Journal of Chromatography, 566 (1991) 383–396
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5809

High-performance liquid chromatographic assays for protoporphyrinogen oxidase and ferrochelatase in human leucocytes

RONG GUO, C. K. LIM* and T. J. PETERS

Division of Clinical Cell Biology, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ (U.K.)

ABSTRACT

Rapid, sensitive and specific high-performance liquid chromatographic assays are described for protoporphyrinogen oxidase and ferrochelatase in human leucocytes. The enzyme reaction products were separated and quantitated by reversed-phase high-performance liquid chromatography with fluorescence detection. The optimal pH for the protoporphyrinogen oxidase assay was 8.6 and the Michaelis constant for protoporphyrinogen IX was 9.78 ± 0.96 μM (mean ± S.D.). The mean (± S.D.) activity of protoporphyrinogen oxidase in fourteen apparently healthy subjects was 0.146 ± 0.023 nmol protoporphyrin IX per min per mg protein. In one patient with variegate porphyria, the activity was 0.028 nmol protoporphyrin IX per min per mg protein. The optimal pH for ferrochelatase was 7.4 and with protoporphyrin and Zn^{2+} as substrates, the Michaelis constants were 1.49 and 8.33 μM, respectively. The mean activity of ferrochelatase in ten control subjects was 0.24 nM Zn–protoporphyrin or 2.05 nM Zn–mesoporphyrin formed per h per mg protein.

INTRODUCTION

Protoporphyrinogen oxidase (EC 1.3.3.4) catalyses the conversion of protoporphyrinogen IX to protoporphyrin IX by removing six hydrogen atoms from the protoporphyrinogen IX nucleus. Ferrochelatase (EC 4.99.1.1) catalyses the next step of reaction, by inserting Fe^{2+} into the protoporphyrin IX to form haem. Protoporphyrinogen oxidase activity has been studied in yeast [1–3], bacteria species [4], rat liver [5–7], human leucocytes [8], lymphocytes [9–11], liver [12] and skin fibroblasts [13]. Ferrochelatase has been assayed in tissues such as liver homogenates [14–20], yeast [21], *Rhodopseudomonas sheroids* [22], human bone marrow and reticulocytes [23], cultured skin fibroblasts [24], lymphocytes [11,25] and leucocytes [8].

No high-performance liquid chromatographic (HPLC) method has been described for measuring protoporphyrinogen oxidase activity in leucocytes and few have been reported for ferrochelatase. The majority of assays described for these two enzymes were based on spectrophotometry, fluorimetry or radioactivity mea-

0378-4347/91/$03.50

surements which lack specificity and hence accuracy. The present paper describes novel HPLC procedures for the determination of protoporphyrinogen oxidase and ferrochelatase activities in human leucocytes.

These two assays are based on the similar HPLC methods described for rat liver homogenates with suitable modifications for leucocytes, which contain much less enzymes than the mitochondria-rich liver cells [7,26].

EXPERIMENTAL

Reagents and chemicals

Protoporphyrin IX, mesoporphyrin, deuteroporphyrin, bovine serum albumin (BSA), dextran (relative molecular mass, M_r 500 000), palmitic acid and glutathione were from Sigma (Poole, U.K.). The concentrations of porphyrin standard solutions were determined spectrophotometrically [27,28].

Ammonium acetate, acetic acid, concentrated HCl, NaOH, KOH, $KHCO_3$, EDTA (disodium salt), zinc acetate, sucrose, dimethyl sulphoxide (DMSO), metallic mercury, metallic sodium, ascorbic acid, Na_2CO_3, potassium sodium tartrate, $CuSO_4$, Folin-Ciocalteu phenol reagent, NH_4Cl and Triton X-100 were from BDH (Poole, U.K.). Tris(hydroxymethyl)aminomethane (Tris) was from Boehringer Mannheim (Lewes, U.K.). Methanol and ethanol (HPLC grade) were from Rathburn (Walkerburn, U.K.). Tween-20 was from Koch-Light Labs. (Colnbrook, U.K.). Heparin was from Paines & Byrne (Greenford, U.K.).

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph was used with a Perkin Elmer (Beaconsfield, U.K.) LS-3 fluorescence detector. A Rheodyne (Cotati, CA, U.S.A.) 7125 injector fitted with a 200-μl loop was used for sample injection. The separation was carried out on a 250 mm × 5.0 mm I.D. Hypersil ODS (5 μm) column (Shandon Scientific, Runcorn, U.K.).

Preparation of human leucocytes

Mixed peripheral blood leucocytes were isolated by erythrocyte sedimentation and NH_4Cl lysis. Heparinised blood (20 ml) was added to an equal volume of 3% dextran solution (M_r 500 000), mixed and centrifuged at 2100 *g* for 10 min at 4°C in a Coolspin centrifuge (MSE, Crawley, U.K.). The leucocyte layer was transferred to another tube and 7–8 ml ice-cold NH_4Cl buffer (8.3 g NH_4Cl, 1 g $KHCO_3$ and 0.04 g EDTA per l) were added. The red cells were lysed by standing on ice for 7 min. The leucocytes were collected by centrifugation as described above and were washed thrice with ice-cold 0.15 *M* NaCl. If red cell contamination was not completely eliminated, NH_4Cl buffer lysis was repeated. The leucocytes were then resuspended in 0.25 *M* sucrose containing 0.05 *M* Tris buffer, pH 7.4, and 5 U of heparin per 20 ml blood. The leucocytes were stored at −30°C if not used immediately. The protein was measured by a modified Lowry method [29] with BSA as standard.

