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Partition coefficients of the iron(III) complexes of pyridoxal isonicotinoyl hydrazone and its analogs and the correlation to iron chelation efficacy

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Pyridoxal isonicotinoyl hydrazone and its analogs are orally effective Fe(III) chelators which show potential as drugs to treat iron overload disease. The present investigation describes the measurement of the partition coefficient of the apochelator and Fe(III) complex of 20 of these ligands. These measurements have been done to investigate the relationship between lipophilicity and the efficacy of iron chelation in rabbit reticulocytes loaded with non-heme ^{59}Fe. The results demonstrate a linear relationship between the partition coefficient (P) of the apochelator and its Fe(III) complex, and a simple equation has been derived relating these two parameters. Experimental data in the literature are in agreement with the equation. The relationship of the partition coefficients of the iron chelators and of their Fe(III) complexes to the effectiveness of the ligands in mobilizing iron *in vitro* and *in vivo* is also discussed.

Keywords: iron chelation, iron overload disease, partition coefficient, pyridoxal isonicotinoyl hydrazone

Introduction

Pyridoxal isonicotinoyl hydrazone (PIH) is a tridentate chelator with high affinity and specificity for Fe(III) (Richardson *et al.* 1989, Vitolo *et al.* 1990). In addition, the apochelator is predominantly neutral at physiological pH (Richardson *et al.* 1990), allowing easy access through biological membranes to intracellular iron pools. Together, these chemical properties probably partly explain the high iron chelation efficacy of PIH observed both *in vitro* and *in vivo* (Hoy *et al.* 1979, Ponka *et al.* 1979a, b, Cikrt *et al.* 1980, Hershko *et al.* 1981, Williams *et al.* 1982). Moreover, PIH can be synthesized economically and is effective after oral administration, in contrast to the drug in current clinical use, desferrioxamine (DFO), which is both highly expensive and requires long subcutaneous infusion (12 h per day, 5–7 days a week; Modell & Berdoukas 1984).

The high activity of PIH has encouraged systematic studies examining the activity of analogs of PIH *in vivo* and *in vitro*. These investigations have identified several analogs of PIH which are more effective than either PIH or DFO (Johnson *et al.* 1982, Baker *et al.* 1985a, Ponka *et al.* 1988, Richardson *et al.* 1988, Baker *et al.* 1992). However, details of the structure–activity relationships in these series of compounds remained largely elusive (Baker *et al.* 1992).

Considering the relationship between chemical structure and biological activity, the lipophilicity of a molecule has been shown to be an important property with regard to its ability to permeate biological membranes. Indeed, following the early work of Meyer, Overton and others (reviewed by Hansch 1969), it seems likely that the rate of passive diffusion of a compound into cells is largely a function of its lipophilicity, conveniently measured by its partition coefficient, P, between *n*-octanol and water. A previous study has exained the relationship between the iron-mobilizing activity and the partition coefficient of PIH and a large number of other aroylhydrazones of similar structure (Ponka *et al.* 1994). This study demonstrated that maximum biological activity of the apochelator occurred when the partition coefficient was approximately 1 ($\log P = 0$). However, it is possible that the relationship between activity and $\log P$ observed for these compounds (Ponka *et al.* 1994), depends not only on the partition coefficients, P_{chel}, of the ligands, but also on the partition coefficients, P_{comp}, of the Fe(III) complexes.

In the present paper we derive a simple equation relating P_{comp} to P_{chel}, and test it with our own data and also the limited amount of data available in the literature. In addition, the relationship of P_{comp} to the efficacy of a chelator at mobilizing iron from cells is also discussed.

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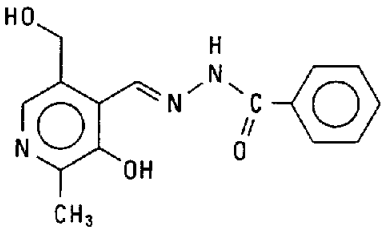
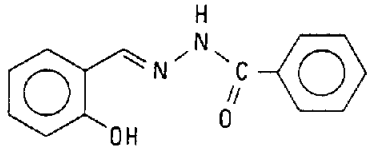
Materials and methods

Preparation of apochelators

Chelators were synthesized by Schiff base condensation between two aromatic aldehydes (pyridoxal-'100 series' and salicylaldehyde-'200 series') and a series of acid hydrazides (Figures 1 and 2) using standard procedures (Wild 1958). Characterization of these chelators with respect to their molecular compositions, melting points and IR spectra was reported previously (Edward *et al.* 1988).



