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DETECTION OF NON-TYPICAL PORPHYRIN ISOMERS IN HUMAN URINES BY ION-PAIR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An improved ion-pair reversed-phase high-performance liquid chromatographic system has been developed for the separation of uroporphyrin isomers I, II and III, whereas the isomers III and IV could not be resolved. Application of this method to the analysis of urines from porphyric patients indicated the presence of small amounts of the non-typical uroporphyrin isomer II. The questionable presence of the isomer IV was confirmed by acid-catalyzed decarboxylation to the corresponding coproporphyrin isomers, which were completely separated by a modified ion-pair method at elevated column temperatures. These procedures enabled the detection of small fractions of the atypical isomers II (1–3%) and IV (8–15%) besides the normal isomers I and III in urines of patients suffering from attacks of acute intermittent porphyria. Because such urines contain large amounts of porphobilinogen, the non-enzymatic self-condensation of porphobilinogen to uroporphyrinogens was studied under mild reaction conditions. In these experiments quite similar isomeric compositions were observed as compared to those in urines of patients with acute intermittent porphyria. Thus the non-typical uroporphyrin isomers II and IV present in human urines originate from a simple non-enzymatic condensation of porphobilinogen.

INTRODUCTION

Porphyrin isomers of types I and III have been found in samples of biological origin, whereas isomers of types II and IV have still not been isolated from such materials[1]. Profiling of urinary porphyrins by high-performance liquid chromatography (HPLC) is of great value for the differential diagnosis of porphyrias[2,3]. The simultaneous determination of the respective porphyrin isomer ratios results in additional information for distinguishing dysfunctions of the porphyrin metabolism.

HPLC analysis of porphyrins and their isomers as free carboxylic acids is more convenient than the analysis of porphyrin esters because extraction and esterification steps can be omitted. The usefulness of reversed-phase HPLC for the separation of free porphyrin carboxylic acid isomers has been demonstrated by others[4–8] and by our group[9,10].

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Recently, we described an ion-pair reversed-phase gradient elution system for the simultaneous separation of porphyrin isomers of types I and III in the series from uro- to coproporphyrin[11]. Applying this method to the routine analysis of urinary porphyrins of patients suffering from severe attacks of acute intermittent porphyria (AIP), we observed occasionally a small unidentified peak besides uroporphyrins I and III in the uroporphyrin region. This additional peak was supposed to consist of a uroporphyrin isomer, the structure of which had not been recognized previously.

Kennard and Rimington[12] had isolated a uroporphyrin from the urine of a case of porphyria cutanea tarda which had been shown, by infrared spectra and X-ray powder photographs, to be identical with synthetic uroporphyrin II prepared by MacDonald and Michl[13]. However, the isolated uroporphyrin II behaved abnormally on partial decarboxylation to the corresponding coproporphyrin. Thus, its structure has not been established.

In this paper we describe an improved ion-pair reversed-phase HPLC method for the separation of uroporphyrin isomers, and the application of this method to the analysis of such isomers from urines of AIP patients. Since these urines normally contain large amounts of porphobilinogen (PBG), we also studied the self-condensation of PBG to uroporphyrinogens by using rather mild reaction conditions. The non-enzymatic condensation of PBG had been investigated by several authors previously[14–18], however, no complete analysis of the isomer proportions of the resulting uroporphyrinogens has been reported. Therefore we studied in detail the isomer ratios of the uroporphyrins formed after autoxidation as well as of the respective coproporphyrins obtained after partial decarboxylation. These experiments should help to clarify the origin of atypical uroporphyrin isomers in human urines.

EXPERIMENTAL

Materials

Porphobilinogen and uroporphyrin III octamethyl ester were obtained from Porphyrin Products (Logan, UT, U.S.A.). A statistical mixture of uroporphyrin isomers I (12.5%), II (12.5%), III (50%) and IV (25%) was prepared by condensation of PBG in 1 mol/l hydrochloric acid according to Mauzerall[16].

Coproporphyrins I, II, III and IV tetramethyl ester and uroporphyrin I octamethyl ester were from H. Fischer's collection. They were kindly provided by the Hans-Fischer-Gesellschaft (München, F.R.G.). Porphyrin free acids were obtained from the methyl esters by hydrolyzing them with 25% hydrochloric acid at 25°C for 72 h.

