Iron Chelators of the Pyridoxal Isonicotinoyl Hydrazone Class Part I. Ionisation Characteristics of the Ligands and Their Relevance to Biological Properties

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Abstract

The orally effective iron chelator, pyridoxal isonicotinoyl hydrazone (PIH), and five analogues, pyridoxal benzoyl hydrazone (PBH), pyridoxal *p*-methoxybenzoyl hydrazone ((PpMBH), pyridoxal *m*-fluorobenzoyl hydrazone (PmFBH), 3-hydroxy-isonicotinaldehyde isonicotinoyl hydrazone (IIH) and salicylaldehyde isonicotinoyl hydrazone (SIH) were synthesised and characterised and their acid dissociation constants measured by glass electrode potentiometry and UV–Vis spectrophotometry. Analysis of the data showed that at physiological pH all of the ligands are predominantly (av. 80%) in the form of the neutral molecule, allowing passage through cell membranes and access to intracellular iron pools. The results are discussed in the context of the development of an orally effective iron chelator for clinical use.

Introduction

The search for high affinity iron chelating agents has been inspired in part by the need to mobilize iron from tissues that are overloaded with iron. An extensive range of structural types has been investigated [1] but, to date, only the tris-hydroxamate desferrioxamine (Desferal, DFO) is in routine clinical use [2]. However, the high cost of subcutaneous administration (currently, in excess of $10 000 per patient per year in Australia) and poor intestinal absorption of DFO [3, 4] are serious limitations and underline the need for an alternative iron chelating compound, especially one which is effective when given orally [4, 5]. This need is felt particularly in tropical areas of Southern Asia, where haemoglobinopathies such as thalassemia are now known to be prevalent [6].

Somewhat unexpectedly, the hydrazone formed from pyridoxal and isonicotinic acid hydrazide (pyridoxal isonicotinoyl hydrazone; PIH; Fig. 1(a)), is an effective chelator of iron(III). The potential of PIH has been examined and confirmed in a variety of biological and biochemical assays [7–18].

Recent studies [15–17] have shown that three analogues of PIH are more active than PIH and DFO in assays using hepatocyte, macrophage and reticulocyte cell types. These compounds are pyridoxal benzoyl hydrazone (PBH), pyridoxal *p*-methoxybenzoyl hydrazone (PpMBH) and pyridoxal *m*-fluorobenzoyl hydrazone (PmFBH) (Fig. 1(b), (c), (d)). Two other analogues, 3-hydroxyisonicotinaldehyde isonicotinoyl hydrazone (IIH) and salicylaldehyde isonicotinoyl hydrazone (SIH) (Fig. 1(e), (f)) also show appreciable activity in the hepatocyte assay [15]. Consideration of the high activity of PIH and these five analogues has prompted systematic studies examining their chemical properties.

This paper is concerned with the determination of the protonation constants for PIH, PBH, PpMBH, PmFBH, SIH and IIH and hence their speciation as a function of pH. This, in turn, is related to the lipophilicity of the ligands and their membrane permeability, a key factor in determining their access to cells. These protonation constants are also required for determination of the formation constants of these ligands with physiologically important metal ions such as Fe(III), Fe(II), Ca(II), Mg(II) and Zn(II) (see following paper p. 171).

A brief account for the pK_a study has appeared elsewhere [19].

Experimental

Preparation of Chelating Agents

All reagents were of the highest purity commercially available and were used without further purification. PBH and PIH were prepared by the method