Pyridoxal Benzoyl Hydrazone (101)



Salicylaldehyde Benzoyl Hydrazone (201)

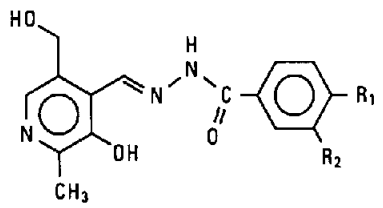
Figure 1. Structures of the two parent compounds: pyridoxal benzoyl hydrazone (100 series) and salicylaldehyde benzoyl hydrazone (200 series).

Determination of partition coefficients

An earlier study by the authors (Ponka *et al.* 1994) used thin layer chromatography via the method of Pliska *et al.* (1981) to determine the partition coefficients of the PIH analogs. This technique was found to be valuable for the smaller chelators, but was not useful to measure the partition coefficients of the larger Fe(III) complexes, due to the adsorption of these compounds to the solid support. Hence conventional extraction techniques were used to determine the partition coefficients of the iron complexes of the PIH analogs.

Extraction using ethyl acetate. Preliminary studies with the PIH analogs demonstrated that ethyl acetate was the best solvent to use to determine the differences in the relative lipophilicity of these chelators. Several solvents including the conventional standard, *n*-octanol (Hansch 1969, 1971), were tried; however, these solvents could not satisfactorily differentiate between the closely related analogs. Hence, we chose to use ethyl acetate instead of *n*-octanol, as we decided that by using this strategy more information could be obtained relevant to analyzing structure–activity relationships.

All chelators were prepared in phosphate buffered saline (PBS; pH 7.4) on the day of the experiment to prevent loss due to hydrolysis (Richardson *et al.* 1989). The Fe(III) chelates were prepared by dissolving the apochelator in PBS containing sufficient iron as ferric nitrilotriacetic acid (NTA) [0.1 mM $Fe_2(NH_4)_2SO_4$ in 0.5 mM NTA at pH 7.4] to saturate the compounds assuming a ligand:iron ratio of 2:1, which is the major iron complex species present at this pH (Vitolo *et al.* 1990). The partition coefficient (P^{EtAc}) was measured as the ratio of the concentration of the compounds



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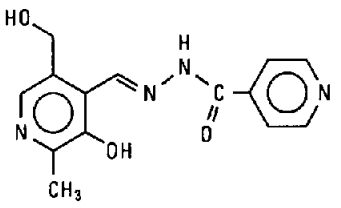

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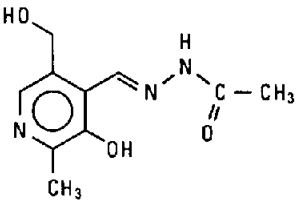

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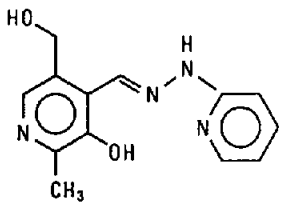

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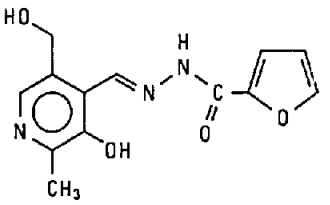

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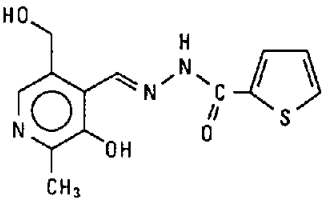

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Figure 2. Structures of analogs derived from Schiff base condensation of pyridoxal (100 series) with the series of acid hydrazides. Number 101: R_1 and R_2 = H; 102–107: R_2 = H; 102: R_1 = –OH; 103: R_1 = $-CH_3$; 104: R_1 = $-NO_2$; 105: R_1 = NH_2; 106: R_1 = $-C(CH_3)_3$; 107: R_1 = $-OCH_3$; 108–110: R_1 = H; 108: R_2 = Cl; 109: R_2 = F; 110: R_2 = Br; 111: isonicotinoyl; 112: acetyl; 113: 2-pyridyl; 114: 2-furoyl; 115: 2-thiophenecarboxyl.

between ethyl acetate and PBS. For each extraction, PBS saturated with ethyl acetate and ethyl acetate saturated with PBS was used. Partitioning of the chelator between the two phases was determined using a Beckman DU-8 spectrophotometer programmed to identify the maximum absorbance peak of the apochelator in the UV-Vis absorbance range of 200–450 nm. To examine the partitioning of the iron complexes of the PIH analogs, the charge transfer band at approximately 475 nm was monitored (Ponka *et al.* 1979a).

