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SEPARATION OF HAEM COMPOUNDS BY REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION IN THE ASSAY OF FERROCHELATASE ACTIVITY

ARILD TANGERÅS

Department of Biochemistry, University of Bergen, Årstadveien 19, N-5000 Bergen (Norway)

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SUMMARY

The separation of haems and porphyrins was achieved in a reversed-phase ion-pair high-performance liquid chromatography system using tetrabutylammonium hydrogen sulphate as the pairing ion. The concentration of methanol and pH in the mobile phase were determinative parameters for the elution pattern of the compounds. Two isocratic systems — one for the assay of protohaem IX and one for deuterohaem IX — were developed.

The chromatographic systems were applied to the assay of ferrochelatase activity in mitochondria using either protoporphyrin or deuteroporphyrin as the substrate. The ferrochelatase activity was also measured in reticulocytes, which contain high levels of endogenous haem.

INTRODUCTION

The final step in the haem biosynthetic pathway is the insertion of ferrous iron into the tetrapyrrole ring of protoporphyrin IX to form protohaem IX [1]. The reaction is catalysed by the enzyme ferrochelatase (protohaem ferrolyase, EC 4.99.1.1) which in eucaryotic cells is located in the mitochondria [2, 3]. Various procedures based on quantitation of porphyrin substrate consumed or haem product formed have been reported for the assay of this enzyme. The utilization of porphyrin can be measured either spectrophotometrically or fluorometrically [2, 4]. However, absorption and fluorescence spectra of porphyrins change both upon aggregation, which occurs readily in aqueous solutions [5], as well as upon binding to various proteins present in plasma [6], cytosol [7] and mitochondria [8]. Thus, an apparent porphyrin consumption was reported to take place several minutes before any

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formation of haem could be detected when the spectrophotometric approach was used [9].

The haem formed is most frequently measured from the reduced minus oxidized absorption spectra of pyridine haemochromogens [10]. However, these spectra will also have contributions from any endogenous haem in the biological material analysed, and often a small increase in the absorption spectrum has to be detected against a high background. This problem is not encountered in radiochemical methods, which, however, require a rather tedious extraction procedure for the separation of the labelled haem from free radio iron [11].

The purpose of the present study was to develop an assay of ferrochelatase activity in which the haem formed is assayed at high sensitivity and precision based on quantitation of haem by high-performance liquid chromatography (HPLC). Although several HPLC systems for the quantitation of porphyrins can be found in the literature, no system has so far been reported for the assay of haem. Ferrochelatase will accept various porphyrin IX derivatives as the substrate in in vitro assay systems [12]. The activity of the enzyme could therefore be measured in samples containing high concentrations of endogenous protohaem when deuteroporphyrin was used as the substrate since the deuterohaem formed was separated from protohaem on HPLC.

MATERIALS AND METHODS

Chemicals

Protoporphyrin IX and deuteroporphyrin IX were obtained from Porphyrin Products (Logan, UT, U.S.A.). Tetrabutylammonium hydrogen sulphate and protohaem IX were from Sigma (St. Louis, MO, U.S.A.).

High-performance liquid chromatography

Separation of haems and porphyrins were obtained by HPLC using a Constametric III pump from Laboratory Data Control (Riviera Beach, FL, U.S.A.), a valve loop injector from Rheodyne (Berkeley, CA, U.S.A.) and a photodiode array spectrophotometric detector Model HP-1040A connected to an HP-85 microcomputer, a Model 3380A recording integrator and a Model 7470A plotter, all from Hewlett-Packard (Avondale, CA, U.S.A.). The column used was a reversed-phase silica supported prepacked Supelcosil LC-18 column (25 cm × 4.6 mm I.D., particle size 5 μm) equipped with a Pelliguard pre-column (2 cm × 4.6 mm I.D.) prepacked with pellicular LC-18 of 40 μm particle size, both from Supelco (Bellefonte, CA, U.S.A.).

