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Note

High-performance liquid chromatographic analysis of "available" hemin in hematin solutions

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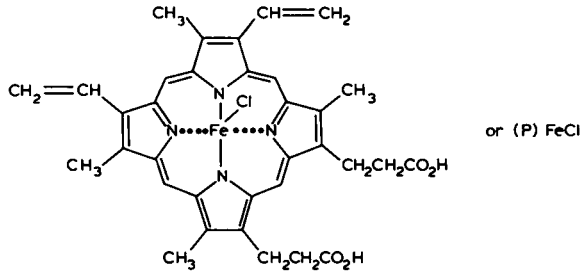
Hemin is an iron porphyrin (I) obtainable from blood cells by extraction procedures. Hemin is practically insoluble in both water and organic solvents. It does,



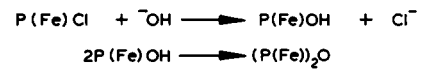
I

however, freely dissolve in aqueous alkali due to the formation of what is traditionally referred to as hematin (II). The structure for hematin which has been historically accepted is the result of replacing the chloride counter-ion of hemin with hydroxide.

(P) FeOH

II

In recent years, however, it has been accepted[1–8] that although the hydroxide may exist as an intermediate, the reaction of hemin is more complicated and results in an oxy-bridged dimer (III) and probably higher order polymers (Scheme 1). This dimerization is reversible upon acidification. However, upon standing in alkaline solutions hemin degrades to other materials not reconvertible to hemin upon acidification.



III

Scheme 1.

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In recent years hematin has become increasingly valuable in the treatment of acute porphyria. Clinicians have been preparing the material by dissolving hemin in dilute sodium carbonate solution and administering the hematin intravenously. Since the patient population is extremely small and each individual requires only two or three treatments per year, hematin has been prepared on demand and shipped to the requesting physician.

Review of the clinical data available and discussions with FDA clinicians[9] indicated that hematin was much more effective when freshly prepared than after storage in solution. In order to study the changes which occur upon standing in alkaline solution it was necessary to develop a stability indicating assay.

There are a small number of high-performance liquid chromatography (HPLC) methods for porphyrin esters reported in the literature, but none is applicable to the metalloporphyrins. The purpose of the work reported herein was to develop an HPLC system for available hemin in alkaline solutions of bulk hemin and to evaluate the stability of such solutions. The data reported herein does not include formulation studies. These will be the subject of a future publication.

EXPERIMENTAL

Hemin used in these studies was obtained from Sigma (bovine) and Abbott Labs. Chemical Division (human).

The HPLC system comprised a DuPont Model 870 chromatographic pump, a Tracor detector with visible lamp operated at 405 nm and a Waters μPorasil column, 30 cm × 4 mm I.D. Data were collected and the chromatograms displayed on a Hewlett-Packard 3388 recording integrator. The mobile phase was methanol–acetonitrile–acetic acid–pyridine (45:45:10:5) and was pumped at 1.0 ml/min.

Standard preparation

Hemin (14 mg) was weighed into a 50-ml volumetric flask and 2.0 ml of 0.5% sodium carbonate were added to dissolve the hemin. The sample was then diluted to volume with 0.6% nitric acid in methanol. This solution was then diluted 1 to 10 with mobile phase.

Sample preparation

A 2-ml aliquot of the alkaline solution of hemin (reconstituted lyophilized product where appropriate) was diluted to 50.0 ml with 0.6% nitric acid in methanol. This was then further diluted 1 to 10 with mobile phase.

RESULTS AND DISCUSSION

In so far as hematin is an undefined and unstable entity, direct quantitation of hematin was impossible. In lieu of this an analysis of "available" hemin was developed since the differences in effectiveness between "old" and fresh solutions imply that this defines the active ingredient. "Available" hemin includes: hemin chloride, hemin hydroxide and the oxy-bridged dimer. Since these compounds are all convertible to hemin by acidification, the first step involves acidification of the solution. This is done by diluting an aliquot of the alkaline solution with 0.6% nitric

acid in methanol. Nitric acid was chosen because the nitrate anion greatly increases the solubility of the metalloporphyrin in organic solvents.

The iron in hemin is complexed by four nitrogens leaving two co-ordination sites, above and below the ring system. The iron co-ordinates rapidly and reversibly with a large number of ligands thus causing a so-called "axial ligand uncertainty". This lability causes much difficulty during chromatography. In order to overcome this problem we have dictated the axial ligand by including pyridine in the eluent. This causes the formation of a pyridine hemochrome (IV) which is preserved throughout the chromatography by an excess of pyridine in the eluent.

This system gives baseline resolution from protoporphyrin (V) which would be formed by demetallation and from two degradation products, as yet unidentified, which are produced upon standing in sodium carbonate solution.