Preparation of protoporphyrinogen IX substrate

A 1.5-ml sample of protoporphyrin IX (200 μM) in freshly prepared 0.01 *M* KOH containing 20% (v/v) ethanol was reduced with freshly prepared powdered 5% (w/w) sodium amalgam (1.5 g) in a 10-ml tube in the dark. This tube was flushed with nitrogen, stoppered and shaken vigorously until little or no UV fluorescence was detected (about 8 min). The resulting solution was mixed with an equal volume of nitrogen-flushed, ice-cold 0.25 *M* Tris buffer containing 5 m*M* glutathione and 5 m*M* EDTA, adjusted to pH 8.6 with 5 *M* HCl. The mixture was transferred to a 10-ml tube, flushed with nitrogen, stoppered and stood on ice in the dark until use (up to 10 min).

If incomplete reduction of protoporphyrin IX occurred, the unreacted protoporphyrin IX may be removed as previously described [7] with a small disposable cartridge packed with a strong anion exchanger (Bond-Elut SAX) from Jones Chromatography (Hengoed, U.K.) or Analytichem International (Harbor City, CA, U.S.A.). The cartridge was conditioned by washing successively with 1-ml aliquots of methanol, water and 0.25 *M* Tris buffer before sample loading. The reaction mixture was mixed with an equal volume of 0.25 *M* Tris buffer containing 5 m*M* EDTA, 5 m*M* glutathione, final pH 9–10, then loaded onto the cartridge. Under nitrogen pressure, protoporphyrinogen IX passed straight through with the buffer and was collected, while protoporphyrin IX was retained on the cartridge. The protoporphyrinogen solution was adjusted to pH 8.6 with 5 *M* HCl and was used immediately. The concentration was determined by HPLC [7].

Incubation procedure for protoporphyrinogen oxidase assay

The incubation mixture [100 μl leucocyte suspension (about 0.5 mg protein), 50 μl of 0.25 *M* Tris–HCl buffer, pH 8.6, containing 5 m*M* EDTA, 5 m*M* glutathione and 1% Tween-20 (w/v)] was preincubated in a stoppered 10-ml tube for 5 min at 37°C in the dark. The reaction was started by adding 100 μl (approximately 35 μM) protoporphyrinogen IX substrate and incubation was continued for another 10 min in the dark without shaking. The reaction was terminated by vortex-mixing with 1 ml of ice-cold methanol–DMSO (8:2, v/v) containing 42 n*M* mesoporphyrin as internal standard and cooled on ice. After centrifugation at 2100 *g* for 10 min at 4°C the supernatant was flushed with nitrogen, stoppered and kept on ice in the dark till HPLC analysis. A parallel blank incubation with boiled leucocyte suspension was set up for all assays to correct for non-enzymic formation of protoporphyrin IX due to auto-oxidation.

HPLC conditions for measurement of protoporphyrinogen oxidase activity

The supernatant, 200 μl, was injected into the HPLC system. The mobile phase was methanol–1 *M* ammonium acetate buffer, pH 5.16 (86:14, v/v). The flow-rate was 1.5 ml/min. The detector was set at excitation and emission wavelengths of 400 and 618 nm, respectively.

A calibration curve for protoporphyrin IX was constructed by plotting the

peak-height ratios of protoporphyrin IX to mesoporphyrin (internal standard) against the concentrations of protoporphyrin IX standard. Protoporphyrinogen oxidase activity was expressed as nmol of protoporphyrin IX formed per min per mg leucocyte protein.

Preparation of Zn–protoporphyrin, Zn–mesoporphyrin and Zn–deuteroporphyrin standards

The porphyrin was dissolved in a minimum amount of DMSO and thoroughly mixed with saturated zinc acetate solution in methanol. The completion of the reaction was monitored by HPLC. When no free porphyrin was detected, sufficient amount of distilled water was added to the mixture to precipitate the Zn complex. The precipitate was washed thoroughly with water to remove the remaining zinc acetate. After centrifugation, the supernatant was discarded and the residue dried in a vacuum desiccator. This can be kept or redissolved in DMSO when it is needed.

Determination of the Zn–porphyrin concentration

The concentration of the zinc complex was determined by demetallation in 2.7 *M* HCl followed by assay of the concentration of the free porphyrin formed [28]. Zn–mesoporphyrin, Zn–protoporphyrin and Zn–deuteroporphyrin in DMSO were diluted with 2.7 *M* HCl (1:10 to 1:50) and kept in the dark for 10 min. After the completion of the reaction, meso- and deuteroporphyrin solutions were further diluted with water to a final concentration of 0.1 *M* HCl. Then the concentrations of meso-, proto- and deuteroporphyrin were measured spectrophotometrically.

