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Preparation and separation of hydroxy derivatives of uroporphyrinogen I by high-performance liquid chromatography with electrochemical detection

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Abstract

The preparation and high-performance liquid chromatography (HPLC) separation of *meso*-hydroxyuroporphyrinogen I, hydroxyacetic acid uroporphyrinogen I and β-hydroxypropionic acid uroporphyrinogen I is described. *meso*-Hydroxyuroporphyrin I, hydroxyacetic acid uroporphyrin I and β-hydroxypropionic acid uroporphyrin I were isolated from the urine of a patient with congenital erythropoietic porphyria. The porphyrins were reduced to the corresponding porphyrinogens with 3% (w/w) Na/Hg amalgam. The hydroxy porphyrinogens were separated on a Hypersil ODS column with 4% (v/v) acetonitrile in 1 *M* ammonium acetate buffer, pH 5.16, containing EDTA (0.27 m*M*) as the mobile phase, and detected electrochemically. Reduction of *meso*-hydroxyuroporphyrin I and hydroxyacetic acid uroporphyrin I, followed by HPLC analysis, showed that, in addition to the expected formation of *meso*-hydroxyuroporphyrinogen I and hydroxyacetic uroporphyrinogen I, respectively, uroporphyrinogen I was also produced. Reduction of β-hydroxypropionic acid uroporphyrin I, however, gave β-hydroxypropionic acid uroporphyrinogen I, acrylic acid uroporphyrinogen I and uroporphyrinogen I as the products. The peaks were identified by conversion into the porphyrin methyl esters and analysed by liquid secondary-ion mass spectrometry.

1. Introduction

We have previously reported the isolation and characterisation of three hydroxy derivatives of uroporphyrin I in the urine and plasma of patients with congenital erythropoietic porphyria (CEP), namely, *meso*-hydroxyuroporphyrin I, hydroxyacetic acid uroporphyrin I and β-hydroxypropionic acid uroporphyrin I [1–3]. CEP is a rare genetic disease characterised by the excretion of an excess amount of uroporphyrin I in the urine, due to uroporphyrinogen III synthase deficiency. As the hydroxy derivatives of uroporphyrin I are most likely to derive from uroporphyrinogen I by, as yet, unknown oxidation mechanisms, the preparation and separation of the hydroxy derivatives of uroporphyrinogen I is important for investigating their origin and metabolisms. We are also interested in studying the potential inhibition of uroporphyrinogen decarboxylase by these compounds, which also required their preparation and separation.

High-performance liquid chromatography (HPLC) with electrochemical detection has been used for the separation and detection of porphyrinogens [4–9]. The preparation and separation of hydroxyuroporphyrinogen derivatives, however, has not been reported. The present

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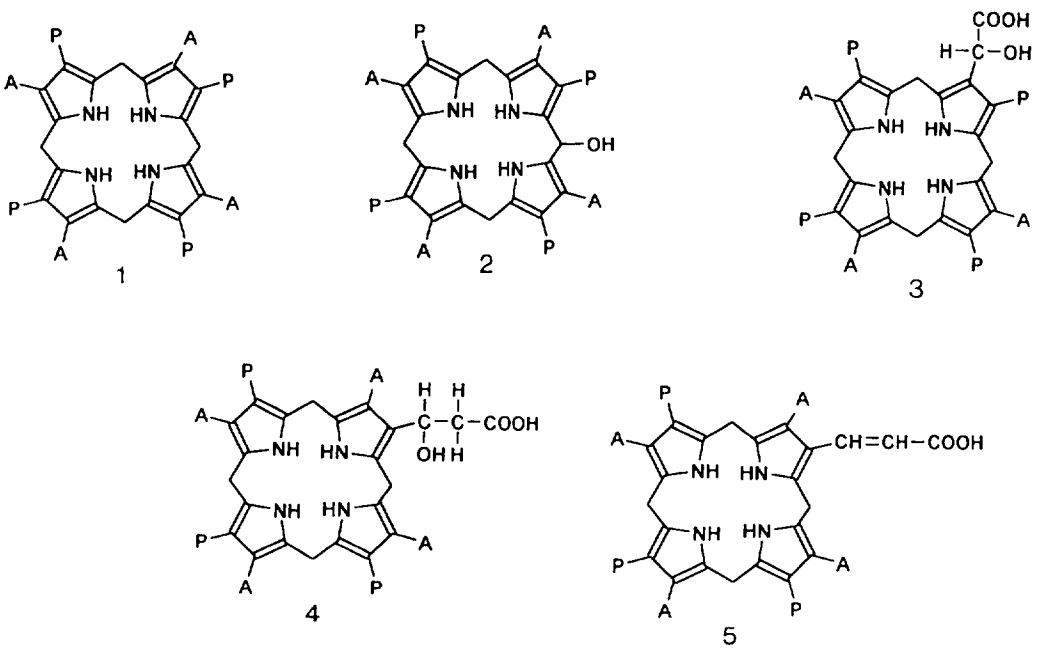

