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Studies on an Oral Iron Chelator: 1,2-Dimethyl-3-hydroxy-pyrid-4-one (DMHP). Mechanism of Intestinal Absorption in Rabbits

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Abstract—Over the last 30 years, desferrioxamine has been the only iron chelator in clinical use. This chelator is expensive and must be given by injection. A new class of chelators, namely 1-alkyl-2-methyl-3-hydroxypyrid-4-ones, have been shown to be orally effective. Using 1,2 dimethyl-3-hydroxy-pyrid-4-one (DMHP), we have carried out a study to clarify the mechanism of intestinal absorption of this new class of drug, using an in-situ system of the intestine from rabbit. The major site of DMHP absorption is in the intestine and is linear with increasing drug concentration. DMHP absorption per unit length of jejunum and ileum is similar; however, due to the larger surface area of jejunum, the absorption by ileum segment is more effective per unit surface. L-Proline, L-tryptophan (amino acids), 2-deoxyglucose, and sodium iodoacetate (metabolic inhibitors) have no effect on DMHP absorption, but L-phenylalanine, an amino acid with a 6-member carbon ring, significantly inhibits the DMHP absorption from the intestinal segment. We conclude that the mechanism of DMHP absorption in the intestine is mainly by simple passive diffusion based on the linear relationship found between drug concentration and absorption. However, the inhibitive effect of L-phenylalanine suggests that the co-existence of a facilitated uptake cannot be ruled out.

Cooley's anaemia (β-thalassaemia major) is a world-wide genetic blood disorder characterized by severe anaemia. In developing countries, due to the lack of effective treatment, the mean life expectancy of thalassaemic patients is only 5 years. Blood transfusion on a continuing basis has been, and remains, the major form of treatment to alleviate the anaemia of thalassaemia (Necheles et al 1974). A complication of transfusion therapy arises from the inefficient excretion and consequent long-term accumulation of iron from catabolized haem leading to iron overload. Iron overload in critical organs is ultimately life-threatening as a consequence of the iron toxicity (Schorr & Radel 1964). Mobilization and removal of excess iron by chelating agents represents the only treatment developed to date for the progressive iron overload in thalassaemia.

In addition to their simple and inexpensive synthesis, the identification of l-alkyl-2-methyl-3-hydroxy-pyrid-4-ones as a class of iron chelators that are orally effective make them an attractive group of compounds for further studies (Kontoghiorghes 1985).

Since 1960, desferrioxamine has been the only effective iron chelator in clinical use; however, it is expensive, and is also required to be administered parenterally. Availability of orally effective iron chelators is therefore a significant advance in clinical therapy of transfusional iron overload.

Kontoghiorghes & Sheppard (1987) reported a simple synthesis of l-alkyl-2-methyl-3-hydroxy-pyrid-4-ones (alkyl = methyl, ethyl, propyl, butyl); their in-vitro and in-vivo studies demonstrated the oral effectiveness and the high iron binding properties of these compounds at physiological pH. Venkataram & Rahman (1990) reported that although the dimethyl compound is active orally, the subcutaneous administration is by far more effective in iron removal from rats with experimental iron loading. In order to understand the mechanism of oral absorption, we have conducted a study testing the intestinal absorption of 1,2-dimethyl-3-hydroxy-pyrid-4-one (DMHP) using an in-situ intestinal system.

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Materials and Methods

Materials

Methylamine (40% aqueous solution) and 3-hydroxy-2-methylpyrid-4-one (maltol) were purchased from Aldrich Chemicals, Milwaukee, WI, USA. Monobasic potassium phosphate and acetonitrile were obtained from Mallinkrodt, Inc., Paris, KY, USA. Butanol and glacial acetic acid were obtained from Fisher Scientific, Fairlawn, NJ, USA, and Columbus Chemical Industries, Columbus, WI, USA, respectively. L-Proline, L-tryptophan, L-phenylalanine, 2-deoxy-D-glucose, and sodium iodoacetate were from Sigma Chemical Co., St Louis, MO, USA. Ketamine and acepromazine were from Parke-Davis, Morris Plains, NJ, USA, and Ayerst Laboratories, Inc., New York, NY, USA, respectively.

