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Note

Improved separation of uroporphyrin isomers by high-performance liquid chromatography

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Of the four uroporphyrinogen (uro'gen) isomers (Fig. 1), type III is the natural precursor of chlorophylls, hemes and vitamin B_{12}, while in biological systems isomer I normally occurs in trace amounts. Enzymatic synthesis of uro'gen III involves porphobilinogen deaminase catalyzed head-to-tail coupling and ring closure of four porphobilinogen molecules to form the unstable intermediate preuroporphyrinogen (Fig. 1) which is either transformed into uro'gen III by uro'gen III cosynthetase or, in the absence of this enzyme, spontaneously rearranges into uro'gen I (refs. 1–3). Under pathological conditions in man and animals suffering from certain porphyrias large amounts of uroporphyrin I (the oxidized form of uro'gen I) are accumulated and excreted[4]. In the case of erythropoietic porphyria this has been linked with the presence of excess porphobilinogen deaminase as compared to uro'gen III cosyn-

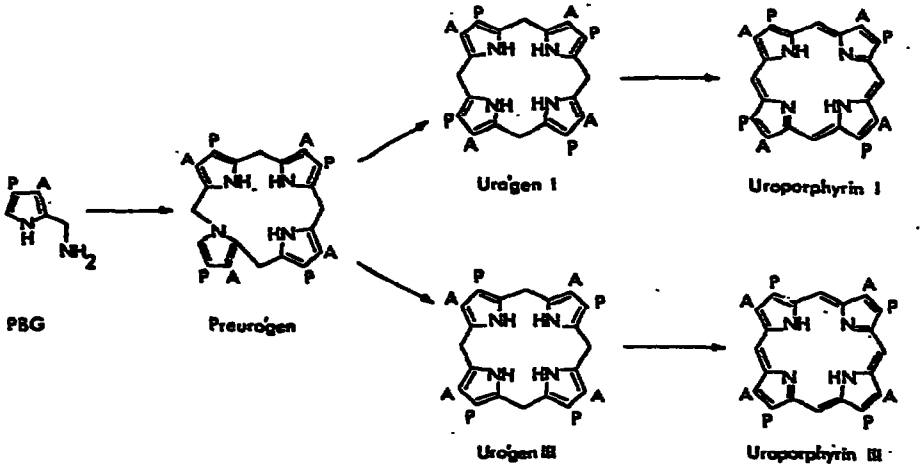

Fig. 1. Structures. A:–CH_2–COOH; P:–CH_2–CH_2–COOH. Uro'gen II: AP-PA-AP-PA; Uro'gen IV: AP-AP-PA-PA.

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thetase[5,6] which makes the latter enzyme unable to consume all the preuroporphyrinogen formed.

When assaying uro'gen III cosynthetase and in the diagnosis of porphyrias it is necessary to separate and quantitate isomers I and III. The first reported separations of octamethyl esters of uroporphyrins I and III (easily available by oxidation and esterification of the uro'gens) using paper chromatography[7,8], apart from being slow and difficult to quantitatate, suffered from cross contamination between the two isomers, probably by formation of molecular complexes with each other[9,10]. Melting point depression and X-ray diffraction pattern analysis[11,12] have not found practical application. In a more reliable method uroporphyrins are decarboxylated to coproporphyrins which as tetramethyl esters can be separated by paper chromatography[13,14]. More recently, high-performance liquid chromatography of coproporphyrin tetramethyl esters has made possible the separation and quantitation of isomers I, II, III and IV (ref. 15). However the decarboxylation step is tedious for routine use. It was therefore a major break-through when high-performance liquid chromatographic (HPLC) separation of uroporphyrin I and III octamethyl esters was reported without cross contamination[16]. Even with this method five recycles through two columns in series is required. We now describe a modification of this method which makes possible base-line separation between uroporphyrin octamethyl esters I, II, and III+IV. Furthermore no recycling is necessary and this relatively fast method has been used successfully for routine assays of uroporphyrinogen III cosynthetase during the purification of this enzyme[17].

EXPERIMENTAL

Chemicals and reagents

All chemicals employed were of reagent grade and were used without further purification. *n*-Heptane was purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.). Glacial acetic acid and acetone were delivered by Fisher Scientific (Pittsburgh, Pa., U.S.A.). Boron trifluoride in methanol was bought from Sigma (St. Louis, Mo., U.S.A.). Uroporphyrin octamethyl esters I and III were supplied by Porphyrin Products (Logan, Utah, U.S.A.) or were synthesized from porphobilinogen (PBG) using deaminase and cosynthetase purified from *Rhodopseudomonas spheroides*[17]. Porphobilinogen was synthesized according to Frydman *et al.*[18]. A statistical mixture of the four uroporphyrin isomers (I–II–III–IV, 12.5:12.5:50:25) was prepared by heating porphobilinogen in acid[19], followed by oxidation and esterification, as described below.

High-performance liquid chromatography

Separations were carried out in a Waters Assoc. liquid chromatograph equipped with a Model 6000 A solvent delivery system, a Model U6K Universal injector, a Model 440 UV detector operated at 405 nm and μPorasil columns (300 × 3.9 mm I.D., 10 μm particle size). A mixture of *n*-heptane–glacial acetic acid–acetone–water (600:300:200:1) was used to equilibrate the column for several hours, after which separations were made using the same eluent without addition of water. The column had to be requilibrated occasionally with the water containing solvent for *ca.* 1 h. Flow-rate was 1 ml/min using one column or 0.5 ml/min when using two columns in series, at a pressure of *ca.* 400 p.s.i.

