Identification of *meso*-hydroxyuroporphyrin I in the urine of a patient with congenital erythropoietic porphyria

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A new porphyrin, *meso*-hydroxyuroporphyrin I, was isolated from the urine of a patient with congenital erythropoietic porphyria. The structure was characterized as the methyl ester, ethyl ester and acetoxy derivatives by fast-atom-bombardment m.s., by conversion into uroporphyrin I and by chemical synthesis.

INTRODUCTION

Congenital erythropoietic porphyria or Gunther's disease is a disorder of haem synthesis inherited in an autosomal recessive manner. The enzyme defect lies at the level of uroporphyrinogen III synthase (Romeo & Levin, 1969; Gidari & Levere, 1977), leading to excessive accumulation of uroporphyrin I and to a lesser extent coproporphyrin I in erythrocytes, plasma and skin, which results in mutilating photo-induced skin lesions. The urinary porphyrin excretion pattern has been studied in detail and was also consistent with that found in the tissues (Gray *et al.*, 1977; Lim & Peters, 1984).

The present paper describes the isolation of a new porphyrin, *meso*-hydroxyuroporphyrin I, from the urine of a 14-year-old boy suffering from Gunther's disease. Because of the small amount of compound isolated, extensive use of micro-scale chemical reactions, h.p.l.c. and f.a.b.-m.s. was made for structural characterization.

EXPERIMENTAL

Materials and reagents

Uroporphyrin I octamethyl ester was from Sigma Chemical Co. (Poole, Dorset, U.K.). Thallic trifluoroacetate was from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Ammonium acetate, acetic anhydride, acetic acid, EDTA, zinc acetate, trichloroacetic acid, I₂, conc. HCl, conc. H₂SO₄, NaHCO₃, KOH, metallic sodium, mercury, chloroform, dichloromethane, tetrahydrofuran, SO₂ gas and pyridine were AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.). Acetonitrile and methanol were h.p.l.c. grade from Rathburn Chemicals (Walkerburn, Borders, Scotland, U.K.). Alumina 90 (70–230 mesh) for column chromatography was from E. Merck (Darmstadt, Germany). C₁₈ Bond Elut extraction cartridge was from Analytichem International (Habor City, CA, U.S.A.).

Isolation of *meso*-hydroxyuroporphyrin I in urine by h.p.l.c.

The h.p.l.c. system consisted of a Varian Associates (Walton-on-Thames, Surrey, U.K.) model 5000 pump and a Varian UV-100 variable-wavelength detector set at 400 nm. Samples were injected by a Rheodyne (Cotati, CA, U.S.A.) 7125 injection valve fitted with a 500 µl

loop. The separation was carried out on a 25 cm × 5 mm Hypersil-ODS column (Shandon Scientific, Runcorn, Cheshire, U.K.) by gradient elution. The solvent mixtures were 10% (v/v) acetonitrile in methanol (solvent A) and 10% (v/v) acetonitrile in 1 M-ammonium acetate buffer, pH 5.16 (solvent B). The elution programme was as follows: 0–14 min, linear gradient from 0 to 7% (v/v) solvent A in solvent B; 14.2–21 min, isocratic elution at 90% (v/v) solvent A in solvent B. The column was then re-equilibrated with 0% solvent A (100% solvent B) for 10 min before injection of the next sample.

Urine was acidified with conc. HCl (5 ml per 100 ml of urine) before injection of 300 µl portions. The peak at retention time 12 min was collected and pooled. The acetonitrile and methanol in the eluate were removed by evaporation at 30 °C under vacuum. The resulting aqueous ammonium acetate solution was loaded by suction on to a C₁₈ Bond Elut cartridge that had been conditioned by washing successively with 5 ml of methanol and 10 ml of 1 M-ammonium acetate buffer, pH 5.16. Up to 500 ml of solution could be loaded on to each cartridge. The porphyrin adsorbed on the cartridge was recovered by elution with five 2 ml portions of 10% (v/v) acetonitrile in methanol or until no fluorescence was detected in the eluate. The combined eluate was evaporated to dryness at 30 °C under N₂. The residue contained *meso*-hydroxyuroporphyrin I.