Using this technique, 20 of the 37 PIH analogs could be assessed to determine their partition coefficient. The remaining 17 chelators were too insoluble to measure accurately their concentration via spectrophotometry.

Calculation of partition coefficients between n*-octanol and water by the additive scheme of Rekker.* The experimentally derived partition coefficients have been compared to values calculated using the additive scheme of Rekker (1977). This scheme was used as a previous study of 30 PIH analogs demonstrated a high correlation between the partition coefficient calculated by the method of Rekker and that derived experimentally (Ponka *et al.* 1994).

Using the procedure of Rekker, log P of an organic molecule between n-octanol and water is obtained by summation of the hydrophobic fragmental constants (f_R) of the fragments (R) into which the molecular structure of the compound can be dissected. Figure 3 shows the f_R values of the groups making up fragment 1′, common to the 100 series of chelators, and fragment 2′, common to the 200 series of compounds. The summation of f_R values yields the hydrophobic contributions (F) of 1′ and 2′ shown in Figure 3. However, it should be noted that these F values require some modification because the summation fails to take account of the fact that the phenolic hydrogen is probably hydrogen bonded to the nitrogen of the aldimine (CH=N) group in all these analogs, as seen for other Schiff bases of similar structure (Dudek & Dudek 1966, Matsushima & Martell 1967). Hence, the phenolic group is probably less hydrophilic than expected from its normal f_R value.

The contribution of hydrogen bonding was analyzed. For o-hydroxybenzaldehyde, where hydrogen bonding is expected between the phenolic group hydrogen and the aldehyde oxygen, the log P value is equal to 1.69 (Hansch & Leo 1979), whereas for m- or p-hydroxybenzaldehyde, where no hydrogen bonding is possible due to stereochemistry, a log P of 1.38 was found (Hansch & Leo 1979). Consequently, on the basis of this analysis, to adjust for the contribution of hydrogen bonding, the F values in Figure 3 must be increased by 0.31. These corrected values of F (given in the legend to Figure 3) are used for calculating log P by the equation:

$$\log P = F + f_R$$

f_R now being the fragmental constant for the R group (listed in Tables 1 and 2) attached to 1′ and 2′.

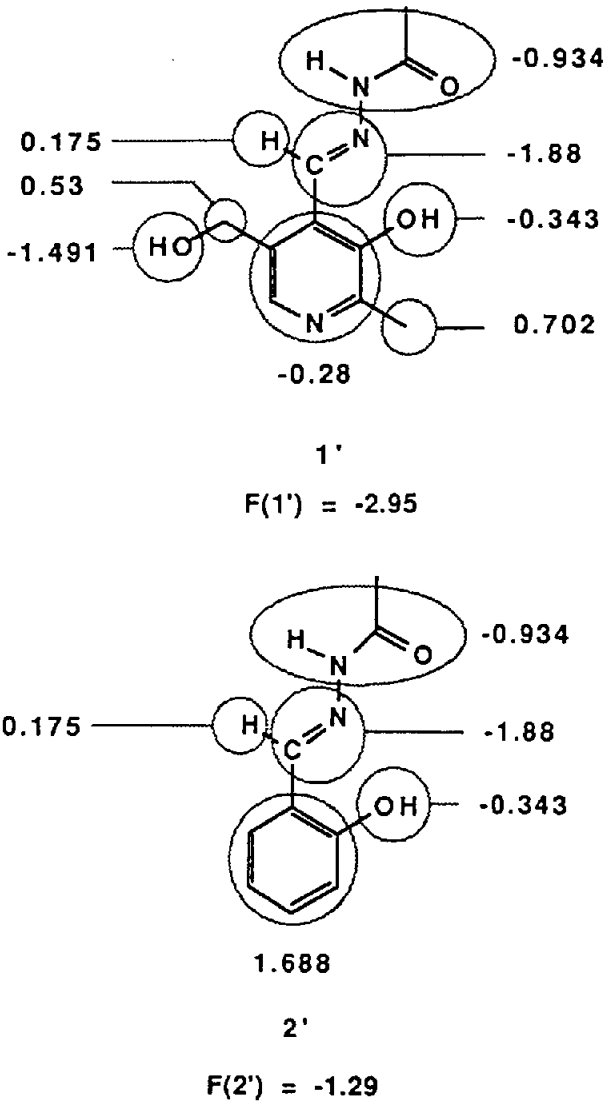


Figure 3. Dissection of the fragments 1′ and 2′ common to the structures of the chelators of the 100 and 200 series, respectively, into smaller fragments with Rekker's hydrophobic fragmental constant f_R for n-octanol/water shown beside the latter. Summation of the f_R values gives the fragmental constants F shown under 1′ and 2′; these need to be corrected by the addition of 0.31 due to intramolecular hydrogen bonding (see text) to give $F(1') = -2.64$ and $F(2') = -0.98$.

