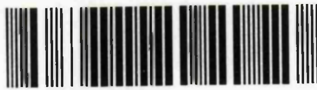

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Oral Iron Chelation Therapy with Deferiprone (L_1)

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A thesis submitted to University of London for the degree of Doctor of Medicine (M.D.)

This work was carried out at the Department of Haematology, Royal Free Hospital School of Medicine, London.

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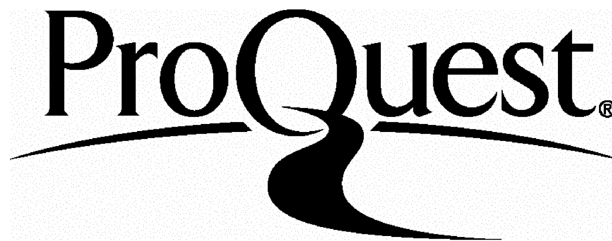

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ABSTRACT

Aims: To evaluate the oral iron chelator deferiprone(L_1) by conducting 2 long-term clinical trials in patients with iron overload and to assess its effectiveness and study its adverse effects both in vivo and in vitro.

Results: L_1 was used long term($\geq$6 months) in 53 patients with iron overload at a dose of approximately 50-100mg/kg/day. Urinary iron excretion(UIE) ranged between 5.3 and 66.8mg/24h. In a significant number of patients a dose in excess of 60mg/kg/day was required to induce a UIE in excess of 0.5mg/kg/day to result in a negative iron balance. No significant change in the mean UIE was observed when L_1 was given with or without food or vitamin C or in 2 or 4 divided daily doses. Serum ferritin fell in 36 of the 53 patients(68%) treated long term. The fall in serum ferritin was more marked in those patients with the highest body iron load as assessed by serum ferritin levels.

Two patients developed agranulocytosis and 3 less severe neutropenia whilst receiving L_1. Studies using liquid culture systems have failed to show an increased susceptibility of the patients' myeloid precursors(CFU-GM) to L_1, alone or bound to iron, compared to normal myeloid precursors. Furthermore, the toxicity of free or iron bound L_1 to normal or the patients' myeloid precursors was less than that of desferrioxamine(DFX).

Joint or musculoskeletal problems was observed in 14 of 53(26%) patients treated long term. The results here show for the first time that L_1 can cause mild zinc deficiency in some patients. This was more marked in patients with diabetes mellitus.

Nine of 63 patients(14%) treated with L_1 developed nausea and 17 of 54(32%) developed a transient fluctuation in the serum level of aspartate transaminase(AST).

L_1 was rapidly absorbed ($abt_{1/2}$: 22.2±17.7min) and eliminated ($elt_{1/2}$: 91.1±33.1min) mainly as L_1-glucuronide(L_1G) but also as free L_1 and L_1-iron complex in urine. L_1 efficiency was 3.8±1.9%. It was found that L_1G can accumulate in patients with impaired renal function. Using urea-polyacrylamide gel electrophoresis L_1 was found to be capable of removing significant proportion of transferrin iron. Assuming that all the iron removed from transferrin by L_1 was excreted in urine in 24 hours, it was found that this comprised 21.3±20.2%(5.8-67.1%) of total UIE. Using an HPLC(high-pressure liquid chromatography)-based method L_1 was found to be capable of causing a significant fall in the serum concentrations of non-transferrin-bound iron in patients with iron overload.

Conclusions: L_1 was shown to be an effective iron chelator in inducing a significant urinary iron excretion in a considerable number of patients leading to a reduction in body iron stores as evident by a fall in serum ferritin. But long-term use was associated with adverse effects the most important of which were agranulocytosis and joint toxicity.

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DECLARATION

The work described in this thesis was performed by myself except the following:

Dr. L.N. Sheppard and Mr. P. Nortey helped in analysing some of the serum samples for the estimation of L_1 level using the HPLC technique described in Chapter 4.

Dr. C.E. DeSilva helped in analysing some of the samples for the estimation of transferrin desaturation using Urea-PAGE method described in Chapter 5.

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The liquid cultures of myeloid progenitors described in Chapter 8 were performed by Dr. S. Wilkes.

PUBLICATIONS

Most of the work described in this thesis has previously been published as follows:

Al-Refaie F N, Hoffbrand AV 1993 Oral iron chelation therapy. *Recent Advances in Haematology* 7:185-216 .

Al-Refaie F N, Hoffbrand AV 1994 Oral iron chelating therapy-the L1 experience. *Baillières Clinical Haematology* 7:941-963.

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ABBREVIATIONS

AHA	anti-histone antibody
ALT	alanine transaminase
ANF	anti-nuclear factor
AST	aspartate transaminase
CFU-GM	colony forming unit-granulocyte/monocyte
CP20	1,2-dimethyl-3-hydroxypyrid-4-one(deferiprone)
CP94	1,2-diethyl-3-hydroxypyrid-4-one
CP96	2-ethyl-3-hydroxy-1-(2-methoxyethyl)-pyrid-4-one
dATP	deoxyadenosine triphosphate
DFX	desferrioxamine
dsDNA	double stranded DNA
DTPA	diethyltriamine pentaacetic acid
dTTP	deoxythymidine triphosphate
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte/monocyte-colony stimulating factor
GTT	glucose tolerance test
HPLC	high pressure liquid chromatography
IMDM	Iscoves Modified Dulbecos Medium
K_{part}	partition coefficient
L_1	1,2-dimethyl-3-hydroxypyrid-4-one
L_1G	L_1-glucuronide
LDH	lactate dehydrogenase
MDA	malonaldehyde
MUGA	multigated scintigraphy
NTA	nitrilotriacetic acid
NTBI	non-transferrin-bound iron
PFA	paraformaldehyde
PHA	phytohaemagglutinine
pM	stability constant
RhF	rheumatoid factor
TIBC	total iron binding capacity
UIE	urinary iron excretion
UPAGE	urea-polyacrylamide gel electrophoresis
UZE	urinary zinc excretion

CHAPTER 1

INTRODUCTION

1.1. IRON OVERLOAD

Transfusion therapy plays an essential role in the management of several blood disorders both inherited and acquired. These include thalassaemia major or intermedia, sickle cell anaemia, red cell aplasia and myelodysplastic syndromes. However, haemosiderosis is an inevitable result of chronic transfusion programmes and if untreated causes considerable morbidity and ultimately death. In some of these disorders iron overload is compounded by increased intestinal iron absorption as a result of increased haemopoietic turnover.

1.1.1. Mechanism of toxicity

The exact mechanisms whereby iron produces cellular injury have not been established but two mechanisms have been proposed. The first is the release of iron from haemosiderin within the acid medium of the lysosomes causing oxidative damage of the lysosomal membrane. This in turn results in the release of hydrolytic enzymes into the cytosol with the consequent cell damage(O'Connell *et al*, 1985). The second is the participation of iron through Haber-Weiss reaction in the formation of free hydroxyl radicals which can cause lipid peroxidation and therefore, membrane damage(Halliwell & Gutteridge, 1984a,b). It has also been suggested that "free iron" or non-transferrin-bound iron which is likely to be present in the serum of iron loaded subjects who have fully saturated transferrin(Hershko *et al*, 1978) is particularly toxic as it is among those species of iron that are most capable of participating in free radical formation.

1.1.2. Assessment of iron status

Each unit of blood contains 200-250mg of iron, therefore a simple and rough estimate of iron overload is to calculate the amount of blood which has been transfused. However, during iron chelation therapy this method becomes less useful.

Measurement of serum iron concentration and transferrin saturation can be useful only in the early stages of iron overload.

Urinary iron excretion in excess of 4mg after an intramuscular administration of 500mg DFX is highly suggestive of parenchymal iron loading, forming the basis of a screening test for hepatocyte iron loading(Pippard, 1989).

Serum ferritin correlates with the amount of blood transfused and with liver iron measured by liver biopsy(Chapman *et al*, 1982; Cazzola *et al*, 1983) or indirectly by magnetic resonance spectroscopy(Dixon *et al*, 1994). However, the use of serum ferritin concentrations in the assessment of iron overload has its limitations. Being an acute-phase reactant serum ferritin concentrations are increased in chronic illnesses, malignancy and inflammatory conditions thereby causing an overestimation of body iron stores. Furthermore, patients with liver disease particularly chronic active hepatitis can have a disproportionately high serum ferritin(DeVirgiliis *et al*, 1981). Ascorbate levels can also influence the level of serum ferritin. Low ascorbate levels suppress the synthesis and release of cellular ferritin and hence causing an underestimation of actual body iron stores(Chapman *et al*, 1982).

Measurement of iron content of serum ferritin has recently been suggested to be more accurate than serum ferritin at assessing iron load because it is not influenced by those factors which affect the ferritin as an acute phase protein(ten-Kate *et al*, 1997). However, this method has not yet been assessed thoroughly for routine use.

Liver biopsy is by far the most accurate method available for estimating the severity of iron overload. Furthermore by doing liver biopsy the degree of iron-induced liver damage can also be assessed.

Other non-invasive techniques have also been tried and shown to have variable degrees

of sensitivity at assessing the severity of iron overload. Among these methods are magnetic resonance imaging of the liver(Houang *et al*, 1979), magnetic susceptibility measurement of hepatic iron stores(Brittenham *et al*, 1982) and magnetic resonance spectroscopy(Dixon *et al*, 1994).

Finally, the use of non-transferrin-bound iron concentrations for assessing iron overload and monitoring the effectiveness of iron chelation therapy was evaluated(Chapter 6). It was found to correlate with the degree of iron load, as assessed by serum ferritin, and may reflect changes in it during iron chelation with L_1.

1.1.3. Clinical manifestations of iron overload

Iron-induced cardiac toxicity may present as a cardiomyopathy with heart failure or as arrhythmias or as both. It is the commonest cause of death in transfusion-dependent thalassaemic patients(Zurlo *et al*, 1989). In a recent study Olivieri *et al*(1994) reported that among factors which affect cardiac disease in thalassaemic patients were age at the start of chelation therapy and serum ferritin concentration in excess of 2500ug/l. Multigated scintigraphy was found to be more sensitive than echocardiogram in assessing the degree of cardiac dysfunction in these patients(Aldouri *et al*, 1990).

Liver disease is the most common complication of transfusional iron overload. Hepatocyte iron deposition produces hepatomegaly, functional abnormalities, fibrosis, and eventually cirrhosis. Hepatocellular carcinoma can be an ultimate complication but rarely develops in non-cirrhotic patients. The severity of hepatic damage is closely correlated with the degree of iron deposition in the hepatocytes and also influenced by the route of iron accumulation. For instance, hepatic fibrosis and cirrhosis develop at levels of hepatic iron concentration higher in transfusional siderosis than in haemochromatosis. This is perhaps because of differences in the duration of exposure

to the iron load or in the distribution of the excess iron between macrophages and parenchymal cells(Risdon *et al*, 1975).

Iron deposition can also damage the endocrine glands. Insulin dependent diabetes mellitus was found to be present in 6.5% of thalassaemia patients over age 10 years(De Sanctis *et al*, 1988). Hypogonadism may result from primary testicular failure or from the effect of iron on the hypothalamic-pituitary axis. During the second decade of life both growth and sexual maturation are usually retarded in unchelated patients with transfusional iron overload. Borgna-Pignatti *et al*(1985) noted that 62% of males and 35% of females over 14 years old were 2SD below the mean for normal height. There was a complete absence of pubertal maturation in 38% of females and 67% of males aged between 12 and 18 years. Primary hypothyroidism was reported in 5.6% and hypoparathyroidism in 2.5% of thalassaemia patients(De Sanctis *et al*, 1992). Adrenal insufficiency is a rare complications of iron overload.

1.2. IRON CHELATION THERAPY

Chronic transfusion programmes have transformed thalassaemia from an anaemic syndrome into a severe disorder of iron overload(Stockman & Oski, 1974). Without an effective iron chelation therapy patients can only survive to their second or third decade to die of one or the other of the complications of iron overload. Similarly patients with other forms of transfusion-dependent anaemia are likely to succumb to the complications of iron overload if not given iron chelation.

1.2.1. Aims

The long-term goal of chelation therapy for iron overload is the reduction and maintenance of the body iron content at a safe level. In a recent review Gabutti & Piga(1996) noted that the maintenance of serum ferritin below 2000ug/l prevents iron

overload related complications. The immediate goal, on the other hand, is to neutralize any harmful species of iron such as non-transferrin-bound iron, that may be present in the serum of the patient at the time of the treatment. Whereas, the former goal can be achieved by inducing iron excretion in excess of the iron administered through blood transfusions and this is dependent on the degree of iron load and on the dose of the chelator, the later goal can only be achieved if the chelator is administered at a dose and duration sufficient to cause a continuous neutralization of the non-transferrin-bound iron.

1.2.2. Desferrioxamine

Desferrioxamine is a siderophore produced by *Streptomyces pilosus*. It is a hexadentate ligand that is minimally absorbed from the gastrointestinal tract and it therefore, has to be administered parenterally. Clearance of the drug is rapid with a half-life after an intravenouse bolus dose of 5-10 minutes(Summers *et al*, 1979). Intravenous infusion of DFX induces both urinary and faecal iron excretion: the proportion of each is dependant on the degree of iron load and the dose of DFX administered(Pippard *et al*, 1989). Slow subcutaneous infusion of DFX for 8-12 hours per day at a dose of 20-50mg/kg/day usually provides adequate therapy(Hussain *et al*, 1977). Administration of ascorbic acid can enhance DFX-induced urinary iron excretion(Hussain *et al*, 1977; Van-der-Weyden, 1984).

The efficacy of regular chelation with DFX has been demonstrated by a study carried out on a large number of thalassaemic patients which showed that survival was significantly improved in patients born after 1970 treated with regular transfusions and subcutaneous chelation(Zurlo *et al*, 1989; Borgna-Pignatti *et al*, 1993).

1.2.3. Desferrioxamine toxicity

DFX is generally a safe drug in iron loaded patients, but systemic complications have

been reported, including allergic anaphylactoid reaction, high-frequency sensorineural hearing loss, growth failure and bone abnormalities, and at very high doses it can cause acute visual loss, night blindness and colour vision abnormalities. Furthermore, infection with Yersinia is another possible complication of the use of DFX. It has been noted that the severe toxic effects on vision, hearing and growth are more frequent at higher doses of DFX and if body iron stores are low(Porter & Huehns, 1989).

1.3. THE NEED FOR AN ORALLY EFFECTIVE IRON CHELATOR

Although DFX is an effective and relatively safe chelator for the treatment of iron overload, an orally active iron chelator is needed. The high expense of DFX and its cumbersome method of administration have been prohibitive to its widespread use, especially in those areas in the world where it is most needed. Several compounds have been developed and screened both in vitro and in vivo for their oral effectiveness and for acute and chronic toxicity. Of the several hundred compounds designed only a few have reached the stage of clinical trials and deferiprone(L_1, 1,2 dimethyl-3-hydroxypyrid-4-one, CP20) is one of a few orally active chelators which have been tested in humans. Shorter term trials have also been undertaken with other compounds including 1,2-diethyl-3-hydroxypyrid-4-one(CP94) N,N'-bis(*o*-hydroxy-benzyl)-ethylenediamine-N,N'-diacetic acid(HBED), desferrithiocin and pyridoxal isonicotionyl hydrazone(PIH) (Fig 1.1). Nevertheless, no single compound has yet emerged as a widely available pharmaceutical preparation.

1.4. 3-HYDROXYPYRID-4-ONES

1.4.1. Physicochemical properties

This class of chelators which was designed by Hider *et al*(1984) possesses a high affinity for iron(III) with a binding constant (log β=37), six orders of magnitude higher than

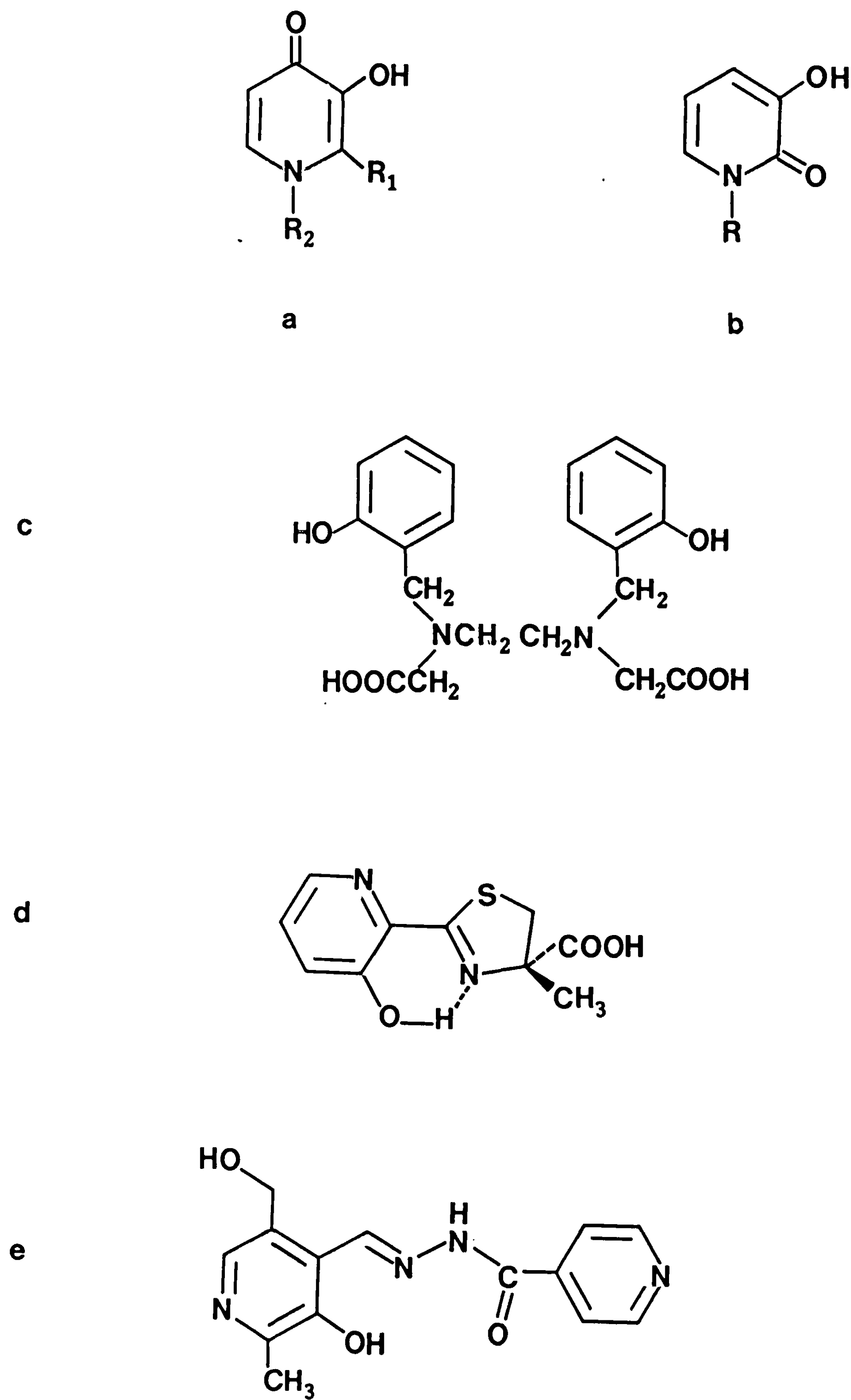


Fig. 1.1. Iron chelators: **a**. hydroxypyrid-4-one; **b**. hydroxypyrid-2-one; **c**. HBED[*N,N'*-bis(*o*-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid]; **d**. DFT(deferrithiocin); **e**. PIH(pyridoxal isonicotinoyl hydrazone).

DFX. These chelators are among the few groups of iron(III) selective ligands that are uncharged both as free ligand and as the iron complex under physiological conditions(Porter *et al*, 1989a). This feature is essential to their success as orally effective chelators. They are bidentate ligands; therefore three molecules are required to form a neutral complex with one atom of iron(III), whereas a hexadentate ligand such as DFX binds iron in a 1:1 ratio. The 3-hydroxypyrid-4-ones are highly specific for Fe(III), lacking any measurable affinity for calcium and magnesium (Stönzi *et al*, 1980) although they do bind aluminum and to a lesser extent zinc. They are able to remove iron directly from ferritin with a greater efficiency than DFX presumably because the lower size of the hydroxypyridones allows entry through the protein pores enabling the bidentate chelator to remove iron from the core(Brady *et al*, 1989).

By modifying the size of the alkyl group at R_1 and R_2(Fig 1.1a,b) positions a wide range of compounds with different lipophilicity can be produced. Studies utilizing primary hepatocyte culture showed that compounds with high lipophilicity are associated with higher mobilisation of intracellular iron with maximum effect achieved by compounds with a partition coefficient(K_{part}, the ratio of the concentrations of the compound between an organic phase usually *n*-octanol and water buffered to pH 7.4) close to one(Porter *et al*, 1988). Those compounds with a low K_{part}(<0.20) are associated with minimal iron mobilization. However, higher lipid solubility(K_{part} >1) is also associated with higher toxicity. It was, therefore, suggested that compounds with a K_{part} between 0.2 and 1 are likely to be most appropriate chelators for in vivo assessment in animals(Porter *et al*, 1989a).

1.4.2. Selection of L_1 for clinical trials

Iron chelation therapy for transfusion dependent refractory anaemias is likely to be over

many years. It was therefore essential to choose a compound for clinical studies unlikely to accumulate in body lipids, especially the central nervous system, after prolonged administration. L_1 has a K_{part} of 0.21 and has been studied both in vitro and in vivo in mice, rats, rabbits and monkeys. It was selected, as a hydrophilic compound, as the most appropriate of the 3-hydroxypyrid-4-ones for initial clinical trials(Kontoghiorghes *et al* 1987a&b).

1.4.3. L_1 Chemistry

L_1 is a white solid compound with a MW of 139 and a melting point of 268°C(Kontoghiorghes *et al* 1990a). Maximum water solubility was estimated to be 1.6-1.8% at 24°C(Kontoghiorghes *et al* 1990a). It is highly stable at pH values ranging from 1-12(Kontoghiorghes *et al* 1990a) and it is resistant to cleavage by digestive enzymes(Porter *et al* 1989a).

1.4.4. Stability of L_1-iron complex

Because L_1 is a bidentate ligand its pM value(-log of the concentration of unchelated Fe(III) in a solution containing 1uM Fe(III) and 10 uM chelator, buffered to pH 7.4) is lower than that of DFX(21 *vs* 27)(Motekaitis & Martell 1991). This implies that partially dissociated L_1-iron complexes(2:1, 1:1) can form at low concentrations of chelator and in turn these complexes in which the iron atom is not completely 'covered' can generate hydroxyl radicals(Porter *et al*, 1989a).

Recently Kline & Orvig(1992) have studied the concentration of various forms of L_1-Fe complexes(1:1, 1:2, 1:3) in solution at pH ranging from 1 to 13 with an iron concentration of 100umol/l and L_1 of 400umol/l. They found that between pH 4 and 11 the predominant species is the 3:1[Fe-$(L_1)_3$] complex. They also determined the stability constant for L_1-Fe complexes by potentiometric titration technique and found it to be

36.3, in agreement with previous reports.

1.4.5. Fate of L_1-iron complex

In 1991 Pippard *et al* reported the injection of a chelate of ^{59}Fe combined with different hydroxypyrid-4-ones into non-iron loaded rats. Neither urinary or biliary excretion of ^{59}Fe could be detected when 0.125umol of ^{59}Fe was injected but at higher doses ^{59}Fe began to appear in the urine. ^{59}Fe did accumulate in red cells, 50% of the injected dose after 5 days. The authors concluded that some of the hydroxypyrid-4-ones form chelates which may allow in vivo transfer of ^{59}Fe to transferrin. A full report of these studies remains to be published. In a similar study performed by Ciba Geigy(1993), the fate of a low concentration of ^{59}Fe-L_1 or ^{59}Fe-DFX complexes(0.125umol iron and 0.250umol iron-binding equivalent of chelator) injected intravenously into normal rats was studied by measuring the amount of radioactivity incorporated in various tissues and excreted in urine. They found that over 70% of radioactivity was incorporated into the skeleton and the red blood cells 3 days after the administration of the ^{59}Fe-L_1 complex. On the other hand, over 70% of ^{59}Fe-DFX was eliminated by the animal in urine and faeces. It was postulated that ^{59}Fe dissociates from the L_1 complex, is picked up by transferrin, which then delivers it to the erythropoietic system. It was, then concluded that in patients with iron overload and saturated transferrin, iron may dissociate from the L_1 complex and feed into the non-transferrin-bound or other iron pools. It is not clear, however, why in these experiments iron loaded animals were not also used in order to test the hypothesis on the fate of L_1 complex in iron overloaded patients.

1.5. CELL CULTURE STUDIES

Several in vitro experiments have been undertaken to study the physicochemical properties of the different compounds in the hydroxypyridone group of oral chelators and

also to determine the relations between these properties and the efficacy and toxicity of these compounds.

1.5.1. Iron mobilisation from hepatocytes

Porter et al(1988) investigated the efficiency of various iron chelators in mobilising iron from ^{59}Fe labelled hepatocytes in culture. Following the addition of chelators to the culture medium the rate of ^{59}Fe release was increased above that of control. There was a significant correlation between the percentage of iron release and lipid solubility of the compound with a maximum mobilization by compounds with a K_{part} close to one. Compounds with higher iron binding constants, [4-one compounds(Fig 1.1a), Log β=36] were more effective than those with lower iron binding constants[2-one compounds(Fig 1.1b), Log β=32] over a wide range of concentrations but particularly at low concentrations of chelator. At higher concentrations of chelator and with more prolonged incubation times, more lipophilic compounds were associated with lower cell viability as determined by higher LDH levels in the culture supernatant.

1.5.2. Iron mobilisation from myocardial cells

Culture of myocardial cells was utilised by Hershko *et al* (1991) to assess the efficiency of several hydroxypyridone compounds and of DFX in mobilising ^{59}Fe and in inhibiting lipid peroxidation induced by iron as indicated by cellular malonaldehyde content(MDA). At a low molar concentration of chelators (0.1 mmol/l), DFX was more effective than the hydroxypyridones in reducing the iron content of the iron loaded heart cell (52% *vs* 9%-16%). At higher concentration (1mmol/l) all hydroxypyrione compounds were as effective or more effective than DFX (87%-89% *vs* 83%).

When DFX and 1,2-diethyl-3-hydroxypyrid-4-one(CP94) were used at a concentration of 1mmol/l, rapid mobilisation of cellular iron was observed reaching a plateau after six

hours. Iron mobilization by both compounds was associated with a reduction in cellular MDA content. However there was no correlation between the MDA level and the amount of iron removed; DFX reduced MDA more than did CP94 despite the greater efficiency of CP94 for iron chelation.

The effect of chelator (CP94 and DFX) concentration on chelating efficiency was also evaluated. At concentrations of 0.1 and 0.25 mmol/l, DFX was more effective than CP94 while at concentrations of 0.75 and 1.0 mmol/l the opposite was true. It was concluded, therefore, that effective mobilization of iron by CP94 and probably by other hydroxypyridones requires a drug:iron ratio exceeding 3:1 permitting the formation of a hexadentate complex.

Recently Link *et al* (1995) reported on the ability of L_1, CP96(1-ethyl-2-methoxyethyl-3-hydroxypyrid-4-one) and DFX to reverse the damage caused by iron loading to heart cell organelles. At a concentration of 1.0 mmol/l, all three chelators were equally efficient in removing iron and restoring the activity of the thiolic sacrolemmal enzyme 5'-nucleotidase and Na,K,ATPase. However, at 0.1mmol/l L_1 and CP96 were less effective than DFX both in iron-mobilizing effect and in promoting thiolic enzyme recovery.

1.5.3. Effect of L_1 on lymphocytes

Patanapanyasat *et al*(1992) studied the effect of hydroxypyridone(CP) compounds (including L_1) and DFX on human lymphocytes. CP compounds and DFX caused a dose dependent inhibition of the proliferative response after 4 hours exposure. At a concentration of 100 umol both DFX and CP compounds caused 70% inhibition and at 200 umol 100% inhibition. These concentrations were not associated with a change in cell viability but higher concentration of chelators or more prolonged exposure (72h) resulted in reduced cell viability. The inhibitory effect of CP compounds was higher than

that of DFX. Greater inhibition was observed among CP compounds with increasing K_{part}. Presaturation of CP and DFX with ferric ion diminished their inhibitory effect on DNA synthesis. It was suggested, therefore, that both DFX and CP compounds exert their effect by chelating iron with subsequent inhibition of DNA synthesis. DFX has previously been shown to inhibit lymphocyte ribonucleotide reductase which is a rate limiting enzyme in DNA synthesis (Hoffbrand *et al* 1976). All chelators studied at these concentrations had no effect, in short term cultures, on protein synthesis, assessed by [^{3}H]leucine incorporation or on T-cell activation markers (transferrin and IL-2 receptors). In a recent study the ability of iron chelators in inducing apoptosis in proliferating cells was studied (Hileti *et al* 1995). Both L_1 and DFX were found to induce apoptosis of proliferating activated T-lymphocytes and of the promyelocytic cell line HL60, but not of resting peripheral blood lymphocytes or granulocytes.

1.5.4. Effect of L_1 on DNA, RNA and protein synthesis

Ganeshaguru *et al* (1991) showed that L_1 at the higher concentration of 1 mmol caused substantial inhibition of DNA, RNA and protein synthesis and cell viability in PHA stimulated lymphocytes. Similar inhibition was observed with DFX at this high concentration. Their actions were, however, different. Whereas L_1 increased both dATP and dTTP concentrations in the cells, DFX, like hydroxyurea (a known ribonucleotide reductase inhibitor) decreased dATP and increased dTTP concentration suggesting DFX inhibits DNA synthesis by chelating iron from the enzyme ribonucleotide reductase. Both L_1 and DFX decreased the cell concentration of dsDNA. However, there was increased recovery of dsDNA with time on removal of L_1 while removal of DFX did not allow substantial recovery. These findings indicate different mechanisms of iron removal and cytotoxicity for L_1 and DFX which remain to be defined.

1.6. ANIMAL STUDIES

A large number of animal studies have been performed in order to assess the effectiveness of L_1 and to determine its acute and chronic toxic side effects. In general L_1 was found to have an efficacy inferior to that of DFX in most of the species studied. These studies have also shown that more lipid soluble compounds tend to be more effective but more toxic. In contrast to the findings in humans(Kontoghiorghes *et al*, 1990b; Olivieri *et al*, 1990a), substantial iron excretion occurs by the faecal route in mice and rats following oral L_1 administration and faecal excretion has also been observed in rabbits and monkeys(Kontoghiorghes & Hoffbrand, 1986; Bergeron *et al*, 1992). The results of these studies summarized in Table 1.1. The major side-effects noted with L_1 have been leucopenia, macrocytosis and hypersalivation (Table 1.2).

1.7. CLINICAL TRIALS IN HUMANS

Initial short term studies were carried out in iron overloaded patients with myelodysplasia. L_1-induced urinary iron excretion was found to be equal to that achieved with comparable doses of DFX. The amounts of iron excreted were related to the dose of L_1 and the iron load of the patients (Kontoghiorghes *et al* 1987a). Similar results were obtained when 4 patients with thalassaemia major were given L_1 at a dose of 100mg/kg/day. The coadministration of oral ascorbic acid further increased urinary iron excretion in most patients (Kontoghiorghes *et al* 1987b). In 1990 other reports of short-term use of L_1 in iron loaded patients emerged(Agarwal *et al* 1990, Töndury *et al* 1990, Olivieri *et al* 1990a). Olivieri *et al* (1990a) found that the mean urinary iron excretion was lower with L_1 therapy than with subcutaneous DFX when both chelators were used at a dose of 50mg/kg/day. However, when L_1 dose was increased to 75mg/kg/day in 5 of the patients; mean urinary iron excretion became comparable to that achieved with DFX

Table 1.1. Results of efficacy studies of L_1 in animals.

Ref	Animals	Study design	Results
Gyparaki *et al* 1987, Kontoghiorghes 1987	Mice	L_1 i.g.	Substantial increase in ^{59}Fe excretion (comparable to that induced by DFX).
Porter *et al* 1990	=	L_1 p.o.	Increased ^{59}Fe excretion with a linear dose response curve over the range 0-750mg/kg. L_1 caused 0.85% increase in ^{59}Fe excretion for each mg/kg administered orally. At low dose L_1 caused mainly faecal ^{59}Fe excretion. UIE increased with the increase of L_1 dose
Porter *et al* 1991	=	L_1(200mg/kg) for 60 days	37% decrease in liver iron (compared to 46% caused by DFX given i.p. at a similar dose).
Kontoghiorghes *et al* 1987c	Rats	L_1(200mg/kg) i.p.	Increased ^{59}Fe excretion (comparable to that induced by DFX). However most of the ^{59}Fe remained in the animals. Liver iron pools was accessible to L_1 given i.p. or p.o.
Venkatram *et al* 1990	=	L_1(200mg/kg)	Chelation efficiency*=1.3%. No increase in faecal iron excretion.
Zevin *et al* 1992	=	L_1 p.o.	L_1 is able to mobilize Fe from both liver and RES stores with efficiency comparable to that of DFX. Iron mobilized from liver was excreted in bile while up to 50% of iron removed from RES was cleared by kidneys.
Florence *et al* 1992	=	L_1 p.o.	Caused iron removal from hepatic ferritin and haemosiderin and significant decrease in ferritin iron and protein content of spleen.
Bergeron *et al* 1992	=	L_1 p.o.	Chelation efficiency*=1.2%. 86% of excreted iron was found in bile.
Kontoghiorghes & Hoffbrand 1986	Rabbits	L_1(200mg/kg)	Caused Predominantly faecal ^{59}Fe excretion comparable to that induced by a similar dose of DFX.
Nortey *et al* 1991	=	L_1(200mg/kg)	Caused increased 59Fe excretion with less than 50% faecal.
Bergeron *et al* 1992	Monkeys	L_1	Chelation efficiency*=2.1%. 70% of iron was excreted in urine and the rest in faeces.

i.g.=intragastric, p.o.=oral, DFX=desferrioxamine, RES=reticuloendothelial system, * ratio of iron excreted to the capacity of L_1 dose to chelate iron by forming 3:1 complexes.

Table 1.2. Animal toxicity of L_1.

Toxicity	Animal species (iron stores)	Dose(mg/kg)/ route	Study
Leukopenia	Mice(normal & iron loaded)	200/i.p.	Porter *et al* (1991)
	Rats(normal)	200/i.p.	Kontoghiorghes *et al*(1989)
	Rats(normal)	300/i.p., i.g.	Grady *et al*(1992)
Anaemia	Mice(normal & iron loaded)	200/i.p.	Porter *et al* (1991)
	Rats(normal)	300/i.p.	Grady *et al*(1992)
	Rats(normal)	200/i.g.	Kontoghiorghes *et al*(1989)
Low RBC	Mice(normal & iron loaded)	200/i.p.	Porter *et al* (1991)
	Rat(normal)	300/i.p.	Grady *et al*(1992)
Low PCV	Rats(normal)	300/i.p.	Grady *et al*(1992)
High MCV	Mice(normal)	200/i.p.	Porter *et al*(1991)
	Rats(normal)	300/i.p.	Grady *et al*(1992)
High serum cholesterol	Rats(normal)	DR/i.g., i.p.	Grady *et al*(1992)
Hyper-salivation	Rats(normal & ironloaded)	200/i.g.,i.p.	Kontoghiorghes *et al*(1987c)
	Rats(normal)	75-300/i.g.	Grady *et al*(1992)
	Rats(normal)*	42-63/i.g.	Bergeron *et al*(1992)
Adrenal enlargement	Rats(normal)	DR/i.g., i.p.	Grady *et al*(1992)
Inclusion body(liver)	Mice(normal &	200/i.p.	Porter *et al* (1991)
Atrophy of Sp., Th.	Rats(normal)	DR/i.g., i.p.	Grady *et al*(1992)
Atrophy of testes	Rats(normal)	DR/i.p.	Grady *et al*(1992)
Reduced weight gain	Rats(normal)	300/i.p.	Grady *et al*(1992)
Death	Mice(normal)**	200/i.p.	Porter *et al*(1991)

Sp.=spleen, Th.=thymus, DR=dose-related, i.p.=intraperitoneal, i.g.=intragastric, RBC=red blood cells, PCV=packed cell volume, MCV=mean corpuscular volume, *40% of animals, **At 60 days:3/9 of animals.

at 50mg/kg/day. Faecal iron excretion rose slightly over baseline in 6 patients studied during L_1 administration. No adverse effect were observed in any of the initial short-term trials of L_1.

