IRON MOBILIZING ABILITY OF 1,2-DIETHYL-3-HYDROXYPYRIDIN-4 -ONE (CP94) IN IRON-OVERLOADED RATS

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ชื่อวิทยานิพนธ์ ความสามารถในการดึงเหล็กของ 1,2-diethyl-3 -hydroxypyridin-4-one (CP94) ในหนูที่ได้รับเหล็กเกิน

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บทคัดย่อ

การศึกษานี้แสดงความสามารถในการดึงเหล็กของ 1,2-diethyl-3-hydroxypyridin -4-one (CP94) ซึ่งเป็นสารขับเหล็กชนิดรับประทานตัวใหม่ ในหนูที่ได้รับเหล็กเกิน หนูที่ได้รับเหล็กเกิน เกิดจากการให้เหล็ก 32 มิลลิกรัม ในรูปของ iron dextran ฉีดเข้าทางช่องท้องแก่หนูปกติเป็นเวลา 1 เดือน พบว่าหนูดังกล่าวมีการสะสม ของเหล็กที่ตับเพิ่มขึ้น 8 เท่า และซีรั่ม TBARs ซึ่งเป็นตัวบ่งชี้ถึงสภาวะการเกิดออกซิเดชั่นของไขมันมีค่าเพิ่มขึ้นด้วย อย่างไรก็ดีเหล็กในซีรั่มไม่มีการเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ

การศึกษานี้เป็นการสร้างรูปจำลองเพื่อใช้อธิบายการกระจายตัวของเหล็ก และความสามารถของ CP94 ในการดึงเหล็ก เมื่อสารนี้เข้าสู่ระบบไหลเวียนของเลือด เมื่อให้ CP94 ขนาด 100 ม ก./ก.ก. ครั้งเดียวทางหลอดเลือดดำ โดยผ่านทาง jugular vein catheter แก่หนูปกติและหนูที่ได้รับเหล็กเกิน ผลการทดลองแสดงว่าทั้งหนูปกติและหนูที่ได้รับเหล็กเกิน มีระดับของ CP94 ในพลาสมาในช่วงเวลาต่างๆ ตลอดจนค่าตัวแปรทางเภสัชจลนศาสตร์ไม่ต่างกัน แต่หลังจากที่ให้ CP94 ไปแล้ว 1 ชั่วโมง พบว่าระดับของเหล็กในซีรั่มในหนูที่ได้

รับเหล็กเกินจะเพิ่มขึ้นมากกว่าหนูในภาวะปกติ จากผลนี้ชี้ให้เห็นว่าการกระจายของเหล็กในซีรั่มนั้นไม่ขึ้นกับระดับความเข้มข้นของ CP94 ในพลาสมาเพียงอย่างเดียวแต่อาจมีปัจจัยอื่นๆมาร่วมกำหนดด้วย ในช่วงที่เหล็กในซีรั่มมีระดับสูงแต่ในขณะเดียวกัน ระดับของ CP94 ในพลาสมามีระดับต่ำ อาจเป็นไปได้ว่าเหล็กในซีรั่มอยู่ในรูปของสารประกอบเชิงซ้อนกับเมตาบอไลท์ของ CP94 ซึ่งยังคงมีความสามารถในการจับเหล็กอยู่

ปริมาณเหล็กที่ถูกขับออกมาในปัสสาวะมีความสัมพันธ์กับระดับของเหล็กในซีรั่ม ซึ่งชี้ให้เห็นว่า CP94 สามารถนำเหล็กออกจากแหล่งเก็บไปยังระบบไหลเวียนเลือดและขับออกทางปัสสาวะ ผลนี้เกิดขึ้นได้อย่างรวดเร็วโดยสามารถเพิ่มการขับเหล็กได้มากที่สุดใน 2 ชั่วโมงหลังการให้ CP94 เมื่อวัดระดับของซีรั่ม TBARs พบว่ามีแนวโน้มที่จะลดลง หลังจากให้ CP94 ไปแล้ว 1 ชั่วโมง ซึ่งอาจจะเป็นผลของการต้านออกซิเดชั่นร่วมกับ คุณสมบัติในการเป็นสารขับเหล็กของ CP94

Thesis Title Iron Mobilizing Ability of 1, 2-Diethyl-3-Hydroxypyridin-4-one (CP94) in Iron-overloaded Rat.

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ABSTRACT

An iron mobilizing ability of a new orally active iron chelator, 1,2-diethyl-3-hydroxypyridin-4-one (CP94) was studied in iron-overloaded rats. The study was performed in both normal and iron-overloaded rats, having the right jugular vein pre-cannulated with a catether 24-hr before the experiment. The catheter was used for drug administration and blood sampling. Iron-overloaded rats were prepared by giving thirty two milligrams of iron (as iron dextran) intraperitoneally to normal rats (2 times per week) over a period of one month. This model was able to build up 8-fold increase of liver iron while the level of serum iron was not changed. Serum TBARs, an indicator of lipid peroxidation, was significantly elevated in iron-overloaded rats.

This study provided an animal model for ascessing the initial iron mobilizing activity of the chelator once it get into the cirrculating blood. A single *iv*-dose of 100 mg/kg CP94 was given directly into the right atrium via the catheter in a concious rat. The result showed that plasma profile of CP94 in iron-overloaded rats was not different from that of normal rats. One hour after the administration of CP94, serum iron was significantly elevated in the

iron overloaded rats while minimal change was observed in normal controls. This evidence indicated that CP94 has a potent iron mobilizing ability once it appeared in the plasma without having the CP94 penetrated into the iron deposited pool. The indifferent of plasma levels and pharmacokinetic parameters of CP94 in both iron-overloaded and normal rats supported this contention. The appearance of higher levels of serum iron when serum CP94 had almost disappeared from the circulating blood suggesting that iron might form complexes with either active metabolites of CP94 eg., hydroxylated metabolite or other plasma factors.

Increased urinary excretion of iron at the first 2-hr coincided with peak plasma level of Fe indicating that CP94 was responsible for attracting iron from the deposited pool to the central (blood) compartment and then excreted into the urine. With the fall of serum TBARs as early as 1 hr after giving the CP94, it was likely that CP94 might have an antioxidant activity in addition to its iron chelating property.

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LIST OF ABBREVIATION

CP20	1,2-Dimethyl-3-hydroxypyridin-4-one
CP94	1,2-Diethyl-3-hydroxypyridin-4-one
DFO	Desferrioxamine
L1	1,2-Dimethyl-3-hydroxypyridin-4-one
g	Gram
hr	Hour
hrs	Hours
K_a	Acid dissociation constant
K_f	Complex formation constant
K_{part}	Partiton coefficient
l	Liter
LD50	Lethal median dose
m	Milli, 10 -3
M	Molar
min	Minute
mol	Mole
MW	Molecular weight
RBC	Red blood cells
$t_{1/2}$	Half-life
μ	Micro, 10-6
%	Per cent
w.wt	wet weight

CHAPTER 1

INTRODUCTION

Thalassemia is the genetic disorder disease, abnormal hemoglobinopathies produce iron loading and tissue damage occure remarkably early in life, particularly if they received blood transfusion regularly. Both blood transfusion and increased dietary iron absorption caused by ineffective erythropoisis in thalassemia often lead to severe iron overload. Iron will accumulate in the body because there is no effective means to excrete iron. The excessive iron causes tissue damage e.g. liver, heart, pancrease, and other organs; death usually from cardiac failure (1). The physiologic limitation that prevents the elimination of accumulated iron can be circumvented by treatment with a chelating agent capable of complexing with iron and permitting its excretion.

The only iron-chelating agent now available for clinical use is a bacterial siderophore first introduced 3 decades ago, deferrioxamine B, a trihydroxamic acid produced by *Streptomyces pilosus.* Follow-up of patients on lifelong transfusion programmes has shown that regular use of the drug prevent the development of iron overload and its patho- logical consequence (2,3,4). However, deferrioxamine suffers from one serious disadvantage of being inactive when it is administratered orally. This only available iron chelator can only causes sufficient iron excretion to keep pace with the transfusion regimens when given either subcutaneously or intravenously over 8-12 hrs several times per week. For this reason, many patients find it difficult to comply with the treatment, and some even stop taking the drug altogether, subsequently the use of DFO has been found to develope also the complications of iron overload. Therefore, an orally-active chelating agent is urgently needed for those pateints on life long transfusion programes.

Hydroxypyridinone represents a group of novel orally active iron chelator, introduced by R.C. Hider and his colleagues. These bidentate 3-hydroxypyridin-4-ones bind to iron in 3:1 ratio with stability constant of 10^{37}, a much higher affinity to iron than that of desferrioxamine (5). The studies on iron mobilizing ability of hydroxypyridones in the *in vitro* models showed that it can mobilize iron from ferritin (6), transferrin (7,8), and haemosiderin (9). In the hepatocyte monolayer cultures, the results showed that chelators could mobilize intracellular iron depending on the lipid solubility of free ligand (10). For hydroxypyridones, 1,2-dimethyl- (CP20) and 1,2-diethyl- (CP94) derivation had been extensively studied in iron overloaded animal models . The excreted iron from iron overloaded animals being treated with these chelators was higher than that with desferrioxamine in the equivalent dose (11,12). Hydroxypyridin-4-ones have a high bioavailability (14) and are relatively more selective to iron(III) *in vivo* (15). CP94 had been claimed to be more effective than CP20 in the iron overloaded animal models (16,17), from the higher lipid soluble nature. Toxicity studies in animals showed no serious toxic effect(13). Recently CP20 was studied in thalassemic pateints and showed that oral dose of 75 mg CP20/kg was as effective as 50 mg desferrioxamine/kg given subcutaneously.

Being a group of highly potent iron chelator, the nature of iron mobilizing ability has been our interest. A model for short term effect for studying the mechanism of iron mobilizing activity was developed. This study was aimed at definding the effect of a single *iv* dose of hydroxypyridin-4-one(CP94) on the distribution of iron in relating to the level of plasma CP94. Serum TBARs, a marker of lipid peroxidation was also followed. A paralleled group of normal rats served as controls.

OBJECTIVE

The aim of this study is to determine the iron mobilizing activity and antioxidant oroperty of 1,2-diethyl-3-hydroxypyridin-4-one(CP94) after a single *iv* dose, in both normal and iron-overloaded rats.

REVIEW OF LITERATURE

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1. Iron Balance

The adult human has a total of 4-5 g of body iron. mostly, about 2500 mg is contained within circulating red cells as hemoglobin. About 1000 mg is held in storage form by ferritin and hemosiderin in the liver, bone marrow, spleen and muscle, serving as a readily available reserve in the event of blood loss. About 300 mg presents in tissues throughout in a variety of heme compounds (myoglobin, cytochromes, etc.) and other iron-dependent enzymes. Only 4 mg iron is bound to the specific iron transport protein, transferrin.

Although only about 4 mg of iron is present in the plasma at any one time, some 20 mg pass into and out of the plasma compartment per day. Most of the iron entering the plasma is delivered to it from the reticuloendothelial system (derived from the breakdown of hemoglobin); considerably smaller amounts enter from the body stores, tissue enzymes, and intestinal absorption. Most of the iron passing out of the plasma is selectively deposited in the bone marrow for hemoglobin synthesis; considerably smaller amounts are deposited into body stores, excreted, or required for cell metabolism throughout the body.

The iron balance in human is largely maintained by regulating absorption to fit the need of the body rather than by changes in excretion. In human with increased iron requirements absorb greater amounts of iron than the normal. The rate of erythropoiesis is also a major determinant of iron absorption with increased erythropoiesis activity links to enhanced iron absorption (19,20). The limited iron absorption and excretion in the normal are about 1-2 mg daily. The obligatory losses were losed via intestine 0.4-0.8 mg, bile 0.2-0.4 mg, urine 0.1 mg and skin 0.2-0.4 mg. Intestinal iron loss due to the extravasation of red cells and exfoliation of epithelial cells. Iron in the skin was lose by the exfoliation of epithelial cells. The turn over of iron pools in normal human were summarized in Figure 1 (21).

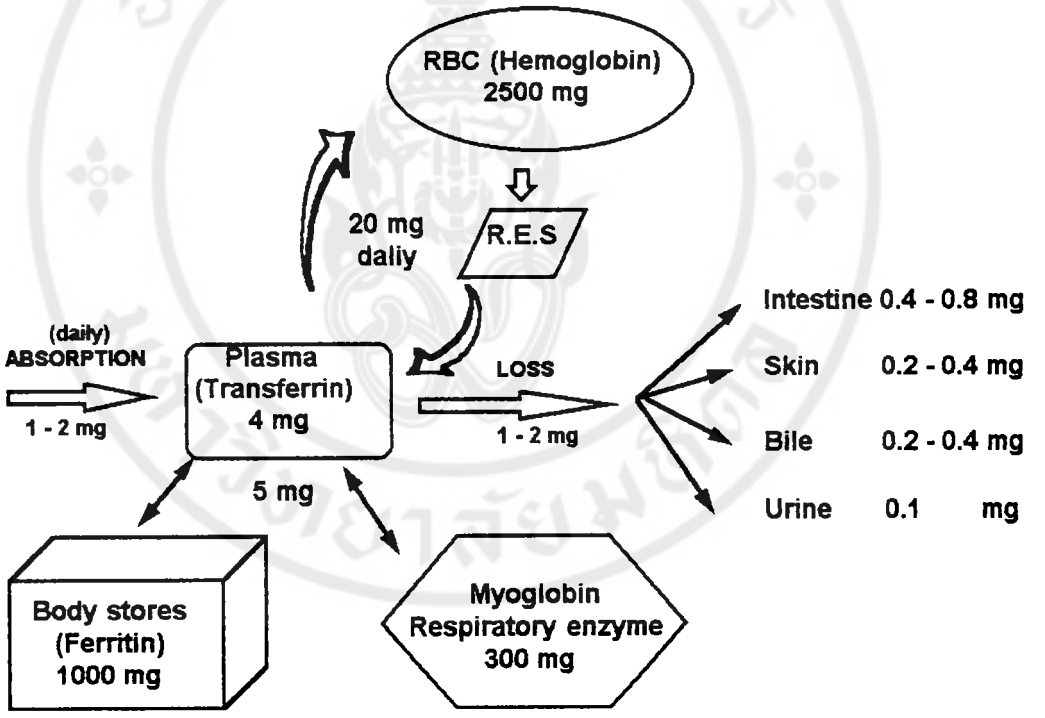


Figure 1. The iron balance in normal adult human. Adepted from Hider RC., et al. 1992 (21)

1.1 Iron transport protein (Transferrin)

The great majority of the iron that passes through the plasma compartment is carried by transferrin. Transferrin plays an important role in iron metabolism in the body. It is a β_1-globulin with the molecular weight of 74000-77000 (22). The two binding sites for ferric iron are located in the N and C terminal halves of the molecule. Under physiological condition, the stability constant for two binding sites are about 10^{31}(23). Because transferrin has two iron binding sites, it may exist as apoferric, monoferric, or diferric transferrin depend on iron loading. The distribution of these various forms is predictable as function of degree of iron saturation. Normally, percent transferrin saturation is about 35-40%. Iron is delivered to tissues by the interaction of the transferrin iron complex with specific membrane receptors. The number of these receptors appears to dictate the relative amount of iron delivered to any particular tissue. The affinity to bind with receptors is higher in diferric transferrin.

1.2 Iron Storage Proteins (ferritin, hemosiderin)

Approximately 25 per cent of the iron in the body is in the storage form. When present in normal total amounts(600 to 1500 mg), iron is stored intracellularly as ferritin and hemosiderin in the liver, spleen, bone marrow, muscle, and other tissues. It is distributed approximately equally between the ferritin and hemosiderin compounds. These compounds are present in both the reticuloendothelial and parenchymal cells of many organs and tissues. About one third of the total stored iron is in the liver, almost that amount is present in the bone marrow, and the remainder disbursed among spleen, muscle, etc.

Ferritin is a water soluble, hollow, spherical shaped protein. It is constructed from 24 structurally and chemically identical subunits of

polypeptide chain(24), each subunit has a molecular weight of about 19,000(25) and has 6 pores that allow access to the interior. This structure can hold up to about 45,000 iron atom, but the average number found in normal individuals is less than 3,000, so that a spare capacity is indeed maintained. Iron in ferritin is in the form of a hydrated iron(III) oxide phosphate complex. Iron enters ferritin as Fe^{2+}, which becomes oxidized by the protein to Fe^{3+} and deposited in the interior. Similarly iron can be removed from ferritin as Fe^{2+} by the action of number a biological reducing agent, include in depend on the present iron in plasma. It increases when transferrin saturation increases.

Small amounts of ferritin are present in normal human serum from 12-300 μg/L. The concentration of serum ferritin parallels the amount of storage iron within the body. The biosyntheses of ferritin molecule has been showed that the increase in synthesis was found to dose-dependent relative to iron and could not be blocked by Actinomycin D. Protein-deficient rats showed no loss in their capacity to respond to iron. These findings were interpreted to indicate the existence of a relatively stable messenger RNA that could be used to produce apoferritin with increased efficiency of template utilization in the presence of iron.(26)

Haemosiderin is the other storage form of intracellular iron which is the partial aggregate of ferritin. It is a normal constituent of mosttissues. Hemosiderin granules are much larger than ferritin molecules and are insoluble in water. Because of these differences, hemosiderin was originally throughout to be a completely different compound from ferritin.

Normally the liver and bone marrow storage iron is composed of approximately equal amounts of ferritin and hemosiderin. With iron overload, however, an increased proportion is hemosiderin. If iron is administered to rabbits, the ferritin iron reaches a peak concentration after

which any further increase in tissue iron is quantitatively reflected by an increase in hemosiderin iron. This is of considerable clinical importance since it supports the interpretation that iron stores and the presence and degree of iron overload can be estimated by the number of hemosiderin granules found in preparations from liver biopsy or bone marrow aspiration.

2. Iron Overload

Iron overload in anemic patients may be consequence of repeated blood transfusion, excessive absorption of dietary iron or a combination of both. Iron overload may be produced by

1. Blood Transfusion

The blood transfusion is necessary to prolong the life of patients. As each unit of blood contains approximately 250 mg of iron, repeated blood transfusion will lead inevitably to an excess accumulation of iron in the body, unless there is equivalent blood loss from haemorrhage. Some patients may accumulate a total in excess of 60 g of iron from repeated blood transfusion.

2. Hyperabsorption of iron via the gut

In humans, the average daily absorption of iron is between 1 and 2 mg which is matched by an equivalent loss of iron by desquamation of cells from the gut. If iron absorption exceeds 1-2 mg/day and if there is no excess blood loss from haemorrhage, then accumulation of iron within the body will ensue.

There are several inherited diseases which require the regular transfusion of red cells and be associated with the gradual excess

accumulation of iron via the gut. One of the most important of these on the worldwide basis is thalassemia. This is an abnormality of the red cells arising from an unbalanced production of the globin chain of haemoglobin. This lead to a shortened red cell survival in the circulation and consequently to anemia. Figure 2 (27) represent the pathophysiology of homozygous β-thalassemia and mechanism of iron loading. Because of the high rate of destruction of the abnormal red blood cell the patients have hyperplastic bone marrow but ineffective erythropoiesis. In β-thalassemia ineffective erythropoiesis resulted from the precipitation of excess α chain in the red blood cell precursor whereas in α thalassemia, the effective erythropoiesis is less because the excess β chain is water soluble and precipitate out in the older red blood cell population. These provide a basis for the difference in severity of α and β thalassemia (28). The iron overload resulted to increased iron absorption secondary from ineffective erythropoiesis. Iron overload also derived from blood transfusion in severe anemia cases, when regular blood transfusion is needed to increase hematocrit level and to suppress erythropoiesis activity.

