Biochemical Pharmacology, Vol. 57, pp. 1305–1310, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00031-3

Investigation into the Correlation between the Structure of Hydroxypyridinones and Blood–Brain Barrier Permeability

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ABSTRACT. Bidentate hydroxypyridinones are under active development as orally active iron chelators. With applications for the treatment of general body iron overload, for instance with thalassaemia, the distribution of the chelators should be limited to peripheral tissue and they should not enter the central nervous system. This study compares the predictive abilities of $LogP_{octanol}$ and $LogP_{cyclohexane}$ and reports the existence of good correlations between blood–brain barrier (BBB) permeability and both values for *N*-alkylpyridinones. 1,ω-Hydroxyalkyl hydroxypyridinones penetrate the BBB much more slowly than the simple 1-alkyl derivatives. This observation is likely to have important toxicological implications. BIOCHEM PHARMACOL **57**;11:1305–1310, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. iron chelators; hydroxypyridinone; blood–brain barrier

Iron overload is a serious clinical condition for which the only available treatment is subcutaneous or intravenous infusions of desferrioxamine, a metal chelating agent [1]. However, due to its oral inactivity and poor pharmacokinetic behaviour, patient compliance is poor. The HPO§ class of iron chelator is currently one of the main candidates for the development of orally active iron-chelating alternatives to desferrioxamine [2]. The primary use of iron chelation therapy is to reduce pathologically high levels of circulating free iron. However, if the metal-chelating compounds are lipid-soluble, they will cross the BBB and may possibly influence the level of iron and other metals in the brain. Such action might be expected to induce toxic effects by either removing or redistributing essential trace metals. On the other hand, lipid-soluble chelators may prove to be useful for removing metals from the CNS when they are present at toxic levels. It is thus of considerable importance to investigate the relationship between the chemical structure of HPOs and BBB permeability in order to identify compounds suited to particular therapeutic tasks.

In a key study, Levin [3] showed that BBB permeability was critically dependent on lipophilicity (octanol/water partition coefficient). The molecular weight of the compound also appeared to influence permeability, with an apparent "cut-off" (low penetration) for compounds of MW $>$ 4–500 [3]. It has subsequently been shown that some of the larger lipophilic agents are substrates for the drug efflux transporter, P-glycoprotein, so that active transport rather than molecular weight may explain their low penetration [4]. However, whether or not there is an upper molecular weight cut-off for drug penetration, there is general agreement that the passive penetration of low molecular weight molecules (MW $<$300) through the BBB is largely dependent on the lipophilicity [5]. It is not clear from previous studies which solvent system for the measurement of partition coefficients most closely resembles the partitioning characteristics of the BBB. Partition coefficients measured in the heptane/water, benzene/water, and chloroform/water solvent system have all been used [6, 7]. More recently, reports on the use of octanol/water partition coefficients in brain penetration studies have appeared [3, 5, 8–10]. In most cases, only partition coefficients measured in a single system were used. However, the combination of K_{part} values in two different solvent systems, such as octanol/water and cyclohexane/water, was also investigated by Young *et al.* [11] and a good correlation was found between the quasi-steady-state brain:plasma distribution ratio of a group of test compounds and the partition parameter, ΔLogP (octanol/cyclohexane) [11].

Hydroxypyridinones can be chemically modified to produce a wide variety of compounds with different physicochemical and pharmacokinetic properties without significantly affecting the affinity for iron [12]. In the current study, a number of bidentate HPOs were selected to investigate the relationship between chemical structure and

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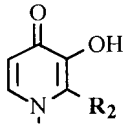
§ *Abbreviations:* HPO, hydroxypyridinone; BBB, blood–brain barrier; K_{part}, partition coefficient; and MOPS, 4-morpholinepropane sulphonic acid.

Received 30 July 1998; accepted 20 November 1998.