Preparation of the substrates for ferrochelatase

Zinc acetate stock solution (2 m*M*) was prepared by dissolving the salt in water. Protoporphyrin IX and mesoporphyrin IX were freshly prepared just before the assay. An appropriate amount of porphyrin was dissolved in 0.01 *M* freshly prepared KOH; the concentration was determined spectrophotometrically after acidification with HCl.

Incubation procedure for ferrochelatase assay

The standard incubation mixture consisted of 100 μl of 0.25 *M* Tris HCl buffer [pH 7.4, containing 1.75 m*M* palmitic acid and 1% (w/v) Tween-20], 50 μl of enzyme preparation (approximately 0.5 mg of protein) and 50 μl of 80 or 100 μM zinc acetate solution, respectively, when meso- or protoporphyrin were used as cosubstrate. The mixture was preincubated for 5 min at 37 °C. The reaction was started by adding 50 μl of 100 μM mesoporphyrin or protoporphyrin. The incubation was continued for 30 min at 37°C in the dark. The reaction was stopped by adding 1 ml of ice-cold methanol DMSO (8:2, v/v) containing 13 n*M* Zn deuteroporphyrin as internal standard and cooled on ice. After centrifuga-

tion at 2100 *g* for 10 min at 4°C, the supernatant was ready for analysis by HPLC. The non-enzymic formation of metal chelate was corrected by a parallel blank with boiled, inactivated enzyme or with sucrose in place of the enzyme solution (0.25 *M* sucrose containing 0.02 *M* Tris–HCl buffer, pH 7.4).

Separation of enzyme reaction product in ferrochelatase assay

The HPLC conditions were the same as those described for protoporphyrinogen oxidase, apart from the detector wavelengths which were 403 and 574 nm, for excitation and emission respectively, for Zn–mesoporphyrin and Zn–deuteroporphyrin when mesoporphyrin was used as the substrate and 410 and 590 nm, respectively, for Zn–protoporphyrin and Zn–deuteroporphyrin when protoporphyrin was used as the substrate. A similar calibration curve for Zn–porphyrin was constructed as described for protoporphyrinogen oxidase. Ferrochelatase activity was expressed as nmol of Zn–porphyrin formed per h per mg leucocyte protein.

RESULTS AND DISCUSSION

Preparation of protoporphyrinogen IX substrate

Relatively pure protoporphyrinogen IX is difficult to obtain because complete reduction of protoporphyrin IX to protoporphyrinogen IX is difficult to achieve and the latter is also very easily auto-oxidised. Excess protoporphyrin IX left in the substrate solution leads to an unacceptable high background and may also inhibit the enzyme activity. Protoporphyrinogen IX has traditionally been prepared from a stock solution of protoporphyrin in 0.01 *M* KOH containing 20% (v/v) ethanol. This solution was reduced with freshly prepared 3% (w/w) sodium amalgam and then the pH was adjusted to 7.5–8.0 with 40% phosphoric acid [1,30]. The yield of protoporphyrinogen formed by this method was approximately 70 80%. When incomplete reduction occurred, removal of excess protoporphyrin IX with a Bond Elut SAX cartridge was necessary [7]. This gives a relatively pure substrate solution for the assay. Protoporphyrinogen may also be generated enzymatically but it involves an addition step of enzyme reaction.

Recovery of protoporphyrin IX from the incubation mixture

Protoporphyrin IX adsorbs strongly to protein. For quantitation by HPLC, protoporphyrin IX must be extracted from the protein precipitate. Trichloroacetic acid is a common precipitant for protein and was successfully used in conjunction with DMSO to extract protoporphyrin IX from protein in the assay of coproporphyrinogen oxidase [31,32]. This extractant is, however, not suitable for the extraction of protoporphyrin IX in the protoporphyrinogen oxidase assay because under acid conditions auto-oxidation of protoporphyrinogen IX in the reaction mixture occurred rapidly. A neutral protein precipitant was needed and 80% (v/v) methanol in DMSO was found to be effective for the simultaneous

precipitation of protein and extraction of protoporphyrin IX from the reaction mixture. Mesoporphyrin in the terminating reagent was used as an internal standard to correct for dilution and sampling errors. A mean (± S.D.) recovery of 96.9 ± 4.2% (n = 9) was obtained when 0.3 nmol of protoporphyrin IX was added to 2 mg of protein followed by the assay and extraction procedures.

The HPLC separation of protoporphyrin IX formed in the incubation mixture is shown in Fig. 1.