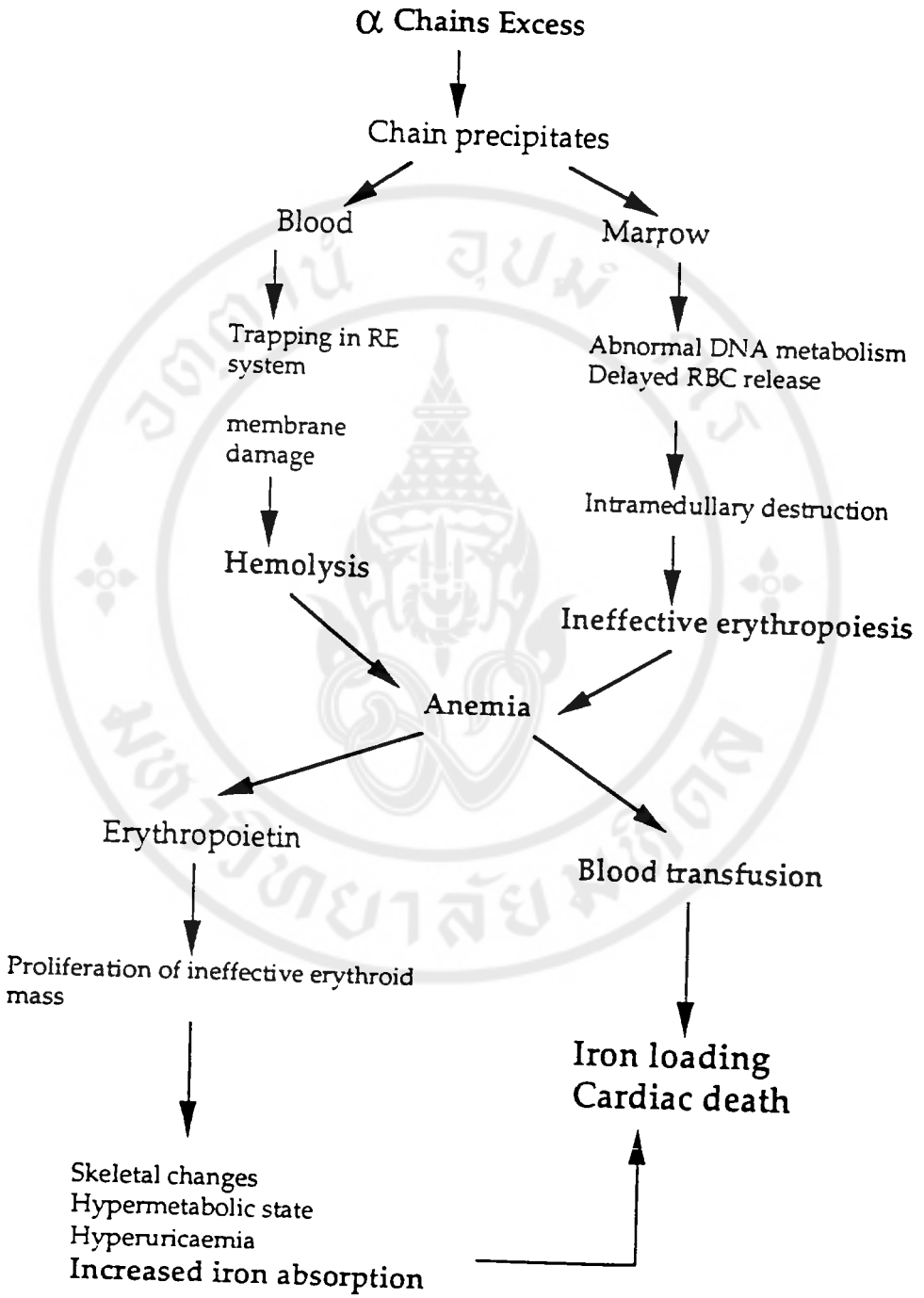


Figure 2. The mechanism of iron loading in homozygous β thalassemia. Modified from Weatherall DJ. and Clegg JB. (27)

Iron toxicity

The overload iron are distributed throughout the body but is found in highest concentrations within the liver as ferriitin and haemosi- derin. The excess iron is also present in a low molecular form within cells and in plasma leading to free radical mediated damage to the heart, endocrine glands, and the liver. The most of patients suffered and died from cardiac malfunction (29). The iron lead to cell damage by catalyzing of free radical formation according to Fenton reaction (i), the redox metal with hydrogen peroxide to yield hydroxyl free radical (30,31) and Haber Weiss reaction (ii).

$$O_2^- + Fe\ (III) \longrightarrow O_2 + Fe\ (II)$$

$$Fe\ (II) + H_2O_2 \longrightarrow Fe\ (III) + OH^- + OH^{\bullet} \quad (i)$$

$$\text{sum}\ O_2^- + H_2O_2 + H^+ \xrightarrow[\text{(catalyst)}]{Fe3+/Fe2+} O_2 + H_2O + OH^{\bullet} \quad (ii)$$

Hydroxyl radical is an important initiator of lipid peroxidation in biological systems which leads to increase membrane rigidity, a reduction of membrane potential, increase permeability to various ions and finally membrane rupture with the release of the content of various cellular organelles such as lysosomal hydrolytic enzymes (32). To eliminate the excessive iron, iron chelation is the only effective way to protect the cell from the iron producing lipid peroxidation.

3. Possible sites of iron chelation in the body

3.1 The Extracellular Iron

The transferrin iron may be the source of iron for the chelator which has higher affinity than transferrin. Although transferrin iron has small amount, it may play the role to reduce the iron storage by mediating the exchanges of iron among various body tissue (33).

Non-transferrin bound plasma iron (NTBI) appears with increasing iron overload which the transferrin iron binding capacity becomes saturated (34,35). Although quantitatively small as 2.7-7.1 μmol/L in thalassemic sera but this iron is not co-ordinates to a ligand with a high affinity for ferric iron, so it is easily chelated. Thus, relatively low concentration of chelators might mop up this iron and produce clinically beneficial effects in term of reducing free radical-mediated tissue damage while it is not necessarily causing much iron excretion.

The release of iron from reticuloendothelial cells following the breakdown of red cells represents approximately 30 mg/24 hr. in normal subjects and is thus a major potential source of chelatable iron , whether this interception occurs extracellularity or within the reticuloendothelial cell is unclear. The magnitude of iron chelation at this site may be proportional to red cell destruction.

An iron chelator might also compete with transferrin at the site of delivery of iron to red cell precursors, or to other cell such as hepatocytes. However, hepatocytes take up transferrin bound iron as well as the unbound iron from plasma and both of these fractions may be available for chelation.

3.2 The Intracellular Iron

The iron in hemoglobin, myoglobin and the various heme-containing enzymes is not chelatable both in iron overloaded condition and normal subject.

Ferritin represents a potentially useful source of chelatable iron if negative iron balance was achieved. However, because of the way in which iron is stored within the ferritin core, it is relatively difficult to chelate directly. Despite its potentially large chelatable source of iron, the slow rate at which this form can be chelated limits its importance. However, smaller chelators may be able to penetrate the protein shell and be more efficient at iron release.

Thus the most important source of rapidly chelatable intracellular iron is neither ferritin nor hemosiderin, but a transient low-molecular-weight iron pool, representing about a quarter of intracellular hepatocytes iron in non-iron-loaded human liver. This material is a relatively stable complex formed between the amino acids glutamate and aspartate and a small iron oxide core, consisting of three or four iron atoms. The complex, molecular weight approximately 1200 is capable of donating iron to ferritin, and yet maintaining iron in a form that does not generate hydroxyl radicals. In normal mammalian liver, the concentration of iron in this form is in the region of 2 μM.

This low-molecular-weight pool represents the major compartment through which all intracellular iron passes, and it is readily chelatable. The iron within any ferritin molecule is regularly turned over with a half-life of about 72 hr. or some what longer in the presence of excess iron, and will passes through this low-molecular-weight iron pool as a consequence. It has also been postulated that when this pool is large the iron may be toxic to cells (36).

4. Iron chelating agent for treatment of iron overload.

4.1 Properties Required for an Ideal Iron Chelators

Clinically iron chelator needs to :

1. be absorbed when given by mouth: It should be uncharged in the iron-free state and be reasonably lipophilic so as to facilitate its diffusion through cell membranes and should not be breakdown in upper GI tract.

2. have a high affinity for iron(III): The chelators should be high charge density because ferric iron has a high charge density and tent to bind tightly to atoms with the same high charge density such as oxygen species including carboxylates, catecholates and hydroxypyridinones.

3. chelate iron specifically without significant affinity for other metals: A high degree of selectivity for iron(III) is required to minimize the long-term toxicity of chelators, resulting from the other metals depletion.

4. penetrate tissues, cells and subcellular sites with abnormal iron accumulation: The lipid solubility of a chelator is affect to the ability to mobilize intracellular iron.

5. be excreted easily via the kidneys and liver only when combined with iron: The rapid elimination of iron chelated in plasma in the urine is desirable to minimize the redistribution of iron to other sites in the body (see below)

6. not redistribute iron to potentially more toxic sites in the body: The redistribution of iron result from the ability of chelator-iron complex to donate iron to unsaturated transferrin.

7. be itself relatively non-toxic: the lipophilicity must be limited because the lipophilic compounds can penetrate blood-brain barrier to CNF and produce neurotoxicity.

8. not be metabolically degraded to non-chelating metabolites: because of the hepatocyte is the major site of iron storage and also be extensive drugs metabolism, therefor the active metabolites are available for mobilize the iron in the hepatocyte.

9. have long plasma half-life of free chelate: The free chelate once absorbed from the gut should have a sufficiently long half-life so that it accesses to the chelatable pools before being eliminated.

10. be sufficiently active to achieve negative iron balance in mildly iron overloaded patients :

11. be unable to encourage bacterial growth: The compounds, closely related bacterial siderophores can enhance the bacterial growth, particularly a high iron requirement such as *Yersinia enterocolitica*.

12. not too expensive, so that the large number of potential patients in poor countries can get benefit. (21,36)

4.2 The candidates for oral iron chelators

Because of desferrioxamine suffers from the disadvantage that it inactive when administered orally, therefore, an orally-active chelating agent is needed.

The hydroxamate

Several compounds were to be oral active iron chelator. Pro-drugs of DFO was synthesized to increase the hydrophobicity by esterifying the labile hydroxamate function. Although some of this derivertives are active orally and would be clinical interest, none has been identified which has comparable activity to DFO when given to animal intraperitoneally. Cholyl-hydroxamic acid a bidentate ligand hydroxamate of cholic acid was actived by the oral route in some animals model. Clinical human trials were undertaken but were complicated by diarrhea at therapeutic doses of 100 mg/kg 24 hr.

The catecholate

Several catecholate bacterial siderophore, such as 2,3 dihydroxybenzoic acid, MECAM (triaminoethylbenzene-tris (2,3 DAB)) are known and these have an extremely high affinity and selectivity for iron (II). The binding iron(III) to catechols results in charged complexes which cannot efflux from cells. Catecholate, unlike hydroxamates, tent to form a mixture of complexes with iron(III) including the 2:1 species rather than the 3:1 complexes. These complexes do not completely protect iron from the solvent, leading to the generation of harmful hydroxyl radicals. 2,3 dihydroxybenzoic acid has been most extensively investigated of catechol. The studies have been shown that it failed to demonstrate adequate iron excretion in thalassemic patients, possibly due to an ability to transform the drug to its active metabolite. MECAM (triaminoethyl-

benzene-tris (2,3 DAB)), which is structurally similar enterobactin, was orally active in rat but was associated with septicemia, presumably mediated by iron delivery to bacteria.

The orthosubstituted phenolated

The phenolates has been studies as potential iron chelator such as desferrithiocin, isonicotinyl hydrazone (PIH). Desferrithiocin is a tridentate molecule which form a 2:1 complex with iron(III). This compound has a variety of advantageous properties including the binding constant and selectivity for iron (III) as well as being three times more effective by mouth than DFO given parenterally to rats. However, this compounds shows significant toxicity in hepatocyte cultures. Isonicotinyl hydrazone (PIH) was reported to be orally active in animal. Unlike other iron chelators, the hydrazone compound has low affinity and selectivity to iron. Some studies showed poor activity in human. Detailed toxicity testing suggest that PIH has low toxicity in animal and human.

The amino carboxylates

The aminocarboxylates, N,N'-Ethylene bis (O-hydroxyphenylglycine) (EHPG), N,N'-bis (O-hydroxybenzyl) ethylene-diaminediacetic acid (HBED), it contains both phenolic and carboxylate functions in order to increase the selectivity for iron(III). However, they are poorly absorbed due to their negative charge.

The hydroxypyridones

Hydroxypyridinone was predicted that, due to their unchanged nature under physiological condition, both as the free ligand and as iron complexes this would give the desirable properties for oral absorption of the free ligand and the rapid excretion of iron complexes. Further

advantageous of the hydroxypyridinones are their high chemical stability and high affinity and specificity for iron (III). Preliminary studies have demonstrated that these compounds have excellent iron scavenging properties and effective mobilizes of iron from hepatocytes in culture as well as from iron-overloaded mice. CP20 (L1) has been selected for humans studies by Kontoghiorghes et al. These compound has been shown to increase iron excretion in thalassemic patients. It is predicted that compounds with higher lipid solubility such as CP94 would be both better absorbed in humans and have superior liver penetration, allowing the increased fecal excretion which is necessary for significant negative iron balance. However, the detail of toxicity must be further studied, because some studies has been shown that it has the correlation between lipid solubility and toxicity (36).

The advantage and disadvantage of candidate oral chelators are summarized in Table 2.

Table 2. Advantages and Disadvantages of orally active iron chelators. (Porter J.(36))

Group	Compound	Advantages	Disadvantages
Hydroxamate	Cholylhydroxamic acid	High selectivity for Iron (III) Acceptable acute and subacute toxicity	Diarrhoea in clinical studies at 100 mg/kg/day
	Prodrug of DFO	Presumed low toxicity	Insufficient activity in animals
Catecholates	2,3-dihydroxybenzoic acid	High affinity and selectivity for Fe (III)	Poor iron excretion in man at 100 mg/kg
	MECAM	High affinity for iron (III)	Septicaemia in animals
Amino Carboxylates	Esters of HBED	Very active in animals	Selectivity for Fe (III) unclear Insufficient toxicity data
Orthosubstituted phenolates	PIH	Low toxicity in animals and man	Poor activity in man at 30 mg/kg/d Low affinity & ? selectivity for Fe (III)
	Desferrithiocin	High oral activity in rats & dogs	Unacceptable subacute toxicity
Bidenate Hydroxypyridine-4-ones	CP20 (L1)	High affinity and selectivity for Fe (III) Orally active in animals and man	Barbiturate interaction in rats Hypersalivation in rats Electroretinographic changes in rats reduced Hemoglobin & White blood cells count in subacute mouse studies No fall in serum ferritin at 1 year Litlle faecal iron excretion in man
	CP21, CP51, CP94	Fe (III) selectivity and affinity as for CP20 More iron excretion in acute and subacute animal models than CP20 (feacal) Lack certain toxicities seen with CP20	Not yet introduced into man

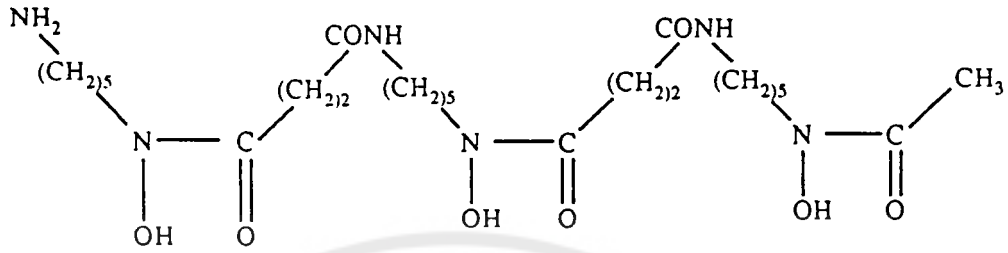
4.3 Desferrioxamine

Desferrioxamine (DFO) has been used in the treatment of iron overload since 1960. It is a siderophore produced by *Streptomyces pilosus.* The structure is a linear trihydroxamte chelator (Figure 3A). The molecular weight 656.8 with three functional hydroxamic acid groups form hexadentate ligand which binds iron in a 1:1 complex. In presence of Fe^{3+} is transformed in a cyclic molecule enclosing the iron called ferrioxamine (Fe A) Figure 3B (37). The complex formation constants of DFO for Fe^{3+} and Al^{3+} are 10^{31} and 10^{25}, respectively, whereas the constant is on 10^{14} and 10^{11} for copper and zinc, respectively (38), whereas the constant for Ca^{2+} is very low 10^{2-5} (39)

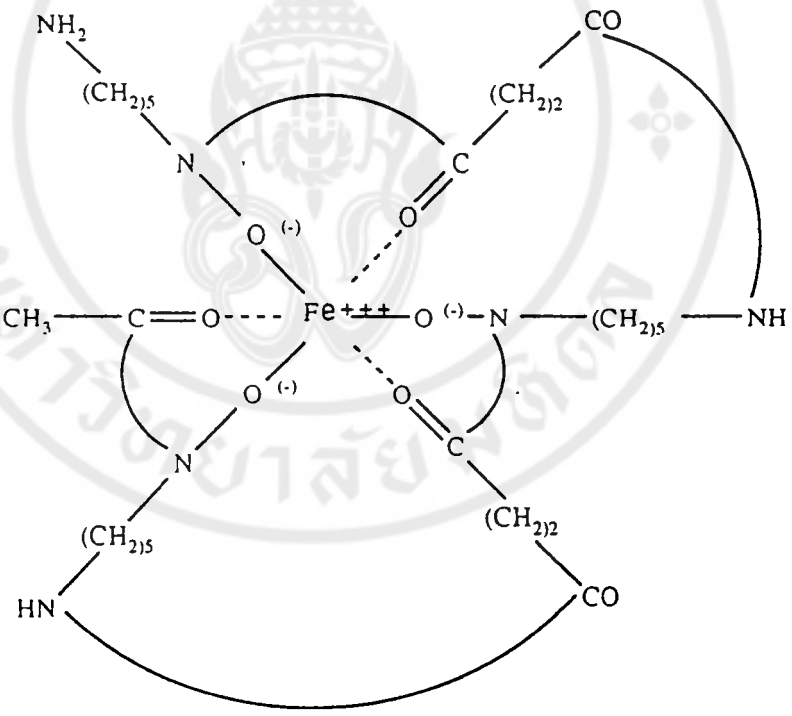
Because of DFO can mobilize iron from various pools including reticuloendothelial system, nontransferrin bound iron (NTBI) and storage pools such as in hepatocyte(40). The interaction of DFO and its main active metabolite (metabolite B) between intracellular and extracellular compartments is illustrated in Figure 4.

The uncomplexed DFO and its metabolites can pass freely between intracellular compartment whereas their iron-complexed counter-parts are unable to do so. The iron complex of DFO (ferrioxamine, FO) and DFO-met B (FO-met B) formed extracellularly can only be excreted via the kidneys into the urine, whereas those that are formed within hepatocytes are excreted into the bile (41).

DFO has been suggested that the rise in urinary iron excretion during active erythropoiesis reflects release of iron in a chelatable form as stores are mobilized for erythropoiesis, or greater deliver of iron to tissues in which it is then available for chelation (42).



A. Structure of desferrioxamine B



B. Structure of ferrioxamine B
(iron-desferrioxamine complex)

Figure 3. Structure of desferrioxamine B (A) and its iron complex or ferrioxamine (B) (Keberle H. (39)).

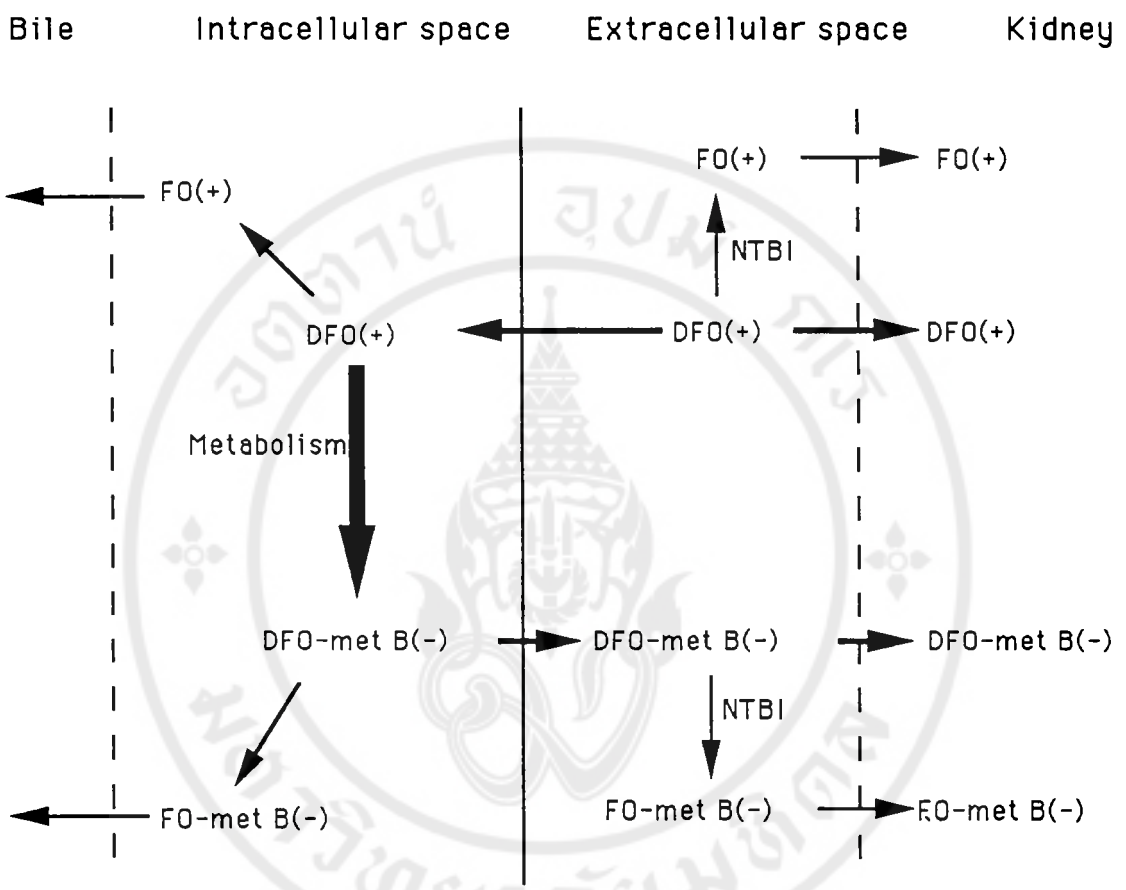


Figure 4. Distribution, metabolism, and elimination of DFO and its main metabolites (free ligand and iron bound) between intracellular and extracellular compartments. FO, ferrioxamine represent DFO-iron complex. Plus and minus refer to the charge of drug / metabolite (Lee P., et al.(41)).

Several studies have been shown that DFO can produce negative iron balance and prolong life expectancy in iron-overloaded patients. (43,44). Modell et al (45) and Smith et al (46) showed that iron excretion could be greatly increased if DFO was given by slow intravenous infusion. The dose of up to 16 g in 24 hr. could then be given without toxicity and remove as much as 200 mg iron in urine of heavily iron-loaded thalassemic patients. DFO has been studied as antioxidant in *in vitro* model by inhibiting MDA production in iron-supplemented hepatocyte cultures, and showed as free radical scavenger by eliminating the hydroxyl radical ($^{\bullet}OH$) and peroxyl radical ($^{\bullet}ROO$) in cell-free model.(47)

Pharmacokinetic studies of DFO

DFO has been shown to be extensively metabolized, forming up to six metabolites, four of which have been previously characterized (Figure 5) (48). The metabolite B is a major active metabolite.