Literature evidence is abundant[1-8] that hemin in sodium hydroxide is converted to the oxy-dimer. Therefore a sample of hemin in 0.1 *N* sodium hydroxide was mixed for 1 week and assayed by the method described herein. It analyzed at 97% of the theory.

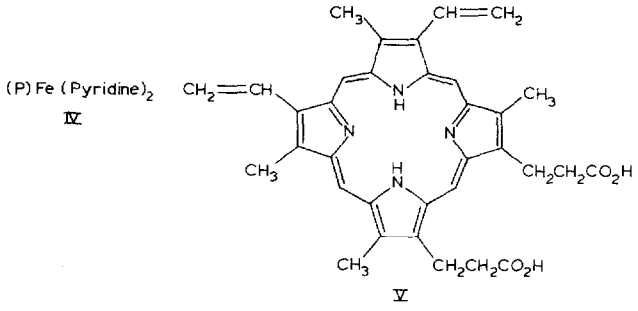

Fig. 1 shows a typical separation of protoporphyrin, hemin and its degradation products. The latter are currently being isolated and identified. The hemin response is linear from 0.07 to 10 mg/ml with a correlation coefficient of 0.9999. Precision was determined on partially degraded solutions and gave relative standard deviations of less than $\pm 1.0\%$. The data are presented in Table I.

This chromatographic system was then used to follow the stability of hemin in sodium carbonate solution. These data are presented in Table II and Fig. 2. The shape of the curve would suggest an oxidative degradation similar to the protohemin oxygenase bio-degradation pathways[9].

CONCLUSION

An HPLC analysis for hemin and for "available" hemin in alkaline solutions has been developed and may be applicable to other metalloporphyrins. The system has been used to study the degradation of hemin in sodium carbonate systems. Work is continuing toward identifying the degradation products and elucidating the kinetics involved.

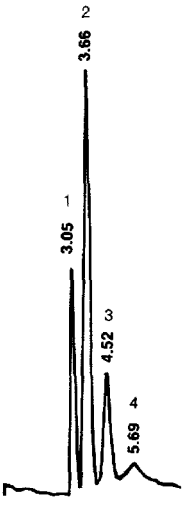

Fig. 1. Typical separation of hemin, protoporphyrin and hemin degradation products. Peaks: 1 = protoporphyrin; 2 = hemin; 3 and 4 = degradation products.

TABLE I

PRECISION DATA FOR "AVAILABLE" HEMIN ASSAY

Values are percentages.

Sample A		*Sample B*	
Analyst I	*Analyst II*	*Analyst I*	*Analyst II*
50.02	50.06	55.01	55.44
50.44	49.36	54.44	53.68
50.08	50.03	54.73	54.40
50.11	50.14	54.40	54.64
49.84		54.47	
Average = 50.01		Average = 54.58	
S.D. = 0.29		S.D. = 0.48	
C.V. = 0.58		C.V. = 0.88	

TABLE II

CHANGES IN "AVAILABLE" HEMIN WITH TIME IN 0.5% SODIUM CARBONATE SOLUTION (7 mg/ml)

Time (h)	*Percentage of initial "available" hemin*
0	100
0.25	98.5
0.50	94.7
1.0	81.6
1.50	71.2
2.0	65.8
2.5	62.9
3.0	55.0
4.0	49.9
5.5	45.97
26.0	17.20

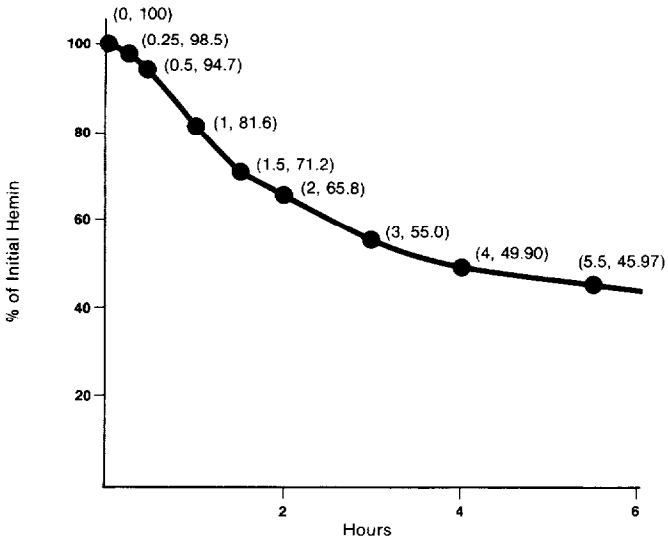

Fig. 2. Graphic representation of decrease of "available" hemin with time in 0.5% sodium carbonate solution.

ACKNOWLEDGEMENT

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