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Note

Determination of porphyrins in tissue: pre-adsorption followed by high-performance liquid chromatography

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In recent years, high-performance liquid chromatography (HPLC) has largely replaced solvent extraction methods for the determination of porphyrins in biological materials. HPLC is most convenient not only for the quantification of total porphyrins, but also for the isolation and quantification of 2- to 8-COOH porphyrins and their isomers [1–7].

Efficient chromatography of porphyrins is commonly performed with porphyrin methyl esters because separation is possible in the isocratic mode. Esterification is generally carried out in methanol with an acid catalyst followed by extraction into chloroform.

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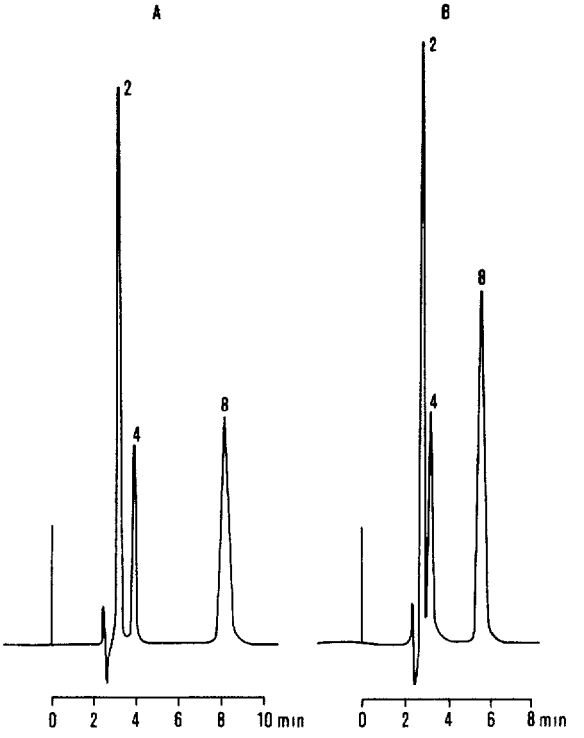

Fig. 1. HPLC separation of a standard mixture of porphyrin methyl esters on CGC glass columns (150×3.3 mm I.D.) packed with (A) Separon SIX NH_2 (5 μm) and (B) LiChrosorb NH_2 (5 μm). Peaks: 2 = protoporphyrin IX dimethyl ester; 4 = coproporphyrin tetramethyl ester; 8 = uroporphyrin octamethyl ester. Mobile phase, *n*-heptane–ethyl acetate–methanol (60 37:3); flow-rate, 1 ml/min; detection, UV absorption at 405 nm.

We have found it convenient to adsorb porphyrin methyl esters from the reaction mixture after esterification on a reversed-phase (ODS) cartridge (PRESEP) where porphyrin esters were quantitatively retained. The porphyrin esters were then eluted from the cartridge with chloroform and evaporated to dryness. The residue was dissolved in a defined volume of chloroform and an aliquot injected into the chromatograph [9].

EXPERIMENTAL

Chemicals

Porphyrin standards were obtained from Sigma (St. Louis, MO, U.S.A.). Ethyl acetate, *n*-heptane, methanol, chloroform and other chemicals were of analytical reagent grade. Water was glass-distilled.

Apparatus and conditions

The self-contained PRESEP cartridge was packed with Separon SIX C_{18} spherical octadecylsilane adsorbent (60–80 μm) (Laboratory Instruments Works,

TABLE I

CAPACITY FACTORS OF PORPHYRIN METHYL ESTERS ON COLUMNS WITH VARIOUS PACKING MATERIALS

Number of carboxyl groups	Capacity factor			
	Separon SIX NH_2	Separon SIX	LiChrosorb NH_2	LiChrosorb Si 60
2	0.36	0.37	0.22	0.20
4	0.72	0.75	0.45	0.50
8	2.60	2.40	1.55	1.75

TABLE II

RECOVERY OF INDIVIDUAL PORPHYRINS FROM PRESEP CARTRIDGES

Amount injected, 0.1–1.0 μg of each substance. C.V. = coefficient of variation ($n=3$), given in parentheses.