For the quantitation of protohaem the mobile phase contained methanol—water (97:3) with tetrabutylammonium hydrogen sulphate (1 mM), acetic acid (10 mM) and adjusted to pH′ 6.5 with 5 M ammonium hydroxide at ambient temperature. For the assay of deuterohaem the mobile phase was similar, except that the methanol—water ratio was 85:15.

The solvent was delivered at a flow-rate of 1 ml/min and the system operated at ambient temperature. The tetrapyrroles were identified from their retention times (t_R) and their peak absorption spectra.

The spectrophotometer was set at a measuring wavelength of 398 nm with

8 nm spectral bandwidth for monitoring protohaem, whereas the setting was 384 nm with 10 nm bandwidth when deuterohaem was monitored. The reference wavelength was set at 550 nm with 100 nm bandwidth for both of the haems.

Assay of ferrochelatase activity

For the assay of ferrochelatase activity the incubation of mitochondria and reticulocytes was performed as described in ref. 13 in a total volume of 1.2 ml. The reaction was quenched by 5 m*M* *p*-chloromercuribenzoate, and 0.2 ml of this solution was mixed with 0.5 ml of ice-cold methanol containing 1 m*M* of tetrabutylammonium hydrogen sulphate. The tubes were then centrifuged for 15 sec in an Eppendorf centrifuge Model 5414; 50 μl of 1 *M* hydrochloric acid and 0.5 ml of the methanol—tetrabutylammonium hydrogen sulphate solution were added to the pellets which were then sonicated in a well type sonicator and centrifuged for 2 min. The combined supernatants were centrifuged for another 2 min, and 20 μl of the final supernatant were injected into the liquid chromatograph. More than 90% of the haem in the sample was extracted by this procedure (see Results).

pH Measurements

The pH was measured using a PHM52 digital pH-meter equipped with a combined electrode GK 2321C, both from Radiometer (Copenhagen, Denmark). The pH values reported are all apparent pH values (pH′) since no correction was made for the varying amounts of methanol present in the solutions.

Preparation of mitochondria and reticulocytes

Rat liver mitochondria (male Wistar rats weighing 200—300 g) were isolated by differential centrifugation as previously described [14, 15]. Reticulocytosis was induced in rats by injecting phenylhydrazine and the red blood cells were prepared essentially as described in ref. 16 except that 0.15 *M* sodium chloride was used as the isolation medium.

Other analytical and preparative methods

Deuterohaem was synthesized from deuteroporphyrin and ferrous sulphate by refluxing in N,N′-dimethylformamide [17].

In the standard solutions porphyrins were quantitated from their absorption spectra in aqueous hydrochloric acid and haems were measured from the reduced minus oxidized difference pyridine haemochromogen spectra [18].

Protein was measured according to the procedure of Bradford [19] using the dye reagent of Bio-Rad Labs. (Richmond, CA, U.S.A.) and bovine serum albumin as a standard.

RESULTS

Chromatographic systems for separation of haems and porphyrins

The retention (expressed as capacity factor k') of the two haem and porphyrin compounds in the reversed-phase ion-pair HPLC system studied, was

influenced by the amount of organic solvent (Fig. 1) and pH′ (Fig. 2) of the mobile phase. The capacity factor for all four compounds decreased when the methanol concentration was increased (Fig. 1). A decrease in the k' value was also observed for the same compounds when the pH′ of the mobile phase was increased from 4 to 7 (Fig. 2). At low pH′ values a shoulder in the peak representing haem appeared (data not shown), and a pH′ of 6.5 in the mobile phase was therefore selected.