TABLE 1. Chemical structure of selected HPO ligands



	R_1	R_2	Molelcular weight
CP20	CH_3	CH_3	139
CP21	CH_2CH_3	CH_3	153
CP22	$CH_2CH_2CH_3$	CH_3	167
CP23	$CH(CH_3)_2$	CH_3	167
CP24	$CH_2(CH_2)_2CH_3$	CH_3	181
CP29	$CH_2(CH_2)_3CH_3$	CH_3	195
CP25	$CH_2(CH_2)_4CH_3$	CH_3	209
CP93	CH_3	CH_2CH_3	153
CP94	CH_2CH_3	CH_2CH_3	167
CP95	$CH_2CH_2CH_3$	CH_2CH_3	181
CP97	$CH(CH_3)_2$	CH_2CH_3	181
CP98	$CH_2(CH_2)_2CH_3$	CH_2CH_3	195
CP40	CH_2CH_2OH	CH_3	169
CP41	$(CH_2)_3OH$	CH_3	183
CP42	$(CH_2)_4OH$	CH_3	197
CP102	CH_2CH_2OH	CH_2CH_3	183
CP106	$(CH_2)_3OH$	CH_2CH_3	197
CP107	$(CH_2)_4OH$	CH_2CH_3	211

BBB permeability. In order to identify a suitable system for estimating the permeability of the BBB, partition coefficients were measured in two different solvent systems, namely octanol/water and cyclohexane/water.

MATERIALS AND METHODS

Iron Chelators

The 3-hydroxypyridin-4-ones used in the present study were synthesized as previously described [12]. The compounds of initial interest are those in which a variable length alkyl chain of 1 to 6 carbons has been substituted at the R_1 position (Table 1). The introduction of a hydroxyl group on the terminal carbon of the R_1 chain influences the overall lipophilicity and hydrogen-bonding potential of the molecules.

Determination of Partition Coefficients (K_{part}) using the Automated Filter Probe Method

The K_{part} of the chelators were determined in two solvent systems, namely octanol/MOPS buffer (pH 7.4) and cyclohexane/MOPS buffer (pH 7.4). A modified automated continuous flow technique [13] was chosen in preference to the traditional shake flask method owing to a greater accuracy and reproducibility of measurements. The system comprised an IBM-compatible PC running the "TOPCAT" program [14, 15], which controls both a Metrohm 665 Dosimat autoburette and a Pye–Unicam Lambda 5 UV/vis spectrophotometer, as well as calculating partition coefficients. All K_{part} determinations were performed using AnalaR grade reagents under a nitrogen atmosphere using a flat-based glass vessel equipped with a sealable lid at 25°. The aqueous and organic phases (octanol or cyclohexane) were presaturated with respect to each other before use. The filter probe consisted of a polytetrafluoroethylene plunger. The aqueous phase (50 mM MOPS, pH 7.4) was prepared using Milli-Q water and was separated from the two-phase system by means of a hydrophilic cellulose filter (5 μm diameter, 589/3 Blauband filter paper, Schleicher and Schuell) mounted in the gel filtration column adjuster (SR 25/50, Pharmacia). A known volume of MOPS buffer (saturated with octanol or cyclohexane) was taken in the flat base mixing chamber. After a base line was obtained, the solution was used for reference absorbance. The compound to be examined was dissolved in MOPS buffer so as to give an absorbance of 0.5–1.5 absorbance units at the preselected wavelength (~280 nm). The flow rate of the aqueous circuit was limited to 1 mL/min. The program calculates the K_{part} for each addition of octanol or cyclohexane.