Methanolic solutions of tetrabutyl-, tetraethyl- and tetramethylammonium hydroxide were from Fluka (Buchs, Switzerland). The tetraalkylammonium phosphate ion-pair reagents were prepared by adjusting the corresponding hydroxides to pH 7.4 with 85% phosphoric acid (Suprapur; E. Merck, Darmstadt, F.R.G.). The pH values of methanolic solutions were measured after a 1:10 dilution in distilled water. Acetonitrile and methanol of LiChrosolv grade were obtained from Merck. Aqueous phosphate buffer solutions were prepared from disodium hydrogenphosphate and potassium dihydrogenphosphate (analytical grade, Merck).

Apparatus

A Model 1090A liquid chromatograph (Hewlett-Packard, Waldbronn, F.R.G.) was used together with a Model F-1000 spectrofluorimeter (excitation 394 nm, emission 624 nm) (Merck-Hitachi, Darmstadt, F.R.G.). A Hewlett-Packard Model 1040A diode-array detector was directly attached to the analytical column for on-line measurement of absorption spectra. This detector allowed recording of spectra in the range 200–590 nm.

Chromatographic conditions

Columns. All separations were performed on LiChrosorb RP-18 columns (7 μm, 250 mm × 4 mm I.D., Merck), which were protected by a guard cartridge LiChroCART (LiChrosorb RP-18, 5 μm, 4 mm × 4 mm I.D.; Merck). The flow-rate was 1 ml/min for all analyses. The column was held at room temperature with exception of the isocratic separation of coproporphyrins which was performed at 38°C.

Gradient elution of urinary porphyrins. Profiles of urinary porphyrins ranging from uro- to coproporphyrins were obtained with the ion-pair multilinear gradient elution system described previously[11].

Isocratic separation of uroporphyrin isomers. The elution system consisted of 60% aqueous phase with a 40 mmol/l phosphate buffer (pH 4.5–5.0, depending on the respective batches of columns used) and 40% methanolic phase containing 12.5 mmol/l tetrabutylammonium phosphate (pH 7.4).

Isocratic separation of coproporphyrin isomers. The elution system consisted of 54% aqueous phase with a 44 mmol/l phosphate buffer (pH 6.6) and 46% organic phase containing 8.9 mmol/l tetrabutylammonium phosphate (pH 7.4) in methanol–acetonitrile–water (72:21:7, v/v/v).

Methods

Preparation of urine samples. A 10-ml sample of urine (pH 3.5) was adsorbed on 300 mg talc, washed twice with 5 ml of water and the porphyrins extracted with 2 ml of a 50 mmol/l solution of tetrabutylammonium phosphate in methanol.

Partial decarboxylation of uroporphyrins. A 1-ml volume of the methanolic solution of the talc extract (see above) was evaporated. The residue was dissolved in 1 ml of 1 mol/l hydrochloric acid. This solution was carefully deaerated with purified nitrogen, and heated in sealed tubes in the dark at 180°C for 7 h. Removal of the hydrochloric acid was performed on Sep-Pak C_{18} cartridges (Waters, Eschborn, F.R.G.) according to Friedmann and Baldwin[19]. Briefly, the Sep-Pak column was rinsed with 10 ml of acetone and 4 ml of 1.2 mol/l phosphoric acid. The acidic porphyrin solution was adsorbed on the column, washed with 10 ml of water and 4 ml of 5% acetone. Porphyrins were eluted with 6–8 ml of pure acetone. After removal of acetone in vacuum, the residue was dissolved in 1 ml of a 50 mmol/l solution of tetrabutylammonium phosphate in methanol.

Non-enzymatic condensation of porphobilinogen. To a 10-ml screw-capped brown vial containing 500 μg PBG, dissolved in 100 μl of 5% sodium hydrogencarbonate, was added 4.9 ml of a 100 mmol/l phosphate buffer of pH 5.0, 7.0 and 8.5, respectively. The pH value of the final mixture was exactly adjusted to the desired value. The reaction was carried out at 23°C for 96 h or at 37°C for 24 h. Separation of the uroporphyrins formed by adsorption on talc at pH 3.5 and partial decar-

boxylation to the respective coproporphyrins were performed as described for urine samples.