Several long-term clinical trials of L_1 have been performed world wide. As a result over 250 patients have been studied in order to determine the efficacy of the drug and its acute and chronic adverse effects. Table 1.3. summarises the major published long-term trials of L_1 in iron overloaded patients including those reported in this thesis.

1.8. THIS THESIS

This thesis contains the results of 2 long term clinical trials of L_1 in patients with iron overload which were designed to assess long term efficacy and toxicity of L_1. It also contains the results of several experiments which were performed during those trials and aimed at elucidating the mechanism(s) of the various adverse effects observed or studying the mechanism of iron chelation by L_1. The pharmacokinetics of both L_1 and L_1-glucuronide in 24 patients with iron overload were examined after a single dose administration of L_1. Correlations between various pharmacokinetic parameters and L_1 efficacy and renal function were explored. The interaction between L_1 and transferrin both in vivo in 16 patients with iron overload and in vitro was studied using urea-polyacrylamide gel electrophoresis. The correlation between NTBI and the degree of iron overload measured by serum ferritin was assessed in 52 patients with thalassaemia major. When this correlation was found to be significant, NTBI was monitored in patients receiving L_1 therapy. In another study the effect of a single oral dose of L_1 on the fate of NTBI and on the kinetics of L_1-Fe complex was examined in 22 patients with iron overload.

Table 1.3. Clinical trials with L_1.

Centre	Number of patients	Diagnosis	L_1 dose mg/kg/day	Duration (months)	Reference
London	63	TM, MDS RCA, CHA	86-150	1-24	Bartlett *et al*, 1990; Kontoghiorghes *et al*, 1990b; Chapter 2,3.
Berne	12	TM	51-93	9-27	Töndury *et al*, 1990; 1992.
Bombay	104	TM, HbE/Thal	50-100	36	Agarwal *et al*, 1992; 1993a&b.
Toronto	22	TM, TI	75-100	9-41	Olivieri *et al*, 1992; 1993; 1995.
Amsterdam	25	MDS, AA, HbE/Thal	31-63	4-16	Goudsmit & Kersten, 1992.
Milan	22	TM	50	1-16	Carnelli *et al*, 1992.
Dusseldorf	6	MDS	2.5g/d	0.7-5.4	Jaeger *et al*, 1992.

TM=thalassaemia major, MDS=myelodysplastic syndrome, RCA=red cell aplasia, CHA=congenital haemolytic anaemia, TI=thalassaemia intermedia, AA=aplastic anaemia.

CHAPTER 2

CLINICAL TRIAL(1)

2.1. INTRODUCTION

Between 1990 and 1991 a long term trial of L_1 in patients with thalassaemia major(TM) and iron overload was carried out and intended to assess the long-term effectiveness of L_1 in patients with TM and to assess the incidence of possible adverse effects. The reason for selecting only patients with TM was that in a previous trial, carried out in 1988/89, a patient with Blackfan-Diamond anaemia developed agranulocytosis whilst receiving L_1 therapy. Therefore, patients with abnormal bone marrow were excluded from this trial to minimise the likelihood for such an incident to happen again.

2.2. PATIENTS AND METHODS

Approval for the trial was obtained from the Royal Free Hospital's Ethical Committee. Eleven patients were initially entered into the trial after giving their written informed consent. Details of their initial clinical findings and doses of L_1 are given in Table 2.1. One patient was taken off the trial after 11 weeks because of the development of agranulocytosis; the long-term follow up data therefore refer to the remaining 10 patients. Detailed clinical examination was performed each time the patient was seen in the out patients and several investigations were carried out at regular intervals(Table 2.2.).

FBC, U&E, LFTs, serum iron, TIBC, PT, PTTK, Coombs test, Igs, RhF, ANF, CD4/CD8 ratio, ECG, CT of adrenals and MUGA scan were performed by standard methods. Serum ferritin was estimated by an ELISA technique(Flowers *et al* 1986). NTBI was measured by an HPLC technique(Singh *et al* 1990, Al-Refaie *et al* 1992), blood samples for this test were collected immediately prior to L_1 intake. Leucocyte ascorbate was measured as described by Denson & Bowers(1961). Retinal function tests were performed as described by Arden *et al*(1984). Lipids were measured by standard fully

Table 2.1. Clinical data of 11 patients with thalassaemia major.

Case	Age/sex	Ethnic origin	Initial serum ferritin (ug/l)	Maximum L_1 dose (mg/kg)	Duration of treatment(days) (all doses)	Anti-HCV status	Other medical problems
1	23/M	Cypriot	8500	93	376	Neg	None
2	23/M	Italian	1400	85	376	Neg	DM
3	21/F	Cypriot	5900	110	348	Neg	2°Amenorrhoea
4	21/F	Cypriot	4350	111	369	Neg	2°Amenorrhoea, DM
5	26/M	Greek	1440	106	355	Pos	CAH
6	24/F	Greek	6200	119	344	Pos	2°Amenorrhoea, Abnormal GTT
7	20/F	Greek	4350	105	61*	Neg	None
8	20/M	Cypriot	1000	85	376	Neg	None
9	25/F	Indian	8300	105	380	Neg	DM, HPT, Sexual Infantilism
10	17/M	Indian	8820	107	281	Pos	DM, Short stature
11	25/M	Indian	9580	98	205	Pos	DM, HPT, CAH, Short stature

HCV:Hepatitis C Virus, DM:Diabetes Mellitus, CAH:Chronic Active Hepatitis, GTT:Glucose Tolerance Test, HPT:Hypoparathyroidism.
* drug was stopped early because of agranulocytosis.

Table 2.2. Investigations performed initially and during the trial.

General investigations:	Full blood count(FBC), blood urea and electrolyte(U&E), creatinine, liver function tests(LFTs), prothrombin time(PT), partial thromboplastin time(PTTK), lipid profile*(triglyceride, cholesterol, LDL and HDL), serum hormones*(TSH, T4, growth hormone, prolactin, luteinizing hormone, follicle stimulating hormone, testosterone, oestradiol and cortisol), 24h urinary cortisol* and computerised tomography(CT) of the adrenals*.
Investigations to assess iron status:	Serum ferritin, serum iron, total iron binding capacity(TIBC), non-transferrin-bound iron(NTBI) and 24 hour urinary iron in response to DFX or L_1.
Investigations to assess potential L_1 toxicity to the immune system:	Serum immunoglobulins(Igs), T cell subsets(CD4/CD8 ratio), rheumatoid factor(RhF) and antinuclear factor(ANF).
Investigations to assess whether L_1 can cause toxicities similar to those observed with DFX:	Retinal function tests(visual acuity, electrooculogram, electroretinogram and pattern, visually evoked responses and colour vision) and audiogram.
Investigations to assess the effect of L_1 on trace elements:	Serum and urinary trace elements(Cu, Zn and Mg) and serum caeruloplasmin.
Other tests:	Direct Coombs test¶, leucocyte ascorbate♣, electrocardiogram (ECG) and multigated scintigraphy (MUGA scan) at rest and with cold stress♠

*The lipid profile, hormones and CT of adrenals were performed because of reports of adrenal hyperplasia and hypercholestrolaemia in animal studies(Table 1.2).
¶In a previous trial a Coombs positive result was observed in a patient receiving L_1(Hoffbrand *et al*, 1989).
♣To assess the effect of iron overload/chelation on ascorbate status.
♠To assess possible iron-induced cardiac toxicity).

enzymatic technique using centrifugal analyzer. Urinary iron and zinc were measured using atomic absorption spectrophotometry (Scudder *et al*, 1978). Hormones were assayed by standard radioimmunoassay technique. Statistical analysis was done using Student's *t*-test.

The patients were seen once weekly for the first four months and then fortnightly. During each visit they received a full clinical examination and had blood tests including FBC, renal and liver function. 24h urine was collected for iron and trace metal once every 1-3 weeks. MUGA scan; retinal function tests; CT of adrenals and audiometry were checked initially and at the end of the Trial. The other tests were repeated once every three months.

L_1 was commenced at a single dose of 0.5g daily with a gradually increasing dose over the first week to a dose of about 40-60mg/kg/day. This was given as a single daily dose for four weeks and then given twice daily. This final dose was chosen because previous dose/response studies showed that at this dose a significant UIE can be induced (Kontoghiorghes *et al*, 1987a, 1990b). All doses were taken on an empty stomach and at least one hour before food. No food was taken for at least one hour after taking the drug. Vitamin C was initially discontinued on all patients except cases 10 and 11. Those who had a fall in leucocyte ascorbate to less than 23ug/10^3 WBC(normal range 23-51) were restarted on vitamin C(200mg daily). Patients 10 and 11 had low pre-trial vitamin C levels and were therefore kept on vitamin C.

2.3. RESULTS

In general the drug was well accepted and compliance was excellent. No change was noted in cardiac function, blood pressure, renal function, lipid profile and hormone levels. No neurological, retinal or audiometry changes were demonstrable throughout the

trial.

2.3.1. Urinary Iron Excretion

Urinary iron excretion varied considerably from day to day in each patient(Table 2.3). The mean excretion on 85-120mg/kg daily ranged from 9.8 to 41.1 mg/24h(mean 23±11.2SD) (Table 2.3). There was a significant correlation between urinary iron excretion and L_1 dose when given at dose of 40-60mg/kg/d($p=0.023$) and when given at this dose but twice daily($p=0.049$)(Fig 2.1a&b). The correlation with serum ferritin was poor($p>0.1$). Prior to the trial the mean urinary iron excretion in these patients at a DFX daily dose of 40-50mg/kg was 22.8±18.3. The effect of the frequency of L_1 administration on UIE was variable. To evaluate it, the same daily dose of L_1 was given in 4 divided doses instead of 2. More frequent administration caused substantial increase in urine iron in patients 2,5 and 8(Fig 2.2).

2.3.2. Serum Ferritin

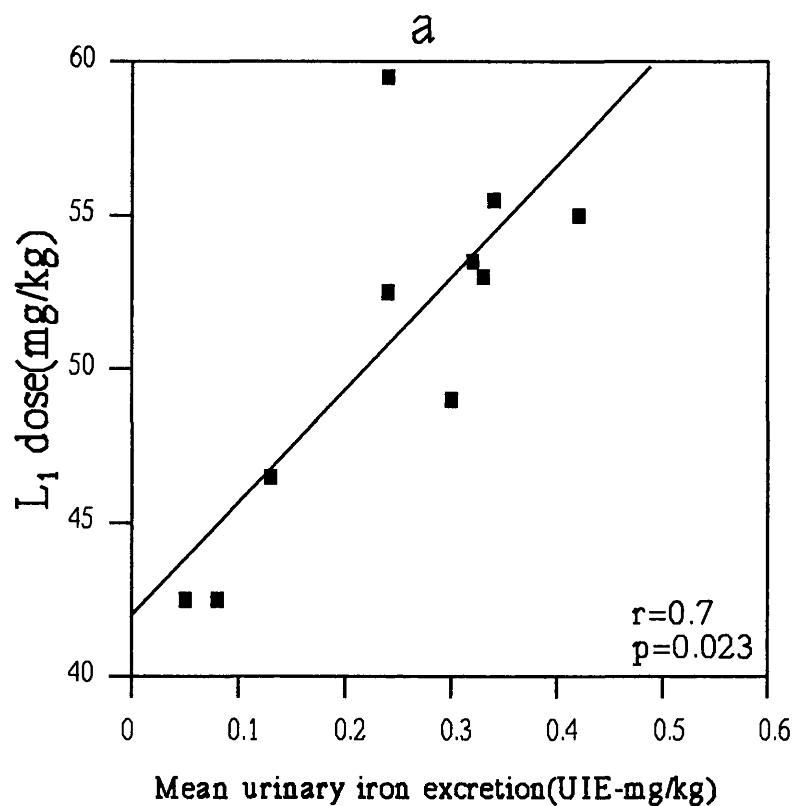
Serum ferritin values dropped in 7 of the 10 patients (1,3,6 & 8-11) by up to 42% of the original pre-trial values, rose by 43% in patient 2 and remained unchanged in patients 4 and 5(Fig 2.3). The mean serum ferritin at the beginning of the trial was 5549ug/l±3333(range=1000-9580) and at the end of the trial: 4126ug/l±2278 (range 738-7435). The overall decline in serum ferritin was significant using *t*-test for paired samples($p<0.05$).

2.3.3. Non-transferrin-bound iron(NTBI)

The initial NTBI values ranged from 3.6 to 9.0umol/l(6.1±1.6). The level fell in 8 patients after 3-6 months of L_1 therapy. The final values ranged from 3.8 to 5.4umol/l(4.5±0.5). This final mean is significantly lower($p<0.005$) than the initial mean NTBI. However both means(initial and final) were significantly higher ($p<0.001$, $p<0.05$

Table 2.3. Urinary iron and zinc excretion.

Case	Chelator	Dose(mg/kg)	No. of 24h urine collections	UIE(mean±SD) mg/24h	UIE(mean±SD) mg/kg/24h	UZE(mean±SD) umol/24h	UZE(mean±SD) umol/kg/24h
1	L_1	3gx2 (93)	6	18.1±5.9	0.28±0.09	11.5±1.6	0.18±0.03
		1.5gx4	3	9.6±8.4	0.15±0.13	7.3±2.2	0.11±0.03
	DFX	3g s/c	2	17.1	0.27	4.4	0.07
2	L_1	3gx2 (85)	8	9.6±5.7	0.14±0.08	22.6±7.6	0.32±0.11
		1.5gx4	4	45.3±18.1	0.64±0.26	42.1±17.8	0.60±0.25
	DFX	3g s/c	2	22.4	0.32	19.3	0.27
3	L_1	3gx2 (110)	7	28.1±11.2	0.52±0.21	13.0±4.5	0.24±0.08
		1.5gx4	5	26.1±8.9	0.48±0.16	35.4±9.5	0.65±0.17
	DFX	3g s/c	2	15.4	0.28	5.9	0.11
4	L_1	3gx2 (111)	8	37.1±10.5	0.69±0.19	23.4±5.4	0.43±0.10
		1.5gx4	5	29.1±6.7	0.54±0.12	18.6±2.6	0.34±0.05
	DFX	3g s/c	2	25.1	0.47	5.0	0.09
5	L_1	3gx2 (106)	5	13.7±11.9	0.24±0.21	15.8±4.7	0.28±0.08
		2gx3	10	20.7±11.6	0.37±0.21	14.1±4.5	0.25±0.08
		1.5gx4	4	29.9±3.9	0.53±0.07	17.1±1.4	0.30±0.03
	DFX	2.5g s/c	2	7.1	0.13	11.6	0.21
6	L_1	3gx2 (119)	7	41.1±7.0	0.82±0.14	12.3±1.3	0.24±0.03
		1.5gx4	4	31.7±3.9	0.63±0.08	9.8±2.0	0.19±0.04
	DFX	3g s/c	2	12.0	0.24	8.2	0.16
8	L_1	2.5gx2 (85)	9	11.4±4.2	0.19±0.07	9.4±1.8	0.17±0.03
		1.25x4	6	15.7±7.8	0.27±0.13	10.3±2.8	0.18±0.05
	DFX	2.5g s/c	2	35.2	0.60	5.7	0.10
9	L_1	2.5gx2 (105)	8	14.5±7.2	0.31±0.15	12.2±6.0	0.26±0.13
		1.25x4	6	15.6±1.5	0.33±0.03	15.7±6.3	0.33±0.13
	DFX	2.5g s/c	2	12.5	0.26	N.D	N.D
10	L_1	2gx2 (107)	10	16.1±6.0	0.43±0.16	4.7±2.8	0.13±0.08
	DFX	2.5g s/c	2	13.8	0.37	4.0	0.11
11	L_1	2gx2 (98)	5	33.4±14.3	0.82±0.35	10.9±8.5	0.27±0.21
	DFX	2g s/c	2	51.5	1.26	26.1	0.64



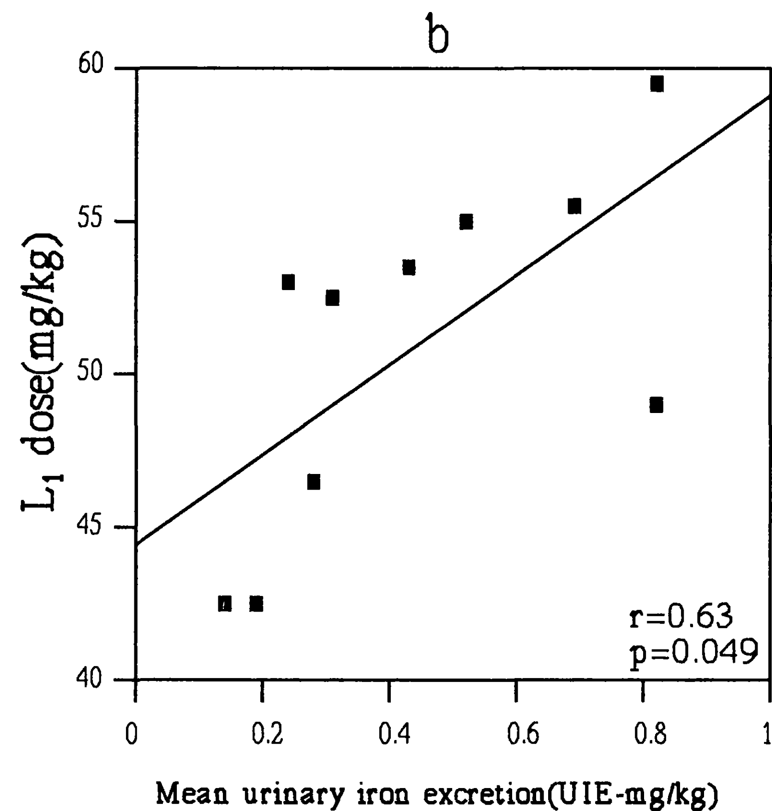


Fig 2.1 The correlation between L_1 dose (a-once daily, b-twice daily) and mean UIE

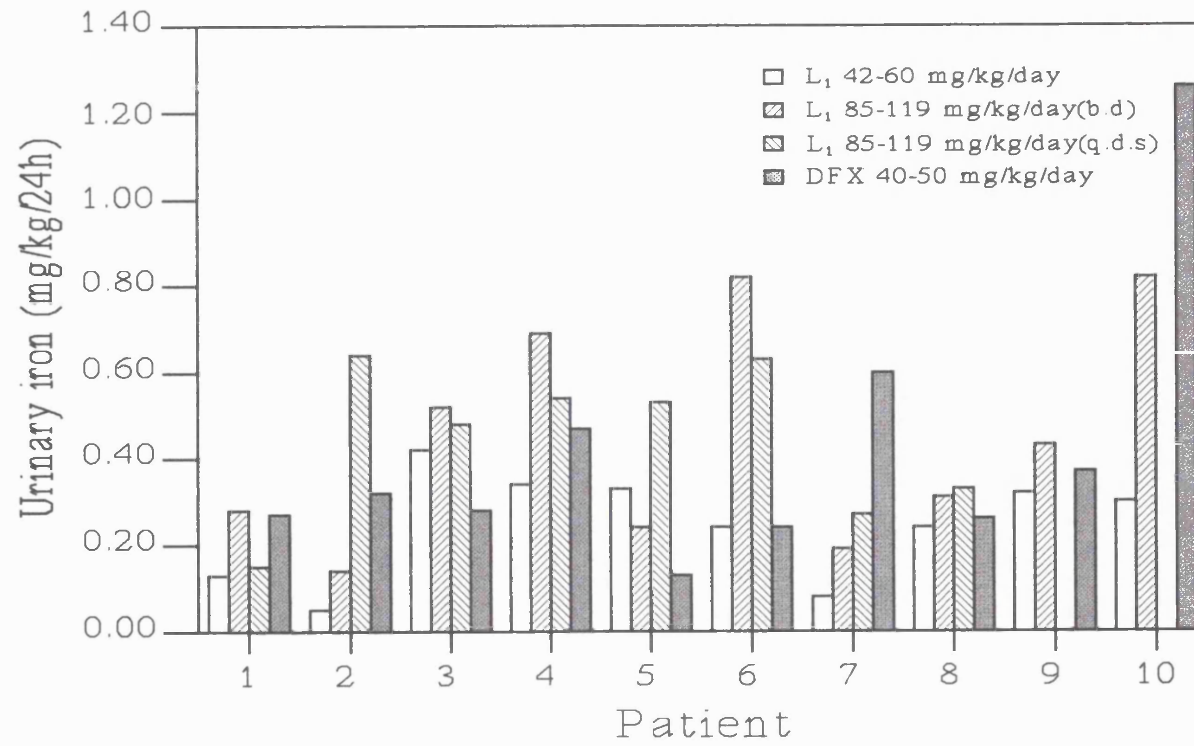


Fig 2.2. The effect of L_1 and desferrioxamine on UIE

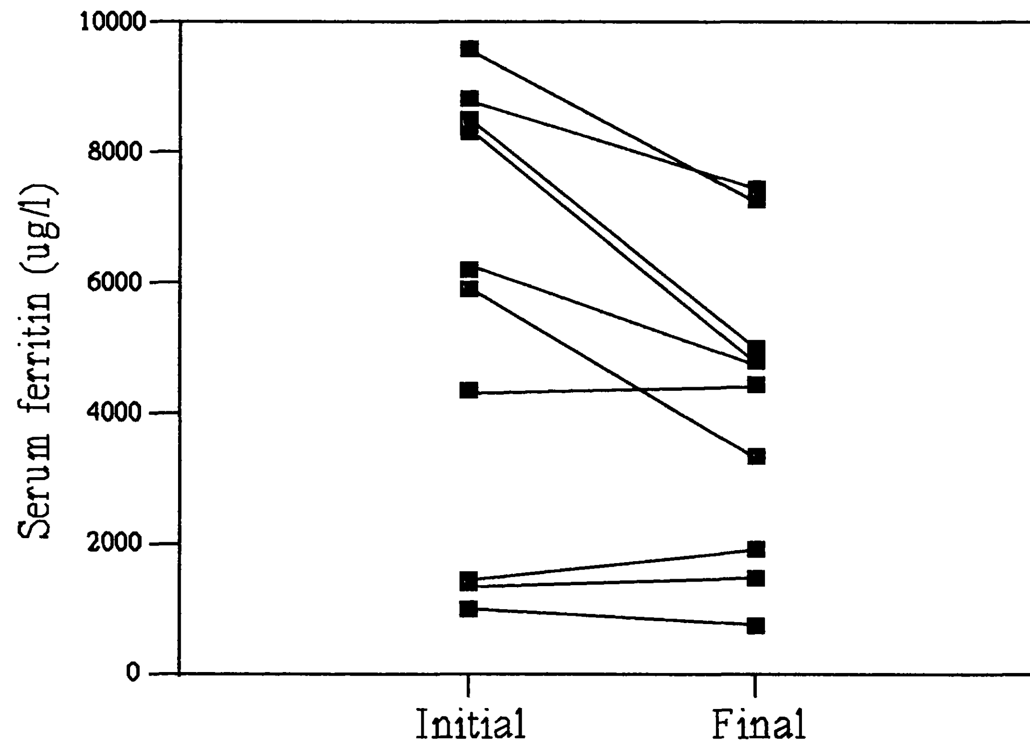


Fig 2.3. Initial and final(7-13 months) serum ferritin

respectively) than the mean NTBI (3.7±2.0) estimated in a group of 38 thalassaemia major patients receiving regular chelation with DFX.

2.3.4 Haematological Changes

All patients maintained normal blood count throughout the trial except patient 7 who developed agranulocytosis of sudden onset in the beginning of the twelfth week of the treatment and 6 weeks after being on a full dose(105mg/kg) of L_1. The patient was being seen once weekly and presented with generalised weakness, low grade fever and sore throat. The drug was immediately withdrawn and she was admitted to hospital and given intensive treatment with broad spectrum antibiotics. She showed progressive clinical improvement over the subsequent three weeks but her neutrophil count remained low($<0.5x10^9$/l) for 7 weeks(Chapter 8, Fig 8.1).

2.3.5. Liver Function

There was no significant difference(paired *t*-test:p>0.05) between serum AST levels at the start of the trial and those measured at the end of the trial. Three patients(3,4&6) showed a substantial drop in serum AST levels from a higher pre-trial values(Fig 2.4). Five patients(1,5,6,7&10) developed a transient fluctuating rise of serum AST shortly after starting the treatment with L_1. This eventually settled in all of them(Fig 2.5).

2.3.6. Serum and urinary zinc

Serum zinc fell in 4 patients to subnormal levels(<11.5umol/l)(Fig 2.6). This was accompanied by an increase(>9umol) in 24 hour urinary zinc excretion in 8 patients(Table 2.3). Two patients became symptomatic requiring treatment with zinc(see below). Urinary zinc excretion in the 10 patients ranged from 4.7-23.4umol/24h (13.1±5.5) (normal range <9umol/l). The correlation between L_1 total dose and urine zinc was poor(p>0.2). The effect of the frequency of L_1 administration on urinary zinc

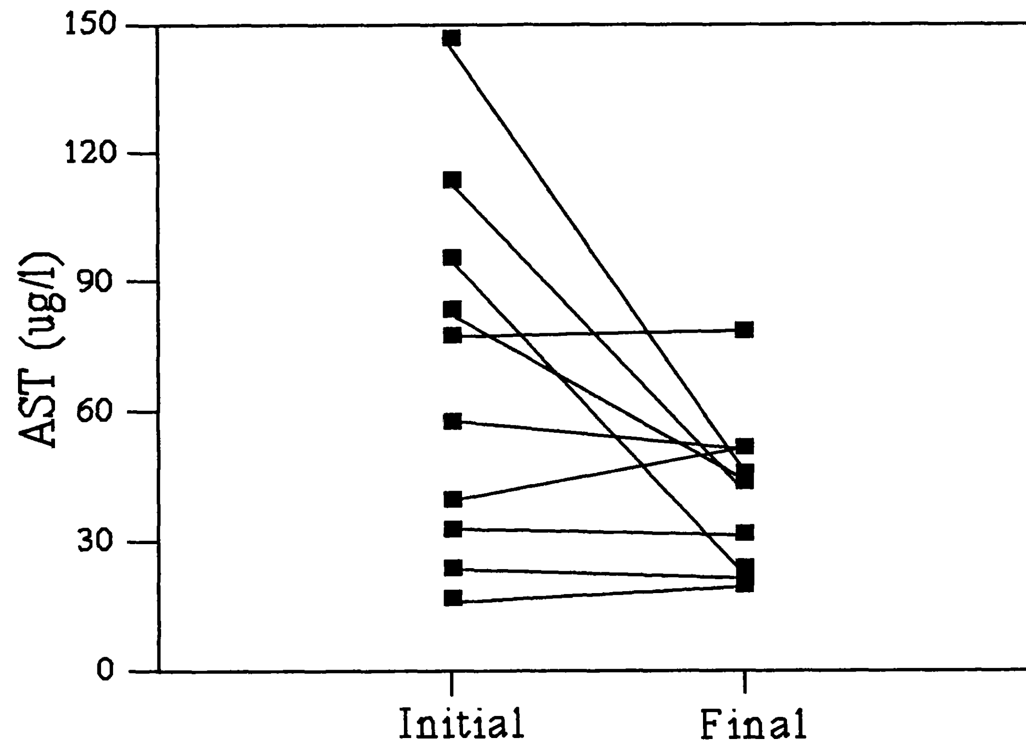


Fig 2.4. Initial and final (7 to 13 months) serum AST levels

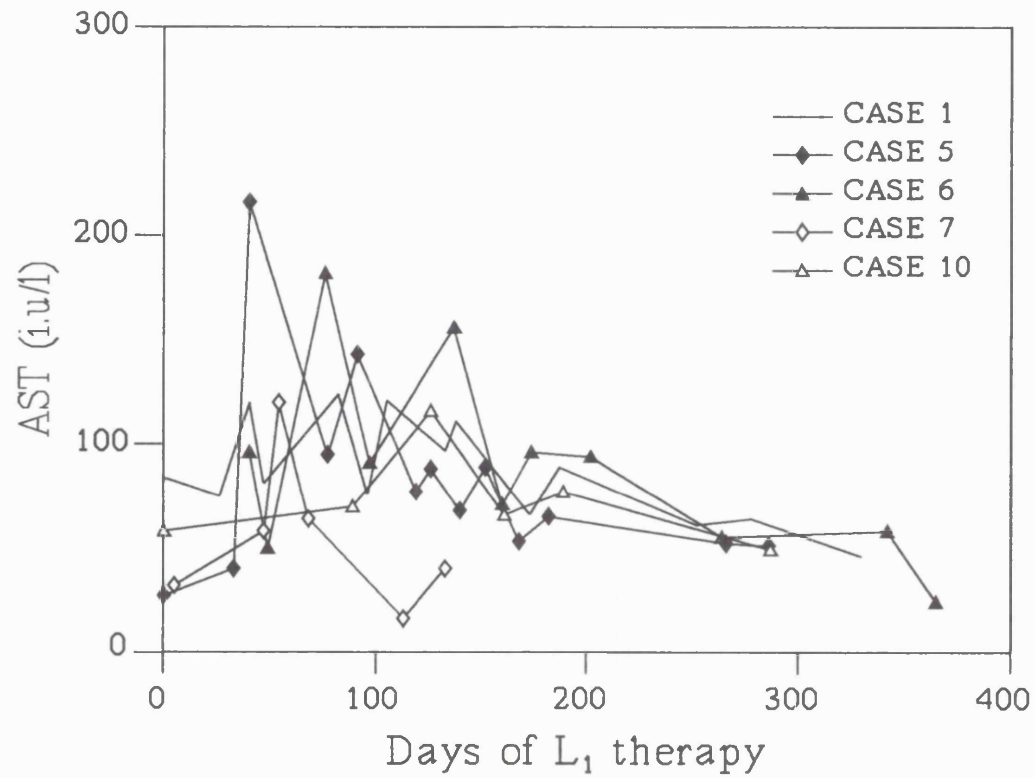


Fig 2.5. Transient and fluctuating increase of serum AST in five patients receiving L_1 therapy

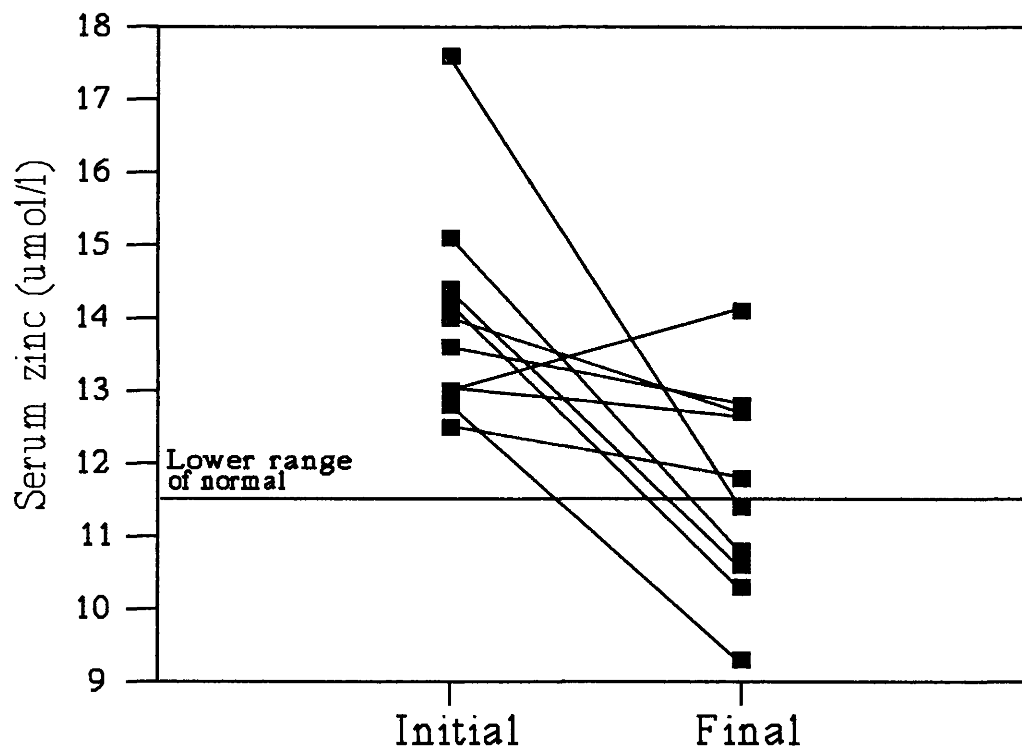


Fig 2.6. Initial and final (7 to 13 months) serum zinc levels

excretion in the individual patient was similar to that observed for urinary iron excretion(Table 2.3). It is of interest that in 3 patients urinary zinc excretion also increased above 9umol when DFX was used by these patients prior to the trial(Table 2.3).

2.3.7. Leucocyte ascorbate levels

Seven patients(1,3-6,9&10) showed a fall in WBC ascorbate level after variable periods(3-5 months) from the commencement of treatment with L_1. None became symptomatic as all were restarted on vitamin C tablets 200mg/day. No significant change in urinary iron excretion was observed in these patients receiving vitamin C therapy.

2.3.8. Gastrointestinal symptoms

The drug was generally well tolerated by the patients. Three patients(3,7&9) however developed nausea on starting the treatment or on increasing the dose of L_1 which lasted for a few days only and required no treatment. One patient complained of loose bowel motions beginning one week after the commencement of L_1. Stools were negative for microorganisms and the problem settled spontaneously six weeks later.

2.3.9. Musculoskeletal symptoms

Three patients(1,6&9) had some form of musculoskeletal symptoms. Patient 1 complained of generalised muscular stiffness, worse in the morning, which began four months after starting treatment and lasted for six weeks and then resolved spontaneously. This patient was negative for RhF and ANF throughout the trial. Patient 6 developed two episodes of generalised musculo-skeletal pain with low grade fever and epigastric pain. The first began four weeks after starting L_1 and the second two weeks later. Each episode lasted for 24 hours only and resolved spontaneously leaving no residual symptoms. RhF was positive at the outset of the trial with a titre of 1/320, which has dropped six months

later to 1/80. Antinuclear factor was negative in the first 8 months and has then become weakly positive with a titre of 1/40. Patient 9 developed generalised muscular pain 9 months after starting the treatment which got progressively worse over the subsequent 4 weeks. When L_1 was temporarily withheld the pain disappeared completely within 48 hours and reappeared but to a lesser degree when L_1 was restarted at a full dose. RhF and ANF were negative throughout the trial.

2.3.10.Autoantibodies

At the beginning of the trial two patients showed positive serum RhF(titres: 1/80, 1/320) and two patients had positive serum ANF (titres: 1/160, 1/40). At the end of the trial, the incidence and titre of RhF was unchanged; four patients were found to have positive ANF (titres: 1/320x1, 1/40x3). To assess the significance of these results, an unselected group of patients with TM(n=51) were checked for the presence of these autoantibodies. Nine(17.6%) were positive for RhF but none were ANF positive.

2.3.11.Dermatological changes

Nine patients were heavily pigmented prior to the start of the trial. This was partly secondary to iron overload. In 7 of them there was a substantial decline in skin pigmentation with noticeable lightening of skin colour. This occurred mainly in the first 3 months of treatment. Two patients(1&2) developed skin eruption on the face and shoulders resembling folliculitis. Microbiological tests on swabs taken from these lesions were negative in both. The eruption lasted for 6 weeks in patient 1 and 2 weeks in patient 2 and required no treatment. Four patients(3,6,7 & 9) complained of dry skin. This was accompanied by hair loss in patients 3 and 9 and later by dry and itchy skin patches in the latter. This was associated with low serum zinc levels in these patients(see above). They were started on Zn tablets 220mg(50mg zinc) daily which was associated with

improvement in the skin lesions within 4 weeks.

2.4. DISCUSSION

In this trial the long term use of L_1 in high dose(up to 120mg/kg/day) in patients with beta thalassaemia major was tested. The drug was generally well tolerated with no detectable changes in renal, cardiac, respiratory, retinal or auditory function.

