A high-performance-liquid-chromatographic method for the assay of coproporphyrinogen oxidase activity in rat liver

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An h.p.l.c. method was developed for the assay of coproporphyrinogen oxidase activity in rat liver. The protoporphyrinogen IX formed is completely oxidized to protoporphyrin IX for separation and quantification by reversed-phase chromatography with mesoporphyrin as the internal standard. The K_m of coproporphyrinogen oxidase is 1.01 ± 0.23 μM. The activities are 4.07 ± 0.40 nmol of protoporphyrin IX/h per mg of mitochondrial protein and 224 ± 19 nmol of protoporphyrin IX/h per g of liver tissue homogenate. The method is sensitive enough for measuring enzyme activity in small amounts of human tissue from needle biopsy.

INTRODUCTION

Coproporphyrinogen oxidase (EC 1.3.3.3) is a mitochondrial enzyme that catalyses the oxidative decarboxylation of coproporphyrinogen III to protoporphyrinogen IX, the precursor for protoporphyrin IX and haem. Its activity has been determined spectrophotometrically (Sano & Granick, 1961; Batlle *et al.*, 1965; Tait, 1972; Poulson & Polglase, 1974) after oxidation of the protoporphyrinogen IX and isolation of the protoporphyrin IX formed by solvent partition. These methods lack sensitivity, specificity and reproducibility. To overcome these problems, radiochemical assays have been developed. Elder & Evans (1978*a*) measured the rate of $^{14}CO_2$ production with [^{14}C]coproporphyrinogen III labelled at the carboxy-group C-atoms of the 2- and 4-propionic acid groups as the substrate. Grandchamp & Nordmann (1982) used [^{14}C]coproporphyrinogen III labelled in the tetrapyrrolic rings as substrate. The protoporphyrinogen IX formed was oxidized to protoporphyrin IX, solvent-extracted and methyl-esterified. Protoporphyrin IX dimethyl ester was then purified by t.l.c. for quantification by radioactivity counting. These procedures are tedious and time-consuming, apart from requiring the preparation of labelled substrate.

The present paper describes a simple and rapid assay for coproporphyrinogen oxidase based on effective h.p.l.c. separation with sensitive fluorescence detection of protoporphyrin IX after complete oxidation of the protoporphyringen IX produced from the enzymic reaction.

EXPERIMENTAL

Materials and reagents

Protoporphyrin IX, coproporphyrin III and mesoporphyrin were from Sigma Chemical Co. (Poole, Dorset, U.K.). The concentrations of standard porphyrin solutions were determined spectrophotometrically by the method of Falk (1964).

Ammonium acetate, acetic acid, concentrated HCl, KOH, EDTA (disodium salt), sucrose, DMSO, trichloroacetic acid, $HClO_4$, metallic sodium and mercury were AnalaR grade from BDH Chemicals Ltd. (Poole, Dorset, U.K.). Methanol was h.p.l.c. grade from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland, U.K.).

Preparation of rat liver homogenate and mitochondrial fraction

Non-fasted Sprague–Dawley rats were anaesthetized by inhalation of methoxyfluorane (C-VET Ltd., Bury St. Edmunds, Suffolk, U.K.) and then killed. The liver was removed immediately. A 2 g portion was cut into small pieces and homogenized in a Dounce homogenizer in 40 ml of 0.25 M-sucrose solution by ten strokes each of a loose- (type A) and a tight- (type B) fitting pestle.

The mitochondrial fraction was isolated by sucrose-gradient centrifugation as follows. The cell debris and nuclei from the liver homogenate were removed by centrifugation at 800 *g* for 10 min in a 4×100 ml swing-out rotor in a Cool-spin centrifuge (MSE, Crawley, Sussex, U.K.). The mitochondrial fraction was isolated by centrifugation at 7000 *g* for 10 min in an 8×50 ml angle-head rotor using a HiSpin 21 MSE centrifuge. After three washes by resuspension and re-centrifugation with 0.25 M-sucrose, the pellet was resuspended in 0.25 M-sucrose with four strokes of the loose-fitting pestle in a Dounce homogenizer and 4 ml was layered on to a discontinuous sucrose gradient of 5 ml each of densities 1.17, 1.22 and 1.28 g/ml, and centrifuged at 100000 *g* for 2 h in a 3×25 ml swing-out rotor in a Superspeed 75 MSE centrifuge. The fraction containing mitochondria (at the 1.17 and 1.22 g/ml interface) was collected.