RESULTS AND DISCUSSION

Urinary porphyrin profiles in acute intermittent porphyria

The urinary HPLC porphyrin profiles of two patients suffering from an attack of AIP are shown in Figs. 1 and 2. The chromatograms were obtained with our ion-pair reversed-phase multilinear gradient system[11]. These patients excreted large amounts of 5-aminolevulinic acid, PBG and especially uroporphyrins. In the uroporphyrin region a small, partially resolved peak (X) can be seen, which might consist of an atypical uroporphyrin isomer. Therefore, a more efficient separation system for uroporphyrin isomers had to be applied in order to enable an identification of this unexpected peak.

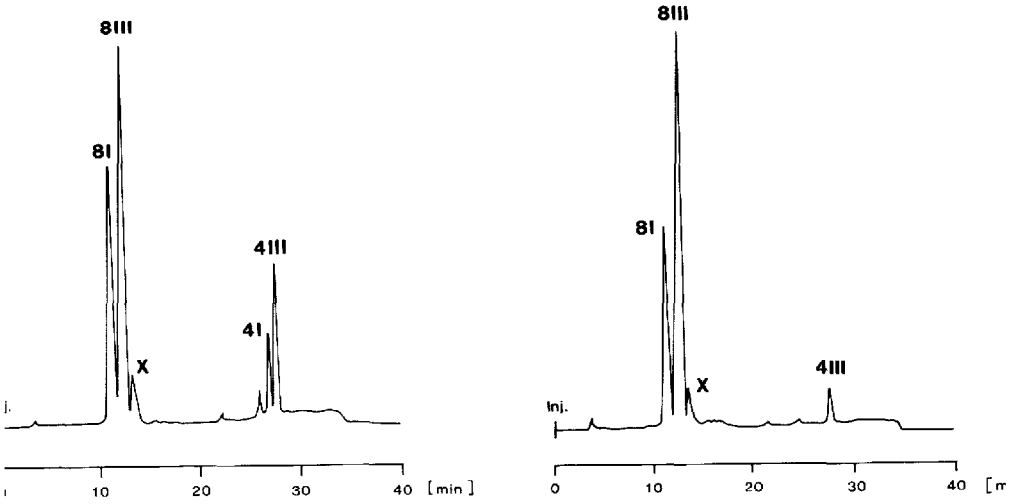

Fig. 1. Urinary porphyrin profile of a patient with acute intermittent porphyria (gradient elution). Fluorescence detection. Peaks: 8I = uroporphyrin I; 8III = uroporphyrin III; 4I = coproporphyrin I; 4III = coproporphyrin III; X = unidentified compound.

Fig. 2. Urinary porphyrin profile of another patient with acute intermittent porphyria. Conditions and peaks as in Fig. 1.

Separation of uroporphyrin isomers

The simultaneous separation of uroporphyrin isomers I, II, III and IV was studied by Wayne *et al.*[20], Jackson *et al.*[21] and Rideout *et al.*[22]. However, no complete separation of all four isomeric uroporphyrins was obtained by these authors. The major problem arises from the difficulty in separating isomers III and IV, which could not be resolved even at the uroporphyrinogen level[23]. Therefore we tried to separate simultaneously all four isomeric uroporphyrins by using ion-pair chromatography.