Fig. 1. Structures of uroporphyrinogen I (1), *meso*-hydroxyuroporphyrinogen I (2), hydroxyacetic acid uroporphyrinogen I (3), β-hydroxypropionic acid uroporphyrinogen I (4) and acrylic acid uroporphyrinogen I (5). A = acetic acid group and P = propionic acid group.

paper describes the preparation and reversed-phase HPLC separation of *meso*-hydroxyuroporphyrinogen I, hydroxyacetic acid uroporphyrinogen I and β-hydroxypropionic acid uroporphyrinogen I (Fig. 1) and their degradation products.

2. Experimental

2.1. Materials and reagents

Ammonium acetate, glacial acetic acid, concentrated HCl, ethylenediamine tetraacetic acid (EDTA, disodium salt), potassium hydroxide, iodine, metallic mercury and sodium were AnalaR grade from BDH Chemicals (Poole, Dorset, UK). Acetonitrile was HPLC grade from Rathburn Chemicals (Walkerburn, Peeblesshire, UK). *meso*-Hydroxyuroporphyrin I, hydroxyacetic acid uroporphyrin I and β-hydroxypropionic acid uroporphyrin I were isolated from the urine of a patient with CEP by the methods described previously [1–3].

2.2. Preparation of porphyrinogens

The porphyrins were dissolved in 0.01 *M* KOH (250 μl), flushed with N_2, and the solution was shaken vigorously with 3% (w/w) Na/Hg amalgam until no fluorescence was detectable under a UV lamp. The porphyrinogen solution was transferred into a clean vial, flushed with nitrogen, capped and kept on ice, in the dark.

2.3. HPLC of porphyrinogens

A Varian Associates (Walnut Creek, CA, USA) Model 9012 liquid chromatograph was used with an LCA-15 electrochemical detector (EDT Research, London, UK) set at an operating potential of +0.60 V with a detector sensitivity of 30 nA. The separation was carried out on a 25 cm × 5 mm ODS-Hypersil (5 μm spherical silica chemically bonded with octadecylsilyl

groups) from Shandon Scientific (Runcorn, Cheshire, UK). The mobile phase was 4% (v/v) acetonitrile in 1 *M* ammonium acetate, pH 5.16 (adjusted with acetic acid), containing 0.27 m*M* EDTA. The flow-rate was 1 ml/min. The mobile phase was degassed before use and a continuous stream of helium was passed during the separation.

2.4. Liquid secondary-ion mass spectrometry (LSIMS)

The porphyrinogen peaks separated by HPLC were collected and then converted into the porphyrin methyl esters by reacting with methanol–concentrated H_2SO_4 (9:1, v/v) overnight. This oxidised the porphyrinogen to porphyrin and esterified the carboxylic acid groups. The porphyrin methyl esters were extracted into chloroform for LSIMS analysis as previously described [1–3]. A VG Analytical (Manchester, UK) ZAB2-E mass spectrometer, operated at 8 keV accelerating voltage and fitted with a caesium gun (35 keV, 0.5 μA emission) was used for LSIMS. Porphyrin esters were dissolved in a small volume (ca. 10 μl) of chloroform + methanol (2:1, v/v) and 1 μl was added to the standard stainless-steel LSIMS target probe previously prepared with thioglycerol (1 μl) as liquid matrix. Mass spectra were acquired in the positive-ion mode, using the VG Analytical 11-250J data system in continuous multichannel analysis mode at a resolution of 1500 RP.