The urinary iron excretion results are consistent with previous reports(Kontoghiorghes *et al* 1990b,Töndury *et al* 1990, Olivieri *et al* 1990a&b). The important determinant of iron excretion was the total L_1 dose. The mean urinary iron excretion at an L_1 dose of 85-120mg/kg is comparable to that achieved with DFX at a dose of 40-50mg/kg(23.0±11.2 *vs* 22.8±18.3) . However some patients achieved iron excretion comparable to that with DFX at similar doses of the two chelators(Fig 2.2). Similar findings have previously been reported(Kontoghiorghes *et al* 1987a&b, Kontoghiorghes *et al* 1990b, Olivieri *et al* 1990a). In the latter study the mean urinary iron excretion was lower with L_1 therapy than with subcutaneous DFX(12.3±6.7 *vs* 18.2±15.3mg/day) at an L_1 or DFX daily dose of 50mg/kg. When the L_1 dose was raised in 5 patients to 75mg/kg/day; mean urinary iron excretion rose from 13.8±7.0 to 26.7±17.8mg/day comparable with that with DFX(50mg/kg/day) 24.9±24.3mg/day (Olivieri *et al* 1990a).

The effect of frequency of L_1 administration on iron excretion in this trial was variable. An increased frequency of smaller doses with the same total daily dose caused a substantial increase in UIE in 3 patients but had little or an opposite effect in the other 7 patients. It remains to be determined whether more frequent L_1 dosing with the resultant lowering of serum peak levels of the drug would also lead to fewer side-effects. Although a correlation between L_1 dose and UIE was observed(Fig 2.1), this was not studied in a single patient and hence does not represent a proper dose/response study. Consequently, UIE in these patients could have been influenced by other factors such as serum ferritin concentrations.

The percentage of patients having a decline in serum ferritin level was higher than what

has previously been reported. Kontoghiorghes *et al*(1990b) reported no overall change in serum ferritin over the period of their study(1-15 months) in 13 patients receiving various doses of L_1. Töndury *et al*(1990) reported a decline in serum ferritin in 3 of the 8 patients who received L_1 at a daily dose of 55-80mg/kg for 4-10 months. It is likely therefore that the high L_1 dose used in this trial for a relatively long period is responsible for this result. There was also a significant fall in the patients' non-transferrin bound iron(NTBI) levels. The final mean NTBI was, however, still significantly higher than that estimated in a comparable group of DFX-treated patients. Further studies are therefore necessary to determine whether more prolonged chelation with L_1 will achieve NTBI values similar to those achieved by prolonged chelation with DFX.

The musculoskeletal adverse effects observed during the course of the trial were mild and transient. These resembled those previously reported by Bartlett *et al* 1990 and Agarwal *et a*l 1990. The changes in the liver enzyme AST were also transient and settled without discontinuation of L_1 therapy. Similar changes have not previously been reported. Bartlett *et al* (1990) reported a rise in AST in one of their patients receiving L_1. This, however, was secondary to alcohol consumption and settled on abstinence.

The occurrence of agranulocytosis in one patient during the early stage of the trial highlights the potential toxicity of this drug. Several experiments have been undertaken to elucidate the role of L_1 in the pathogenesis of agranulocytosis in this patient(Chapter 8). These studies did not show the presence of anti-neutrophil or anti-myeloid precursor antibodies in the blood of the patient during agranulocytosis or convalescence. This was the second case of L_1-induced agranulocytosis to be reported. The first was a woman of 28 with Blackfan-Diamond anaemia who developed agranulocytosis 6 weeks after receiving L_1 at a dose of 105mg/kg. She had previously received the drug for 5 months,

largely at the lower dose of 50mg/kg. Similarly in that case no evidence for L_1-induced anti-neutrophil or anti-myeloid antibodies was found nor were the marrow myeloid progenitors particularly sensitive to L_1(Hoffbrand *et al*, 1989).

Although L_1 is an avid iron chelator with a high binding constant, it can, nevertheless, bind other cations although with a much lower affinity. No change in serum zinc levels or increase in urinary zinc excretion in patients receiving L_1 has been reported previously. In the present trial, however L_1 administration was associated with a fall in serum zinc levels in 4 of the patients. There was an increase in urinary zinc excretion in 8 of the patients and symptoms attributable to zinc deficiency in 2 of them. These symptoms improved after zinc administration. However the correlation between the amount of zinc excreted in urine and the severity of serum zinc decline was poor. Zinc excretion also increased when subcutaneous desferrioxamine was given to these patients at a daily dose of 40-50mg/kg prior to the trial and by a large group of patients(n=39) with thalassaemia major receiving subcutaneous DFX(Chapter 9). However none of the L_1-treated patients had low serum zinc levels prior to the trial and only 8 of the 39 non-L_1 patients showed subnormal serum zinc levels and none had symptoms attributed to zinc deficiency.

CHAPTER 3

CLINICAL TRIAL(2)

3.1. INTRODUCTION

L_1 effectiveness has now been shown in many reports (Chapters 1,2, Kontoghiorghes *et al*, 1990b; Olivieri *et al,* 1993, 1995; Agarwal *et al,* 1993) to be comparable to that of DFX. However, long term use of L_1 has been associated with adverse effects the most important of which are agranulocytosis and joint toxicity. A longer term trial was, therefore, needed in order to establish the incidence of adverse effects and further assess L_1 efficacy. In June 1992 a third long term trial of L_1 in patients with iron overload due to various aetiologies was launched at the Royal Free Hospital. In this chapter the results of the first 2 years of this trial are reported.

3.2. PATIENTS AND METHODS

This study had the approval of the Royal Free Hospital Ethical Committee. Initially 52 patients were entered into the trial after giving their written informed consent. Subsequently 14 patients had to be taken out of the trial(in 9 before 6 months) because of unacceptable adverse effects(Table 3.1).

Table 3.1. Causes of trial termination in 14 of 52 patients with iron overload receiving L_1 therapy.

Cause	Number of patients	Duration on L_1 (months)	Incidence
Nausea	5	3.9±4.6	9.6%
Non-compliance	3	9.0±7.8	5.8%
Neutropenia(<1.5x10^9/l)	2	14,19	3.9%
Arthritis	1	7	1.9%
Arthritis/Neutropenia	1	12	1.9%
Tachycardia	1	4	1.9%
Agranulocytosis	1	1.5	1.9%

Forty three patients received L_1 for ≥6 months and their clinical details are summarized in Table 3.2.

Table 3.2. Clinical details of patients who received L_1 therapy long term($\geq$6 months).

Number of patients (long-term)	43
Age	27.7±14(13-81)y
Sex	26♂, 17♀
Diagnosis	TM(32), SS(3), CSA(2), PKD(2), MDS(2), HbE/βthal(1), S/βthal(1)
Initial serum ferritin	4276±3031(976-13900)ug/l
Initial AST level	50±34(16-175)i.u/l
HCV status	7 with positive anti HCV test

TM=thalassaemia major, SS=sickle cell disease, CSA=congenital sideroblastic anaemia, PKD=pyruvate kinase deficiency, MDS=myelodysplastic anaemia, HCV=hepatitis C virus.

L_1 was initially given at an oral dose of 83.8±17.1(45-109)mg/kg/day for 12.1±6.1 months then reduced to 69.1±16.8(30-96)mg/kg/day for the rest of the trial duration. The initial dose was chosen lower than the dose used in the previous trial in an attempt to reduce the incidence of myelotoxicity, assuming that this adverse effect was dose-dependent. However, when side effects were still observed the dose was further reduced to the final one. The total duration of L_1 therapy at any dose ranged from 6 to 27(17.7±6.3)months.

L_1 was obtained from different sources. From October 1992 to February 1993 it was synthesised at the Royal Free Hospital by a previously established technique (Kontoghiorghes & Sheppard, 1987) and was obtained from Siegfried AG, Switzerland from February 1993 to September 1993, and from Lipomed, Switzerland from September 1993 to May 1994 and from Vitra Pharmaceutical, U.K. from June 1994 onward.

Patients were initially seen weekly for 12 weeks and had their full blood count and liver function intested and subsequently they were seen monthly. The investigations performed during the trial are listed in Table 3.3.

Table 3.3. Investigations performed initially and during L_1 trial.

Investigation	Frequency
FBC	weekly in first 12 weeks then monthly
LFT	= = = = = = = = = = = = = = = =
UIE, UZE	= = = = = = = = = = = = = = = =
U/E	monthly
Serum ferritin, iron, TIBC	2 monthly
RhF, ANA, dsDNA, AHA,	3 monthly
CD4/CD8 ratio	= = = = =
Serum zinc	= = = = =
WBC ascorbate	= = = = =
MUGA scan	initially then 2 yearly
GTT	= = = = = = = = =

FBC=full blood count, LFT=liver function tests, UIE=24h urine iron excretion, UZE=24h urine zinc excretion, U/E=urea, creatinine and electrolytes, TIBC=total iron binding capacity, RhF=rheumatoid factor, ANA=antinuclear antibody, dsDNA=anti-double-stranded-DNA antibody, AHA=anti-histone antibody, MUGA=multigated acquisition scintigraphy, GTT=glucose tolerance test(using 75g of glucose).

The reason for choosing these investigations was as follows: FBC and LFT, to monitor L_1-induced myelotoxicity and changes in AST respectively, observed in the previous trial. Iron studies and UIE to monitor L_1 effectiveness. Serum zinc and UZE to monitor changes in zinc status observed previously. Immunological studies particularly, ANA, dsDNA and AHA were performed because SLE-like syndrome was claimed to be induced by L_1 in a previous report(Mehta *et al*, 1991). MUGA scan and GTT to observe whether L_1 has an effect on iron-induced cardiac toxicity and changes in GTT. Serum ferritin was measured using an ELISA technique(Flowers *et al*, 1986). Full blood count(FBC), serum iron, TIBC, LFT, U&E, autoantibodies(RhF, ANA, dsDNA, antihistone-AHA), CD4/CD8 ratio and Multigated acquisition(MUGA) scan were performed using standard methods. WBC ascorbate was measured as described by Denson and Bowers(1961). Oral glucose tolerance tests was performed by administering 75g of glucose after an overnight fast and sampling blood at zero time and every 30min

for two hours. Serum zinc and urine iron and zinc were measured using atomic absorption spectroscopy. Statistical analysis is performed using Student's *t*-test. Values are expressed as mean±SD.

3.3. RESULTS

In general the drug was well tolerated with good compliance. No change was noted in neurologic, cardiac, renal or immunologic functions of the patients throughout the trial. Forty three patients have received the drug long term(>6months) and their data are analysed below.

3.3.1. Serum ferritin

The final mean serum ferritin(3657±2479ug/l) was significantly lower than the initial mean serum ferritin(4276±3031ug/l) using the paired *t*-test(p=0.038) in the 43 patients who received L_1 long term. Serum ferritin concentrations fell significantly in 29 patients from an initial mean of 5000±3350ug/l to a final mean 3498±2471ug/l. In the remaining 14 patients a rise in serum ferritin was observed(initial mean=2687±1385ug/l, final mean=3450±1678ug/l).

3.3.2. Urinary iron excretion

L_1-induced UIE(mean of initial 4 collections) in all the patients ranged between 5.3-66.8 (29.6±15.2)mg/24h significantly higher than that induced by 40-50mg of subcutaneous DFX(23.2±10.0mg/24h, p=<0.05). No correlation was found between L_1 dose and UIE. However in a significant number of patients a dose in excess of 60mg/kg/day was required in order to induce a UIE in excess of 0.5mg/kg/day to result in a negative iron balance, assuming patients transfusion requirement is 1ml/kg/day of whole blood(Fig. 3.1).

At the beginning of the trial the effect of food, the co-administration of vitamin C(200

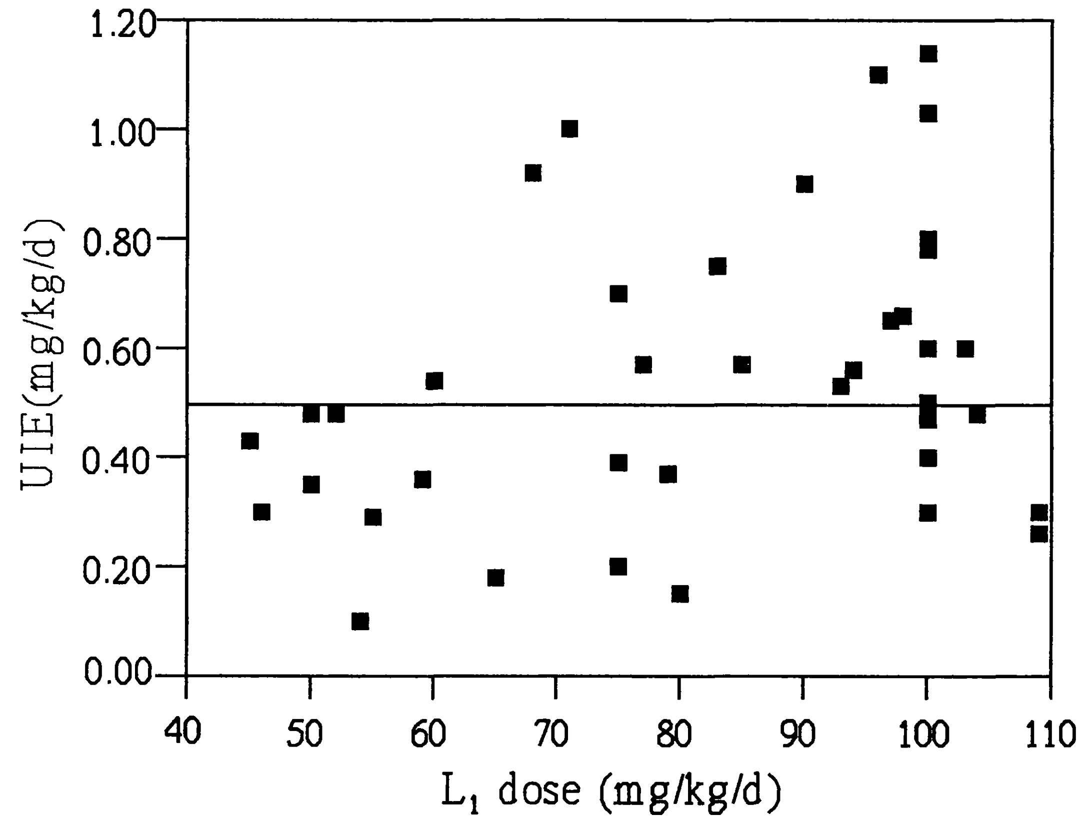


Fig 3.1. UIE versus L_1 dose in 43 patients with iron overload

mg/day) and of giving the total dose of L_1 in four divided doses instead of two on UIE was examined(Fig. 3.2). No significant differences were found between these means. However all of them were significantly higher than the mean UIE induced by 40-50mg/kg of DFX($p<0.05$).

3.3.3. AST fluctuation

No significant difference was observed between the initial and final mean AST levels(initial: 50.0±34.2, final: 44.4±25.3, $p>0.05$). However 12 patients developed a rise or fluctuation in serum AST levels within 2.9±3.5(0.25-12)months of starting L_1 therapy. In 11 patients AST levels settled within 4.5±3.4months of onset, spontaneously and without altering L_1 dose. In one patient AST levels are still unsettled. He is positive for HCV test.

3.3.4. Joint symptoms

Eleven patients developed joint problems. Two patients had to be taken out of the trial because of severe arthritis affecting both knees in one and both ankles in the other(Table 3.1). The first patient continued to complain of pain and swelling of both knees despite being off L_1 for 12 months. Clinical, X-ray and MRI examination of his knees were consistent with the diagnosis of chondromalacia patellae. The other patient became asymptomatic within 2 weeks of stopping L_1. During the trial 9 more patients developed joint manifestations. The knees were involved in 8 patients, the ankles in 2 and interphalangeal joints in one. Joint involvement manifested as pain only in 7 patients and with swelling in the other 4. L_1 dose, serum ferritin and UIE were higher in patients with joint problems than those without. However, these differences were not statistically significant. Clinical details of these patients are shown in Table 3.4.

In the 9 patients who continued L_1 therapy symptoms subsided, in 3 spontaneously

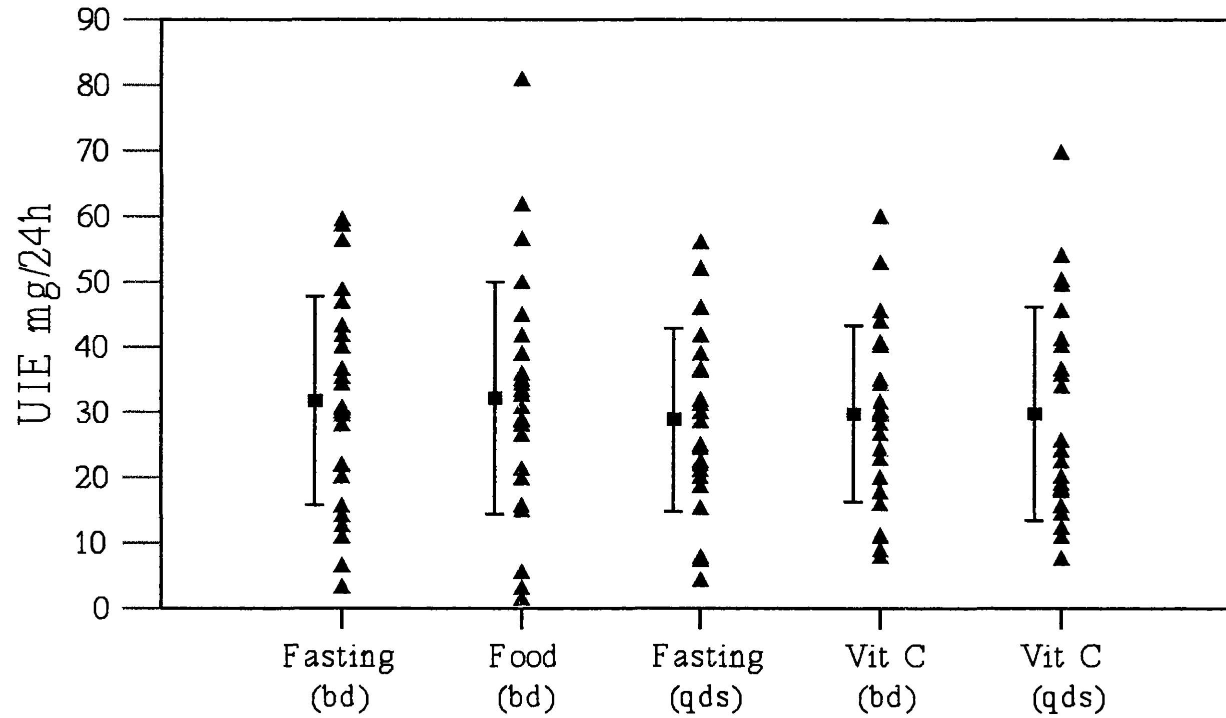


Fig 3.2. The effect of food, the co-administration of vitamin C (200mg/d) and of the frequency of L_1 administration on UIE

despite L_1 continuation, in 3 after temporary withdrawal of L_1, and in 2 after reducing L_1

Table 3.4. Details of patients with Joint symptoms whilst receiving L_1 therapy.

Incidence	11/43(25.6%)*
Age	31.6±11.2y (vs 29.9±14.6)**
Sex	6♂, 5♀
Time of onset	8.2±6.0(0.5-18)months
L_1 dose	86.9±20.5mg/kg/day (vs 82.2±19.5)**
Serum ferritin	3782±2218ug/l (vs 3640±4041)**
UIE	0.68±0.31mg/kg/day (vs 0.52±0.34)**

*the incidence is in 43 patients who received L_1 long term.
**values for unaffected patients are given in parenthesis for comparison. They are not significantly different($p>0.05$) from those of the affected patients.

dose. In the ninth patient milder symptoms persisted despite discontinuation of L_1 for 3 months. He was then restarted L_1 on a reduced dose(55mg/kg/day) with no worsening of symptoms.

3.3.5. Gastrointestinal symptoms

Six patients developed nausea and anorexia within one week of commencing L_1 therapy. This was severe in 5 patients and as a result they had to be taken out of the trial(Table 3.1). One of these 5 patients aged 81 with myelodysplasia was found to have impaired renal function with accumulation of L_1-glucuronide, the principle metabolite for L_1, in his blood. When this patient was kept off the drug for a few days his serum L_1-glucuronide fell to undetectable levels and his tolerance to L_1 improved only to deteriorate after a few days of L_1 therapy(Chapter 4). In the other patient with mild nausea, symptoms disappeared within one week.

3.3.6. Autoantibodies

Initially 5/43 patients were positive for RhF (2x1/160, 3x1/40) and finally 3/43 were positive (3x1/40). Initially 2/43 patients were positive for ANA(2x1/80) and finally 3/43 were positive(2x1/80, 1x1/160) No change was noted in the incidence or titre of dsDNA

or AHA.

3.3.7. Zinc deficiency

L_1-induced 24 hour urinary zinc excretion was increased to 15.1±7.3(4.4-34.2)umol significantly higher than that associated with DFX therapy(11.1±6.0umol, $p=0.01$) and both were significantly higher than the normal range for UZE ($p<0.001$, $p=0.04$ respectively). The increase in zinc excretion was found to be related to the patient's blood glucose status(Chapter 9). The initial mean serum zinc concentration in 43 patients was not significantly different from the final mean serum zinc concentration. However, in 10 patients a fall in serum zinc level was observed from an initial 13.1±1.7umol/l to a final 9.6±0.9umol/l. Six of these patients had abnormal glucose tolerance and four have diabetes mellitus. This fall in serum zinc level was associated in one patient with symptoms of dry skin and itchy skin patches requiring treatment with oral zinc sulphate.

3.3.8. Haematologic changes

All patients maintained normal blood counts throughout the trial except four. One patient of 63 with myelodysplasia developed agranulocytosis six weeks after the commencement of L_1 therapy at a daily dose of 78mg/kg. The patient presented with sore throat, fever and generalised weakness. His neutrophil count was $0x10^9/l$. He recovered one week after L_1 withdrawal and the commencement of G-CSF. In another three patients a fall in neutrophil count($<1.5x10^9/l$) during L_1 therapy was observed. L_1 withdrawal was followed, within 2 weeks, with complete normalisation of neutrophil count. Re-challenge of these three patients with L_1 was associated with similar fall in neutrophil count after 4 weeks which again recovered rapidly after stopping L_1(Chapter 8).

3.3.9. Death

One patient, a man of 63 with myelodysplasia, died of intractable heart failure and

arrythmia 2 months after the discontinuation of L_1 therapy. L_1 therapy had previously been discontinued after 6 weeks because of the development of agranulocytosis and septicaemia(see above). Postmortem examination revealed cardiomyopathy secondary to iron overload as the cause of death.

3.4. DISCUSSION

The data confirm previous studies showing that L_1 is capable of inducing significant UIE comparable to that induced by therapeutic doses of subcutaneous DFX. However, in this study and in contrast to the previous one(Chapter 2), no correlation was found between L_1 dose and UIE. This maybe due to the fact that larger number of patients with wider range of serum ferritin and hence iron load were studied this time and this might have skewed the correlation between L_1 dose and UIE.

Despite the lack of significant difference in the mean UIE whether L_1 is given with food or without with vitamin C or without or in 2 or 4 daily subdoses, there were individual variations. Therefore in individual patients these different regimes have to be tried if maximum UIE is to be achieved.

A substantial fall in serum ferritin was observed in 29 patients. Although a significant overall fall in serum ferritin using the paired *t*-test was found, no significant difference between the initial and final mean serum ferritin concentrations was observed. This lack of significant overall fall in serum ferritin, in contrast to the results from previous trials is due to the rise in serum ferritin observed in 14 patients during the trial which is attributed to poor compliance in 6 and lack of effectiveness(low UIE) in 2. In the other 6 patients no clear cause was found, however 4 of them were HCV positive with marked fluctuation in serum AST levels.

Agranulocytosis in a patient with myelodysplasia and neutropenia in 3 with thalassaemia major have been observed during this trial bringing the total number of cases reported in the literature to 9(4 agranulocytosis and 5 neutropenia) with 2 additional unreported cases(Olivieri 1992 personal communication). The mechanism underlying this problem remains obscure. No evidence for an increased L_1 toxicity to normal or patients

myelopoiesis has been elicited using liquid or semisolid myeloid progenitor cultures(Chapter 8).

Joint symptoms were first described as an adverse effect of L_1 by Bartlett *et al*(1990). The incidence of joint toxicity found here(25.6%) is similar to those found in the previous trials(Chapter 2; Bartlett *et al,* 199; Agarwal *et al,* 1992; Berkovitch *et al,* 1994). The mechanism of this adverse effect remains obscure. Although there was a slight increase in the incidence of ANA no correlation was observed between this and the joint manifestations. No change in the incidence or titre of AHA or dsDNA was observed throughout the trial. Similarly, Fassos *et al*(1993) observed no change in the incidence or titre of these two antibodies in their long-term clinical trial of L_1. The recent suggestion by some workers(Berkovitch *et al*, 1994) that it may be due to partial L_1-Fe complexes(2:1, 1:1) remains to be substantiated. Agarwal *et al*(1992) reported that patients with higher iron load and receiving larger dose of L_1 are more prone to joint problems. This is not substantiated by this study as there was no significant difference in the L_1 dose or iron load between those with and without joint toxicity(Table 3.4).

Although the incidence of gastrointestinal symptoms in this trial is similar to previous reports, its severity was more than previously reported. As a result of this five of six patients with gastrointestinal symptoms had to be taken out of the trial at 3.9±4.6 months. A correlation between the accumulation of L_1-glucuronide in serum and severe nausea is suggested by observations on the oldest patient in this study(male, 81y) who had impaired renal function(Chapter 4). Furthermore, a similar problem of severe nausea has recently been observed in an elderly patient with myelodysplasia, iron overload and impaired renal function, receiving L_1 therapy. His L_1-glucuronide clearance has not been determined yet but is likely to be impaired as a result of his renal impairment.

CHAPTER 4

PHARMACOKINETICS

4.1. INTRODUCTION

Any new drug such as L_1 requires a detailed pharmacokinetic profile in order to assist in designing the optimum regimen for drug administration. Furthermore, drug pharmacokinetics help to evaluate the effect of various parameters such as bioavailability, speed of absorption, metabolism, and elimination on the efficacy and possible side effects of the drug. Such studies are also important to assess the pharmacokinetics of drug metabolites and their possible effects on the efficacy and toxicity of the drug. Only a few small studies on L_1 pharmacokinetics have now been published(Kontoghiorghes *et al* 1990a&b, Olivieri *et al* 1990a, Matsui *et al* 1991), but no similar reports on the pharmacokinetics of L_1G have so far emerged. In none of the previous studies has the relation between the pharmacokinetics of L_1 or L_1G and the renal function of the patients been determined. In this study the pharmacokinetics of both L_1 and L_1-glucuronide in 24 patients with iron overload were examined. Correlations between various pharmacokinetic parameters and L_1 efficacy and renal function have been explored.

4.2. PATIENTS

This study had the approval of the Ethical Committee of the Royal Free Hospital. Twenty four patients were included. Their clinical details are summarized in Table 4.1. L_1 was withheld for 24 hours prior to the study in the 17 patients who had already received L_1(50-100mg/kg/day) for durations ranging from 4-32(16.9±10.3) weeks (Table 4.1). L_1(approximately 50mg/kg) was given orally after an overnight fast. Blood samples were collected at regular intervals(0, 10, 20, 30, 45, 60, 75, 90, 120, 180, 240, 300, 360 minutes) and serum was immediately separated and stored at -20°C until the time of analysis. Twenty four hour urine collections were obtained simultaneously from 14

Table 4.1. Clinical details of 24 patients with iron overload.

Case	Age(y) /sex	Diagnosis	Duration on L_1 prior to study(weeks)	Serum ferritin (ug/l)	Transferrin saturation (%)	Serum creatinine (umol/l)	Creatinine clearance (ml/min)	Serum AST (iu)	UIE (mg/ 24h)
AA	21/F	BTM	0	8130	95	76	54	31	16.0
AD	23/F	BTM	10	7400	83	82	ND	61	ND
AE	22/M	BTM	32	2127	97	52	ND	15	ND
AM	13/M	CSA	0	3131	95	62	76	23	30.3
BJ	60/F	SS	0	4000	100	130	35	33	9.2
CH	35/M	BTM	22	2922	90	46	ND	239	ND
CSo	29/M	BTM	30	2108	85	60	99	26	ND
CSt	81/M	MDS	11	5650	83	168	24	42	12.5
DI	15/M	SS	9	5950	36	78	90	58	8.1
EM	26/M	BTM	9	2393	87	78	ND	92	ND
FM	25/M	BTM	32	1365	63	72	ND	20	ND
FV	20/M	BTM	28	4122	100	58	ND	128	ND
GZ	21/M	BTM	16	7875	100	66	ND	69	ND
JT	46/M	CSA	12	2055	82	66	89	35	17.3
LL	26/M	BTM	4	9060	79	88	96	104	11
MH	17/M	PKD	0	3050	94	73	128	55	32.7
MM	22/M	BTM	28	3350	100	65	ND	49	ND
MS	23/M	BTM	8	3520	100	82	114	56	16.5
PD	27/F	BTM	25	3980	100	64	57	41	ND
SA	30/F	BTM	6	3850	100	61	100	49	8.9
SB	43/F	MDS	0	1285	83	78	78	36	9.8
SG	15/M	BTM	0	1320	100	57	124	22	4.7
TR	51/M	PKD	0	3131	96	92	87	28	21.2
TT	31/F	BTM	6	4000	88	80	84	68	7.3
mean±D	30.1±16.0		16.9±10.3*	3991±2236	89.0±14.7	76.4±25.7	83.4±29.3	57.5±47.5	14.7±8.4

ND=not done, AST=aspartate transaminase, UIE=urine iron excretion, BTM=β-thalassaemia major, CSA=congenital sideroblastic anaemia, SS=sickle cell disease, MDS=myelodysplastic syndrome, PKD=pyruvate kinase deficiency. *Mean±SD of durations >0.

patients; in 8 of them urine was collected at intervals (2, 4, 6, 12, 24 hours), whereas the other 6 produced one single 24 hour collection.

4.3. MATERIALS AND METHODS

L_1 was synthesised at the Royal Free Hospital as described previously(Kontoghiorghes & Sheppard 1987). L_1 and L_1-glucuronide(L_1G) concentrations were estimated in serum samples and urine using a previously described high pressure liquid chromatography(HPLC) technique(Goddard & Kontoghiorghes 1990) with the following modification: Amicon Centrifree filters were used instead of perchloric acid for protein removal from the serum samples. When serum samples were analyzed using either Amicon filters or perchloric acid no significant difference was found. Iron in 24 hour urine samples was estimated using atomic absorption spectrophotometry. Serum ferritin was assayed by an ELISA technique(Flowers *et al* 1986). Transferrin saturation, serum creatinine levels and creatinine clearance were measured using routine laboratory techniques. $T_{1/2}$ of absorption and elimination($abt_{1/2}$, $elt_{1/2}$), areas under the curve(AUC) of L_1 and L_1G concentrations versus time and $t_{1/2}$ of L_1G appearance($appt_{1/2}$) in the blood were calculated using a computer programme specially designed for pharmacokinetic analysis(Johnston & Woollard 1983). Data are expressed as range and mean±SD.

4.4. RESULTS

All patients but one had undetectable serum levels of L_1 or L_1G in their base line serum samples. The exception, a man of 81 with myelodysplastic syndrome, was found to have a high base line serum level of L_1G(346umol/l) and a maximum concentration(C_{max}) of 698umol/l observed 4 hours following L_1 administration(Fig 4.1a). He had commenced L_1 therapy eight weeks earlier and his last dose was taken 24 hours before the study. His L_1G pharmacokinetic results are not, therefore, included in the statistical analysis of the

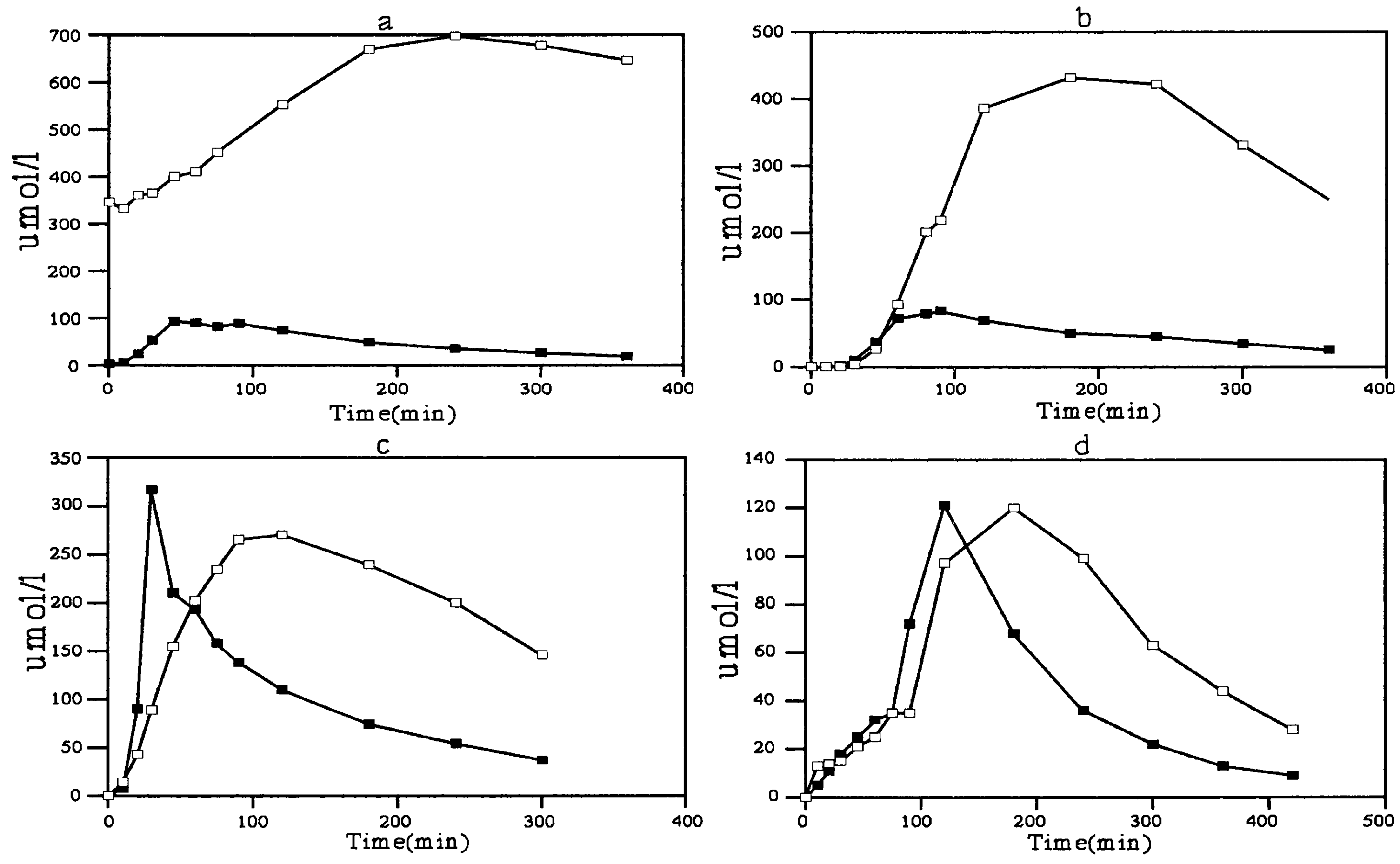


Fig 4.1. Pharmacokinetic profile of L_1(■) and its glucuronide(□) in the serum of 4 patients with iron overload following L_1 administration at an oral dose of approximately 50mg/kg.

data obtained from other 23 patients.

The details of the L_1 and L_1G pharmacokinetics are given in Table 4.2 and the changes in serum concentration of L_1 and L_1G versus time in 4 of the patients are shown in Figure 4.1.

When the pharmacokinetics of L_1 and L_1G in patients who had taken L_1(n=17) prior to the study were compared with the pharmacokinetics of L_1 and L_1G in those who had not taken L_1(n=7), no significant differences were observed($p > 0.05$).