Brain Perfusion in Rats

The uptake into brain of the HPOs was determined using a short duration brain perfusion technique modified from that described by Takasato *et al.* [16]. Male adult Wistar rats (weight range 240–350 g) were anaesthetized with urethane (25% solution w/v, 0.7 mL/100 g body weight). Once fully anaesthetised, the right common carotid artery was exposed and cannulated in the direction of the head for perfusion of the right internal carotid artery. Blood circulation to the right hemisphere is maintained during the surgical procedure by collateral blood flow from the left carotid artery and the circle of Willis. The right external carotid artery was then ligated close to its bifurcation from the common carotid artery. The saline-filled cannula was connected to an infusion pump (Pump 22, Harvard Instruments) and via a T connector to a pressure transducer to monitor the infusion pressure. Immediately the cannula was in place, the heart was exposed and stopped by severing the ventricles and the infusion started. Perfusions were carried out using a HEPES-buffered saline (20 mM, pH 7.4) to which the test compound was added (5 mM). A marker that does not cross the BBB ([^{14}C]sucrose, 2 μCi/100 mL buffer, Amersham) was included in the perfusate to monitor the integrity of the BBB and to calculate the residual vascular volume in each brain tissue sample. The rate of the infusion pump was adjusted to maintain an infusion pressure of between 100 and 120 mm Hg during the course of the perfusion. After 60 sec, the infusion cannula was pulled from the carotid artery to stop the perfusion to the brain. The brain was then rapidly dissected out and frozen at −30°. This technique allows a constant concentration of drug to be maintained within the cerebral vasculature so that BBB permeability can be measured from a protein-free, pH-controlled buffer. Thus, it is possible to obtain kinetic data on the rates of brain uptake for each of the HPOs, which is more closely related to BBB permeability.

Assay of HPOs in Rat Brain

Weighed rat brain tissue was homogenized in 50 mM phosphate buffer (pH 7.4) using a glass homogenizer. Biological homogenate (2 mL) was transferred to an extraction tube containing 100 μL of internal standard (various HPOs). After the addition of 10 mL dichloromethane, the resulting solution was thoroughly mixed. The organic layer was subsequently removed and evaporated to dryness in a water bath. The residue obtained was reconstituted with 100 μL phosphate buffer and then analyzed by HPLC. A calibration curve was constructed by spiking known quantities of HPO into pooled brain homogenate obtained from control rats. These samples were then processed and analyzed as described above. Peak area ratios were plotted against concentration. The calibration curves obtained were linear over the entire range with correlation coefficient values >0.99.

A Hewlett Packard Model 1090 M series-II HPLC system, complete with an auto injector, auto sampler, and diode array detector and linked to an HP 900-300 data station, was used to measure drug concentration. Brain sample extracts were analyzed by reverse-phase HPLC using Hypercarb PGC® column (10 cm × 0.46 cm) packed porous graphatized carbon. An isocratic system containing different amounts of acetonitrile (% v/v) (12% for CP20 and CP21, 18% for CP94, 30% for CP24 and CP29, and 40% for CP25) in 10 mM sodium dihydrogen phosphate (pH 3.0) containing 2 mM EDTA was used. The analyses of CP102, CP41, and CP107 were conducted using a polymer PLRP-S column (15 cm × 0.46 cm). The mobile phase consisted of 8% (v/v) acetonitrile. The eluate was detected at 280 nm and the flow rate was 1 mL/min.

Calculation of BBB Permeability

Permeability × surface area (PS) products ($mL \cdot sec^{-1} \cdot g^{-1}$, Eqn 1) were calculated for the right cerebral hemisphere using the single point method of Ohno *et al.* [17]. The short perfusion time (60 sec) was selected to minimize back diffusion of the more lipid-soluble chelators.

$$PS = -F_{pf}\ln(1-(C_{br}/tF_{pf}C_{pf})) \quad (1)$$

where F_{pf} is the perfusate flow rate ($mL \cdot sec^{-1} \cdot g^{-1}$), C_{br} the solute concentration in brain ($\mu g \cdot g^{-1}$, corrected for the residual vascular space, see below), C_{pf} the solute concentration in the perfusate ($\mu g \cdot g^{-1}$), and t is total infusion time (sec). For solutes where PS is less than 10% of the perfusion fluid flow rate, as in this study, Eqn 1 can be reduced to a brain uptake space Eqn 2 [18]:

$$PS = C_{br(t)}/C_{pf(t)} \quad (2)$$

Because a small quantity of perfusate containing 5 mM HPO remains within the blood vessels (vascular space) in each brain tissue sample, it is necessary to subtract this from the total brain concentration of HPO to determine the actual amount that has crossed the BBB. The volume of the residual vascular space is given by the following equation [16]:

$$\text{Residual vascular space} = [\text{sucrose}_{brain}]/[\text{sucrose}_{perfusate}] \quad (3)$$

where $[\text{sucrose}_{brain}]$ is the concentration of sucrose in the brain tissue and $[\text{sucrose}_{perfusate}]$ is the concentration of sucrose in the perfusate.