Amount retained (μg)	Mean recovery (%)		
	2-COOH	4-COOH	8-COOH
500	98.0 (0.77)	96.6 (0.7)	94.9 (1.3)
50	97.7 (4.5)	97.4 (0.9)	96.2 (0.9)
5	96.3 (2.4)	95.9 (1.4)	95.2 (2.3)

TABLE III

LINEARITY AND PRECISION OF PORPHYRIN DETERMINATION IN TISSUE

Actual concentration (ng/ml)	Measured concentration (mean ± S.D.) (ng/ml)	Coefficient of variation ($n=3$) (%)
Intra-assay		
10	9.8 (0.5)	5.1
54	55 (1.5)	2.7
107	103 (3.1)	3.1
209	201 (5.1)	2.5
391	400 (5.8)	1.5
Overall		2.2
Inter-assay		
10	10.2 (0.4)	3.9
54	53 (2.2)	4.1
107	105 (4.1)	3.9
209	209 (13.0)	6.2
391	399 (8.5)	2.1
Overall		3.6

TABLE IV

MEASUREMENT OF PORPHYRIN CONTENT IN TISSUE HOMOGENATES

Subjects	Tissue	Porphyrin content			
		Total (μg/g of tissue)	Individual homologues (%)		
			2-COOH	4-COOH	Unknown
Mice fed griseofulvin for 72 h*	Harderian glands	562.0 ± 157.4	99.75 ± 0.32	0.23 ± 0.19	0.50 ± 0.26
	Liver	116.4 ± 4.7	99.36 ± 0.57	0.66 ± 0.43	0.60 ± 0.20
	Kidneys	12.7 ± 1.3	95.99 ± 2.19	0.62 ± 0.50	3.75 ± 2.04
			2-COOH	7-COOH	8-COOH
Rats fed hexachorobenzene for 5 months**	Harderian glands	416	97.6	–	–
	Liver	660	–	28.8	70.3
	Kidneys	73	–	12.6	75.8

*Each value is the mean ± S.D. ($n=5$). Significance was calculated according to Student's t-test ($p<0.001$).