We studied the effects of type and concentration of ion-pairing reagents (tet-

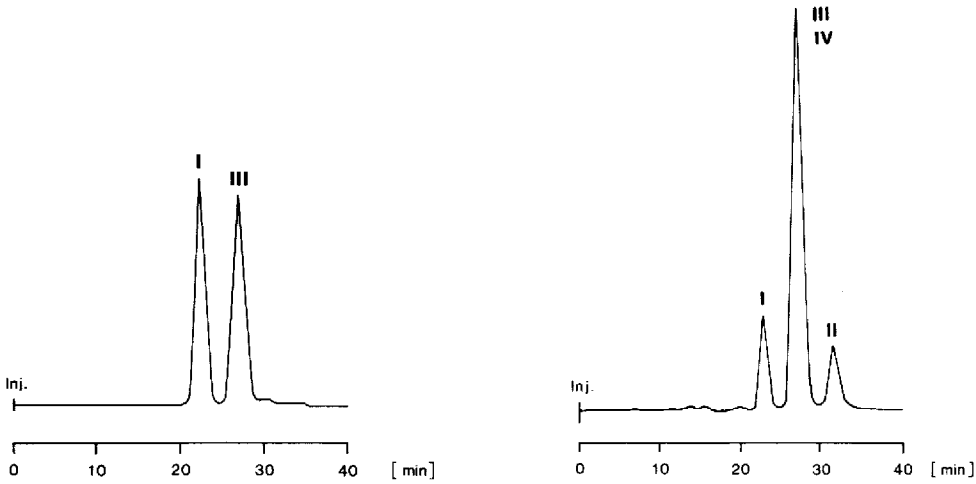

Fig. 3. Isocratic separation of uroporphyrins I and III at pH 5.0 of the aqueous phosphate buffer. Fluorescence detection.

Fig. 4. Isocratic separation of a synthetic mixture of uroporphyrins I, II and III + IV. Conditions as in Fig. 3.

raalkylammonium phosphates), organic solvent concentration, pH value and ionic strength of the phosphate buffer on the retention behaviour of uroporphyrin isomers. The optimum conditions for the separation of these isomers on a 7-μm RP-18 LiChrosorb column were 60% aqueous phosphate buffer (40 mmol/l, pH 4.5–5.0) and 40% methanolic phase with 12.5 mmol/l tetrabutylammonium phosphate (pH 7.4). This eluent system enables a baseline separation of uroporphyrins I and III (Fig. 3), and the complete separation of the isomers I, II and III (Fig. 4). At pH values below 3.5 extremely prolonged retention and peak broadening are observed, whereas at pH values above 6 the resolution of these isomers is sharply decreased (Fig. 5). Unfortunately the isomers III and IV cannot be separated under these conditions.

Fig. 6 shows the isocratic ion-pair separation of the uroporphyrin fraction of a urine sample from an AIP patient. Co-chromatography with authentic substances revealed the presence of uroporphyrins I, II and III. These isomers were positively identified via their absorption spectra in the range between 300 and 590 nm by using a diode-array detector, which was directly coupled to the HPLC column (Fig. 7). The low concentration of isomer II precluded recording of the complete spectrum, and therefore only the typical Soret band at 398 nm can be observed. The unidentified peak U in Fig. 6 had an absorption maximum at 410 nm and its spectrum did not resemble that of a porphyrin. The questionable presence of uroporphyrin IV cannot be checked directly at this moment, whereas coproporphyrin isomers can be completely resolved by HPLC methods.

Separation of coproporphyrin isomers

The isocratic ion-pair separation method for coproporphyrin isomers I–IV[11] was improved by a slightly modified eluent composition and an elevated column