Pharmacokinetic investigation of DFO has been studied in 11 thalassemic patients by P. Lee, et al (41). They showed that after stopping intravenous infusion at a dose of 50 mg/kg/24 hr over 48 hr. Plasma levels of DFO decreased in a biexponential fashion. It was cleared rapidly from the systemic circulation with a clearance of 0.5 ± 0.24 liters/kg/hr, and initial half-life of 0.28 ± 0.10 hr, and terminal half-life of 3.05 ± 1.30 hr. DFO was best described by a two compartment model. The initial phase indicates that rapid distribution of DFO occur that is followed by a slower β-elimination phase. DFO was also found to be extensively distributed in the body with an apparent volume of distribution during the terminal phase (Vd) of 1.88 ± 1.0 liters/kg. The studies of Bentur Y., et al, They suggested that the rapid decreasing at initial phase result from rapidly metabolized mainly in the plasma, probably by an enzyme belonging to the α2-globin (49).

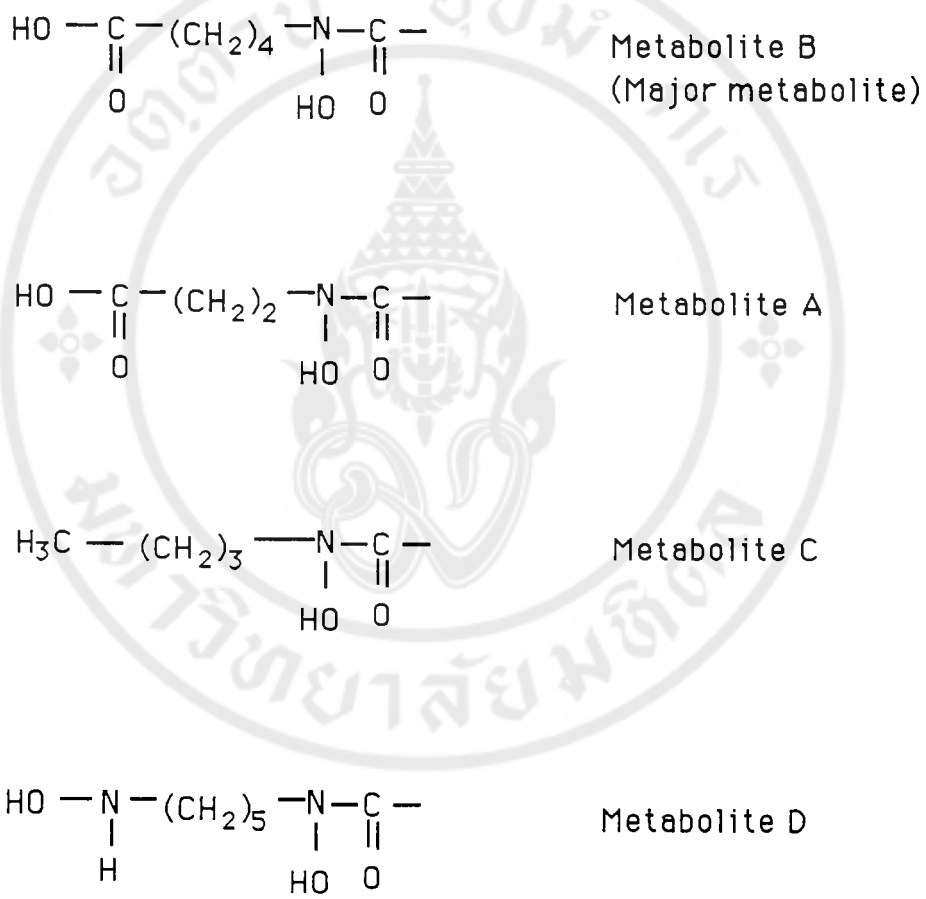


Figure 5. Structure of various metabolites of DFO (Singh S., et al. (48)).

Side effect of DFO

The main side effect are divided to short term and long term effect:

- short term effect are include allergy, anaphylaxis, local erythema, tenderness, abdominal discomfort and renal insufficiency. Hypotension may occur after rapid infusion of DFO, venous dilatation and histamine release may be the possible mechanism.

- long term effect is visual and auditory neurotoxicity. The decrease visual acuity is generally accompanied by ophthalmologic signs such as optic atrophy and abnormal pigmentation of the macular area. The degree of hearing loss could be mild or profound with sometimes acute deafness. Since these visual and auditory symptoms seem dependent on the doses of DFO.

Many studies have been showed that DFO increase the risk of microbial infection in iron-overloaded patients. Especially *Yersina enterocolitica* which much synthesize siderophores for an efficient iron intake. Iron availability is absolutely necessary for the growth but in body fluid of healthy subjects the level of free iron is too low to allow the requirments. Thus, iron overload increase the risk of infection but DFO can also increase this risk (37).

The main limitation in the use of DFO is its lack activity by the oral route; treatment compliance in many patients being a major problem and leading to significant reduction in life expectancy. A second drawback particularly in developing countries is the high cost of DFO. Despite these disadvantages, it is clear that DFO is a valuable drug, achieving negative iron balance in iron overloaded patients, with acceptably few side effects considering that it is given continuously

throughout life. DFO therefore is the standard with which all new iron chelators must be compared in their evaluation as iron chelating agents (36).

5. Hydroxypyridin-4-one

The oral iron chelators, 3-hydroxypyridin-4-one (CP compounds), were synthesized by a group of sciences led by Hider and Kontoghiorghes of the department of pharmacy, King's college and the department of hematology, the Royal Free Hospital, University of London.

5.1 Physiochemical properties of 3-hydroxypyridin-4-ones.

The structure of these chelators an based on the structure of natural siderophores, the low-molecular weight compounds manufactured by microorganism to facilitate the uptake of Fe^{3+}. The functional groups that act as strong bidentate for Fe^{3+} of siderophores one hydroxamate and catecholates moieties (Figure 6A). Cathecol is oral orally active and forms charged iron complexes whereas hydroxamate is not orally active and forms neutral iron complexes. To synthesize the potent oral iron chelators which are neutral complexes, the functionalities of hydroxamate and catechol were incorporated. 3-Hydroxypyridin-4-ones have two possible mesomeric forms (Figure 6B), the zwitterionic form makes a major contribution. Thus, the 3-hydroxypyridin-4-ones protonate on the carbonyl oxygen, the pKa values is 3.6 and the pKa of hydroxyl oxygen is 9.9 (Figure 6C) (52). From acid dissociation constants and stepwise complex formation constant of the ligands with iron, the neutral tris complexes form at the wide range of pH (Figure 7) (53). The formation constant (K_f) with the ferric ion and other cations are shown in Table 3 (50,54). These indicated that at physiological pH 3-hydroxypyridin-4-ones posses a high affinity and high selectivity for ferric ion compare to other chelators.

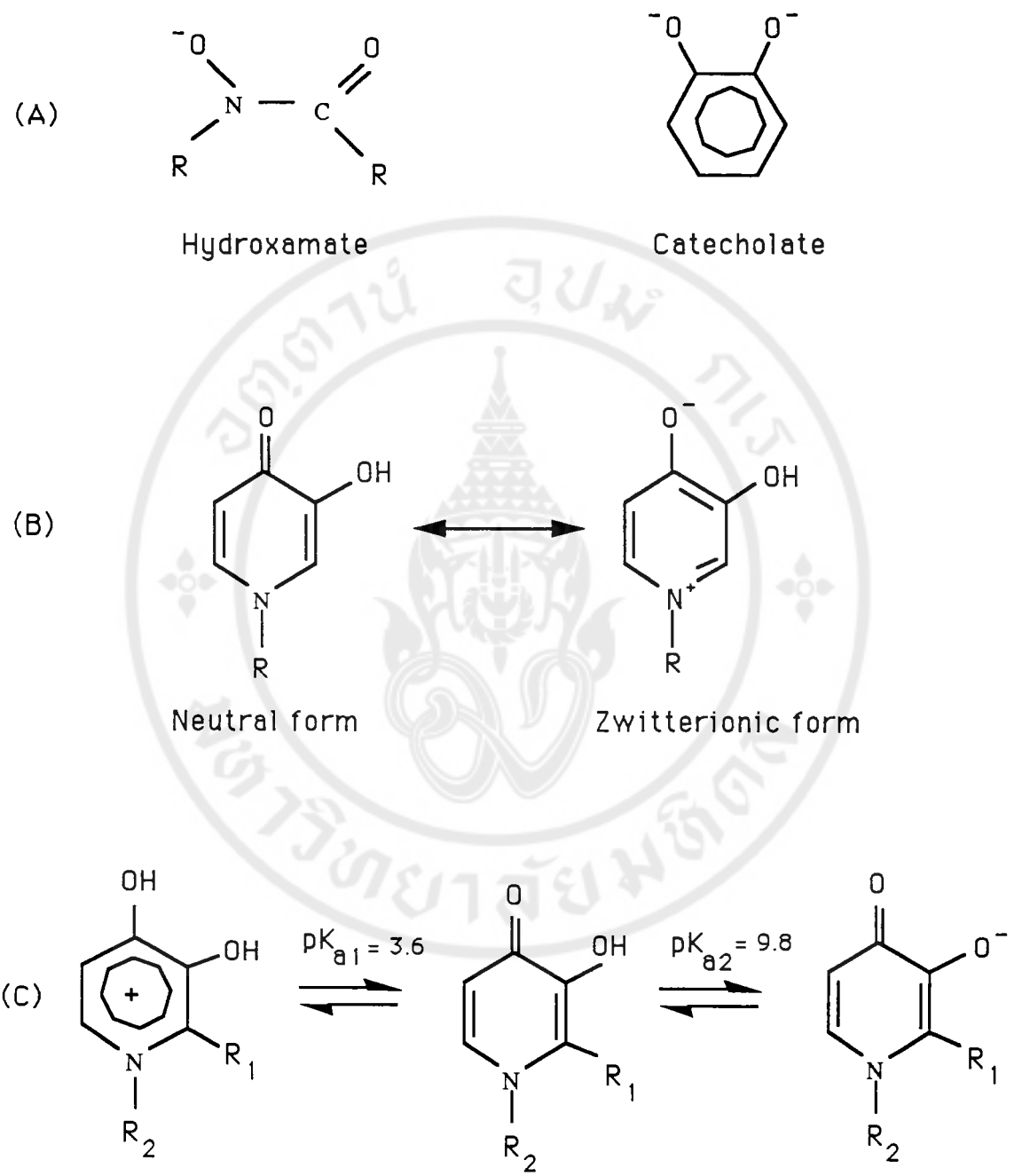


Figure 6. The physiochemical properties of 3-hydroxypyridin-4-ones. The function groups of the siderphores are the hydroxamate and cathecolate moieties (A) (Scarrow RC. et al (50)). The mesomers of 3-hydroxypyridin-4-ones (B) (Hider RC. and Hall AD. (51)). The two stepwise protonation of 3-hydroxypiridin-4-ones (C) (Clevevette DJ. et al. (52)).

$$Fe^{3+} + L^{-} \rightleftharpoons FeL_1 \qquad \log k_{f1} = 15.1$$

$$(FeL_2)^{2+} + L^{-} \rightleftharpoons (FeL_2)^{1+} \qquad \log k_{f2} = 12.1$$

$$(FeL_2)^{1+} + L^{-} \rightleftharpoons FeL_3 \qquad \log k_{f3} = 9.8$$

$$Fe^{3+} + 3L \rightleftharpoons FeL_3 \qquad \log k_{\beta} = 37$$

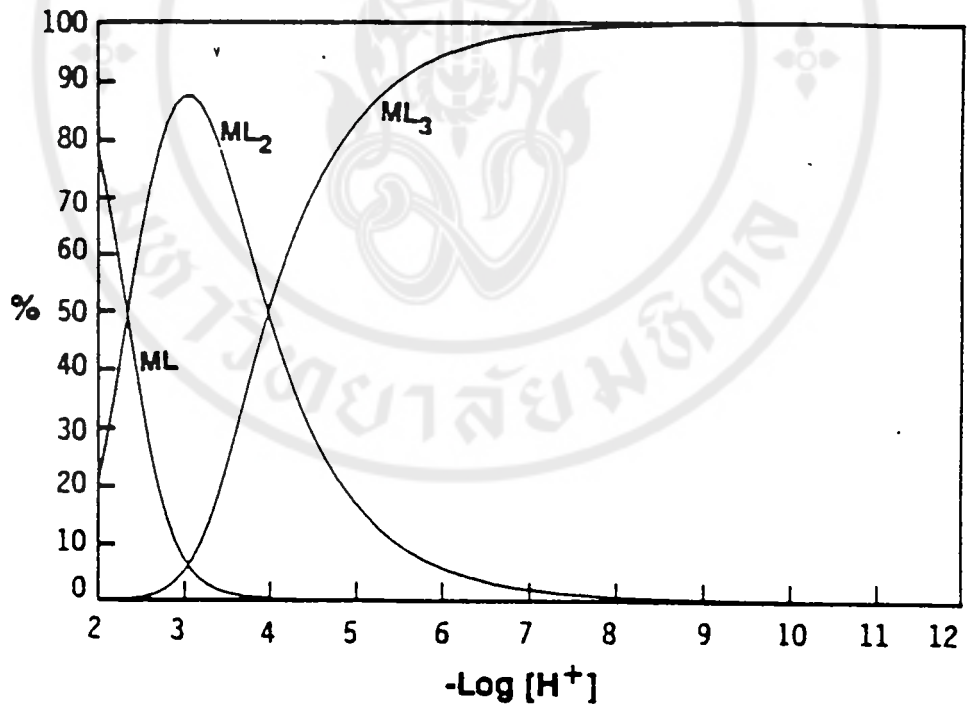


Figure 7. Stepwise complex formation constant of 3-hydroxypyridin-4-one and Fe^{3+} (Scarrow RC.,et al (50)) (A). Species distribution curves of the complex of 3-hydroxypyridin-4- ones(L) and ferric iron(M) at various pH (Martell AE., et al. (53)) (B).

Table 3. Logarithms of complex formation constants (log K_f).

Metal ion	EDTA	Desferrioxamine B	3-Hydroxypyridin-4-one (log β_n)	Catechol (log β_n)
Fe3+	25	31	37	40
Cu2+	18	14	17	25
Zn2+	16	11	12.5	17
Mg2+	9	4	7	6
Ca2+	11	2.5	4.5	-

$\beta_n = K_{f1} \cdot K_{f2} \cdot K_{fn}$

The number of this family of chelators that has received the most extensive evaluation in animal and human studies is 1,2-dimethyl-3-hydroxypyrid-4-one (known as L1, CP20 and DMHP). Physiochemically the drug is a white crystalline solid, readily soluble in water (16 to 18 mg/ml at 24 C). L1 is a neutral molecule that forms a neutral red 3:1 chelator-iron complex at pH 7.4. The shelf life of the agent has been estimated to be more than 3 years and the drug is stable in both acidic (pH <1) and basic (pH > 12) solutions; the chelator-iron complex is also stable in solution for prolonged periods. (55)

5.2 The efficacy and toxicity of hydroxypyridinone

5.2.1 *In vitro* model

The iron mobilization from transferrin has been investigated in *in vitro* model (7). The results showed that hydroxypyridinone remove iron from transferrin in a biphasis manner. It was suggest that the two phase correspond to the differential iron release from each sites of diferric transferrin. The site specificity of the removal of iron from diferric transferrin, at pH 7.4 has been investigated (8). The result has been shown that the removal of iron is not a random process. CP20 remove iron preferentially from the C-terminal site than N-terminal site.

The iron mobilization from ferritin has been investigated in *in vitro* model (6). The result showed that hydroxypyridinone mobilized iron from ferritin at pH 7.3 in a slow reaction taking up to 3 days to reach completion. The iron mobilization from ferritin depends on several factors such as: ability of chelators to mobilize polar nuclear iron, the presence of mediators such as ascorbic acid which enhance iron mobilization, the size, charge and other structure features such as lipid/water solubility of the chelator and its iron complex.

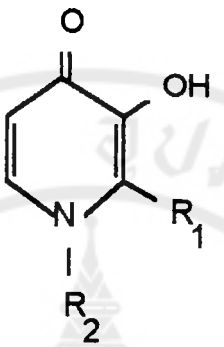
Kontoghiorghes G.J. has suggested that iron mobilization from ferritin and transferrin at physiological pH is a great importance to those designing iron chelator for the treatment of iron overload in thalassemia and other diseases of iron imbalance (56).

The iron-chelating activity has been investigated in hepatocytes (10,57), macrophage (58) and myocardial cells culture (59). The chelating ability of these compounds depend on the lipid solubility altering the length of substitutent groups (R groups). The partition coefficient of free ligand and iron complex are shown in Table 4 (60)

The relationship between lipid solubility and iron mobilization has been investigated in hepatocyte cultures. The chelators that were approximately equally soluble in lipid and aqueous phases (K part ~ 1) were most active compounds to mobilize intracellular iron. Highly hydrophilic chelators did not mobilize intracellular iron pools, whereas highly lipophilic compounds were toxic to hepatocytes (10). Moreover, lipophilic compounds may redistribute iron to the other cells or tissues and produce toxicity. Compounds with intermediate lipid solubilities (K part between 0.2-1.0) have greater ability to mobilize hepatocyte iron than DFO over a wide concentration range (33 μmol/l - 1 mmol/l) (21)

In ironloaded myocardial cells culture(58), the result has shown that the chelating ability and the efficiency to inhibit lipid peroxidation depend not only on lipid solubility of free ligand and complex but also on dose of the chelators. The proportion of neutral tris iron complex, unavailable Fe^{3+} for Fenton reaction, was increased at high dose.

Table 4. Structure and partition coefficient of 3-hydroxypyridin-4-ones both free form and iron complex form. (Hider et al (60))



	Structure		Partition Coefficient (K*part*)	
Compound	R_1	R_2	Free ligand	Iron complex
CP25	CH_3	$(CH_2)_4CH_3$	>20	>20
CP24	CH_3	$(CH_2)_3CH_3$	1.98	7.7
CP22	CH_3	$(CH_2)_2CH_3$	1.35	0.65
CP52	CH_3	$(CH_2)_3CH_3OCH_2CH_3$	1.0	0.39
CP94	CH_2CH_3	CH_2CH_3	0.85	0.07
CP96	CH_2CH3	$(CH_2)_2OCH_3$	0.83	0.046
CP93	CH_2CH_3	CH_3	0.5	0.03
CP21	CH_3	CH_2CH_3	0.4	0.03
CP51	CH_3	$(CH_2)_2OCH_3$	0.3	0.005
CP55	CH_3	$(CH_2)COOC_2H_5$	0.24	0.003
CP20	CH_3	CH_3	0.21	0.0009
CP40	CH_3	$(CH_2)_2OH$	0.002	0.002

The structures of chelators are shown in order of decreasing lipid solubility (Kpart). The partition coefficient (K*part*) was measured as the ratio concentration of compounds between n-octanol and Tris HCl (20 mmol/l, pH7.4) at 20°c

The hydroxypyridinones have been studies as antioxidant in *in vitro* model. The result showed that it can act as antioxidant by inhibiting malondialdehyde production in iron-supplemented hepatocyte culture. The free radical scavenging property was investigated in a cell-free model. The result showed that hydroxypyridinone can eliminated the hydroxyl radical (•OH) which generated from UV photolysis of H_2O_2 and eliminate the peroxyl radical (•ROO) which generate from autooxidation of linoleic acid micelles (47).

5.2.2 Animal Model

The efficacy of hydroxypyridinones have been evaluated in rodents and primate. In the mouse, a single dose of 200 mg/kg of intraperitoneal and oral CP20 each produce a similar increase in iron excretion (16). The investigation in the hypertransfused rat found that CP20 and desferrioxamine were comparable in chelating efficacy. Using selective radioiron probes, urinary iron excretion of iron mobilized by CP20 was found to be derived from reticuloendothelial cells while a portion of the iron mobilized from reticuloendothelial cells and all of the iron derived from hepatocytes was excrete through the bile (61).

The effect of CP20 was examined in the Cebus monkey in which iron overload (500 mg Fe/kg) had been produced by *IV* iron dextran. A comparison was made between doses of equivalent iron binding capacity of deferroxamine administered subcutaneous (150 μmol/kg or 100 mg/kg) and of CP20 administered orally (450 μmol/kg or 63 mg/kg). Chelation efficiency (observed total iron excretion/theoretical total iron excretion with chelator, expressed as a percent) with CP20 (2.1%) was less than half that observed with desferrioxamine (5.5%). In contrast to the rodent studies, where both urinary and biliary iron excretion were observed, iron excretion produced by CP20 in the monkey was exclusively urinary (62).

The relationship between the oral efficiency and acute toxicity has been investigated in mice by Porter et al. (16). The results showed that there is a linear relationship between the K_{part} of each chelator and iron excretion following oral administration (Figure 8A), unlike that K_{part} and acute toxicity ($1/LD_{50}$) which trend to increased acute toxicity with increased lipid solubility (Figure 8B). However, the hydroxypyridin-4-ones with K_{part} values less than 1 show relatively small differences in LD_{50} values, that below a critical lipid solubility (K_{part} approximately equal to 1) acute toxicity is relatively independent of K_{part}. In Figure 8C shows the relationship between the relative oral efficacy of each compound (expressed as the increase in radioiron excretion per unit increase in dose administration in milligrams per kilogram) and relative acute toxicity. CP51 and CP94 lie to the left of this line, indicating that these compounds have a more favorable efficacy to toxicity ratio than others which lie closer to or to the right of the line. These suggest that the potential use of CP51 and CP94 in chelating therapy.

