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An h.p.l.c. assay for protoporphyrinogen oxidase activity in rat liver

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An h.p.l.c. method is described for the assay of protoporphyrinogen oxidase activity in rat liver. A relatively pure protoporphyrinogen IX substrate was obtained by selectively removing any protoporphyrin IX unreduced by sodium amalgam on a small disposable cartridge packed with a strong anion-exchanger. The protoporphyrin IX formed was extracted with dimethyl sulphoxide/methanol (3:7, v/v) containing mesoporphyrin as the internal standard for separation and quantification by reversed-phase chromatography. The K_m for protoporphyrinogen was 9.5 ± 1.6 μM, and the enzyme activities were 0.59 ± 0.11 nmol of protoporphryin IX produced/min per mg of mitochondrial protein and 33.5 ± 2.7 nmol protoporphyrin IX produced/min per g of liver tissue homogenate. The method is applicable to the determination of enzyme activity in small amounts of human liver biopsy.

INTRODUCTION

Protoporphyrinogen oxidase (EC 1.3.3.4), the penultimate enzyme in the haem-biosynthetic pathway, catalyses the oxidation of protoporphyrinogen IX to protoporphyrin IX by removing six hydrogen atoms from the porphyrinogen nucleus (Jackson *et al.*, 1974; Poulson & Polglase, 1975; Poulson, 1976; Smith *et al.*, 1976). The activity of protoporphyrinogen oxidase has been measured spectrophotometrically (Poulson & Polglase, 1975; Poulson, 1976) and fluorimetrically (Brenner & Bloomer, 1980; Jacobs & Jacobs, 1982). These methods lack specificity and accuracy, as no separation of enzyme reaction product (protoporphyrin IX) from possible interfering substances was carried out. The present paper describes the first chromatographic method for measuring protoporphyrinogen oxidase in rat liver using h.p.l.c. for efficient separation with u.v. or fluorescence detection providing a highly sensitive assay. The precision and accuracy of assay were greatly improved by the inclusion of mesoporphyrin as the internal standard in quantitative measurements. A relatively pure protoporphyrinogen IX substrate was obtained by selectively removing unreduced protoporphyrin IX on a small disposable cartridge packed with a strong anion-exchanger. This led to a much more sensitive assay, as the background was largely eliminated.

EXPERIMENTAL

Materials and reagents

Protoporphyrin IX, mesoporphyrin, BSA, Hepes and mannitol were from Sigma Chemical Co. (Poole, Dorset, U.K.). The concentrations of standard porphyrin solutions were determined spectrophotometrically (Falk, 1964).

Ammonium acetate, ascorbic acid, DMSO, acetic acid, concentrated HCl, trichloroacetic acid, metallic mercury, metallic sodium, NaH_2PO_4, KOH, Tris, sucrose and Triton X-100 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

Fasted adult male Sprague–Dawley rats were anaesthetized by inhalation of methoxyflurane (C-VET Ltd., Bury St. Edmunds, Suffolk, U.K.) and killed. The liver was removed immediately and washed with an ice-cold isolation medium consisting of sucrose (70 mM), D-mannitol (220 mM), Hepes (2.0 mM) and BSA (0.5 mg/ml) prepared as described by Greenawalt (1974). The liver (8 g) was cut into small pieces and homogenized in 16 ml of isolation medium with a glass Potter–Elvehjem homogenizer by five passes of a motor-driven (1000 rev./min) pestle. The homogenate was then diluted with the medium to 0.1 g of liver/ml.

Mitochondria were isolated as described by Greenawalt (1974). The homogenate was centrifuged at 700 *g* for 15 min in a 4 × 100 ml swing-out rotor in a Cool-Spin centrifuge (MSE, Crawley, Sussex, U.K.). The supernatant was centrifuged at 7000 *g* for 15 min in a 8 × 50 ml angle-head rotor in a Hi-Spin 21 MSE centrifuge. The pellet was washed with the isolation medium by means of five strokes of the loose-fitting pestle in a Dounce homogenizer and re-centrifugation as described above. After two washes the mitochondrial pellet was resuspended by means of five strokes of a loose-fitting pestle in 20 mM-sodium phosphate buffer, pH 7.4, containing BSA (0.2 mg/ml). Protein was determined by a modified Lowry method, with bovine serum albumin as standard (Schacterle & Pollack, 1973).

