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Note

Characterization and purification of iron porphyrins by high-performance liquid chromatography and column chromatography

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Iron porphyrins are important as biological cofactors and regulatory signals. In addition to serving as a prosthetic group for many enzymes[1–3], iron porphyrins regulate many biosynthetic pathways[4–16].

Many workers have investigated the high-performance liquid chromatographic (HPLC) characterization of iron-containing natural porphyrins[17–25]. Lim *et al.*[18] were able to achieve excellent separation of *meso* and protoporphyrins and their corresponding Fe(III) derivatives using a SAS-Hypersil (C_1) column with a methanol–1 *M* ammonium acetate gradient; Bonkovsky *et al.*[19] have used a reversed-phase C_{18} column and similar solvent conditions. Tangerås[20] has separated protoporphyrin and protohemin (**1**) using a reversed-phase C_{18} column and tetrabutylammonium hydrogen sulfate in a water–methanol mixture. Protohemin and protoporphyrin have also been separated on a silica column using methanol–acetonitrile–acetic acid–pyridine as the mobile phase[21]. HPLC techniques have been used to quantitate protoporphyrin and protohemin in mitochondria of mice with porphyria induced by griseofulvin[22] and to analyze levels of heme synthesis in mitochondria[23]. There has been far less study of non-biological iron-containing porphyrins although it has been found that Fe(III) tetrakis(N-methyl-4-pyridyl)porphyrin chloride gives a sharp peak on a LiChrosorb RP-18 column using acetone[24] or ethanol[25] as the mobile phase.

As described above, most of the HPLC methods development work to date centers on protohemin and related iron porphyrins, because these are the most important *in vivo*. However, there is a need to extend this work to a larger variety of iron-containing porphyrins for two reasons. First, there is a good deal of current work involving the isolation and characterization of hemes from a variety of proteins which would be aided by improved isolation and characterization techniques[26–30]. Second, there is an increasing interest in the reconstitution of heme proteins with iron-containing porphyrins to probe structure-function relationships in these proteins. Studies include those of cytochrome b_5[31,32], myoglobin[33], hemoglobin[34], cyto-

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chrome *c* peroxidase[35,36] and horseradish peroxidase[37,38]. Finally, the use of positively-charged porphyrins for imaging *in vivo*[39,40], as DNA complexing agents[41] and as electron transfer agents in model systems for photosynthesis and energy storage[42,43] has led us to investigate the purification of these species.

In this paper we report the HPLC characteristics of a variety of ferric porphyrins, both natural (derived from protoporphyrin IX) and synthetic (derived from tetraphenylporphyrin).

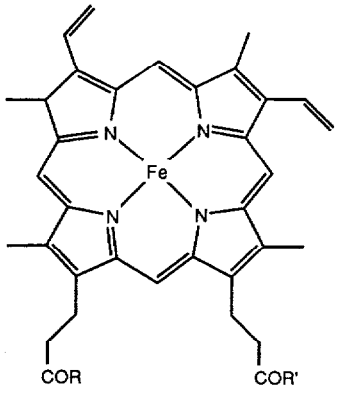
EXPERIMENTAL

Apparatus

Chromatography was performed with Beckman 110A/110B solvent delivery modules equipped with a Beckman 160 UV absorbance detector (visible lamp operating at 405 nm), an Axxion 710 HPLC controller, a Hewlett-Packard 3392A integrator and Econosphere silica gel 5-μm or C_8 5-μm columns (150 × 4.4 mm I.D., Alltech). All HPLC solvents were filtered and degassed. The mobile phase flow-rate was 1 ml/min. Sephadex LH-20-100 was from Sigma, 25–100 μm. Optical spectra were taken on a Varian DMS 200 spectrometer.

Porphyrins

Protohemin (**1**) was obtained from Aldrich. The Fe(III) bismethyl esters of the natural hemins were made by inserting iron into the commercially available porphyrins (Midcentury) using the $FeCl_2$–propionic acid method[44]. The Fe(III) bis(glycine ethyl ester) (**3**), biscyclohexylmethyl ester (**4**), cyclic ester (**5**) and monomethyl ester (**6**)[35] protohemin derivatives were made from protohemin with pivaloyl chloride as the condensing reagent[45]. The bispositively-charged porphyrin (**8**) was synthesized by condensing protohemin and $H_2NCH_2CH_2CH_2N(CH_3)_2$ using carbonyldiimidazole to give **7** followed by alkylation of **7** with methyl iodide. Details of the syntheses will be described elsewhere[46]. All compounds based on the protoporphyrin ring system were characterized by thin-layer chromatography (TLC) and NMR[47–49]. Tetrakis (N-methyl-4-pyridyl)porphyrin (TMPyP) tosylate, Fe(III)TMPyP chloride and *trans*-5,10-diphenyl-15,20-di-(N-methylpyridyl)porphyrin chloride (Midcentury) were used as received. The other Fe(III) tetraphenylporphyrin (TPP) derivatives were