RESULTS

The residual vascular spaces in each brain sample, determined from the [^{14}C]sucrose activity, were very low (mean = 0.014 $mL \cdot g^{-1}$ ± 0.003 SD, N = 21, range = 0.011 to 0.019), indicating an intact blood–brain barrier in each perfused brain. The measured partition coefficients of selected HPO ligands are shown in Table 2. As expected, an increase in the length of the alkyl chain on the heterocyclic nitrogen resulted in an increase in the K_{part} values. The overall lipid solubility (Log $P_{octanol}$) was markedly influenced by and proportional to the number of carbons in the R_1 alkyl chain (Fig. 1). The replacement of a hydrogen atom with a hydroxyl group to the terminal carbon of the R_1 chain greatly reduced the lipophilicity of the molecules (Table 2). However, a correlation between Log $P_{octanol}$ and the length of the R_1 chain was still apparent (Fig. 1), albeit at a much reduced level compared to the non-hydroxylated compounds. There was a good correlation between the Log P value of ligands in the octanol/water system and the Log P value of corresponding ligands in cyclohexane/water (Fig. 2). However, the linear relationship between Log $P_{octanol}$ and the ΔLog P values was relatively poor (data not shown).

Nine compounds, CP20, CP94, CP21, CP24, CP29, CP25, CP102, CP41, and CP107, were selected to investigate BBB permeability in rats and the results are summarized in Table 2 and Fig. 3. Clearly, more lipophilic compounds achieve higher brain penetration (LogPS). Regression analysis of the physicochemical data for the six *N*-alkyl HPOs in Table 2 on LogPS demonstrated that there was a good linear correlation between BBB permeability and both Log $P_{octanol}$ ($r = 0.956$) (Fig. 3A) and Log $P_{cyclohexane}$ ($r = 0.956$) (Fig. 3B). A poor correlation was observed with ΔLog P (data not presented). Under the experimental conditions used, *N*-hydroxyalkyl derivatives of HPOs, such as CP102, CP41, and CP107, showed negligible permeability across the blood–brain barrier.

DISCUSSION

A number of HPOs have been investigated in order to establish a physicochemical model for the prediction of BBB permeability. The results reveal that brain penetration is strongly dependent on lipophilicity of HPOs (Fig. 3). The lipid solubility (Fig. 1) and BBB permeability (Fig. 3) of the

TABLE 2. Measured partition coefficients in octanol/MOPS (pH 7.4), cyclohexane/MOPS (pH 7.4) (mean ± SD, N = 5) and blood–brain barrier permeability (LogPS) (mean ± SD, N = 3) of selected HPOs

Chelator	K_{part}(octanol)	K_{part}(cyclohexane)	LogP(octanol)	LogP(cyclohexane)	LogPS
CP20	0.17 ± 0.01	0.0016 ± 0.0004	−0.770	−2.796	−1.89 ± 0.04
CP21	0.49 ± 0.01	0.007 ± 0.001	−0.310	−2.155	−1.48 ± 0.05
CP22	1.51 ± 0.04	0.011 ± 0.004	0.179	−1.959	ND
CP23	1.12 ± 0.01	0.009 ± 0.001	0.049	−2.046	ND
CP24	5.05 ± 0.02	0.025 ± 0.006	0.703	−1.602	−0.64 ± 0.05
CP29	17.4 ± 0.20	0.037 ± 0.003	1.241	−1.432	−0.38 ± 0.02
CP25	79.0 ± 5.10	0.100 ± 0.020	1.898	−1.000	−0.36 ± 0.02
CP93	0.62 ± 0.01	0.007 ± 0.0002	−0.208	−2.155	ND
CP94	1.70 ± 0.02	0.014 ± 0.003	0.230	−1.854	−1.03 ± 0.08
CP95	5.04 ± 0.03	0.044 ± 0.004	0.702	−1.357	ND
CP97	5.40 ± 0.10	0.039 ± 0.005	0.732	−1.409	ND
CP98	16.6 ± 0.30	0.055 ± 0.005	1.220	−1.260	ND
CP40	0.08 ± 0.002	0.0010 ± 0.0004	−1.097	−3.000	ND
CP41	0.13 ± 0.001	0.0013 ± 0.0003	−0.886	−2.886	<−3.00*
CP42	0.18 ± 0.001	0.002 ± 0.0002	−0.745	−2.699	ND
CP102	0.22 ± 0.01	0.0013 ± 0.0004	−0.658	−2.886	<−3.00*
CP106	0.36 ± 0.01	0.003 ± 0.0002	−0.444	−2.523	ND
CP107	0.52 ± 0.01	0.006 ± 0.0003	−0.284	−2.222	<−3.00*