Preparation of protoporphyrinogen IX substrate

Protoporphyrin IX (400 μM) in 0.01 M-KOH containing 20% (v/v) ethanol and sodium amalgam (Na/Hg) were freshly prepared. Sodium amalgam (3 g)

Abbreviations used: BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; a.u.f.s., absorption unit full scale.

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was ground to a fine powder and transferred into 1.5 ml of protoporphyrin IX solution in a tube. The tube was flushed with N_2, stoppered and shaken vigorously for 10 min in the dark. A 1 ml portion of the resulting solution was mixed with 1 ml of N_2-flushed 0.25 M-Tris buffer, pH 7.2, containing 5 mM-EDTA and 0.2 M-ascorbic acid, adjusted to pH 8.5 with 5 M-HCl, and then used immediately in the enzyme assay.

Incomplete reduction of protoporphyrin IX may occur; if this happened, the unchanged protoporphyrin IX was removed by passing the reaction mixture through a small disposable cartridge packed with a strong anion-exchanger (Bond-Elut SAX) from Jones Chromatography (Hengoed, Mid-Glamorgan, Wales, U.K.) or Analytichem International (Harbor City, CA, U.S.A.) as follows. The cartridge was conditioned by washing successively with 1 ml aliquots of methanol, water and 0.25 M-Tris buffer, pH 7.2. The reaction mixture was mixed with equal volume of Tris buffer, pH 7.2, and then loaded on to the cartridge. Protoporphyrin IX was retained on the cartridge, whereas protoporphyrinogen IX passed (under N_2 pressure) straight through with the buffer and was collected. The concentration of protoporphyrinogen IX in the eluate was determined by h.p.l.c. and the solution was used as the enzyme substrate after adjusting it to pH 8.5.

H.p.l.c. of protoporphyrinogen IX and protopophyrin IX

A Varian Associates (Walton-on-Thames, Surrey, U.K.) model-5000 liquid chromatograph was used with a Varian UV-100 variable-wavelength detector. The separation was carried out on a 25 cm × 5 mm stainless-steel column packed with 5 μm Hypersil ODS (Shandon Southern, Runcorn, Cheshire, U.K.). A Rheodyne 7125 injector (Rheodyne, Cotati, CA, U.S.A.) fitted with a 10 μl loop was used for sample injection. Protoporphyrinogen IX and protoporphyrin IX were separated by stepwise elution. The initial eluent was 83% (v/v) methanol in 1 M-ammonium acetate buffer, pH 5.16, with the detector set at 240 nm and 0.05 a.u.f.s. After 7 min the mobile phase was changed to 95% methanol in the buffer. The detector was changed to 400 nm and 0.1 a.u.f.s. after 10 min. Baseline correction was provided by the auto-zero function of the detector. A 6 min re-equilibration was allowed between injections. The flow rate was 1.5 ml/min.

For the separation of protoporphyrin IX and mesoporphyrin (internal standard) in the determination of protoporpyrinogen oxidase, isocratic elution with 88% methanol in the ammonium acetate buffer was used. The detector was set at 400 nm and 0.01 a.u.f.s.

Construction of a calibration curve for the determination of protoporphyrinogen IX concentration by h.p.l.c.

An accurately known concentration (approx. 400 μM) of protoporphyrin IX was prepared as described above. After reduction to protoporphyrinogen IX the concentration of the unchanged protoporphyrin IX was measured by h.p.l.c. from a standard calibration curve of peak heights versus protoporphyrin concentrations. The initial minus the final concentration of protoporphyrin IX is the concentration of protoporphyrinogen IX stock solution, and dilutions (with 0.2 M-Tris buffer, pH 7.2, containing 0.2 M-ascorbic acid) of 1:3, 1:4, 1:6 and 1:12 were made to provide a range of known protoporphyrinogen IX concentrations. By using h.p.l.c., the peak height of each concentration was measured and a calibration curve was constructed and used to determine the protoporphyrinogen IX substrate concentrations in enzyme assays. Protoporphyrin IX was stoichiometrically converted into protoporphyrinogen IX, as no intermediate peaks were detected by h.p.l.c.