Synthesis of DMHP

DMHP was synthesized according to the method described by Kontoghiorghes & Sheppard (1987). The yield ranged from 60 to 65%. Proton NMR and mass spectra were used to confirm the purity of DMHP.

HPLC analysis of DMHP

The concentration of DMHP was measured by an HPLC method developed in our laboratory. The HPLC system consisted of a solvent delivery module (Model 110B), a variable wavelength UV-visible detector (Model 163), a system controller (Model 421A), a sample injection valve

(Model 210A), all from Beckman Instruments, San Ramon, CA, USA. An integrator/plotter (model CR3-A, Shimadzu Electronics Corporation, Columbia, MD, USA) was used for data collection. For DMHP analysis, a 25 cm anion exchange column (Ultrasil-Sax, Beckman Instruments) and a 2 cm guard column (Adsorbosphere-Sax, Alltech Associates, Inc., Waukeegan, IL, USA) were used.

The mobile phase was acetonitrile: glacial acetic acid: butanol: 0·2 M monobasic potassium phosphate, at a ratio of 2:2.1:5:4 volume percent in distilled water. The mobile phase was freshly prepared and filtered through a 0·2 μm membrane filter before use. The flow rate was 1·0 mL min^{-1} at ambient column temperature and the eluant was monitored at 280 nm. These conditions gave a retention time of 4·6 min for DMHP.

Stock solution for DMHP standard was prepared at 100 μg mL^{-1} in distilled water. Serial dilutions from this stock solution were then made in the concentration range of 1–20 μg mL^{-1}. A standard curve (peak area vs concentration) was obtained on the day of each experiment and used for calculating the drug concentrations of all drug solutions.

For plasma calibration, a standard curve of DMHP in plasma was also prepared every day. For this calibration, plasma (0·2 mL) was mixed with 0·05 mL aqueous DMHP solutions (30–500 μg mL^{-1}). Acetonitrile (0·2 mL) was then added to precipitate the protein in the plasma samples. The mixture was vortexed for 30 s and centrifuged at 14000 rev min^{-1} for 2 min. The supernatant was injected and peak area plotted as a function of concentration. The final DMHP concentration in this calibration curve ranged from 1·2 to 20 μg mL^{-1}. The coefficient of variation of DMHP measurement in plasma was less than 10% for concentrations ranging between 3–30 μg mL^{-1}.

Intestinal absorption study in an in-situ system

Male New Zealand White rabbits were used (3·23 ± 0·22 kg). The procedure for the in-situ system is described by Sawchuk & Awni (1986). Briefly, a marginal ear vein was cannulated for blood sampling. The animal was anaesthetized by administering ketamine (45 mg kg^{-1}) intramuscularly in the thigh and muscle relaxation was achieved using acepromazine (2 mg kg^{-1}). These doses were administered a second time after an interval of 10 min to achieve adequate anaesthetic effect over a period of 2 h.

A midline abdominal incision was made following removal of hair from the abdominal region. A segment of the small intestine (about 30 cm long) was cannulated at proximal and distal ends using surgical silk to place the connecting catheters. The proximal end was connected to a syringe on a variable speed infusion pump and the segment cleaned by passing saline through it. The pH of the infusion solution was close to 7·0 (6·93–7·04). The drug (DMHP) solutions with or without other compounds were prepared in saline (0·9% NaCl) and infused through the segment at a fixed rate of 0·28 mL min^{-1}. The segment was kept moist with a saline-soaked gauze during the entire experiment. Samples from intestinal effluent were collected at fixed intervals of 10 min over a period of 120 min. Blood samples were taken at the midpoint of these intervals. The length of the isolated segment was measured accurately at the end of the experiment. The flow rate was determined from slope of the plot of the volume remaining in the syringe vs time. At the end of the experiment, the plasma was separated and stored in the freezer before analysis.