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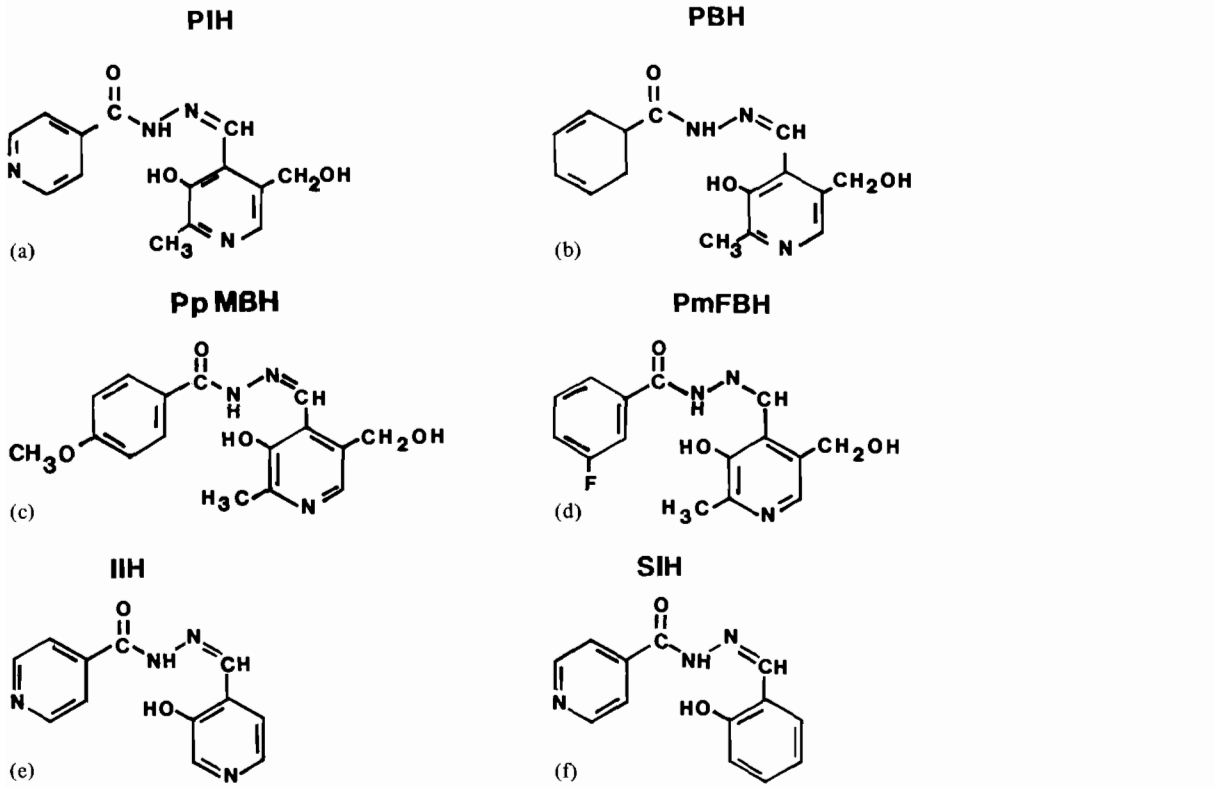

Fig. 1. Structures of the chelating agents: (a) pyridoxal isonicotinoyl hydrazone (PIH), (b) pyridoxal benzoyl hydrazone (PBH), (c) pyridoxal *p* methoxybenzoyl hydrazone (PpMBH), (d) pyridoxal *m*-fluorobenzoyl hydrazone (PmFBH), (e) 3-hydroxyisonicotinaldehyde isonicotinoyl hydrazone (IIH), (f) salicylaldehyde isonicotinoyl hydrazone (SIH).

of Johnson *et al.* [12] and were recrystallised from methanol/petroleum ether and ethanol/hexane, respectively. Yields of PIH and PBH were 65% and 80%, respectively. In the case of PpMBH and PmFBH, the *p*-methoxybenzoic acid hydrazide and *m*-fluorobenzoic acid hydrazide were synthesised by a modification of the method of Manoussakis *et al.* [20]. The appropriate benzoic acid (30 mmol) was refluxed with absolute ethanol (1 mol) and 2 ml of sulfuric acid (100%) for 14–20 h. The ethyl ester produced was separated as an oil before being extracted with diethyl ether. Absolute ethanol (50 ml) and hydrazine hydrate (7 ml, 150 mmol) were added and the mixture refluxed for 5–7 h. Subsequent crystallisation resulted in needle-like white crystals of the *m*-fluoro compound (yield 23%). The *p*-methoxybenzoic acid hydrazide was crystallised, also as needle-like white crystals (yield 15%), by the addition of petroleum ether and standing at 4 °C for 24 h.

The chelating agents PpMBH and PmFBH were produced by condensation of these hydrazides with pyridoxal and crystallised from ethanol/petroleum ether at 4 °C, 24 h. Yields and melting point (m.p.) data were 68%, 204–205 °C and 70%, 220–221 °C, respectively.

The compound IIH (m.p. 260 °C) was synthesised by condensation of isonicotinoyl hydrazine with 3-hydroxyisonicotinaldehyde prepared by bromination of 4-picoline in fuming H_2SO_4 and subsequent oxidation, generally following literature methods [21–23]. The compound SIH was prepared as white crystals (m.p. 242 °C) as reported elsewhere [24, 25].

All of the above chelating agents were characterised by conventional spectroscopies and data are in good agreement with previously reported values, e.g. for PIH and PBH [14, 20]. Data were recorded variously on a Pye-Unicam SP2000 IR spectrophotometer, a Hewlett-Packard 8450 UV–Vis spectrophotometer, a Hewlett-Packard 5986 glc-mass spectrometer operating at 35 eV and a Bruker HX-90 (90 MHz) NMR spectrometer in NaOD.