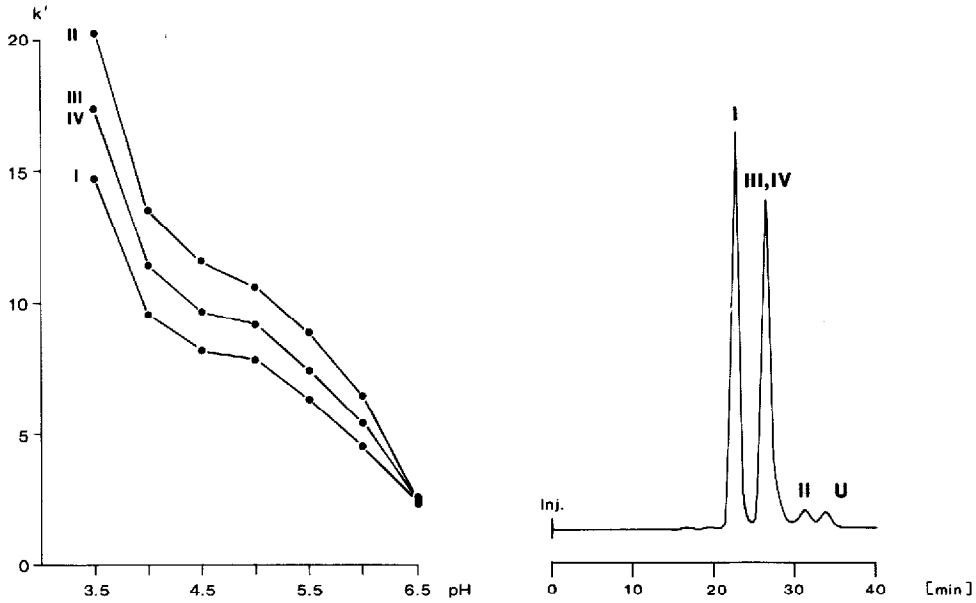

Fig. 5. Effect of pH of the aqueous phosphate buffer on the capacity factors, k', of uroporphyrins I, II and III + IV (isocratic conditions).

Fig. 6. Isocratic separation of the uroporphyrin fraction of a patient with acute intermittent porphyria. Conditions as in Fig. 3. Peaks: I, II, III + IV = uroporphyrin isomers; U = unidentified compound.

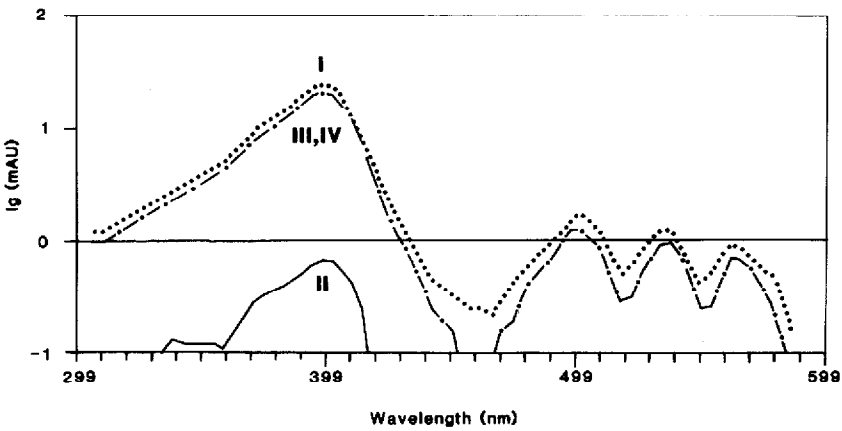

Fig. 7. Absorption spectra of uroporphyrins I, II and III + IV obtained after isocratic separation from the urine of a patient with acute intermittent porphyria. The spectra were obtained by on-line diode-array detection (300–590 nm).

temperature. The temperature dependence of the capacity factors for all four isomeric coproporphyrins is given in Fig. 8. The retention times are markedly reduced with increasing temperatures. The resolution of the individual isomers, however, is not

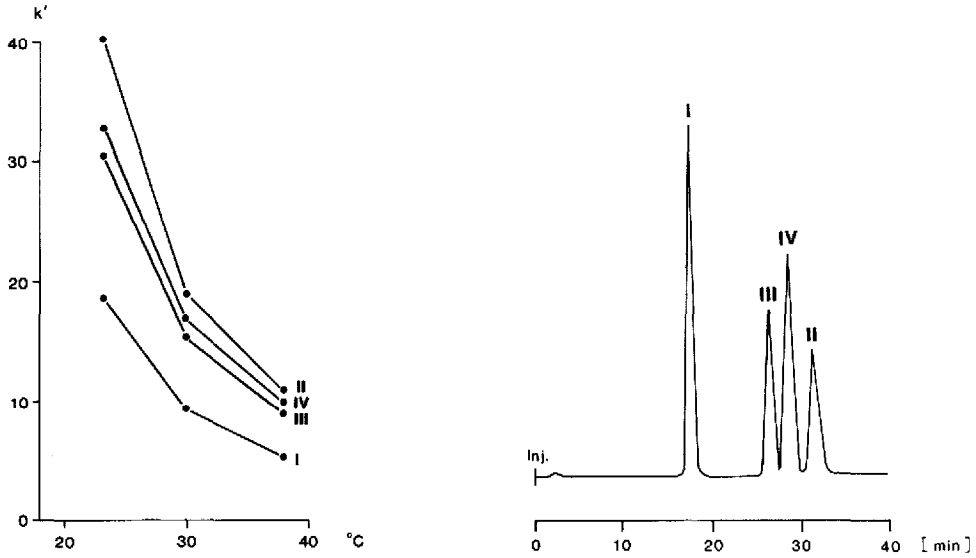

Fig. 8. Effect of temperature on the capacity factors, k', of coproporphyrins I, II, III and IV at pH 6.6 of the aqueous phosphate buffer (isocratic conditions).