In-vivo pharmacokinetics of DMHP

Male New Zealand White rabbits were used in this study. The ear vein was cannulated using an intravenous placement catheter for dosing and blood sampling. The animal was anaesthetized using ketamine (45 mg kg^{-1}) and muscle relaxation was achieved using acepromazine (1–2 mg kg^{-1}). DMHP in saline was administered at a dose of 100 mg kg^{-1} over a period of 2 min. Blood samples were collected into EDTA-containing tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ, USA) before DMHP injection at 5, 10, 20, 40, 60, 90, 120, 180, 240, 360 and 480 min after dosing. Plasma was separated immediately by centrifugation and stored in a freezer until used for analysis.

Experimental design

The experimental design for this study is summarized in Table 1.

Group I. Rabbit numbers 1–16. Experiments were conducted to determine the site of maximal absorption, if any, and the effect of various drug concentrations on absorption. For determination of site of maximal absorption, two intestinal sites were selected, jejunum and ileum. The length of the intestinal segment was kept at approximately 30 cm throughout the entire experiment. The actual measured length in each group of rabbits is indicated in Table 1. The absorption through jejunal and ileal segments was studied in 6 and 3 rabbits, respectively, using 106 mM DMHP solution. For determination of the effect of drug concentration, the

Table 1. Experimental design.

Group	Rabbit no.	Average weight (kg ± s.d.)	Treatment	Length of jejunum (cm ± s.d.)
I	1–6	3·12 ± 0·12	106 mM DMHP	31·3 ± 4·2
	7–9	3·23 ± 0·32	106 mM DMHP	25·0 ± 2·2
	10–12	3·23 ± 0·15	72 mM DMHP	29·8 ± 5·9
	13–16	3·30 ± 0·19	36 mM DMHP	30·5 ± 3·3
II	17–19	3·01 ± 0·01	72 mM DMHP + 217 mM L-proline	32·5 ± 1·2
	20–22	3·22 ± 0·32	72 mM DMHP + 49 mM L-tryptophan	28·5 ± 2·7
	23–25	3·23 ± 0·07	72 mM DMHP + 151 mM L-phenylalanine	29·9 ± 2·6
III	26–28	3·16 ± 0·05	72 mM DMHP + 10 mM 2-deoxy-D-glucose	26·7 ± 0·7
	29–31	3·33 ± 0·28	72 mM DMHP + 0·1 mM sodium iodoacetate	29·0 ± 3·0

Table 2. Effect of intestinal site on DMHP absorption.

Number of rabbits	Site	Amount disappearing from intestinal segment (mg) ± s.e.m.	Cumulative amount in plasma after intestinal administration (mg) ± s.e.m.
6	Jejunum	365·12 ± 36·17	272·99 ± 31·9
3	Ileum	382·65 ± 27·36	199·40 ± 9·18

experiment was carried out at two more concentrations (72 and 36 mM) using the jejunal segment. A comparison, therefore, was made at three drug concentrations (106, 72 and 36 mM) for absorption through the jejunal segment.

Group II. Rabbit numbers 17–25. Experiments were conducted to determine the mechanism of absorption. For this purpose, three different amino acids were added to the DMHP solution (72 mM) to evaluate inhibition or enhancement in drug absorption. Three amino acids were chosen and added to the drug solution before the start of the experiment. Three rabbits were used in each set of studies with L-proline (217 mM), L-tryptophan (49 mM) and L-phenylalanine (151 mM).

Group III. Rabbit numbers 26–31. Experiments were conducted using metabolic inhibitors along with DMHP to determine if the absorption process was energy-dependent. Sodium iodoacetate and 2-deoxyglucose were used in a concentration of 0·1 and 10 mM, respectively, with the DMHP solution (72 mM) and infused through the jejunal segments in a set of three rabbits each.

The set of rabbits in group I (Table 1) that received 72 mM DMHP solution (jejunal segment) was used as control for all comparisons in groups II and III.