Effect of reducing agent on protoporphyrinogen oxidase activity

In the protoporphyrinogen oxidase assay, autooxidation of protoporphyrinogen IX to protoporphyrin IX always occurs to some extent and a reducing agent must be used to minimise this effect. Ascorbic acid (0.04 mM) in the incubation mixture was shown to significantly decrease auto-oxidation of protoporphyrinogen IX and 0.08 M was used in the assay for rat liver protoporphyrinogen oxidase [7]. However, when used in the assay of protoporphyrinogen oxidase in human leucocytes, it was found to give very low enzyme activity. Thiol-reducing agents were reported to increase protoporphyrinogen oxidase activity at low concentration but inhibited the activity at concentrations above 5 mM [5]. The inhibitory effect may be due either to competition with the thiol group in the enzyme mole-

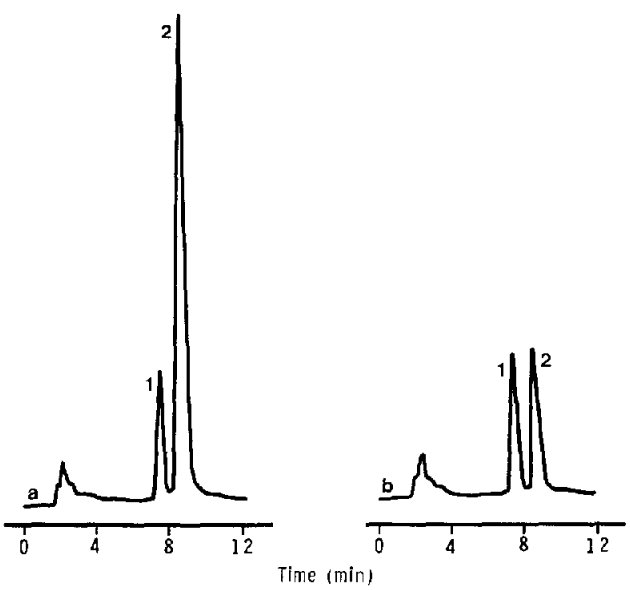

Fig. 1. HPLC for the determination of protoporphyrinogen oxidase activity in human leucocytes. (a) Enzyme incubation mixture; (b) blank incubation with boiled enzyme. Column, ODS-Hypersil (250 mm × 5.0 mm I.D.); eluent, 88% (v/v) methanol in 1 M ammonium acetate, pH 5.16. Flow-rate, 1.5 ml/min; fluorescence detection, excitation at 400 nm and emission at 618 nm. Peaks: 1 = mesoporphyrin (internal standard); 2 = protoporphyrin IX.

cule for the substrate or to direct binding with the vinyl groups of protoporphyrinogen IX, thereby diminishing the substrate concentration [5]. In the present assay, a much higher activity was obtained when buffer containing 5 m*M* glutathione instead of 0.2 *M* ascorbic acid was used (the final concentrations in the incubation mixture are 2 m*M* and 0.08 *M*, respectively). Another thiol-reducing agent, dithiothreitol, was tried but very low activity was obtained. The effect of glutathione, dithiothreitol and ascorbic acid on protoporphyrinogen oxidase activity is shown in Table I.

Optimum pH for protoporphyrinogen oxidase assay

Under acid conditions, auto-oxidation of protoporphyrinogen IX is very fast. If the assay is carried out below pH 7.0, the protoporphyrin IX formed was largely caused by auto-oxidation, resulting in a high blank. The optimal pH was 8.6, close to the value (pH 8.7) reported for human lymphocytes [9], fibroblasts [13] and leucocytes [8], but different from the value of pH 7.2 for human liver reported by Camadro *et al.* [12].

Time course and linearity of protoporphyrinogen oxidase assay

Protoporphyrin IX formation increased linearly up to 10 min under the present assay conditions. After 10 min, protoporphyrin IX production slowed down as the oxygen tension in the incubation medium dropped. This is because the enzyme requires oxygen for activity. There is, therefore, no advantage in prolonging the incubation time as this does not increase the activity and can actually lead to higher blank values because of auto-oxidation.

Protoporphyrin IX production was also proportional to leucocyte protein concentration up to 1 mg, and 0.5 mg protein was used in the present assay.

TABLE I

EFFECT OF REDUCING AGENTS ON PROTOPORPHYRINOGEN OXIDASE ACTIVITY

Reducing agent	Concentration in incubation mixture (m*M*)	Activity (%)
Glutathione	3	100
	1.2	22.6
	5	73.8
Dithiothreitol	1.8	24.1
	3	9.6
	5	10.1
Ascorbic acid	60	6.9
	90	7.9
	120	14.2
	240	6.2

Michaelis constants (K_M) of protoporphyrinogen oxidase and substrate concentration

Kinetic constants were calculated from the direct linear-plot method [33]. The K_M for protoporphyrinogen was 9.78 ± 0.96 μM (mean ± S.D. for three preparations). Because of the difficulty in reducing a high concentration of protoporphyrin IX, 200 μM protoporphyrin IX was used in the reduction step with a resulting substrate concentration in the incubation mixture of approximately 35 μM.

Activity of protoporphyrinogen oxidase

Leucocyte protoporphyrinogen oxidase was assayed in fourteen apparently healthy persons, between the age of 20 and 57 years. The mean activity was 8.73 nmol of protoporphyrin IX formed per h per mg protein. No significant differences between ages and sexes were found. A 2 S.D. reference range was established as 4.36–13.10 nmol protoporphyrin IX per h per mg protein. A leucocyte sample was assayed six times, and the mean was 8.75 ± 1.38 nmol/h/mg protein.