Incubation procedures for the assay of protoporphyrinogen oxidase activity

The incubation medium, 50 μl of a 0.25 M-Tris/HCl buffer solution, pH 8.5, containing 5 mM-EDTA, 0.2 M-ascorbic acid and 0.5% (v/v) Triton X-100, was preincubated with 100 μl of enzyme solution (liver homogenate or mitochondrial fraction) for 5 min at 37 °C in a water bath in the dark. A 100 μl portion of protoporphyrinogen IX solution was then added and the mixture was incubated for 10 min without shaking. The enzyme reaction was terminated by the addition of 1 ml of DMSO/methanol (3:7, v/v) containing mesoporphyrin (400 pmol) and vortex-mixed for 10 s. After centrifugation at 1800 *g* for 10 min the supernatant was transferred into a clean tube and flushed with N_2. A 100 μl sample of this solution was injected into the h.p.l.c. for measurement of protoporphyrin IX formed. A blank assay with protoporphyrinogen IX in the incubation medium without enzyme or with an enzyme solution deactivated by boiling was carried out simultaneously to correct for the non-enzymic formation of protoporphyrin IX. Enzyme activity was expressed as nmol of protoporphyrin IX formed/min per mg of protein for mitochondria and as nmol/min per g of liver for homogenates.

RESULTS AND DISCUSSION

Preparation and purification of protoporphyrinogen IX

The major problem in the assay of protoporphyrinogen oxidase is the difficulty in preparing a substrate relatively free of protoporphyrin IX. The reduction of protoporphyrin IX with sodium amalgam is often incomplete and variable. Substrate containing excessing protoporphyrin IX is unsatisfactory for the accurate quantification of protoporphyrin IX formed and may also inhibit the enzyme. Complete reduction of protoporphyrin IX is possible by prolonging the reaction time and/or at higher reaction temperatures. This, however, may lead to the formation of undesired by-products of an unknown nature.

Protoporphyrinogen IX and protoporphyrin IX can be easily separated by h.p.l.c. (Fig. 1). The method, however, was not adaptable to the preparative isolation of proporphyrinogen IX, because the eluent contained a high proportion of methanol. Removal of methanol by evaporation of extracting protoporphyrinogen IX with an organic solvent resulted in substantial autoxidation to protoporphyrin IX. Since h.p.l.c. purification of protoporphyrinogen IX was impractical, we therefore investigated the possibility of removing protoporphyrin IX selectively by solid-phase sorption. Of the wide range of sorbents examined, including alumina, silica, talc and the bonded reversed phases, only the strong anion-exchanger was found to be suitable. When a solution of protoporphyrin IX and protoporphyrinogen IX was loaded on to a small cartridge packed with a strong anion-exchanger (Bond-Elut SAX), the former was almost totally 'sorbed', whereas the latter passed

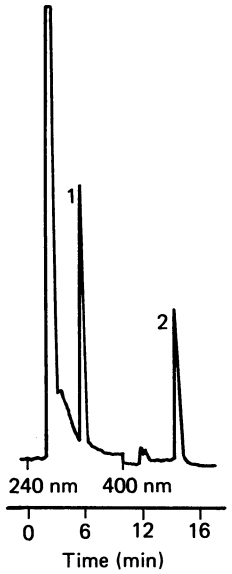

Fig. 1. H.p.l.c. chromatogram for the separation of protoporphyrinogen IX (peak 1) and protoporphyrin IX (peak 2)

The preparation 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 the eluent for 7 min, which was then changed to 95% methanol in the buffer. The flow rate was 1.5 ml/min. The detector was set at 240 nm for 10 min and then automatically changed to 400 nm.

straight through with the loading buffer. This simple and rapid procedure allows protoporphyrinogen IX virtually free from protoporphyrin IX contamination to be obtained. As protoporphyrinogen IX was collected in the buffer used for enzyme assay (apart from pH adjustment), the possibility of autoxidation was minimized. The recovery of protoporphyrinogen IX by this process was greater than 70%.