1 R = R′ = OH
2 R = R′ = OCH_3
3 R = R′ = $NHCH_2COOC_2H_5$
4 R = R′ = CH_2-cyclo-C_6H_{11}
5 R - R′ = OCH_2CH_2O
6 R = OH, R′ = OCH_3
7 R = R′ = $NH(CH_2)_3N(CH_3)_2$
8 R = R′ = $NH(CH_2)_3N^+(CH_3)_3$

either purchased from Midcentury Chemicals or synthesized by pyrrole–benzaldehyde condensation in a propionic acid reflux[50]. Iron was inserted using $FeCl_2$ in dimethylformamide[51].

RESULTS AND DISCUSSION

Analysis of hemin esters and Fe(III)TPP derivatives

Some hemins separate well on silica columns. Following earlier work of Bauer and Fornnarino[21], we used an acetonitrile–methanol–acetic acid–pyridine (240:240:1:40) solvent mixture on the silica column. The pyridine in this solvent mixture serves to ligate the positively charged iron; ligation appears to be necessary to move the Fe(III) porphyrins off the column. For complexes with propionic acid side chains, it is necessary to add acid to the eluent to protonate the CO_2^- groups of the porphyrin. Acetic acid served well, retention times decreased (and peaks sharpened) as the amount of acetic acid was increased. The effect of acetic acid on retention time was general, however, and observed also for Fe(III) porphyrins without acid side chains. Loss of iron under HPLC conditions was not observed for any of the compounds investigated. Table I gives the retention times for ferric porphyrins under these conditions.

For protohemin, the longer the complex remained on the column, the broader the peak, as expected for a system undergoing protonation/deprotonation. Under the conditions described in this paper, iron porphyrin diacids remaining on the column longer than about 20 min gave peaks that were so broad as to be almost indistinguishable from baseline. Design of an individual separation scheme must balance the better peak separation at longer times with the increased broadening. Fig. 1 shows the separation of protohemin(**1**) and protohemin bismethyl ester (**2**) under gradient conditions.

Although the acetic acid is necessary to protonate any porphyrin acid side chains in the mixture, it must be noted that the acetic acid can add itself, or promote

TABLE I

RETENTION TIMES OF Fe(III) PORPHYRINS ON SILICA WITH ACETONITRILE–METHANOL–ACETIC ACID–PYRIDINE (240:240:1:40, v/v) MOBILE PHASE

Fe(III) Porphyrins	*Retention time (min)*
Natural Fe(III) porphyrins	
Protohemin bismethyl ester, **2**	19.2
*Meso*hemin bismethyl ester	18.8
Deuterohemin bismethyl ester	20.1
Protohemin bis(glycine ethyl ester), **3**	9.4
Protohemin bis(cyclohexylmethyl) ester, **4**	18.4
Protohemin cyclic ester, **5**	20.4
Tetraphenyl Fe(III) porphyrins	
Tetraphenylporphyrin	18.2
(4-Methyl) tetraphenylporphyrin	17.1
(4-Isopropyl) tetraphenylporphyrin	14.5
(4-Methoxy) tetraphenylporphyrin	16.7
Tetraphenylporphyrin μ-oxodimer	18.5

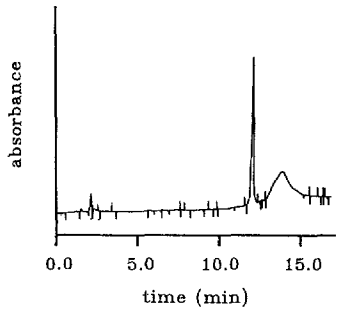


Fig. 1. HPLC trace of Fe(III) protoporphyrin (**1**, 13.7 min) and the corresponding bismethyl ester (**2**, 11.8 min). The solvent system was acetonitrile–methanol–acetic acid–pyridine, held at a ratio of 240:240:1:40 for 5 min and ramped to a ratio of 45:45:10:5 over a 5-min period.

the addition of methanol, to the vinyl groups at the 2 and/or 4 positions. Addition is most easily verified by taking the optical spectrum of a reduced sample in a solution containing enough pyridine to ligate the Fe(II) center fully. These pyridine hemochrome spectra show shifts of the bands that are characteristics of the extent of delocalization of the ring system with the vinyl side chains[52].

