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Determination of hydroxy and peroxy acid derivatives of uroporphyrin in the plasma of patients with congential erythropoietic porphyria by high-performance liquid chromatography

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method is described for the determination of hydroxy and peroxy acid derivatives of uroporphyrin in the plasma of patients with congenital erythropoietic porphyria. The porphyrins were extracted from the plasma with 20% trichloroacetic acid – dimethyl sulphoxide (1:1, v/v). The supernatant after centrifugation was chromatographed on a Hypersil-ODS column by gradient elution with 9% (v/v) acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16) (solvent A) and 10% (v/v) acetonitrile in methanol (solvent B) as the gradient mixture. The method was also suitable for the preparative isolation of the porphyrins.

INTRODUCTION

We have previously isolated and characterized three hydroxy and one peroxy acid uroporphyrin I derivatives [1,2] in the urine of patients with congenital erythropoietic porphyria (CEP), namely *meso*-hydroxyuroporphyrin I, β-hydroxypropionic acid uroporphyrin I, hydroxyacetic acid uroporphyrin I and peroxyacetic acid uroporphyrin I (Fig. 1). CEP is a rare genetic disease inherited in the autosomal recessive trait. Patients with CEP characteristically excrete high concentrations of uroporphyrin I in the urine because of uroporphyrinogen III synthase deffect [3,4]. Uroporphyrin I also accumulates in the plasma and skin of these patients, resulting in multilating photo-induced skin lesions. As hydroxy and peroxy acid derivatives of uroporphyrins must be derived from uroporphyrin or uroporphyrinogen by as yet unknown oxidation mechanisms, the origin and distribution of these compounds were further investigated in the plasma of patients with CEP. This paper describes a reversed-phase high-performance liquid chromatographic (HPLC) method for the isolation and determination of hydroxy and peroxy acid derivatives of uroprophyrin in human plasma and reports for the first time the detection of these compounds in plasma of CEP patients.

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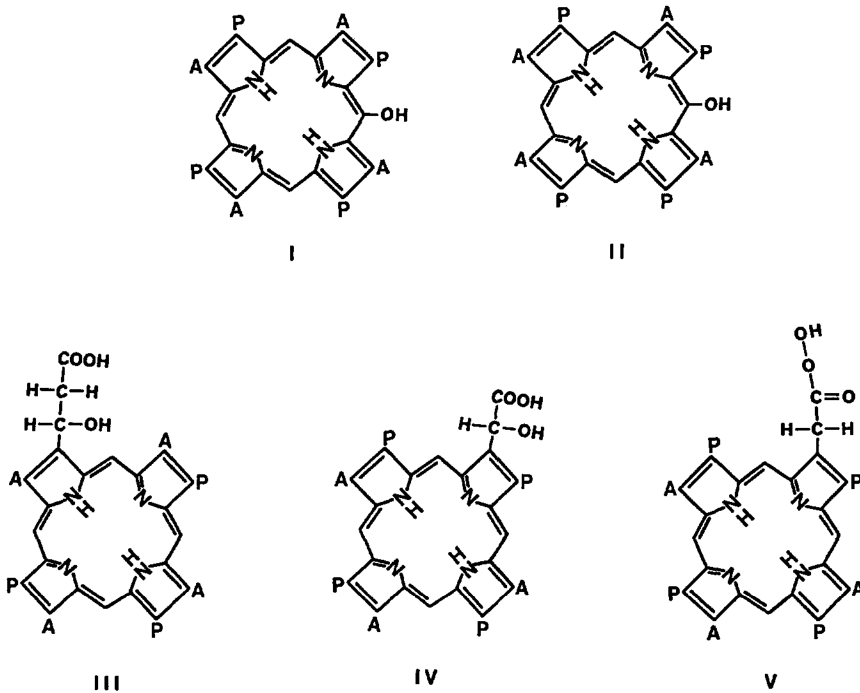

Fig. 1. Structures of hydroxy and peroxy acid derivatives of uroporphyrin. I, *meso*-Hydroxyuroporphyrin I; II, *meso*-hydroxyuroporphyrin III (only one of the four possible isomeric forms is shown); III, β-hydroxypropionic acid uroporphyrin I; IV, hydroxyacetic acid uroporphyrin I; V, peroxyacetic acid uroporphyrin I. A = Acetic acid and P = propionic acid.