AUC of L_1 and L_1G beyond the 6 hour period of the study (6h-∞)comprised 9.9±8.5(0.4-29.5)% and 24.4±15.1(6.8-56.3)% respectively of the total AUC(0-∞). The AUC of L_1 and L_1G beyond 12h(12-∞) were found to comprise 1.3±2.1(0-6.4)% and 5.3±6.0(0.5-23.5)% respectively of the total AUC.

Creatinine clearance correlated significantly with L_1G-$elt_{1/2}$(r=-0.79,p=0.002,Fig 4.2) but not with L_1-$elt_{1/2}$(r=0.44, p=0.087). No significant correlations were observed between the serum AST levels of the patients and AUC of L_1G and the half life of L_1G appearance(L_1-$appt_{1/2}$) in the serum($p > 0.05$).

Twenty four hour UIE during the L_1 clearance study in the 14 patients studied ranged from 4.7-32.7(14.7±8.4)mg. The amounts of L_1 and L_1G excreted in urine over the same period were 329±326(62-1191)mg and 1927±423(1167-2516)mg respectively. Hydrolysis of L_1G in 24h urine collections resulted in a total urine L_1 of 2257±383(1635-3000)mg. The total amount of L_1 recovered in the urine over the first 24h, therefore, comprised 77.9±13.3(46.7-100)% of L_1 dose. When the 24h UIE was divided by the amount of iron the oral dose of L_1 was capable of binding, the L_1 efficiency was found to be 3.8±1.9(1.4-7.5)%.

Table 4.2. Pharmacokinetics of deferiprone(L_1) and its metabolite L_1-glucuronide(L_1G) in 24 patients with iron overload.

Case	L_1-*ab*$t_{1/2}$ min	L_1-*el*$t_{1/2}$ min	L_1-C_{max} umol/l	L_1-T_{max} min	L_1-$AUC_{0-\infty}$ min.umol/l	L_1G-*app*$t_{1/2}$ min	L_1G-*el*$t_{1/2}$ min	L_1G-C_{max} umol/l	L_1G-T_{max} min	L_1G-$AUC_{0-\infty}$ min.umol/l
AA	51.8	101.1	99	90	20005	55.4	283.1	150	240	79133
AD	41.2	138.5	85	120	24361	53.6	212.1	146	240	66876
AE	26.6	55.5	163	60	11248	30.2	106.4	133	120	28662
AM	5.3	91.8	240	45	34880	51.9	205.1	90	240	40250
BJ	12.5	165.1	83	90	22357	44.4	220.8	432	180	185077
CH	6.3	83.8	189	30	24211	30.2	90.0	113	90	26615
CSo	68.5	82.8	222	120	28518	81.4	153.6	140	240	48981
CSt	18.2	134.9	94	45	20977	**	**	**	**	**
DI	15.6	79.0	73	60	9737	ND	ND	ND	ND	ND
EM	*	80.0	356	20	43968	39.3	99.9	225	120	60305
FM	*	58.0	241	20	14524	21.3	93.0	173	90	34241
FV	*	57.5	128	30	21898	23.6	140.6	121	120	34266
GZ	4.5	56.5	104	30	26384	21.7	184.2	119	120	39054
JT	*	64.7	143	20	12548	36.6	147.5	187	120	63057
LL	25.4	89.1	101	75	14246	45.3	147.6	167	180	52246
MH	24.3	166.5	96	90	29072	29.2	111.4	135	120	33326
MM	*	52.9	310	20	19616	18.2	86.3	147	90	26784
MS	15.8	82.6	119	60	13272	33.3	111.3	159	120	39392
PD	14.6	95.4	317	30	35259	26.8	205.6	270	120	102691
SA	20.0	91.1	87	75	14450	49.4	104.2	134	180	37356
SB	8.1	94.4	212	30	26889	29.5	169.8	193	120	63196
SG	13.0	59.3	112	60	11364	31.1	139.4	120	120	33629
TR	4.1	126.8	116	45	23205	41.4	126.6	137	180	42107
TT	45.0	78.9	121	120	18465	61.8	111.8	120	180	31739
X±SD	22.2±17.7	91.1±33.1	158.8±82.9	57.7±33.2	21727±8625	38.9±15.5	147.7±52.0	164.1±72.0	151.4±51.9	53136±35093

$t_{1/2}$=half-life, *ab*=absorption, *el*=elimination, *app*=appearance, C_{max}=maximum concentration, T_{max}=time to maximum concentration, AUC=area under the serum concentration *vs* time curve, ND=not done, *not enough points to calculate *ab*$t_{1/2}$, **results omitted because of high base line values.

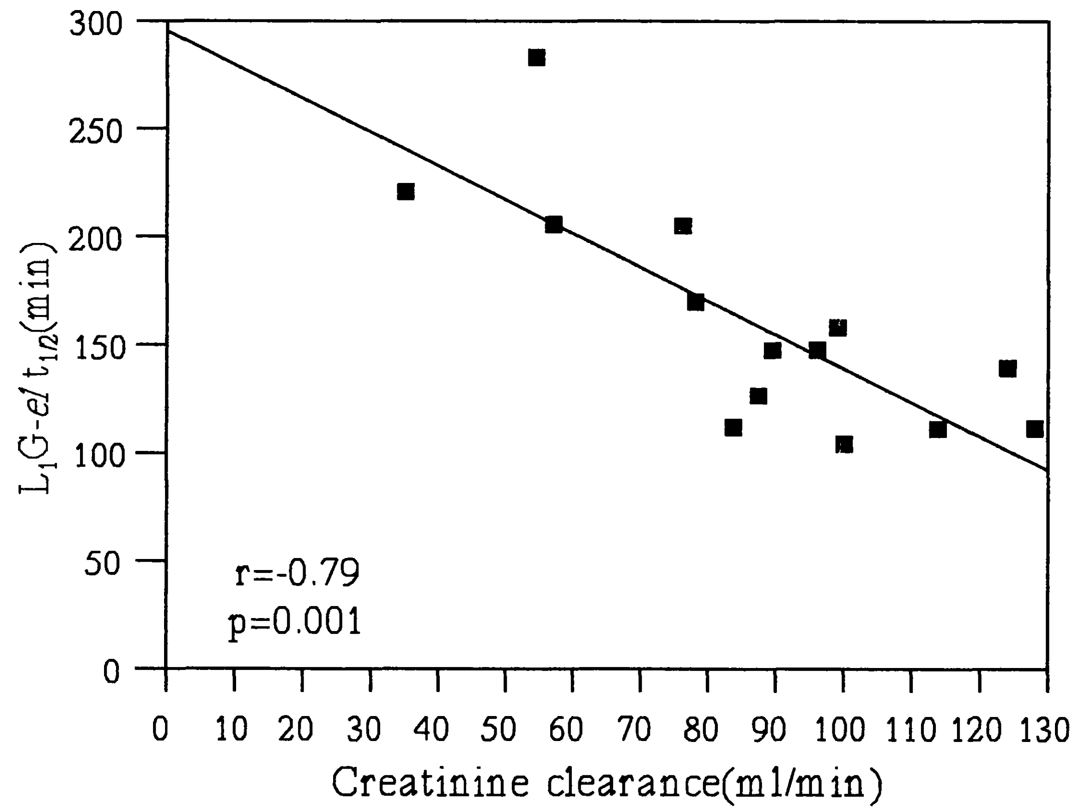


Fig 4.2. The correlation between creatinine clearance and elimination half life of L_1G(L_1G-el$t_{1/2}$) in 14 patients with iron overload

UIE in the 14 patients was found to correlate significantly with L_1-AUC(r=0.68, p=0.007, Fig 4.3), but not with L_1-Cmax or L_1-*el*$t_{1/2}$ nor with L_1G-C_{max}, L_1G-*app*$t_{1/2}$ or L_1G-AUC(p>0.05).

Analysis of the different fractions of urine collected in 8 patients over a period of 24 hours showed that 75.4±20.7% of L_1, 68.4±16.5% of L_1G and 70.7±13.0% of iron excreted in 24 hours following L_1 ingestion were excreted in the first six hours following L_1 administration.

4.5. DISCUSSION

The results here confirm that L_1 absorption is rapid in most patients(Fig 4.1a-c). Some patients, however, showed a delayed absorption despite taking L_1 on an empty stomach(Fig 4.1d). The mean absorption $t_{1/2}$ observed in this study is higher than that reported by Kontoghiorghes *et al*(1990a) in six iron overloaded patients and one normal volunteer(7.1±11.3min)(Table 4.3). The speed with which L_1 is absorbed suggests that it occurs through the upper part of the gastrointestinal tract but whether from the stomach or duodenum or both remains to be established.

Table 4.3. Comparison between the pharmacokinetics of L_1 observed in this study with those previously reported in two major studies.

Study	Number of patients	*ab*$t_{1/2}$ (minutes)	*el*$t_{1/2}$ (minutes)	L_1 recovery (%)	L_1 efficiency (%)
Current study	24	22.2±17.7	91.1±33.1	77.9±13.3	3.8±1.9
Kontoghiorghes *et al*(1990b)	7	7.1±11.3	74.3±28.7	90.5±8.2	6.8±5.6
Matsui *et al* (1991)	14	NA	159.6±20.5	NA	NA

$t_{1/2}$=half-life, *ab*=absorption, *el*=elimination, NA=not available.

L_1 elimination occurred mainly via the kidneys with an average of over 75% of the drug recovered in the urine in the first 24 hours mainly as L_1G. The mean elimination $t_{1/2}$ in this study is higher than that reported by Kontoghiorghes *et al*(1990a)(74.3±28.7min) but

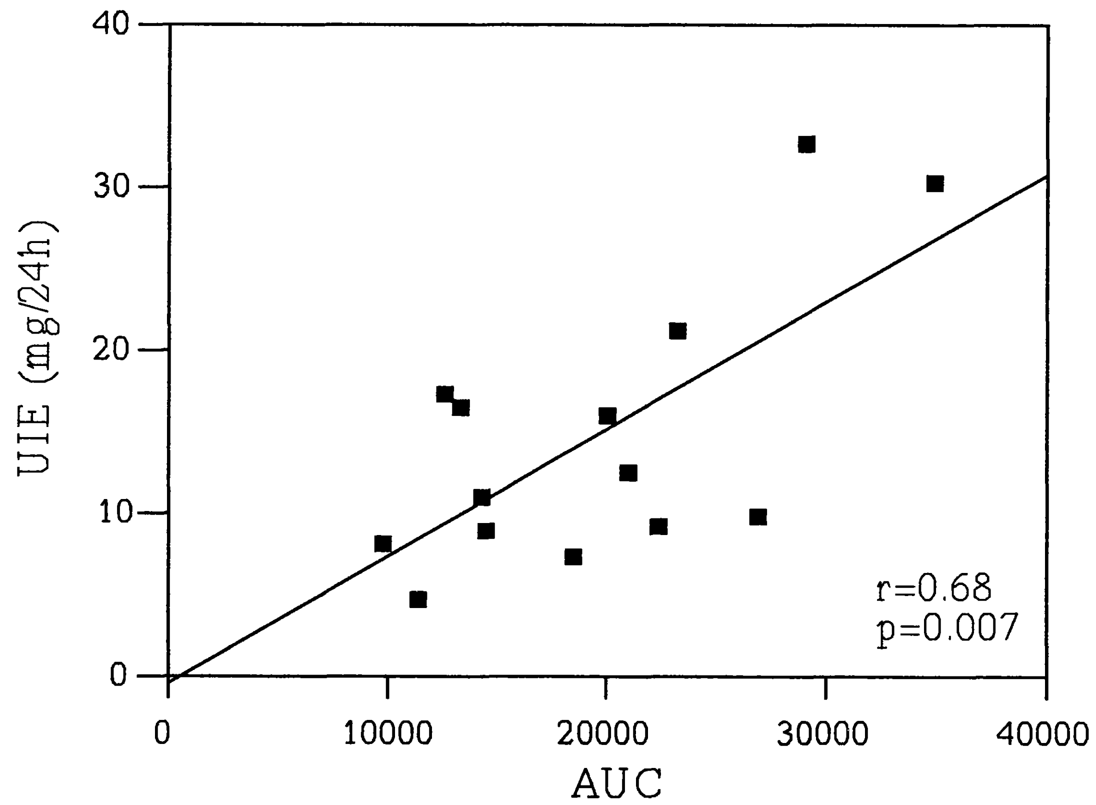


Fig 4.3. The correlation between urine iron excretion(UIE) and area under the curve of L_1 serum concentration versus time(AUC) in 14 patients with iron over load.

lower than the one reported by Matsui *et al*(1991)(159.6±20.5min)(Table 4.3). The amount of total L_1 recovered in urine showed that about 20% of the ingested dose remained unaccounted for at 24 hours(Table 4.3). This could be due to faecal excretion due to incomplete absorption of the drug or its re-excretion into the gastrointestinal tract as a free form or complexed with iron. Reports on faecal L_1 excretion are contradictory. Kontoghiorghes *et al*(1990b) reported no increase in iron excretion and no evidence of L_1 in the stools of two patients with iron overload given L_1 at an oral dose of 50mg/kg. On the other hand Olivieri *et al*(1990a) and Collins *et al*(1992) reported an increase in faecal iron excretion in 10 patients with iron overload following the oral administration of L_1 accounting for up to 28% of the total iron excretion. Although, in the latter study no attempt was made at measuring faecal L_1, their data suggest that L_1 is excreted in stool at least in some patients following L_1 ingestion.

In a few patients studied here 24h urine L_1 and L_1G accounted for 100% of the oral dose of L_1 indicating that the main and possibly only route of L_1 metabolism in man is glucuronidation. L_1G elimination in the urine was found to be slower than that of L_1(Fig 4.1, Table 4.1). This is probably due to the difference in size and physicochemical properties of the two molecules.

The chelation efficiency of L_1 at 24 hours in this study(3.8±1.9%) is lower than that reported by Kontoghiorghes *et al*(1990a)(6.8±5.6%-Table 4.3) and higher than reported previously in animal studies. Bergeron *et al*(1992) reported an L_1 chelation efficiency in iron overloaded monkeys of 2.1%, whereas Venkataram & Rahman(1990) found the chelation efficiency of L_1 in iron overloaded rats to be 1.3% which is not significantly different from that found in normal rats(1.2%)(Bergeron *et al* 1992).

The lack of significant difference between the pharmacokinetics of L_1 and L_1G in

patients who had received L_1 prior to the study and those who had not, implies that L_1 does not induce its own metabolism as has previously been suggested(Matsui *et al* 1991). Because L_1G cannot bind iron, it seemed possible that the speed and degree of L_1 glucuronidation would inversely correlate with L_1 efficacy as reflected by UIE. However, no such correlation was found between UIE and C_{max}, $appt_{1/2}$ or AUC of L_1G.

About 70% of the L_1, L_1G and iron excreted in the first 24 hours were excreted in the urine within 6 hours of L_1 ingestion. In a smaller group of patients, Kontoghiorghes(1990) reported that 85-90% of L_1 and L_1G were eliminated in the first 6 hours of L_1 ingestion.

The $elt_{1/2}$ of L_1 was in the range of 1 to 3 hours and this suggests that the L_1 dose has to be given every few hours if continuous levels of L_1 in the serum are essential to achieve higher efficacy. However, data from the clinical trial described in Chapter 3 show no overall difference in UIE whether the total daily dose of L_1 is divided into two or four. As L_1 is given at a maximum dose of about 50mg/kg twice daily, it was necessary to assess whether this dose would lead to accumulation of L_1 or L_1G. The mean AUC of both L_1 and L_1G after the first 12 hours of L_1 administration constituted only a small fraction(1.3±2.1%, 5.3±6.0% respectively) of the total AUC suggesting that, in most of the patients, no significant accumulation of L_1 or L_1G occurs. However the range of AUC after first 12 hours for L_1G was wide(0.5-23.5%) implying that in a minority of patients L_1G accumulation is possible(Fig 4.1a,b).

The present study has shown that the elimination of L_1G is influenced by the renal function of the patient as there is a significant correlation between the speed of the elimination of L_1G and the creatinine clearance of the patients. The lack of a similar correlation between L_1-$elt_{1/2}$ and creatinine clearance may be due to the difference in size

of the two compounds.

Severe gastro-intestinal tract symptoms, especially nausea and anorexia developed in the patient(CS) who was found to have high base line levels of L_1G associated with previous L_1 therapy and in whom L_1G reached a very high level after a single dose(Fig 4.1a). This patient, the oldest studied, was also found to have impaired renal function(Table 4.1) which may explain the accumulation of L_1G. When this patient was kept off the drug for a few days his serum L_1G fell to undetectable levels and his tolerance to L_1 improved only to deteriorate after a few days of L_1 therapy. Whether there is a true association between high L_1G levels and gastro-intestinal symptoms remains to be established in other patients. Mild degree of nausea was also observed in a few more patients during long term L_1 therapy(Chapter 3) but with no correlation to L_1G clearance.

CHAPTER 5

INTERACTION WITH TRANSFERRIN

5.1. INTRODUCTION

The use of L_1 or DFX is associated with a wide variation between patients and even, from day to day in individual patients in the amount of iron chelated and excreted in urine. The exact body pools of iron available for chelation by L_1 or DFX are not established. However, transferrin(Tf) has been suggested as one source of iron available for chelation by L_1 but not by DFX(Kontoghiorghes & Evans 1985, Evans *et al* 1992). Tf has a molecular weight of 80000 and two binding sites for iron, one at each of its two terminals. In normal individuals, Tf is only one third saturated whereas in patients with iron overload it is usually completely saturated. Tf saturation is tested routinely by estimating the difference between the total iron content of serum before and after the addition of a saturating concentration of iron. Although this method is adequate for most purposes, it can be inaccurate. Other forms of iron such as non-transferrin-bound or ferritin iron can cause over estimation of Tf saturation(Chapter 6, Pootrakul *et al* 1988). Therefore, if minor changes in Tf saturation are to be estimated, such as those caused by an iron chelator, a more sensitive method is required. Furthermore the presence of an excess of the chelator in a serum sample might interfere with the routine measurement of Tf saturation.

Macky and Seal(1976) showed that partially saturated human Tf separates into four bands on urea-polyacrylamide gel electrophoresis(UPAGE). The slowest and fastest represent the iron-free Tf(Apo-Tf) and diferric TF(Tf-Fe_2) respectively, whereas the two intermediate bands represent the C- and N-terminal monoferric forms(Evans & Williams 1978).

In contrast to DFX, L_1 at concentrations similar to those observed in plasma of patients receiving L_1 therapy can remove an appreciable amount of iron from transferrin in

vitro(Kontoghiorghes & Evans 1985). Evans *et al* (1992) recently observed a progressive fall in transferrin saturation after the administration of L_1 to an iron overloaded patient. In this study the interaction between L_1 and transferrin both in vivo in a larger group of patients and in vitro was studied. The results help to establish the degree of Tf desaturation in vivo after L_1 administration and its relation to the amount of iron excreted in urine.

5.2. MATERIALS AND METHODS

This study had the approval of the Ethical Committee of the Royal Free Hospital. L_1 was synthesised at the Royal Free Hospital as described before(Kontoghiorghes & Sheppard 1987). Acrylamide/Bisacrylamide(19:1) as a ready-made solution of 40%(w/v) and rivanol were obtained from Sigma. Blood samples were obtained from 16 patients with iron overload(Table 5.1) at different time intervals(0, 10, 20, 30, 45, 60, 75, 90, 120, 180, 240 and 300min) following the oral administration of L_1 (50mg/kg), separated within 30 minutes of obtaining the blood and stored at -20°C until the time of analysis. Serum samples were thawed within four weeks of collection and immediately analyzed using 6M-Urea/polyacrylamide gel electrophoresis (UPAGE) as described by Williams *et al*(1978). Serum samples were also obtained from 10 normal volunteers and used as normal controls with each run of UPAGE.

At the time of analysis samples were treated with rivanol as described before(Evans & Williams 1980) and applied to the gel. After staining and destaining, gels were scanned using a laser densitometer(Molecular Dynamics). The L_1 level in these samples was estimated using high pressure liquid chromatography(HPLC) as described before(Goddard & Kontoghiorghes 1990).

A further experiment was performed: zero time samples(T_0) from 10 patients were

incubated with 150uM of L_1 or normal saline either at room temperature (RT) or at 37 °C for 30 minutes and 24 hours and also at -20°C for 6 weeks. This concentration of L_1 was chosen to be comparable to the mean of peak L_1 concentration observed in patients' sera(Chapter 4). Samples were then treated with rivanol and analyzed using UPAGE as above.

Table 5.1. Clinical details of patients

Patient	Age(y)/Sex	Dx	Serum ferritin (ug/l)	TIBC (umol/l)	Tf saturation(%)*	UIE (mg/24h)
1	21/F	BTM	8130	33	78.8	15.8
2	23/F	BTM	7400	NA	NA	NA
3	13/M	CSA	3131	39	100	30
4	60/F	SCD	4006	36	100	8.7
5	81/M	MDS	5650	36	83	12.5
6	15/M	SCD	5950	66	36	8.1
7	46/M	CSA	2055	38	81.6	17.3
8	26/M	BTM	9060	48	79	11
9	17/M	PKD	3050	36	94.4	32.7
10	22/M	BTM	3350	NA	NA	NA
11	23/M	BTM	3520	30	100	16.5
12	27/F	BTM	3980	33	100	11.5
13	30/F	BTM	3850	54	100	5.2
14	43/F	ASA	1285	36	83.3	9.8
15	15/M	BTM	1320	27	100	3.1
16	31/F	BTM	4000	47	100	NA

BTM=β-thalassaemia major, CSA=congenital sideroblastic anaemia, SCD=sickle cell disease, MDS=myelodysplastic syndrome, PKD=pyruvate kinase deficiency, ASA=acquired sideroblastic anaemia. *measured by routine laboratory technique.

Serum iron and TIBC were measured by routine laboratory techniques(ICSH Expert Panel on Iron 1978 a&b). Urinary iron was measured using atomic absorption spectrophotometry. Serum ferritin was estimated by an ELISA technique(Flowers *et al* 1986). Statistical significance was assessed using Student's *t*-test.

5.3. RESULTS

Using the UPAGE method three bands were observed in patients' sera following L_1

administration. These represent the iron-free transferrin(Apo-Tf), the C-terminal monoferric transferrin [Tf-Fe(C)] and the diferric transferrin (Tf-Fe_2), in the order of increasing mobility(Evans & Williams 1978). Adding an excess of iron to the sera caused complete disappearance of the first two bands and an increase in the density of the third band.

L_1 was capable of removing iron from transferrin (Fig 5.1). The mean transferrin saturation at T_0 in patients' sera ranged between 57.8-100%(X±SD: 93.0±10.6) compared with 12.7-20.5%(16.4±3.0) for normal volunteers(n=10). The correlation between the transferrin saturation measured by UPAGE and that obtained by using the routine laboratory method (88.3±17.6) was significant (r=.83, p=.0003). Following L_1 administration there was a progressive fall in the degree of transferrin saturation and the percentage of Tf-Fe_2 and the appearance or rise in the percentage of Apo-Tf and Tf-Fe(C)(Fig 5.2). The lowest transferrin saturation observed following L_1 administration was 54.5±17.2%(range, 16.0-74.7%) occurring 72.5±50.0 minutes(10-180 min) after L_1 administration. Tf desaturation(difference between T_0 and lowest values) was 39.5±17.4%(range, 14.8-84.0%). In six patients Tf saturation returned to the T_0 values after 1.6-6h (4.5±2.0h) of L_1 administration whereas in the rest it returned to 86.0-98.0% of its T_0 values after the 6h of follow up. In patient 6 Tf saturation rose to 170% of the T_0 value at 6h (Fig 5.1a) and in patient 15 the desaturation of Tf was biphasic(Fig 5.1d). L_1 concentrations observed at the time of the lowest transferrin saturation ranged between 57 and 310 umol/l (128.4±69.6). In 11 patients the lowest transferrin saturation coincided with the peak L_1 concentration. There was a significant correlation between maximum Tf desaturation and the simultaneous L_1 concentration(r=.56, p=.02, Fig 3). No significant correlation was found between the degree of transferrin desaturation and

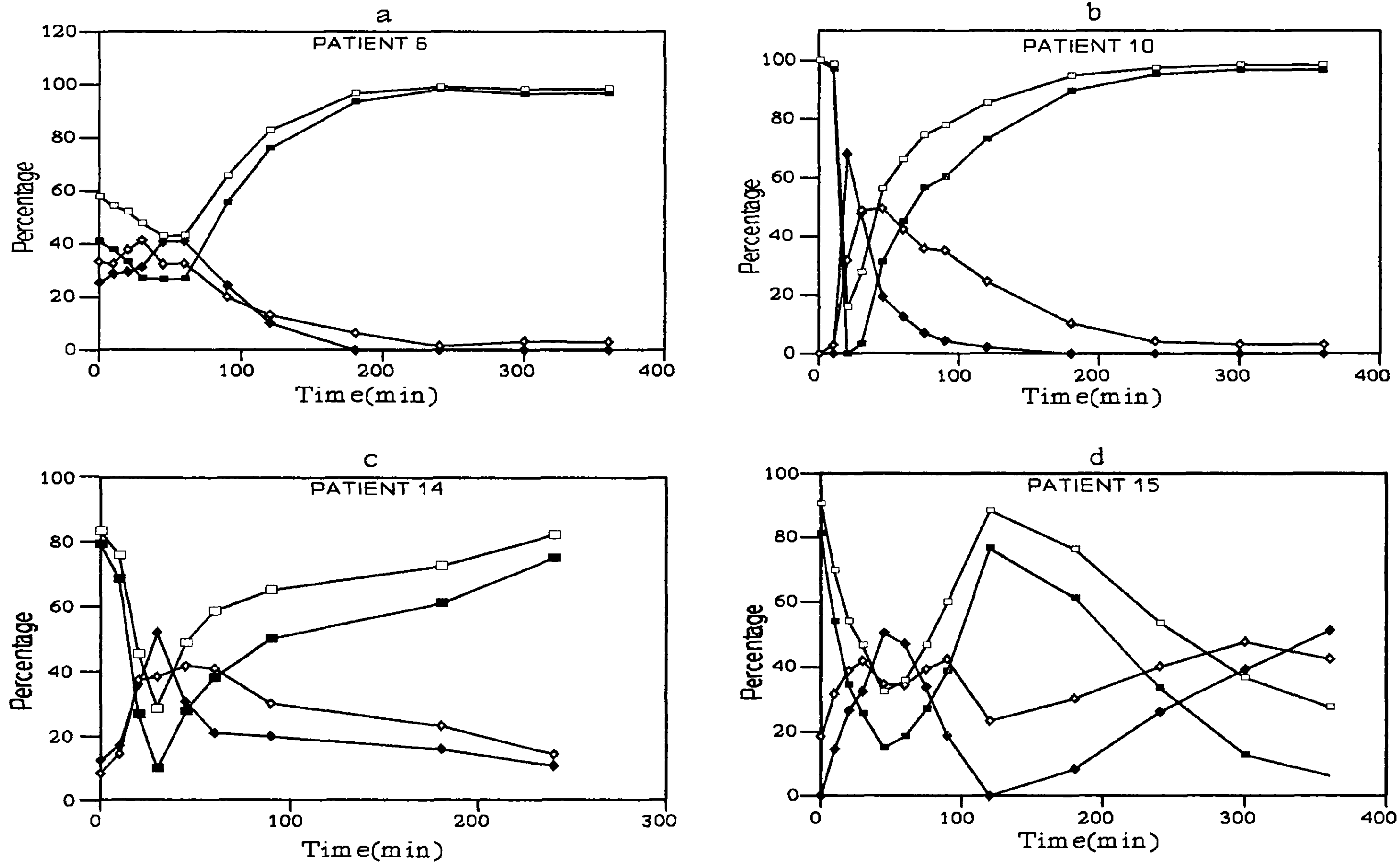


Fig 5.1. Changes in serum Tf saturation(---□---) and in the percentage of Apo-Tf(---◆---), Tf-Fe(C)(---◇---) and Tf-Fe_2(---■---) in 4 patients(6, 10, 14, 15) following the oral administration of L_1(50mg/kg)

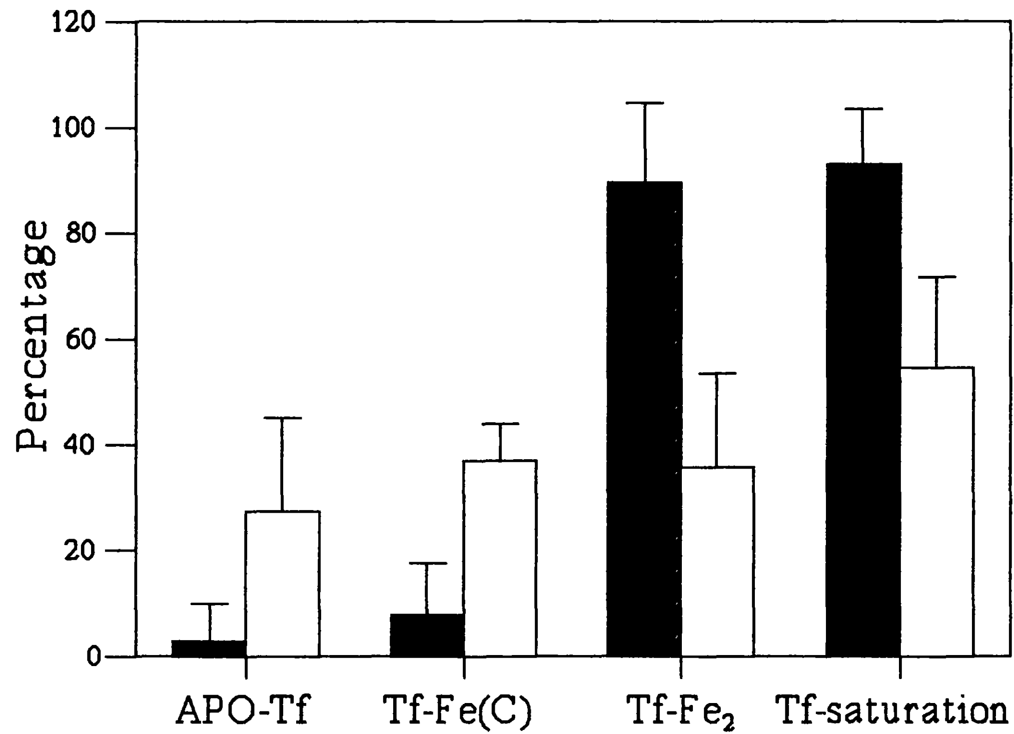


Fig 5.2. The percentages of the various forms of Tf at zero time(T_0)(■) and at the time of maximum change(□) in 16 patients with iron overload following the oral administration of L_1(50mg/kg). Data shown as mean±SD

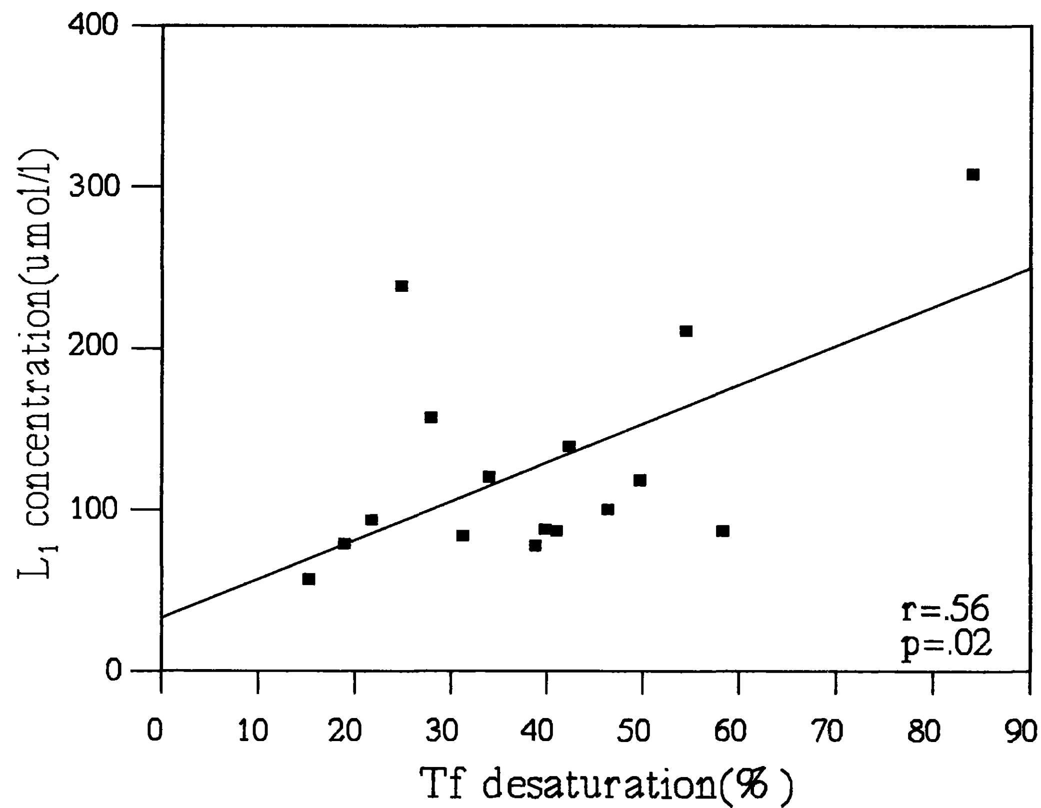


Fig 5.3. The correlation between the maximum Tf desaturation and the associated L_1 concentration in 16 patients following the oral administration of L_1(50mg/kg).

serum ferritin concentration(p=.25) or 24 hour urinary iron excretion(p=.64). The amount of iron removed from Tf in vivo was 13.8±5.1umol/l(6.7-22.1umol/l) estimated by multiplying patient's TIBC value by maximum Tf desaturation. The total amount of iron chelated from Tf by L_1 was approximately estimated by multiplying the amount of iron removed from Tf by each patient's predicted plasma volume[weight(kg) x 70(ml/kg) x (1-PCV)] and was 35.0±15.3umol(13.9-67.1umol). Urinary iron excretion measured during the study was 250.3±156.4umol(55.4-583.9umol). No significant correlation was found between UIE and the total amount of iron removed from Tf(p=.9).

The incubation of T_0 serum samples (n=10) with 150uM of L_1 for 30 minutes at RT caused a small fall in transferrin saturation (4.8±4.4%) and when the incubation was continued for 24 hours this fall was more pronounced(21.7±8.2%, p=.004). By contrast, at 37°C a significant drop in transferrin saturation was observed at 30 minutes (28.6±11.7%, p=.04) comparable to that achieved in 24h at RT(p=.31) but this fall did not significantly increase after 24 hours of incubation(25.6±10.5%,p=.68). Only a small fall in Tf saturation was observed when the samples were incubated at -20°C for 6 weeks(7.8±1.7%)(Fig 5.4). In this experiment a fourth band was observed in the UPAGE gels, corresponding to Tf-Fe(N), after incubating the samples with L_1 at RT and 37°C for 30min and 24h. No similar band was found after 6 week incubation at -20°C. The percentages of this form of Tf were 10.8±4.9%, 29.3±7.4%, 25.1±1.6% and 26.7±1.7% respectively. These changes were associated with a rise in the Tf-Fe(C) level of 10.8±4.9%, 4.9±3.0%, 8.2±3.3% and 8.2±5.3% respectively.