3. Results and discussion

The porphyrinogens are the reduced forms of porphyrins or hexahydroporphyrins. Porphyrinogens do not fluoresce and have weak UV absorption at around 220 nm, in contrast to the strongly fluorescent porphyrins, which have very strong absorption in the 400 nm region. Porphyrinogens are, therefore, difficult to detect with a UV detector. However, they are easily oxidised back to the porphyrins with the loss of 6 protons and the concurrent generation of 6 electrons, and are, therefore, ideal for sensitive (pmol) electrochemical detection. Sensitive detection is important in the present study, because the hydroxylated uroporphyrin I derivatives are only minor components of the total urinary porphyrin excretion in patients with CEP and only small amounts have been isolated.

When a mixture containing *meso*-hydroxyuroporphyrin I, hydroxyacetic acid uroporphyrin I and β-hydroxypropionic acid uroporphyrin I isolated from CEP urine was reduced with 3% sodium amalgam to the corresponding porphyrinogens, and the products separated on an ODS-Hypersil column with 4% (v/v) acetonitrile in 1 *M* ammonium acetate buffer (pH 5.16) as eluent, five peaks were detected (Fig. 2). The peaks are identified as described below.

Reduction of *meso*-hydroxyuroporphyrin I, followed by HPLC analysis, showed the formation of peaks 1 and 4. Peak 4 had an identical retention time to authentic uroporphyrinogen I and was, therefore, identified as uroporphyrinogen I. Peak 1, which was eluted faster (more polar) than peak 4, is most probably the expected *meso*-hydroxyuroporphyrinogen I. This was confirmed by collecting the peak and then reacting it with I_2, when uroporphyrin I was formed. It has been reported [10] that aromatisa-

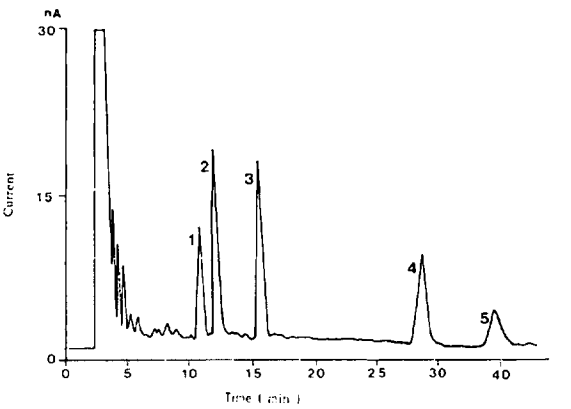

Fig. 2. Separation of uroporphyrinogen I and derivatives. Column, ODS-Hypersil (5 μm, 25 cm × 5 mm I.D.); eluent, 4% (v/v) acetonitrile in 1 *M* ammonium acetate, pH 5.16; flow-rate, 1 ml/min; detector, electrochemical, +0.60 V, 30 nA. Peaks: 1 = *meso*-hydroxyuroporphyrinogen I, 2 = hydroxyacetic acid uroporphyrinogen I, 3 = β-hydroxypropionic acid uroporphyrinogen I, 4 = uroporphyrinogen I, 5 = acrylic acid uroporphyrinogen I.

tion of *meso*-hydroxyporphyrinogen resulted in the formation of porphyrin with the elimination of the *meso*-hydroxy group.

Reduction of hydroxyacetic acid uroporphyrin I gave peak 2 and peak 4 (uroporphyrinogen I). Peak 2 could be oxidised with I_2 to the corresponding porphyrin without the loss of the hydroxy group. LSIMS analysis of the porphyrin methyl ester gave an $[M+H]^+$ ion at m/z 959 (Fig. 3), corresponding to the molecular mass of hydroxyacetic acid uroporphyrin I octamethyl ester. Peak 2 is, therefore, identified as the hydroxyacetic acid uroporphyrinogen I.