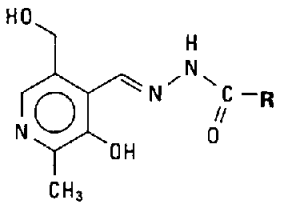
Results and discussion

Relationship of the partition coefficients between ethyl acetate and PBS to the calculated partition coefficient between n*-octanol and water*

The partition coefficient between n-octanol and water has become the standard measure of lipophilicity when discussing structure–activity relationships (Hansch 1969). However, for reasons of practical necessity, as described previously (see Materials and methods), the partition

Table 1. Partition coefficients between ethyl acetate and PBS (pH 7.4) of apochelators of the 100 series (P^{EtAc}_{chel}) and of their Fe(III) complexes (P^{EtAc}_{comp})

PIH analogs (100 series)

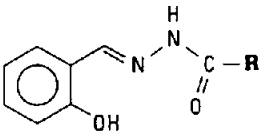


No.	Hydrazone R =	Chelator		Fe(III) complex	
		% extracted	log P	% extracted	log P
101	Benzoyl	81 ± 1[a]	0.93	50 ± 2	0.30
102	p-Hydroxybenzoyl	47 ± 5	0.25	17 ± 3	−0.39
103	p-Methylbenzoyl	78 ± 3	0.85	87 ± 2	1.03
105	p-Aminobenzoyl	53 ± 2	0.35	10 ± 3	−0.65
106	p-t-Butylbenzoyl	64 ± 2	0.55	22 ± 3	−0.25
107	p-Methoxybenzoyl	68 ± 4	0.63	62 ± 1	0.51
108	m-Chlorobenzoyl	92 ± 1	1.36	99 ± 1	2.30
109	m-Fluorobenzoyl	85 ± 1	1.05	83 ± 2	0.99
110	m-Bromobenzoyl	91 ± 5	1.30	98 ± 2	1.99
111	Isonicotinoyl	23 ± 2	−0.21	8 ± 1	−0.76
113	2-Pyridyl	54 ± 7	0.37	26 ± 7	−0.15
114	2-Furoyl	56 ± 6	0.41	49 ± 5	0.28
115	2-Thiophenecarboxyl	52 ± 11	0.33	30 ± 10	−0.07

[a] Extraction results are mean ± SEM (three or four experiments).
Concentration of the apochelator and its Fe(III) complex = 0.1 mM.

Table 2. Partition coefficients between ethyl acetate and PBS (pH 7.4) of apochelators of the 200 series (P^{EtAc}_{chel}) and of their Fe(III) complexes (P^{EtAc}_{comp})

Salicylaldehyde benzoyl hydrazone analogs (200 series)



No.	Hydrazone R =	Chelator		Fe(III) complex	
		% extracted	log P	% extracted	log P
201	Benzoyl	93[a]	1.42	>98	>1.99
202	p-Hydroxybenzoyl	96	1.68	>98	>1.99
203	p-Methylbenzoyl	93	1.42	>98	>1.99
205	p-Aminobenzoyl	79	0.88	>98	>1.99
208	m-Chlorobenzoyl	98	1.99	>98	>1.99
209	m-Fluorobenzoyl	93	1.42	>98	>1.99
210	m-Bromobenzoyl	98	1.99	>98	>1.99

[a] Extraction results are the means of two experiments.
Concentration of apochelator and its Fe(III) complex = 0.1 mM.

coefficients of the compounds were measured between ethyl acetate and PBS (pH 7.4). Values of P_{chel} of 13 chelators of the 100 series are given in Table 1 and of seven compounds of the 200 series in Table 2, along with values of P_{comp} of the 20 Fe(III) complexes formed by these ligands.