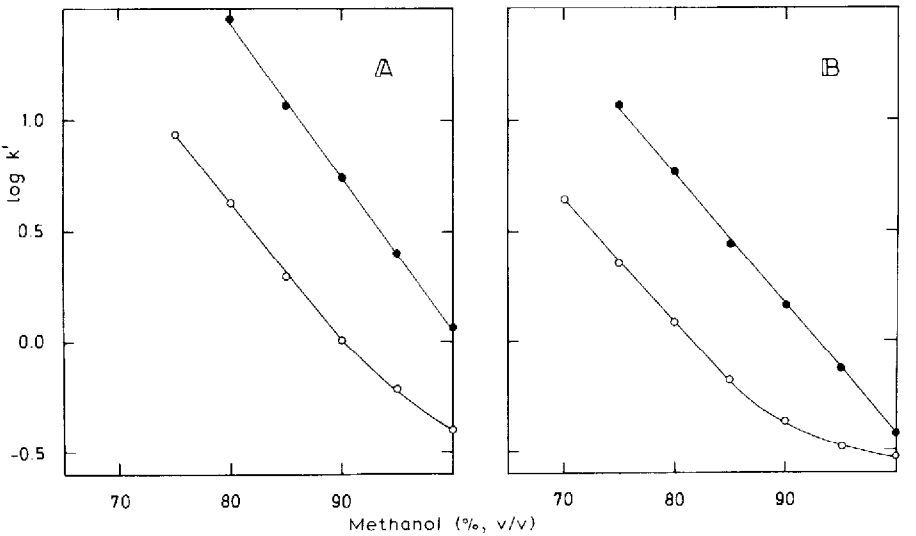

Fig. 1. The effect of the concentration of methanol on the capacity factor (k') in HPLC of porphyrin (•) and haem (○); (A) proto compounds, (B) deutero compounds. The mobile phase contained methanol—water in varying ratios, tetrabutylammonium hydrogen sulphate (1 mM), acetic acid (10 mM), and the pH′ was adjusted to 6.5 with 5 M ammonium hydroxide. The correlation coefficient for the linear regression line was −0.999 for both protoporphyrin (A) and deuteroporphyrin (B).

When the concentration of tetrabutylammonium hydrogen sulphate in the mobile phase was varied between 0 and 5 mM, the symmetry of the peaks improved and showed an optimum at approximately 1 mM, whereas the retention times were only marginally affected (data not shown).

The calibration curves for both protohaem and deuterohaem using the standard HPLC conditions were linear with a correlation coefficient > 0.999, and the detection limit was about 2 pmol at a signal-to-noise ratio of 2.

The recovery of protohaem when extracted from mitochondria containing variable amounts of added haem is shown in Fig. 3. When corrected for the endogenous haem a straight line through the origin with a slope of 0.907 was obtained, indicating that 90% of the added haem was recovered.

The chromatogram of a mixture of protohaem and protoporphyrin, using the conditions for protohaem quantitation (see Materials and Methods), is shown in Fig. 4A, I. When a mixture of deuterohaem, protoporphyrin and deuteroporphyrin was chromatographed using the conditions for deuterohaem quantitation, the chromatogram shown in Fig. 4A, II was obtained.