Both homogenate and mitochondrial fraction were frozen and thawed three times and then divided into 0.5 ml aliquots and stored at −30 °C. Immediately before assay the fractions were diluted with 0.15 M-NaCl. Protein was determined by a modified Lowry method with bovine serum albumin as standard (Schacterle & Pollack, 1973).

Abbreviations used: DMSO, dimethyl sulphoxide.

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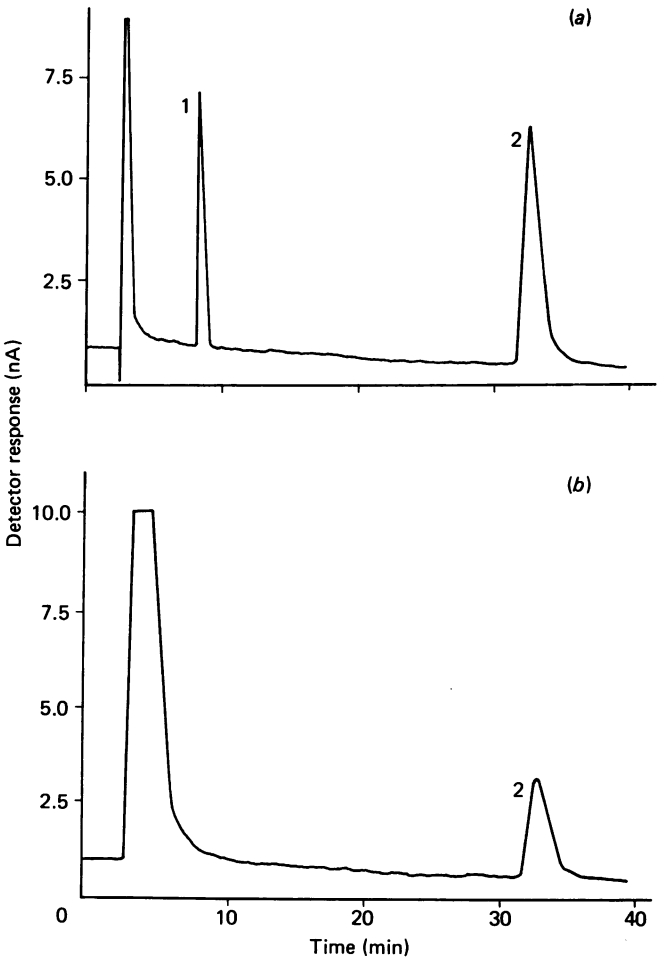

Fig. 1. H.p.l.c. chromatograms for determining the extent of conversion of protoporphyrinogen IX into protoporphyrin IX

(*a*) A standard mixture of protoporphyrinogen IX (peak 1) and protoporphyrin IX (peak 2). (*b*) Enzyme incubation mixture as described in text. H.p.l.c. separation was carried out on a 25 cm × 5 mm ODS-Hypersil (5 μm) column with 83% methanol in 1 M-ammonium acetate buffer, pH 5.16, as mobile phase, at a flow rate of 1 ml/min; detection was amperometric at +0.75 V.

Preparation of coproporphyrinogen III

A 100 μl portion of coproporphyrin III (300 μM) in 0.05 M-KOH was reduced with 3%(w/w) sodium amalgam. Reduction was carried out in the dark under N_2. When the solution no longer fluoresced under u.v. light, 900 μl of 0.25 M-Tris/HCl buffer containing ascorbic acid (0.1 M) and EDTA (1 mM), pH 7.2, was added. After mixing, the coproporphyrinogen III solution was transferred into another tube placed on ice, flushed with N_2, and was used immediately.

Enzyme assay

The assay was carried out in 10 ml tubes. The incubation mixture included 100 μl of 0.25 M-Tris/HCl buffer, pH 7.2, containing 1 mM-EDTA, and 100 μl of enzyme solution. The mixture was preincubated at 37 °C for 5 min and 50 μl of buffered coproporphyrinogen III substrate added. The incubation was then carried out for 1 h in the dark under aerobic conditions. The reaction was stopped by cooling on ice and immediately vortex-mixed with 250 μl of 10% (w/v)trichloroacetic acid (or $HClO_4$)/DMSO (1:1, v/v) containing mesoporphyrin (100 pmol) as internal standard. After centrifugation at 1800 *g* for 10 min at 4 °C, 100 μl of the supernatant was injected into the h.p.l.c. apparatus.