EXPERIMENTAL

Materials and reagents

Ammonium acetate, glacial acetic acid, dimethyl sulphoxide (DMSO), trichloroacetic acid (TCA) and hydrochloric acid were of Analar grade from BDH (Poole, UK). Acetonitrile and methanol were of HPLC grade from Rathburn Chemicals (Walkerburn, UK). *meso*-Hydroxyuroporphyrin I and III were synthesized by the thallium(III) trifluoroacetate method [5], which has been adapted by us for the preparation of *meso*-hydroxyuroporphyrin [1], and β-hydroxypropionic acid uroporphyrin I and hydroxyacetic acid uroporphyrin I were isolated from CEP patients as described previously [1,2].

Extraction of porphyrins from plasma

Plasma (200 μl) from patients with CEP was vortex mixed for 1 min with 200 μl of 20% TCA–DMSO (1:1, v/v) and centriguged at 2000 *g* for 10 min. The supernatant was injected into the HPLC system.

For larger scale extraction, 15 ml of plasma were used. The plasma was vortex mixed with an equal volume of 20% TCA–DMSO (1:1, v/v). The supernatant after centrifugation was collected. The residue was again vortex mixed with 20% TCA–

DMSO (1:1, v/v) (5 ml), centrifuged and the supernatant collected. The pooled supernate was diluted 1:15 (v/v) with 0.5 *M* ammonium acetate buffer (pH 5.16) and loaded under suction into a C_{18} Bond-Elut sorbent extraction cartridge (Analytichem International, Habor City, CA, USA) that had been preconditioned by washing successively with methanol (2 ml) and 0.5 *M* ammonium acetate buffer (pH 5.16) (10 ml). The cartridge was washed with 3 ml of 0.5 *M* ammonium acetate buffer (pH 5.16) and the adsorbed porphyrins were eluted with 1-ml portions of 10% (v/v) acetonitrile in methanol until very little fluorescent was detected on the cartridge under a UV lamp. The eluate was pooled and then evaporated to dryness at 40°C under nitrogen. The residue was dissolved in 1 ml of 0.3 *M* hydrochloric acid for HPLC separation.

HPLC separation of uroporphyrin I and its hydroxylated derivatives in plasma extract

The HPLC system consisted of a Varian Associates (Walton-on-Thames, UK) Model 5000 pump and a Varian fluorescence detector with an excitation wavelength of 400 nm and a 580-nm cut-off filter for the emission. Samples (250 µl) were injected by a Rheodyne (Cotati, CA, USA) Model 7125 injection valve fitted with a 500-µl loop. The separation was carried out on a 25 cm × 5 mm I.D. Hypersil-ODS column (Shandon Scientific, Runcorn, UK) by gradient elution. The solvent mixtures were 9% (v/v) acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16) (solvent A) and 10% (v/v) acetonitrile in methanol (solvent B). The elution programme was as follows: time 0 to 30 min, 0% solvent B (100% solvent A) to 90% solvent B (10% solvent A); time 30 to 40 min, isocratic elution at 90% solvent B; time 40.1 min, re-equilibrate column at 0% solvent B (100% solvent A). The flow-rate was 1 ml/min throughout.

For the preparative isolation of individual porphyrin, the peak was collected and pooled. The organic solvents were removed by evaporation under nitrogen. The porphyrin in the aqueous solution was then concentrated and recovered by the cartridge extraction technique described above.

RESULTS AND DISCUSSION

The chromatogram of porphyrins in the plasma of a patient with CEP is shown in Fig. 2. Of the eight peaks, six have been positively identified.

Peak 2 co-eluted with synthetic *meso*-hydroxyuroporphyrin I under all HPLC conditions. It is therefore probably *meso*-hydroxyuroporphyrin I (Fig. 1, structure I). This was confirmed by sodium amalgam reduction of the compound followed by aromatization with iodine, which eliminated the hydroxyl group to give uroporphyrin I [1].