The activities of protoporphyrinogen oxidase in various human tissues are summarized in Table II. The activity using the present HPLC method was similar to that in liver, higher than those in lymphocytes and fibroblasts, but much lower than that in leucocytes obtained by the method of Viljoen *et al.* [8]. The enzyme activity is stable for up to six months when stored at −30°C.

Selection of substrates for ferrochelatase assay

Other bivalent metal ions such as Zn^{2+}, Co^{2-}, Cu^{2+} and Mn^{2+} are also substrates for ferrochelatase. The natural substrate, Fe^{2+}, is easily oxidised to Fe^{3+}, which is not a substrate for ferrochelatase but a strong inhibitor [21]. Thus, with Fe^{2+} as the substrate the assay must be carried out under strictly anaerobic conditions. Zn^{2+} is one of the alternative and effective substrates for ferrochela-

TABLE II

PROTOPORPHYRINOGEN OXIDASE ACTIVITY IN VARIOUS HUMAN TISSUES

Tissue	Activity (nmol protoporphyrin/h/mg protein)	Ref.
Lymphocyte	6.20	3
	4.39	11
	0.82	9
Leucocyte	820	8
	8.73	Present method
Fibroblast	1.97	13
Liver	8.30	12
	10.8	20

tase and is also stable to oxidation. Assays with Zn^{2+} as substrate can therefore be carried out under aerobic conditions. Zn^{2+} has also been shown to be the main endogenous metal ion entrapped within the mitochondrial membranes [19,21]. In patients with iron deficiency aneamia and lead intoxication, Zn^{2+} can be chelated into protoporphyrin *in vivo* [34,35]. Thus, Zn^{2+} has greater pathophysiological relevance in porphyrin metabolism than other non-iron metals. Furthermore, Zn–porphyrins are fluorescent, which makes the direct and highly sensitive detection of the enzymic product possible. Zn–porphyrin can also be easily separated from haemin by HPLC.

Apart from protoporphyrin, other decarboxylic porphyrins are also substrates for ferrochelatase. Among them, mesoporphyrin and deuteroporphyrin show activities which are several times greater than that of protoporphyrin [15,18]. Mesoporphyrin was therefore chosen for the present assay. However, if excessive endogenous protoporphyrin is present in the enzyme preparation, as in erythrohepatic protoporphyria, protoporphyrin IX should be used as the substrate.

Effects of fatty acids and detergents on ferrochelatase activity

Fatty acids play an important role in ferrochelatase activity [36–39]. In crude enzyme preparation, the activity was stimulated about three-fold by fatty acids, while in purified enzyme preparation a fifteen-fold increase was obtained. This may be because of the presence of endogenous fatty acids in the crude enzyme preparation. It was also found that the effect of the stimulation was varied when the position of double bond in the C_{18} acid was changed, as well as when the fatty acid carbon chain length was altered [39]. The stimulation was specific for fatty acids because oleic acid methyl ester, oleyl alcohol and short-chain carbonic acid had no effects. This strongly suggested that the carboxyl groups of fatty acids provide suitable conditions for the approach of porphyrin molecules to the catalytic site in ferrochelatase [39]. In the present study, palmitic acid was used in the reaction. As previously reported [26], maximum activity was obtained when 0.25 *M* Tris–HCl buffer, pH 7.4, containing both palmitic acid and detergent was used. Palmitic acid not only stimulates the activity but also diminishes the non-enzymic formation of Zn–porphyrin in the presence of a detergent [26]. The detergent Tween-20 used in the present assay has several functions: (1) to prevent porphyrin from forming aggregates [40]; (2) to keep palmitic acid in solution [26]; (3) to inhibit the non-enzymic formation of Zn–porphyrin [15,39,41]; and (4) to release enzyme from the mitochondrial membrane [42], thus increasing the activity. Although 0.5% Tween-20 gave the highest activity, it was unable to keep palmitic acid completely in solution: 1% Tween-20 and 1.75 m*M* palmitic acid were therefore chosen in the standard assay. Triton X-100, 1% (w/v), was also effective. It was reported recently that Tris–HCl buffer combined with Bicine buffer could also reduce the non-enzymic formation of Zn–porphyrin [25].

Recovery of Zn–porphyrin from enzyme incubation mixture

To quantitate Zn porphyrin by HPLC, the important step is to release Zn–porphyrin effectively from the protein. Zn–porphyrin is very easily demetallated under mild acid conditions, and a neutral extractant is therefore essential. As with the assay of protoporphyrinogen oxidase, DMSO–methanol (2:8, v/v) containing the internal standard Zn–deuteroporphyrin was used to stop the reaction. Under neutral extraction conditions, a haemin-free solution can be obtained [26,43]. To check the extraction efficiency, Zn–mesoporphyrin (0.282 n*M*) or Zn–protoporphyrin (0.216 n*M*) was added to the incubation mixture followed by the same incubation and extraction procedures as described for the enzyme assay. The recovery of Zn–mesoporphyrin in the presence of 0.5 mg leucocyte protein was 98.5 ± 0.01% (n = 12, mean ± S.D.). The recovery of Zn–protoporphyrin was 101.6 ± 2.98% (n = 10, mean ± S.D.). The internal standard further improved the precision and accuracy of the assay. Figs. 2 and 3 are the chromatograms for the measurement of ferrochelatase activities in leucocytes with mesoporphyrin and protoporphyrin as substrates, respectively.