5.4. DISCUSSION

The results obtained following L_1 administration showed that L_1 is effective in removing an appreciable amount of iron from Tf in most of the patients studied. These data are

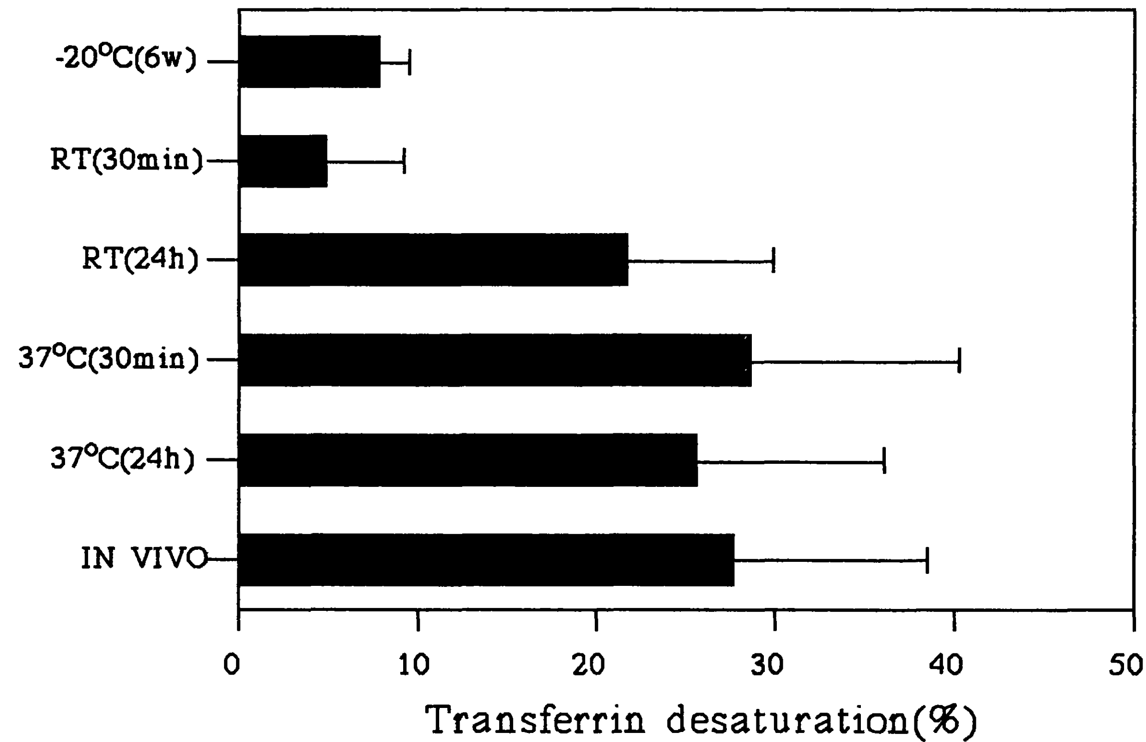


Fig 5.4. Tf desaturation in 10 patients with iron overload following the incubation of their zero time(T_0) samples with L_1(150umol/l) for 30 min and 24h at room temp(RT) and 37°C and for 6 weeks at -20°C, compared with the maximum in vivo desaturation of Tf(in vivo) observed in their sera following the oral administration of L_1(50mg/kg). Data shown as mean±SD.

comparable to previously reported result(Evans *et al* 1992). The degree of Tf desaturation was dependent on L_1 concentration as there was a significant correlation between maximum Tf desaturation and the associated L_1 concentration.

The significant correlation between Tf desaturation and L_1 concentration may suggest that these changes could have occurred in vitro after obtaining the serum samples rather than in vivo. Experiments were, therefore, carried out to establish the speed of iron removal from Tf by L_1 at different temperatures. The results indicate that this process is much faster at 37°C than at RT or -20°C, being accomplished within 30 minutes at 37°C (Fig 5.4). There was no significant difference($p>.05$) between the degree of Tf desaturation achieved in vitro after 30min or 24 hours at 37°C or 24 hours at RT and the maximum Tf desaturation observed in vivo(27.7 ± 10.8)(Fig 5.4). These results suggest that the observed changes in Tf saturation are more likely to have occurred in vivo than to be purely caused by the interaction between L_1 and Tf in the stored serum sample. Furthermore changes in Tf saturation observed in patients 6 and 15(Fig 5.1a,d) imply that Tf saturation is not entirely dependent on L_1 concentration and other in vivo factors may influence the interaction between Tf and L_1.

The absence of Tf-Fe(N) from the serum samples is consistent with previous reports(Williams and Moreton 1980, Evans *et al* 1982). Williams and Moreton(1980) found that storage of serum samples at -15°C for several days causes redistribution of iron between Tf-Fe(C) and Tf-Fe(N), resulting in the disappearance of the latter from serum but with no effect on overall Tf saturation. Therefore, it was not possible to examine, in vivo, the distribution of iron between the four different forms of Tf following L_1 administration. However, a fourth band representing Tf-Fe(N) was observed in vitro after incubating serum samples taken from 10 patients with L_1. The changes in

the concentration of this form of Tf during incubation with L_1 were more marked than the concurrent changes in the concentration of TF-Fe(C). In keeping with previous reports(Kontoghiorghes & Evans 1985, Evans *et al* 1992), this suggests that L_1 preferentially binds the iron atom present in the C-terminal site of diferric Tf.

Following L_1 administration Tf saturation usually returned to the T_0values within 6 hours whereas in patient 6 Tf saturation continued to rise reaching 170% of its T_0 value at six hours(Fig 1a). As this patient's T_0 Tf saturation was relatively low(54%) (measured by UPAGE), it is possible that L_1 at an initial high concentration caused a fall in Tf saturation whereas at a later stage when L_1 concentration fell it participated, instead, in the transport of iron to Tf. Previous studies have shown that L_1 given to a normal volunteer can cause a progressive rise in Tf saturation(Evans *et al* 1992). The cause of the biphasic Tf desaturation observed in patient 15(Fig 5.1d) is unclear.

The degree of the iron load of the patients had no bearing on the degree of Tf desaturation as is evident from the lack of significant correlation between the latter and the serum ferritin concentration.

Assuming that all the iron removed from Tf by L_1 was excreted in urine in 24 hours, it was found that this comprised 21.3±20.2%(5.8-67.1%) of total UIE. This calculation does not allow for possible interruption of iron flux from tissues by unsaturated Tf when free L_1 is present in plasma.

CHAPTER 6

NON-TRANSFERRIN-BOUND IRON

6.1. INTRODUCTION

In haemochromatosis or transfusion dependent refractory anaemia, plasma transferrin is saturated leading to excessive accumulation of iron in various tissues. Moreover, some iron released from tissues to plasma may remain 'free'. The precise nature of this non-transferrin-bound iron(NTBI) is uncertain. Hershko *et al*(1978) suggested that NTBI is a low molecular weight iron complex probably loosely bound to albumin, while others postulated that NTBI exists mainly as iron citrate(Grootveld *et al* 1989). Whatever its form, NTBI is known to be effective in the formation of potentially toxic oxygen derivatives(Gutteridge *et al* 1985, Halliwell & Gutteridge 1986).

Two other factors have been suggested which may have a bearing on the mechanism of NTBI-induced tissue injury. First, plasma is deficient in antioxidant enzymes, such as superoxide dismutase(SOD), that are capable of inactivating toxic oxygen species and normally exist inside cells(Hershko 1987) and second, NTBI is more readily taken up by tissues than transferrin-bound iron(Craven *et al* 1987, Fawwaz *et al* 1967).

Several methods are currently in use for measuring NTBI(Batey *et al* 1980, Gower *et al* 1989, Gutteridge *et al* 1981, Gutteridge *et al* 1986, Hershko *et al* 1978, Singh *et al* 1989, Singh *et al* 1990). The most recent one has been claimed to be simple and sensitive with high reproducibility(Singh *et al*, 1990).

In this study, NTBI levels were measured in 52 patients with TM to determine its relation to degree of iron overload. When NTBI was found to correlate significantly with iron overload in these patients its measurements were used to monitor the efficacy of L_1.

6.2. MATERIALS AND METHODS

The method used in this study was modified from that described by Singh *et al*(1990) as follows: First, the more convenient and rapid Amicon Centrifree Micropartition system

(Amicon, Beverly, MA) was used instead of Amicon Centriflo-CF 25 cone membranes to prepare the serum ultrafiltrates. Second, 5 mmol of L_1 was substituted for the 3mmol/l of L_1NPr(CP-22)(1-propyl-2-methyl-3-hydroxypyrid-4-one) in the HPLC mobile phase since L_1-iron complexes elute faster than the corresponding L_1NPr-iron complexes. The change in polarity caused by the use of L_1 instead of L_1NPr necessitated the reduction of acetonitrile in the HPLC mobile phase from 20% to 5%.

The modified method was, therefore, as follows. To 0.9ml of serum was added 0.1ml 800mmol/l nitrilotriacetic acid (NTA: Aldrich, Gillingham, Dorset, UK), which had previously been adjusted to pH 7.0 with 1mol/l NaOH, and allowed to stand at room temperature for 30min. The solution was then ultrafiltered with an Amicon centrifree micropartition system with an applied centrifugal force of 1000g for 30min using a fixed-angle rotor (MSE GF-8 centrifuge, Crawley, Sussex, UK). A 20ul aliquot of the ultrafiltrate (approx. total volume 500ul) was injected directly onto the HPLC system using an iron-free syringe. The HPLC system comprised of an LDC constrametric III pump, LDC spectromonitor 3000 uv-visible detector, LDC model 308 integrator (Milton-Roy, Stone, Staffs, UK) and Rheodyne 7125 injector (Cotati, CA). A 100 x 3mm Chromspher-ODS (Chrompack, The Netherlands) glass column, filled with 5um material, was used with a mobile phase of 5% acetonitrile and 5mmol/l L_1 in 5mmol/l MOPS, pH 7.0, at a flow rate of 0.6ml/min. Detection was at 460nm. A standard curve was generated by injecting 0, 2, 4, 6, 8 and 10umol/l iron prepared in 80mmol/l NTA (adjusted to pH 7.0). All injections were performed in duplicate with a standard after each sample.

NTBI results obtained by the modified and original procedures were initially compared. In 38 patients with beta thalassaemia major the correlation coefficient between the NTBI

values from the 2 methods was 0.926($y=0.823x + 0.465$)($p<0.0001$). The within- and between-batch coefficient of variation (CV) for the modified procedure was 7 and 11% respectively. This was determined by multiple (10) analyses of a pooled sample. The modified HPLC conditions, as with the original conditions, allows the separation of DFX-bound iron from other NTBI in patient samples. Control serum samples were included in each batch and showed consistent values.

L_1 was prepared and characterised as previously described(Kontoghiorghes & Sheppard 1987). L_1NPr was generous gift of Professor RC Hider.

Serum ferritin was estimated by ELISA technique as described by Flowers *et al*(1986). Serum iron and TIBC were measured by routine laboratory techniques(ICSH Expert Panel on Iron 1978 a&b). Statistical analysis was performed using Student's *t*-test.

6.3. PATIENTS

Serum samples were obtained from 52 patients with β thalassaemia major. Their ages ranged from 3 to 34 years (19.0±7.3), 27 males and 25 females. Serum samples were also obtained from 12 normal volunteers(34.0±3.6 years).

The patients were receiving regular blood transfusions to keep their haemoglobin above 10 g/dl. They were using subcutaneous DFX(40-50mg/kg/night) for chelation with variable degree of compliance (1-5 nights/week). Subsequently 10 of the patients were entered into a trial of the oral chelator L_1(Chapter 2).

To avoid contamination of samples with external iron, all handling was performed in disposable iron-free equipment. Blood samples were separated within one hour of collection to avoid the possible release of iron from haemolysis of erythrocytes. The serum was stored at -20°C until analysis. Samples used in this study were obtained immediately before the start of DFX infusion or L_1 ingestion and approximately 12 hours

after the last administration to ensure complete clearance of L_1(Chapter 4, Kontoghiorghes *et al* 1990 a&b).

6.4. RESULTS

The NTBI levels obtained with the modified method in the 12 normal controls ranged from -0.7umol/l to -2.3umol/l(-1.5±0.6). In the 52 patients the NTBI values ranged from -1.5 to 9.0 umol/l(3.6±2.3). As these negative values are artifactual(see below) we have assigned them zero values when statistical analyses were performed.

The patients' serum ferritin concentrations ranged from 207 to 11400ug/l(2674±2538). In 35 of the patients the total serum iron concentrations ranged from 20 to 61 umol/l (39.5±9.6) and transferrin saturation ranged from 44% to 110% (84.5±13.8). There was a significant correlation between serum ferritin and NTBI values ($r=0.502$, $p<0.001$, Fig 6.1). There was also a significant correlation between total serum iron and NTBI ($r=0.608$, $p<0.001$, Fig 6.2) and between transferrin saturation and NTBI ($r=0.481$, $p=0.004$, Fig 6.3).

Ten of the patients were entered into a trial of the oral iron chelator L_1. They, therefore, stopped using DFX and commenced chelation with L_1, initially at a total daily dose of about 40-60mg/kg and 4 weeks later at a total daily dose of 80-120mg/kg both were divided into 2 doses/day. Their ages ranged from 16 to 26 years(22.3±2.7) and their serum ferritin concentrations, at the start of the trial, ranged from 1000 to 9580ug/l (5549±3333). Their NTBI levels were measured at the beginning of the trial and 3 and 6 months later (Fig 6.4). The initial NTBI ranged from 3.6 to 9.0umol/l (6.1±1.6) and the final NTBI ranged from 3.8 to 5.4umol/l (4.5±0.5). The difference between the 2 mean values is significant ($p<0.001$) as was the overall fall in NTBI values when paired *t*-test was used($p=0.007$). On the other hand when measured at 6 months serum ferritin levels

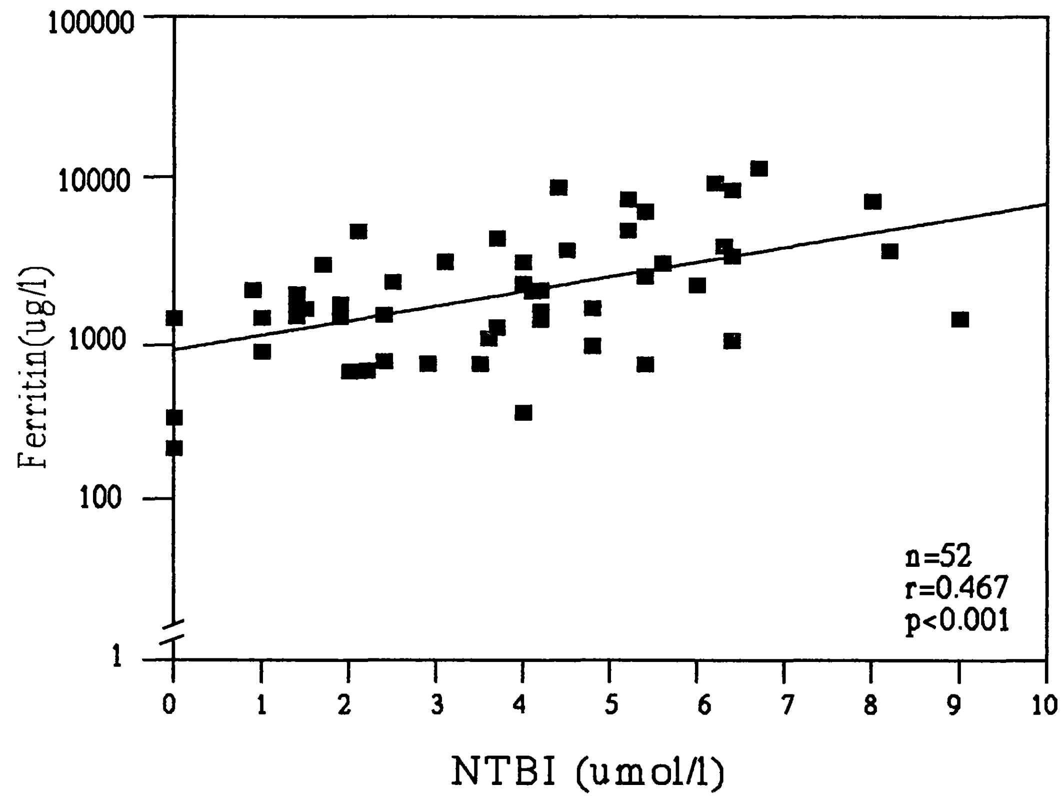


Fig 6.1. The correlation between NTBI and serum ferritin concentrations.

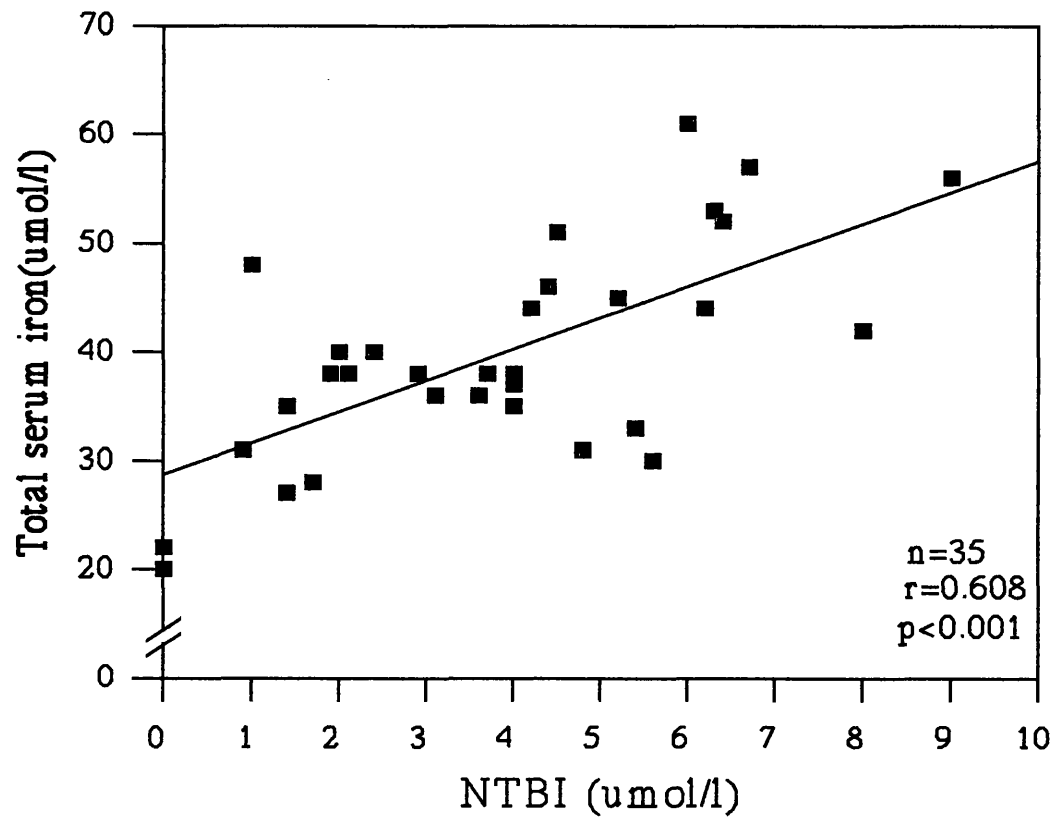


Fig 6.2. The correlation between NTBI and total serum iron concentrations.

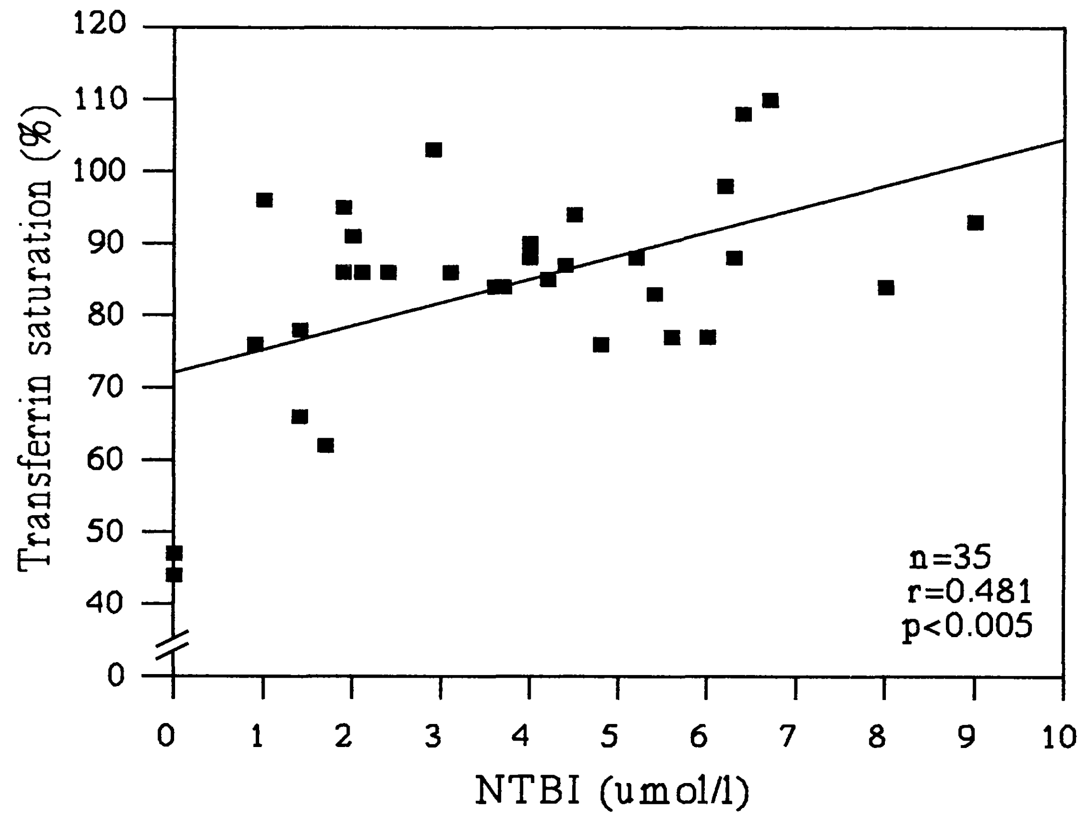


Fig 6.3. The correlation between NTBI concentration and transferrin saturation.

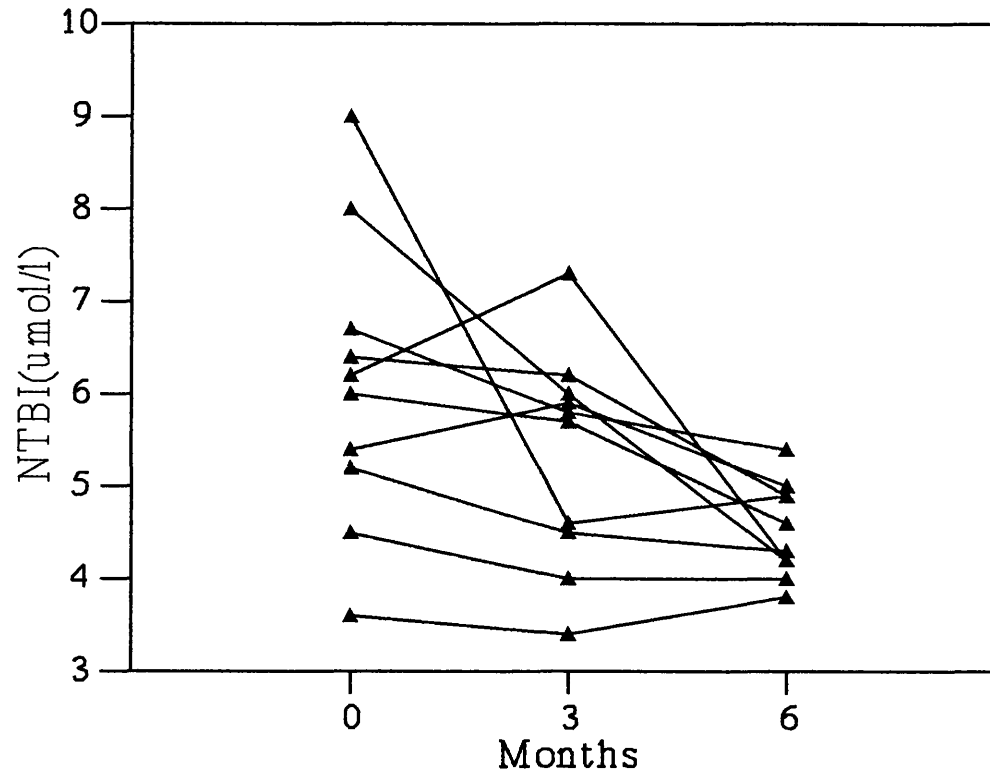


Fig 6.4. The change in serum NTBI levels in 10 patients with iron overload whilst receiving L_1 over a period of 6 months.

showed no significant fall(paired *t*-test: $p=0.55$).

Both initial and final mean NTBI of L_1-patients were significantly higher ($p<0.001$, $p=0.005$ respectively) than the mean NTBI of the DFX-patients(3.3 ± 2.0 umol/l, $n=42$). The difference between the mean serum ferritin of L_1-patients at the beginning of the trial and the mean serum ferritin of the DFX-patients was also significant(5745 ± 3578 ug/l *vs* 2006 ± 1562 ug/l, $p=0.003$), indicating a significant difference in the degree of iron overload and therefore, DFX chelation of the 2 groups.

6.5. DISCUSSION

The results obtained with the modified procedure for the estimation of NTBI correlates well with the results achieved with the original Singh *et al*(1990) procedure. The Amicon centrifree micropartition system used in this study allows samples to be prepared more rapidly than with the Amicon centriflo-CF25 cone membranes used by Singh *et al*(1990). Furthermore as the centrifree micropartition system is disposable, no lengthy washing, with the possible introduction of iron contamination, is required. The chelator in the HPLC mobile phase was changed to L_1 from L_1NPr to enable faster elution of the iron complex.

Several other methods have been developed for the measurement of NTBI(Batey *et al* 1980, Gower *et al* 1989, Gutteridge *et al* 1981, Gutteridge & Hou 1986, Hershko *et al* 1978, Singh *et al* 1989, Singh *et al* 1990). Many of them are either insufficiently sensitive(Gutteridge *et al* 1981, Singh *et al* 1989), oversensitive(Gower *et al* 1989, Jack *et al* 1991, Hershko *et al* 1978) or have poor reproducibility(Gutteridge *et al* 1981). Others are difficult to perform and therefore not suitable for routine use. Some of them have also shown the presence of NTBI in the serum of normal individuals. As transferrin has an extremely high affinity for free iron, NTBI should not exist in plasma in the

presence of substantially unsaturated transferrin. Therefore, it is likely that detection of NTBI in normal individuals is an artefact due to oversensitivity of some of these methods. NTBI has, however, been detected in many iron overloaded patients who have partly unsaturated transferrin. This finding may possibly be due to the limitations of routine clinical methods for the measurement of transferrin saturation(Hershko 1987). Wagstaff *et al*(1986) have, however, suggested that either NTBI is not in a form available to transferrin or that transferrin in these patients is damaged, possibly due to free radical attack of the carbohydrate or protein moieties. In this work 3 patients had transferrin saturation marginally in excess of 100%. This again probably reflects limitations in the transferrin saturation assay although ferritin iron(Pootrakul *et al* 1988) or NTBI might be responsible.

In our current study normal individuals have consistently shown "negative" NTBI values and this was also noted in some of the well chelated patients as well as by Singh *et al* (1990). These "negative" values are clearly artefactual and are presumably due to the presence of unsaturated transferrin in their sera which is capable of removing iron contamination from NTA. The efficacy of transferrin in removing iron from NTA-iron is well recognised(Bates *et al*, 1967). By comparison with Chelex-treated deionised water and standards made-up therein, the 80mmol/l NTA used in this study was shown to contain 3umol/l Fe-NTA as a contaminant. When sera, with significant unsaturated iron-binding capacity, are incubated with NTA the unsaturated transferrin is able to bind some of the iron contamination thereby producing a "negative" NTBI result when compared to iron standards also diluted in 80mmol/l NTA. Although this situation is unsatisfactory, comparison against standards made-up in Chelex-treated deionised water would overestimate the NTBI by up to 3umol/l, particularly in thalassaemic patients in

whom the transferrin is fully saturated. Clearly the most satisfactory method would be to use NTA without iron contamination but we were unable to achieve this.

The NTBI values estimated in our thalassaemia major patients are within the range reported by previous workers(Anuwatanakulchai *et al* 1984, Hershko *et al* 1978, Singh *et al* 1989). The significant correlation between NTBI values and serum ferritin and between the NTBI and total serum iron are also consistent with previous reports(Anuwatanakulchai *et al* 1984, Wagstaff *et al* 1986). These significant correlations suggest that NTBI is related to body iron stores.

The likely explanation for the difference observed between the initial mean NTBI of L_1-patients and the mean NTBI of the DFX-patients is the fact that 7 of the 10 L_1 -patients were poorly complying with DFX before commencing the treatment with L_1 while only 13 of the other 42 DFX-patients were poorly compliant. This was also reflected by the significant difference between the mean serum ferritin of L_1-patients and DFX-patients. Once L_1-patients complied with the new chelator for 6 months their NTBI values fell significantly although their serum ferritin remained unchanged. This suggests that NTBI is more sensitive than ferritin to short term chelation. It is, however, not clear why there was still a significant difference ($p=0.005$) between the final NTBI values and those of the DFX-patients. This difference may be related to the difference in iron mobilising properties of the two chelators or to the difference in the mode of administration of the 2 chelators. DFX is infused over 8-12 hours while L_1 was given orally twice a day leading to a higher but less sustained rise in the serum level(Chapter 4) with the resultant briefer contact between L_1 and NTBI. It is however important to determine whether more prolonged chelation with L_1 will eventually lead to as low NTBI values as found in compliant patients on DFX.

CHAPTER 7

EFFECT OF L_1 ON SERUM FREE IRON AND KINETICS OF L_1-Fe COMPLEX

7.1. INTRODUCTION

In patient with gross iron overload, several years of regular iron chelation are required in order to reduce the body iron burden to a safe level. During this time the patient is continuously exposed to the damaging effect of NTBI excluding those few hours when the level of the iron chelator in the serum is sufficient to neutralise any NTBI. Therefore, a chelation therapy that is associated with a more sustained neutralising effect on NTBI is likely to be more effective in preventing those complications that can arise from iron overload. Therefore, iron chelation therapy has two important roles to play. The first is to reduce the body iron burden and the second to neutralise the NTBI and convert it to a harmless complex. The former role can be achieved by the regular use of the chelator at an effective dose, whereas it is not very clear how to achieve the latter role. For a parenteral chelator such as desferrioxamine(DFX) with a constant serum level following subcutaneous infusion the fate of NTBI is predictable. If DFX is given at a sufficient dose, the NTBI is likely to disappear as long as the infusion continues. The effectiveness of continuous chelation with DFX on the outcome of patients with severe iron-induced cardiomyopathy has previously been demonstrated(Aldouri *et al* 1990). After an oral chelator such as L_1 with variable serum levels the fate of NTBI can not be predicted.

The effectiveness of both L_1 and DFX at reducing body iron load has been confirmed(Chapters 2,3, Al-Refaie *et al* 1993). However, the effect of any of these chelators on the level of NTBI has not been thoroughly studied. It has previously been shown that compliance with DFX or L_1 resulted in a significant reduction in the level of NTBI(Chapter 6). But this was, most likely, due to the effect of these chelators on body iron load.

In this study the effect of a single oral dose of L_1 on the fate of NTBI and on the kinetics

of L_1-Fe complex has been examined in a large group of patients with iron overload.

7.2. PATIENTS

Twenty two patients with iron overload were studied. Their clinical details are given in Table 7.1.

Table 7.1. Clinical details of 22 patients with iron overload.

Case	Age(y) /sex	Diagnosis	Serum ferritin (ug/l)	Transferrin saturation (%)	UIE (mg/ 24h)
AA	21/F	BTM	8130	95	16.0
AD	23/F	BTM	7400	83	ND
AM	13/M	CSA	3131	95	30.3
BJ	60/F	SS	4000	100	9.2
CH	35/M	BTM	2922	90	ND
CSo	29/M	BTM	2108	85	ND
CSt	81/M	MDS	5650	83	12.5
DI	15/M	SS	5950	36	8.1
EM	26/M	BTM	2393	87	ND
FM	25/M	BTM	1365	63	ND
FV	20/M	BTM	4122	100	ND
JT	46/M	CSA	2055	82	17.3
LL	26/M	BTM	9060	79	11
MH	17/M	PKD	3050	94	32.7
MM	22/M	BTM	3350	100	ND
MS	23/M	BTM	3520	100	16.5
PD	27/F	BTM	3980	100	ND
SA	30/F	BTM	3850	100	8.9
SB	43/F	MDS	1285	83	9.8
SG	15/M	BTM	1320	100	4.7
TR	51/M	PKD	3131	96	21.2
TT	31/F	BTM	4000	88	7.3
mean±D	30.9±16.5		3899±2141	88.1±15.0	14.7±8.4

ND=not done, UIE=urine iron excretion, BTM=β-thalassaemia major, CSA=congenital sideroblastic anaemia, SS=sickle cell disease, MDS=myelodysplastic syndrome, PKD=pyruvate kinase deficiency.

L_1 was given at an oral dose of 50mg/kg to each patient after an overnight fast and blood samples were withdrawn at 0, 10, 20, 30, 45, 60, 90, 120, 180, 240, 300, 360min intervals separated immediately and stored at -20°C until the time of analysis.

Twenty four hour urines were collected simultaneously from 14 patients and analyzed for total iron excretion.

7.3. MATERIALS AND METHODS

L_1 was synthesised at the Royal Free Hospital as previously described(Kontoghiorghes & Sheppard 1987). Serum L_1 level was measured using a previously established high pressure liquid chromatography(HPLC) technique(Goddard & Kontoghiorghes 1990). Urine iron was estimated using atomic absorption spectroscopy and serum ferritin by an ELISA technique(Flowers *et al* 1986). Serum total iron binding capacity was measured using routine laboratory techniques(ICSH Expert Panel on Iron 1978 a&b).

NTBI and L_1-Fe complexes are measured by the method described in Chapter 6. This method, however, can not distinguish between NTBI and L_1-Fe complex which can co-exist in the serum samples. As L_1 bind to iron in 3:1 ratio to make $(L_1)_3$-Fe complex which is stable at physiological pH(Kline & Orvig 1992), NTBI and L_1-Fe complex levels were calculated as follows: if 1/3 of L_1 concentration(to reflect the effective concentration of L_1 capable of binding to Fe in 3:1 ratio) was in excess of the concentration of iron measured by the above method, this iron concentration was considered to be that of L_1-Fe complex. On the other hand, if 1/3 of L_1 concentration was lower than the concentration of iron measured, this 1/3 of L_1 concentration was considered to be the level of L_1-Fe complex and the difference between this concentration and the concentration of the total iron measured by the method represents the concentration of NTBI(Fig 7.1).

Changes in the serum concentration of L_1-Fe complex, thus measured and of L_1 were plotted against time and analyzed for kinetics using a computer programme specially designed for pharmacokinetic measurements(Johnston & Woollard 1983). Statistical

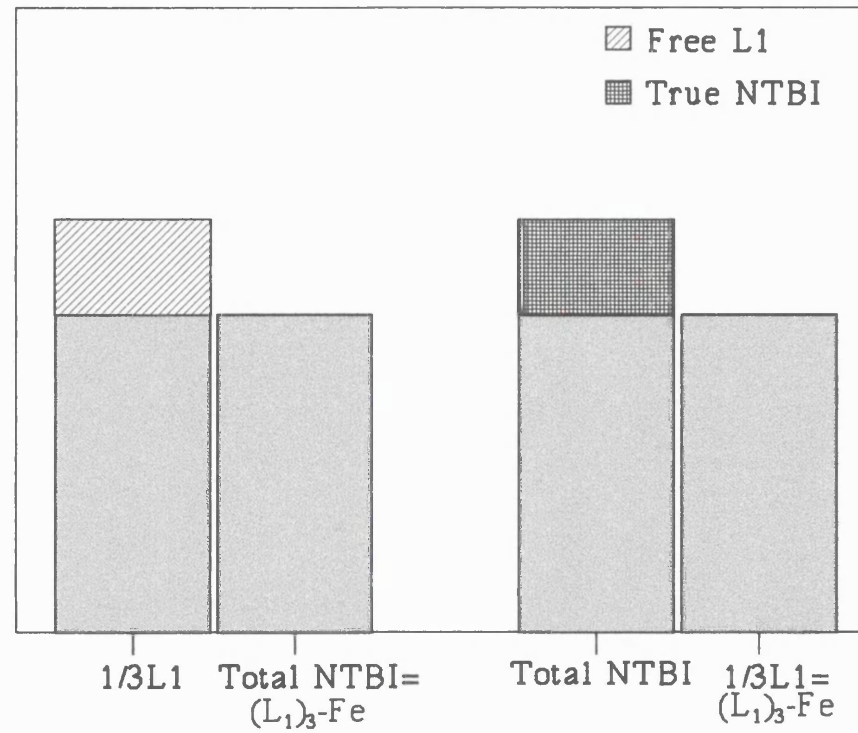


Fig 7.1. This figure demonstrates the method used to estimate the concentration of true NTBI and $(L_1)_3$-Fe complexes in the sera of the patients.

analysis was performed using Student's *t*-test. Results are expressed as mean±SD.