*No hydroxypyridinone was detected in brain; ND, not determined.

N-alkyl HPOs show strong positive correlation with carbon chain length/molecular size, each compound having the same number of hydrogen-bonding sites. Furthermore, the much lower lipid solubility and BBB permeability of the *N*-hydroxyalkyl HPOs (which have an increased number of hydrogen-bonding sites) suggests that this positive effect of molecular size on lipid solubility and BBB permeability can be largely outweighed by a negative effect of increased hydrogen bonding. It is possible that for simple *N*-alkyl-substituted HPOs, intramolecular hydrogen bonding (Fig. 4) restricts the number of hydrogen-bonding sites, which in turn facilitates transfer of such molecules across the BBB. With *N*-hydroxyalkyl derivatives, although the same intramolecular hydrogen bonding will occur, the hydrogen-bonding ability of the *N*-substituted groups is unaffected by such an interaction. Clearly, this functional group has a dominant influence on the brain penetration of such molecules. These observations are consistent with the hypothesis suggested by Stein [19]: if the rate-limiting step in the transport of a polar solute across a cell membrane is desolvation, there should be a correlation between the total number of hydrogen bonds the solute can form with water and its permeability. The apparent impermeability of *N*-hydroxyalkyl HPOs suggests that the additional energy required for breaking the hydrogen bonds between hydroxyalkyl HPOs and water presents a significant barrier.

The solubility data for the *N*-alkyl HPOs indicate that the strength of the intermolecular drug–solvent hydrogen bonds is not the same for all compounds and that the size of the non-hydrogen-bonding part of the molecule may have a direct influence on the hydrogen-bonding ability of the polar moieties. Thus, it may not be appropriate to relate