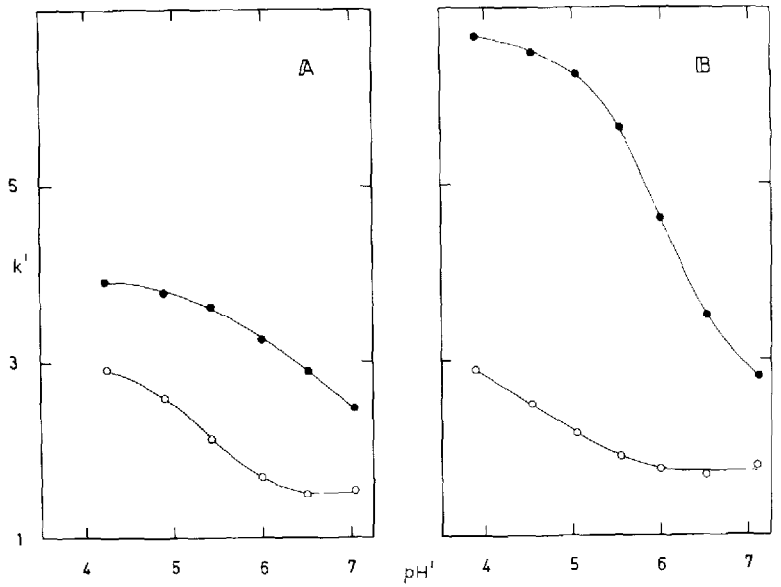

Fig. 2. The effect of pH' on the capacity factor (k') in HPLC of porphyrin (•) and haem (○); (A) proto compounds, (B) deutero compounds. The mobile phase contained tetrabutylammonium hydrogen sulphate (1 mM), acetic acid (10 mM) and methanol—water at a ratio of 97:3 for the proto compounds and 85:15 for the deutero compounds. The pH' of the mobile phase was adjusted with 5 M ammonium hydroxide.

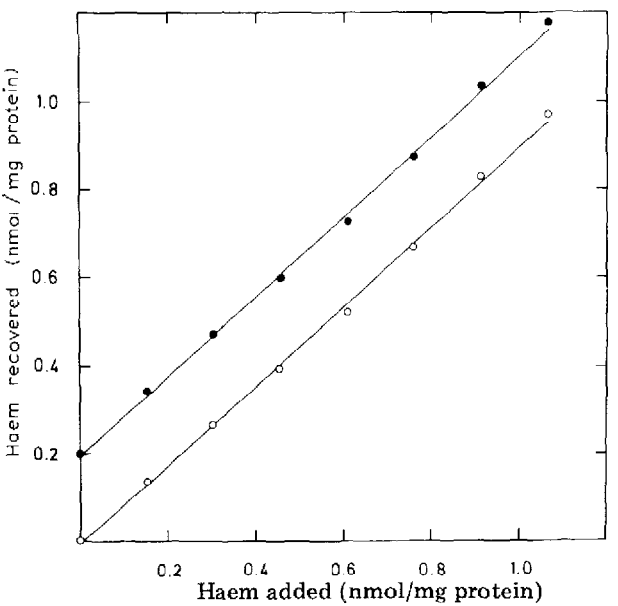

Fig. 3. The recovery of protohaem IX extracted from mitochondria with variable amounts of added protohaem. The total amount of protohaem found (•) and the amount recovered after correcting for the endogenous content of protohaem in mitochondria (○) are shown. Various amounts of protohaem were added to a fixed amount of mitochondria (3 mg of protein) in a total volume of 0.135 ml. This mixture was then extracted and analysed for protohaem as described in Materials and methods. The linear regression line for the data points, representing the recovery of the added protohaem, was $Y = 0.907X - 0.01$ and the correlation coefficient was 0.999.