Preparation of Ligand Solutions

Ligands were prepared in a mixture of 0.1 potassium nitrate (as an inert background electrolyte) and nitric acid (0.6–5.0 mM) diluted from a 0.1000 M nitric acid stock solution prepared from concentrated volumetric ampules (BDH, U.K.). All solutions were prepared using distilled water that was then passed through a Millipore, Milli-Q

System, henceforth referred to as millipore water.

Initially, solutions at a concentration of 1 mM (PIH), 0.5 mM (PBH and PmFBH) and 0.25 mM (PpMBH) were prepared by dissolving the ligands in an excess of nitric acid (5 mM) by heating on a steam bath for 20–30 min. However, results from such titrations indicated some ligand loss via acid-catalysed hydrolysis. This was later confirmed by spectrophotometry. To minimise ligand hydrolysis, the acid concentration was decreased, generally to less than 2 mM and dissolution was achieved by sonication for 30–45 min without heating. Examination of the ligand solutions spectrophotometrically during sonication revealed no significant hydrolysis. Nevertheless, all ligand solutions were prepared immediately (≤2 h) prior to titration. Ligand solutions for spectrophotometric measurements were prepared in succinic acid–succinate or Tris–HCl buffer systems adjusted to an ionic strength of 0.1 M with potassium nitrate.

Determination of Protonation Constants

Potentiometric (pH) titrations were used to determine the protonation constants for all ligands. The poor solubility of SIH limited the use of potentiometry to the pH region 9.0–11.5. Hence the protonation constants for this ligand were obtained by a combination of potentiometry and UV–Vis spectrophotometry.

Potentiometric titration data were obtained using a purpose-built automatic titrator [26] consisting of a 5 ml piston burette (Metrohm, Model 655), a digital electrometer accurate to ±0.1 mV and stirrer, which were all under the control of a programmable calculator (Hewlett-Packard Model HP41C). Potentiometric measurements were carried out in a capped, jacketed, titration vessel (initial volume 50 ml), fitted with a glass electrode (Metrohm, Model AG9100) and a fibre-tipped saturated calomel electrode. The titration vessel was connected via insulated tubing to a Haake Model FK circulator which maintained the temperature of the vessel contents at 25.0 ± 0.025 °C. The entire apparatus was kept in a thermostatted room at 23 ± 1 °C.

Ligand solutions were titrated with standard 0.1 M potassium hydroxide prepared from concentrated volumetric ampules (BDH, U.K.) diluted with fresh, degassed millipore water. All titrations were performed under a high purity nitrogen atmosphere.

The glass electrode was calibrated for each titration using strong acid (0.01 M HNO_3)–strong base (0.1 M KOH) titrations at $I = 0.1$ (KNO_3). The program library ESTA (see below) was used to determine the Nerstian intercept ($E^{\circ\bullet}$) ionic product of water at 25 °C, $I = 0.1$ (KNO_3). The glass electrode was then re-calibrated in 0.01 M HNO_3 immediately before and after each titration, and the $E^{\circ\bullet}$ obtained by comparison with the strong acid–strong base titration data.

The UV–Vis spectra were recorded with a HP8450 spectrophotometer fitted with thermostatted (25.0 ± 0.1 °C) cell holders. The pH of the solutions, thermostatted at 25 °C and kept under positive argon pressure, was determined using a Radiometer PHM64 research pH meter equipped with a Radiometer EA107 glass electrode and saturated calomel electrode.

Calculation Procedures

Analysis of the titration data for PIH, PBH, PpMBH and PmFBH was generally performed using the computer program library ESTA (Equilibrium Simulation for Titration Analysis) [27].

Protonation constants were calculated from titration data by using the ZBAR task. This defines the number of ionisable protons bound to the ligand, e.g. PIH has four ionisable protons: at low pH (≤2) four sites are protonated ($Z_H = 4$) while at pH ≥12 it is totally deprotonated ($Z_H = 0$). Plots of Z_H versus pH were used to illustrate the protonation of the ligands. Spectrophotometric data were processed by standard procedures [28] using the Marquart non-linear regression method [29].

Results and Discussion

Before discussing the significance of the results a few general comments on the precision and accuracy of the titration data are required. PIH and its analogues are, in their neutral forms, sparingly soluble in water (≤1 mM). This is at least an order of magnitude lower than the optimal concentration for a pH titration [30]. Consequently, the precision of the equilibrium constants calculated from such data are somewhat lower than usual. Further uncertainty is introduced by the possibility of acid- and base-catalyzed hydrolysis of the ligands at extreme pH values. Nevertheless, the potential significance of these ligands is such that the lower than usual precision is more than justified by the possible utility of the data.