To determine if the partition coefficient derived using ethyl acetate (P^{EtAc}) gave a reliable estimate of relative lipophilicity of the chelators, these values were plotted against the partition coefficient, P, between n-octanol and water, which were calculated using the additive scheme of Rekker

(Figure 4). Collander (1951) has shown that the partition coefficients (P^X) of organic solutes distributed between an immiscible organic solvent X and water are related to their partition coefficients (P^Y) between another immiscible organic solvent Y and water by the equation:

$$\log P^X = a \log P^Y + b \quad (1)$$

where a and b are constants. Our data plotted in Figure 4 fits equation (1) for $P^X = P^{EtAc}$, $P^Y = P$, with $a = 0.47$, $b = 1.08$ and $r = 0.93$. It should be noted that the *p*-t-butyl derivative, chelator 106, showed abberant behaviour which cannot be explained and this point is left out of the equation. As there is a linear relationship between the partition coefficient measured in ethyl acetate, P^{EtAc}, and P calculated using the scheme of Rekker, it can be suggested that the use of ethyl acetate to measure the partition coefficient gave a reliable indication of the relative lipophilicity of the chelators.

It is of relevance to note that Leo & Hansch (1971), using experimental data for nine solute molecules, found that with X = ethyl acetate and Y = *n*-octanol, equation (1) applied well, with $a = 0.93$ and $b = 0.05$ (correlation coefficient $r = 0.97$). The considerable differences between our a and b values and those of Leo & Hansch probably arise because the latter authors examined monofunctional solute molecules, whereas our studies have investigated polyfunctional molecules.

Relation between the size and nature of a molecule and its value of P

In order to develop an equation to relate P_{comp} to P_{chel}, we consider first the effect of changes in the size and constitution of a molecule upon P generally.

The solubilities in water of the purely hydrophobic alkanes decrease with increasing size, and, consequently, their partition coefficients, P, between *n*-octanol and water increase. This has been explained by the increasing energy required to form cavities in water to accommodate the hydrocarbon molecules as they become larger. However, there has been disagreement as to whether this energy, and hence log P, is proportional to the volume, $\bar{V}$, or the surface area of the cavity (McAuliffe 1966, Jencks 1969, Harris *et al.* 1973, Hermann 1973, Edward *et al.* 1982). Taft *et al.* (1985) found that experimental log P values of 102 aliphatic and aromatic compounds fitted well to the equation:

$$\log P = 0.2 + 2.74\bar{V}/100 - 0.92\pi - 3.49\beta \quad (2)$$

where $\bar{V}$ is the molar volume, π is a solvatochromic parameter that measures solute dipolarity/polarizability and β is another solvatochromic parameter that measures the hydrogen-bond-acceptor basicity of the solute. The most important terms are $\bar{V}$, which makes a positive contribution to log P, and β, which makes a negative contribution.

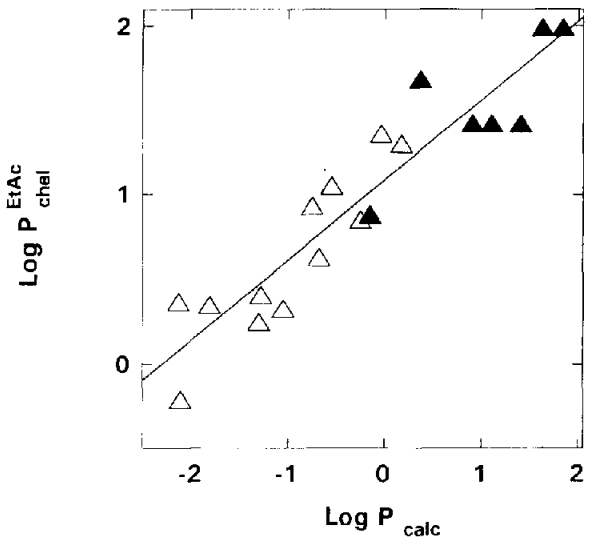


Figure 4. Plot of log P_{chel}^{EtAc} (ethyl acetate/PBS) of chelating compounds of the 100 series (△) and 200 series (▲) against their log P (*n*-octanol/water) values calculated from Rekker's fragmental constants.

Relation of P_{chel}^{EtAc} *of compounds of the 100 series and* P_{comp}^{EtAc} *of their Fe(III) complexes*

These concepts described above can now be applied to derive the relation of P_{comp}^{EtAc} of a series of Fe(III) complexes of structure **2** (see Figure 5; Vitolo *et al.* 1990) differing in the nature of the R groups (see Figures 1 and 2; Tables 1 and 2), to P_{chel} of the corresponding chelating molecules of structure **1** (Figure 5).