Preparation of octamethyl and octaethyl esters of *meso*-hydroxyuroporphyrin I

meso-Hydroxyuroporphyrin I (about 200 µg) was dissolved in 2 ml of methanol/conc. H₂SO₄ (9:1, v/v) or ethanol/conc. H₂SO₄ (9:1, v/v) and the mixture was left standing for 20 h in the dark. The solution was poured into a separating funnel containing 3 ml of chloroform, and distilled water (6 ml) was added immediately. After extraction, the chloroform layer was washed successively with 5 ml of saturated NaHCO₃ solution and two 5 ml portions of distilled water. The organic layer was filtered through a filter paper pre-wetted with chloroform. The filtrate was evaporated to dryness at 30 °C under N₂. The product was analysed by f.a.b.-m.s.

Preparation of *meso*-acetoxyuroporphyrin I octamethyl ester

The method of Jackson *et al.* (1968) for the preparation of *meso*-acetoxyuroporphyrin III was used.

Abbreviation used: f.a.b.-m.s., fast-atom-bombardment m.s.

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meso-Hydroxyuroporphyrin I octamethyl ester (approx. 100 μ g) in pyridine (200 μ l) and acetic anhydride (50 μ l) were thoroughly mixed and stood at room temperature for 10 min. The mixture was evaporated to dryness under N_2 . The product was analysed by f.a.b.-m.s.

Conversion of *meso*-hydroxyuroporphyrin I into uroporphyrin I

The method was a modification of that reported by Jackson *et al.* (1968). *meso*-Hydroxyuroporphyrin I in 0.01 M-KOH (500 μ l) was flushed with N_2 , stoppered and then shaken vigorously with 3% (w/w) sodium amalgam until no fluorescence was detected under u.v. light. The solution was removed from the mercury into a clean tube, and 50 μ l of a saturated solution of I_2 in trichloroacetic acid was added. The mixture was stood on ice for 5 min and then separated by h.p.l.c. on 0.5% Hypersil-ODS with 13% (v/v) acetonitrile in 1 M-ammonium acetate buffer, pH 5.16, as mobile phase (Lim *et al.*, 1983). The peak corresponding to uroporphyrin I was collected, esterified and analysed by f.a.b.-m.s.

Preparation of *meso*-hydroxyuroporphyrin I

The procedure used was similar to that described for the synthesis of *meso*-hydroxycoproporphyrin I (Barnett *et al.*, 1973). Zinc(II) uroporphyrin I octamethyl ester

was prepared by treating the porphyrin ester in chloroform with zinc acetate in methanol as described by Fuhrhop & Smith (1975). A mixture of the zinc complex (approx. 2 mg) in tetrahydrofuran (0.5 ml) and dichloromethane (2 ml) was treated with a solution of thallic trifluoroacetate (3 mg) in tetrahydrofuran (0.5 ml) and then stirred for 1 min. Water (20 μ l) in tetrahydrofuran (1 ml) was added, and the mixture was stirred for a further 10 min. The solution was treated briefly with SO_2 gas, and conc. HCl (200 μ l) was added. After being stirred for 5 min the mixture was poured into water (20 ml) and extracted with dichloromethane (10 ml). The organic layer was filtered through a filter paper pre-wetted with chloroform. The filtrate was evaporated to dryness under N_2 . The residue was redissolved in chloroform and loaded into a Pasteur pipette half-filled with alumina 90. The column was eluted with chloroform (10 ml) and then 10 ml of chloroform/methanol (1:1, v/v). The chloroform/methanol fraction contained *meso*-hydroxyuroporphyrin I octamethyl ester. The solvent was removed by evaporation under N_2 . The residue was dissolved in 25% (w/w) HCl and left standing at room temperature in the dark for 96 h to hydrolyse the methyl ester. The porphyrin was then purified by h.p.l.c. with the system described above for the isolation of the compound from urine.

F.a.b.-m.s.