**Data are mean values from series determination.

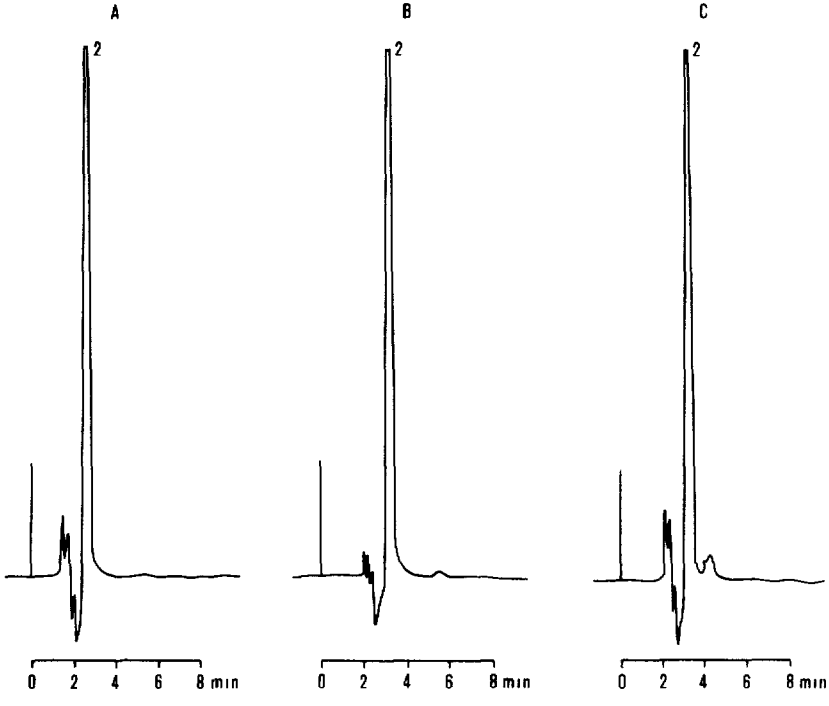

Fig. 2. HPLC separation of tissue homogenates from male mice after feeding for 72 h with griseofulvin [2.5% (w/w) in Larsen mixture]. (A) Harderian glands; (B) liver; (C) kidneys. The main substance in all tissues is protoporphyrin (peak 2), in Harderian glands and liver more than 99% and in kidneys more than 95%. Column: CGC (150 × 3.3 mm I.D.) packed with Separon SIX NH_2 (5 μm). HPLC conditions as in Fig. 1.

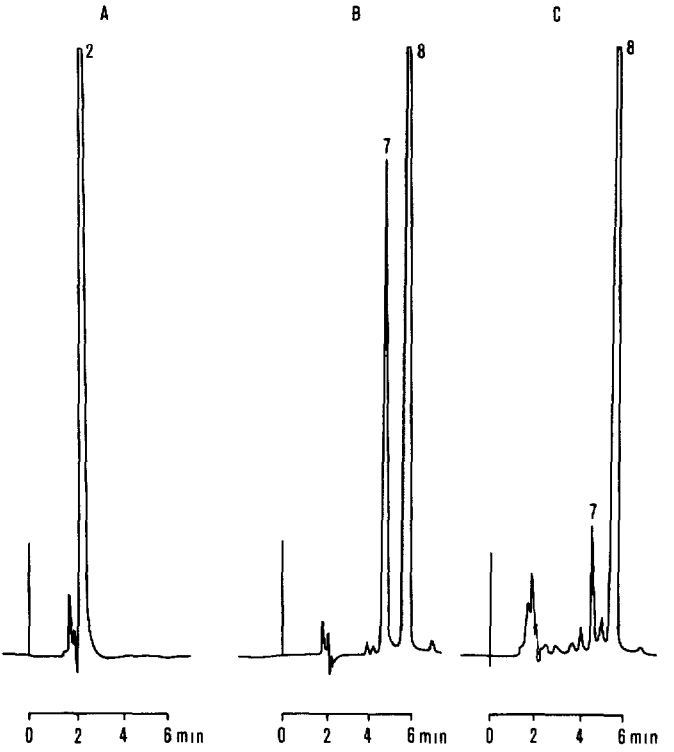

Fig. 3. HPLC separation of tissue homogenates from female rats after feeding for five months with hexachlorobenzene [0.8% (w/w) in Larsen mixture]. (A) Harderian glands [97.6% protoporphyrin of peak 2)]; (B) liver [28.8% 7-COOH porphyrin (peak 7) and 70.3% uroporphyrin (peak 8)]; (C) kidneys [12.6% 7-COOH porphyrin (peak 7) and 75.8% uroporphyrin (peak 8)]. Column: CGC (150×3.3 mm I.D.) packed with Separon SIX (5 μm). HPLC conditions as in Fig. 1. Mobile phase: *n*-heptane–ethyl acetate–methanol (60.34:6).

Prague, Czechoslovakia; distributed by Senetek, Åarhus, Denmark).

Chromatography was carried out on 150 mm × 3.3 mm I.D. glass columns packed with the silica amino bonded phase Separon SIX NH_2 and Separon SIX, particle size 5 μm (Laboratory Instruments Works), and with LiChrosorb NH_2 and LiChrosorb Si 60, particle size 5 μm (Merck, Darmstadt, F.R.G.) [8]. Absorbance was detected with a UV variable-wavelength absorbance detector (Optronica, Frankfurt, F.R.G.) at 405 nm.

The solvent system in the isocratic mode at ambient temperature was *n*-heptane–ethyl acetate–methanol (60:37:3 for amino bonded phase and 60:34:6 for silica, v/v/v) at a flow-rate 1 ml/min.