Log P_{chel}^{EtAc} will be an additive function of the varying contribution of the R group and of the constant contribution of the rest of the molecule. The electrically neutral complex (Figure 5; Webb & Vitolo 1988, Vitolo *et al.* 1990) has two tridentate chelating molecules octahedrally coordinated to Fe(III), so that it will have twice the volume and twice the area of the ligand alone (the contribution of the iron atom to the volume and hence to log P is negligible; Edward 1970).

Consequently we can expect:

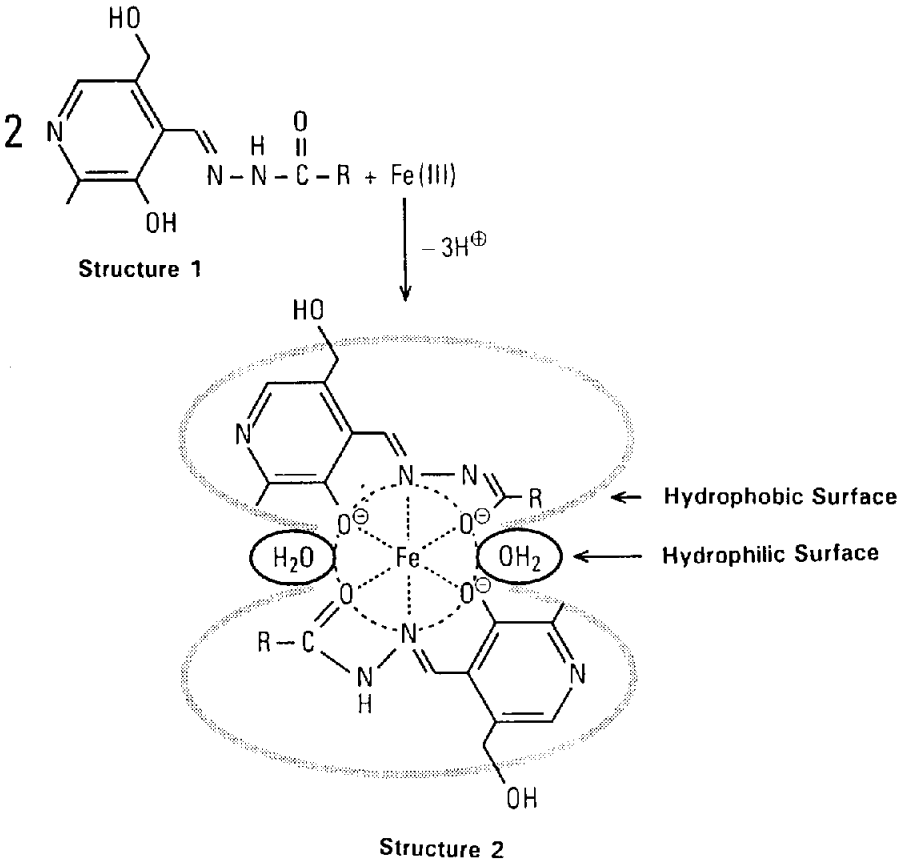
$$\log P_{comp}^{EtAc} = 2 \log P_{chel}^{EtAc} + k \quad (3)$$

where k is a constant representing the change in hydrophobicity in convering the six hydrophilic ligating groups of the two molecules of structure **1** (Figure 5) into the coordination sphere of the Fe(III) complex. The coordinated iron atom may not be completely shielded by the ligands and hence water molecules may be expected to interact (schematically shown in Figure 5) with this part of the complex, resulting in a decrease in lipophilicity. In fact, for the 100 series chelators, log P_{comp}^{EtAc} plotted against log P_{chel}^{EtAc} (Figure 6) gives rise to a straight line of slope 1.94 (theoretical = 2) and intercept $k = -0.83$ ($r = 0.92$). It should be noted that the data in Table 2 has not been used to derive this equation because the iron complexes of the 200 series ligands were almost entirely extracted into the solvent and, hence, the log P_{comp}^{EtAc} values can be expected to have large experimental errors (Table 2).

For the more general case of one iron atom complexed by n ligand molecules, we may expect:

$$\log P_{comp} = n \log P_{chel} + k \quad (4)$$

Figure 5. Schematic representation of the FeL_2 Fe(III) complex of PIH illustrating hydrophilic and hydrophobic surfaces.