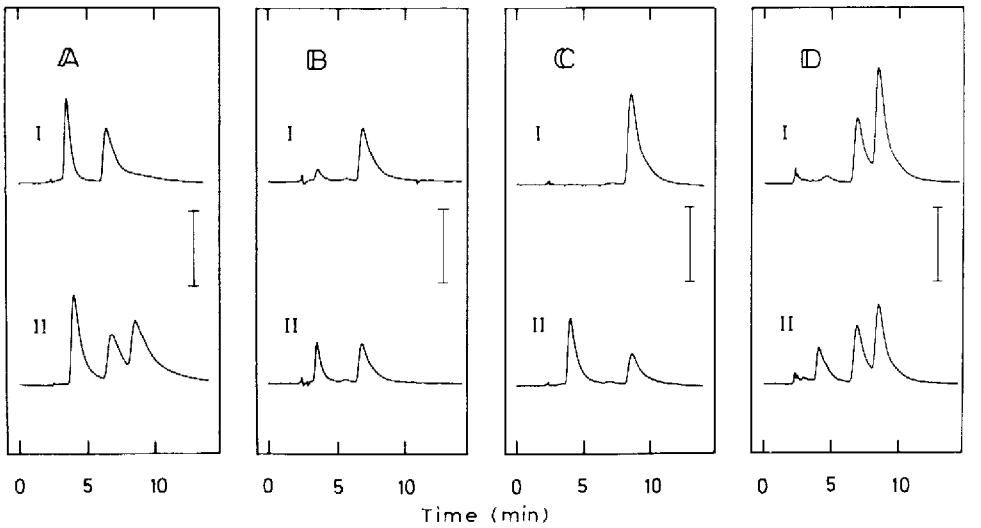

Fig. 4. Chromatograms demonstrating the separation of tetrapyrrole standards (A) and the assay of ferrochelatase activity in isolated liver mitochondria (B and C) and reticulocytes (D). (A) Chromatograms obtained when a mixture of protohaem (80 pmol) and protoporphyrin (200 pmol) was analysed (A, I) and that obtained (A, II) for a mixture of deuterohaem (190 pmol), protohaem (315 pmol) and protoporphyrin (215 pmol). (B) Chromatograms of extracts from mitochondria prior to incubation (I) and following 20 min incubation (II) of the ferrochelatase assay mixture. The porphyrin substrate used was protoporphyrin, and the incubation temperature was 22° C. The amount of mitochondria present in the incubation mixture was 10 mg of protein. For details, see Materials and Methods. (C) Same as B except that deuteroporphyrin was used as substrate and the incubation time was 6 min (II). (D) Chromatograms of extracts from reticulocytes prior to incubation (I) and following 45 min incubation (II) of the ferrochelatase assay mixture. The amount of red blood cells used in the assay mixture contained approximately 60 nmol of protohaem, and the incubation temperature was 37° C. The bars represent an absorption of $A = 0.01$. The chromatographic conditions for the analysis of protohaem were employed in A (I) and B whereas in A (II), C and D the conditions for the analysis of deuterohaem was used (see Materials and methods).

Application of the HPLC procedure in the assay of ferrochelatase

The application of the chromatographic system for the assay of ferrochelatase activity in rat liver mitochondria, using protoporphyrin as the substrate, is shown in Fig. 4B. Three compounds were detected in the chromatogram at zero time (Fig. 4B, I). The peak at t_R = 3.5 min represented endogenous protohaem, whereas the peak at t_R = 6.8 min represented the protoporphyrin substrate. The peak at t_R = 5.6 min is not yet identified, but may represent haem *a* from cytochrome *a* + a_3. The chromatogram of the tetrapyrroles extracted from the assay mixture incubated for 20 min, showed that the peak of protohaem was increased whereas that of protoporphyrin was decreased (Fig. 4B, II). The amount of protohaem synthesized was 0.7 nmol/mg protein.

When deuteroporphyrin was the substrate and mitochondria the enzyme source, one distinct peak (t_R = 8.6 min), representing the porphyrin substrate, appeared in the chromatogram at zero time (Fig. 4C, I). A small peak at t_R = 7.1 min was also seen, representing endogenous protohaem of the

mitochondria. A new peak of t_R = 4 min appeared in the chromatogram following an incubation period of 6 min (Fig. 4C, II) whereas the peak representing the porphyrin substrate decreased. The new peak represented deuterohaem, and the amount formed was 4 nmol/mg protein.

Ferrochelatase activity could also be measured in biological materials containing high amounts of endogenous haem, e.g. reticulocytes, when deuteroporphyrin was used as the substrate (Fig. 4D). In the control chromatogram (Fig. 4D, I) two prominent peaks were observed, one (t_R = 6.7 min) representing the endogenous protohaem from haemoglobin in the red blood cells, and one (t_R = 8.2 min) representing deuteroporphyrin. A small unidentified peak (t_R = 4.5 min) was also seen. Following a reaction period of 45 min, a new peak (t_R = 4 min) appeared in the chromatogram (Fig. 4D, II), representing deuterohaem formed, i.e. 13 nmol/mg haemoglobin (haemoglobin major protein component). The small peak with t_R = 4.5 min in Fig. 4D (I) now appeared as a shoulder in the deuterohaem peak. However, this did not interfere with the estimation of the amount of haem formed when based on peak height, rather than on peak area.