Toxicological evaluation has been studies in mice, rat and dog. In acute toxicity studies in mice, the LD50 after intraperitoneal injection has been estimated to be about 1,000 mg/kg (16). At a dose of 200 mg/kg daily by intraperitoneal injection, anemia, leukopenia, and thrombocytopenia have been reported in mice(16,62,63), anemia and leukopenia, but not thrombocytopenia, were reported in rats(61). Other side effects of CP20 administration that have been noted but not reported in full are hypersalivation, prolonged anesthesia with barbiturates, and electroretinographic changes.(15,17,62).

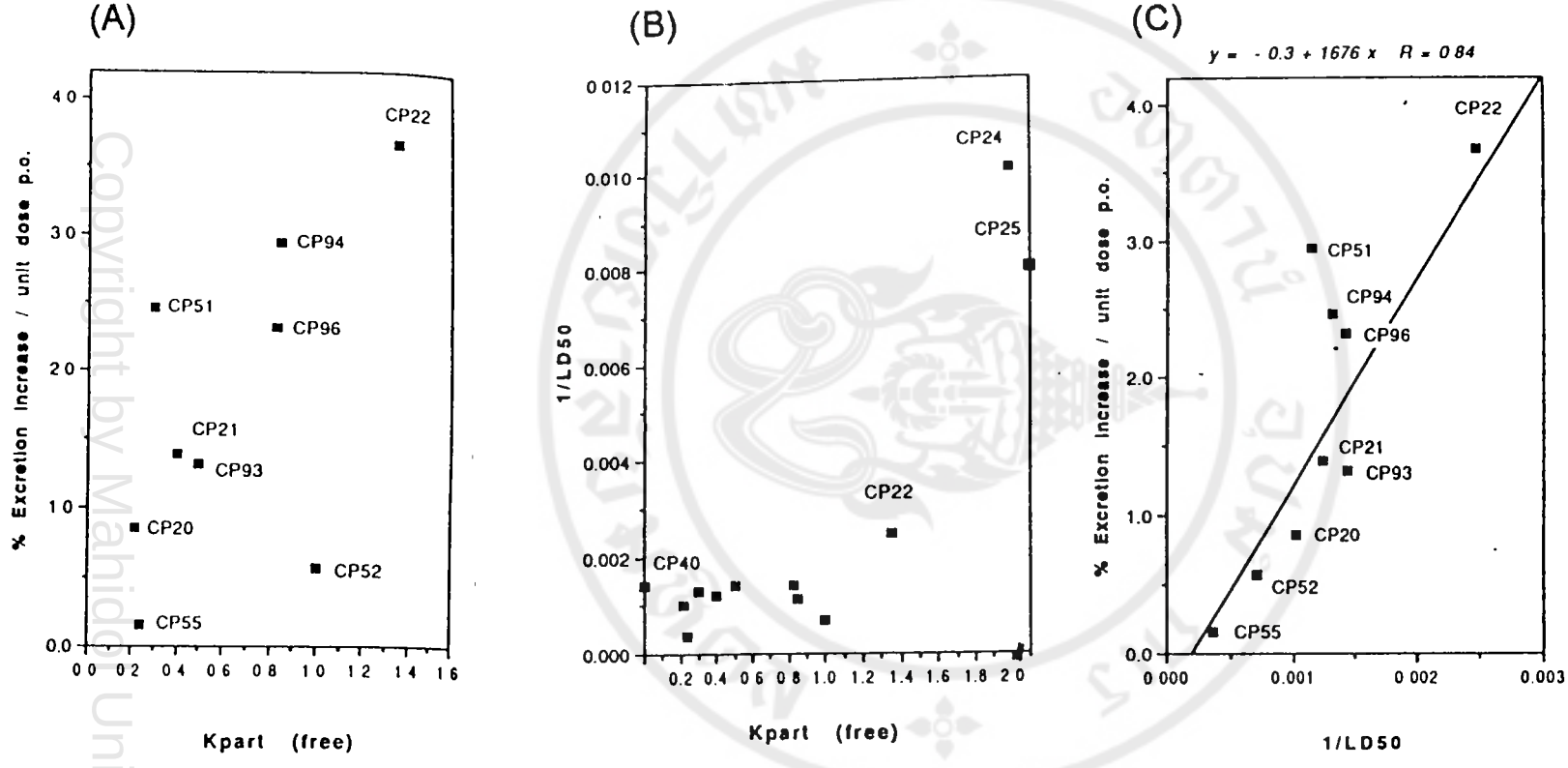


Figure 8. The relationship between the Kpart and the relative efficacy of each chelator following oral administration is shown in (A). The relationship between the Kpart and the reciprocal of the LD50 value via the intraperitoneal route for each chelator (B). The relationship between the reciprocal of the LD50 and the relative effectiveness of each compound by the oral route (expressed as percentage of 59Fe excretion increase per unit dose) is shown in(C). (Porter J., et al. (16))

In a formal toxicity study in dogs which were administered doses of 0, 25, 50, 100, 200, 400, and 600 mg/kg each, once daily by gelatin capsule for 14 days. The deaths occurred only at the highest dose of 600 mg/kg/d. At doses 400 mg/kg/d or more, gastrointestinal lesion and adrenal hypertrophy was observed. At doses 200 mg/kg/d or more. growth retardation, bone marrow atrophy, reticulocytopenia, leukopenia, and thrombocytopenia developed (64).

5.2.3 In Human (clinical trial)

CP20 has been selected for human studies since 1987. The therapeutic effect is usually judged by measuring the increased urine iron excretion or the change in parameters of iron metabolism, e.g., serum ferritin (65-73). In a comparative study of urinary iron excretion in five patients, a dose of 75 mg CP20/kg (180 µmol iron binding equivalents/kg) resulted in a urinary iron excretion of 26.7 mg Fe/d that was similar to the 24.9 mg Fe/d produced by a dose of 50 mg **deferr**ioxamine/kg subcutaneously (76 µmol iron-binding equivalents/kg) (18). This study found that, in terms of iron binding equivalents, more than twice the dose of CP20 was required to produce the same urinary iron excretion as desferrioxamine. This comparison of urinary iron excretion did not take into account any fecal iron excretion produced by desferrioxamine, which may constitute a substantial fraction of the total desferrioxamine-induced iron excretion with desferrioxamine is considered, then the effect on iron excretion of a dose of 75 mg CP20/kg may correspond to a dose of subcutaneous desferrioxamine of about 30 to 40 mg/kg.

Many studies have been shown the efficacy as decrease in the serum ferritin concentration after treatment with CP20. In long term study by Kontoghiorghes et al., (13). They found no consistent decrease in the serum ferritin concentration after treatment with CP20 in 13 patients treated with a variety of doses of CP20 for from 1 t o 5 months, serum

ferritin fluctuated but were unchanged overall. The study by Al-Refaie et al. (73), using a high dose of about 100 mg/kg/d, showed statistically significant decrease in serum ferritin concentration in 10 patients.

However, urine iron excretion and serum ferritin give no reliable information on the amount and changes of the storage iron under treatment especially in severely iron-loaded patients. More recently, the non-invasive liver iron quantitation by biomagnetometry or magnetic resonance tomography, as well as a ^{59}Fe labeling technique were shown to be potent new methods to measure more directly changes in the iron stores during iron depletion therapy in patients (74).

Direct evidence that CP20 can decrease body iron has been obtained by Olivieri et al.,(71). In a 29-year-old man with thalassemia intermedia CP20, 75 mg/kg/d administered for 9 months resulted in a reduction in hepatic iron from 14.6 mg Fe/g liver, dry weight, to 1.9 mg Fe/g liver; the serum ferritin decreased from 2,174 μg/L to 251 μg/L.

The toxicity of CP20 was included from the studies and case report. Mild adverse effects associated with CP20 administration described by Al-Refaie et al (73) include dermatologic changes associated with zinc depletion, minor gastrointestinal complaints, transient liver function abnormalitits, and musculoskeletal symptoms. The series adverse reaction, agranulocytosis has been occurred in long term trial, in 28-year-old woman with Blackfan-Diamond anemia who had received CP20 at a dose of 105 mg/kg/d for 6 weeks, developed agranulocytosis, with septicemia and shock (71,75).

5.3 Pharmacokinetic studies of hydroxypyridones

5.3.1 in animal

The pharmacokinetic studies of 1,2-dimethyl-3-hydroxypyridin-4-one(CP94) and its 2-(1-hydroxyethyl) metabolite (metabolite A) were examined in male Wistra rats (76) using a chronically cannulated concious-rat model. Following *iv* doses of 25,50 and 100 mg/kg, the parent compound was eliminated from blood in a biexponential fashion with an average systemic clearance of 1.5 liters/hr/kg. The mean terminal elimination half-life was 2.02 hr and the mean volume of distribution at steady state was 2.69 liters/kg. The area under the curve (AUCs) for the 25,50 and 100 mg/kg iv doses were 15,36, and 72 μg/ml/hr, respectively , suggested that the disposition of CP94 in rats obeys linear kinetics.

Pharmacokinetic studies of CP20 and CP94 have been investigated in rabbit (77). The studies showed these compounds are fairly well absorbed from the gastrointestinal tract. CP94 is more rapidly eliminated by the rabbit than in CP20.

The urinary metabolic profile of CP20 and CP94 in rat have been illustrated by Singh et al. The metabolism of CP20 was also studied in humans (78). The route of metabolize of CP94 differ from CP20 as show in Figure 9 & 10. CP94, unlike CP20 does not form an O-methylate metabolite, the formation of this compound suggests that CP20 is a substrate for methyl transferase enzymes, that most likely being catechol -O-methyl transferase. This is undesirable, as being a substrate CP20 may complete for enzyme activity site with natural substrate such as noradrenaline and dopamine. CP20 is also extensively conjugated (~44% in rats and >85% in man with glucuronic acid. Both these metabolic changes lead to a marked loss in iron binding capacity due to inactivate of the 3-hydroxyl functional group. CP94 in contrast is less efficiency

conjugated (13.8%) in rats, resulting in a much lower loss of iron binding capacity. This is largely because the hydroxylated metabolites of CP94 (metabolite A and B), in contrast to the hydroxylated metabolite of CP20, do not form glucuronide conjugates. This non-conjugative pathway is crucial as approximately 40% of the drug is metabolized in this manner. This leads to the formation of a hydrophilic iron chelator with similar metal binding ability as that of the parent drug. This difference in metabolism possibly explains the relatively poor ability of CP20 to mobilize iron. Therefore, CP94 has opportunity of chelating hepatic iron when it access to liver which also function as major metabolizing organs.

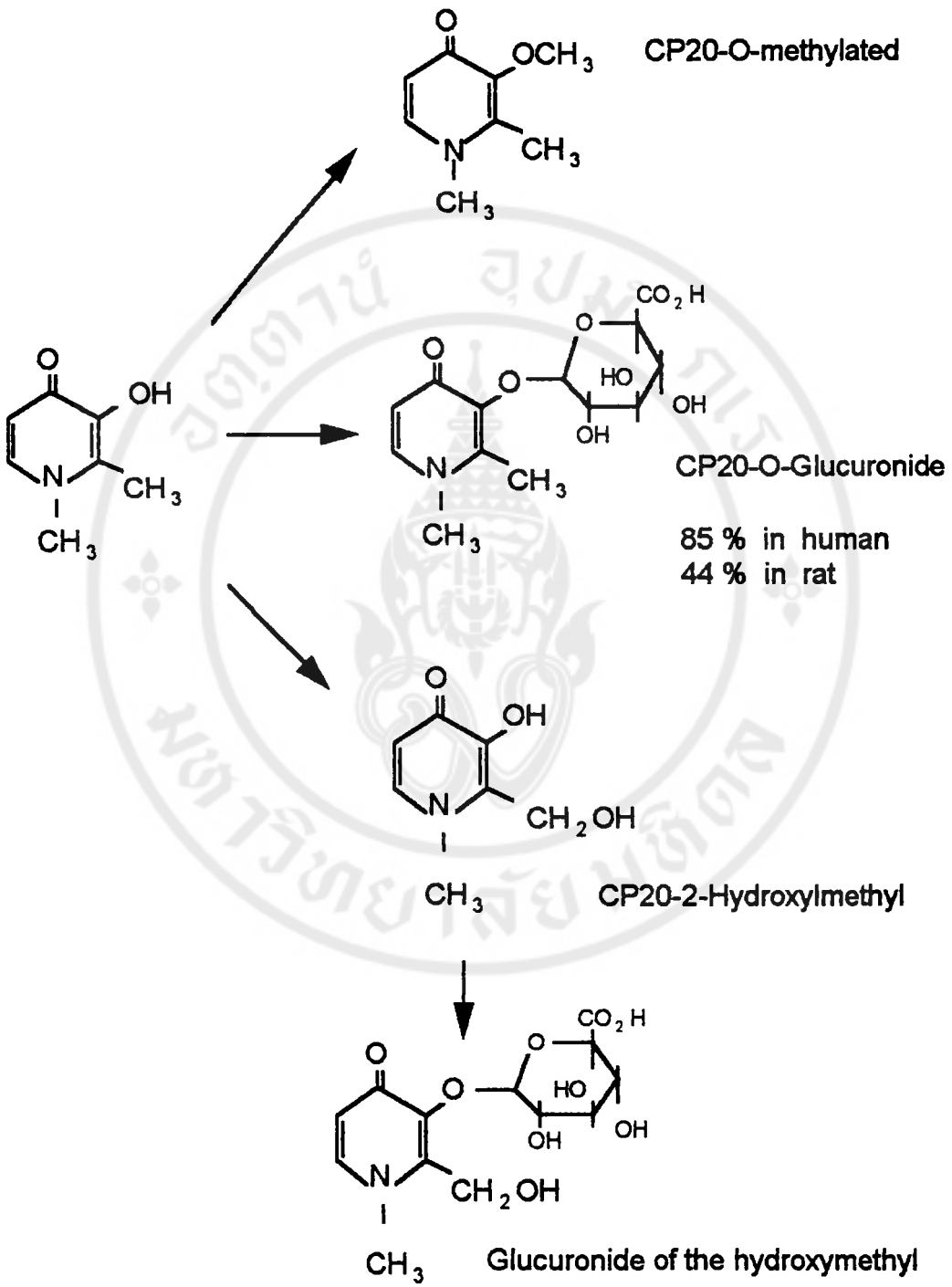


Figure 9. Scheamtic representation of metabolic pathway of CP20. The major metabolite is the non-chelating glucoronide. Percent of metabilite indicated were measured in rat and human urine. (Singh S. et al (78)).

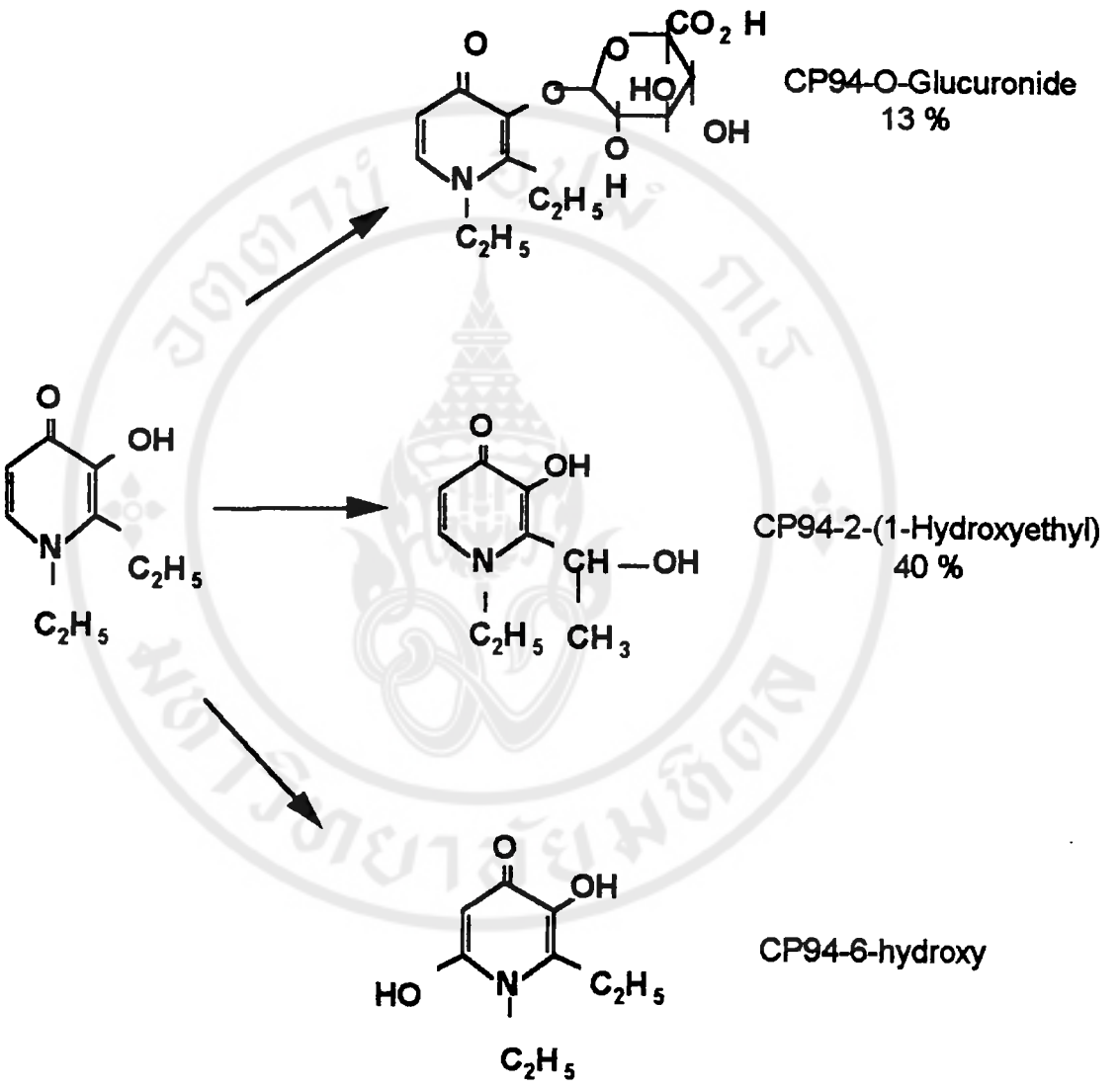


Figure 10. Scheamtic representation of metabolic pathway of CP94. The major metabolite is the hydroxylated metabolite which is itself capable of chelating iron (III). Percent of metabilite indicated were measured in rat urine. (Singh S. et al (78)).

5.3.2 in human

The pharmacokinetic of 1,2-diethyl-3-hydroxypyridin-4-one (CP20) has been studied in humans (13,18,79,80). Kontgiorghes GJ et al has been monitored CP20 plasma concentration in thalassemia patients by using the HPLC method which was developed by Goddard JG et al (81). The results shown that after ingest 3 g dose, the absorption is apparently from the stomach (half-life 1 to 5 minutes) with the drug appearing in the blood within 5 to 10 minutes. Importantly, glucuro-nationis the major route of CP20 metabolism and almost all the drug administered is recovered in the urine as the glucuronide (which is not and an iron chelator) or as the iron-CP20 chelate (12). The half-life of serum elimination has been reported as 47 to 134 minutes. Within 5 to 6 hours, 85% to 90% of the drug was eliminated from the serum. Maximum serum concentrations were 132$\pm$44 µmol/L with 3 g dose (35 to 75 mg/kg) in one study (13).

Pharmacokinetic details of CP20 in thalassemic patients have been studied by Klein J et al. (80). They used HPLC method which was developed by Epemolu RO et al. (82). The results shown that after a single oral dose of 25 mg/kg, the peak plasma concentration (Cmax) was 93.42-112.15 µmol/L which was achieved 45-60 min (Tmax). The elimination half-life(T1/2) was 115.5-173.2 min. Similar in another study (18) with a dose of 25 mg/kg, the peak serum concentration (Cmax) ranged from 51 to 150 µmol/L which was achieved 45-90 min. Elimination half-life varied between 117 and 237 minutes (73).

The study of Matsui D showed effect of food on the bioavailability of CP20 (82). Since the iron presence in the food can theoretically decrease the drug's bioavailability because a fraction of the drug may be bound to iron in the gastrointestinal tract and may not be availability for systemic iron chelation. They indicated that food delays absorption of

CP20, evidenced by lower peak height; however, it does not affect the extent of absorption, measured by AUC. They also indicated that CP20 can be given with food without affecting iron excretion.

CHAPTER 2

MATERIALS AND METHODS

Animal

Male Spraque-Dawley rats, weighing 80-100 g were obtained from National Laboratory animal center, Mahidol university, Nakorn Pathom, Thailand. They were acclimatized at the animal center, faculty of science for 1 week before starting the experiment. Animal were caged in group of 5 each, food and water were freely available.

Chemicals

Hydroxypyridinone derivertive (CP94, CP95) were kindly provided by Prof. R.C. Hider, Pharmacy department, King's College, University of London, United Kingdom. Other chemicals were obtained from commercial sources as follows: trichloroacetic acid, sodium acetate anhydrous, L-ascorbic acid (vit.C), thioglycolic acid, 2,2'-dipyridyl,3-(2-pyridyl)-5, 6-bis(4-phenylsulfonic acid) 1,2,4 triazine (ferrozine), sodium hydrosulfite and iron dextran, 100 mg iron/ml; dextran content 116 mg/ml, from Sigma chemical company, St Louis. MO 63178USA. Heparin sodium (Leo^{R})Leo Pharmaceutical products, Ballerup, Denmark (Each vial contains heparin sodium 5,000 i.u./ml), Pentobarbital sodium injection (Nembutal^{R}) from Abbott Laboratories International Co.,Ltd., North Chicago, Illinois 60064 USA, absolute ethanol, hydrochloric acid fuming 37%, Di-sodium hydrogen phosphate dihydrate (Na_2HPO_4 .$2H_2O$) from E. Merck, D-6100 Darmstadt, F.R. Germany, monopotassium phosphate (KH_2PO_4) from May&Barker LTD Dagenham England, and the HPLC grade solvents (acetonitrile and ethanol) from Baker analyzed J.T.Baker Inc phillips- berg, NJ 08865USA.