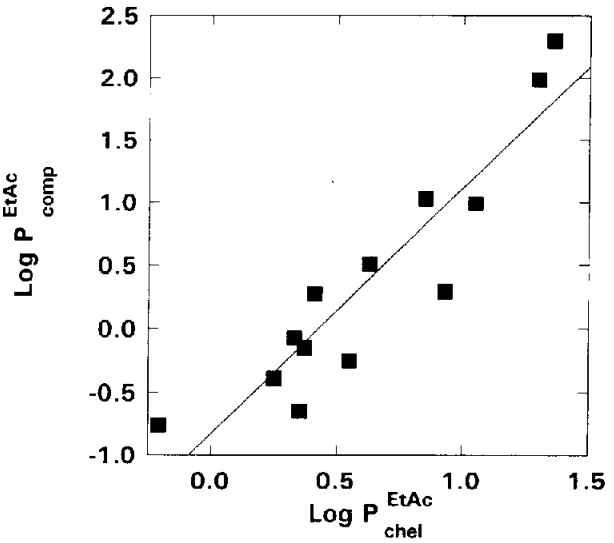


Figure 6. Plot of log P_{chel}^{EtAc} of ligands of the 100 series against their respective log P_{comp}^{EtAc} values.

It is of importance to note that when a metal ion forms complexes with a series of closely related ligands, equation (4) offers a simple method for determining the stoichiometry of the complexes. Interestingly, Suzuki and associates (Omeri *et al.* 1964, Suzuki *et al.* 1968a, b, Wakahayashi *et al.* 1968, Akiba *et al.* 1969), using different arguments, have derived an identical equation to relate log P_{comp} of a single metal complex to log P_{chel} in different immiscible organic phase/water systems.

Complexes of Fe(III) with N-alkyl-3-hydroxypyridin-4-ones

To further test the validity of equation (4), a literature search was performed to examine whether the partition coefficient of a series of structurally similar Fe(III) chelators and their iron complexes had been determined in previous studies. The only suitable data found were from a study examining the relationship between the lipophilicity and the biological activity of the bidentate hydroxypyridone class of iron chelators (Porter *et al.* 1988). These chelators coordinate with Fe(III) in a 3:1 ratio (Porter *et al.* 1988, Taylor *et al.* 1988) to form octahedral complexes. The *n*-octanol/water partition coefficient (P_{comp}) of the complexed iron should be given by equation (4) with $n=3$ and k being negative.

In fact the values of log P_{comp} plotted against log P_{chel} of the 10 chelators having R_2 = alkyl (circles, Figure 7) or alkoxyalkyl (closed triangles, Figure 7) (Porter *et al.* 1988), fit to a straight line ($r=0.95$) required by equation (4) having $k=-0.61$, and $n=3.34$. The deviation of n from the theoretical value of 3 may be explained by an 11% association of the iron complex, FeL_3, to form the dimer, Fe_2L_6. The dimerization reaction could be driven by the tendency of the hydrophobic areas on the outer surface of FeL_3 to associate to avoid contact with water.

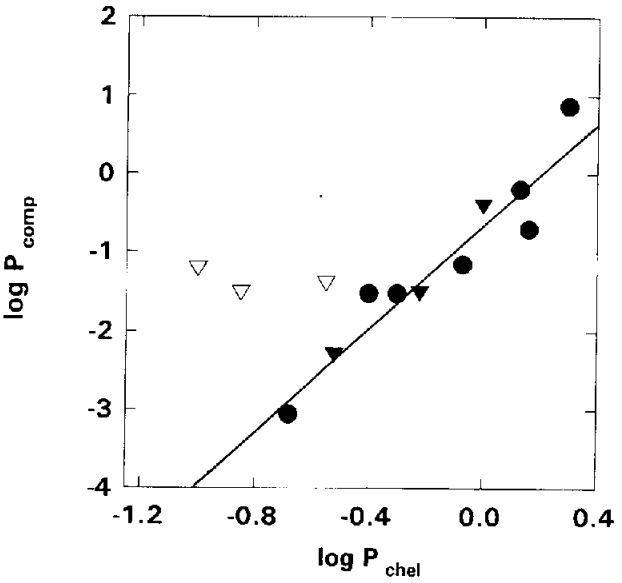


Figure 7. Plot of the log P_{comp} of Fe(III) complexes of *N*-alkyl-3-hydroxypyridine-4-ones against log P_{chel} of the chelating agents. R_2 = alkyl (●); R_2 = alkoxyalkyl (▼); R_2 = hydroxyalkyl (▽).