The precision of the ferrochelatase assay is shown in Table I. Of the three steps involved, i.e. anaerobic incubation, haem extraction and assay of haem, the incubation step contributed most to the variation. Thus, the relative standard deviation was 7.8% for the complete assay, 2.0% for the combined extraction and HPLC assay, and 1.0% for this last step alone.

TABLE I

PRECISION OF THE VARIOUS STEPS OF THE ASSAY OF FERROCHELATASE ACTIVITY

Mitochondria were incubated at 25°C for 10 min under the conditions given in Materials and methods using deuteroporphyrin as the substrate. Multiple aliquots of one of the assay mixtures were extracted for haem (parallel extractions) and for one of the extracts multiple HPLC runs were performed (parallel HPLC runs). The mean and S.D. for each series is given.

Deuterohaem found (nmol/mg protein per min)		
Parallel assays	Parallel extractions	Parallel HPLC runs
1.03 ± 0.08 (n = 6)	1.01 ± 0.02 (n = 6)	1.01 ± 0.01 (n = 6)

DISCUSSION

Free porphyrin carboxylic acids have already been separated by HPLC using either ion-exchange, normal-phase, reversed-phase or reversed-phase ion-pair chromatographic systems (see refs. 20–23). In the present study a reversed-phase ion-pair system was selected for the separation of haem and porphyrin compounds since this method is reported to be the most reproducible one for the analysis of tetrapyrroles [24, 25]. The retention time of both haem and porphyrin could be modified either by varying the polarity or the pH of the

mobile phase. A linear relationship between the logarithm of the capacity factor (log k') and the amount of methanol in the mobile phase was found for the porphyrins studied, and this relationship was also obtained for the haem compounds up to a methanol concentration of about 85% (Fig. 1). Such a correlation is frequently observed in reversed-phase ion-pair systems as well as in pure reversed-phase systems [26].

A decrease in k' value was found for both haems and porphyrins when the pH′ of the mobile phase was increased (Fig. 2), in agreement with previous studies on porphyrins [25]. From the reported pK values of the tetrapyrroles [27] the effect of pH on the k' values suggests that the retention of the compounds is mainly through hydrophobic forces rather than through an ion-exchange mechanism [26]. A decrease in k' for porphyrins with increasing pH is also found in a reversed-phase system [22]. Thus, for the reversed-phase ion-pair system both the influence of pH′ as well as the negligible effect of pairing ion in the mobile phase on the k' value suggest that the separation is largely due to hydrophobic interactions. However, even though an adequate separation of the compounds could also be achieved in a reversed-phase system, an ion-pair system was selected since this improved the peak symmetry.

Under the selected chromatographic conditions, haem was well separated from porphyrin and so was deuterohaem from protohaem. Thus, by using deuteroporphyrin as the substrate for ferrochelatase, the enzyme could also be measured in red blood cells containing high concentrations of protohaem in haemoglobin (Fig. 4D). Ferrochelatase activity in erythroid cells has previously been measured using the pyridine haemochromogen method with meso-porphyrin as the substrate [28]. However, due to a considerable overlap of the spectra for mesohaem and protohaem, this method could only be used when the amount of haem synthesized represented more than 20% of the total amount of haem in the sample [29]. Such a limitation is not present in the method reported here.

The radiochemical method [11] is still the method of choice when very high sensitivity is required. This method can, however, be made more convenient when combined with the present HPLC method. Thus, the tedious extraction procedure used traditionally [11] can be replaced by the much simpler procedure reported here, followed by HPLC. By using protohaem as an internal standard, the recovery of the extraction can be estimated from the same HPLC run.

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