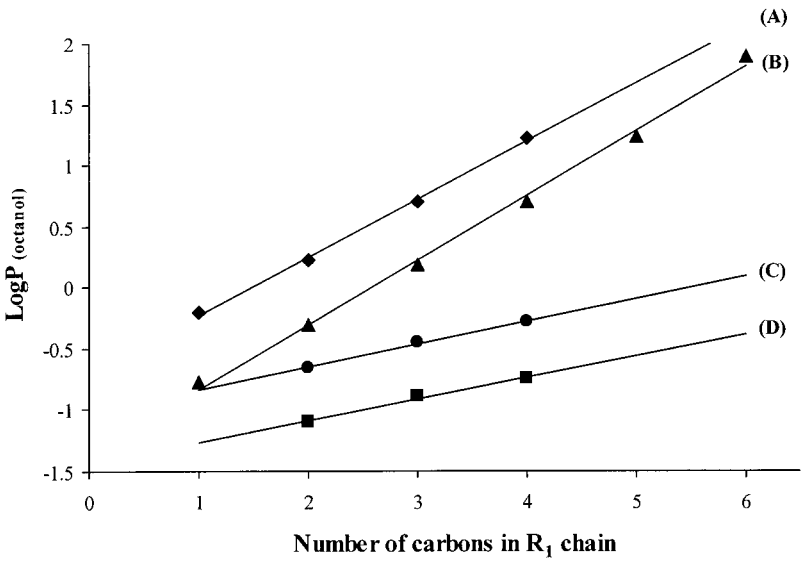


FIG. 1. Log $P_{octanol}$, as a function of the number of carbon atoms in the R_1 chain for the 3-hydroxypyridin-4-one family of iron chelators. Line A (–◆–): $R_2 = C_2H_5$ and $R_1 = CH_3$ to $(CH_2)_3CH_3$; Line B (–▲–): $R_2 = CH_3$ and $R_1 = CH_3$ to $(CH_2)_5CH_3$; Line C (–●–): $R_2 = C_2H_5$ and $R_1 = (CH_2)_nOH$; Line D (–■–): $R_2 = CH_3$ and $R_1 = (CH_2)_nOH$.

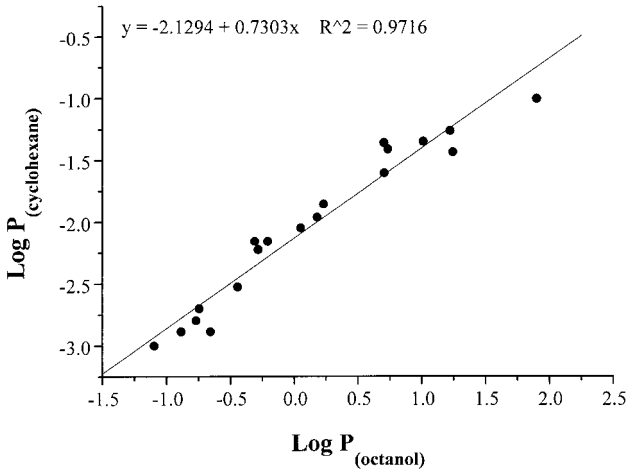


FIG. 2. Correlation between Log $P_{octanol}$ and Log $P_{cyclohexane}$ for 3-hydroxypyridin-4-ones.

lipid solubility, or BBB permeability, to a simple arithmetic sum of a drug's hydrogen-bonding sites. To better understand how a particular drug will partition between an aqueous and a lipid environment, or between the blood and the brain, it is necessary to separate out and quantify the interactions occurring between drug molecules and surrounding solvent molecules (e.g. hydrogen bond acidity and basicity, and dipole–dipole attractions). The overall lipid solubility, or BBB permeability, of a particular drug is thus likely to be a function of all of these interactions (both positive and negative). Abraham and colleagues have recently shown that the lipid solubility [20] and BBB permeability [21, 22] of a drug can be described in terms of five main solvation descriptors: excess molar refraction, dipolarity/polarizability, overall hydrogen bond acidity, overall hydrogen bond basicity, and molecular volume. Their work shows that an increase in molecular volume has a positive influence on both lipid solubility and BBB permeability, whereas hydrogen bond basicity and dipolarity both exert negative influences. Our results confirm their findings, showing a strong positive correlation between BBB permeability and the molecular size of *N*-substituted HPOs, and reduced BBB permeability for the *N*-hydroxylated HPOs. This is in contrast to previous treatments showing a negative influence of molecular weight on BBB permeability [3, 4]. These previously published reports have largely been based on BBB permeability data for structurally unrelated compounds that not only cover a wide range of molecular size, but also show a diverse range of molecular dipolarity and hydrogen-bonding ability. Thus, it is not clear to what extent differences in the total number of hydrogen bonds, hydrogen bond acidity/basicity, and the overall molecular dipolarity of these compounds contribute to the effect of molecular weight (e.g. the larger molecules are likely to contain more hydrogen-bonding groups than the smaller molecules).

