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Note

High-performance liquid chromatographic differentiation of urinary free porphyrins

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Naturally occurring porphyrins in biological material have rather similar physicochemical properties which make their separation difficult.

Several extraction, chromatographic, and fluorometric methods have been presented; however, only a combination of them allows a precise but time-consuming identification of the different carboxylic compounds and of their isomeric forms [1—7].

So we welcome the opportunity to separate and quantitate the different porphyrins in biological materials by high-performance liquid chromatography (HPLC). This technique has become important in recent years and is becoming as widely used for relatively involatile substances as gas chromatography is for volatile ones [8].

A good separation of porphyrin polycarboxylic acids may be obtained by HPLC with a preliminary conversion of free acids into their methyl esters, but this procedure, although simple, requires several hours, it may be incomplete, and it introduces some problems as further reactions occur at vinyl groups, which finally may lead to a loss of material [9—11]. On the other hand, few papers in the literature present HPLC methods available for the separation of porphyrins as free acids in biological material: some deal with porphyrin detection in concentrations far greater than the normal range of urinary excretion [11, 12], others employ time-consuming gradient elution devices coupled with pre-running of sample [13].

We propose here a new simplified HPLC method of separating the free urinary porphyrins, followed by fluorometric detection to optimize their esti-

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mation within the range of normal human excretion. This procedure is then compared with a traditional solvent extraction method in normal subjects and liver patients.

EXPERIMENTAL

Instrumentation

The experiments for the solvent extraction method were performed on a Zeiss PQM II spectrophotometer. HPLC was performed on a Varian Aerograph 8500 high-pressure liquid chromatograph equipped with a Varian fluorimetric detector (Fluorichrom); the excitation filter was a 400-nm interference filter, and the emission filter was a 490-nm cut-off filter; the lamp was in the HI position, with gain × 1, attenuation × 10; a Varian Model A 25 recorder, a stop-flow injector, and a 10-μl S.G.E. syringe were also used.

Solvent extraction procedure

For many years we have commonly detected urinary coproporphyrin and uroporphyrin by the solvent extraction method of Fernandez et al. [3], described in detail elsewhere. The copro- and uroporphyrin HCl eluates were spectrophotometrically quantitated with zero absorbance set with distilled water.

HPLC procedure

The column was a stainless steel (25 × 0.2 cm) RP-18 MCH (Varian, Palo Alto, CA, U.S.A.). As mobile phase 20% acetonitrile (Carlo Erba RPE, Milan, Italy) in a solution of 0.25 *M* methanesulfonic acid (Merck, Darmstadt, G.F.R.) in bidistilled water, was used. The flow-rate was 60 ml/h; and the column temperature 25°C.

Quantitative evaluation was obtained by correlating peak areas, obtained by the height times width at half-height, to known concentrations of relative reference solutions.

Reference standard

We prepared five portions of standard solutions containing uro- and coproporphyrin in 3 *M* hydrochloric acid at a known scalar concentration ranging from 5 to 300 μg/l (see Fig. 3A, B).

Sample preparation

A 5-ml volume of urine, previously treated with 0.1 ml of a solution containing 1 mg/ml chloranil (Carbo Erba RPE) in glacial acetic acid [14], was passed through a column of anion-exchange resin AG 1-X8 (Cl^-) 100—200 mesh, 4 × 0.7 cm (Prefilled Econo-Column™, Bio-Rad Labs, Richmond, CA, U.S.A.); after three washings with 4 ml of distilled water, porphyrins were eluted with two portions of 2 ml of 3 *M* hydrochloric acid (Carlo Erba RPE). (We used a sample/eluate ratio of 5:4 to make up for losses during purification [4].) Then, 10 μl of the eluate were injected into the HPLC column.

RESULTS AND DISCUSSION

As porphyrins in a strongly acidic solution show their maximum relative

quantum yield by fluorescence, we decided to employ a mobile phase of pH near 1, so as to ionize pyrrolic nitrogens, and to use $CH_3SO_3^-$, as a counterion; thus, a sensitivity greater than 5 μg/l for coproporphyrin and 1 μg/l for uroporphyrin can be obtained.

To evaluate column durability in such extreme conditions, we fluxed solvent continuously through it for 48 h and measured its efficiency before and after treatment. Column efficiency did not diminish appreciably.