A Waters Associates (Milford, MA, U.S.A.) liquid chromatograph with a Perkin Elmer (Beaconsfield, Bucks., U.K.) LS3 fluorescence detector set at excitation and emission wavelengths of 400 and 618 nm respectively was used. Sample injection was via a Rheodyne 7125 injector fitted with a 100 μl loop. The separation was performed on a 25 cm × 5 mm ODS-Hypersil (5 μm) column with 88% (v/v) methanol in 1 M-ammonium acetate buffer, pH 5.16, as the elution solvent. The mobile-phase flow rate was 1.5 ml/min.

The enzyme activities were expressed as nmol of protoporphyrin IX formed/h per mg of protein for mitochondria or nmol/h per g of liver tissue for homogenate.

H.p.l.c. with electrochemical detection of protoporphyrinogen IX and protoporphyrin IX

The separation was carried out on the ODS-Hypersil column with 83% methanol in 1 M-ammonium acetate buffer, pH 5.16, containing 0.27 mM-EDTA as eluent. The mobile phase was continuously degassed with a stream of helium during the separation. An LCA-15 electrochemical detector (EDT Research, London NW10 7LU, U.K.) set at an operating potential of +0.75 V and a detector sensitivity of 10 or 30 nA was used for solute detection.

RESULTS AND DISCUSSION

Oxidation of protoporphyrinogen IX and recovery of protoporphyrin IX

There are two important factors governing the successful assay of coproporphyrinogen oxidase by h.p.l.c. with fluorescence detection: the effective oxidation of the non-fluorescent enzymic product (protoporphyrinogen IX) to the fluorescent protoporphyrin IX and the complete recovery of protoporphyrin IX from the protein precipitate.

Protoporphyrinogen IX is unstable and is susceptible to oxidative degradation. It is therefore difficult to achieve quantitative conversion into protoporphyrin IX by chemical means without significant losses caused by degradation and side reactions. Ideally protoporphyrinogen IX should be detected directly after termination of the enzyme reaction. This can be achieved by h.p.l.c. separation with electrochemical detection. The ease of protoporphyrinogen IX oxidation makes it an ideal compound for high-sensitivity detection when the electrochemical detector is operated in the oxidative mode. Protoporphyrin IX is less electroactive than protoporphyrinogen IX but can still be detected in the range of 1–5 ng injected. For protoporphyrinogen IX, sub-nanogram detection is possible at an operating potential of +0.75 V and detector sensitivity of 10 nA full scale. This is more than adequate for the detection of protoporphyrinogen IX in the enzyme incubation mixture.

Fig. 1 shows the separation of protoporphyrinogen IX

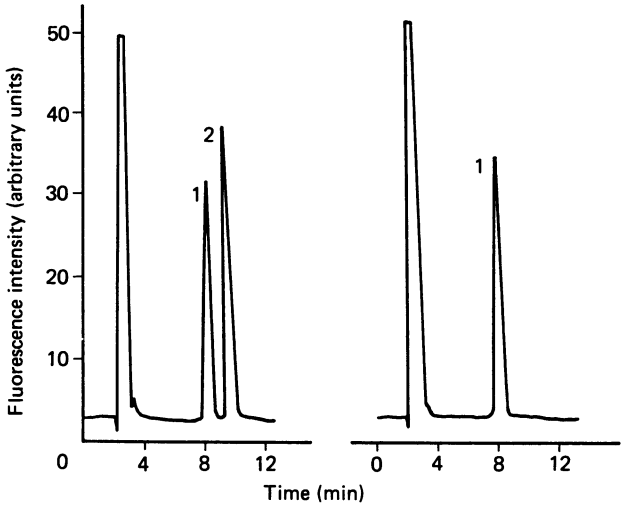

Fig. 2. H.p.l.c. chromatograms for the determination of coproporphyrinogen oxidase activity in rat liver homogenate

(*a*) Enzyme incubation mixture; (*b*) zero-incubation-time blank. The column contained ODS-Hypersil and the eluent was 88% methanol in 1 M-ammonium acetate buffer, pH 5.16; the flow rate was 1.5 ml/min; the detector measured fluorescence excitation at 400 and emission at 618 nm. Peaks: 1, mesoporphyrin; 2, protoporphyrin IX.