Optimum pH for ferrochelatase assay

The highest activity of leucocyte ferrochelatase was found when the pH of the Tris–HCl buffer was 7.4. This is the same value as that reported for human bone

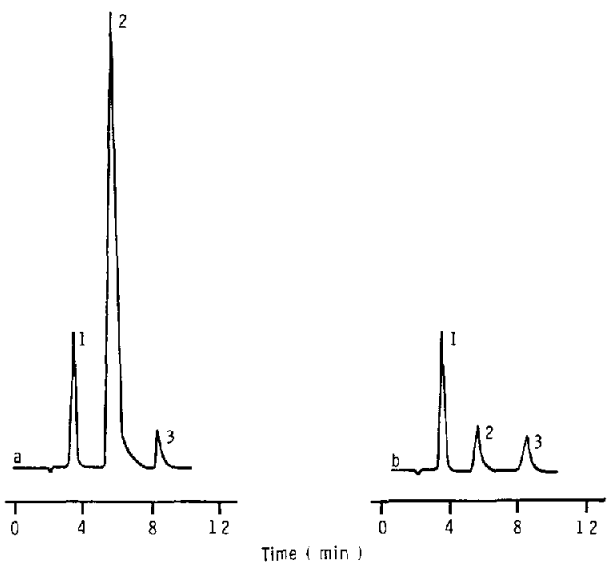

Fig. 2. HPLC for the measurement of ferrochelatase activity in human leucocytes with mesoporphyrin and Zn^{2+} as substrates. (a) Enzyme incubation mixture; (b) blank incubation with boiled leucocytes. Column, ODS-Hypersil (250 mm × 5.0 mm I.D.); eluent, 88% (v/v) methanol in 1 *M* ammonium acetate, pH 5.16. Flow-rate, 1.5 ml/min; fluorescence detection, excitation at 403 nm and emission at 574 nm. Peaks: 1 = Zn–deuteroporphyrin (internal standard); 2 = Zn–mesoporphyrin; 3 = mesoporphyrin.

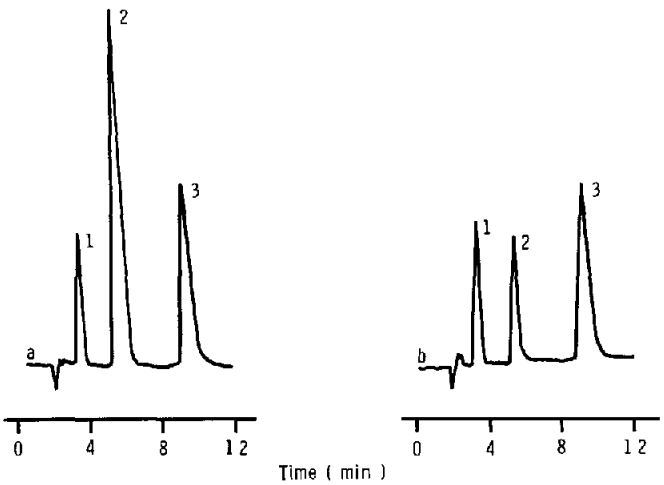

Fig. 3. HPLC for the measurement of ferrochelatase activity in human leucocytes with protoporphyrin and Zn^{2+} as substrates. (a) Enzyme incubation mixture; (b) blank incubation with boiled leucocytes. Column, ODS-Hypersil (250 mm × 5.0 mm I.D.); eluent, 88% (v/v) methanol in 1 *M* ammonium acetate, pH 5.16. Flow-rate, 1.5 ml/min; fluorescence detection, excitation at 410 nm and emission at 590 nm. Peaks: 1 = Zn–deuteroporphyrin (internal standard); 2 = Zn–protoporphyrin; 3 = protoporphyrin.

marrow [44] and similar to the values of pH 7.5 for human skin fibroblasts [15], 7.3 for lymphocytes [11] and 7.6 for liver [39], but lower than the value of 8.0 for lymphocytes reported by Rossi *et al.* [25]. This may be caused by the use of Tris–HCl combined with Bicine buffer in their assay.

Kinetic constants of ferrochelatase

It was reported that the chelation of metals into porphyrin was a random bi–bi mechanism [19,21,22,45,46]. In the present assay, the kinetic parameter was calculated from direct linear plots [33] and double reciprocal plots. The K_M for protoporphyrin was 1.49 μM, and for Zn^{2+} it was 8.33 μM.