The extent of addition of nucleophiles to the heme vinyl groups depends both on the compound and on sample preparation. The vinyl groups of protohemin itself remained intact even after storage of the compound in the HPLC solvent (acetonitrile–methanol–acetic acid–pyridine, 240:240:1:40) for many days (Table II). Protohemin bismethyl ester, however, added one, but not two, nucleophiles during storage in the HPLC solvent (Table II). This type of addition has been observed before[53,54]. When a freshly prepared sample of protohemin bismethyl ester in acetonitrile was subjected to HPLC, the vinyl groups remained intact for the 30 min that the compound remained on the column, as shown by the maxima at 415.6 and 555.6 nm in the visible spectrum of the pyridine hemochrome. Addition of the solvent to the vinyl groups can on occasion make interpretations of results difficult. Changes in substituent at the 2 and 4 positions of the heme periphery can be visualized by HPLC in some instances, *e.g.* deuteroporphyrin bismethyl ester and *meso*porphyrin bismethyl ester can be differentiated readily (Fig. 2).

TABLE II

UV–VIS SPECTRAL CHARACTERISTICS OF SELECTED Fe(III) PORPHYRINS

Compounds	*α*	*β*	*Soret*
Freshly prepared protohemin*	555.6	522.8	416.7
Protohemin solution**	554.5	522.8	416.7
Freshly prepared protohemin bismethyl ester*	555.6	522.8	415.6
Protohemin bismethyl ester after HPLC***	555.6	522.8	415.6
Protohemin bismethyl ester solution**	550.8	520.3	413.5
*Meso*hemin bismethyl ester solution**	545.8	516.7	410.1

* Solvent acetonitrile–methanol–acetic acid–pyridine (240:240:1:40, v/v).
** Solid sample dissolved in acetonitrile.
*** Sample in HPLC solvent for 5 days or more.

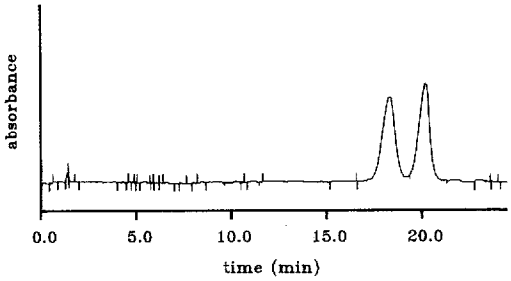


Fig. 2. HPLC trace of Fe(III) *meso*porphyrin bismethyl ester (18.9 min) and Fe(III) deuteroporphyrin bismethyl ester (20.1 min). The solvent system was acetonitrile–methanol–acetic acid–pyridine (240:240:1:40).

Another concern in the ferric porphyrin series is that these molecules can exist either associated with a counterion (*e.g.* chloride) or in an oxygen-bridged dimer, the μ-oxo dimer. Treatment of the ferric porphyrin with acid gives the former species; treatment with aqueous base gives the latter species. We observed no difference in retention time between the Fe(III)Cl and μ-oxo dimer complex of tetraphenylporphyrin, probably because one is converting to the other on the column. Table I gives the retention times for selected derivatives of Fe(III)TPP.

Separation of protohemin, the monoacid monomethyl ester and bismethyl ester

Because the role of the 6- and 7-propionic acid side chains in heme proteins continues to be of interest[31–38,55–57], we were especially interested in using HPLC to characterize the monoacid monomethyl esters of protohemin, **6**. These can be synthesized either by partial esterification of protohemin or by partial hydrolysis of the protohemin bismethyl ester[45]. Either of these techniques gives a mixture of protohemin, the two monoacid monoester derivatives (the 6,7 and 7,6 positional isomers) and the bismethyl ester. All three were separated cleanly using a reversed-phase column, as shown in Fig. 3. No separation of the 6,7 positional isomers were observed on either the silica or reversed-phase columns.

Purification of positively-charged porphyrins and hemins

Initial studies were performed with 5,10-diphenyl-15,20-di-(N-methylpyridyl) porphyrin chloride because this porphyrin has only two positive charges and no central metal. No clear peak was seen on the C_8 column with a gradient between

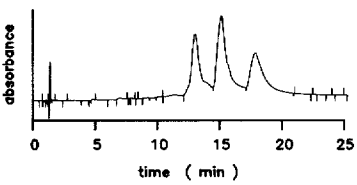


Fig. 3. Separation of protohemin (**1**, 12.9 min), the monoacid monomethyl esters (**6**, 14.9 min) and bismethyl ester (**2**, 17.7 min) on a C_8 5-μm column. A solution of methanol–0.1 *M* ammonium phosphate (30:70) adjusted to pH 3.5 (using concentrated phosphoric acid) was run in a 15-min gradient to 100% methanol and held at 100% methanol for 15 min.