7.4. RESULTS

Figure 7.2 shows the changes observed in the serum concentration of L_1, total NTBI(including L_1-Fe complex) and NTBI(free iron) in 2 patients.

7.4.1. NTBI

L_1 administration caused the complete disappearance of NTBI from the serum of patients studied within 28.5±26.2(1-95)min of L_1 ingestion. It reappeared after 156.6±77.9(52-304)min following L_1 ingestion.

The final(360min) mean NTBI concentration (5.0±3.0, range:0-11)umol/l was significantly lower than the initial mean NTBI(7.1±1.7, range:3.8-10.4)umol/l (paired *t*-test, p=0.0003)(Table 7.2).

7.4.2. L_1-Fe complex

Area under the curve($AUC_{0-\infty}$) of the concentration of L_1-Fe complex versus time was 5581±2132(2230-8907)min.umol/l, comprising 76.9±13.3(49.7-99.6)% of 1/3 of L_1-$AUC_{0-\infty}$(Table 7.2) and correlating significantly with UIE(r=0.66, p=0.015, Fig 7.3) and L_1-$AUC_{0-\infty}$(r=0.88, p<0.0001).

The elimination half life($elt_{1/2}$) of L_1-Fe complex was 101.9±51.5(35.0-252.5)min. The duration of NTBI disappearance correlated significantly with $AUC_{0-\infty}$ of L_1(r=0.66, p=0.0009, Fig 7.4a), the initial NTBI concentration(r=-0.69, p=0.0005, Fig 7.4b) and also with the difference between L_1-$AUC_{0-\infty}$ and $AUC_{0-\infty}$ of L_1-Fe complex(r=0.5, p=0.018).

Following L_1 administration two concentration peaks of L_1-Fe complex were observed in the sera of 9 patients and one in the others(Fig 7.2). The first peak measured 24.3±7.4umol/l occurring at 79.5±46.5min whereas the second peak measured 23.9±6.2umol/l occurring at 93.3±38.1min following L_1 administration.

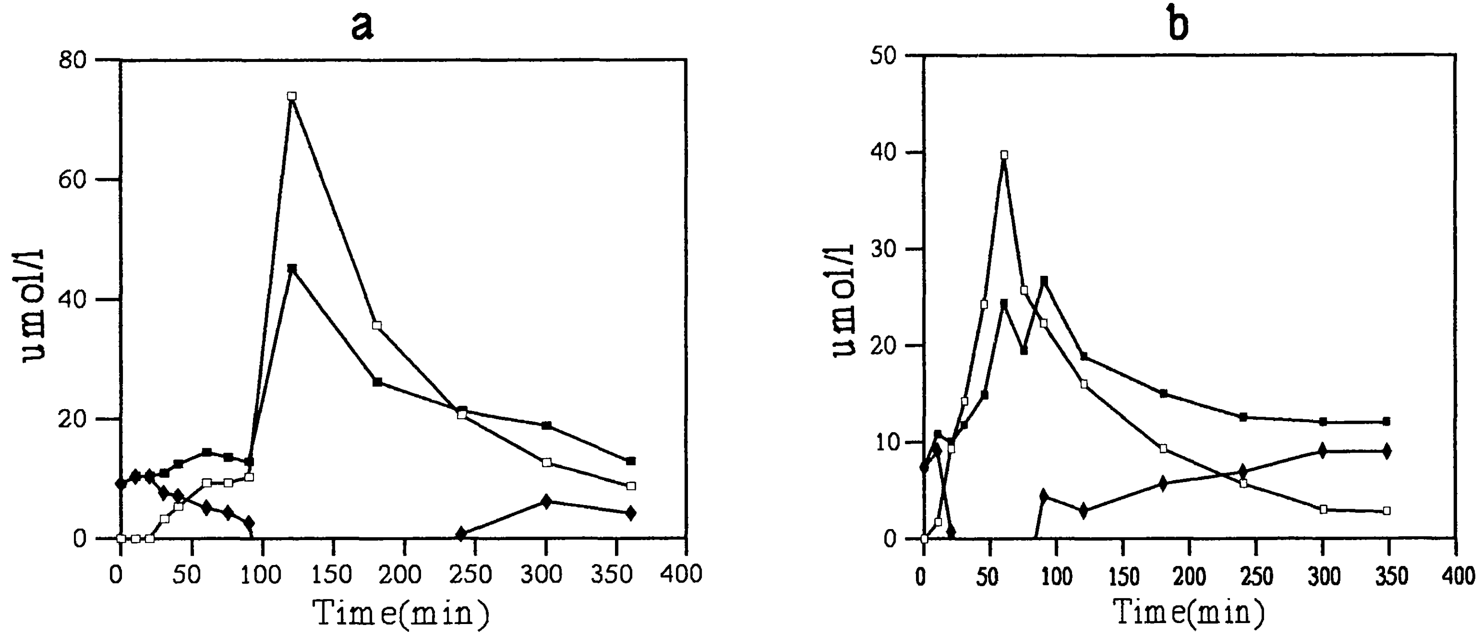


Fig 7.2. Changes in the serum concentrations of 1/3 L_1(-□-), total NTBI(L_1 and non-L_1 bound)(-■-) and true NTBI(-♦-) in 2 thalassaemic patients with iron overload. The first (a) showing a single peak concentration of total NTBI and the second (b) showing 2 peaks.

Table 7.2. Changes in NTBI concentration and pharmacokinetics of deferiprone(L_1) and $(L_1)_3$-Fe complex in 22 patients with iron overload.

Case	Initial NTBI (umol/l)	Final NTBI (umol/l)	Duration of NTBI absence(min)	L_1-$elt_{1/2}$ min	L_1-$AUC_{0-\infty}$ min.umol/l	L_1-Fe-$elt_{1/2}$ min	L_1-Fe-$AUC_{0-\infty}$ min.umol/l	L_1-Fe-$AUC_{0-\infty}$/ 1/3L_1-$AUC_{0-\infty}$(%)
AA	5.3	4.0	135	101.1	20005	83.8	5625	84.4
AD	4.7	0	292	138.5	24361	181.8	6756	83.2
AM	7.3	3.0	193	91.8	34880	138.7	8907	76.6
BJ	7.3	1.1	282	165.1	22357	252.5	7256	97.4
CH	8.4	6.4	155	83.8	24211	97.1	6130	76.0
CSo	9.2	4.2	145	82.8	28518	106.2	7833	82.4
CSt	7.9	7.3	80	134.9	20977	129.7	6486	92.8
DI	8.6	4.0	138	79.0	9737	108.7	2230	68.7
EM	5.1	5.9	304	80.0	43968	36.9	8389	57.2
FM	6.4	7.9	100	58.0	14524	65.9	3262	67.4
FV	5.6	7.7	162	57.5	21898	34.8	3626	49.7
JT	6.9	4.7	113	64.7	12548	68.2	2783	66.5
LL	7.2	5.8	145	89.1	14246	68.5	4048	85.3
MH	7.2	3.3	160	166.5	29072	147.3	8740	90.2
MM	9.7	11	60	52.9	19616	59.0	3811	58.3
MS	7.4	9.1	63	82.6	13272	74.4	3697	83.6
PD	3.8	0	294	95.4	35259	172.6	8347	71.0
SA	10.4	6.9	52	91.1	14450	93.2	4798	99.6
SB	5.3	2.9	212	94.4	26889	126	6796	75.8
SG	6.8	1.1	163	59.3	11364	74.4	2576	68.0
TR	7.4	5.4	110	126.8	23205	91.2	5184	67.0
TT	7.5	8.2	86	78.9	18465	84	5523	89.7
X±SD	7.1±1.7	5.0±3.0	156.6±77.9	94.3±32.8	21992±8669	104.3±51.3	5582±2133	76.9±13.3

NTBI=non-transferrin-bound iron(free iron), $t_{1/2}$=half-life, *el*=elimination, AUC=area under the serum concentration *vs* time curve.

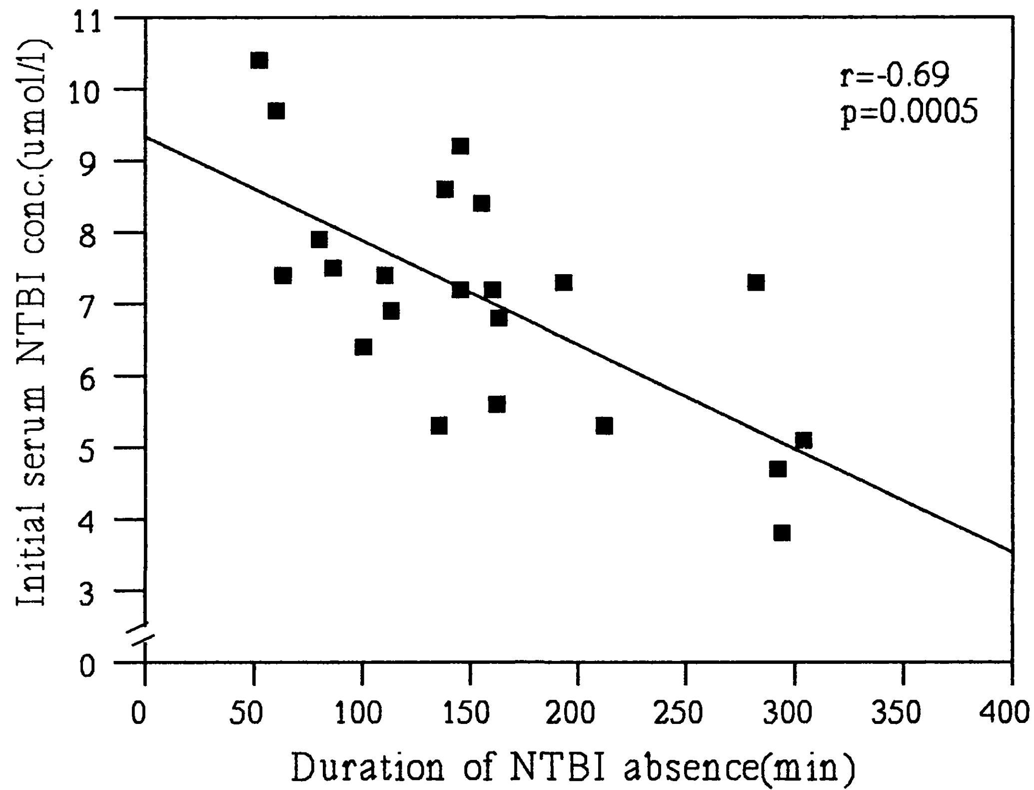
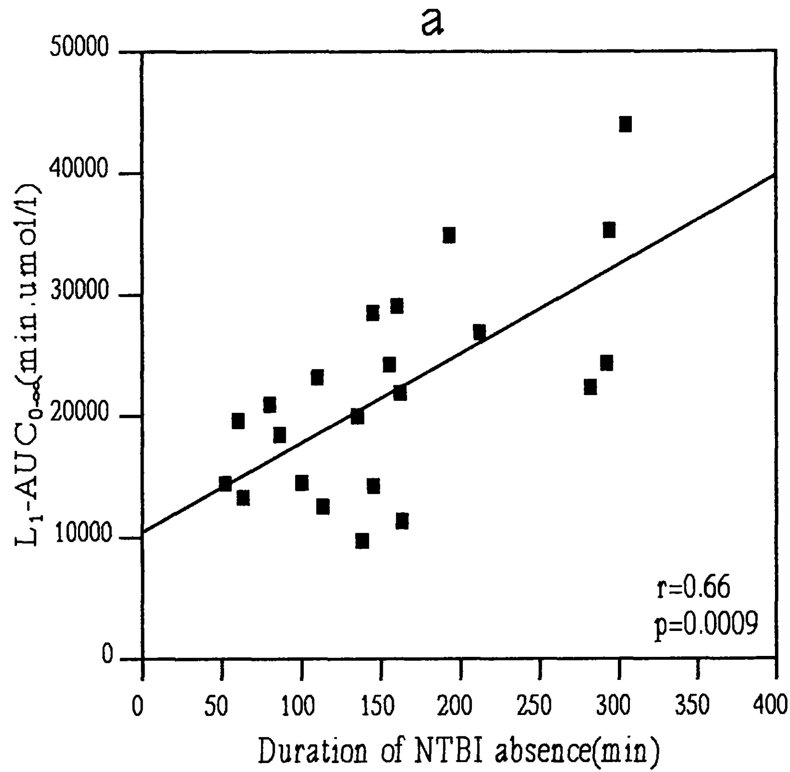


Fig 7.3. The correlation between the initial serum NTBI concentration and the duration of NTBI absence in 22 patients with iron overload following the administration of L_1(50mg/kg).



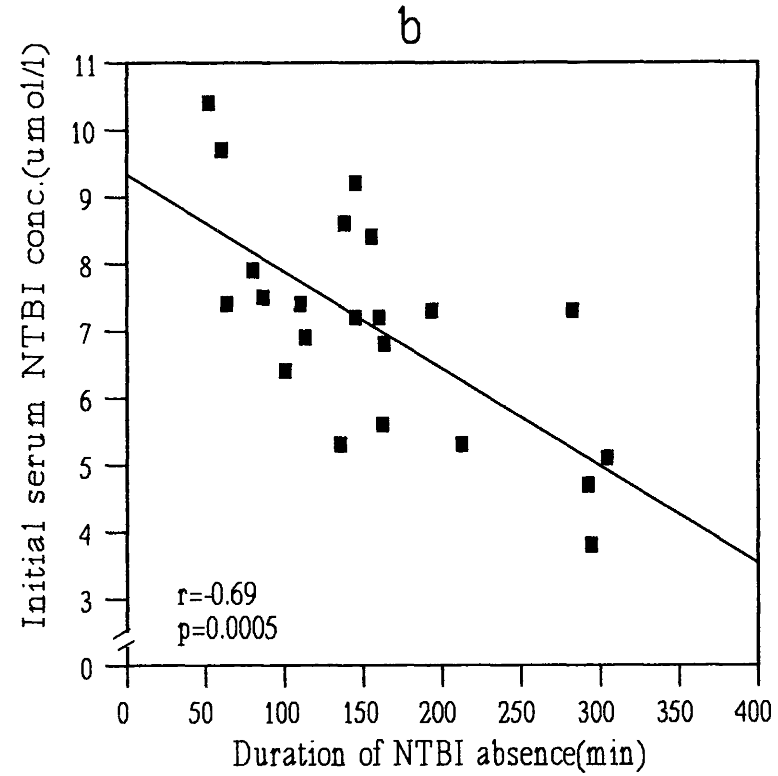


Fig 7.4. The correlation between the duration of NTBI absence and L_1-AUC(a) and the initial serum NTBI concentration(b) in 22 patients with iron overload following the oral administration of L_1 (50mg/kg).

The peak serum concentration of L_1 coincided with the peak concentration of L_1-Fe complex in 8 of the 13 patients who had one peak of L_1-Fe complex and with the first peak in 7 of those with 2 peaks.

There was a significant correlation between the peak serum concentration of L_1-Fe complex and that of L_1($n=15$, $r=0.59$, $p=0.019$).

7.5. DISCUSSION

Currently there are a few methods to measure the level of NTBI in the serum. The easiest to perform and most reproducible is the one designed by Singh *et al*(1990). Although this method can distinguish between NTBI and DFX-Fe complex it cannot do so between NTBI and L_1-Fe complex and no method is, at present, available for this purpose. Therefore, in patients receiving L_1 therapy this method measures the total NTBI in serum including L_1-Fe complex. Therefore, an indirect estimation of the concentration of NTBI and of L_1-Fe complex was necessary. Although, the method utilised here to measure NTBI is an indirect one and does not take into account the possible dissociation of L_1-Fe complex at low concentrations(Mottekaitis & Martell 1991), this dissociation only happens at very low concentrations of L_1-Fe complex which only occur shortly after L_1 intake and also towards the end of L_1 elimination from the body. This comprises a short interval of the total duration of L_1 presence in serum. Therefore, for most of the L_1 life span in the serum L_1 is present at high concentrations compared to NTBI resulting in a stable L_1-Fe complex.

The results of this study show that L_1 is capable of removing all of NTBI for variable periods of time with a mean duration of about 2.5 hours and a maximum exceeding 5 hours. Therefore, in patients receiving L_1 twice a day at the same dose used in this study, NTBI will be absent from their sera for a total mean duration of about 5 hours and

maximum exceeding 10 hours. These figures could be even higher. The data show that the duration of NTBI absence is correlated to the initial NTBI concentration. Therefore, the second dose of L_1 would probably cause longer absence of NTBI if its concentration has not reached the initial concentration by the time the second dose of L_1 was administered. Furthermore, regular chelation with L_1 will cause a reduction in the body iron load resulting in lower initial NTBI concentrations(Chapter 6) and therefore, a more prolonged absence of L_1. The situation with DFX is totally different. NTBI is likely to be absent as long as DFX was infused(usually 8-12 hours/day), assuming that DFX dose was sufficient to cause a complete neutralization of NTBI. Once DFX infusion is stopped NTBI reappears within short time(1-2 hours) in the serum(Gower *et al*, 1989).

The $AUC_{0-\infty}$ of the L_1-Fe complex comprised over 75% of 1/3 of that of L_1 suggesting that L_1 was efficiently utilised by combining with iron. However, as most of L_1 is metabolised to L_1-glucuronide the efficiency of an oral dose of L_1 in human was previously shown to be low at only 3.8%(Chapter 4). Therefore, reducing the rate of L_1 glucuronidation will probably lead to an improved L_1 efficiency.

The significant correlation between L_1-AUC and the disappearance duration of NTBI indicates that bigger doses of L_1 will be associated with a longer absence of NTBI from the serum.

One peak of L_1-Fe complex were observed in 13 patients and 2 in 9. The first peak is probably caused by an L_1 peak in those patients in whom an L_1 peak coincided with the first peak of L_1-Fe complex. The cause of the appearance of the second peak in 9 patients is unclear. It may represent a delayed efflux of L_1-Fe complex from iron loaded tissues. It was anticipated that the elimination of a molecule such as $(L_1)_3$-Fe(M.W 473) would be slower than that of L_1(M.W 139). This is also supported by the previous reports which

showed that elimination $t_{1/2}$ of L_1 is higher in the iron overloaded than in the normal animals or humans(Stobie *et al* 1993, Rahman *et al* 1992). However, the elimination $t_{1/2}$ of L_1-Fe complex in this study(101.9±51.5min) is not significantly different from that of L_1(91.1±33.1min).

A direct method for measuring NTBI and L_1-Fe complex during L_1 therapy is needed. Until such a method becomes available, only indirect estimation is currently possible.

CHAPTER 8

MYELOTOXICITY

8.1. INTRODUCTION

One patient with TM(Chapter 2) and another with MDS(Chapter 3) developed agranulocytosis. The mechanism underlying this adverse effect was investigated in both patients. Three other cases of less severe neutropenia have also been observed during L_1 trial(Chapter 3).

8.2. AGRANULOCYTOSIS-CASE 1

8.2.1. Case history

A twenty-year old Greek female(AM) was diagnosed as having beta-thalassaemia major at the age of 6 months. She commenced regular blood transfusion at the age of 9 months and has subsequently received over 300 units of packed red cells. She began using occasional intramuscular DFX at the age of one year. At the age of 5 years she developed 2 episodes of haemolytic anaemia following blood transfusions when anti-D and anti-Kell antibodies were found in her blood. She did not respond to steroid therapy alone and eventually responded to azathioprine which was then withdrawn gradually. She has subsequently been transfused with D negative, Kell negative blood. She has used DFX subcutaneously since the age of 5, and currently was taking 2.5 gm on 2-5 nights each week with poor compliance.

In September 1990 she volunteered to participate in the clinical trial of the oral chelator L_1(Chapter 2).

At that time she was asymptomatic with normal physical examination. The liver and spleen were both impalpable and she had normal blood count (total white cells 4.6×10^9, granulocytes 2.6×10^9, platelets 185×10^9) and normal renal and liver function. Serum ferritin was 5700ug/l, serum iron 42 umol/l, TIBC 36 umol/l, and she had multiple red cell alloantibodies(anti-D, C, Kell and Kp^a) and diffusely positive antinuclear

antibody(ANA) with a titre of 1/160.

She started oral L_1 therapy at a single daily dose of 3g, increased 4 weeks later to 3g twice daily equivalent to 105mg/kg body weight. The white cell and platelet counts remained normal during the first 11 weeks of treatment (Fig 8.1).

At the beginning of the 12th week she presented with generalised weakness, easy fatigability and a sore throat. On examination she was found to have low grade fever (38°C) and enlarged and congested tonsils. The rest of the clinical examination was normal. Her total white cell count was 2.0×10^9/l, neutrophils 0.1×10^9/l and platelets 253×10^9. She had negative anti-viral antibody screen and her auto antibody status was unchanged.

L_1 was discontinued and she was admitted to hospital and commenced on intravenous broad spectrum antibiotics(Vancomycin and Amikacin). Bone marrow aspirate was performed next day and showed increased cellularity with erythroid hyperplasia. Megakaryocytes were plentiful with normal morphology. Myelopoiesis was markedly suppressed with absence of promyelocytes, myelocytes and mature neutrophils. In an attempt to speed up marrow recovery a single dose(5ug/kg) of GM-CSF/SANDOZ was given subcutaneously three weeks after the onset of agranulocytosis. This was immediately followed by a systemic reaction with pyrexia, rigor, shortness of breath, hypotension and peripheral cyanosis. This was successfully managed with intravenous fluid, antihistamine and hydrocortisone. It was, therefore decided not to administer further GM-CSF.

In the subsequent days she made a progressive clinical recovery but her neutrophil count remained low ($<0.5 \times 10^9$) for 7 weeks (Fig 8.1). She was then discharged from hospital and has since been asymptomatic. She has resumed chelation with subcutaneous DFX.

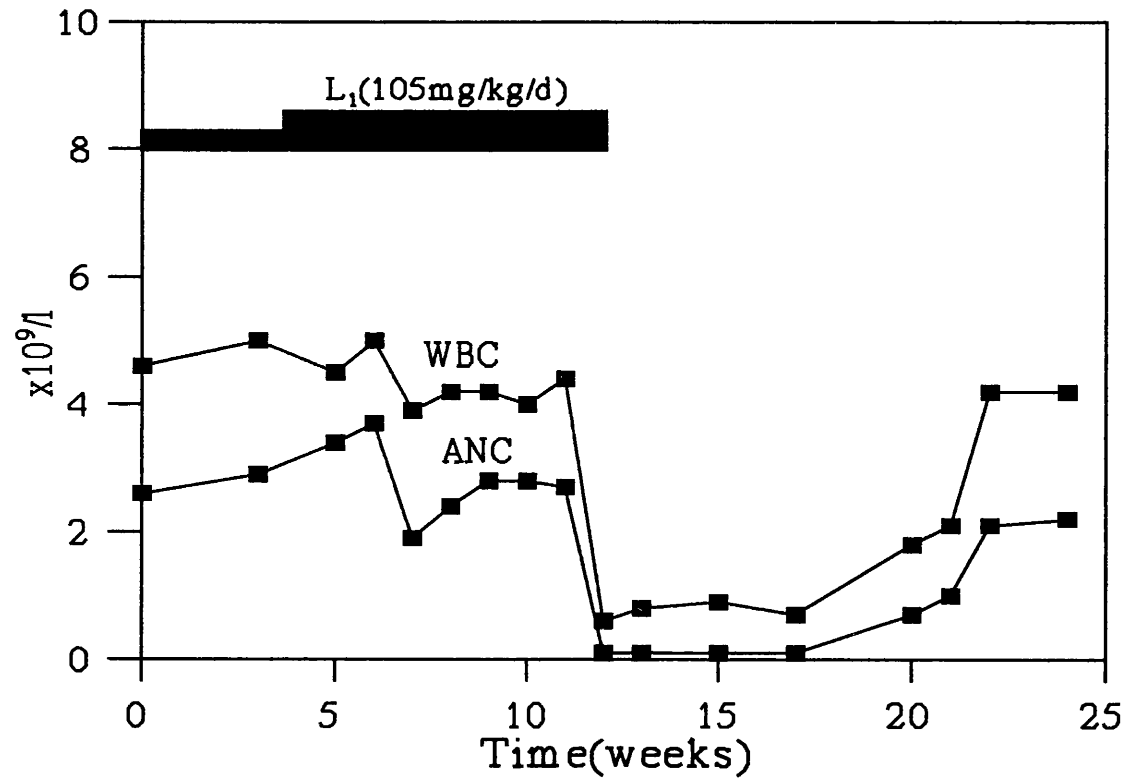


Fig 8.1. Course of white blood cell(WBC) and absolute neutrophil(ANC) counts in patient(AM) who experienced agranulocytosis whilst receiving L_1.

8.2.2. Laboratory experiments

Preservative-free heparinised samples of bone marrow were obtained from a normal volunteer and from the patient whose blood count had recovered to normal several months earlier.

Experiment 1: Tests for direct inhibition of myeloid growth

Mononuclear cells were isolated using Lymphoprep centrifugation. Cells other than myeloid precursors were removed by adherence on a petri dish and immunological selection using My8 and glycophorin A antibodies(Nagler *et al* 1990). The myeloid progenitors were then cultured in triplicate in 1 ml liquid cultures of Iscoves Modified Dulbecos Medium (IMDM) with 15% foetal calf serum (FCS) and 10ng/ml granulocyte colony stimulating factor (G-CSF) (Amgen) at a cell concentration of 1 x 10^6/ml. 10% heat treated autologous serum taken prior to the neutropenic episode (pre) or serum taken during the neutropenic episode (Day 2) were incorporated into the culture with and without the addition of a previously established non-myelotoxic concentration of L_1 (31uMolar)(Cunningham *et al* 1994). Myeloid cell growth was then measured by analysing expression of membrane CD16, a marker of late myeloid differentiation, as previously described(Veys *et al* 1992).

Experiment 2: Test for complement dependent inhibition of myeloid growth

Tests for a complement dependent antibody were performed by removing aliquots from each culture established as described above. The cells were centrifuged in IMDM at 1200 rpm for 5 minutes, incubated with equal volumes of fresh normal serum and either pre-serum or neutropenic serum for 1 hour at 37° C. Cells were then washed in IMDM and resuspended in liquid culture with IMDM and 15% FCS and myeloid growth analysed as above.

Experiment: 3 Immunofluorescent tests for immune complex/auto-antibody mechanisms

The non-adherent bone marrow mononuclear cells were incubated for 12 hours in IMDM and 15% FCS, washed and fixed with 1% paraformaldehyde(PFA), washed again and used as target cells for equal volumes of a premixture of pre or neutropenic serum and 31uMolar L_1. After a 30 minute incubation at 37°C deposition of an immune complex or antibody on the cell surface was detected using rabbit anti FITC Fab_2 anti-human IgG and IgM as described previously(Veys *et al* 1989).

Experiment: 4 Immunofluorescent tests for antibody to myeloid cells: hapten mechanism

The non-adherent bone marrow mononuclear cells were incubated with 31uMolar L_1. At 12 hours the cells were washed, fixed with 1% PFA washed again and incubated for 30 minutes at 37°C with equal volumes of either pre or neutropenic serum. The cells were then washed again and analysed for the presence of IgG and IgM using rabbit anti-FITC Fab_2 anti-human immunoglobulin polyclonal antibodies as above.

8.2.3. Results

The neutropenic serum enhanced growth of autologous myeloid cells to a mean value of 125% of the pre serum. The addition of L_1 to the pre serum reduced mean myeloid growth to 70% of its original value whereas the addition of L_1 to the neutropenic serum reduced mean growth to 44% of its original value. These results together with the absolute values are shown in Table 8.1.

The complement studies failed to show the presence of a complement dependent antibody with mean myeloid cell growth in the presence of neutropenic serum achieving 104% of the myeloid growth in the presence of pre serum.

Table 8.1. The effect of the neutropenic serum of first patient with agranulocytosis and L_1 on myeloid cell growth.

Sample(serum)	Mean myeloid growth(No. of CD16+ cells counted in 5000 cells)	
	Absolute value	%
pre	574	100
neutropenic	715	125
pre + L_1(31 uMolar)	400	70
neutropenic + L_1(31uMolar)	316	44

No excess of IgG or IgM immune complexes were deposited on the myeloid precursor cells in the presence of the neutropenic serum compared to the pre-serum.

A small increase in myeloid membrane IgM was detected following incubation of the neutropenic serum as opposed to the pre serum with cells primed in culture with L_1. This may reflect a weak IgM antibody. No increase in membrane IgG was found.

8.3. AGRANULOCYTOSIS-CASE 2

8.3.1. Case history

A 63 year old Caucasian male(JF) was generally well until 1989 when he developed deep vein thrombosis complicated by pulmonary embolism. At this time he was found to be moderately anaemic with a haemoglobin of 9.5g/dl the rest of the blood counts were normal. This anaemia was associated with a positive direct antiglobulin test and reticulocytosis (6.4%). A bone marrow aspirate at that time showed increased megakaryocytes and a small population of plasma cells and changes consistent with myelodysplasia with almost complete absence of erythroid activity. A diagnosis of myelodysplastic syndrome and pure red cell aplasia was made. A paraprotein (IgG) of 11g/l was found in the serum but there was no immunosuppression or Bence-Jones proteinuria. He became transfusion dependent requiring approximately four units of

packed red cells every four weeks. Both the positive antiglobulin test and reticulocytosis disappeared very early in the course of the disease. He became iron overloaded with a serum ferritin 5000ug/l and commenced subcutaneous DFX infusion 2g/day for 4-5 nights per week in April 1990. In February 1991 he begun reacting to DFX with high temperature (39-40℃) and malaise. Changing the DFX batch or using local and systemic steroid had no effect on this reaction. He, therefore, commenced L_1 therapy in November 1992. At that time, he was asymptomatic. There was generalised pigmentation of the skin with an enlarged liver 3 cm and spleen 4 cm below the costal margin. The blood count was Hb 11.1g/dl, white cells $8.0x10^9/l$, neutrophils $5.1x10^9/l$, platelets $441x10^9/l$; renal and liver function were normal apart from raised AST (63 U/l) and ALT (132 U/l). Serum ferritin was 3700ug/l, serum iron 49umol/l and TIBC 48umol/l. Tests for hepatitis B surface antigen and antibody, hepatitis C antibody, direct antiglobulin test, ANA and dsDNA antibody were negative; RhF was weekly positive(1/80). Multigated acquisition (MUGA) scan revealed a resting LVEF at the lower limit of normal (52%) with some deterioration on cold stress to 46%, consistent with a mild cardiomyopathy.

He started oral L_1 therapy 2g twice daily, increased after 7 days to 3g twice daily equivalent to 79mg/kg. Both the white cell and platelet counts remained normal on weekly testing during the first six weeks (Fig 8.2). At the beginning of the seventh week he presented with malaise and sore throat. On examination he was febrile (temperature 38.5℃) with bronchial breathing and crepitations over the left lower zone. The liver and spleen were both palpable as before. The total white cell count was $1.9x10^9/l$ with no granulocytes and platelets $319x10^9/l$. Chest X-ray showed collapse of the left base with a left basal pleural effusion. The bone marrow was hypocellular with markedly decreased erythropoiesis and absent myelopoiesis. The autoantibody status was unchanged. L_1 was

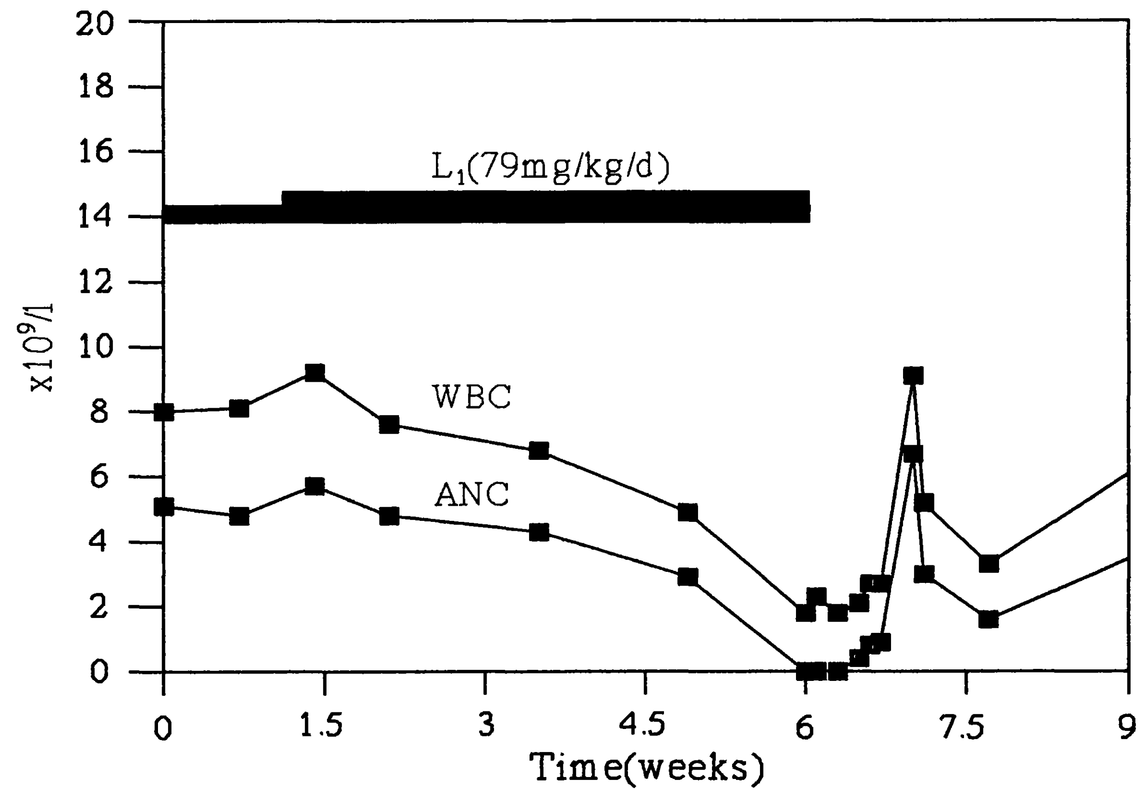


Fig 8.2 Course of peripheral white cell(WBC) and absolute neutrophil (ANC) counts in patient(JF) who experienced agranulocytosis whilst receiving L_1.

discontinued and he was admitted to hospital and commenced on intravenous fluids and broad spectrum antibiotics (vancomycin and ceftazidime). As he was still pyrexial three days later intravenous metronidazole and acyclovir were added and he was also commenced on G-CSF at a dose of 300ug daily. Next day he developed a rapid and irregular pulse with an ECG showing atrial fibrillation. He was therefore commenced on oral digoxin. A day later he felt unwell with pleuritic chest pain and central cyanosis with an O_2 saturation of 88% and a ventilation perfusion scan suggesting bilateral pulmonary embolism. He was therefore started on O_2 therapy and intravenous heparin infusion and as he was still febrile his antibiotics were changed to imipenem, metronidazole and liposomal amphotericin. Four days later he became afebrile with an O_2 saturation of 97% on air and improved chest signs. At this time his neutrophil count was $0.4x10^9/l$. The liposomal amphotericin was stopped and four days later imipenim, metronidazole and G-CSF were also stopped and he was converted to warfarin for anticoagulation. His total white cell count at this time was $4.3x10^9/l$, neutrophil count $2.3x10^9/l$ and platelets $213x10^9/l$ (Fig 8.2).

He was then commenced on daily subcutaneous DTPA infusion for iron chelation, initially at a dose of 1g which was then increased to 2g per day over 10 hours together with zinc sulphate(220mg q.d.s).

8.3.2 Laboratory experiments

Preservative-free heparinised samples of bone marrow were obtained from normal volunteers and the patient three months after complete recovery from agranulocytosis. Serum samples were obtained from the patient before (pre) and during agranulocytosis (day 2) and during convalescence (recovery 1) and 3 months after complete recovery (recovery 2). These samples were stored at -20℃ until the time of analysis. All samples

were heat treated at 56°C for 30 minutes to destroy complements shortly before use.

