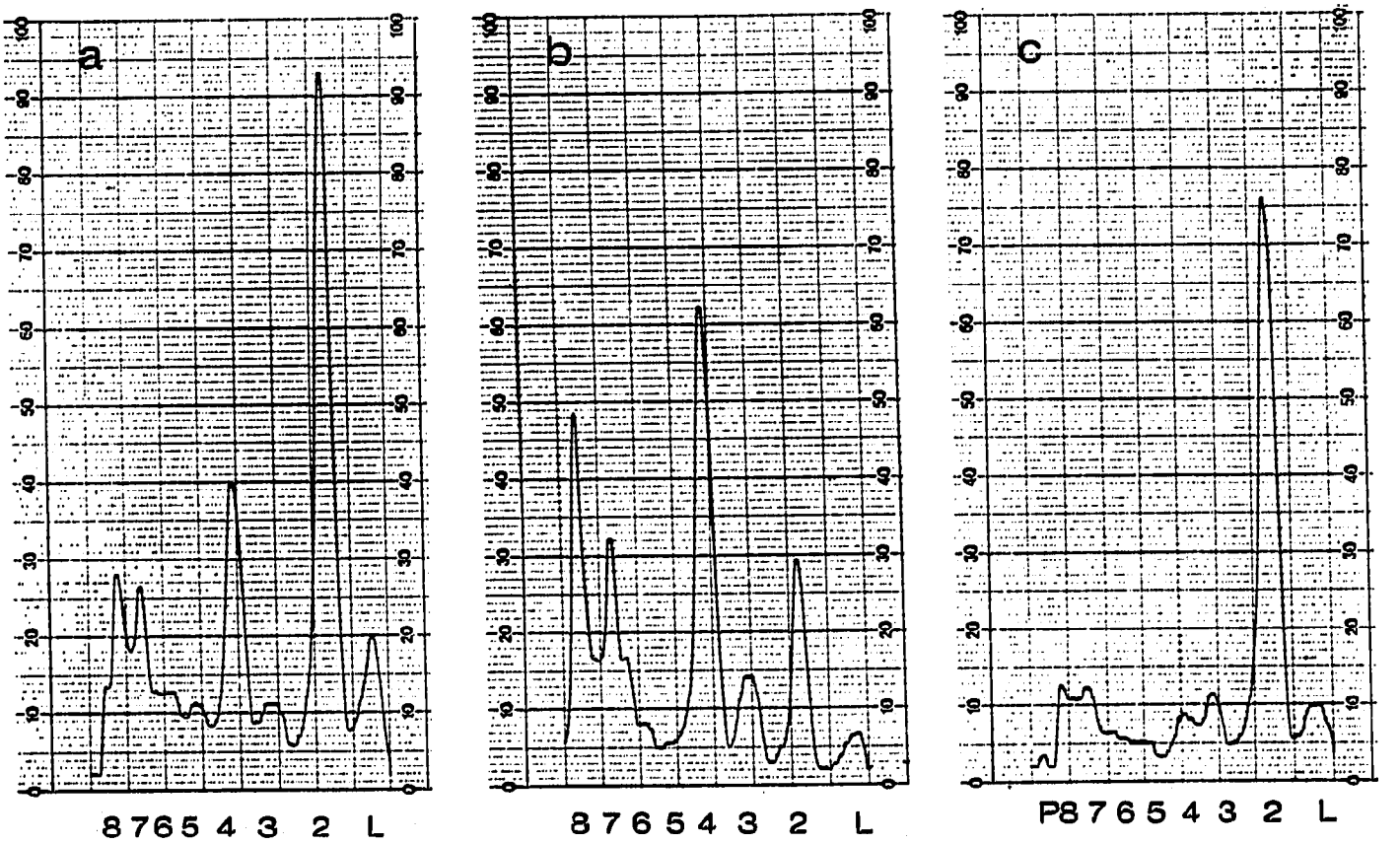

Fig. 9. Fluorescence scanning charts with porphyrin patterns of *Escherichia coli* (a), *Pseudomonas aeruginosa* (b), and *Achromobacter metalcaligenes* (c). The scans represent the porphyrin composition of cells grown on lactate from endogenous δ-aminolaevulinic acid. The numbers 8–2 indicate the number of carboxylic acid side chains in each porphyrin, in the order of the biosynthetic sequence; P is so-called porphyrin 650. L represents the lipid zone at the front of the chromatogram. The excitation slit width is 3.0 and the velocity of the scanner 1 cm/min.

fluorometric method is particularly suitable for estimations of coproporphyrin isomers I and III after their separation as free acids in a silica gel layer[7] (Fig. 8), if total urinary coproporphyrin is determined spectrophotometrically in a parallel assay. Under the conditions described no differences were observed between the fluorescence emission of the two isomers of coproporphyrin.

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