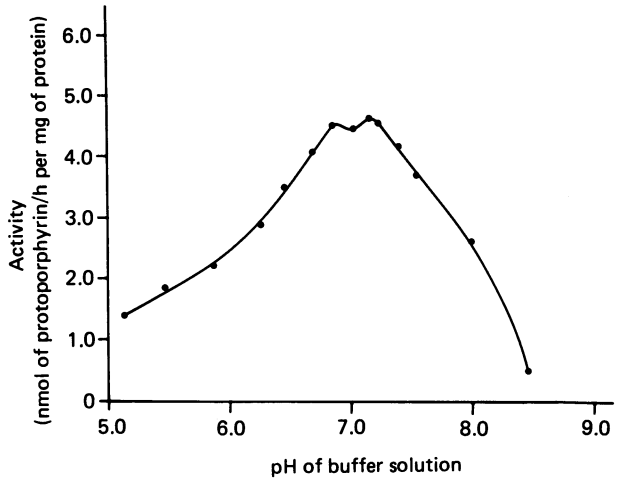

Fig. 3. pH–activity graph for coproporphyrinogen oxidase in mitochondrial preparations in 0.25 M-Tris/HCl buffer

and protoporphyrin IX in (*a*) the standard mixture and (*b*) the incubation mixture for coproporphyrinogen oxidase assay. Protoporphyrinogen IX was not detected in the incubation mixture, indicating total oxidation to protoporphyrin IX, which was easily detectable. The complete oxidation of protoporphyrinogen IX to protoporphyrin IX must be brought about by protoporphyrinogen oxidase also found in the mitochondria during the incubation. This was confirmed by performing the assay with purified mitochondrial intermembrane space, the soluble protein fraction between the inner and outer membranes (Elder & Evans, 1978*b*) containing negligible or no protoporphyrinogen oxidase, when only protoporphyrinogen IX was detected with the electrochemical detector. In separate experiments the pH optimum (7.2) used for the assay of coproporphyrinogen oxidase was also found to be suitable (although not optimum) for the protoporphyrinogen oxidase reaction. Further conversion of protoporphyrin IX to protohaem by ferrochelatase was prevented by the presence of EDTA in the incubation medium.

In a spectrophotometric method (Sano & Granick, 1961) and one of the radiochemical assays (Grandchamp & Nordmann, 1982), the incubation mixture was exposed to weak light in the presence of dilute acid to oxidize the protoporphyrinogen IX formed. We found this treatment was unnecessary and actually led to a decrease in protoporphyrin IX formation.

There was a low and variable recovery of protoporphyrin IX if 10% trichloroacetic acid was used to terminate the enzyme reaction, presumably attributable to the adsorption of protoporphyrin IX on to the protein precipitate. This problem was solved by using a 1:1 (v/v) mixture of 10% trichloroacetic acid and DMSO

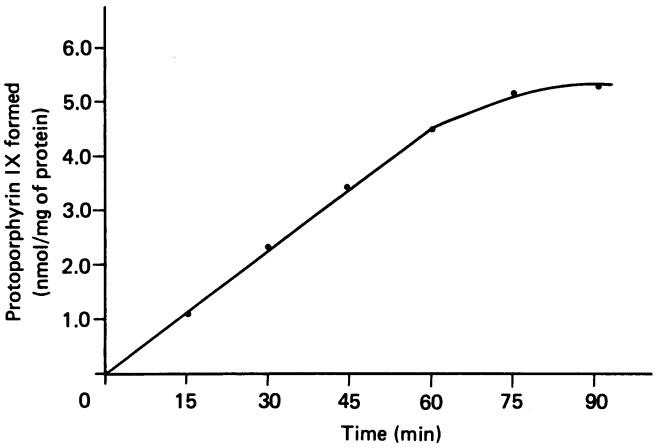

Fig. 4. Time course of protoporphyrinogen formation

The initial concentration of coproporphyrinogen III was 6.0 μM.

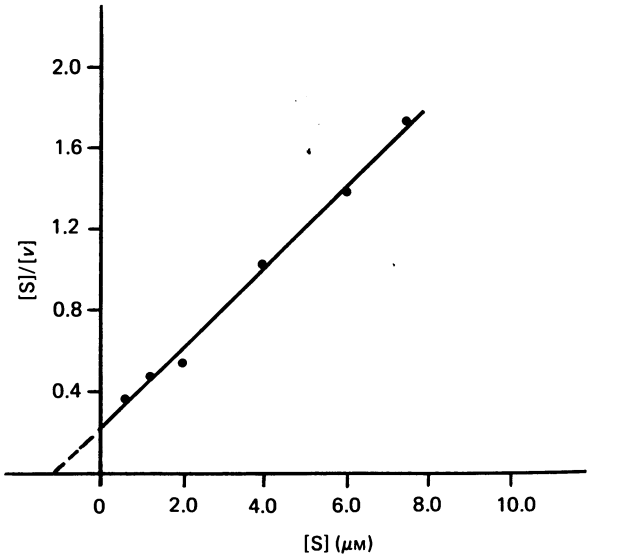

Fig. 5. Coproporphyrinogen oxidase activity at various substrate concentrations (direct linear plot)

v is expressed as nmol of protoporphyrin IX/h per mg of mitochondrial protein.