Peak 3 was identified as *meso*-hydroxyuroporphyrin III (Fig. 1, structure II; there are four possible isomers of which only one is shown here) as it had a retention time identical with that of an authentic standard. The four *meso*-hydroxyuroporphyrin III isomers could not be resolved under the HPLC conditions used. Reduction of the compound followed by re-oxidation gave uroporphyrin III, confirming that the assignment was correct. The detection of *meso*-hydroxyuroporphyrin III in the plasma is interesting, as the compound had not been isolated from the urine of CEP patients despite the fact that uroporphyrin III excretion was also elevated, although this was not as high as uroporphyrin I. In the plasma, however, uroporphyrin III was barely detectable. Whether *meso*-hydroxyuroporphyrin III in its reduced form can

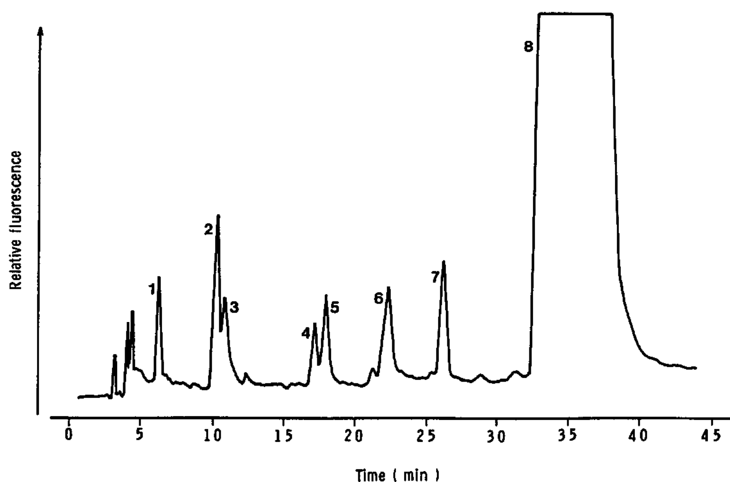

Fig. 2. Separation of porphyrins in the plasma of a patient with congenital erythropoietic porphyria. Peaks: 1, unidentified; 2, *meso*-hydroxyuroporphyrin I; 3, *meso*-hydroxyuroporphyrin III; 4, unidentified, 5, β-hydroxypropionic acid uroporphyrin I; 6, hydroxyacetic acid uroporphyrin I; 7, peroxyacetic acid uroporphyrin I; 8, uroporphyrin I.

give rise to uroporphyrinogen III *in vivo* is not known. If it can, then this could represent an alternative pathway to the biosynthesis of uroporphyrinogen III and hence haem.

Peak 5 was identical in chomatographic and chemical behaviour with that of the β-hydroxypropionic acid uroporphyrin I isolated from the urine of CEP patients [6] and was therefore assigned structure III in Fig. 1. This compound can be easily dehydrated by heating in dilute hydrochloric acid to give the monoacrylic derivative as the main product [6].

The chromatographic and chemical properties of peak 6 were identical with those of hydroxyacetic acid uroporphyrin I (Fig. 1, structure IV). This compound had also been isolated from the urine of CEP patients. On heating in dilute hydrochloric acid no dehydration product was formed [6], indicating that the hydroxyl group is attached to an acetic acid group and is therefore difficult to dehydrate. Some partial decarboxylation products were formed instead.

Peak 7 was peroxyacetic uroporphyrin I (Fig. 1, structure V). It had a characteristic greenish colour with a typical red porphyrin fluorescence under UV light. As with the same compound isolated from urine of CEP patients, it gave uroporphyrin I when treated with 0.1 *M* potassium or calcium hydroxide but was stable under acidic conditions [2]. It also co-eluted with the authentic standard under all HPLC conditions.

The presence of hydroxy and peroxy acid derivatives of uroporphyrins in the plasma of patients with CEP rules out of the possibility that they were formed in the kidney prior to excretion in the urine where they were originally isolated [1,2,6]. These compounds must be derived from uroporphyrin or uroporphyrinogen *in vivo* by unknown hydroxylation or oxidation processes which require further investigation.

Studies on the metabolism and the biological and toxicological importance of

these compounds are also needed. Hydroxylated and particularly peroxylated derivatives can easily generate free radicals which can lead to cell damage. The reduced forms (porphyrinogens) are structurally very similar to uroporphyrinogen III. They may therefore compete with or inhibit the decarboxylation of uroporphyrinogen III to coproporphyrinogen III by uroporphyrinogen decarboylase. It may be significant that we have found normal uroporphyrinogen decarboxylase activity in the red cells of patients with CEP while these patients always excrete elevated level of uroporphyrin III in the urine. Inhibition of uroporphyrinogen decarboxylase by any one or all of these compounds is a possible explanation.

Peaks 1 and 4 were not identified as these two compounds were not found in the urine of CEP patients and sufficient amounts have not been isolated for positive identification. Peak 8 was the large amount of uroporphyrin I present in the plasma of CEP patients.

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