Liquid cultures of myeloid progenitors were used in all the experiments. These cultures were performed as previously described(Veys *et al* 1992). At the end of the seven-day incubation period CD16+ cells (CD16=mature granulocyte marker) and CD33+ cells (CD33=myeloid progenitor marker) were counted using FACS scanning and the results were compared to a parallel control. Results obtained utilising the CD16 marker were comparable to those obtained with the CD33 marker and data presented here were obtained with the former marker.

The inhibitory concentration 50(IC_{50}) is defined as the concentration of the chelator associated with 50% inhibition of myeloid growth compared to a normal control. Statistical significance was evaluated using the Student's *t*-test. The following four experiments were performed:

Experiment 1: In order to assess the possibility that patient serum might contain an inhibitory factor (L_1- or non-L_1 dependent) to myelopoiesis, patient's sera (pre, day 2 of agranulocytosis, recovery 1 & 2) were incorporated into myeloid cultures obtained from the patient's recovery bone marrow with and without the addition of a non-myelotoxic concentration of L_1 (31uM). These sera were used at 10% concentration and the pre and day-2 sera were also used at 40% concentration.

Experiment 2: L_1 toxicity to normal and patient myelopoiesis was compared. L_1 at concentrations 0-1000uM was added to myeloid cultures obtained from the patient's recovery marrow and from a normal volunteer. A saturating concentration of iron (1 Fe:3 L_1) was added to parallel cultures.

Experiment 3: L_1 toxicity to normal myeloid progenitors was compared to that of DFX. L_1 or DFX with or without saturating concentrations of iron (1 Fe : 3 L_1, 1 Fe : 1 DFX)

was added at concentrations 0-1000uM to myeloid cultures obtained from a normal volunteer.

Experiment 4: In order to assess the reversibility of chelator (DFX or L_1) inhibitory effect on myelopoiesis, an inhibitory dose (250uM, see below) of L_1 or DFX was separately incorporated into cultures of normal myeloid progenitors and saturating concentrations of iron (as above) were added to the cultures at different time intervals (0, 4, 6, 10, 24 hours) after the addition of the chelator. Separate controls for each time interval were used to which similar concentrations of iron were added. Results from each time interval at the end of the incubation period were compared to the specific control.

8.3.3. Results

When patient's recovery myeloid cultures where incubated with autologous sera(pre, day 2, recovery 1 or 2) for 7 days, no significant inhibition of myeloid growth was observed in any of the cultures in the presence or absence of L_1. Indeed, the neutropenic serum at both concentrations tested (10%, 40%) enhanced the growth of the patient's myeloid cells in the presence of L_1 to a mean value of 137% and 244% respectively whereas less marked enhancement compared to control was observed in the absence of L_1 (104% and 137% respectively) (Fig 8.3).

No significant difference between the IC_{50} of L_1 for normal and patient's myelopoiesis was found (150uM *vs* 172uM respectively). The addition of a saturating dose of iron was associated with complete abolition of toxicity in both cultures(Fig 8.4).

DFX was found to be more toxic to myelopoiesis than L_1 with an IC_{50} of 9uM compared to 150uM for L_1 ($p<0.05$). The addition of a saturating concentration of iron was again associated with complete suppression of inhibitory effects to myelopoiesis of both L_1 and DFX(Fig 8.5).

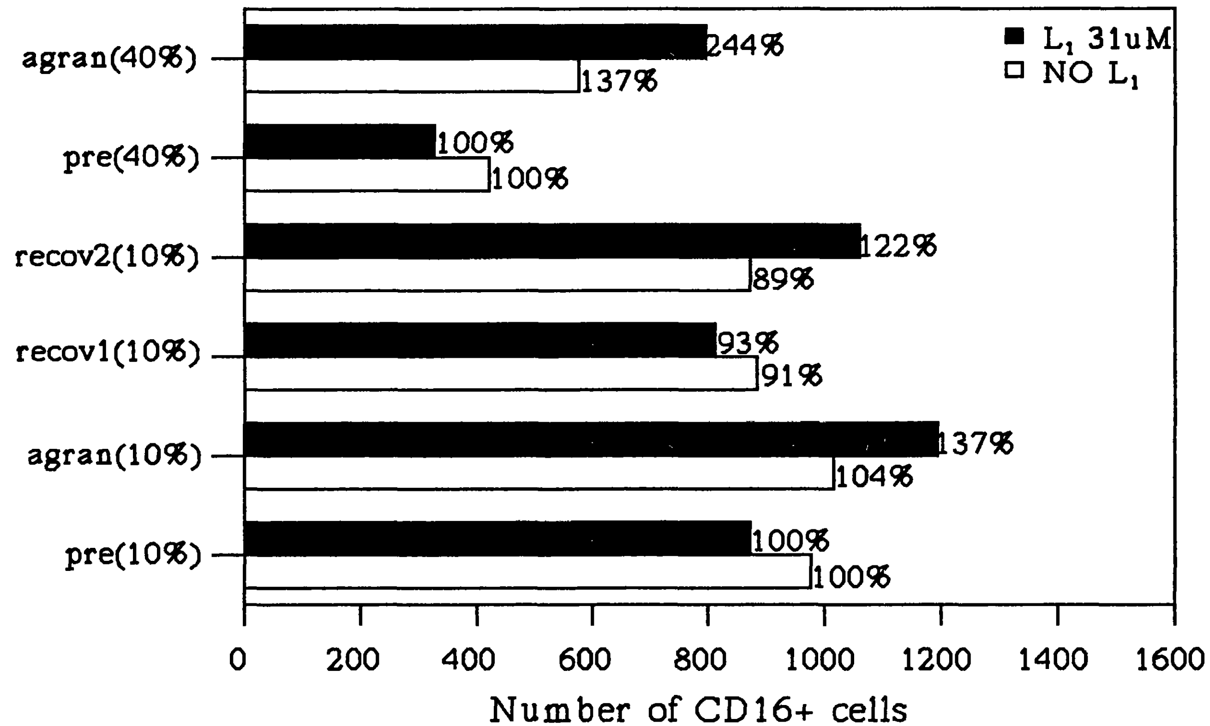


Fig 8.3 The effect of patient's sera obtained before(pre), during agranulocytosis (agran), during convalescence(recovery 1) and 3 months after complete recovery (recovery 2) on the growth of myeloid progenitors obtained from the patient's recovery bone marrow in the presence(solid columns) or absence(open columns) of a non-myelotoxic concentration of L_1(31μM).

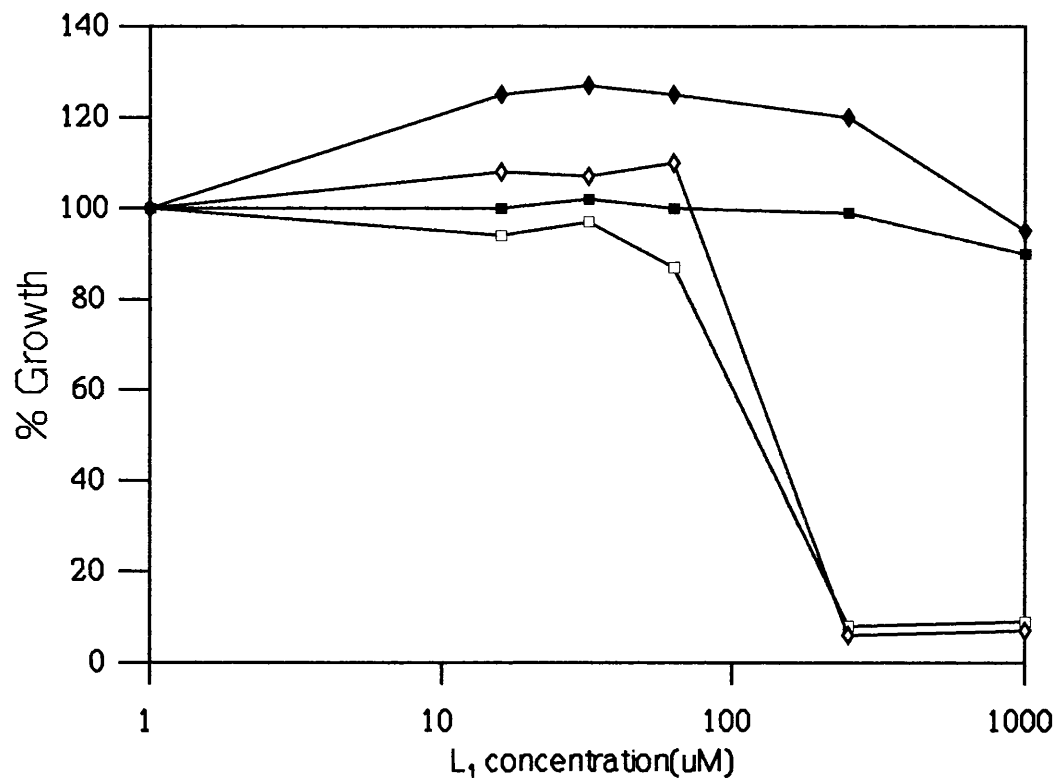


Fig 8.4 Comparison between L_1 toxicity to normal and patient's myelopoiesis in the presence (-■-, -♦-) or absence (-□-, -◊-) respectively of saturating concentrations of iron.

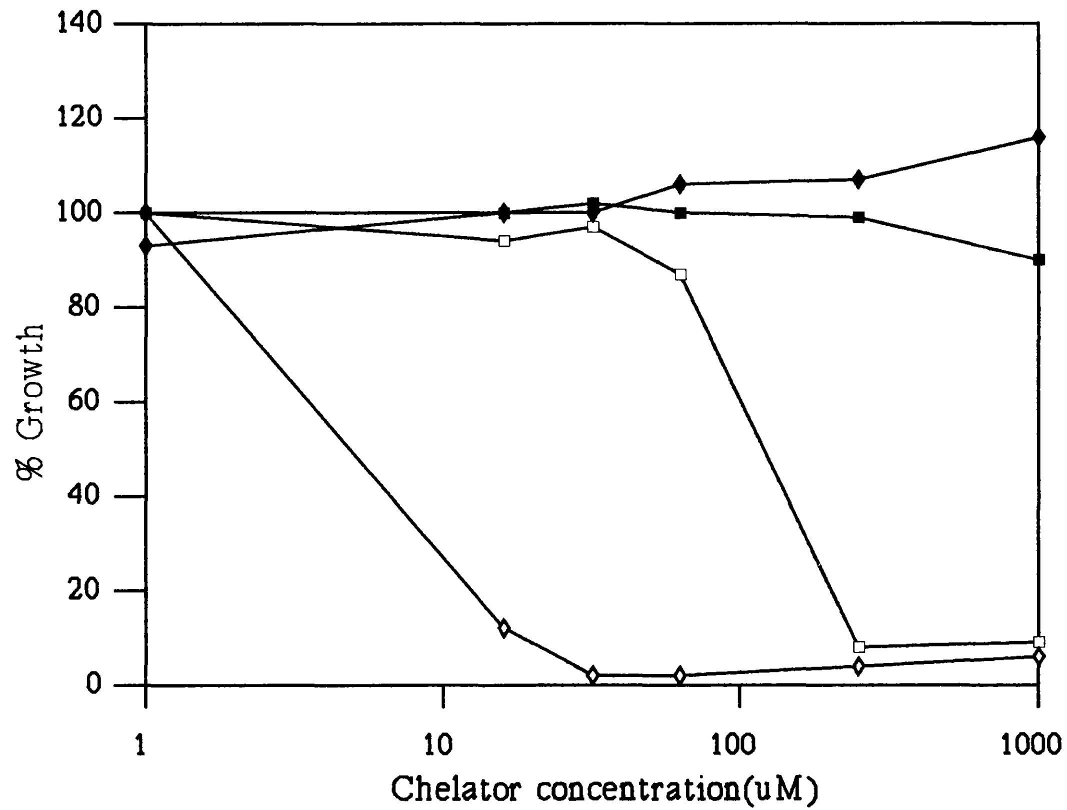


Fig 8.5 Comparison between L_1 and DFX toxicity to normal myelopoiesis in the presence (-■-, -♦-) or absence (-□-, -◊-) respectively of saturating concentrations of iron.

When iron was added at intervals of up to 6 hours to cultures containing L_1 it was still capable of abolishing the inhibitory effect of L_1 on myeloid cell proliferation whereas at 10 hours L_1-induced inhibition was only partially reversible resulting in myeloid growth of only 55%. At 24 hours L_1 inhibition was not recovered by iron. Similarly the DFX inhibitory effect on myelopoiesis was reversible when iron was added to the cultures in the first few hours. However, by 6 hours the addition of iron failed to completely reverse the DFX effect and by 24 hours addition of iron had no effect (Fig 8.6).

8.4. CASES OF L_1 INDUCED NEUTROPENIA

8.4.1. Case 1(AD)

A 23 year old female patient with thalassaemia major, started L_1 (90mg/kg/day) therapy in June 1992. She had previously used DFX (50mg/kg/day) with poor compliance. Her total white cell count was $5.9x10^9$/l, neutrophils $2.7x10^9$/l, serum ferritin 3770ug/l, serum iron 34umol/l and TIBC 40umol/l. She was not splenectomized and anti-HCV negative. In August 1993 she became neutropenic with a total white cell count of $2.7x10^9$/l(neutrophils $0.3x10^9$/l) but was asymptomatic. L_1 was withdrawn and two weeks later her neutrophils were $2.5x10^9$/l. In September 1993 (neutrophils $3.2x10^9$/l) L_1(40mg/kg/day) was re-introduced but four weeks later her neutrophils dropped to $0.4x10^9$/l. L_1 was, therefore, stopped and her neutrophil count returned to normal two weeks later(Fig 8.7).

8.4.2. Case 2(DH)

A 20 year old male patient with thalassaemia major, had been chelated with DFX 40-50mg/kg 4-5 nights per week. He was splenectomized at the age of 12 years and was anti-HCV negative. At the beginning of 1992 he became allergic to DFX with urticarial skin reaction and he, therefore, commenced L_1(97mg/kg/day) in May 1992. His white

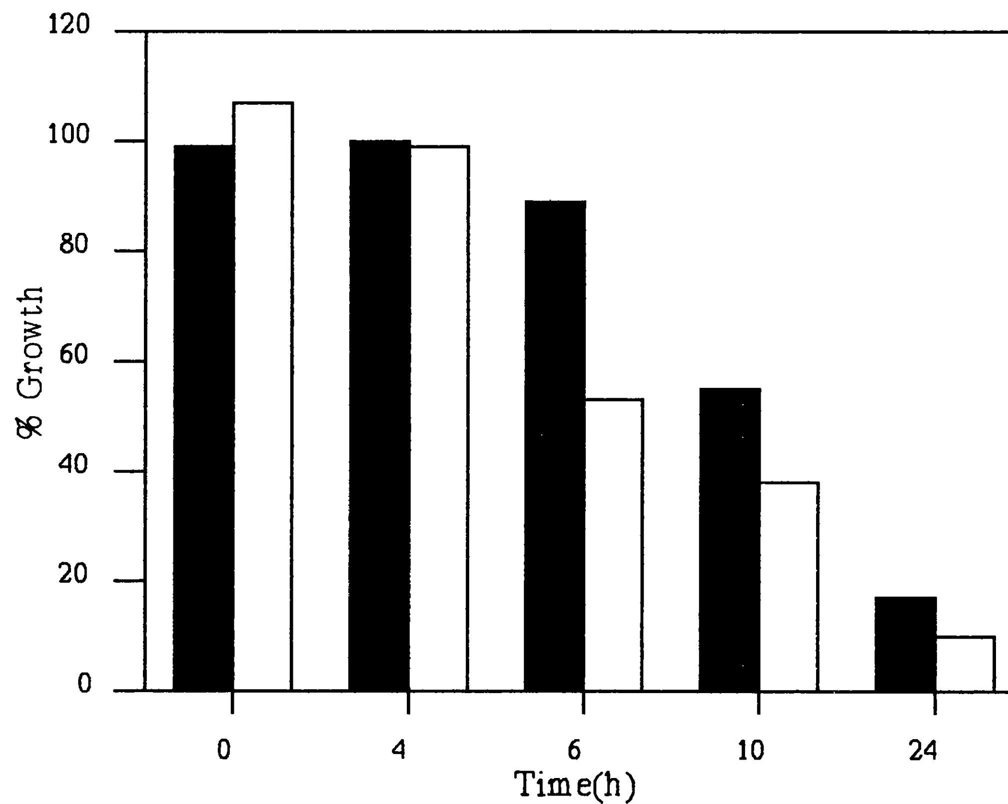


Fig 8.6 Effect of delayed addition of saturating concentrations of iron to normal myeloid cultures in the presence of an inhibitory concentration(250μM) of L_1(solid columns) or DFX(open columns).

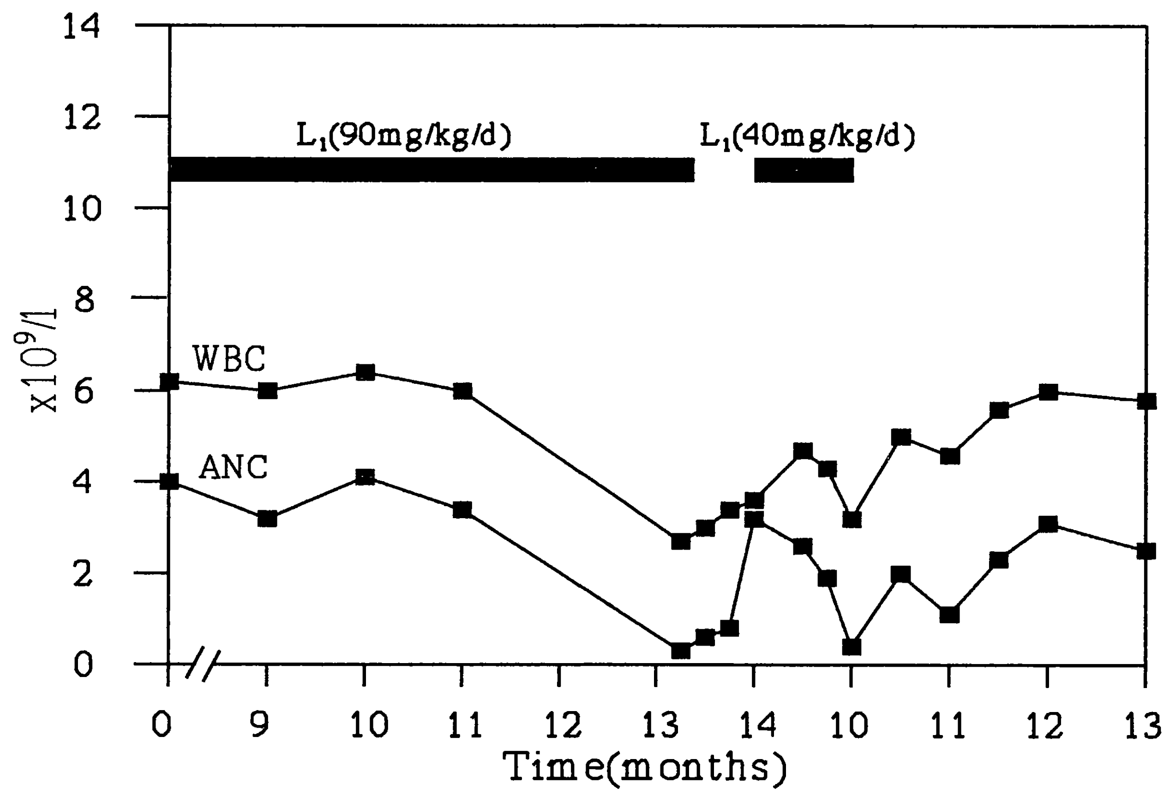


Fig 8.7 Course of peripheral white cell(WBC) and absolute neutrophil (ANC) counts in patient(AD) during L_1 therapy.

cell count was 12.8x10^9/l, neutrophil count 2.6x10^9/l, serum ferritin 2168ug/l, serum iron 31umol/l and TIBC 36umol/l. In July 1993 his neutrophil count fell to 1.8x10^9/l and the L_1 was stopped and then restarted 2 months later at a dose of 86mg/kg/day. In December 1993 his neutrophil count fell to 0.6x10^9/l. He was asymptomatic and the neutrophil count rose to 4.6x10^9/l two weeks after stopping L_1. In February 1994 when the neutrophil count was 2.8x10^9/l L_1(43mg/kg/day) was re-administered but the neutrophil count fell again to 1.2x10^9/l four weeks later and reversed to normal two weeks after the discontinuation of L_1(Fig 8.8).

8.4.3. Case 3(MM)

A 24 year old, male patient with thalassaemia major started L_1 therapy(103mg/kg/day) in May 1992. He had previously received DFX(50mg/kg/day) for iron chelation but with poor compliance. His white cell count prior to L_1 therapy was 7.1x10^9/l, neutrophil 3.0x10^9/l, serum ferritin 3350ug/l, serum iron 33umol/l and TIBC 42umol/l. He was not splenectomized and was anti-HCV positive with abnormal liver function. In April 1993 the neutrophil count fell to 1.4x10^9/l. L_1 was discontinued and the neutrophil count recovered two weeks later to 2.3x10^9/l. In June 1993 when the neutrophil count was 3.9x10^9/l, L_1(58mg/kg/day) was restarted but the neutrophil count fell again to 1.2x10^9/l four weeks later and rose back to normal two weeks after the withdrawal of L_1(Fig 8.9).

8.5. DISCUSSION

The neutropenic serum of both patients with agranulocytosis caused an enhanced myeloid cell growth. This probably reflects the presence of increased concentrations of circulating myeloid growth factors as a result of the profound peripheral neutropenia. It also suggests that no direct myelotoxic action of L_1 or L_1-metabolite was present in the serum. However, the addition of a low concentration of the drug to the myeloid cultures

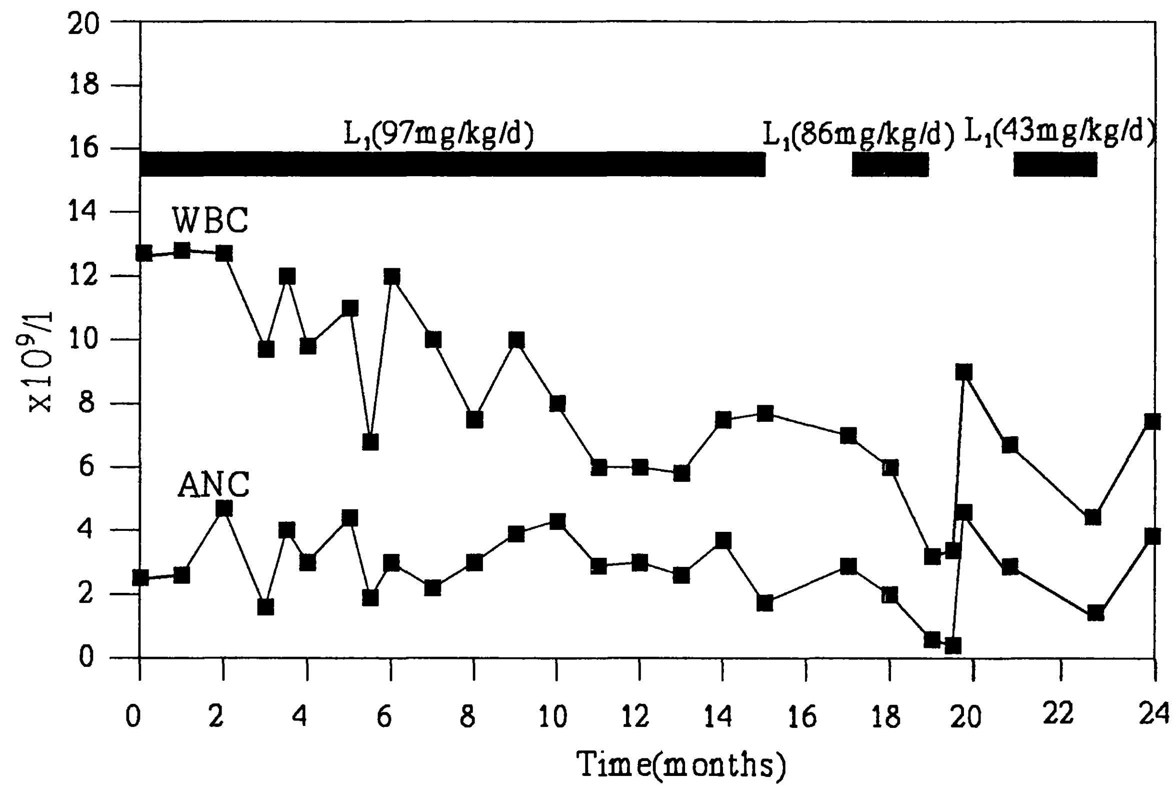


Fig 8.8 Course of peripheral white cell(WBC) and absolute neutrophil (ANC) counts in patient(DH) during L_1 therapy.

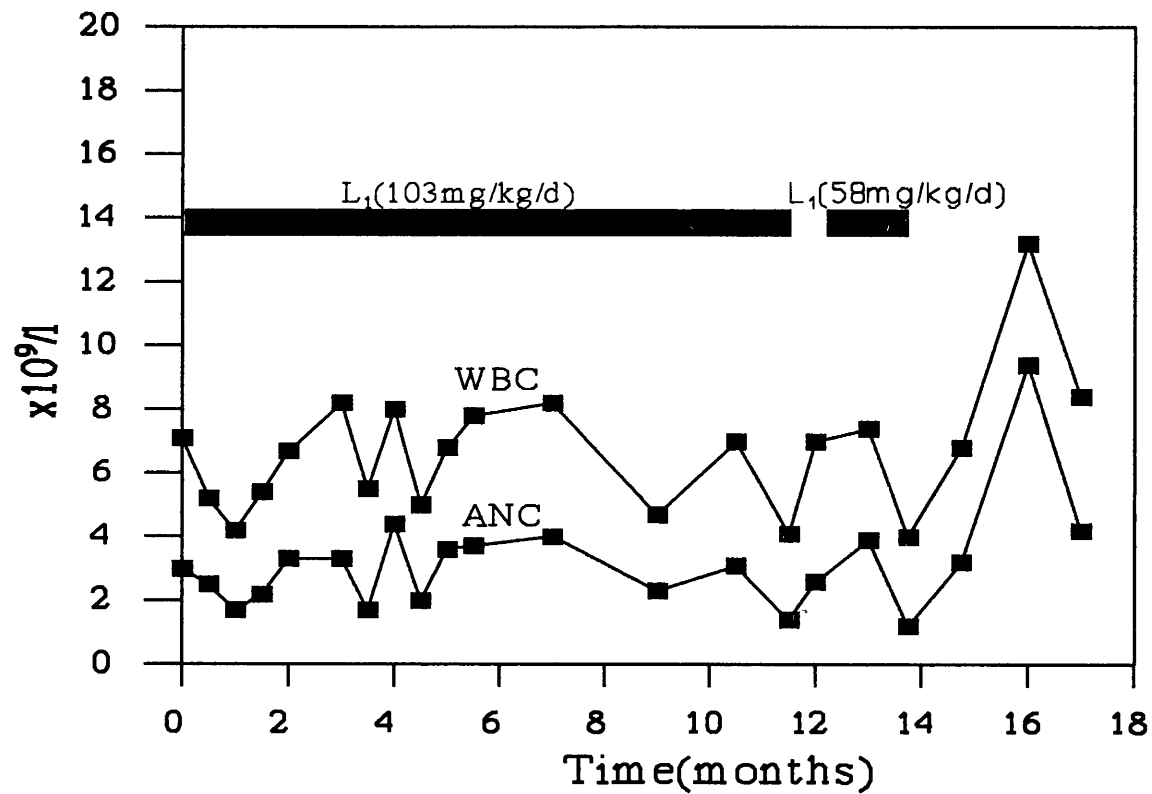


Fig 8.9 Course of peripheral white cell(WBC) and absolute neutrophil (ANC) counts in patient(MM) during L_1 therapy.

of the first patient caused toxicity greater in the neutropenic serum than pre serum. This could be explained by the formation of a drug antibody immune complex or the formation of a hapten with recognition by an antibody present in the neutropenic serum. It is also possible that the increase in myeloid turnover induced in culture by the neutropenic serum may have potentiated a myelotoxic effect due to iron deprivation at the low concentration of L_1 in this system. Such inhibition was not seen in the second patient. The significance of a weak IgM antibody found in the screen for a hapten mechanism is uncertain.

No significant difference was observed between L_1-induced inhibition of normal and second patient myelopoiesis, implying that patients' myelopoiesis is not unduly sensitive to L_1 in vitro.

Toxicity of L_1 to normal myelopoiesis was found to be much lower than that of DFX (IC_{50}: DFX 9uM *vs* L_1 150uM, $p < .05$). These results are consistent with the previous report by Cunningham *et al* (1994) who used semisolid rather than liquid myeloid cultures (IC_{50}: DFX 7.9uM *vs* L_1 130uM). In both studies the myelotoxicity of L_1 and DFX at doses up to 1000uM was abolished by a saturating concentration of iron added at the beginning of culture indicating that the in vitro toxicity of L_1 or DFX at these concentrations is due to chelation of iron in cultures. These in vitro observations are in contrast to in vivo studies in animals which have shown that iron overload has no bearing on L_1-induced suppression of myelopoiesis(Porter *et al* 1989b, Grady *et al* 1992). Moreover, the patients developing L_1 associated agranulocytosis have all been heavily iron overloaded. Thus it seems that L_1 induced agranulocytosis in vivo is not a direct consequence of iron depletion. Both L_1 and DFX can inhibit iron-containing enzymes including ribonucleotide reductase which is involved in DNA synthesis (Hoffbrand *et al*

1976, Dezza *et al* 1989, Porter *et al* 1992). In view of its different effects from DFX or hydroxyurea on supply of deoxyribonucleotides for DNA synthesis, L_1 may have other actions than that on ribonucleotide reductase in inhibiting DNA synthesis (Ganeshaguru *et al* 1991,1992).

Delayed addition of iron to myeloid cultures containing an inhibitory concentration of L_1 or DFX showed that chelator-induced inhibition of myelopoiesis in vitro is still reversible by iron for six hours but not after 24 hours of incubation.

It appears that there are two patterns of myelotoxicity associated with L_1 therapy in humans. The first is a severe agranulocytosis usually occurring about 6 weeks from the commencement of high dose L_1 therapy. The second is less severe neutropenia, occurring at any time during L_1 therapy. Reintroduction of L_1 appears to cause neutropenia to recur, but at the same pace as for the original development of neutropenia. This pattern is more in favour of a toxic than immune mechanism for L_1-induced myelotoxicity. Table 8.2 summarizes the cases of L_1-induced neutropenia and agranulocytosis reported, so far, in the literature.

Table 8.2. Clinical details of 9 patients* developing agranulocytosis or neutropenia during L_1 therapy.

Age/Sex	Diagnosis	ANC at diagnosis at diagnosis	Dose of L_1 (mg/kg/day)	Duration on L_1 therapy (months)	Time to recovery (weeks)	Centre (reference)
28/F	BDA	0x10^9/l	105	1.5	3	London (Hoffbrand *et al* 1989)
20/F	TM	0x10^9/l	105	1.5	7	London (Chapter 2,8)
63/M	MDS	0x10^9/l	79	1.5	1	London (Chapter 3,8)
23/F	TM	0.3x10^9/l	90	14	2	London (Chapter 8)
20/M	TM	0.6x10^9/l	86	19	2	London (Chapter 8)
24/M	TM	1.4x10^9/l	103	12	2	London (Chapter 8)
10/F	TM	0x10^9/l	75	10	<1	Bombay (Agarwal *et al* 1993a,b)
16/F	TM	0.2x10^9/l	75	21	<1	Bombay(Agarwal 1994 personal communication)
40/M	MDS	0.3x10^9/l	50	12	<1	Amsterdam (Goudsmit& Kersten 1992)

ANC=absolute neutrophil count, BDA= Blakfan-Diamond anaemia, TM=thalassaemia major, MDS=myelodysplastic syndrome.
* two more cases of agranulocytosis have been reported by Olivieri *et al*(1994 personal communication)

CHAPTER 9

EFFECT ON ZINC STATUS

9.1. INTRODUCTION

Zinc is an essential trace metal for the normal function of many enzymes involved in cell division, DNA and protein synthesis in humans(Prasad & Oberleas 1974). Zinc deficiency is associated with several clinical manifestations such as growth retardation, delayed wound healing, skin changes, hypogonadism, glucose intolerance, anaemia and abnormal leucocyte function(Mahajan 1989). Patients with diabetes mellitus(DM) and particularly those with insulin-dependent DM(IDDM) are at risk of developing zinc deficiency(Kinlaw *et al* 1983, Mooradian & Morley 1987). Although these patients excrete more zinc in urine than normal individuals(Kinlaw *et al* 1983, Martin *et al* 1991, Honnorat *et al* 1992) serum zinc levels may be normal, increased or decreased(Nakamura *et al* 1991). Furthermore, only few of DM patients develop clinical manifestations of zinc deficiency. Although several mechanisms for hyperzincuria in diabetics have been suggested such as a non-osmotic process mediated by glucose and an alteration in gastrointestinal absorption of zinc(Kinlaw *et al* 1983, Craft & Faila 1983), the exact mechanism remains obscure. The low incidence of zinc deficiency among patients with hyperzincuria is probably due to an adequate intake or compensatory increased absorption of zinc. Furthermore the estimation of serum zinc level has its technical and interpretive limitations(Committee on Nutrition 1978), so patients with normal serum zinc levels can be zinc deficient. On the other hand a subnormal serum zinc level is only suggestive but alone not diagnostic of zinc deficiency(Mahajan 1989).

Patients with thalassaemia major not receiving regular chelation therapy or blood transfusions have also been found to have serum zinc levels lower than normal individuals and to have increased UZE(Çavdar 1991). It is unclear whether these findings

are due to the presence of DM among these patients.

The effect of iron chelation therapy on trace metals in patients with iron overload depends on the affinity of the chelator to these metals. DFX has now been used for many years with no previous reports of an associated zinc deficiency. This is in contrast to the well known severe zinc loss associated with the iron chelator diethyltriamine penta-acetic acid(DTPA)(Pippard *et al* 1986, Wonke *et al* 1989) necessitating substantial oral supplements of zinc.

None of the earlier short- and long-term trials of L_1 reported a change in serum zinc levels or increased urinary zinc excretion. Recently, however, an increase in UZE and subnormal serum zinc levels were found in several patients on regular chelation therapy with L_1. This was associated with symptoms of dry itchy skin patches which resolved on zinc supplementation in two patients(Chapters 2,3).

In this study zinc status was monitored closely in patients receiving L_1 therapy and correlated with the presence of DM or more subtle biochemical abnormalities of glucose metabolism.

9.2. MATERIALS AND METHODS

DFX was obtained from Ciba Geigy and L_1 was synthesised as previously described(Kontoghiorghes & Sheppard 1987). Serum ferritin was estimated by enzyme linked immunosorbent assay(ELISA) technique(Flowers *et al* 1986). Urinary iron and zinc and serum zinc were measured using atomic absorption spectrophotometry(Scudder *et al* 1978). Oral glucose tolerance tests were performed by administering 75g of glucose after overnight fasting and sampling blood every 30min for two hours. Statistical significance was evaluated using the Student's *t*-test. Data will be expressed as mean±standard deviation.

9.3. PATIENTS

Thirty nine patients (24 males, 15 females) were studied. Their ages ranged between 13-60(27.1±11.0)years. Initial serum ferritin ranged between 733-9060(3551±2123)ug/l. 31 patients had β-thalassaemia major, 2 sickle cell disease, 2 congenital sideroblastic anaemia, 1 myelodysplastic syndrome, 1 pyruvate kinase deficiency, 1 haemoglobin E/β-Thalassaemia and 1 sickle/β-Thalassaemia. Serum zinc level was assayed initially and two monthly thereafter. Two to four 24 hour urine collections were obtained from each patient whilst receiving subcutaneous infusion of DFX at an approximate dose of 50mg/kg/day and four or more collections of urine were obtained during L_1 therapy (50-100mg/kg/day). These urine samples were analyzed for both the total iron and zinc content. Normal values for serum zinc concentration and 24-hour UZE are 11.5-17.0umol/l and 4.5-9.0umol/24h, respectively.