Recovery of protoporphyrin IX from the incubation mixture

Protoporphyrin IX has a tendency to be adsorbed strongly to proteins. In the method for coproporphyrinogen oxidase determination, we described (Li *et al.*, 1986) the use of 10% (w/v) trichloroacetic acid/DMSO (1:1, v/v) for the simultaneous precipitation of proteins (by trichloroacetic acid) and extraction of protoporphyrin IX (by DMSO). This method is unsuitable here, as trichloroacetic acid promotes the autoxidation of protoporphyrinogen IX. A neutral protein precipitant is therefore required. We have achieved effective protein precipitation and protoporphyrin IX release with methanol mixed with 20–60% (v/v) DMSO. In the standard assay, 1 ml of methanol/DMSO (7:3, v/v), containing mesoporphyrin (400 pmol), was added. The proteins were removed by centrifugation and the supernatant was analysed by h.p.l.c. (Fig. 2). The internal standard (mesoporphyrin) corrects for sampling errors. The recovery of 1.2 nmol of protoporphyrin IX after the whole incubation and termination procedures was 101.3 ± 3.3% (mean ± S.D., $n = 15$) for 100 μg of mitochondrial protein and 95.2 ± 2.2% ($n = 10$) for 2 mg of liver tissue homogenate.

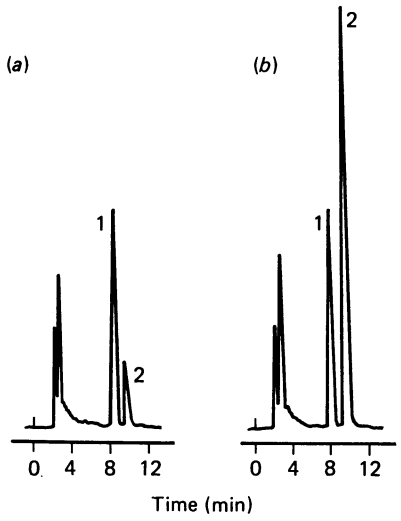

Fig. 2. H.p.l.c. chromatogram for the determination of protoporphyrinogen oxidase activity in rat liver homogenate

(*a*) Blank incubation of protoporphyrinogen IX in medium without enzyme; (*b*) enzyme incubation mixture. The column was 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 was set at 400 nm, 0.01 a.u.f.s. Peaks: 1, mesoporphyrin; 2, protoporphyrin IX.

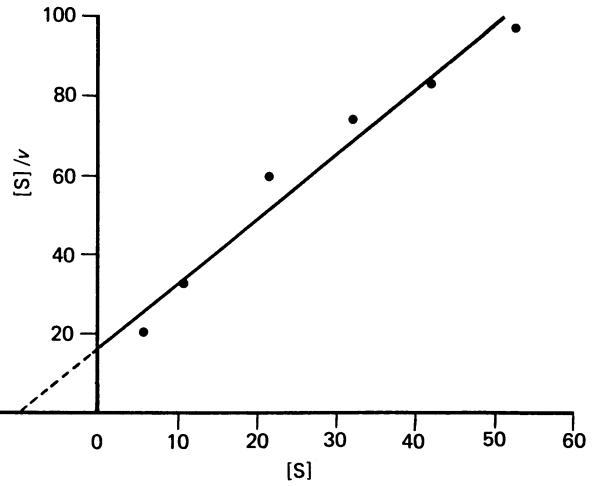

Fig. 3. Protoporphyrinogen oxidase activity at various substrate concentrations (direct linear plot)

Velocity (v) is expressed as nmol of protoporphyrin IX/min per mg of mitochondrial protein, and substrate concentration ([S]) is expressed in μM.

Autoxidation control and correction for non-enzymic formation of protoporphyrin IX

In the protoporphyrinogen oxidase assay, autoxidation of protoporphyrinogen IX to protoporphyrin IX always occurs and must be minimized and corrected for. The presence of about 40 mM-ascorbic acid in the incubation mixture significantly decreased autoxidation of protoporphyrinogen IX. The enzyme reaction was rapid and linear up to 10 min. This was followed by a slow, but gradual, increase in activity as the O_2 partial pressure in the incubation mixture decreased. There is therefore no

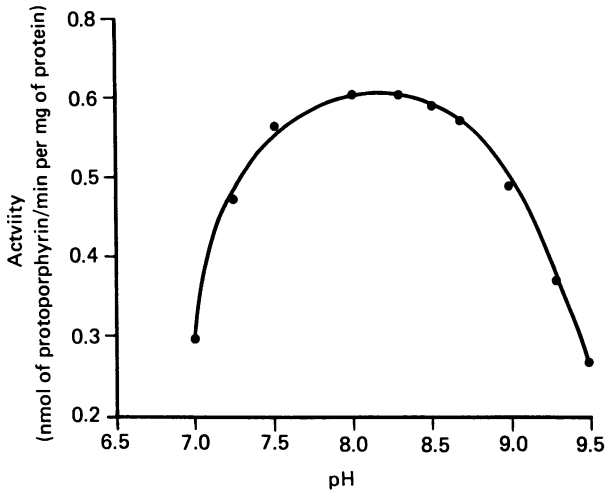

Fig. 4. pH–activity graph for protoporphyrinogen oxidase in a mitochondrial fraction in 0.25 M-Tris/HCl buffers