The results are summarized in Figs. 1—3 and Table I and II.

The uroporphyrin values in both Table I and Table II are indicated as "traces" when the amount ranged between 0 and 0.5 μg/l. In Table II we have reported only the octa-, hepta- and tetracarboxylic porphyrins recorded in the HPLC chromatograms; however, in all the porphyria cutanea tarda patients we

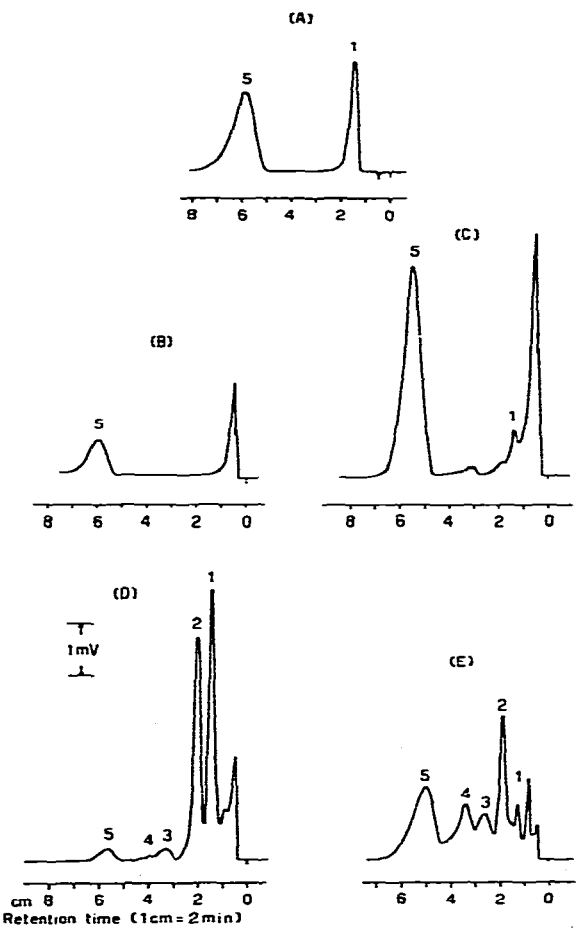

Fig. 1. Examples of porphyrin HPLC chromatograms of (A) standard solution, and the urine of (B) control subjects, (C) liver cirrhosis + cholestasis, (D) porphyria cutanea tarda patient, (E) HCl coproporphyrin extract obtained by solvent extraction method from the same porphyria cutanea tarda patient's urine. 1 = uroporphyrin; 2 = heptacarboxyporphyrin; 3 = hexacarboxyporphyrin; 4 = pentacarboxyporphyrin; 5 = coproporphyrin.

TABLE I

URINARY PORPHYRIN EXCRETION IN CONTROLS, DETECTED BY SOLVENT EXTRACTION AND HPLC

No.	Age	Sex	Coproporphyrin (μg/l)		Uroporphyrin (μg/l)	
			Solvent extraction	HPLC	Solvent extraction	HPLC
1	48	F	68	9	5	Traces
2	42	F	28	20	11	14
3	48	M	52	35	22	5
4	25	M	31	28	3	Traces
5	30	F	38	12	5	Traces
6	55	F	46	25	6	Traces
7	53	M	35	6	13	Traces
8	45	M	71	40	19	Traces
9	50	M	29	25	12	1
10	40	F	79	23	23	Traces
$\bar{x}$	43.6		47.7	22.3	11.9	
S.D.	9.6		18.9	10.9	7.3	
S.E.	3.0		5.9	3.4	2.3	

TABLE II

URINARY PORPHYRIN EXCRETION IN PATIENTS WITH DIFFERENT LIVER DISEASES

CAH = Chronic active hepatitis; LC = liver cirrhosis.