Sample preparation from tissues

A 100–500 mg amount of wet animal tissues (*e.g.*, liver, Harderian glands, kidneys) was weighed out according to the expected porphyrin concentration and homogenized with a small amount of methanol. To esterify the porphyrins the homogenate samples were placed in a 100-ml erlenmeyer flask containing 45 ml of 10% sulphuric acid in methanol, shaken occasionally and left to stand for 24 h

at 37°C in the dark. After cooling to 20°C the volume was made up to 50 ml and the solution was filtered. The clear filtrate was used for the determination of total porphyrin methyl ester using a spectrofluorimeter (Perkin-Elmer 203) with an excitation wavelength of 407 nm and an emission wavelength of 605 nm.

An aliquot from the supernatant (5–10 ml) was diluted with water (to a 20% methanol solution) and injected with a syringe into the cartridge (PRESEP), prepared by washing with 5 ml of methanol and 5 ml of water. The porphyrin esters were quantitatively retained.

The PRESEP was then washed with 10 ml of water, dried with a stream of nitrogen, eluted with 5 ml of chloroform and the eluate was then evaporated to dryness with nitrogen. The residue was dissolved in a defined volume of chloroform (0.2–1 ml) and injected into the HLC system (injection volume 2–5 μl).

The extent of sorption and desorption on the cartridge was checked on the spectrofluorimeter.

RESULTS

The isocratic elution of porphyrin methyl esters on glass columns packed with Separon SIX NH_2 demonstrated a very good separation efficiency for 2- to 8-COOH porphyrin methyl esters in less than 10 min (Fig. 1). We found that different columns from the same manufacturer provided nearly identical separation. We also found that similar separation results can be achieved with CGC glass columns packed with LiChrosorb NH_2 and with glass columns packed with LiChrosorb Si 60 silica and with Separon SIX silica with a slightly modified mobile phase. The capacity factors (k') are given in Table I. None of the columns was appreciably degraded with time and the resolutions and retentions remained remarkably stable.

Determination of the total porphyrin content was carried out on a spectrofluorimeter with an excitation wavelength of 407 nm and an emission wavelength of 605 nm. A calibration graph used for the quantitation of total porphyrins in tissue homogenates was constructed by plotting relative fluorimetric response against the sample concentration. The graph was linear in the concentration range 10–400 ng/ml. The following regression equation was obtained: $y=2.2967x+0.025$; $r=0.9996$.

Recoveries of porphyrins were determined by comparing the peak areas of 2-, 4- and 8-COOH porphyrin methyl esters before adsorption and after elution on the PRESEP cartridge. The recovery varied between 95 and 98%. (Table II).

The precision data for the method are summarized in Table III. The intra-assay variability of porphyrins was determined by analysing samples of various concentrations in triplicate by the fluorimetric method described under Experimental. The mean coefficient of variation was 2.2%. The inter-assay variability of porphyrins was determined by analysing samples of various concentrations in triplicate within thirty days on three separate days. The mean coefficient of variation was 3.6%.

As an example of the practical use of the described method, the determination of total and individual porphyrins in tissue homogenates from griseofulvin-fed

mice is given. In our experiments (Table IV and Fig. 2) virtually only protoporphyrin was present: in Harderian glands and liver more than 99% and in kidneys more than 95% of total porphyrins.

Another example (Fig. 3) demonstrates typical chromatograms of tissue porphyrins from hexachlorobenzene-fed rats. Protoporphyrin was present only in Harderian glands (97.6%). In the liver and kidneys 7-COOH and 8-COOH porphyrins were present.

The results obtained are in agreement with the general porphyrogenic behaviour of griseolfulvin and hexachlorobenzene.

The quantification of separated 2- to 8-COOH porphyrin homologues can be achieved by expressing it as the percentage of the total content of porphyrins measured with the spectrofluorimeter according to the peak areas obtained by HPLC on the integrator.

CONCLUSION

It is possible to replace the extraction of porphyrin methyl esters after esterification in chloroform by their sorption on an ODS cartridge and elution with a small amount of chloroform. This method is easier, faster, requires less solvent and provides reproducible results. The quantification of total porphyrins can be achieved directly in the reaction mixture after esterification using spectrofluorimetry. Quantification of the porphyrin homologues can be achieved by HPLC.

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