Reduction of β-hydroxypropionic acid uroporphyrin I gave peaks 3, 4 (uroporphyrinogen I) and 5. Treatment of peak 3 with I_2 gave the original β-hydroxypropionic acid uroporphyrin I, the methyl ester of which had the $[M+H]^+$ signal at m/z 959, that was expected for β-hydroxypropionic acid uroporphyrin I octamethyl ester. This peak must, therefore, be β-hydroxypropionic acid uroporphyrinogen I.

Oxidation of peak 5 to the corresponding porphyrin followed by LSIMS analysis of the porphyrin methyl ester gave the $[M+H]^+$ ion at m/z 941 (Fig. 4). This is 18 mass units less than β-hydroxypropionic acid uroporphyrin I and indicates dehydration of the β-hydroxypropionic acid group to an acrylic acid group. Peak 5 is, therefore, identified as acrylic acid uroporphyrinogen I (Fig. 1). This is consistent with the fact that of the three hydroxyuroporphyrinogen I derivatives, only that with a hydroxypropionic acid function can be dehydrated. The dehydration reaction probably took place during reduction with sodium amalgam, when considerable heat was generated.

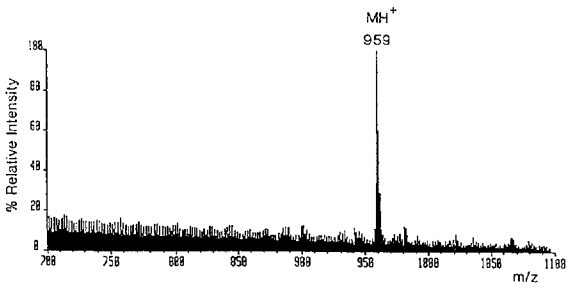

Fig. 3. Positive-ion LSIMS of hydroxyacetic acid uroporphyrin I octamethyl ester.

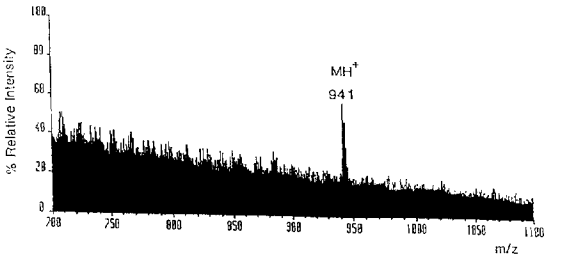

Fig. 4. Positive-ion LSIMS of acrylic acid uroporphyrin I octamethyl ester.

The stability of the porphyrinogens has been investigated. They were stable to oxidation for at least two hours, if kept on ice, in the dark. The stability can be further improved by flushing the solution with N_2, and by adding a reducing agent such as ascorbic acid to the solution. The porphyrinogens are, therefore, stable for a sufficient length of time, for use in the study of their metabolisms and potential inhibition of uroporphyrinogen decarboxylase.

References

[1] R. Guo, J.M. Rideout and C.K. Lim, Biochem. J., 264 (1989) 293.
[2] R. Guo, J.M. Rideout, W. Chai, A.M. Lawson and C.K. Lim, Biomed. Chromatogr., 5 (1991) 53.
[3] R. Guo and C.K. Lim, J. Chromatogr., 550 (1991) 603.
[4] C.K. Lim, F. Li and T.J. Peters, Biochem. J., 234 (1986) 629.
[5] F. Li, C.K. Lim and T.J. Peters, Biochem. J., 239 (1986) 481.
[6] F. Li, C.K. Lim and T.J. Peters, Biochem. J., 243 (1987) 621.
[7] F. Li, C.K. Lim and T.J. Peters, Biochem. J., 243 (1987) 863.
[8] C.K. Lim, F. Li and T.J. Peters, Biochem. J., 247 (1987) 229.
[9] F. Li, C.K. Lim and T.J. Peters, Chromatographia, 24 (1987) 421.
[10] A.H. Jackson, G.W. Kenner, G. McGillivray and K.M. Smith, J. Chem. Soc. C, (1968) 294.