Pharmacokinetic analysis

The concentration of drug solutions exiting the internal segment was estimated as the average of the concentrations (C_l) in samples collected between 30 and 90 min. These times were chosen since they represented attainment of steady-state. The average concentration C_l was corrected for differences between the volume of solution collected and infused due to water influx for each experimental segment. The fraction of the drug remaining to be absorbed at steady-state within a specified intestinal length C_l/C_o was calculated for all experiments where C_l is the drug concentration leaving the intestinal segment and C_o is the original drug concentration entering the intestinal segment. The amount of DMHP disappearing from the intestinal segment was calculated by mass balance. The amount which had disappeared within a specific interval was taken as the difference between the amounts entering and leaving the segment within that interval. The cumulative amount disappearing from the intestine over all the intervals was then calculated. The cumulative amount of DMHP absorbed into systemic circulation (A_b) was estimated as

$$A_b = CL_p \times AUC_{intestinal}$$

where $AUC_{intestinal}$ is the area under the blood concentration time curve for DMHP after intestinal administration and plasma clearance (CL_p) was estimated using data from rabbits in which DMHP was given as a 100 mg kg^{-1} intravenous bolus.

The total area under the curve (AUC) and the total area under the moment curve (AUMC) up to the last time point were estimated by the trapezoidal rule. The terminal region was calculated using the blood concentration at the last time point and the terminal elimination half-life. The half-life was estimated by nonlinear regression analysis of the final segment of the blood-concentration time data. Plasma clearance (CL_p) was calculated by dividing dose by the AUC. The mean residence time (MRT) was calculated as the ratio of AUMC and AUC. The volume of distribution at steady-state was estimated by multiplying MRT by CL_p.

Results

Effect of intestinal site of DMHP absorption

As can be seen from Table 2, there is no significant difference between amounts disappearing from the jejunum and ileum. Fig. 1 shows the amount of drug that disappeared from the intestinal tract and appearing in plasma as a function of time from the two intestinal sites. Again, the curves obtained from the jejunum and the ileum are superimposable.

Effect of DMHP concentration on intestinal absorption

The absorption of DMHP was found to be concentration dependent and did not show saturation over the range of 36–106 mM (Table 3). A plot of amount disappearing from the intestinal segment as a function of concentration is shown in Fig. 2.

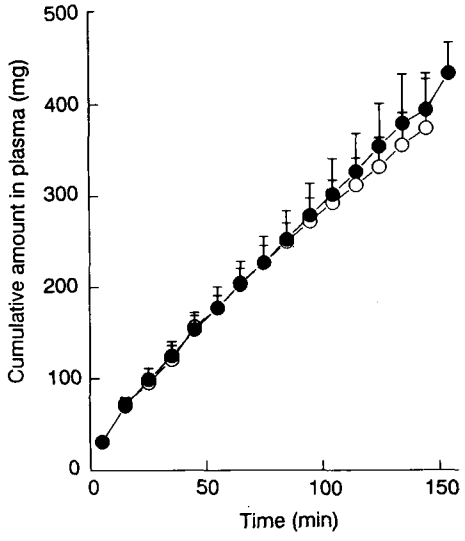

FIG. 1. Cumulative amount of DMHP appearing in plasma after intestinal administration. Each point represents the mean (± s.e.m.) of the cumulative amount of DMHP in plasma. ● Jejunum, ○ ileum.

Table 3. Effect of DMHP concentration on intestinal absorption.

Number of rabbits	Concentration (mM)	Amount disappearing from intestinal segment (mg) ± s.e.m.	% Absorbed*	Cumulative amount in plasma after intestinal administration (mg) ± s.e.m.
6	106	365·12 ± 36·17	59·9	272·99 ± 31·95
3	72	233·34 ± 54·14	54·1	124·31 ± 43·28
4	36	149·59 ± 42·73	59·6	47·22 ± 11·07

* Percent absorbed is calculated from the amount disappearing from the lumen and the total amount infused.