Fig. 9. Isocratic separation of a synthetic mixture of coproporphyrins I, II, III and IV at 38°C and pH 6.6 of the aqueous phosphate buffer. Fluorescence detection.

influenced adversely. Baseline separation of these isomers is obtained at a column temperature of 38°C (Fig. 9).

Uroporphyrins from urines of AIP patients were separated by adsorption on talc in order to remove PBG[14] and uroporphyrinogens[24]. Acid-catalyzed partial decarboxylation to the corresponding coproporphyrins was performed at 180°C. The chromatogram of the resulting coproporphyrins from the urine of an AIP patient shows the presence of all four isomeric coproporphyrins (Fig. 10). These isomers were identified by co-chromatography with authentic substances and their absorption spectra obtained via on-line diode-array detection (Fig. 11).

The atypical isomers II and IV were found only in urines of AIP patients in small amounts ranging from 1 to 3% for the type II isomer and 8 to 15% for the type IV isomer. Urines from other porphyric patients, *e.g.*, porphyria cutanea tarda, contained exclusively the normal isomers I and III as shown in Fig. 12. Because the isomers II and IV were found only in urines of patients excreting large amounts of PBG, we studied the isomeric composition of uroporphyrinogens formed from PBG by self-condensation under mild experimental conditions.

Non-enzymatic condensation of porphobilinogen

Non-enzymatic condensation of PBG at room temperature was reported to yield uroporphyrins I and III, the proportions of which varied with the pH of the reaction solution[15,17]. The isomers II and IV could not be observed under these conditions, probably due to the analytical techniques available at that time.

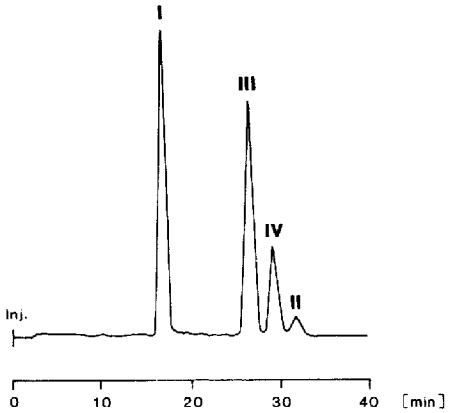

Fig. 10. Isocratic separation of coproporphyrins I, II, III and IV obtained after decarboxylation of the uroporphyrin isomers from the urine of a patient with acute intermittent porphyria. Conditions as in Fig. 9.

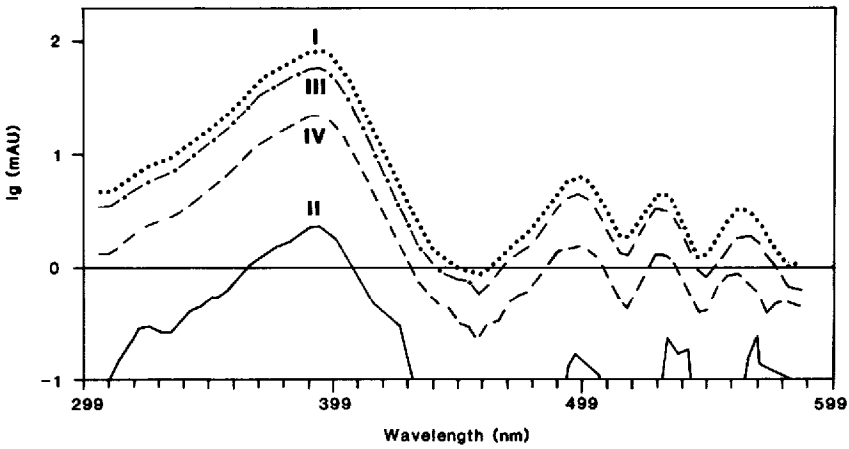

Fig. 11. Absorption spectra of coproporphyrins I, II, III and IV obtained after decarboxylation of the corresponding uroporphyrin isomers and isocratic separation at 38°C from the urine of a patient with acute intermittent porphyria. The spectra were obtained by on-line diode-array detection (300–590 nm).