Analysis of the titration curves yielded four pK_a values for PIH and IIH, and three each for PBH, PpMBH, PmFBH and SIH. The assignment of pK_a values to ionisable sites on the ligands was made by comparison with pyridoxal, the acid hydrazide and related compounds (Table 1). Thus, since the pyridinium proton of isoniazide has a pK_a of 3.54 and the phenolic proton of pyridoxal a pK_a of 4.20 it seems reasonable to assign the first and second ionizations to the corresponding sites in PIH. However, some caution is necessary

TABLE 1. Protonation constants of compounds related to pyridoxal isonicotinoyl hydrazone (PIH)

Compound	pK_a	Assignment[a]	Reference
Pyridoxal	4.20	–OH	33
	8.66	ring N	
Pyridoxal-5'-phosphate	6.4	–OH	33
	8.05	ring N	
5-Desoxypyridoxal	3.8	–OH	34
	10.0	ring N	
Isoniazide	1.85	$-NH_2$	35
	3.54	ring N	
	10 77	–NH-N	

[a]From refs. 27–29.

TABLE 2. Protonation constants of pyridoxal isonicotinoyl hydrazone (PIH) and analogues

Ligand	pK_i (±s.d.)	Assignment[a]
PIH	2.95 ± 0.02	INH ring N
	4.41 ± 0.01	PYR ring OH
	7.86 ± 0.01	PYR ring N
	10.25 ± 0.01	–NH-N
PBH	4.24 ± 0.02	PYR ring OH
	8.15 ± 0.01	PYR ring N
	11.19 ± 0.01	–NH-N
PpMBH	4.14 ± 0.07	PYR ring OH
	8.28 ± 0.06	PYR ring N
	11.06 ± 0.03	–NH-N
PmFBH	4.378 ± 0.008	PYR ring OH
	8.087 ± 0.007	PYR ring N
	10.828 ± 0.005	–NH-N
IIH	2.97 ± 0.002	INH ring N
	3.97 ± 0.002	PYR ring OH
	7.93 ± 0.15	PYR ring N
	10.0 ± 0.9	–NH-N
SIH	3.43 ± 0.11	INH ring N
	8.29 ± 0.04	PYR ring OH
	9.8 ± 1.6	–NH-N

[a]INH and PYR refer to the pyridine rings derived from isoniazide and pyridoxal respectively.

because both pyridoxal-5'-phosphate and 5-desoxypyridoxal appear to lose their phenolic protons first (Table 1). Careful spectroscopic studies as a function of pH, although difficult due to low solubility, would resolve this ambiguity.

From examination of the pK_a values of PIH and PBH (Table 2), it is evident that the extra ring nitrogen in PIH acts in an analogous way to a nitro group by withdrawing electron density. In the case of PIH, a pK_a of 10.25 is associated with the ionisation of the NH-N proton which is almost one unit lower than that of PBH (11.19). A comparable, although less dramatic increase, in acidity of the NH-N group is seen when comparing PBH and PmFBH, due to the electron withdrawing fluoro group [31]. On the other hand, the *p*-methoxy group does not have any substantial effect on the pK_a values obtained (compare PBH and PpMBH, Table 2).

A plot of Z_H versus pH for PIH is presented in Fig. 2. At pH <3, three to four sites on the molecule are protonated, while at physiological pH the molecules have predominantly two protons bound, i.e. are largely in the neutral form H_2L. PIH is virtually fully deprotonated at pH >11. The distribution of the various forms of PIH as a function of pH are shown in Fig. 3.

The protonations of PBH, PpMBH and PmFBH are very similar. All three sites on these molecules are protonated at pH <3, two sites at physiological pH and none at pH >11. The species distribution plot for PBH is given in Fig. 4, and clearly shows the predominance of the neutral species H_2L near neutral pH. At near-physiological pH (7.5), all the chelating agents are present as a mixture of neutral and a singly charged anionic species (Figs. 3 and 4). The proportions are shown in Table 3. These results would indicate that the chelating agents would be lipophilic, as indicated by their relative ease of