Time course and linearity of Zn–porphyrin formation

When mesoporphyrin and Zn^{2+} were used as substrate, the Zn–mesoporphyrin formation was linear for up to 70 min. A 30-min incubation time was chosen for the standard assay. The formation of Zn–mesoporphyrin was proportional to leucocyte protein concentration up to 0.7 mg per incubation; therefore, approximately 0.5 mg was used in the standard assay.

Effect of reducing agents on ferrochelatase

Previous studies have shown that reductants such as glutathione, cystein, dithiothreitol and ascorbic acid were very important for ferrochelatase activity [15,39,44,47,48]. It was suggested that low concentrations of these reductants could keep iron the ferrous state, protect the enzyme against auto-oxidation and

stabilize or stimulate the enzyme by activation of its sulphydryl groups [39,44]; ascorbic acid was found to be more effective than glutathione [15,44]. However, when ferrochelatase activity was determined by the pyridine haemochromogen method, glutathione, ascorbic acid and cysteine were found to cause degradation of haem [14] and dithiothreitol interfered with the extraction of haem into organic solvent [15].

Under the present assay conditions with freshly prepared enzyme low concentrations of ascorbic acid, glutathione and dithiothreitol had minimal effect on enzyme activity while high concentrations inhibited the activity. With stored enzyme (−30°C, about six months), low concentrations of these reductants did enhance the enzyme activity to some extent. The probable explanation is that the gradual loss of enzyme activity in stored sample was reactivated by the reducing agent.

Ferrochelatase activity and stability

The activity of ferrochelatase was investigated in ten apparently healthy persons aged from 24 to 59 years. The mean activity was 2.05 nmol Zn–mesoporphyrin or 0.24 nmol Zn–protoporphyrin formed per h per mg protein, respectively. No significant difference between sexes was noticed. The 2 S.D. reference ranges were established as 1.11–3.00 nmol Zn–mesoporphyrin or 0.21–0.28 nmol Zn–protoporphyrin per h per mg protein, respectively. Since different substrates were used, the activities were difficult to compare. The activity of the enzyme in various human tissues is summarized in Table III. The enzyme activity is stable for up to four months when stored at −30°C.

TABLE III

FERROCHELATASE ACTIVITY IN VARIOUS HUMAN TISSUES

Tissue	Activity	Ref.
Lymphocyte	12.9 nmol mesohaem/h/mg	11
	2.09 nmol haem/h/mg	11
	3.25 nmol Zn–meso/h/mg	25
Neutrophil	0.340 nmol Zn–meso/h/mg	25
Leucocyte	54.7 nmol haem/h/mg	8
	0.240 nmol Zn–proto/h/mg	This study
	2.05 nmol Zn–meso/h/mg	This study
Skin	0.214 nmol deuterohaem/h/mg	13
fibroblast	0.032 nmol/haem/h/mg	13
	0.031 nmol haem/h/10^7 fibroblasts	49
	0.159 nmol deuterohaem/h/mg	24
	0.141 nmol deuterohaem/h/mg	50
	0.024 nmol haem/h/mg	50
Liver	0.352 nmol haem/h/mg	50
	3.72 nmol Zn–meso/h/mg	20

Clinical applications

Although protoporphyrinogen oxidase and ferrochelatase activities are relatively low in leucocytes, compared to liver, they can be measured by the present sensitive and accurate HPLC methods. Measuring the enzyme activities in leucocytes is much more convenient than in liver in clinical work, because it is very difficult to establish the normal range for enzyme activities in liver tissues as they are not readily available. These assays enable the precise diagnosis of porphyrias, especially in the detection of latent carriers in a family study.

Protoporphyrinogen oxidase activity was measured in a patient clinically diagnosed as variegate porphyria. The activity (1.70 nmol/h/mg) was markedly reduced compared with the normal range (4.36–13.1 nmol/h/mg). This is consistent with many other observations that protoporphyrinogen oxidase activity is reduced by more than 50% in patients with variegate porphyria [8–11,13].

Patients with erythrohepatic protoporphyria where ferrochelatase is defective were unfortunately not available for the present study.