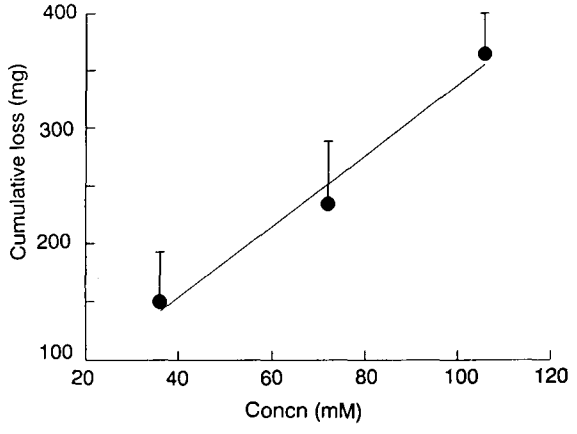

FIG. 2. Cumulative amount of DMHP disappearing from jejunal segment as a function of DMHP concentration. Each point represents mean (±s.e.m.) disappearing from a 30-cm segment of jejunum.

Effect of amino acids and metabolic inhibitors on DMHP absorption

As can be seen from the data summarized in Table 4, addition of 0·1 mM sodium iodoacetate or 10 mM 2-deoxyglucose to the drug solution did not significantly inhibit DMHP absorption. The data in Table 4 also indicate that the presence of 49 mM L-tryptophan and 217 mM L-proline did not cause any significant inhibition of DMHP absorption. However, the amount disappearing from the intestine was found significantly decreased in the presence of 151 mM L-phenylalanine.

Discussion

The half-life of DMHP is approximately 60 min. The plasma clearance is 0·92 ± 0·04 L kg^{-1} h and the volume of distribution at steady-state is about 1·31 ± 0·13 L kg^{-1}. In this study, the experiment was repeated in the presence of an anaesthetic, ketamine, and a muscle relaxant, acepromazine. The pharmacokinetic parameters are not different from our earlier experiments when these agents were not used.

In the studies of intestinal absorption, blood level of drugs cannot always be used as a measure of relative absorption rates because of the variables of drug distribution, metabolism and excretion. Accordingly, since the drug is neither degraded nor precipitated in the intestinal lumen, their relative rates of absorption can be more reliably assessed from the rates at which they disappear from the intestine. (Schanker 1959, 1962). In the present study, drug solution has been perfused for up to 2·5–3 h and we expressed our results as a fraction of the total amount of drug being absorbed based on the initial amount used for intestinal infusion.

Effect of intestinal site on DMHP absorption

The functional and morphological characteristics of the jejunum and ileum may be different in the same animal model and may further vary in different species. Rats, rabbits and dogs have been used in the studies of intestinal absorption of drugs (Robinson et al 1977; Carr & Toner 1984). As indicated by the studies in the rat and dog model, there are differences in the jejunal and ileal mucosa. It has been demonstrated that there are marked differences in characteristics between the two regions in the dog and they are different from those in the rat. The differences can be in density of the tissue, size of the villi, number of enterocytes and crypts. The levels of acid phosphatase, absorption of sodium, potassium, and water can also be significantly different. All these factors can significantly contribute to retarding or enhancing drug absorption (Robinson et al 1977).

Table 4. Effect of amino acids and metabolic inhibitors on DMHP absorption.

Group	Number of rabbits	Compound infused with DMHP (72 mM)	Amount disappearing from intestinal segment (mg) ± s.e.m.	% Absorbed*	Cumulative amount in plasma after intestinal administration (mg) ± s.e.m.
II	3	Control	233·34 ± 45·14	54·1	124·31 ± 43·28
	3	L-Proline, 217 mM	226·74 ± 8·49	56·0	97·98 ± 0·66
	3	L-Tryptophan, 49 mM	142·00 ± 25·53	32·9	53·79 ± 7·33
	3	L-Phenylalanine, 151 mM	87·96 ± 6·43	21·9	64·48 ± 11·59
III	3	2-Deoxy-D-glucose, 10 mM	159·84 ± 23·10	39·5	73·96 ± 18·15
	3	Sodium iodoacetate, 0·1 mM	171·97 ± 11·27	40·6	65·49 ± 5·99

* Percent absorbed is calculated from the values of amount infused and amount recovered in the intestinal effluent.