Method

1. Preparation of iron overloaded rats

Iron overloaded rats were prepared using rats weighing 80-100 g. They were loaded with iron by intraperitoneally injection of 0.5 ml iron dextran, 8 mg iron/ml twice a week for four weeks. Iron dextran was prepared by diluting 2 ml iron dextran (100 mg iron/ml) with sterile normal saline solution to make the final volume of 25 ml. The iron overloaded rats were used 10-14 days after the last injection iron dextrans.

2. Cannulation procedure

One day before the experiment, rats were anesthetised with sodium pentobarbital (30mg/kg) intraperitoneally and polyethylene catheter (PE 50, I.D. 0.58 mm O.D. 0.965 mm, Clay Adams USA.) filled with heparinized saline was injected into the right external jugular vein. The catheter was exteriorized through the skin of the scruff, sealed with heat and kept in a small box attached at the back of the animal. The chelator was given via this catheter.

3. Measurement of serum iron

To 1 ml of serum, standard iron solution, and 1 ml water (the 'blank') in separate iron-free tubes were added before the adding of 1 ml mixed acid reagent (20% trichloroacitic acid, 10% (w/v) thioglychloric acid, 2.76%(v/v) hydrochloric acid). The tube was mixed throughly with a vortex mixer for 30 seconds and, then centrifuged at 1,000X g for 10 min to give a clear supernatant for iron measurement.

Color development: To 1 ml of the supernatant or the standard or blank in the tube, one milliliter of chromogen (50 mM sodium ascorbate, 1.0 mM ferrozine, 1.05 M sodium acetate) was added. Mix throughly, leave for 10 min. before the measurement was performed at 562 nm against the distilled water. (83)

4. Quantitation of liver iron

The liver were weighed before cutting two pieces of the main lobe and dried by keeping in the oven at 95 degree celceus for 48 hrs. The dried liver were weighed and grinded with mortar and pestle. The powderized liver were extracted the non-heam iron by acid extraction. The grinded liver were weighed to 20 mg and transfer to a test tube followed by the addition of 1 ml of acid mixture (3 mol hydrochrolic acid and 10% trichloroacetic acid). The tube was closed with a marble and keep in an oven for 20 hr at 65 degree celceus. The iron content of each sample was determined by color formation with ferrozine as describe in the serum iron.

5. Quantitation of urine iron

The urine were centrifuged to remove the precipitates at 1,500Xg for 20 min. Transfer 2 ml urine to the iron-free tubes then add 2 ml phosphate buffer solution 0.067 M, pH 7.0 (Sorensen; monopotassium phosphate, KH_2PO_4 9.08g/liter, 39.2 ml and disodium phosphate, $Na_2HPO_4.2H_2O$ 11.87g/liter, 60.8ml) followed by 5 mg dithionite powder. Make up the final volume to 5 ml with iron-free water and mix throughly. Pipette 2 ml aliquots of the solution into two clean test tubes. The first tube was the sample urine performed by adding 0.2 ml α, α'-bipyridyl 64 mM,(1 percent wt/vol in 0.05 N sulfuric acid), mixed well, and let it stand for 30 minutes. The second tube was the urine-blank and 0.2 ml 0.05 N sulfuric acid was added.

The reagent blank and the standard iron were prepared by substituting 2 ml urine with iron-free water and standard iron, respectively. Reading the optical density of all tubes at 515 nm.

Calculation

The absorbance of the urine blanks were subtracted from the absorbance of urine test to obtain the corrected absorbance of urine test. The urinary iron content was read as µmol concentration from the calibration curve.(84)

6. Determination of TBARs

Thiobarbituric acid reaction substance (TBARs) a common metabolites of lipids was determined by a spectrofluorometric method (85).

7. The quantitation of hydroxypyridinones in plasma

7.1 Sample preparation

The quantitation was performed in 100 µl of the deproteinized rat plasma by using 1 ml absolute ethanol. The ethanol extracts were evaporated to dryness by blowing nitrogen over the sample tube and the residues then reconstituted in 500 µl of the mobile phase (16% acetonitrile in phosphate buffer pH3). Aliquots (50µl) of these samples were injected into the HPLC column.

7.2 Chromatography

The HPLC system consisted of a Water 6000A pump, a UV spectrophotometer (UVIDEC-100-III Jasco) and a Millinium 2010

chromatography solfware version 2.0. Sample were introduced into the column by a Water 717-plus Autosampler.

HPLC was carried out on a Hypercarb PGC column (10X0.46 cm) from Shandon Scientific Ltd. Runcorn, Cheshire WA7 1PR, England. The mobile phase consisted of 16:84% V/V acetonitrile: $Na_2HPO_4.2H_2O$ buffer (10mM pH 3.0 adjusted with phosphoric acid). The eluent was monitored at 280 nm and the flow rate was 1.0 ml/minute (86).

The HPLC chromatogram showed single asymmetrical peak (tailing peak) for both CP94 and CP95(internal standard), with complete base line resolution between those components. The injected contents were completely eluted within 15 minutes. The retention time of CP94 and internal standard CP95 were 5.47 and 9.87 minutes, respectively (Figure 11).

7.3 Assay variation

The accuracy and precision of the assay were determined by spiking CP94 into 100 µl control rat plasma at two concentration: 1 µg and 40 µg. The study was carried out on four separate occasions. The assay precision, as indicated by the CV% and the accuracy as shown by mean percent deviation (M%D) are given in Table 5.

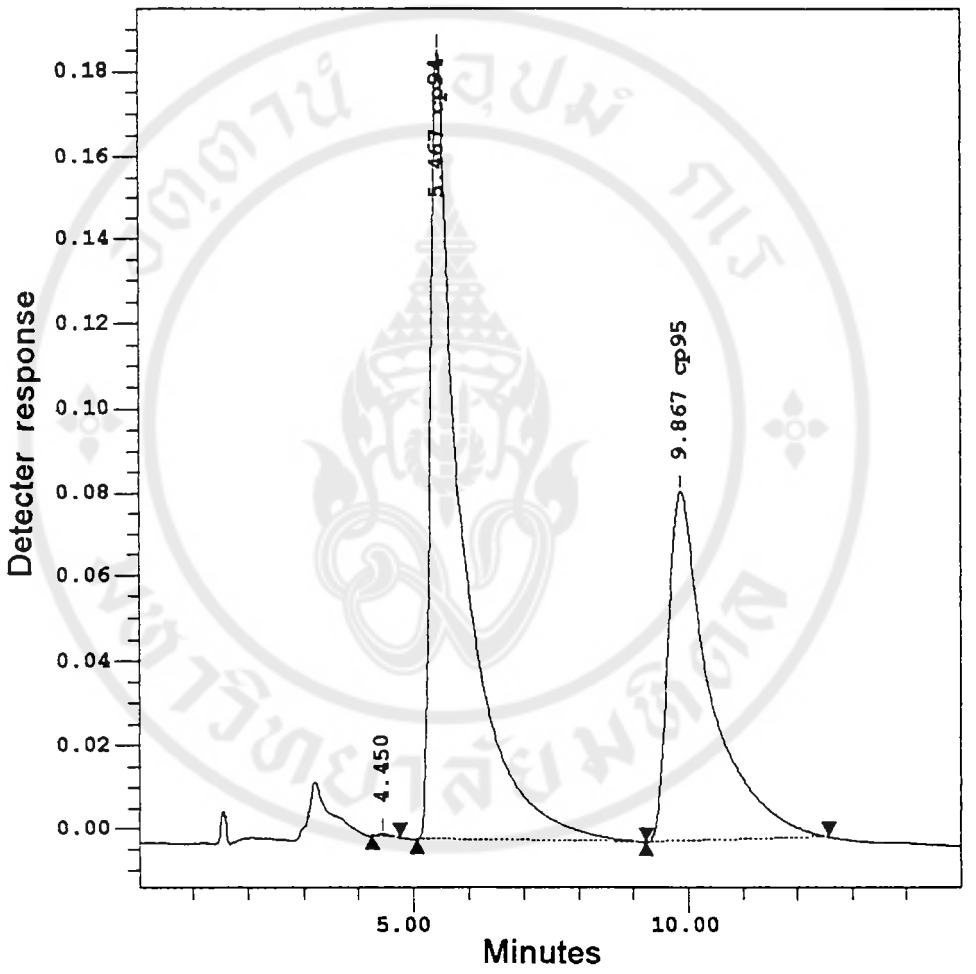


Figure 11. Chromatogram of the extract of rat plasma (containing CP94 and CP95).

Table 5. Precision and accuracy of the high performance liquid chromatography assay for CP94 in rat plasma.

Spike Conc^{n} (µg/100µl .(%Recovery)	Mean plasma)	SD	CV*	M%D**	n
1.0	0.94(94.42%)	0.13	13.7	6	4
40.0	38.7 (96.75%)	1.49	3.45	3.25	4

* CV% = S.D/MEAN x 100

**M%D = (MEAN-SPIKED CONC.) / SPIKD CONC. X 100

7.4 Preparation of calibration standards

The calibration curve should cover at low concentration for the latter phase post drug administration, and high concentration for the early phase. The calibration curves were constructed by adding various amounts of CP94, 1.0, 2.0, 3.0, 4.0, 5.0, 10.0, 20.0, 30.0 Figure 12. Calibration curve of CP94 in the rat plasma. µg, into extraction tubes containing 100 µl of rat plasma. The internal standard CP95 3 µg (30 µl of 0.1 µg/µl) was added to each tube before 1 ml absolute ethanol extraction which was decribed in sample preparation. After HPLC analysis, the peak height ratios were plotted against CP94 concentration.

The calibration curve showed good linearity over the concentration range of 1-30 µg/100µl plasma, and the regression coefficient was greater than 0.99 as shown in Figure 12.

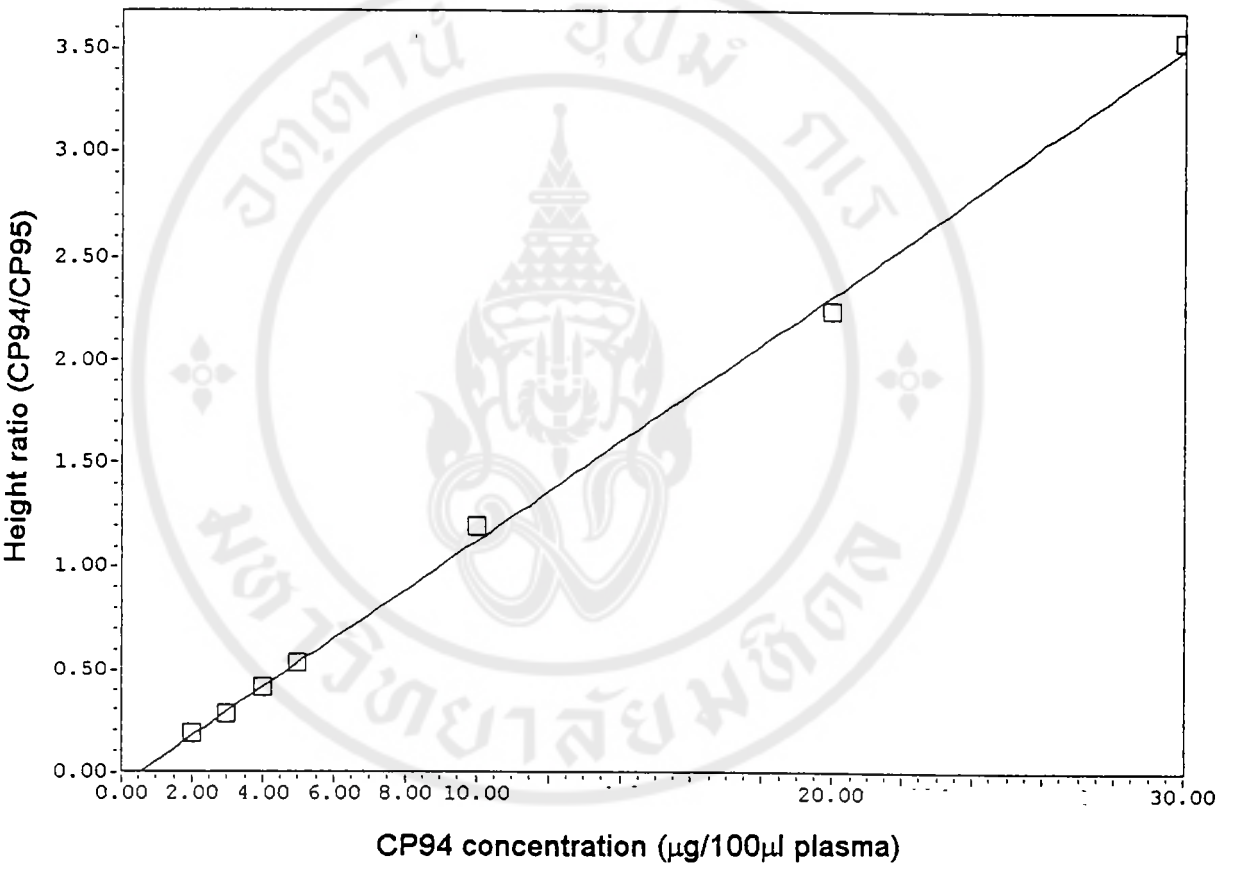


Figure 12. Calibration curve of CP94 in the rat plasma.

Experimental procedure

The studies were performed in both normal and iron-overloaded rats. The untreated group was determined of all the control values such as serum iron, serum TBARs, liver iron and urine iron. At the sacrificed time, the animals were killed by exsanguination from abdominal aorta. Six millileter of blood was collected then followed by perfusion with 20 ml of normal saline before removing the liver. Urine was collected individually kept in metabolic cage. The iron content in liver, serum and urine were determined by spectrophotometric method.

For the CP94 treated group, CP94 plasma levels were determined as well. After the animal were recovered from operation (overnight) a CP94 the dosage of 100 mg/kg with saline was administered intravenously via the catheter at time 0. About 0.25 ml blood samples were collected via catheter at various time intervals. The plasma CP94 concentrations were determined for 12 points within 8 hours at 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360 and 480 mins. The rats were divided into 4 groups as A, B, C and D. Each group blood samples were collected for only 3 times as designing in the Table 6.

Table 6. The sample collecting time of 0.25 ml blood for determining CP94 plasma concentration.

	Blood sampling (min.)		
group	1st	2nd	3rd
A	5	10	60
B	20	45	120
C	30	90	240
D	180	360	480

Each groups were sacrificed after the 3rd blood sampling. Therefore, the samples were collected at 60, 120, 240, 480 min. Six milliliter of blood was collected via abdominal aorta for determining serum iron and serum TBARs. The urine in the bladder were collected and pooled together with the urine collecting from the metabolic cage. The liver was removed and weighed after perfusion with 20 ml of normal saline.

The procedures are summarized in Figure 13.

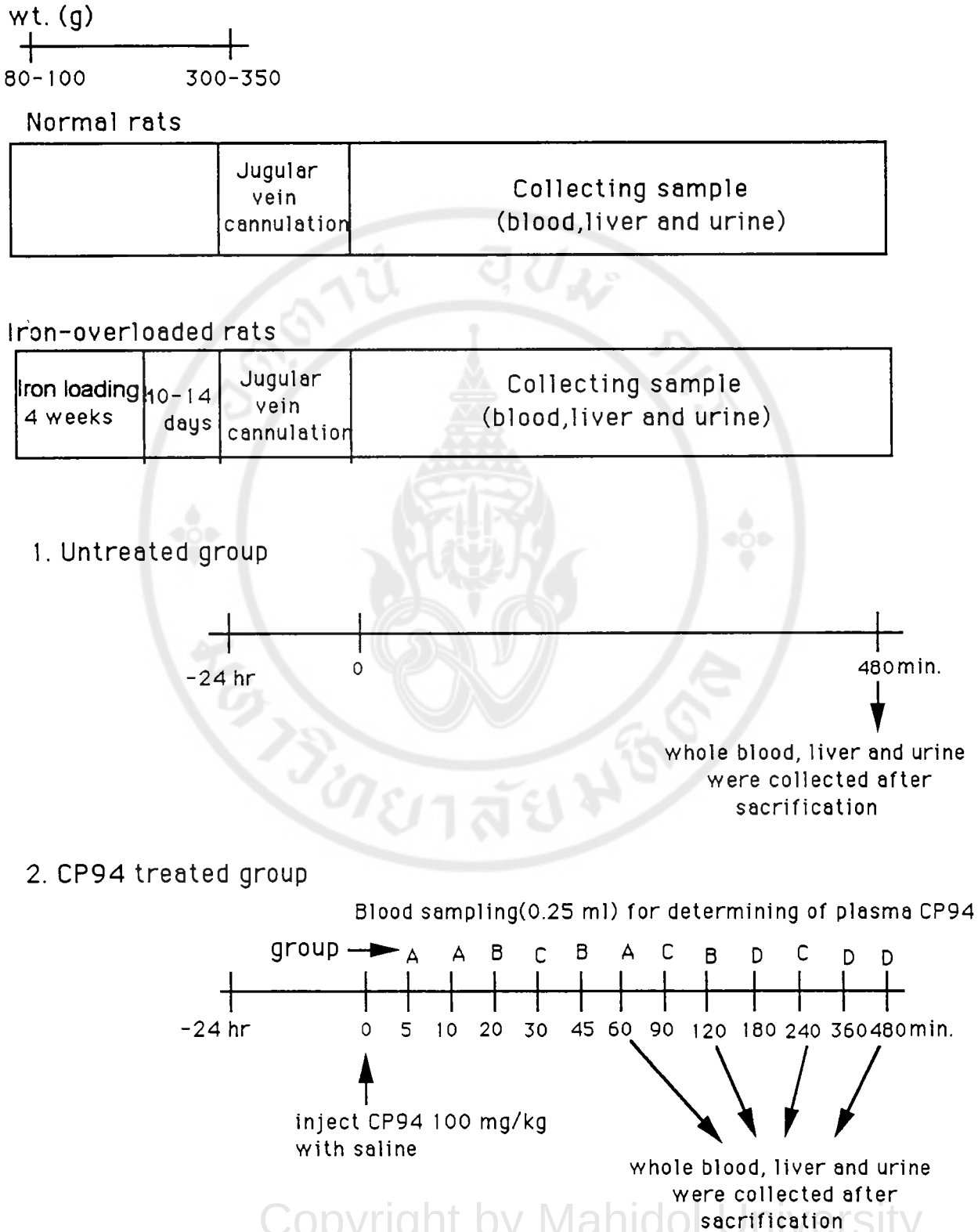


Figure 13. A schematic diagram of the experimental design.

The estimation of the pharmacokinetic parameters by MK model

The pharmacokinetic parameters were calculated by MK model, an extended least squares modeling program (87). The two compartment model of the plasma level-time curve is described as bi-exponential fashion (Figure 14)

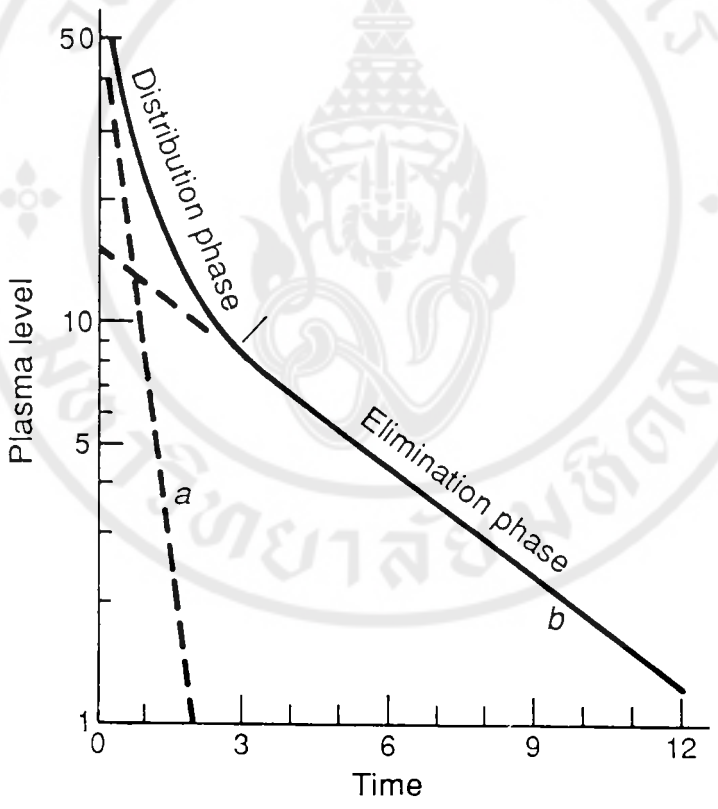


Figure 14. Plasma level-time curve for the two compartment open model (single *iv* dose). (Shargel L. (88))

MK model can estimate both linear and/or non-linear parameters of the model e.g. slope and intercept of a straight line were used least square linear regression. The slope of straight line was represented the rate of disappearance of drug. This model was obtained the two rate constants which are initial rate constant (a or α) for distribution phase and terminal rate constant (b or β) for elimination phase. The half-life($t_{1/2}$) of each phases were derived from the following relationship.

$$t_{1/2\alpha} = \frac{0.693}{\alpha}$$

The area under the blood time curve (AUC) and the area under the moment curve(AUMC) was calculated by trepazoidal rule. Mean residence time (MRT) decribes the average time for all drug molecules to reside in the body, it was determined by the following.

$$MRT = \frac{AUMC}{AUC}$$

The systemic clearance (CL) was calculated from the following :

$$CL = \frac{Dose}{AUC}$$

The volume of distribution at steady state (Vd_{SS}) was calculated from the following :

$$Vd_{SS} = \frac{CL}{MRT}$$

CHAPTER 3

RESULTS AND DISCUSSION

1. CP94 plasma concentration-time profile.

The plasma drug concentrations after a single bolus *iv* injection of 100 mg/kg CP94 were determined at various time-interval. At the first sampling time (5 min.), concentrations of CP94 in normal and iron overloaded rats were 294.5±60.8 and 216.5±55.9 μg/ml, respectively, the values was not significant difference. The rapid fall of plasma drug concentration in both normal and iron-overloaded rats were indifferent. Both showed biphasic drops in plasma levels (Figure 15). Kinetically, this could be described by a two compartment model. CP94 was rapidly distributed by showing the short initial half-life ($t_{1/2\alpha}$), and cleared from the plasma about 98% within 8 hr. The distribution and elimination of CP94 from the blood in iron-overloaded rats was not different from normal rats by showing the similar profiles (Figure 15), and the close value of the initial half-life ($t_{1/2\alpha}$) which were 0.156 hr for normal rats and 0.149 hr for iron-overloaded rats, and terminal half-life ($t_{1/2\beta}$) which were 4.46 hr and 4.94 hr, respectively. The others pharmacokinetic parameters did not show the significant difference between normal and iron-overloaded rats, were presented in Table 7.