It is clear that for prediction of BBB permeability of a new HPO, a simple measurement of lipid solubility is not

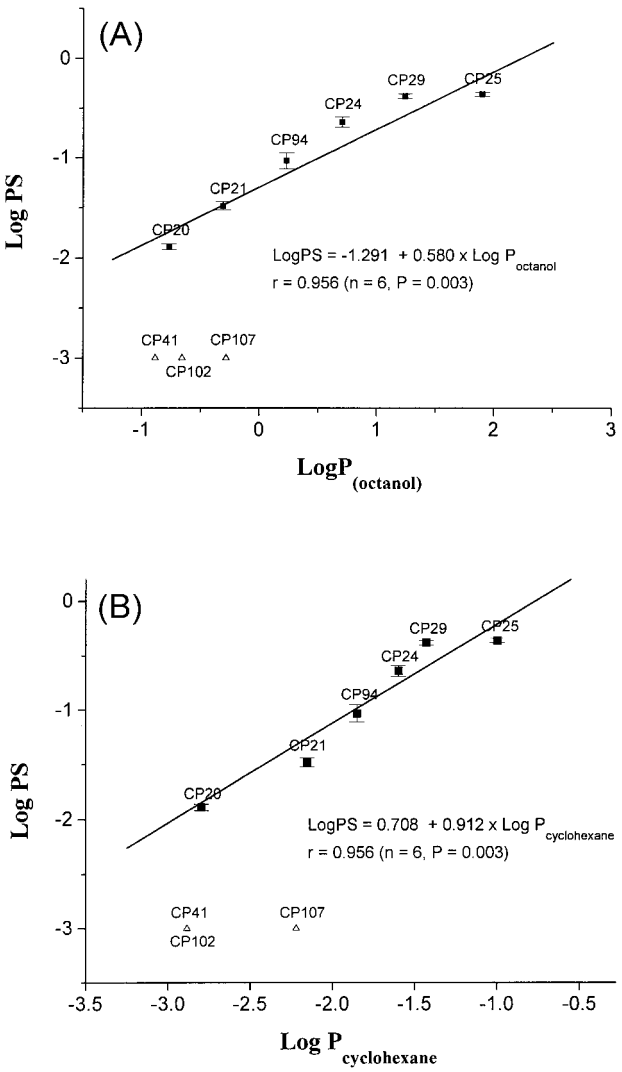


FIG. 3. Relationship between blood–brain barrier permeability (LogPS) of the HPO family of iron chelators with (A): Log $P_{octanol}$ and (B): Log $P_{cyclohexane}$ in adult Wistar rats. Comparison of chelators with a hydroxyl group on the terminal carbon of the R_1 chain (△) and simple *N*-alkyl chelators (■). The vascular perfusion time was 1 min. Values are expressed as means ± SD (N = 3).

sufficient. For example, CP21 and CP107 both have similar Log $P_{octanol}$ values, but the BBB permeability of CP21 is more than one order of magnitude greater than that of CP107. The presence of the hydroxy group on the *N*-

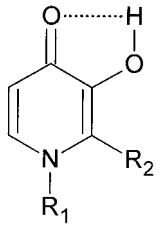


FIG. 4. Possible intramolecular hydrogen bonding of the 3-hydroxypyridin-4-ones.

substituted alkyl chain clearly has a greater effect on BBB permeability that it does on Log $P_{octanol}$ values. To be able to predict the BBB permeability of HPOs that are more structurally diverse, and to determine how different structural elements affect BBB permeability, it may be necessary to determine "Abraham" solvation descriptors for these compounds.

In summary, there is a good relationship between BBB permeability and both Log $P_{octanol}$ and Log $P_{cyclohexane}$ for *N*-alkyl substituted HPOs, which offers a reliable predictive method for simple alkyl HPOs. In contrast, appreciable permeability of the BBB was not observed for *N*-hydroxyalkyl HPOs using the short duration vascular perfusion method adopted in this study. This is an important observation since the hydroxypyridinone currently in clinical trial (Deferiprone, CP20) does cross the BBB, whereas monohydroxylated derivatives of *N*-alkyl HPOs would be expected to penetrate much more slowly. These results also demonstrate that the biological distribution pattern of the HPOs can be significantly altered by simple changes in the chemical structure of these compounds without compromising their pharmacological function (selective iron chelation).

M. D. H. was supported by a LINK initiative grant from MRC, Merck, Sharp & Dohme, Yamanouchi and Smith Kline Beecham.

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