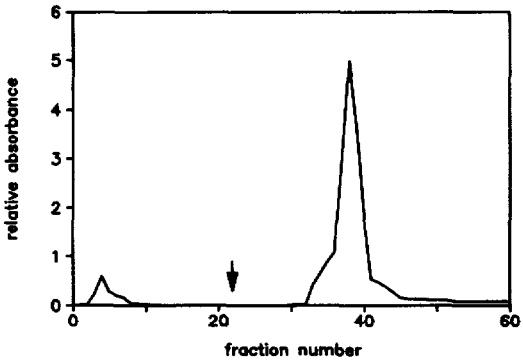


Fig. 4. Separation of a mixture of TMPyP and Fe(III)TMPyP on a Sephadex LH-20 column. Methanol was used as the eluent for the first 22 fractions (elution of TMPyP); saturated aqueous $NaHCO_3$–methanol (15:85) thereafter [elution of Fe(III)TMPyP]. The absorbance was measured at 420 nm. The maximum absorbances for TMPyP and Fe(III)TMPyP are 420.7 nm and 424.4 nm (methanol), respectively.

solution A containing 0.1 *M* potassium acetate adjusted to pH 3.0 with formic acid and solution B containing equal amounts of acetonitrile and water[39] nor with a gradient between aqueous ammonium phosphate and methanol[19]. Similar negative results were obtained using water–acetic acid (50:50) containing 0.35 *M* sodium dodecyl sulfate[58]. A gradient of water–acetic acid (9:1) to water–acetic acid (3:1) ramped over 15 min again gave the same results not only for this porphyrin but also for TMPyP and Fe(III)TMPyP.

Excellent results were obtained, however, using Sephadex LH-20 in standard column chromatography. This has been reported to work very well for TPP quaternized with 1–4 hexadecyl chains[42] and for mixtures derived from the self-condensation of hematoporphyrin[59], but poorly for TMPyP and InTMPyP[39]. We have achieved very good separation of positively charged porphyrins eluting with mixtures of methanol and saturated aqueous $NaHCO_3$. For example, Fig. 4 shows the elution profile of a mixture of the tetrapositively charged TMPyP and its corresponding Fe(III) chelate eluted with 15% saturated aqueous $NaHCO_3$ in methanol; baseline separation is observed, with the porphyrin eluting first. It should be noted that TMPyP can chelate metals and that reproducible results necessitate metal-free solvents.

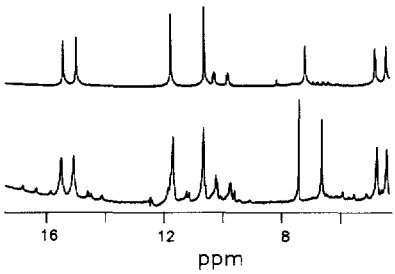


Fig. 5. ^1H NMR of the reaction of **7** with methyl iodide to give the bispositively charged **8**. (Bottom) Reaction mixture after purification by column chromatography on Sephadex LH-20 using only methanol as the eluent. (Top) Reaction mixture after purification on Sephadex LH-20 as described in the text. Spectra were taken of the low-spin biscyano complexes in dimethyl sulfoxide-d_6.

Sephadex LH-20 chromatography is also very useful in purifying positively-charged derivatives of natural porphyrins. For example, methylation of **7** to give **8** is expected to give the protohemin bearing two quaternary ammonium side chains. However, if methylation is incomplete, some of the compound bearing one $-N(CH_3)_3^+$ and one $-N(CH_3)_2$ will be formed. Because there are two positional isomers (6,7 and 7,6 substitution), the ^1H NMR of the methyl region of the low-spin Fe(III) complex shows eight methyl peaks, four each for the two isomers. The bottom trace of Fig. 5 shows a reaction mixture after alkylation and one attempt at purification. The four methyl singlets of the desired bisquaternary salt are seen between 10 and 16 ppm but other peaks in this region indicate the presence of other hemin derivatives as well. The top trace of Fig. 5 shows the same sample after passage though a Sephadex LH-20 column eluting first with methanol and then with 20% saturated aqueous $NaHCO_3$ in methanol; the presence of only four methyl peaks indicates that only the desired bisquaternary salt is present.

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