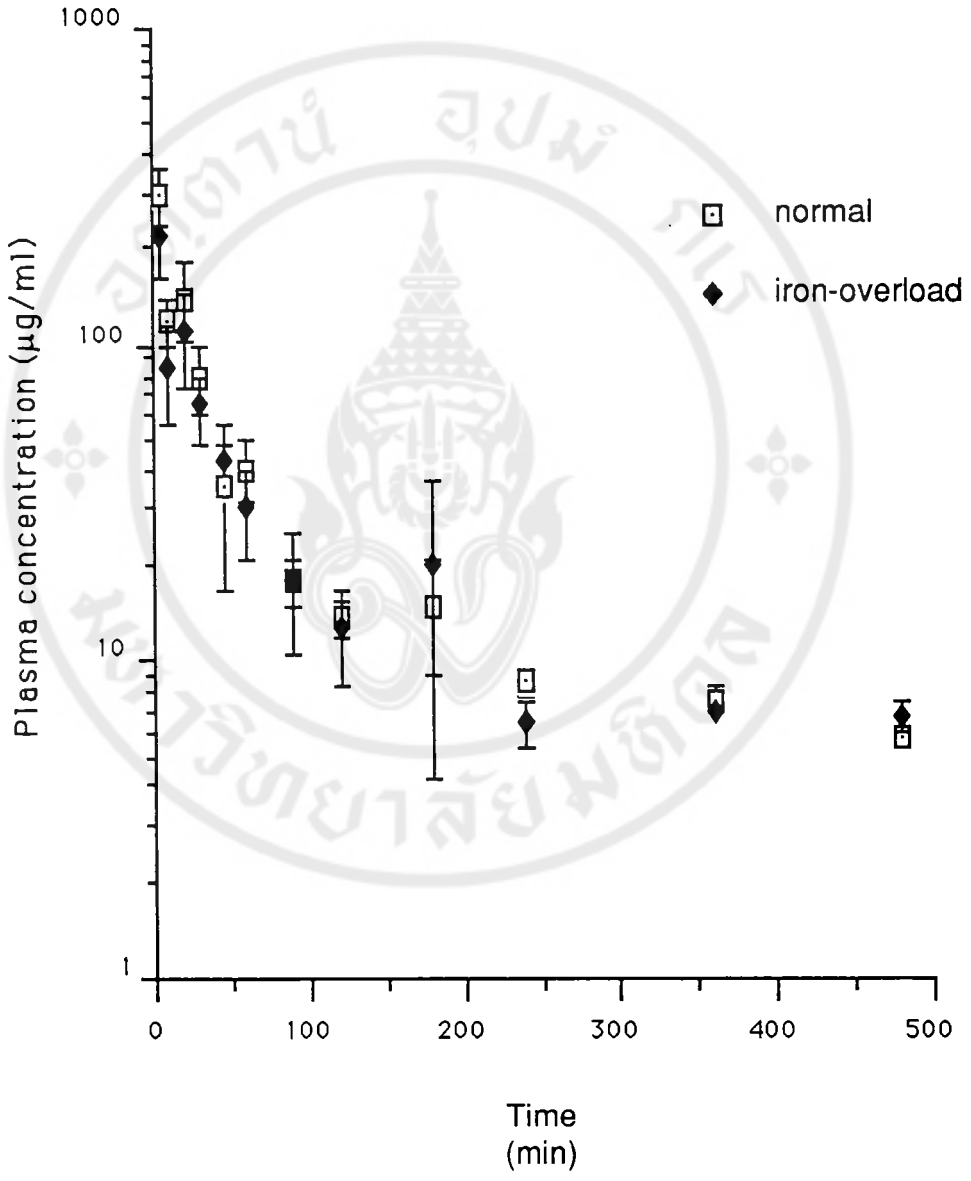


Figure 15. "Plasma time-concentration profile" of CP94 after a single (*iv*) dose of 100 mg/kg in normal and iron overloaded rats.

Table 7. Pharmacokinetic parameter of CP94 in normal and iron-overloaded rats after intravenously dose 100 mg/kg.

Pharmacokinetic Parameter	Normal rat (n =3-5)	Iron-overloadeded rat (n = 3-7)
Initial elimination rate constant (α) (hr^{-1})	4.44	4.62
Terminal elimination rate constant (β) (hr^{-1})	0.155	0.140
Initial half-life ($t_{1/2\alpha}$) (hr)	0.156	0.149
Termiinal half-life ($t_{1/2\beta}$) (hr)	4.46	4.94
Cl(liters/hr/kg)	0.523	0.538
Vd (liters/kg)	2.28	2.89
AUC (μg/ml/hr)	191.67	185.98
MRT (hr)	4.36	5.48

2. Effect of single(*iv*) dose, 100 mg/kg of CP94 on the distribution of iron in serum and liver, and on the serum TBARs level

The serum iron, liver iron and serum TBARs were determined at 1, 2, 4, 8 hr after CP94 administration. The effect of CP94, 100 mg/kg single(*iv*) dose on the serum TBARs and distribution of iron in serum and liver were shown in Table 8. Without CP94, the serum iron concentration in normal and iron-overloaded rats was similar, the mean serum iron concentration were 37.68±3.67 and 36.45±5.19 μM, respectively. At 1 hr after CP94 administration, serum iron was significantly increased ($p<0.02$) in iron-overloaded rats (67.85±15.03 μM) , and slightly increased in normal rats (43.71±1.63 μM). This result showed that the increasing of serum iron in iron-overloaded rats was significantly higher than normal rats ($p<0.07$). The median values of maximum serum iron concentration in normal rats were 43.67 μM, occurred at 1 hr after administrating CP94, and iron-overloaded rats was 80.35 μM, occurred for 2 hr after CP94 injection. It was approximately 2-times more in iron-overloaded than that of normal. The serum iron in normal rats, was decreased and closed to the control value (20 μM) at 4 hr and remained to 8 hr. In iron overloaded rats, it was decreased and closed to control at 8 hr, but the level still higher (46.05±11.27 μM) than control.

Measurement of the iron content in the liver found that after loading with iron (ferric iron in iron dextran form) 8 mg weekly for 4 weeks, the liver iron was approximately 8-times more in iron-overloaded (16.23±3.17 μmol/g w.wt) than normal (2.15±0.75 μmol/g w.wt) rats. The effect of CP94 on the liver iron was shown in Figure 16. After CP94 injection, the liver iron was not significantly changed in both normal and iron-overloaded rats, but tend to decrease in iron-overloaded rats.

Table 7. Effect of single(iv) dose of CP94 on the serum TBARs and distribution of serum and liver iron in normal and iron overloaded rats.

Time after CP94 injection (hrs)	Normal				Iron overloaded			
	n	Serum iron (μM)	Serum TBARs (μM)	Liver iron (μmol/g w.wt)	n	Serum iron (μM)	Serum TBARs (μM)	Liver iron (μmol/g w.wt)
0	6	37.68±3.67 [x] (38.01)*	0.23±0.03 [a] (0.23)	2.15±0.75 (1.97)	6	36.47±5.19 [y] (37.90)	0.39±0.15 [a] (0.41)	16.23±3.17 (16.37)
1	5	43.71±1.63 [b,x] (43.67)	0.16±0.06 (0.17)	2.59±0.76 (2.09)	5	67.85±15.03 [b,y] (71.52)	0.28±0.12 (0.22)	14.02±3.31 (13.48)
2	3	42.2±12.79 [c] (35.86)	0.24±0.02 (0.24)	2.03±0.26 (2.10)	6	72.25±21.97 [c] (80.35)	0.31±0.12 (0.27)	16.47±5.94 (14.19)
4	4	30.18±2.67 [d] (29.72)	0.20±0.05 (0.20)	1.91±0.36 (2.00)	7	57.30±11.91 [d] (54.88)	0.26±0.14 (0.18)	17.13±7.06 (18.12)
8	3	37.04±1.68 (36.78)	0.20±0.02 (0.20)	1.94±0.10 (1.95)	4	46.05±11.27 (44.35)	0.25±0.14 (0.19)	15.71±4.03 (14.90)

The value were expressed as Mean±SD

* Value in the parenthesis was the median value of each groups.

a,d....p < 0.01 x,y....p < 0.02 b,c....p < 0.07

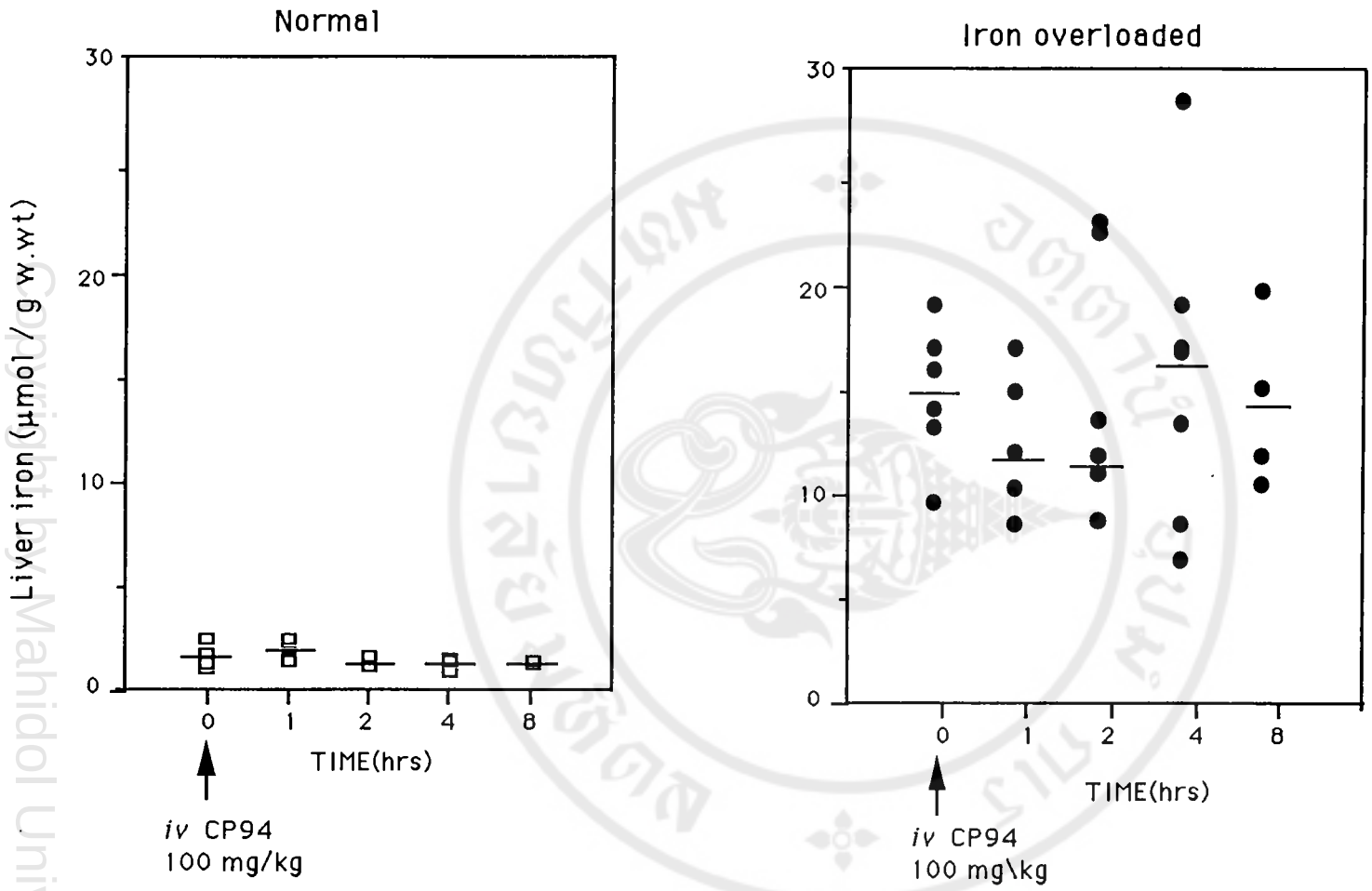


Figure 16. Effect of CP94 on liver iron in normal and iron-overloaded rats (CP94 was injected as single *iv* bolus dose). Each point was an individual and median of eanh groups was showed.

Studies on the levels of serum TBARs, an indicative marker of lipid peroxidation in both normal and iron-overloaded rats were shown in Table 8. It indicated that iron-overloaded rats with 8-times higher liver iron were under greater oxidative stress by showing the mean serum TBARs approximately 2-times higher significantly in iron-overloaded rats (0.39 ± 0.15 nmol/ml) than that of normal rats (0.23 ± 0.03 nmol/ml) The effect of CP94 on serum TBARs in normal and iron-overloaded rats were shown in Figure 17. In normal rats, there is no significant change of affect the on the serum TBARs level at any time after CP94 injection. However, iron-overloaded rats, CP94 seem to decrease the serum TBARs closely to the normal value in 1 hr (median = 0.22 nmol/ml, n=5) and until 8 hr, the serum TBARs level is still lowered than control (median = 0.19 nmol/ml, n=4).

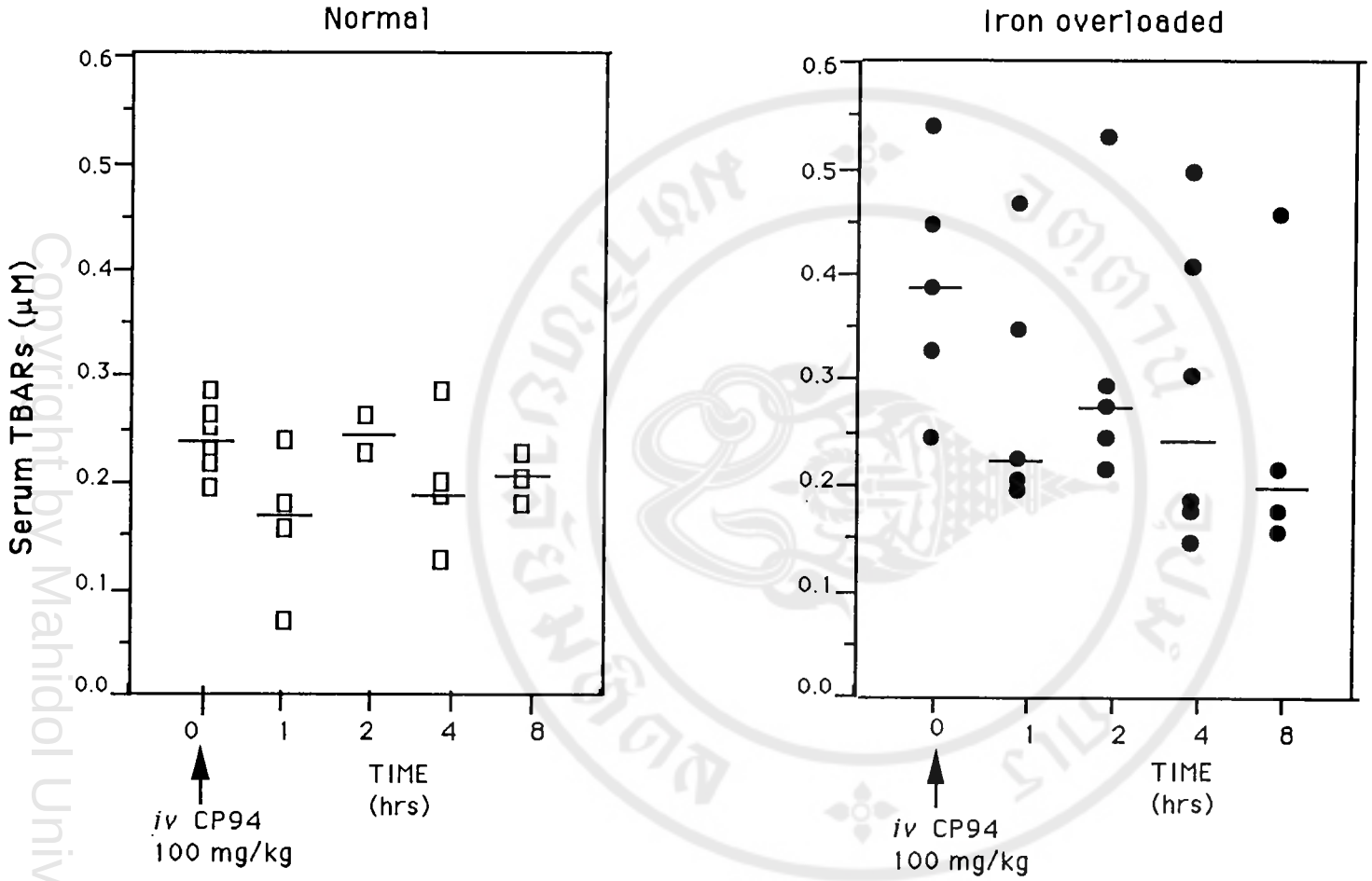


Figure 17. Effect of CP94 on serum TBARs in normal and iron-overloaded rats (CP94 was injected as single *iv* bolus dose). Each point was an individual and median of eanh groups was showed.

3. Effect of single(*iv*) dose of CP94 on the urinary iron excretion.

Measurement of cumulative urine iron excretion at various time interval, 0-2, 0-4, 0-8 after CP94 injection was shown in Figure 18. The results shown that CP94 has the most effect on urinary iron excretion at first 2 hr in both normal and iron-overloaded rats by showing the highest urine iron concentration 55.79±0.84 μM for normal and 193.82± 23.89 for iron-overloaded rats, approximately 4-times higher in iron-overloaded rats than that normal rats. For normal rats, it has less effect by presenting lower in cumulative urine iron excretion at time interval 0-4 hr. (28.83±3.17 μM) and 0-8 hr. (17.58±0.36 μM.) In iron-overloaded rats, CP94 still has effect at first 4 hr. by presenting high iron concentration at time interval 0-4 hr, 166.40±16.54 μM, it has less effect by showing the lower urine iron concentration at time interval 0-8 hr. (82.99±3.16 μM). Analysis of urinary iron concentration with respect to the serum iron concentration (Figure 19) revealed that the decrease of the serum iron concentration parallels to the decrease of urine iron concentration in both normal and iron-overloaded rats.

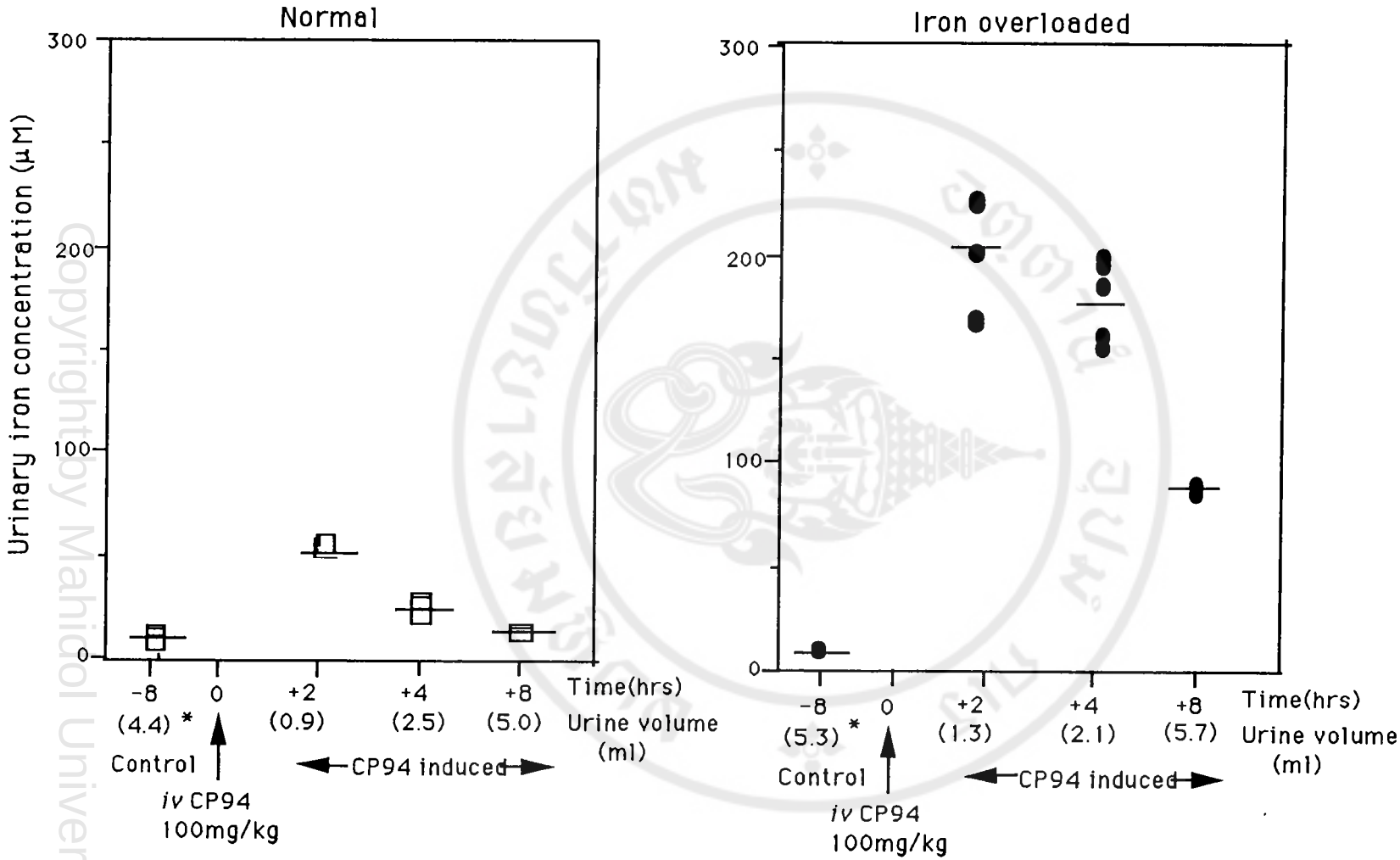


Figure 18. Effect of CP94 on the cumulative concentration of urinary iron in normal and iron-overloaded rats at various time intervals (CP94 was injected as single *iv* bolus dose). Each point was an individual and median of eanh groups was showed. ( )* represents the cumulative urine volume in ml.