In a series of experiments we studied the chemical conversion of 0.44 mmol/l solutions of PBG in phosphate buffer of pH 5.0, 7.0 and 8.5 at reaction temperatures of 23 and 37°C, respectively. Similar concentrations of PBG are present in urines of patients suffering from severe attacks of AIP. The reactions were carried out in the dark and in the presence of air. Thus, the resulting uroporphyrinogens were immediately oxidized to uroporphyrins, which are stable to further isomerization. Control experiments with an additional oxidation step by treatment with iodine did not increase the yield of uroporphyrins isolated. After separation on talc, the total amount of uroporphyrins formed as well as the proportions of isomers I, II and

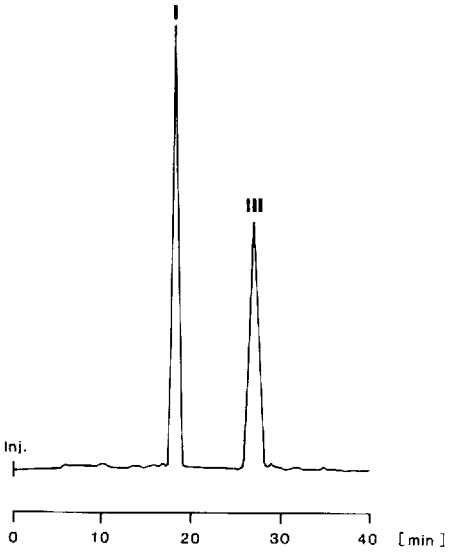

Fig. 12. Isocratic separation of coproporphyrins I and III obtained after decarboxylation of the corresponding porphyrin isomers from the urine of a patient with porphyria cutanea tarda. Conditions as in Fig. 9.

III/IV were measured by HPLC analysis using isocratic conditions. Separate determination of the isomers III and IV was performed after partial decarboxylation to the corresponding coproporphyrins and HPLC analysis at a column temperature of 38°C. A maximum yield of total uroporphyrins was found at pH 7 for both reaction temperatures (Table I). Increasing proportions of isomer I were obtained at higher pH values, whereas the proportions of the isomers II, III and IV decreased markedly from pH 5 to 8.5. The composition of the individual uroporphyrin isomers correlated well with that of the respective coproporphyrin isomers obtained after decarboxylation (Tables II and III). Using these mild reaction conditions the amount of uroporphyrin II varied from 0.3 to 2.5%, and that of isomer IV from 4 to 18%. These are of the same order as those found for the atypical isomers II and IV in urines of AIP patients.

TABLE I

YIELD OF TOTAL UROPORPHYRINS OBTAINED AFTER NON-ENZYMATIC CONVERSION OF PORPHOBILINOGEN

Temperature (°C)	*Time (h)*	*pH*	*Yield (%)*
23	96	5.0	1.4
		7.0	5.5
		8.5	5.2
37	24	5.0	10.3
		7.0	14.6
		8.5	12.3

TABLE II

ISOMERIC COMPOSITION (%) OF UROPORPHYRINS (U) AND COPROPORPHYRINS (C) OBTAINED AFTER NON-ENZYMATIC CONVERSION OF PORPHOBILINOGEN AT 23°C AND PARTIAL DECARBOXYLATION

pH	*U I*	*U II*	*U III + IV*	*C I*	*C II*	*C III*	*C IV*
5.0	41	2.5	56	40	2.3	39	18
7.0	54	1.6	44	53	1.5	35	10
8.5	68	0.6	31	67	0.4	26	6