REFERENCES

1 R. Poulson and W. J. Polglase, *J. Biol. Chem.*, 250 (1975) 1269.
2 J.-M. Camadro, D. Urban-Grimal and P. Labbeew, *Biochem. Biophys. Res. Commun.*, 106 (1982) 724.
3 P. Labbe, J.-M. Camadro and H. Chambon, *Anal. Biochem.*, 149 (1985) 248.
4 N. J. Jacobs and J. M. Jacobs, *Arch. Biochem. Biophys.*, 211 (1981) 305.
5 P. Poulson, *J. Biol. Chem.*, 251 (1976) 3730.
6 J. Kolarov, B. D. Nelson and S. Kuzela, *Biochem. Biophys. Res. Commun.*, 116 (1983) 383.
7 F. Li, C. K. Lim and P. J. Peters, *Biochem. J.*, 243 (1987) 863.
8 D. J. Viljoen, R. Cummins, J. Alexopoulos and S. Kramer, *Eur. J. Clin. Invest.*, 13 (1983) 283.
9 P. N. Meissner, R. S. Day, M. R. Moore, P. B. Desler and E. Harley, *Eur. J. Clin. Invest.*, 16 (1986) 257.
10 L. J. Siepker and S. Kramer, *Br. J. Haematol.*, 60 (1985) 65.
11 J. Ch. Deybach, H. De Verneuil and Y. Normann, *Hum. Genet.*, 58 (1981) 425.
12 J.-M. Camadro, N. G. Abraham and R. D. Levere, *Arch. Biochem. Biophys.*, 242 (1985) 206.
13 D. A. Brenner and J. R. Bloomer, *N. Engl. J. Med.*, 302 (1980) 765.
14 R. J. Porra, K. S. Vitols, R. F. Labbe and N. A. Newton, *Biochem. J.*, 104 (1967) 321.
15 J. R. Bloomer and K. O. Morton, *Enzyme*, 28 (1982) 220.
16 B. M. Harbin and H. A. Dailey, *Biochemistry*, 24 (1985) 366.
17 A. Tangeras, *Biochim. Biophys. Acta*, 882 (1986) 77.
18 C. L. Honeybourne, J. T. Jackson and O. T. G. Jones, *FEBS Lett.*, 98 (1979) 207.
19 J.-M. Camadro, N. G. Abraham and R. D. Levere, *J. Biol. Chem.*, 259 (1984) 5678.
20 F. Li, C. K. Lim, K. J. Simpson and T. J. Peters, *J. Hepatol.*, 8 (1989) 86.
21 J.-M. Camadro and P. Labbe, *Biochim. Biophys. Acta*, 707 (1982) 280.
22 M. S. Jones and O. T. G. Jones, *Biochem. J.*, 119 (1970) 453.
23 S. S. Bottomley, M. Tanaka and M. A. Everett, *J. Lab. Clin. Med.*, 86 (1975) 126.
24 J. R. Bloomer, *J. Clin. Invest.*, 65 (1980) 321.
25 E. Rossi, K. A. Costin and P. Gracia-Webb, *Clin. Chem.*, 34 (1988) 2481.
26 F. Li, C. K. Lim and T. J. Peters, *Biomed. Chromatogr.*, 2 (1987) 164.
27 J. E. Falk, *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam, London, New York, 1964, p. 231.
28 J. H. Fuhrhop and K. M. Smith, in K. M. Smith (Editor), *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam, 1975, p. 782.
29 G. R. Schaterle and R. L. Pollack, *Anal. Biochem.*, 139 (1973) 714.

30 D. A. Brenner and J. R. Bloomer, *Clin. Chim. Acta*, 100 (1980) 259.
31 F. Li, C. K. Lim and T. J. Peters, *Biochem. J.*, 239 (1986) 481.
32 R. Guo, C. K. Lim and T. J. Peters, *Clin. Chim. Acta*, 177 (1988) 245.
33 R. Eisenthal and A. Cornish-Bowden, *Biochem. J.*, 139 (1974) 715.
34 A. A. Lamola and T. Yamana, *Science*, 186 (1974) 936.
35 A. A. Lamola, *Acta Dermato-Venereol.*, 100 (Suppl.) (1982) 57.
36 A. M. Mazanowska, A. Neuberger and G. H. Tait, *Biochem. J.*, 98 (1966) 117.
37 H. Sawada, M. Takeshita, Y. Sugita and Y. Yoneyama, *Biochim. Biophys. Acta*, 178 (1969) 145.
38 D. M. Simpson and R. Poulson, *Biochim. Biophys. Acta*, 482 (1977) 461.
39 S. Taketani and R. Tokunaga, *J. Biol. Chem.*, 256 (1981) 12 748.
40 R. J. Porra and O. T. G. Jones, *Biochem. J.*, 87 (1963) 181.
41 R. J. Kassner and H. Walchak, *Biochim. Biophys. Acta*, 304 (1973) 294.
42 A. M. Mazanowska, A. M. Dancewics, T. Malinowska and E. Kowalski, *Eur. J. Biochem.*, 7 (1969) 583.
43 E. Rossi and P. Garcia-Webb, *Biomed. Chromatogr.*, 1 (1986) 163.
44 S. S. Bottomley, *Blood*, 31 (1968) 314.
45 H. A. Dailey, Jr. and J. Lascells, *Arch. Biochem. Biophys.*, 160 (1974) 523.
46 H. A. Dailey and J. E. Fleming, *J. Biol. Chem.*, 258 (1983) 11 453.
47 Y. Yoneyama, A. Tamai, T. Yasuda and H. Yoshikawa, *Biochim. Biophys. Acta*, 105 (1965) 100.
48 S. Taketani and R. Tokunaga, *Biochem. Biophys. Res. Commun.*, 93 (1980) 1343.
49 D. J. Viljoen, E. Cayanis, D. M. Becker, S. Kramer, B. Dawson and R. Bernstein, *Am. J. Hematol.*, 6 (1979) 185.
50 H. L. Bonkowsky, J. R. Bloomer, P. S. Ebert and M. J. Mahoney, *J. Clin. Invest.*, 56 (1975) 1139.