	Diagnosis	Age	Sex	Coproporphyrin (μg/l)		Uroporphyrin (μg/l)		
				Solvent extraction	HPLC	Solvent extraction	HPLC	
							8C*	7C**
1	CAH	17	F	105	25	31	Traces	—
2	CAH	44	M	84	20	7	4	—
3	LC	50	F	86	26	6	3	—
4	LC	46	M	46	17	5	1	—
5	LC	40	F	42	30	6	1	—
6	LC	49	M	87	47	22	18	—
7	CAH + cholestasis	35	M	144	70	17	11	—
8	LC + cholestasis	24	M	230	117	29	Traces	—
9	LC + cholestasis	54	M	429	232	72	26	—
10	LC + cholestasis	42	F	181	90	49	14	—
11	LC + cholestasis	59	M	235	47	29	Traces	—
12	LC + cholestasis	54	M	200	93	30	Traces	—
13	Porphyria cutanea tarda	52	M	130	20	762	316	101
14	Porphyria cutanea tarda	48	M	62	23	828	576	149
15	Porphyria cutanea tarda	50	M	73	15	1025	348	287

*8C = uroporphyrin.
**7C = heptacarboxyporphyrin.

easily recorded minor amounts of hexa- and pentacarboxylic porphyrins as Fig. 1D shows.

Fig. 2 summarizes the statistical analysis of urinary coproporphyrin results obtained with both methods.

The mean (± S.E.) of urinary coproporphyrin in controls is 47.7 (± 5.9) μg/l detected by solvent extraction method, and 22.3 (± 3.4) μg/l detected by HPLC. The corresponding values in patients are as follows: 75.0 (± 10.2) μg/l in chronic liver disease without cholestasis, 236.5 (± 40.8) μg/l in chronic liver disease with cholestasis, and 88.3 (± 28.0) in porphyria cutanea tarda patients detected by the solvent extraction method; and 27.5 (± 4.3) μg/l in chronic liver diseases without cholestasis, 108.1 (± 26.5) μg/l in chronic liver disease with cholestasis, and 19.3 μg/l (± 2.3) in porphyria cutanea tarda detected by the HPLC method.

The analysis of variance (ANOVA) of controls vs. each group of patients shows a significant increase of coproporphyrin excretion by the solvent extraction method in all the three groups, mainly in cholestatic liver disease, while we documented a significant increase by HPLC only in the last group.

The coefficient of variation of the proposed method has been calculated from series of ten replicate experiments at four different concentrations of coproporphyrin I ranging from 5 to 300 μg/l, the coefficient of variation was always within 10%, agreeing with the results of Doss and Schmidt [4]. The coefficient of variation at 5, 50 and 300 μg/l was 9.1, 3.5 and 8.0%, respectively. Also the coefficient of variation of ten replicate analyses of uroporphyrin I at 50 μg/l was 8.5%.

The coefficient of variation of the solvent extraction method was 8% (ten replicate analyses) in our laboratory.

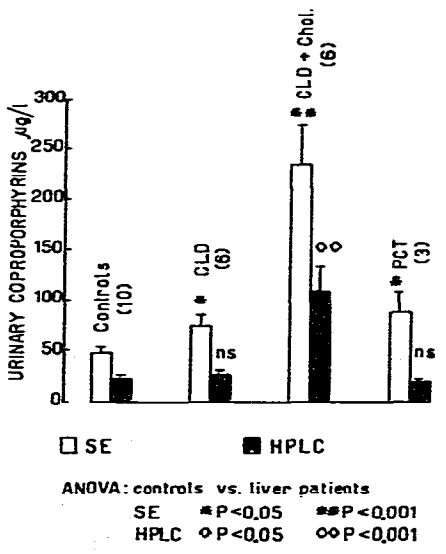

Fig. 2. Statistical analysis of urinary coproporphyrin results ($\bar{x}$ + S.E.; ANOVA = analysis of variance). CLD = chronic liver disease; PCT = porphyria cutanea tarda; SE = solvent extraction.