In our study, the amount of DMHP absorbed from the jejunum and the ileum is the same (Table 2) when the results are compared on the basis of intestinal unit length. The amount appearing in plasma over time is shown in Fig. 1 and is clearly seen to be very similar. However, when comparison is made on the basis of intestinal surface area, absorption per unit surface area is more efficient in the ileum since the surface of the jejunal mucosa is significantly greater than that of the ileum. In the absence of more information on mucosal area or weight or number of absorptive cells per unit weight of the jejunal and ileal segments, we can only express the absorption of DMHP as per unit length of the intestine. In reality, adequate DMHP absorption should largely occur through the proximal intestine, i.e. jejunum, this being the principal site of contact after oral drug administration and the fast transit through the stomach. Partial absorption in the stomach also cannot be ruled out (Schanker et al 1957).

Effect of DMHP concentration on absorption

The degree of ionization and lipid solubility of a drug are important factors in determining the rate of absorption. The organic acids and bases are, in general, readily absorbed in their lipid-soluble, undissociated form. If the drug is absorbed from the gut by a specialized transport mechanism, the process will approach saturation if high concentration is attained in the intestinal lumen (Jackson 1987). There are two pK_a values for DMHP, 3·56 and 9·64 (Hider et al 1990), which means that DMHP is in undissociated form at the neutral pH of the solution infused in this study. The relationship between lipid solubility and chelating efficacy of several chelators including DMHP has also been discussed by Porter et al (1990), who found a weak linear correlation between the lipid solubility of the iron-free form of each chelator and the amount of iron excreted per unit dose. However, the relationship between lipid solubility and intestinal absorption of these chelators has not been examined. Such a study would greatly enhance our understanding of the required structural parameters of a chelator for optimal intestinal absorption on the one hand, and for iron chelating efficacy on the other.

Using high initial concentrations for our drug absorption studies, the intestinal absorption of DMHP showed no evidence of saturation when the drug concentration is increased 6-fold. The absolute amount of drug disappearing from the intestinal segment was directly proportional to the concentration of the initial perfusate (Fig. 2). The values of amount disappeared (mean ± s.e.m.) as a function of concentration were fitted using linear regression analysis. The percent absorption of drug at various concentrations was the same and is shown in Table 3. Higher DMHP concentration in infused solutions exhibit greater DMHP absorption indicating that passive diffusion is a dominant mechanism involved in DMHP absorption and is driven by its concentration gradient across the intestinal epithelium.

Effect of amino acids and metabolic inhibitors

DMHP absorption is not altered significantly in the presence of L-proline or L-tryptophan. However, there is significant inhibition in the presence of L-phenylalanine. The structural similarity between DMHP and L-phenylalanine could be responsible for the competitive inhibition of DMHP in the presence of this amino acid. However, we should point out that the concentration of L-phenylalanine used in this study is high. Further experiments testing a decreasing concentration of L-phenylalanine should be carried out in order to clarify the mechanism of this apparent inhibition.

DMHP absorption is not inhibited in the presence of two metabolic inhibitors and two neutral amino acids. However, inhibition of L-phenylalanine suggests that there may be a contribution from a facilitated transport system.

When the experiments were carried out in the presence of these amino acids, the volume of the fluid recovered was found to be much greater than the volume infused. This observation indicated that the outflow of fluid into the lumen was increased. The transmucosal fluid flow was found to be an important factor in previous studies by Kitazawa et al (1975) and Schedl & Clifton (1963), who reported that the drug absorption could be enhanced or reduced if the inflow of fluid is high (from the luminal side to serosal side) or low, respectively. In the present study, the increased outflow of the fluid did not seem to negatively influence the drug absorption when L-proline or L-tryptophan is present, but it did in the presence of L-phenylalanine. The existence of this solvent drag effect can only be ascertained if solutions of different tonicities have been compared and some correlation is found. DMHP is also known to form dimers (Chan et al 1992), and enhanced absorption may occur since dimers are less polar than the ionized drug form.

In conclusion, DMHP is absorbed to the same extent by the jejunum and the ileum in the rabbit and the absorption is predominantly by passive diffusion.

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