It should be noted that three chelators of the hydroxypyridone series having R_1 = hydroxyalkyl (open triangles; Figure 7), deviate strongly from the straight line required by equation (4). In these three compounds, the hydrophobic surface of the ligand also possesses a hydrophilic hydroxyl group, lessening log P_{chel}. Hence, it can be suggested that the association of these hydrophobic areas may be enhanced by intermolecular hydrogen bonding and, hence, log P_{comp} will be greater than that expected from the log P_{chel} value.

Relationship of P_{chel} *and* P_{comp} *to the efficiency of iron mobilization* in vitro *and* in vivo

Lipophilicity is an important criterion for membrane permeability. Indeed, the high biological activity of some of the PIH analogs compared with PIH itself may be at least partly related to their increased lipophilicity (Baker *et al.* 1985a, 1992).

Considering the role of lipophilicity in the biological activity of the PIH analogs, the 10 most effective chelators at mobilizing iron from reticulocytes, seven of them from the 100 series and three from the 200 series, had log P_{chel} (octanol/water) values varying between −1.28 and 0.63 (Ponka *et al.* 1994). The corresponding log P_{comp} (octanol/water) values of their Fe(III) complexes can be calculated by equation (4) (with $n = 1.94$, $k = -0.83$) and equation (1) (with $a = 0.47$, $b = 1.08$), as varying between −2.09 and 1.62. Hence, both P_{chel} and P_{comp} values occur about unity. This is about the value to be expected if the chelating agent is able to diffuse through an aqueous medium and lipoidal barrier in one direction, and the iron complex in the opposite direction. Moreover, these data are in agreement with the results of Porter *et al.* (1988), examining the effect of a series of 3-hydroxypyridin-4-one chelators on mobilizing iron from hepatocytes in culture. In their study, the most effective hydroxypyridones had log P_{chel} values between −2.30 and −0.19, and log P_{comp} values between −0.52 and 0.16 (Porter *et al.* 1988). While Porter and associates concluded that P_{comp} was less critical than P_{chel} in determining iron release, in fact both their results and ours indicate that not only P_{chel}, but also P_{comp}, are important.

The relevance of considering the lipophilicity of both the apochelator and iron complex in the design of effective iron chelators is demonstrated by examining the properties of the drug in current clinical use, DFO. Several studies have shown that this ligand can diffuse easily into cells, whereas the iron complex is far more hydrophilic, resulting in an accumulation of the iron chelate within the cell and a decrease in iron chelation efficacy (Richardson *et al.* 1994, Bottomley *et al.* 1985). In contrast, other iron chelators such as cholylhydroxamic acid, which can easily diffuse into cells to form a highly lipophilic iron complex, can also result in little iron mobilization due to the partitioning of the iron complex into the cell membrane (Baker *et al.* 1985b). Hence, the often quoted concept of an apochelator having an optimal hydrophilic/lipophilic balance (Ponka *et al.* 1994, Porter *et al.* 1988) can be equally well applied to the iron complex.

Furthermore, it is prudent to note that the lipophilicity of an iron complex can also significantly effect the distribution of iron within the body and the subsequent route of excretion. Noteworthy is the dependence on liposolubility of biliary excretion of ferrioxamines (Meyer-Brunot & Keberle, 1968). In addition, in an attempt to increase the efficacy of 2,3-dihydroxybenzoic acid (2,3-DHB), several lipophilic analogs of the chelator were synthesized and tested *in vivo* in the rat model. Methylation of the acid groups of 2,3-DHB failed to change the overall effectiveness of the chelator, but the excretion pattern of the iron complex was altered to the fecal route (Grady, 1976). Indeed, it is of significance that the appropriate lipophilicity of the PIH Fe complex may explain its bilary excretion in the rat (Cikrt *et al.* 1980). Moreover, the enhanced excretion of iron in the bile after the administration of pyridoxal *m*-fluorobenzoyl hydrazone (109) in comparison to PIH (Richardson *et al.* 1992), may be due to the greater lipophilicity of the iron complex of the former compound (Table 1).

In conclusion, the results of the present study imply that when designing new analogs of the PIH class, attention should be focussed on ligands having both P_{chel} and P_{comp} close to unity. Also, it can be suggested, that before initiating the expensive and time consuming process of screening analogs of PIH in biological models, the lipophilicity of the apochelator and iron complex should be determined so that compounds with low activity due to inappropriate lipophilicity can be excluded. This can be conveniently achieved by utilizing the scheme of Rekker (Figure 3) to calculate the partition coefficient of the apochelator, followed by the use of equation (4) to calculate the partition coefficient of the iron complex.

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