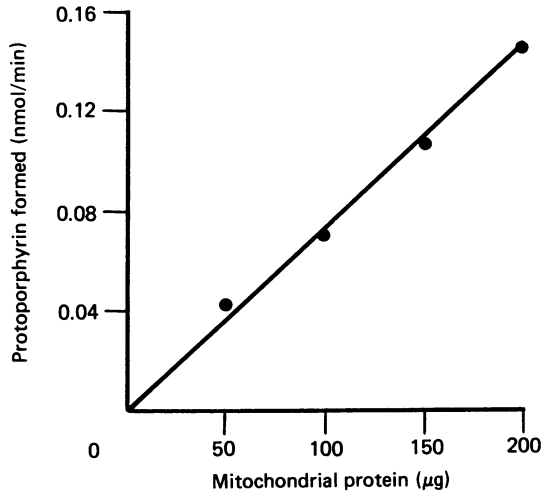

Fig. 5. Relationship between protoporphyrin IX produced and amount of mitochondrial protein

advantage in using a longer reaction time. A short incubation time (10 min) coupled with the use of neutral organic solvents for terminating the reaction also minimize the extent of autoxidation. Furthermore, all solutions for h.p.l.c. separation were transferred into clean tubes, thoroughly flushed with N_2 and then kept in the dark to inhibit autoxidation. In spite of the above precautions, however, some autoxidation did still occur, and this has to be corrected for by setting up parallel blank assays. The rate of autoxidation was 4.0 ± 1.0 pmol/min (mean $\pm$ S.D.; $n = 25$) in the present assay.

K_m and pH optimum

The K_m of protoporphyrinogen oxidase was determined with the mitochondrial fraction of rat liver (Fig. 3), and a value of 9.5 ± 1.6 μM (mean $\pm$ S.D.) for three preparations was obtained. This agrees with the value (11 μM) obtained with partially purified protoporphyrinogen oxidase (Poulson, 1976). Protoporphyrinogen oxidase is active over the pH range 7.0–9.5. The optimum pH was between 7.5 and 8.7 in Tris/HCl buffer (Fig. 4), a pH of 8.5 being chosen for the standard assay.

Activity and linearity of assay

Under the standard assay conditions the protoporphyrinogen oxidase reaction was rapid, and only a 10 min incubation time was necessary. The activity of protoporphyrinogen oxidase in rat liver was 0.59 ± 0.11 nmol of protoporphyrin IX/min per mg of mitochondrial protein for five preparations. The concentration of protoporphyrin IX formed was proportional to the amount of enzyme up to 200 μg of mitochondrial protein per incubation (Fig. 5). For rat liver homogenate the activity was 33.5 ± 2.7 nmol of protoporphyrin IX/min per g of tissue, and the linear range is for up to 5 mg of tissue.

The activity of protoporphyrinogen oxidase obtained by this method is three to four times higher than that reported previously (Poulson, 1976; Jacobs & Jacobs, 1982). This is most probably attributable to the much purer protoporphyrinogen IX substrate being used. Thus possible enzyme inhibition by excess protoporphyrin IX is eliminated, and a 'clean' background allows high-sensitivity detection of the protoporphyrin IX formed. Replacing glutathione or dithiothreitol with ascorbate in the enzyme assay may also have contributed to increased sensitivity, since thiol-reducing agents are known to react with the vinyl groups of protoporphyrinogen IX (Sano & Granick, 1961) and to inhibit protoporphyrinogen oxidase activity (Poulson, 1976).

Conclusions

H.p.l.c. with u.v. detection is a highly sensitive and specific method for measuring protoporphyrinogen oxidase activity. Under identical assay conditions the sensitivity can be further improved by using fluorescence instead of spectrophotometric detection. The assay is applicable to the determination of the enzyme in milligram quantities of human liver biopsy.

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