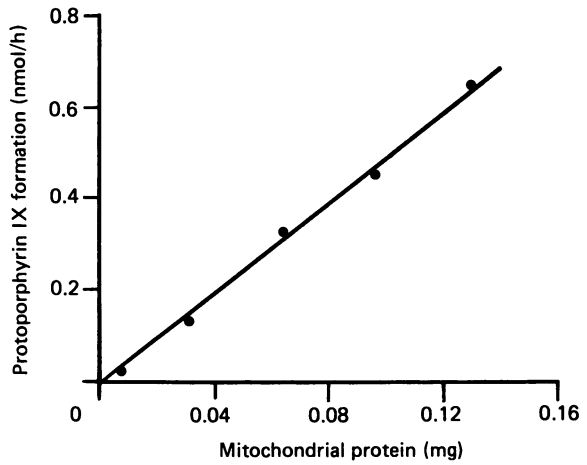

Fig. 6. Relationship between protoporphyrinogen production and amount of mitochondrial protein

containing mesoporphyrin as the internal standard to stop the reaction. The presence of DMSO effectively released protoporphyrin IX from the protein precipitated by trichloroacetic acid, thus achieving simultaneous protein precipitation and quantitative extraction. The supernatant of the extract can be injected directly into the h.p.l.c. for separation and quantification (Fig. 2). $HClO_4$ may replace trichloroacetic acid as the protein precipitant, but a high ratio of DMSO to trichloroacetic acid $HClO_4$ (e.g. 3:2), however, should be avoided, as this resulted in incomplete protein precipitation.

To assess the recovery, a known amount of protoporphyrin IX was added to the incubation mixture and then similarly extracted with 10% trichloroacetic acid/DMSO (1:1 v/v) and analysed by h.p.l.c. The recovery was $101.6 \pm 4.6\%$ (mean $\pm$ S.D., $n = 14$) for 80 μg of mitochondrial protein. For liver homogenate the recovery was over 90% with 3 mg of tissue.

Optimum incubation pH

The optimal activity was obtained when buffers from pH 6.8 to 7.3 were used (Fig. 3). Tris/HCl buffer, pH 7.2, was chosen for our standard assay. At this pH the highest rate of protoporphyrinogen IX formation was reached. This agrees with the pH optimum reported for purified coproporphyrinogen oxidase from rat liver mitochondria by Batlle *et al.* (1965).

Time course of protoporphyrinogen formation

The incubation time course is shown in Fig. 4. Protoporphyrinogen IX formation was linear with time up to 60 min when 160 μg of mitochondrial protein was the enzyme source.

K_m and substrate concentration

The K_m of coproporphyrinogen oxidase was determined with a mitochondrial fraction of rat liver (Fig. 5). The K_m value was estimated by the direct-linear-plot method (Eisenthal & Cornish-Bowden, 1974) and was 1.01 ± 0.23 μM (mean $\pm$ S.D. for three preparations). This is in good agreement with the value (1.21 μM) reported by Elder & Evans (1978*a*). The substrate concentration under standard incubation conditions was 6 μM.

Activities and linearities

Under standard assay conditions the mean ($\pm$S.D.) coproporphyrinogen oxidase activity was 4.07 ± 0.40 nmol of protoporphyrin IX/h per mg of mitochondrial protein for five preparations; the linear range with respect to protein concentration was up to 160 μg per incubation (Fig. 6). For rat liver homogenate the enzyme activity was 224 ± 19 nmol of protoporphyrin IX/h per g of tissue ($n = 7$) and the linearity was satisfactory up to 4 mg of tissue per incubation.

The enzyme activity of rat liver mitochondria obtained by the h.p.l.c. assay described here and that reported by Elder & Evans (1978*b*), 120 pmol of CO_2/min per mg of protein, were similar, but much higher than the value of 0.24 or 1.089 nmol of protoporphyrin IX/h per mg of protein reported by other authors (Batlle *et al.*, 1965; Grandchamp & Nordmann, 1982). These latter values were obtained by methods based on solvent extraction and partition or t.l.c. It is difficult to achieve a high and reproducible recovery of protoporphyrin IX with those techniques.

Conclusion

The h.p.l.c. assay with fluorescence detection is a highly sensitive and specific method for measuring the activity of coproporphyrinogen oxidase. This simple and rapid assay gives high and reproducible recovery of protoporphyrin IX. The method can be applied to the assay of coproporphyrinogen oxidase activity in milligram quantities of needle-biopsy specimens from human liver.

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