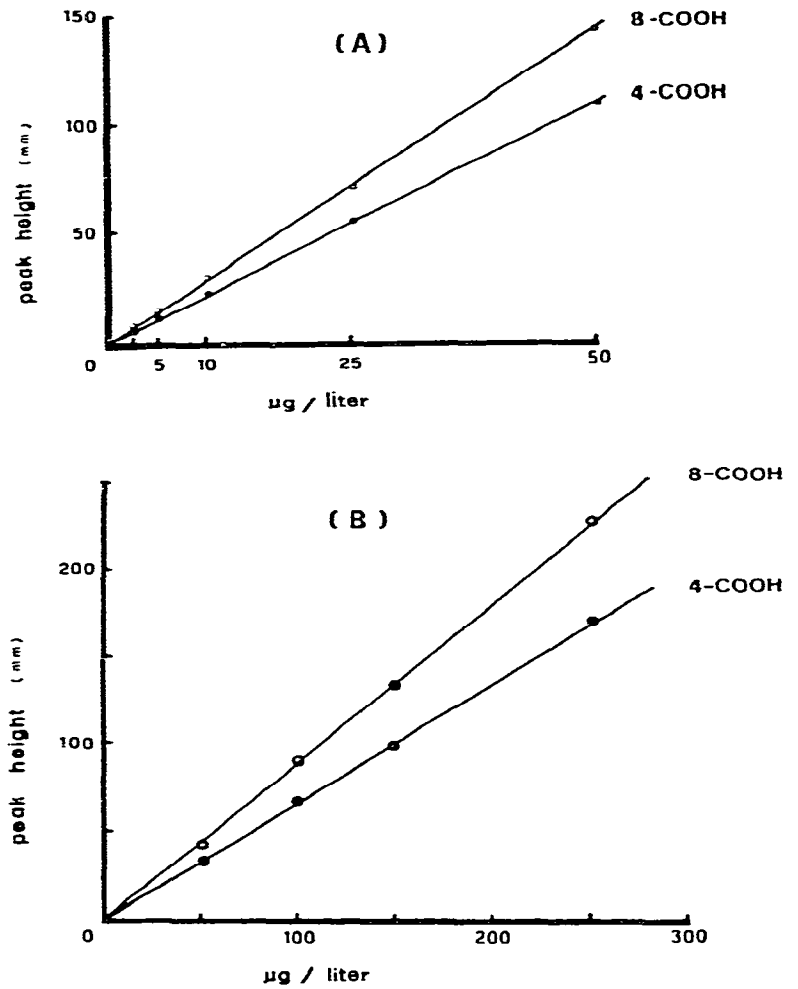

Fig. 3. Plots of peak height vs. concentration of uroporphyrin (8-COOH) and coproporphyrin (4-COOH) in normal range (A) and in pathological range (B).

The amount of urinary coproporphyrin is almost double when detected by the solvent extraction method for both the controls and the liver patients (see Fig. 2).

As to recovery, the two methods overlapped and the fluorometric scanning of HPLC free-porphyrin eluate at an acid pH greatly increases the sensitivity of the proposed method. We believe that 50% of the coproporphyrin detected by the traditional technique may be due to the imprecision of the method itself.

For the same reason the uroporphyrin excretion seems usually within a few µg/l of urine in healthy subjects, and in chronic liver disease without cholestasis (Tables I and II); so far only a small number of early-morning urine samples have been studied.

In pigment-rich urines we observed a "true" increase of coproporphyrins (with both methods) as an effect of impaired biliary secretion of cholephil anions; this agrees with our and other previous observations [15].

As regards the coproporphyrin excretion in porphyria cutanea tarda patients, we recorded increased values with the solvent extraction method according to our and other previous reports [16]. However, we found a "true" coproporphyrin (4-COOH) excretion within the normal urinary range in our three porphyria cutanea tarda patients, and some penta- or hexacarboxylic porphyrins in the HPLC chromatograms (Fig. 1D). Moreover, the HCl coproporphyrin extract from a porphyria cutanea tarda patient's urine obtained by solvent extraction showed a predominance of hepta- and uroporphyrin on subsequent HPLC analysis (Fig. 1E). This confirms the lack of specificity of the solvent extraction method in detecting coproporphyrin when a predominance of uroporphyrin is present in the sample.

The "uroporphyrin class" determined by the solvent extraction method represents the sum of octa- and heptacarboxylic porphyrins, which, on the contrary, are easily differentiated by the HPLC method.

The usefullness of measuring the ratio of uro- to heptacarboxylic compounds in the differentiation of porphyria cutanea tarda from other chronic hepatic porphyrias have been widely emphasized [17].

We therefore agree that the solvent extraction and other similar methods may be regarded as useful, as in the past, for screening or monitoring porphyric patients, but more complex and time-consuming chromatographic techniques are required for the differentiation of porphyrias.

The HPLC of porphyrin free acids and fluorometric scanning which we propose fulfils the need for an easy and quantitative analysis of individual compounds even within the normal range. Further investigations are in progress on a larger number of patients and controls to differentiate individual carboxylic porphyrins and their isomers in other biological materials such as faeces, plasma and liver homogenates.

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