Sample preparation

Uroporphyrinogens were oxidized with 0.1% I_2 in HCl–methanol (1:1). Uroporphyrins in 100 μl water were esterified with 2 ml boron trifluoride–methanol for 30 min at 60° or alternatively freeze-dried and esterified overnight with 5% H_2SO_4 in methanol. The methyl esters were extracted into chloroform. When necessary they were purified by thin-layer chromatography on silica gel plates (Merck) using benzene–ethyl acetate–methanol (80:16:4) as eluent. After evaporation a small amount of chloroform or the HPLC solvent was added and *ca.* 0.1 μg of uroporphyrin octamethyl ester was injected in a volume of 10 μl.

RESULTS AND DISCUSSION

A baseline separation between uroporphyrin octamethyl esters I and III could be achieved in 3 h using two μPorasil columns in series and eluting with *n*-heptane–glacial acetic acid–acetone (6:3:2) at a flow-rate of 0.5 ml/min (Fig. 2a). Under these conditions, mixtures with less than 1% of either isomer could be analyzed (Fig. 2b, c). Most routine separations can be done in less than 40 min using only one column and a flow-rate of 1 ml/min; in this case the separation factor is 0.7 and either isomer can be quantitated down to *ca.* 3%. Usually 0.1 μg sample was loaded onto the columns, but in the range 0.01–1 μg the ratio between isomers I and III was constant. Provided the uroporphyrin octamethyl ester samples were pre-purified by thin-layer chromatography it was possible to inject a sample once every 6 min allowing up to 7 analyses per hour. When a Waters Wisp autoinjector was used it was more convenient to omit the prepurification and inject every 40 min giving a capacity of 20 analyses overnight.

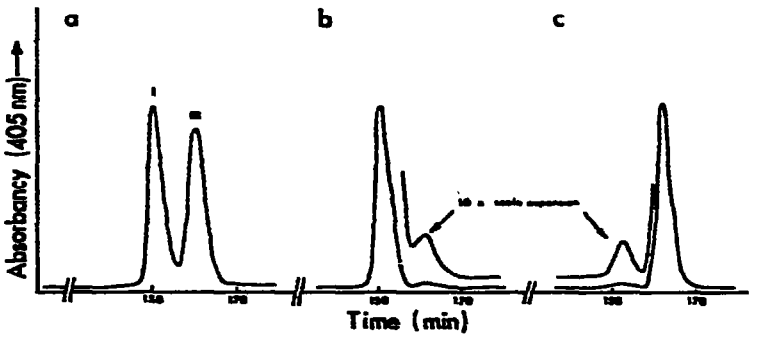

Fig. 2. Separation of *ca.* 0.1 μg uroporphyrin octamethyl esters I and III on two μPorasil columns in series using *n*-heptane–glacial acetic acid–acetone (6:3:2) as eluent at a flow-rate of 0.5 ml/min. (a) *ca.* 50% of each isomer; (b) *ca.* 2% of isomer III; (c) *ca.* 2% of isomer I.

In an effort to separate all four uroporphyrin octamethyl ester isomers, the statistical mixture (I–II–III–IV, 12.5:12.5:50:25) prepared by heating porphobilinogen in acid followed by oxidation and esterification was injected into two columns in series and eluted at a flow-rate of 0.5 ml/min. The chromatogram obtained appears in Fig. 3, where a complete separation between isomers I, II, and III+IV is observed. The non-symmetry of the III+IV peak indicates a partial separation of these isomers which however, was not improved by recycling. The areas of the peaks

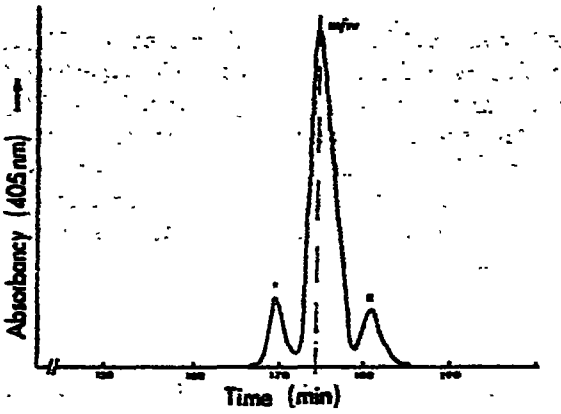

Fig. 3. Separation of a statistical mixture of the four uroporphyrin octamethyl ester isomers. Conditions as in Fig. 2.

showed good agreement with the statistically expected ratios (I–II–III+IV, 12.8: 12.6:74.6).

The chromatographic separation described above is a non-equilibrium one as the stationary phase must be first equilibrated with a water-saturated solvent before the separation can take place using a water deficient mobile phase (commercial acetone containing 0.5% water was used since use of dry acetone did not improve the separation). However after an initial stabilization time of *ca.* 1 h the system remains stable during several days of continuous use. When necessary, reequilibration is easily performed by a 1-h flushing with water supplemented mobile phase.

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