TABLE III

ISOMERIC COMPOSITION (%) OF UROPORPHYRINS (U) AND COPROPORPHYRINS (C) OBTAINED AFTER NON-ENZYMATIC CONVERSION OF PORPHOBILINOGEN AT 37°C AND PARTIAL DECARBOXYLATION

pH	*U I*	*U II*	*U III + IV*	*C I*	*C II*	*C III*	*C IV*
5.0	54	2.0	44	55	1.5	32	11
7.0	61	1.0	38	60	1.1	30	8
8.5	73	0.3	26	74	0.4	21	4

Similar isomeric compositions and yields of uroporphyrins were observed, when the non-enzymatic condensation of PBG was performed in 0.9% sodium chloride solutions or in normal urine samples.

CONCLUSION

Ion-pair reversed-phase HPLC allows the separation of the isomeric uroporphyrins I, II and III/IV, whereas the isomers III and IV can be resolved only after conversion into the corresponding coproporphyrins.

In urines of patients suffering from acute attacks of AIP, small fractions of the non-typical uroporphyrin isomers II and IV were found occasionally. Control experiments at low temperatures and physiological pH values suggest that these isomers are easily formed by a simple non-enzymatic self-condensation of PBG either in the human body or during sampling and storage of such urines.

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REFERENCES

1 A. R. Battersby and E. McDonald, in K. M. Smith (Editor), *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam, New York, Oxford, 1975, p. 75.
2 C. H. Gray, C. K. Lim and D. C. Nicholson, *Clin. Chim. Acta*, 77 (1977) 167.
3 C. K. Lim and T. J. Peters, *Clin. Chim. Acta*, 139 (1984) 55.
4 E. Englert, Jr., A. W. Wayne, E. E. Wales, Jr. and R. C. Straight, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 570.
5 M. Udagawa, Y. Hayashi and C. Hirayama, *J. Chromatogr.*, 233 (1982) 338.
6 M. Chiba and S. Sassa, *Anal. Biochem.*, 124 (1982) 279.
7 C. K. Lim, J. M. Rideout and D. J. Wright, *J. Chromatogr.*, 282 (1983) 629.
8 D. J. Wright, J. M. Rideout and C. K. Lim, *Biochem. J.*, 209 (1983) 553.
9 H. D. Meyer, K. Jacob, W. Vogt and M. Knedel, *J. Chromatogr.*, 199 (1980) 339.
10 H. D. Meyer, W. Vogt and K. Jacob, *J. Chromatogr.*, 290 (1984) 207.
11 K. Jacob, W. Sommer, H. D. Meyer and W. Vogt, *J. Chromatogr.*, 349 (1985) 283.
12 O. Kennard and C. Rimington, *Biochem. J.*, 55 (1953) 105.
13 S. F. MacDonald and K.-H. Michl, in G. E. W. Wolstenholme and E. C. P. Millar (Editors), *Porphyrin Biosynthesis and Metabolism*, J. & A. Churchill, London, 1955, pp. 285–293.
14 G. Westall, *Nature (London)*, 170 (1952) 614.
15 G. H. Cookson and C. Rimington, *Biochem. J.*, 57 (1954) 476.
16 D. Mauzerall, *J. Am. Chem. Soc.*, 82 (1960) 2605.
17 A. T. Carpenter and J. J. Scott, *Biochim. Biophys. Acta*, 52 (1961) 195.
18 R. B. Frydman, S. Reil and B. Frydman, *Biochemistry*, 10 (1971) 1154.
19 H. C. Friedmann and E. T. Baldwin, *Anal. Biochem.*, 137 (1984) 473.
20 A. W. Wayne, R. C. Straight, E. E. Wales and E. Englert, Jr., *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 2 (1979) 621.
21 A. H. Jackson, K. R. N. Rao and S. G. Smith, *Biochem. J.*, 203 (1982) 515.
22 J. M. Rideout, D. J. Wright and C. K. Lim, *J. Liq. Chromatogr.*, 6 (1983) 383.
23 C. K. Lim, F. Li and T. J. Peters, *Biochem. J.*, 234 (1986) 629.
24 P. Martasek, M. Jirsa and V. Kordac, *J. Clin. Chem. Clin. Biochem.*, 20 (1982) 113.