F.a.b.-m.s. was carried out on a Finnigan MAT-371 double-focusing mass spectrometer. Porphyrin methyl esters for analysis were dissolved in the matrix on a stainless-steel target stage and bombarded by a xenon atom beam (10 kV, 10 μ A) from an atom gun supplied by M-Scan (Ascot, Berks., U.K.). Positive-ion spectra were obtained from samples dissolved in chloroform and introduced into a thin film of thioglycerol, coated on the target.

RESULTS AND DISCUSSION

The h.p.l.c. elution program was specifically optimized for the isolation of the new porphyrin from urine, as shown in Fig. 1. The compound was eluted much faster and is therefore more polar than uroporphyrin. F.a.b.-m.s. of the methyl and ethyl esters gave the $M+1$ signals at 959 (M_r 958) and 1071 (M_r 1070) respectively, showing the presence of eight carboxylic acid groups and an increase of 16 mass units over uroporphyrin octamethyl ester (M_r 942) and octaethyl ester (M_r 1054) respectively. This indicated that the compound is closely related to uroporphyrin and strongly pointed to *meso*-substitution of uroporphyrin by a hydroxy group. Acetylation of the methyl ester with pyridine/acetic anhydride gave an acetoxy derivative with the f.a.b.-m.s. signal at $M+1 = 1001$ (M_r 1000), an increase of 42 mass units and confirming the presence of a hydroxy group.

Reduction of the porphyrin free acid with 3% (w/w) sodium amalgam followed by aromatization with I_2 gave uroporphyrin I as the only product, as shown by h.p.l.c. analysis (Lim *et al.*, 1983). The reaction proved that the hydroxy group is located at the *meso*-position and established that the porphyrin is of the type I isomer. The structure is shown in Fig. 2.

That the assigned structure was correct was confirmed by synthesis of *meso*-hydroxyuroporphyrin I by means of the thallic trifluoroacetate method (Barnett *et al.*, 1973).

Fig. 1. Separation of *meso*-hydroxyuroporphyrin I (peak 1) in urine of a patient with congenital erythropoietic porphyria

Peak 2 contains mainly uroporphyrin I. H.p.l.c. conditions are described in the Experimental section.

Fig. 2. Structure of *meso*-hydroxyuroporphyrin I

A and P are acetic acid and propionic acid groups respectively.

described for the preparation of oxophlorins. The synthetic compound had identical h.p.l.c. behaviour and gave similar chemical reactions and identical f.a.b.-m.s. spectra for the methyl ester, ethyl ester and acetoxy derivatives compared with the natural product.

It is well known that uroporphyrin I in congenital erythropoietic porphyria is derived from non-enzymic cyclization of excessive hydroxymethylbilane (Battersby *et al.*, 1982) accumulating as a result of uroporphyrinogen III synthase deficiency. Whether the presence of a larger than usual concentration of hydroxymethylbilane in the body can also lead to the formation *in vivo* of *meso*-hydroxyuroporphyrin I is not clear. *In vitro* chemical cyclization of hydroxymethylbilane and oxidation of uroporphyrinogen I with I_2 , however, gave the expected uroporphyrin I, without a trace of *meso*-hydroxyuroporphyrin I being detected by h.p.l.c. The urine of a patient with acute intermittent porphyria during acute attack, where the uroporphyrin concentration was highly elevated, was also analysed for the presence of *meso*-hydroxyuroporphyrin, but none was detected. This would also suggest that *meso*-hydroxyuroporphyrin was not derived from the non-enzymic chemical oxidation of uroporphyrin or uroporphyrinogen.

From the evidence above, it appears that *meso*-hydroxyuroporphyrin I was synthesized *in vivo*. Since hydroxylation increases the water-solubility of uroporphyrin, this may be a way of increasing the excretion of excess uroporphyrin I in congenital erythropoietic porphyria. Hydroxylation of uroporphyrin by the kidney is another possibility. *meso*-Hydroxycoproporphyrin I, however, could not be found in the same urine sample, where the coproporphyrin I concentration was also elevated, although not to the same extent as uroporphyrin I. It was estimated that about 500 μ g of the porphyrin was isolated from 1 litre of urine. This was approx. 2% of the total urinary porphyrin.

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