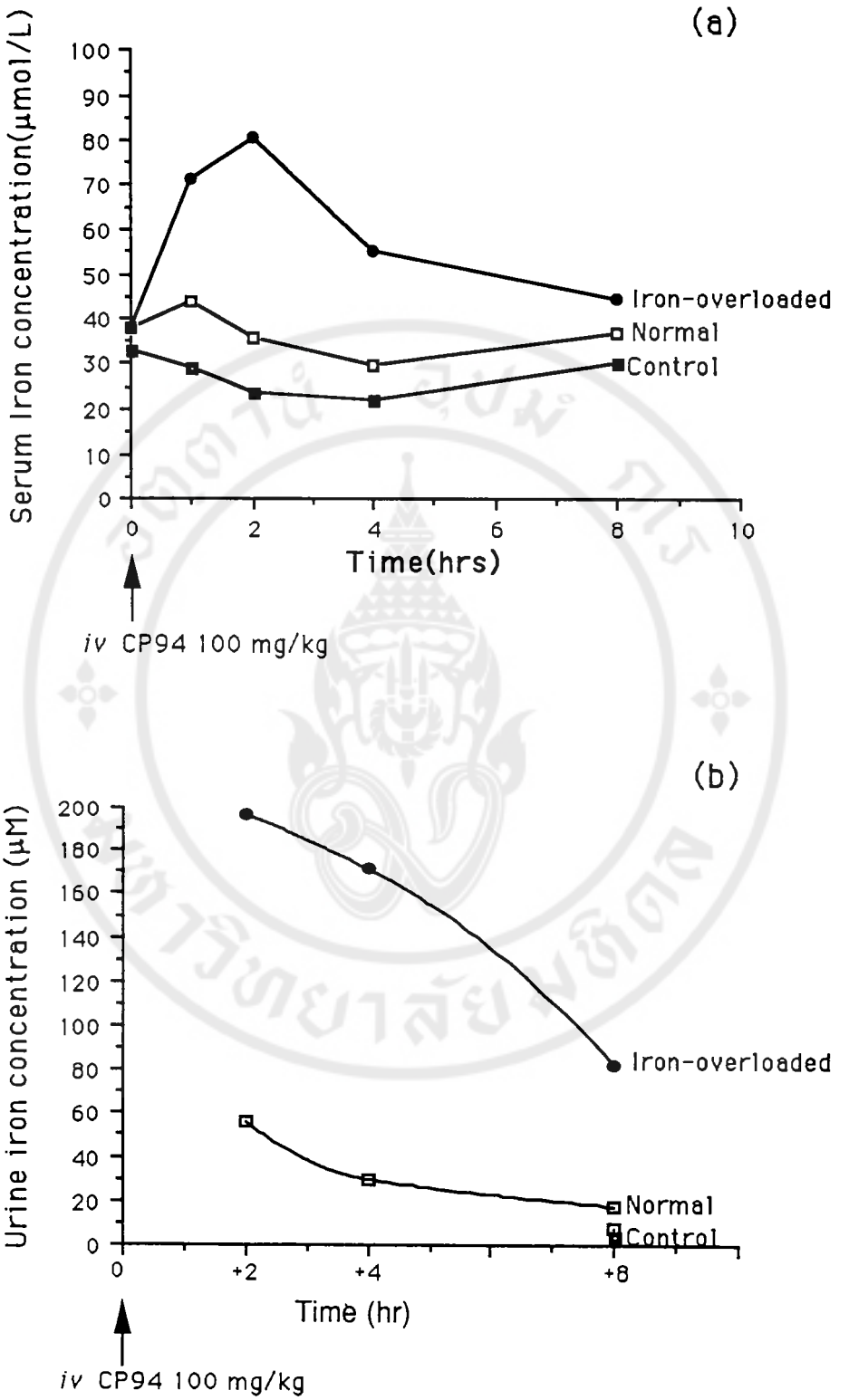


Figure 19. Effect of single (*iv*) dose of CP94 on serum iron concentration (a) and urine iron concentration (b) in normal and iron-overloaded rats. The control is the untreated rats. Each point is the median values.

DISCUSSION

Assessment of the initial iron-mobilizing ability of CP94 from the circulating, 'plasma' pool was made possible using a rat model having a catheter cannulated in the right jugular vein. The catheter was precisely placed at the right atrium and served as a convenient route for direct introduction of drug and sampling of blood in the concious animal without using anesthesia during the experiment. The model was ideal for studying the pharmacokinetic parameters, especially those values at the initial or distribution phase that could be accurately derived. From the plasma profiles of CP94, it was evident that CP94 was rapidly distributed and/or eliminated from the blood in a bi-exponential fashion that could be best described with a two-compartment open model in both normal and iron-overloaded rats. The time-plasma concentration profiles of CP94 in both normal and iron-overloaded rats were not different; i.e. no significant change in those pharmacokinetic parameters tested. This might suggest that the overloaded iron, at this stage had no influence on the disposition of CP94. Kontoghiorghes JG. et al. (1990) had conversely demonstrated that plasma concentration profile of CP20, a predecessor of CP94 in the iron overloaded patients was quite different from that of normal volunteers. They suggested that level of serum iron might be an important controlling factor on the plasma profile of hydroxypyridones (CPs). Thus it's yet to be defined if the varying degrees of iron overload has any significant impact on these pharmacokinetic parameters. CPs in the serum of iron-overloaded subject was found mostly in the form of CP-iron complex of which was cleared slower than the unbound form (79). The slower clearance of the iron complex might be due to its 3-time larger in size than those unbound form of drug.

Approximately, some 32 mg of iron in the form of iron-dextran was preloaded to an animal over a period of 4 weeks. Content of iron in the liver was commonly taken as an indicative marker of iron overload (89) and was found to have some eight-fold increased in these 4-wk iron-dextran treated rats. Though

serum iron was not elevated in these animals, but the increase of serum TBARs indicated that these 4-wk iron dextran treated rats had significant degree of oxidative damage. These data clearly revealed that iron overloaded rats used in this study was moderately overloaded by iron at which a significant degree of iron overloaded had already attained. Analysis of liver iron with respect to the serum TBARs showed that 8-times of liver iron higher in iron-overloaded rats contributed to the increased serum TBARs of 2-time higher than the normal control. This indicated that the iron induced damages or lipid peroxidation occurred in the hepatocyte and gave rise to an increased serum TBARs. Numerous evidences had suggested that the most important source of toxic iron in hepatocyte was neither ferritin nor haemosiderin, but a transient low molecular weight iron pool (90) while ferritin iron was relatively stable did not generate hydroxyl radical (91). For these reasons, new chelators should have access to the toxic pool.

The iron mobilizing ability of the CP94 was evident from the increase of serum iron as early as 1 hr after the CP94 injection in both normal and iron overloaded rats, this might be resulted from the high lipid solubility of CP94 in crossing the biological membrane to mobilize the intracellular iron. Certain parts of CP94 were metabolized to active metabolites which had a good intracellular iron binding capacity and mobilizing activities. Once intracellular iron had been chelated by the chelator, the chelated iron might be excreted in the bile and then reabsorbed by intestine back into the serum. This enterohepatic cycle had been commonly known. However the elevated levels of serum iron during the first 2 hr, was coincided with the continuing decline of the plasma CP94 concentration, this might explain the iron mobilizing activity of CP94 at the initial phase followed by the consequential binding of the CP94-metabolites. Singh S. et al.(1992) showed that most of the metabolites of CP94 did not loss the iron binding capacity (78). Therefore it was possible that CP94 might be chelating hepatic iron when certain fraction was mobilized. The increase in serum iron in iron overloaded rats was higher than these in normal rats as more of liver iron in the iron overload was removed. The mobilized iron could also be derived from the

enterohepatic circulation as the complex with hydroxylate metabolite. Rats were known to be extensive biliary iron excretor. Studies of Bergeron RJ, et al. (1992) (62) showed that bile duct-cannulated rat, treated with 75 mg/kg of CP94 was able to induce complete iron excretion within 24 hr in normal rats. Most of the iron was excreted by biliary excretion (69%) with a 31% in the urine. Once intracellular iron had been chelated by the chelator, the chelated iron may had first been excreted in the bile and then reabsorbed by intestine and appeared again in the serum.

Following a single dose of CP94, an initial elevation of serum iron in the iron-overloaded rats was rapid and steadily elevated during the first 2 hours, but the reverse was true for serum CP94. Urine sample collected at 2 hour showed the highest concentration of iron excreted, over the other periods during the 8-hr studies. Being a highly potent iron chelator the presence of CP94 in the central pool set forth a gradient flow of iron from the overloaded tissues back into the central 'plasma' pool which was likely responsible for the initial phase of action of the CP94 and thus the urinary excretion of these iron could evidently followed. This finding presents the first evidence of the initial effect of CP94 in mobilizing iron from the plasma pool and immediately excreted into the urine. One might argue if CP94 was playing the role in mobilizing iron since serum iron after the initial 2-hr was still high but the level of CP94 was almost cleared from the plasma. The evidence that Singh et al. (1992) (78) had demonstrated that CP94 was metabolized to an active iron chelating metabolite (Hydroxylated metabolite) which might support our finding that in the absence of plasma CP94, active metabolite of CP94 which was not detected in our HPLC system could be responsible for the still elevated level of serum iron after the first initial 2-hr.

Beside the iron mobilizing ability of CP94 an antioxidant property of the CP94 could also be demonstrated. With the initial 2-hr elevation of serum iron there was no increase in serum TBARs level at that particular time indicating that lipid peroxidation was inhibited. Moreover, serum TBARs trend to decrease at 1-hr after giving the CP94 suggesting that CP94 probably had an antioxidant

or free radical scavenging activities. Studies *in vitro* clearly substantiated this point as Morell et al (1992) showed that CP94 decreased both TBARs production in iron-loaded hepatocyte and eliminate the hydroxyl radical ($^{\bullet}OH$), generated from UV photolysis and peroxyl radical ($^{\bullet}ROO$), generated from autooxidation of linoleic acid micelles (47). Though our finding provided significant data ofthe initial iron mobilizing ability and its potential antioxidant property, thus further studies using a repeated doses would be complementary.

The single (iv) dose of CP94 showed a declining trend of liver iron in both iron-overloaded and normal rats. Being the largest iron storage pool an 100 mg/kg single dose of CP94 was not enough to cause a significant reduction of liver iron. Porter JB (63) had shown that giving a daily of 200 mg/kg ip. CP94 for 60 days was able to cause a significant reduction of liver iron in iron-overloaded mice (loaded by iron dextran).

The single dose of 100 mg/kg CP94 showed significant increase in urinary iron excretion at the first 2 hr and a 5-fold increase of urinary iron excretion in iron-overloaded rats than those of normal rats, indicating that CP94 could rapidly remove iron from the storage pools with consequent increase in serum iron and the excretion into the urine.

This study revealed that CP94 was able to mobilize the excessive iron in iron-overloaded rats from the storage pool into the serum and immediately excreted into the urine. Therefore the negative iron balance may occurred following a repeated dosage regimen or a long-term use may be anticipated.

CHAPTER 4

CONCLUSION

This study provided an animal model for the assessment of initial iron-mobilizing activity and antioxidative property of CP94 in iron-overloaded rats.

The results showed that

1. CP94 was able to mobilize iron from the storage pools into the serum and excrete in the urine at the greatest extent during the first 2-hour

2. the plasma profile and pharmacokivetic parameters of CP94 were not affectd by the overloaded iron and

3. the degree of oxidative damage in iron-overloaded rats could be inhibited by CP94.

BIBLIOGRAPHY

1. Engel MA. Cardiac involement in Cooley's anemia. Ann NY Acad Sci 1964; 119: 694-702.
2. Giardina PJ, Grady RW, Ehlers KH, et al. Current therapy of Cooley's anemia: a decade of experience with subcutaneous desferrioxamine. Ann NY Acad Sci 1990; 162:275-284.
3. Model CB. Long-term desferrioxamine in thalassemia.Ann NY Acad Sci 1974; 232:201-210.
4. Barry M. long-term chelation therapy in thalassemia major effect on liver iron concentration, liver histology and clinical process. Br Med J 1974, 2:16-20.
5. Hider RC, Kontoghiorghes GJ, Silver J. UK pateint NO GB 21187176 1982.
6. Kontoghiorghes GJ. Iron mobilization from ferritin using α-ketohydroxyheterometic chelators. Biochem J 1986; 233: 299-302.
7. Kontoghiorghes GJ. The study of iron mobilization from transferrin using α-ketohydroxyheterometic chelators. Biochim. Biophys Acta. 1986; 869:141-146.
8. Kontoghiorghes GJ, Evans RW. Site specificity of iron removal from transferrin by α-ketohydroxypyridine chelators. FEBS; 189(1): 141-144.
9. Kontoghiorghes GJ, Chamber S, Hoffbrand AV. Comparative study of iron mobilization from haemosiderin, ferritin and iron(III) precipitates by chelators. Biochem J 1987; 241: 87-92.
10. Porter JB, Gyparaki M, Burke LC, etal. Iron mobilization from hepatocyte monolayer culture by chelators. The importance of membrane permeability and the iron binding constant. Blood 1988; 7(5): 1497-1503.
11. Kontoghiorghes GJ. Oral active alpha ketohydroxypyridine iron chelators: Study in mice. Molecular Pharm 1986; 30: 670-673.
12. Kontoghiorghes GJ, Hoffbrand AV. Orally active alpha ketohydroxypyridine iron chelators intended for clinical use: *in vivo* studies in rabbits. Br J Haematol 1986; 62: 607-613.

13. Kontoghiorghes GJ, Bartlett AN, Hoffbrand AV. Long term trail with the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one(L1). Br J Haematol 1990; 76: 295-300.
14. Hider RC, Hall AD. Iron chelating agents in medicine, the application of bidentate hydroxypyridin-4-ones. Persp Bioinor Chem 1991; 1.
15. Hider RC, Singh S, Porter JB, Huehns ER. The development of hydroxypyridin-4-ones as orally active iron chelator. Ann NY Acad Sci 1990; 612: 327-338.
16. Porter JB, Morgan J, Hoyes KP, Burke LC, Huehns ER, Hider RC. Relative oral efficacy and acute toxicity of hydroxypyridin-4-ones iron chelators in mice. BLood 1990; 76(11): 2383-2396.
17. Kontoghiorghes GJ, Nasseri-Sina P, Goddard JG, Barr JM, Nortey P, Sheppard LN. Safety of oral iron chelator L1(letter). Lancet 1989 ; 2 : 4578.
18. Olivieri NF, Koren G, Hermann C, et al. Comparison of oral iron chelator L1 and desferrioxamine in iron-loaded patients. Lancet 1990; 336: 1275.
19. Finch CA, Huebers HA. Perspective in iron metabolis. N Engl J Med 1982; 306: 1520-1528.
20. Weintraub LR, Connad ME, CROSby WH. Regulation of the intestinal absorption of iron by the rate of erythropoisis. Br J Haematol 1965; !!:432-438.
21. Hider RC, Singh S, Porter JB. Iron chelating agents with clinical potential. Proc Royal Soci Edenb 1992 ; 99B(1/2) : 137-168.
22. Roberts RC, Makey DG, Seal US. Human transferrin: molegular weight and sedimentation properties. J Bio Chem 1966; 241:4907-4913.
23. Asa R, Malmstrom GD, Saltman P, Vanngard T. Thespecific binding of iron(III) and copper(II) to transferrin and conalbumin. Biochim Biophys Acta 1963; 75:203.
24. Harrison PM. Ferritin and Iron-storage Molecule. Semin in Hematol weight and sedimentation porperties. J Biol Chem 1966; 241:4907-4913.
25. Aise P, Listowsky I. Iron transport and storage proteins. Ann Rev Biochem 1980;49:357-359.

26. Chu L LH, Finebug RA. On the mechanism of iron-induced synthesis of apoferritin in Hela cells, J Biol Chem 1969;244:3847-3854.
27. Weatherall DJ, Clegg JB. The thalassemia syndromes. 3rd ed. Oxford: Black well Scientific Publication 1981: 133-319.
28. Bank A, Rifkind RA, Marks PA. The thalassemia syndromes. In: The red blood cell vol.2. Edit by Surgenor D Mac N. Academic press New York, 1975: 909-931.
29. Schafer AI, Cheron RG, Dluhy R, et al. Clinical consequences of accquired transfusional iron overload in adults. N Engl J Med 1981; 304:319-324.
30. Saltman P. Oxidative stress : a radical view. Semin in Hematol 1989; 27(4):249-256.
31. Halliwell B, Gutteridage JMc. Oxigen toxicity, oxygen radicals, transition metal and disease. Biochem J 1984; 219: 1-14.
32. Hershko C. Mechanism of iron toxicity and its possible role in red cell membrane damage. Semin In Hematol 1989;26(4):277-285.
33. Phumala N. Effect of 3-hydroxypyridn-4-ones (CP20 and CP94) on the disposition of ^{59}Fe-transferrin in normal and iron-overloaded rats. MS thesis of Pharmacology, Mahidol university 1992.
34. Hershko C, Graham G, Bates GW, Rachmilewitz EA. Nonspecific serum iron in thalassemia : an abnormal serum fraction of potential toxicity. Br J Haematol 1978; 40 : 225-263.
35. Anuwatanakulchai M, Pootrakul P, Thuvasethakul P, Wasi P. Non-transferrin plasma iron in β-thalassemia/HbE and HbH disease. Scand J Haematol 1984; 32 : 153-158.
36. Porter J. Oral iron chelators : Prospects for future development. Eur J Haematol 1989; 43: 271-285.
37. Allain P, Mauras Y. Deferoxamine. Drug of today 1992,28(suppl. A) : 89-94.
38. Moeschlin S, Schnider U. Treatment of primary and secondary hemocheomatosis and acute iron poisoning with a new potent iron eliminating agent(desferrioxamine B).New Engl J Med 1963; 269:57-66.

39. Keberle H. The biochemistry of desferrioxamine and its relation to iron metabolism. Ann NY Acad Sci 1964; 119: 758-768.

40. Hersko C, Rachmilewitz EA. Mechanism of desferrioxamine induced iron excretion in thalassemia. Br J Haematol 1979; 42: 125-132.

41. Lee P, Mohammed N, Marchall L, et al . Intravenous infusion pharmacokinetic of desferrioxamine in thalassemic patients. Drug Metab. Dispos.1993; 21(4): 640-644.

42. Pippard MJ, Callender ST, Finch CA. Ferrioxamine excretion in iron loaded man. Blood 1982; 60: 288-294.

43. Giardiena PJ, Grady Rw,Ehlers KH, et al. Current therapy of Cooleys anemia: A decade of experience with subcutaneous desferrioxamine. In: Sixth Cooley's Anemia Symposium. Edit by Bank A. Ann NY Acad Sci 1990; 612: 286-292.

44. Martin T, Nathan F, Nathan DG,. Treatment of Cooley's anemia. Blood 1990;76(3): 435-444.

45. Modell CB, Beck J. Long term desferrioxamine therapy in thalassemia. Ann NY Acad Sci 1974; 232: 201.

46. Smith RS. Chelating agents in the diagnosis and treatment of ironoverload in thalassemia. Ann NY Acad Sci 1964; 119: 776-778.

47. Morel I, Cillard J., Lescoat G,. et al. Antioxidant and free radical scavenging activities of the iron chelators pyoverdin and hydroxypyrid-4-ones in iron-loaded hepatocytes cultures :comparison of their mechanism of protection with that of desferrioxamine. Free Rad. Biol. Med. 1992; 13: 499-508.

48. Singh S, Hider RC, Porter JB. Separation and identification of desferrioxamine and its iron chelating metabolites by high performance liquid chromatography and fast atom bombardment mass spectrometry choice of complexing agent and application to biological fluids. Anal. Biochem 1990; 187: 212-219.

49.BenturY,Mc Guigan M, Koren G. Desferoxamine (desferrioxamine): new toxicities for an old drug. Drug Safety 1991; 6(1):37-46.

50. Scarrow RC, Riley PE, Abu-dari K, White DL, Raymond KN. Ferric iron sequestering agents.13. Synthesis, structure and hermodynamic of complexation of Cobalt(III) annd iron(III) complexs of several chelating hydroxypyridinones. Inorg Chem 1985;24: 954-967.