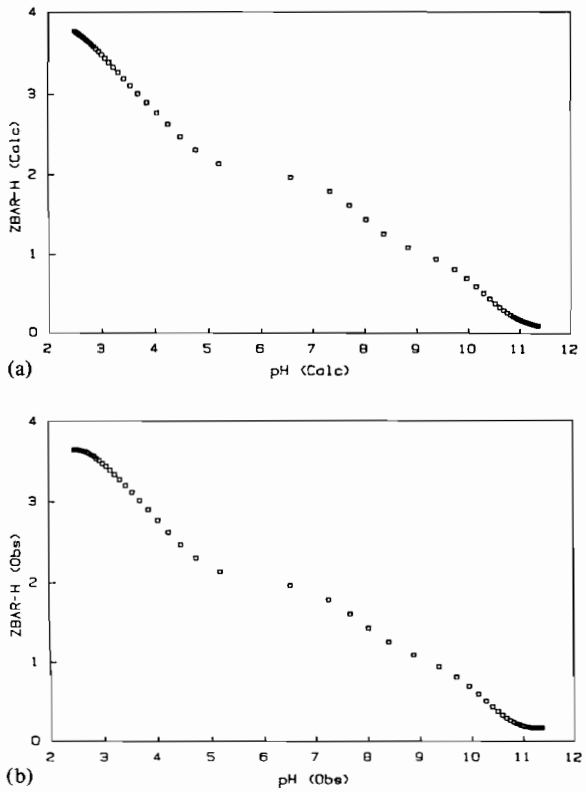

Fig. 2. For PIH, plots of (a) calculated values of $\bar{Z}_H$ versus pH; (b) observed values.

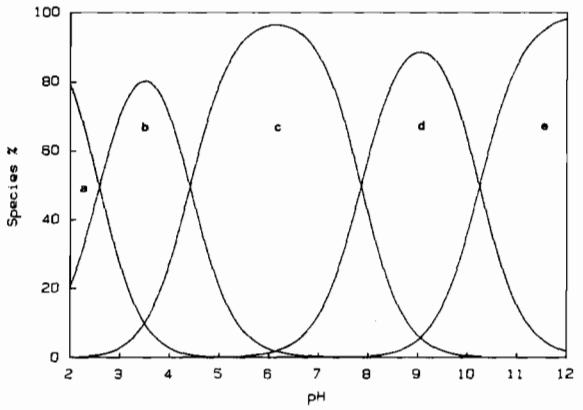

Fig. 3. Species distribution plot over pH 2–12 for PIH. (a) H_4L^{2+}; (b) H_3L^{+}; (c) H_2L; (d) HL^{-}; (e) L^{2-}.

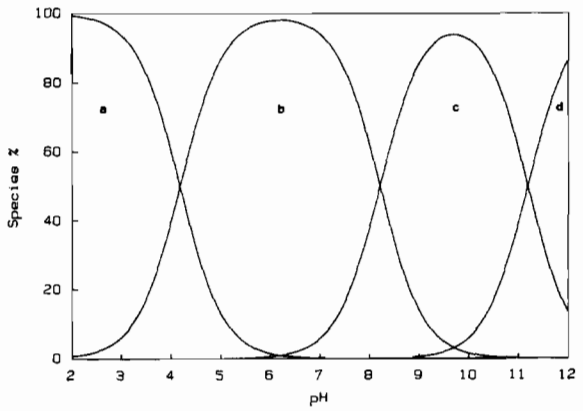

Fig. 4. Species distribution plot over pH 2–12 for PBH. (a) H_3L^{+}; (b) H_2L; (c) HL^{-}; (d) HL^{2-}.

TABLE 3. Relative percentage of neutral and singly charged anionic species at pH 7.5 for PIH and derivatives

Chelating agent	Neutral species (H_2L)	Anionic (HL^{-})
PIH	73	27
PBH	80	20
PpMBH	78	22
PmFBH	78	22
IIH	73	27
SIH	86	14

extraction into ethyl acetate [15, 16] and, furthermore, would be able to diffuse through cell membranes, allowing absorption from the gut and access to intracellular iron pools. From examination of the above data, the presence of the neutral species is maximal at pH 6 and hence maximal absorption of these ligands would probably occur in the small intestine where the pH is in this range [32].

In addition, due to the similarities in protonation and the increased lipophilicity of PpMBH and PmFBH when compared to PIH and PBH [17], absorption from the gut should be similar to, if not better than, that which has been reported for these latter two chelating agents [9, 11, 13]. Since the ratio of neutral to charged species is approximately the same for all four chelating agents, the differences in activity seen in the fetal hepatocyte screen [15, 16] and *in vivo* in rats [12] cannot be attributed simply to a difference in the charged state of the ligands.

Supplementary Material

Tables of IR, UV, NMR and mass spectral data for the six compounds are available from the authors on request.

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