51. Hider RC, Hall AD. Clinically useful chelators of tripositive elements. Prog Med Chem 1991; 28: 2079-2081.

52. Clevette DJ, Nelson WO, Nordin A, Oevig C, Sjoberg S. Complexation of aluminium with N-substituted-3-hydroxypyridin-4-ones. Inorg Chem 1989; 28: 2079-2981.

53. Martell AE, Motekaitis RJ, Clarke ET, Sun Y. Comparison of the drugs use for the treatment of iron overload with drugs that have potential for the same purpose. Drug of Today 1992; 28(Suppl. A): 11-18.

54. Dobbin PS, Hider RC. Iron chelating therapy. Chem in Britain 1990; 26(12): 565-568.

55. Brittenham GM. Development of iron-chelating agents for clinical use. Blood 1992; 80(3): 569-574.

56. Kontoghiorghes GJ. The design of orally active iron chelator for the treatment of thalassemia, PhD thesis, University of Essex. 1982.

57. Porter JB, Gyparahi M, Huehns ER, HIder RC. The relationship between lipophilicity of hydroxypyridine-4-one iron chelations and cellular iron mobilization, using an hepatocyte culture model. Biochem Soc Trans 1986; 14: 1180.

58. Brock JH, Liceaga J, Arthus HML, Kontoghiorghes GJ. Effect of novel 1-alkyl-3-hydroxy-2-methylpyrid-4-one chelators on uptake and release of iron from macrophages. Am J Hematol 1990; 34: 21-25.

59. Hershko C, Link G, Pinson A, Peter HH, Dobbin P, Hider RC. Iron metabolization from myocardial cells by 3-hydroxypyridin-4one chelators:studies in rat heart cells in culture. Blood !991; 77(9): 2049-2053.

60. Porter JB, Hider RC, Huehns ER. Update on the hydroxypyridinone oral iron-chelating agents. Semin in Hematol 1990; 27(2): 95-100.

61. Zevin S, Link G, Grady RW, Hider RC, Peter HH, Hershko C. Origin and fate of iron mobilized by the 3-hydroxypyridn-4-one oral chelators : studies in hypertransfused rats by selective radioiron probes of retiiculoendothelial and hepatocellular iron stores. Blood 1992; 79(1) : 248-253.

62. Bergeron RJ, Streiff RR, Wiegand J, et al . A comparison of the iron clearing proporties of 1,2-dimrthul-3-hydroxypyridin-4-one, 1,2-diethyl-3-hydroxypyridin-one and deferoxamine.Blood 1992; 79(7): 1882-1890.

63. Porter JB, Hoyes KP, Abeysinghe RD, Brooks PN, Huehns ER,Hider RC. Comparison of the subacute toxicity and efficacy of 3-hydroxypyridin-4-one iron chelators in overloaded and non overloaded mice. BLood 1991;78(10): 2727-2734.

64. Biesemeier JA, Laveglia J. 14-day oral toxicity study in dogs with 1,2-dimethyl-3-hydroxypyrid-4-one (DHMD, L1). Food and Drug Reserch Laboratories, Waverly, NY, 1991, p1, Contract No. NO1-DK-4-2255,NIDDK, NIH.

65. Kontoghiorghes GJ, Aldouri MA, Sheppard LN, Hoffbrand AV. 1,2-dimethyl-3-hydroxypyrid-4-one, an orally active chelator for treatment of iron overload. Lancet 1987; i: 1294-1295.

66. Olivieri NF, Koren G, Freedman MH, St louis P, Panicucci R Mc clelland RA. Effective iron chelation with oral agent 1,2-dimethyl-3-hydroxypyrid-4-one (L1)(Abs). Clinical Res 1989; 37(2):385(a).

67. Olivieri NF, Koren G, St. louis P, Freedman MH, Mc Clelland RA, Templeton DM. Studies of the oral chelator 1,2-dimethyl-3-hydroxypyrid-3-one in thalassemia pateints. Semin in Hematol 1990;27(2): 101-104.

68. Tundury P, Kontoghiorghes GJ, Ridrofl Luthy A, et al. L1(1,2-dimethyl-3-hydroxypyrid-3-ones) for oral iron chelation in pateints with beta thalassemia major. Br J Haematol 1990; 76: 550-553.

69. Schnebli HP. Notes from the L1 work shop Aug 30,1991 Basal Oct 23, 1991.

70. Kontoghiorghes GJ, Sina NP, Goddard JG, Barr JM, Norty P, Sheppard LN. Safety of oral iron chelator L1. Lancet 1989; ii: 458.

71. Hoffbrand AV, Bartlett AN, Veys PA, J O' Coner NT, Kontoghiorghes GJ. Agranulocytosis and trombocytopenia in patient with Blackfan-Diamond anaemia during oral chelator trial. Lancet 1989; ii:457.

72. Olivieri NF, Koren G, Matsui D, et al. Reduvtuin of tissue iron stores and normalization of serum ferritin during treatment with oral iron chelator L1 in thalassemia intermedia. Blood 1992; 79(10): 2741-2748.

73. Al-Refaie FN, Wonke B, Hoffbrand AV,Wickens DG,Nortey P,Kontoghiorghes GJ. Efficacy and possible adverse effects of the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one(L1) in thalassemia major. Blood 1992; 80(3): 593-599.

74. Kaltwasser JP, Gottschalk R, Schalk K{, Hartl W. Non-invasive quantification of liver-overload by magnetic resonance imaging . Brit J Haematol 1990;74: 360-363.

75. Bartlett AN, Hoffbrand AV, Kontoghiorghes GJ. Long term trial with the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one(L1) II clinical observatiions. Br J haematol 1990; 76:301-304.

76. Epemolu OR, Singh S, Hider RC, Damani LA. Pharmacokinetics of 1,2-diethyl-3-hydroxypyrid-4-one (CP94) in rats. Drug Metab. Dispos. 1992; 20(5): 736-741.

77. Fredenburg AM, Wedlund PJ, Skinner TL, et al. Pharmacokinetics of representative 3-hydroxypyrid-4-ones in rabbits : CP20 and CP94. Drug Metab. Dispos. 1993;21(2): 255-258.

78. Singh S, Epemolu RO, Dobbin PS, et al. Urinary metabolic profiles in human and rat of 1,2-dimethyl and 1,2-diethyl substituted 3-hydroxypyrid-4-ones. Drug Metab. Dispos. 1992;20(2): 256-261.

79. Kontoghiorghes GJ, Goddard JG, Bartlett AN, Shappard L. Pharmacokinetic studies in human with the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one. Clin Pharm Ther 1990; 48(3): 225-261.

80. Klein J, Damani LA, Chung D, Epemolu O, Olivieri N, Koren G. A high performance liqiud chromatographic method for measurement of the iron chelator 1,2-dimethyl-3-hydroxypyridin-4-one in human plasma. Ther Drug Monit 1991; 13: 51-54.

81. Goddard JG, Kontoghiorghes GJ. Development of an HPLC method for measuring orally administered 1-substitute 2- alkyl-3-hydroxypyrid-4-one iron chelators in biological fluids. Clin. Chem 1990; 36(1):5-8.

82. Matsui D, Klein J, Hermann C, et al. Relationship between the pharmacokinetic and iron excretion pharmacodynamics of the new oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one in patients with thalassemia. Clin. Pharmacol. Ther. 1991; 50:294-298.

83. Simpson RJ, Cooper CE, Raja KB, et al. Non-transferrin-bound iron species in the serum of hypotransferric mice. Biochim. Biophys. Acta 1992; 1156: 19-26.

84. Cook JD. Iron. 1st ed Cherchill Livingstone Inc. 1980: 90-115.

85. Asakawa T, Matsushita S. Coloring conditions of thiobarbituric acid test for detecting lipid hydroperoxides. Lipids 1980; 15: 137-140.

86. Epemolu RO, Singh S, Hider RC, Damani LA. High performance liquid chromatographic determination of 1,2-diethyl-3-hydroxypyridin-4-one(CP94) and its metabolite in rat blood. J Chromatogr 1992; 573(1):178-182.

87. Holford N. MK MODEL, An extented least squares modelling program. Elsevier Science Plublisher BV Netherland 1986.

88. Shargel L, Yu Andrew B.C. Applied biopharmaceutics and pharmacokinetics. 3rd edition. Prentice-Hall International Editions 1992 :61-76.

89. Van Wyk CP, Linder-Horowitz M, M&Munro N. Effect of iron loading on non-heme iron compounds in different liver cell populations. J Biol Chem 1971; 246: 1025-1031.

90. White GP, Jacobs A, Grady RW, Cerami A. The use of change cells cultured in vitro to evaluate potential iron chelating drugs. Brit J Haem ;33: 487-494.

91. Deighton N, Hider RC, Purification and characterization of a low molecular weight iron complex isolated rat liver. Bioc Soc Trans 1988. (in press).

APPENDIX-1

CP94 plasma concentration in µg/ml of normal rats
after single(*iv*) dose 100 mg/kg.

	Time (min)											
Rat No.	5	10	20	30	45	60	90	120	180	240	360	480
7	249.1	100.9				25.9						
8	327.4	148.8				42.5						
9	237.5	118.0				50.6						
10	383.9	100.2				39.3						
11	274.8	120.8				43.2						
Mean	294.5	117.8				40.3						
SD	60.8	19.8				9.0						
12			140.0		13.9			11.4				
13			178.6		42.6			15.4				
14			101.1		50.8			14.1				
Mean			140.0		35.8			13.6				
SD			38.8		19.4			2.0				
15				103.3			ND			ND		
16				57.2			14.60			7.65		
17				84.3			18.40			8.92		
18				71.0			20.10			9.04		
Mean				79.0			17.7			8.54		
SD				19.6			2.8			0.77		
19									14.1		8.40	5.53
20									9.20		7.66	6.02
21									21.1		6.78	5.61
Mean									14.80		7.61	5.72
SD									5.98		0.81	0.26

APPENDIX-2

CP94 plasma concentration in ug/ml of iron-overloaded rats after single(iv) dose of 100 mg/kg

	Time (min)											
Rat No.	5	10	20	30	45	60	90	120	180	240	360	480
28	225.0	76.8				25.0						
29	294.3	100.4				27.4						
30	142.9	108.5				30.6						
31	189.4	104.3				46.9						
32	231.1	36.8				23.5						
Mean	216.5	85.4				30.7						
SD	55.9	29.8				9.5						
33			96.5		39.2							
34			169.3		47.7			18.0				
35			77.7		47.0			12.7				
36			113.9		43.5			9.1				
37			86.3		36.4			7.7				
38			ND		45.5			15.0				
Mean			108.7		43.2			12.5				
SD			36.4		4.5			4.2				
39				64.5			12.4			ND		
40				93.6			27.7			5.68		
41				50.7			13.5			5.73		
42				54.5			11.0			8.05		
43				ND			13.6			6.92		
44				67.1			27.9			7.00		
45				55.0			20.0			5.26		
Mean				64.2			18.0			6.44		
SD				15.7			7.3			1.06		
46									6.86		7.00	7.22
47									14.1		7.08	ND
48									37.7		6.93	6.13
49									22.7		ND	ND
Mean									20.34		7.00	6.68
SD									16.13		0.08	0.77

APPENDIX-3

The control values in normal rats.

Rat No.	Body weight (g)	Hct. (%)	liver weight (g)		Serum TBARs (nmol/ml)	Serum Iron (umol/L)	Liver Iron (umol/g wet)
			wet	dry			
1	340	38	14.87	3.56	0.19	42.84	1.47
2	365	41	15.22	3.85	0.22	39.92	1.50
3	335	40	14.64	3.48	0.24	37.78	2.07
4	345	41	14.03	3.25	0.27	34.84	2.48
5	320	36	13.82	3.14	0.25	38.24	3.48
6	325	40	13.52	3.28	0.21	32.46	1.87
Mean	338	39	14.35	3.43	0.23	37.68	2.15
SD	16.0	1.9	0.66	0.26	0.03	3.67	0.75
Median	338	40	14.34	3.38	0.23	38.01	1.97

APPENDIX-4 (a)

Effect of single(iv) dose ofCP94on
serum TBARS, serum iron and liver iron in normal rats.

Rat No.	Body weight (g)	Hct. (%)	liver weight (g)		Serum TBARs (nmol/ml)	Serum Iron (umol/L)	Liver Iron (umol/g wet)
			wet	dry			
1hr after treated							
7	365	36	13.92	3.30	0.089	41.67	3.30
8	365	33	13.87	3.24	ND	45.71	3.53
9	340	43	12.32	3.17	0.18	42.65	2.09
10	345	40	12.27	2.95	0.16	43.67	2.08
11	335	39	12.84	3.06	0.23	44.87	1.96
Mean	350	38	13.04	3.14	0.16	43.71	2.59
SD	14.1	3.8	0.81	0.14	0.06	1.63	0.76
Median	345	39	12.84	3.17	0.17	43.67	2.09
2 hr after treated							
12	340	33	12.41	3.03	ND	33.82	1.75
13	360	42	12.51	3.08	0.22	56.92	2.10
14	340	41	11.48	2.95	0.25	35.86	2.25
Mean	347	38	12.13	3.02	0.24	42.20	2.03
SD	11.6	4.9	0.57	0.07	0.02	12.79	0.26
Median	340	41	12.41	3.03	0.24	35.86	2.1

APPENDIX-4 (b)

Effect of single(iv) dose ofCP94 on
serum TBARS, serum iron and liver iron in normal rats.

Rat No.	Body weight (g)	Hct. (%)	liver weight (g)		Serum TBARs (nmol/ml)	Serum Iron (umol/L)	Liver Iron (umol/g wet)
			wet	dry			
4 hr after treated							
15	335	41	12.02	3.07	0.19	27.45	1.41
16	360	40	13.37	3.37	0.14	33.82	1.95
17	340	39	12.64	3.14	0.27	29.33	2.24
18	345	40	13.04	2.96	0.20	30.11	2.05
Mean	345	40	12.77	3.14	0.20	30.18	1.91
SD	10.8	0.8	0.58	0.17	0.05	2.67	0.36
Median	343	40	12.84	3.11	0.20	29.72	2.00
8 hr after treated							
19	340	38	12.24	3.25	0.18	35.51	1.84
20	315	37	11.89	3.19	0.22	38.84	1.95
21	330	40	13.04	3.30	0.20	36.78	2.04
Mean	328	38	12.39	3.25	0.20	37.04	1.94
SD	12.6	1.5	0.59	0.06	0.02	1.68	0.10
Median	330	38	12.24	3.25	0.20	36.78	1.95

APPENDIX-5

The control values in iron-overloaded rats.

Rat No.	Body weight (g)	Hct. (%)	Liver weight (g)		Serum TBARs (nmol/ml)	Serum Iron (umol/L)	Liver Iron (umol/g wet)
			wet	dry			
22	320	43	13.18	3.51	0.53	ND	15.46
23	335	44	13.31	3.65	0.44	35.18	11.24
24	320	43	12.78	3.27	0.44	38.58	17.28
25	345	40	12.81	3.60	0.38	42.31	20.30
26	325	35	12.01	3.08	0.32	37.9	14.68
27	345	43	13.08	3.56	0.24	28.39	18.41
Mean	332	41	12.86	3.45	0.39	36.47	16.23
SD	11.7	3.4	0.47	0.22	0.10	5.19	3.17
Median	330	43	12.95	3.54	0.41	37.9	16.37

APPENDIX-6 (a)

Effect of single(*iv*) dose of CP94 on
serum TBARS, serum iron and liver iron in iron-overloaded rats.

Rat No.	Body weight (g)	Hct. (%)	liver weight (g)		Serum TBARs (nmol/ml)	Serum Iron (umol/L)	Liver Iron (umol/g wet)
			wet	dry			
1hr after treated							
28	355	40	13.62	3.12	0.34	82.05	10.18
29	330	39	12.89	3.03	0.46	69.82	18.28
30	315	43	13.07	3.55	0.2	71.52	16.40
31	295	40	12.5	3.32	0.19	73.56	13.48
32	345	40	12.48	3.27	0.22	42.31	11.78
Mean	328	40	12.91	3.26	0.28	67.85	14.02
SD	23.9	1.5	0.47	0.20	0.12	15.03	3.31
Median	330	40	12.89	3.27	0.22	71.52	13.48
2 hr after treated							
33	330	42	13.96	3.59	ND	ND	12.43
34	315	40	14.31	3.23	0.52	99.03	10.25
35	355	35	12.27	3.62	0.27	91.89	24.12
36	295	33	12.95	3.62	0.21	56.24	23.63
37	345	43	13.27	3.64	0.29	80.35	14.98
38	345	38	13.16	3.49	0.24	48.76	13.39
Mean	331	38	13.32	3.53	0.31	75.25	16.47
SD	22.4	3.9	0.73	0.16	0.12	21.97	5.94
Median	338	39	13.22	3.61	0.27	80.35	14.19

APPENDIX-6 (b)

Effect of single(*iv*) dose of CP94 on
serum TBARS, serum iron and liver iron in iron-overloaded rats.

Rat No.	Body weight (g)	Hct. (%)	liver weight (g)		Serum TBARs (nmol/ml)	Serum Iron (umol/L)	Liver Iron (umol/g wet)
			wet	dry			
4 hr after treated							
39	320	36	12.97	3.17	0.17	53.86	8.56
40	320	40	13.44	3.53	0.40	49.44	14.81
41	335	40	13.71	3.38	0.49	80.35	18.30
42	365	33	11.24	2.94	0.14	57.59	29.69
43	300	34	13.62	3.15	0.18	54.88	18.12
44	330	41	13.91	3.78	0.30	42.65	10.10
45	350	34	13.8	3.7	0.17	62.35	20.35
Mean	331	37	13.24	3.38	0.26	57.30	17.13
SD	21.4	3.4	0.93	0.31	0.14	11.91	7.06
Median	330	36	13.62	3.38	0.18	54.88	18.12
8 hr after treated							
46	315	34	13.46	3.33	0.15	34.16	21.07
47	330	36	13.85	3.57	0.45	44.01	16.45
48	295	35	13.08	3.18	0.17	61.33	13.35
49	325	38	13.39	3.59	0.21	44.69	11.97
Mean	316	36	13.45	3.42	0.25	46.05	15.71
SD	15.5	1.7	0.32	0.20	0.14	11.27	4.03
Median	320	36	13.13	3.45	0.19	44.35	14.9

APPENDIX-7 (a)

Effect of single(*iv*) dose of CP94 on the cumulative urinary iron excretion at various time intervals in normal rats.

Time intervals (hrs)	Rat No.	Urine volume (ml)	Iron concentration (μM)	Total amount (μmol)
Control excretion				
0-8	1	5.0	11.60	0.058
	3	4.2	7.14	0.030
	4	4.4	9.09	0.040
	6	3.8	6.58	0.025
Mean		4.4	8.60	0.038
SD		0.5	2.27	0.014
Median		4.3	8.12	0.035
CP94 -induced excretion				
0-2	12	0.8	55.00	0.044
	13	1.2	56.67	0.068
	14	0.7	55.71	0.039
Mean		0.9	55.79	0.050
SD		0.3	0.84	0.016
Median		0.8	55.71	0.044

APPENDIX- 7 (b)

Effect of single(*iv*) dose of CP94 on the cumulative urinary iron excretion in at various time intervals in normal rats.

Time intervals (hrs)	Rat No.	Urine volume (ml)	Iron concentration (μM)	Total amount (μmol)
0-4	15	2.5	29.60	0.074
	16	2.7	31.48	0.085
	17	2.3	30.00	0.069
	18	2.6	24.23	0.063
Mean		2.52	28.83	0.073
SD		0.17	3.17	0.009
Median		2.55	29.80	0.072
0-8	19	5.4	17.78	0.096
	20	5.0	17.80	0.089
	21	4.6	17.17	0.079
Mean		5.0	17.58	0.088
SD		0.4	0.36	0.008
Median		5.0	17.78	0.089

APPENDIX-8 (a)

Effect of single(*iv*) dose of CP94 on the cumulative urinary iron excretion at various time intervals in iron-overloaded rats.

Time intervals (hrs)	Rat No.	Urine volume (ml)	Iron concentration (μM)	Total amount (μmol)
Control excretion				
0-8	22	6.1	4.26	0.026
	23	4.6	2.17	0.010
	24	4.2	2.14	0.009
	25	5.0	4.40	0.022
	26	5.6	5.00	0.028
	27	6.5	6.31	0.041
Mean		5.3	4.05	0.023
SD		0.9	1.63	0.012
Median		5.3	4.33	0.024
CP94 -induced excretion				
0-2	33	0.9	196.67	0.177
	34	1.2	170.00	0.204
	35	1.5	216.00	0.324
	36	1.4	168.57	0.236
	38	1.4	217.86	0.305
Mean		1.3	193.82	0.249
SD		0.2	23.89	0.064
Median		1.4	196.67	0.236

APPENDIX- 8 (b)

Effect of single(*iv*) dose of CP94 on the cumulative urinary iron excretion in at various time intervals in iron-overloaded rats.

Time intervals (hrs)	Rat No.	Urine volume (ml)	Iron concentration (μM)	Total amount (μmol)
0-4	39	2.0	151.50	0.303
	40	2.3	146.50	0.337
	42	1.9	171.00	0.325
	43	2.1	182.90	0.384
	45	2.4	180.0	0.432
Mean		2.1	166.40	0.356
SD		0.2	16.54	0.052
Median		2.1	171.00	0.337
0-8	46	5.5	82.27	0.480
	47	6.1	82.62	0.504
	48	5.4	82.41	0.455
	49	5.8	79.66	0.462
Mean		5.7	82.99	0.473
SD		0.3	3.16	0.025
Median		5.6	82.52	0.471