9.4. RESULTS

Twenty four hour UZE in 39 patients receiving L_1 therapy was 15.1±7.3umol (range: 4.4-34.2) significantly higher than that associated with DFX therapy(11.1±6.0umol, range: 2.6-26.5, $p=0.01$) and both were significantly higher than the normal range for UZE ($p<0.001$, $p=0.04$ respectively). There was significant correlation between L_1- and DFX-associated UZE($r=0.74$, $p<0.001$). Different regimes of L_1 administration (bd, qds) showed no significant difference in their effect on UZE in 19 patients studied nor had the co-administration of vitamin C. Also taking L_1 with food or fasting did not significantly alter UZE.

No correlation was found between UZE and L_1 dose($p=0.11$) or urine iron excretion(UIE)($p=0.1$) or between UZE and serum ferritin levels($p=0.92$).

UZE was significantly higher in patients receiving L_1 with DM(24.6±7.9, n=8) than

patients with a normal GTT(NGTT)(13.1±6.2, n=18, p=0.0006) or those without DM but with an abnormal GTT(AGTT)(16.3±7.3, n=13, p=0.02). No significant difference was observed between the latter two groups of patients(p=0.2)(Fig 9.1). Comparable results were observed with DFX. DM patients receiving DFX(n=7) excreted more zinc than non-diabetics(n=17) or those with AGTT(n=9) [16±6.4 *vs* 9.7±4.6(p=0.01), 8.2±3.7(p=0.008) respectively](Fig 9.2). Again no significant difference was found between the latter two group of patients (p=0.59).

There were significant differences between UZE of DM or AGTT patients receiving L_1 therapy and corresponding patients receiving DFX(p=0.04 and 0.03 respectively) but no significant difference was observed between NGTT patients receiving L_1 and those receiving DFX(p=0.59). However when the paired *t*-test was used to compare the excretion of zinc in the individual patients in the latter two groups a significant difference was obtained (p=0.003). All three groups of patients receiving L_1 therapy have significantly increased UZE compared to normal (DM:p=0.0009; AGTT:p=0.0036; NGTT:p=0.01). Among patients receiving DFX only those with DM have significantly increased UZE compared to normal (p=0.03).

9.5. DISCUSSION

The results observed in this study confirm previous observation that in patients receiving L_1 therapy zinc excretion in the urine is increased(Chapters 2,3). This was significantly higher than the zinc excretion found in patients receiving DFX therapy, although this was also significantly increased compared to normal subjects. Neither the L_1 dose nor iron load of the patients correlated significantly with UZE. The significant correlation observed between L_1- and DFX-associated UZE suggests that individual susceptibility for increased zinc excretion is the same with both chelators.

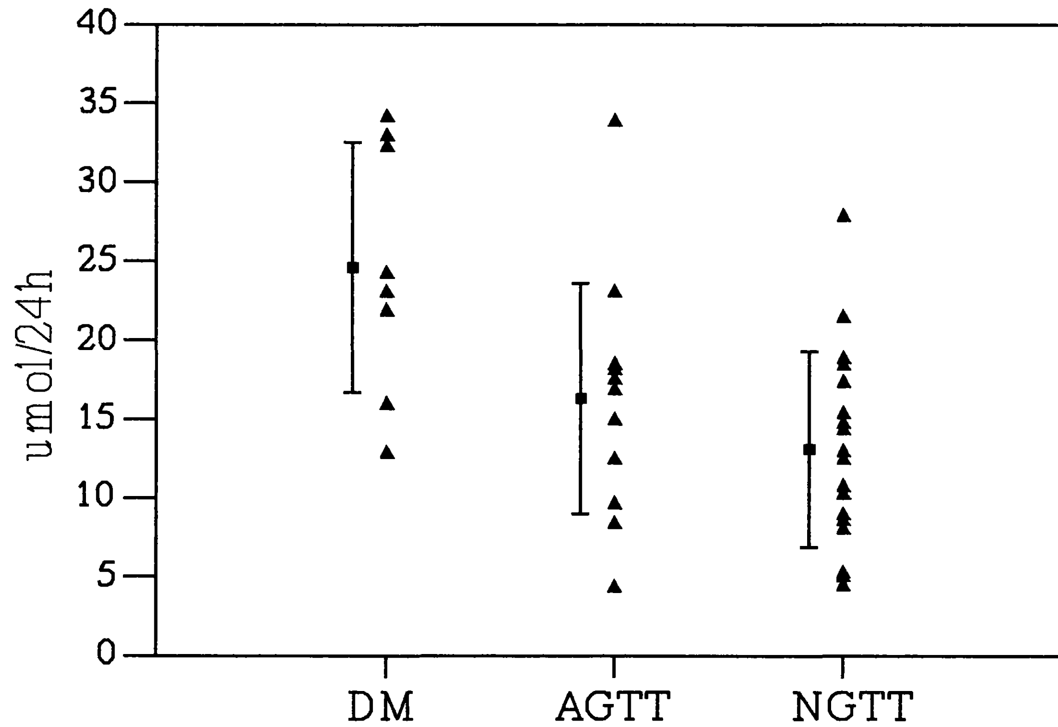


Fig 9.1. Urinary zinc excretion in 39 patients with iron overload receiving L_1 therapy, segregated into 3 groups: diabetes mellitus(DM, n=8), abnormal glucose tolerance test(AGTT, n=13), and normal glucose tolerance test(NGTT, n=18). X±SD for each group is shown.

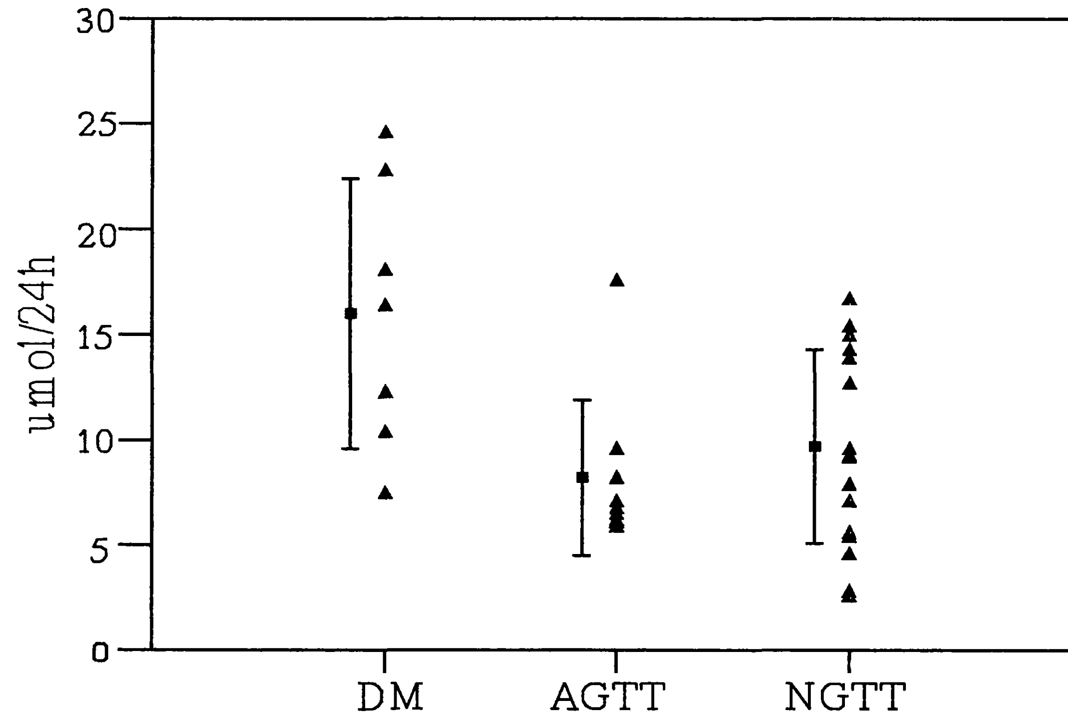


Fig 9.2. Urinary zinc excretion in 33 patients with iron overload receiving DFX therapy, segregated into 3 groups: diabetes mellitus(DM, n=7), abnormal glucose tolerance test(AGTT, n=9), and normal glucose tolerance test(NGTT, n=17). X±SD for each group is shown.

Patients with transfusion dependent refractory anaemias are at risk of developing DM as a result of iron overload. As DM patients excrete more zinc in urine than normal individuals it was essential to assess the UZE of patients receiving iron chelation therapy in relation to their blood glucose status. When patients receiving L_1 or DFX were segregated according to their glucose tolerance into normal, DM and those with AGTT, it was found that patients with DM excreted significantly more zinc than the others. All three groups of patients receiving L_1 therapy excreted raised amount of zinc compared to normal. Although the UZE in diabetics receiving L_1 is higher than the mean(18.4umol/24h) of previously reported values for UZE in patients with IDDM not on chelation therapy: 21.4±9.5(Martin *et al* 1991); 18.3±4.1(Honnorat *et al* 1992); 15.4±5.5(Kiilerich *et al* 1990), the difference was not statistically significant(p=0.06). This may be due to the small number of patients with DM receiving L_1 therapy studied here. On the other hand, patients receiving DFX with NGTT or AGTT did not excrete increased amounts of zinc and in diabetics DFX was not associated with increased UZE compared to diabetics not receiving chelation therapy(p=0.64). The increase in UZE in patients receiving DFX compared to normal appears to be mainly if not entirely due to the presence of DM in some of them.

The lack of significant difference between the mean UZE of L_1- and DFX-treated patients with NGTT(13.1±6.2 vs 9.7±4.6, p=0.59) may also be due to the small number of patients studied since a significant difference(p=0.003) was obtained when the paired *t*-test was used.

CHAPTER 10

CONCLUSION

The two clinical trials described here show that L_1 is effective in inducing significant urinary iron excretion in heavily iron loaded patients leading to a negative iron balance and a fall in serum ferritin. Despite the lack of significant overall variation in dose/regime-response results, there were individual differences. Therefore, the dose and regime need to be determined in each patient if maximum UIE is to be achieved. The psychological impact of using L_1 on the patients was excellent as was their compliance with the drug throughout the trials.

Serum ferritin fell in 36 of the 53 patients (68%) treated long term. The fall in serum ferritin was more marked in those patients with the highest body iron load and the highest initial serum ferritin levels. Most of those who showed no decline in their serum ferritin were either poorly compliant or had liver disease or were positive for anti-HCV antibody. Several other trials reported on the effectiveness of L_1 in reducing body iron stores. Agarwal *et al*(1992) reported a fall in serum ferritin in 46 of the 52 patients (89%) treated long term with L_1 at a dose of 75-100mg/kg/day. They also found that 100% of patients excreting >0.5mg/kg/day of iron but only 50% of those excreting <0.5mg/kg/day showed a fall in serum ferritin(Agarwal *et al* 1993). Olivieri *et al*(1992, 1993, 1995) reported a significant overall decline in serum ferritin levels in 21 patients with iron overload treated long-term. In a 29-year-old man with thalassaemia intermedia, the serum ferritin fell to normal after 9 months of treatment with L_1 with the normalization of liver iron content(Olivieri *et al* 1992).

Further evidence that L_1 can reduce body iron stores effectively came from Toronto where 21 patients with iron overload were treated with L_1 long-term(3.1±0.3 years) and had their liver iron stores determined yearly(Olivieri *et al* 1995). It was found that in 10 patients in whom previous chelation therapy with DFX had been ineffective, liver iron

concentrations decreased from 125.3±11.5 to 60.3±9.6 umol/g during L_1 therapy. By contrast in the other 11 patients in whom DFX therapy had been effective, L_1 therapy maintained liver iron concentrations below 80 umol/g.

The pharmacokinetic study shows that L_1 is rapidly absorbed and eliminated mainly as L_1G but also as free L_1 and L_1-iron complex in urine. L_1 efficiency was found to be better than reported previously in several animal models. The fast absorption and elimination of L_1 suggest that more than one dose of L_1 can be administered daily without significant accumulation of the drug. L_1G may, however, accumulate if high doses of L_1 are given repeatedly to patients with impaired renal function. This was the first time that the relation between L_1G excretion and renal function of the patients was studied. Whether, L_1G accumulation can cause adverse effects such as nausea and vomiting remains to be established. Although one patient with L_1G accumulation had severe nausea and vomiting during L_1 therapy, most of the other patients with mild to moderate nausea had normal renal function and therefore did not accumulate L_1G during L_1 therapy.

Whether L_1 is capable of causing a significant faecal iron excretion or not remains controversial. Kontoghiorghes *et al*(1990b) reported no increase in iron excretion and no evidence of L_1 in the stools of two patients with iron overload given L_1. On the other hand Olivieri *et al*(1990a) and Collins *et al*(1992) reported an increase in iron excretion in the stool of 9 patients with iron overload following the oral administration of L_1 amounting to up to 28% of the total iron excretion. When L_1 pharmacokinetics were studied(Chapter 4) the amount of total L_1 recovered in urine showed that about 20% of the ingested dose remained unaccounted for at 24 hours. This suggests that faecal iron excretion may occur during L_1 therapy. Clearly more carefully controlled metabolic studies are needed to resolve this issue.

As yet only a few side effects have been noted during L_1 therapy. L_1 has the potential of causing agranulocytosis or neutropenia in some of the patients. Initially, this adverse effect was only observed in London trials. Subsequently, other centres have reported similar problems. It is possible that the higher dose used, initially, in London trials has led to the higher incidence of this adverse effect observed in this centre.

The cause for the individual susceptibility to L_1 remains obscure. There is no in vitro or in vivo evidence for an immune mechanism. In vivo studies in animals showed that L_1-induced myelotoxicity was dose dependent and not influenced by the iron load of the animal (Porter *et al* 1991, Grady *et al* 1992). In vitro studies using either semi-solid agar or liquid culture systems have failed to show an increased susceptibility of the patients' myeloid precursors(CFU-GM) to L_1, alone or bound to iron, compared to normal myeloid precursors. Furthermore, in these in vitro studies, the toxicity of free or iron bound L_1 to normal or the patients' myeloid precursors was less than that of DFX.

Recently Hoyes *et al*(1993) studied the in vivo and in vitro effects of L_1 and other 3-hydroxypyridin-4-one chelators on murine haemopoiesis and compared these to those of DFX. Administration of L_1 resulted in anaemia, lymphopenia and granulocytopenia accompanied by bone marrow hypocellularity. DFX at similar dose caused lymphopenia but marrow cellularity was unaffected. In the in vitro studies using murine marrow both L_1 and DFX caused a dose dependent inhibition of the marrow progenitors. The addition of saturating concentrations of iron abrogated the effect of DFX but not of L_1. The effect of L_1 was abrogated only when iron was added in sufficient amount to saturate the transferrin in the medium. These results in mice are in contrast to those observed in humans, when the addition of saturating concentrations of iron caused complete reversal of both L_1 and DFX toxicity to human myeloid progenitors (Cunningham *et al* 1994,

Chapter 8). The relevance of these results to L_1-associated myelotoxicity in humans is yet to be determined.

Although rechallenge with L_1 has not been carried out in cases with agranulocytosis, re-exposure of the three London cases with milder degree of neutropenia(Chapter 8) and one case of Goudsmit & Kersten (1992) has not led to a fulminant neutropenia as may be seen when a drug dependant antibody is present. It seems most likely, therefore, that L_1 agranulocytosis is due to an individual susceptibility to toxic effects of L_1.

The results here showed for the first time that L_1 can cause mild zinc deficiency in some patients. Subsequently, other studies have reported zinc deficiency in patients receiving L_1 therapy(Jaeger *et al* 1992, Goudsmit & Kersten 1992, Agarwal *et al* 1993). It was also noted that patients with diabetes mellitus and receiving L_1 are more prone to develop this adverse effect. The zinc study was therefore, performed to establish the effect of L_1 compared to DFX and diabetes on the patients zinc status. It was found that patients with iron overload receiving DFX do not excrete more zinc than normal individuals unless they have DM when their increased zinc excretion is comparable to diabetics not receiving DFX. On the other hand, patients receiving L_1 therapy excrete more zinc than similar patients receiving DFX or normal individuals. By contrast diabetic patients receiving L_1 therapy showed the highest urinary zinc excretion. The overall increase in zinc loss accompanying L_1 therapy is modest and in most patients is presumably balanced by increased absorption of dietary zinc. In a few patients negative zinc balance leads to zinc deficiency. Fortunately It is much less severe than that encountered in patients receiving the iron chelator DTPA(Pippard *et al* 1986) and easily corrected with zinc supplementation.

The clinical data also show that although L_1-associated joint toxicity is reversible in most

of the patients it can, in a few patients, cause prolonged disability despite the discontinuation of L_1 therapy. The incidence of joint or musculoskeletal problems observed during the 2 trials reported here was 26% (14 of 53 patients). The first report of joint involvement came from London where 4 of 13 patients (31%) treated with L_1 long term developed joint pains and muscle stiffness occurring as early as 3 weeks after starting L_1(Bartlett *et al* 1990). In Bombay Agarwal *et al*(1992) observed similar problems in 20 of 52 (39%) patients treated long term. Sixteen patients had pain and four also developed joint effusion. Berkovitch *et al*(1992) also reported knee joint pain and swelling in 3 of 15 (20%) patients treated long term.

The incidence of joint involvement appears to be related to the degree of iron load of the patient with the heavily iron loaded patients being more prone to develop this problem. Consistent with this, the incidence observed in Bombay, where larger numbers of grossly iron overloaded previously underchelated patients were included in the trials, has been higher than else where in the world. The dose of L_1 may also have a bearing on the incidence. The incidence of joint involvement in Bombay was found to be 20 of 52, 4 of 40 and 3 of 30 at L_1 doses of 100, 75 and 50 mg/kg/day respectively(Agarwal *et al* 1992), suggesting that with doses in excess of 75mg/kg/day the incidence rises sharply. In none of the reported trials has a clear correlation been found between joint problems and a change in the incidence or titre of autoantibodies.

The cause of joint involvement in patients treated with L_1 remains obscure. Arthroscopy in seven affected patients in Bombay revealed excess of iron in the synovium, cartilage and joint fluid but no L_1(Agarwal *et al* 1993b), implying that iron is probably involved in the aetiology of this problem. Berkovitch *et al*(1992) found no L_1-iron complexes in the synovial fluid of their affected patients but synovial biopsy revealed extensive iron

deposition.

Further studies are clearly needed in order to elucidate the mechanism(s) involved in the causation of this problem. Careful and early assessment of the severity of joint involvement in patients receiving L_1 therapy is mandatory in order to identify those who require early withdrawal of the drug.

A transient fluctuation in the serum level of AST during L_1 therapy was observed in 17 of 54 patients(Chapters 2,3). Similar problem has also been reported by other groups(Agarwal *et al* 1992, Carnelli *et al* 1992). This was attributed in some patients to post transfusion hepatitis(Agarwal *et al* 1992) or HCV infection(Carnelli *et al* 1992). The mechanism of AST fluctuation in patients receiving L_1 therapy remains unclear. It is possible that L_1, in the presence of high concentration of iron in the nearby hepatocytes, can lead to the local formation of partial complexes with iron(1:1 or 2:1) capable of catalysing free radical formation with the resultant toxicity of hepatocytes. The lack of significant difference between initial and final mean AST levels suggests that there was no chronic liver toxicity ascribed to long term use of L_1.

The serum concentration of NTBI in patients with iron overload and receiving DFX or L_1 was also examined. It was found that NTBI concentrations correlate with body iron pools. L_1 caused a significant fall in NTBI concentrations during a period of 6 months. The interaction between L_1 and NTBI and the kinetics of the L_1-Fe complex was examined. Although this study was based on some assumptions, it does however, show that when L_1 is administered orally it is capable of removing NTBI from the serum of iron overloaded patients for a significant duration of time the length of which was mainly dependent on the initial NTBI concentration and L_1-$AUC_{0-\infty}$ and therefore L_1 dose. As L_1 is, usually, given several times per day(usually 4) it is likely to cause more sustained

effect on NTBI than that caused by DFX given in the usual way.

The sources of iron available for chelation by L_1 are not clearly known. The data here suggest that transferrin is one such source. Its rapid resaturation within six hours of L_1 administration indicate that it could act efficiently in recruiting more iron for chelation should further doses of L_1 be given. However, it is not the sole source of chelatable iron. Non-transferrin-bound iron may be another form of iron readily available for chelation. However, its contribution to the total UIE is yet to be evaluated. Other sources of iron such as tissue iron stores presumably also contribute to the iron chelated by L_1 either directly or indirectly through an intermediate vehicle such as transferrin.

It has now become clear that iron chelation can be carried out with an orally active drug. It has also been shown that L_1 possesses a high efficacy in reducing body iron stores despite the need for continuing blood transfusions. However, the safety issue of using L_1 remains a major obstacle to its wide spread use. DFX should, therefore, continue to be used wherever possible. However, if patients are incapable of using DFX because of side effects, poor compliance or unavailability of the drug due to economical reasons, the use of an alternative chelator such as L_1 becomes imperative.

There is now some evidence to suggest that L_1 given at a low dose maybe associated with less adverse effects especially those involving myeloid progenitors and joints. Therefore, patients with other forms of iron loading anaemias such as thalassaemia intermedia and pyruvate kinase deficiency may benefit from L_1 therapy. In these conditions iron loading is a slow process and L_1 administered at a low dose maybe sufficient to induce negative iron balance.

At all events patients receiving L_1 must continue to be subjected to regular monitoring of their blood counts, particularly during the first few months of therapy, and also of any

other serious side effect.

Future in vitro and in vivo studies of L_1 need to address the following issues:

-The safety and efficacy of L_1 when administered at a low dose.

-The mechanism(s) involved in L_1-induced myelotoxicity. Although the preliminary studies carried out, sofar, in order to elucidate the mechanism(s) involved in this adverse effect have all been negative, there is still a room for more studies. It is quite possible that an in vitro study which is carefully designed to resemble more accurately the in vivo situation would lead to more insight into the mechanism(s) behind this problem. The in vitro studies have shown that both L_1 and DFX are toxic to myeloid progenitors by iron deprivation. Although the patients who developed myelotoxicity whilst receiving L_1 therapy were all iron overloaded, it maybe that the mechanism involved in redistributing iron from stores to the myeloid progenitors was defective. Furthermore, L_1 may impede this mechanism by simultaneously removing iron from transferrin (Chapter 5). Therefore, future studies on this problem need to address all these issues.

-The mechanism(s) involved in L_1-induced joint toxicity.

-The efficacy and safety of using other oral preparation of L_1 such as a slow release form.

-Whether L_1 toxicity is due to the fact that L_1-Fe complexes dissociate at low concentrations. This is likely to happen at the two ends of L_1 life time in the circulation. In patients receiving multiple doses of L_1 this only happens if L_1 is administered with long intervals allowed between the doses. Therefore, if multiple doses of L_1 are given at shorter intervals so that the serum concentration following one dose overlaps with that of the subsequent dose, L_1 trough concentration will not fall to such a level that would lead to the dissociation of L_1-Fe complexes. This maybe achieved by giving multiple small doses. Alternatively a slow-release preparation may lead to similar results.

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APPENDIX

COPIES OF PUBLICATIONS

Efficacy and Possible Adverse Effects of the Oral Iron Chelator 1,2-Dimethyl-3-Hydroxypyrid-4-One (L_1) in Thalassemia Major

By F.N. Al-Refaie, B. Wonke, A.V. Hoffbrand, D.G. Wickens, P. Nortey, and G.J. Kontoghiorghes

Eleven patients with β thalassemia major were entered into the trial of the oral chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L_1). Their ages ranged from 17 to 26 years (mean ± SD, 22.3 ± 2.7). Six were male and five were female. L_1 was administered at an initial daily dose of 42.5 to 60 mg/kg as a single dose. After 4 weeks, the dose was increased to 85 to 119 (102 ± 10.7) mg/kg for 191 to 352 days divided into either two or four doses daily, except for one patient who developed agranulocytosis after 11 weeks and was taken off the trial. Initial serum ferritin values in the remaining 10 patients ranged between 1,000 and 9,580 (5,549 ± 3,333) μg/L and at end of the trial their mean serum ferritin was significantly lower (4,126 ± 2,278; $P < .05$ using the paired *t*-test). Urinary iron excretion at a daily dose of 85 to 119 mg/kg administered as two divided doses ranged between 0.14 and 0.82 (0.44 ± 0.26) mg/kg/24 h. In three patients, the four doses per day schedule caused substantially more iron excretion than the same total dose divided into two. During the course of the trial, several possible adverse effects have been encountered. One patient (female, aged 20) developed agranulocytosis 11 weeks after starting treatment and 6 weeks after beginning treatment with a daily dose of 105 mg/kg. This patient's neutrophil count recovered spontaneously 7 weeks after the discontinuation of L_1. A decrease in serum zinc levels to subnormal levels was observed in four patients with symptoms of dry skin, with an itchy scaly rash in two that was associated with low serum zinc levels that responded to zinc therapy. Urinary zinc levels ranged from 4.7 to 23.4 (13 ± 5.5) μmol/24 h and were above 9 μmol/24 h (upper limit of normal) in eight patients. Mild nausea occurred in three patients and transient diarrhea in a fourth. Mild musculoskeletal symptoms occurred in three patients but settled without discontinuation of L_1 therapy in two and with temporary discontinuation of L_1 in the third. A transient increase in serum aspartate transaminase was also noted in five patients, but serum aspartate transaminase levels subsequently decreased in all of them. No cardiovascular, neurologic, renal, or retinal toxicities were demonstrable. These results confirm that L_1 is an effective oral iron chelator. Further clinical trials are needed to determine the incidence and severity of adverse effects.
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THE CHRONIC transfusion program in patients with β thalassemia major (TM) is associated with iron overload and widespread organ damage. Desferrioxamine (DFX) has proved to be a highly effective and reasonably safe iron chelator and with the introduction of chronic subcutaneous administration there has been a decline in morbidity and mortality in those on regular chelation.[1,2] However, the use of DFX is hindered by two major obstacles: high cost and poor compliance. Therefore, there is an urgent need for an orally acting iron chelator that is safe and inexpensive. Many compounds have been developed, of which 1,2-dimethyl-3-hydroxypyrid-4-one (L_1) has now been tested in several clinical trials[3-11] and shown to be effective. This present trial was designed to further assess the long-term effectiveness of L_1 in patients with TM and the incidence of possible adverse effects.

PATIENTS AND METHODS

Approval for the trial was obtained from the Royal Free Hospital's Ethical Committee. Eleven patients were initially entered into the trial after giving their written informed consent. Details of their initial clinical findings and doses of L_1 are given in Table 1. One patient was taken off the trial after 11 weeks because of the development of agranulocytosis; the long-term follow-up data, therefore, refer to the remaining 10 patients. Initial investigations included detailed clinical examination, full blood count (FBC), blood urea and electrolyte (U&E), creatinine, liver function tests (LFTs), and tests of iron status, including serum ferritin, serum iron, total iron binding capacity (TIBC), non–transferrin-bound iron (NTBI), and 24-hour urinary iron in response to DFX or L_1. Other tests included prothrombin time (PT), partial thromboplastin time (PTTK), direct antiglobulin test, leukocyte ascorbate, electrocardiogram (ECG), multigated scintigraphy (MUGA scan) at rest and with cold stress, retinal function tests (visual acuity, electrooculogram, electroretinogram and pattern, visually evoked responses, and color vision), audiogram, serum Igs, T-cell subsets (CD4/CD8 ratio), rheumatoid factor (RhF), antinuclear factor (ANF), serum and urinary trace elements (Cu, Zn, and Mg), serum caerruloplasmin, lipid profile (triglyceride, cholesterol, low-density lipoprotein [LDL], and high-density lipoprotein [HDL]), serum hormones (thyroid-stimulating hormone [TSH], T4, growth hormone, prolactin, luteinizing hormone, follicle-stimulating hormone, testosterone, oestradiol, and cortisol), 24-hour urinary cortisol, and computerized tomography (CT) of the adrenals. FBC, U&E, LFTs, serum iron, TIBC, PT, PTTK, Coombs test, Igs, RhF, ANF, CD4/CD8 ratio, ECG, CT of adrenals, and MUGA scan were performed by standard methods. Serum ferritin was estimated by an enzyme-linked immunosorbent assay (ELISA) technique.[12] NTBI was measured as described by Singh et al.[13] Blood samples for this test were collected immediately before L_1 intake and at least 12 hours after the previous dose of L_1, or at least 12 hours after the end of the previous DFX infusion. Leukocyte ascorbate was measured as described by Denson and Bowers.[14] Retinal function tests were performed as described by Arden et al.[15] Lipids were measured by standard fully enzymatic technique using a centrifugal analyzer. Urinary iron and zinc were measured using atomic absorption spectrophotometry.[16] Hormones were assayed by standard radioimmunoassay technique. Statistical analysis was performed using the Student's *t*-test.

The patients were seen once weekly for the first 4 months and then once every 2 weeks. During each visit they received a full

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Table 1. Clinical Data: 11 Patients With TM

Case No.	Age/Sex	Ethnic Origin	Initial Serum Ferritin (μg/L)	Maximum L_1 Dose (mg/kg)	Duration of Treatment (d) (all doses)	Anti-HCV Status	Other Medical Problems
1	23/M	Cypriot	8,500	93	376	Neg	None
2	23/M	Italian	1,400	85	376	Neg	DM
3	21/F	Cypriot	5,900	110	348	Neg	2°Amenorrhoea
4	21/F	Cypriot	4,350	111	369	Neg	2°Amenorrhoea, DM
5	26/M	Greek	1,440	106	355	Pos	CAH
6	24/F	Greek	6,200	119	344	Pos	2°Amenorrhoea, abnormal GTT
7	20/F	Greek	4,350	105	61*	Neg	None
8	20/M	Cypriot	1,000	85	376	Neg	None
9	25/F	Indian	8,300	105	380	Neg	DM, HPT, sexual infantilism
10	17/M	Indian	8,820	107	281	Pos	DM, short stature
11	25/M	Indian	9,580	98	205	Pos	DM, HPT, CAH short stature

Abbreviations: DM, diabetes mellitus; CAH, chronic active hepatitis; GTT, glucose tolerance test; HPT, hypoparathyroidism; Neg, negative; Pos, positive.

*Drug was stopped early because of agranulocytosis.

clinical examination and had blood tests, including FBC and renal and liver function. Urine was collected for 24 hours for iron and trace metal once every 1 to 3 weeks. MUGA scan, retinal function tests, CT of adrenals, and audiometry were checked initially and at the end of the trial. The other tests were repeated once every 3 months.

L_1 was commenced at a single dose of 0.5 g daily, with a gradually increasing dose over the first week to a dose of about 50 mg/kg/d. This was administered as a single daily dose for 4 weeks and then twice daily or at the same total dose administered four times daily. All doses were taken on an empty stomach and at least 1 hour before eating. No food was taken for at least 1 hour after taking the drug. Vitamin C was initially discontinued on all patients, except cases 10 and 11. Those who had a decrease in leukocyte ascorbate to less than 23 μg/10^3 white blood cells (WBC; normal range, 23 to 51) were restarted on vitamin C (200 mg daily) with breakfast given separate from L_1. Patients 10 and 11 had low pretrial vitamin C levels and were therefore kept on vitamin C 200 mg daily with breakfast.

RESULTS

In general, the drug was well accepted and compliance was apparently excellent as assessed by the patient's history, parental evidence, and useage of capsules provided in just-sufficient quantities between hospital visits. No change was noted in cardiac function, blood pressure, renal function, lipid profile, and hormone levels. No neurologic, retinal, or audiometry changes were shown throughout the trial.

Urinary iron excretion (UIE). UIE varied considerably from day to day in each patient (Table 2). The mean excretion in response to 85 to 119 mg/kg daily administered as two divided doses ranged from 9.6 to 41.1 (mean ± SD 23.0 ± 11.2) mg/24 h (0.14 to 0.82, 0.44 ± 0.26 mg/kg/24 h, Table 2). Excretion depended mainly on the dose of L_1 ($P = .003$; Fig 1). The correlation with serum ferritin was poor ($P > .1$). Before the trial the mean UIE in these patients at a DFX daily dose of 40 to 50 mg/kg was 0.42 ± 0.32 mg/24 h (Table 2). The effect of the frequency of L_1 administration on UIE was variable. To evaluate it, the same daily dose of L_1 was administered in four divided doses instead of two. More frequent administration caused substantial increase in urine iron in patients 2, 5, and 8 (Fig 2), but in four of the other patients tested, excretion was slightly greater with the twice daily regime.

Serum ferritin. Serum ferritin values decreased in 7 of the 10 patients (1, 3, 6, 8, 9, 10, and 11) by up to 42% of the original pretrial values, increased by 43% in patient 2, and remained unchanged in patients 4 and 5 (Fig 3). The mean serum ferritin at the beginning of the trial was 5,549 ± 3,333 (range, 1,000 to 9,580) μg/L and at the end of the trial was 4,126 ± 2,278 (range, 738 to 7,435) μg/L. The overall decline in serum ferritin was significant using *t*-test for paired samples ($P < .05$).

NTBI. The initial NTBI values ranged from 3.6 to 9.0 (6.1 ± 1.6) μmol/L. The level decreased in eight patients after 3 to 6 months of L_1 therapy. The final values measured at least 12 hours after the previous dose of L_1 ranged from 3.8 to 5.4 (4.5 ± 0.5) μmol/L. This final mean is significantly lower ($P < .005$) than the initial mean NTBI. However, both means (initial and final) were significantly higher ($P < .001$ and $P < .05$ respectively) than the mean NTBI (3.7 ± 2.0 μmol/L) estimated on blood samples taken at least 12 hours after the end of the previous DFX infusion in a group of 38 TM patients receiving regular chelation with DFX.

Hematologic changes. All patients maintained normal blood count throughout the trial except for patient 7, who developed agranulocytosis of sudden onset in the beginning of week 12 of the treatment and 6 weeks after being on a full dose (105 mg/kg) of L_1. The patient was being seen once weekly and presented with generalized weakness, low-grade fever, and sore throat. The drug was immediately withdrawn and she was admitted to a hospital and administered intensive treatment with broad spectrum antibiotics. She showed progressive clinical improvement over the subsequent 3 weeks, but her neutrophil count remained low ($< 0.5 \times 10^9$/L) for 7 weeks. Details of this patient are to be reported elsewhere.[17]

Liver function. There was no significant difference (paired *t*-test, $P > .05$) between serum aspartate transaminase (AST) levels at the start of the trial and those measured at the end of the trial. Three patients (3, 4, and 6) showed a substantial decrease in serum AST levels from higher pretrial values (Fig 4). Five patients (1, 5, 6, 7, and

Table 2. UIE and UZE

Case No.	Chelator	Dose (mg/kg)	No. of 24-h Urine Collections	UIE (mean ± SD) mg/24 h	UIE (mean ± SD) mg/kg/24 h	UZE (mean ± SD) μmol/24 h	UZE (mean ± SD) μmol/kg/24 h
1	L_1	3 g × 2 (93)	6	18.1 ± 5.9	0.28 ± 0.09	11.5 ± 1.6	0.18 ± 0.03
		1.5 g × 4	3	9.6 ± 8.4	0.15 ± 0.13	7.3 ± 2.2	0.11 ± 0.03
	DFX	3 g SC	2	17.1	0.27	4.4	0.07
2	L_1	3 g × 2 (85)	8	9.6 ± 5.7	0.14 ± 0.08	22.6 ± 7.6	0.32 ± 0.11
		1.5 g × 4	4	45.3 ± 18.1	0.64 ± 0.26	42.1 ± 17.8	0.60 ± 0.25
	DFX	3 g SC	2	22.4	0.32	19.3	0.27
3	L_1	3 g × 2 (110)	7	28.1 ± 11.2	0.52 ± 0.21	13.0 ± 4.5	0.24 ± 0.08
		1.5 g × 4	5	26.1 ± 8.9	0.48 ± 0.16	35.4 ± 9.5	0.65 ± 0.17
	DFX	3 g SC	2	15.4	0.28	5.9	0.11
4	L_1	3 g × 2 (111)	8	37.1 ± 10.5	0.69 ± 0.19	23.4 ± 5.4	0.43 ± 0.10
		1.5 g × 4	5	29.1 ± 6.7	0.54 ± 0.12	18.6 ± 2.6	0.34 ± 0.05
	DFX	3 g SC	2	25.1	0.47	5.0	0.09
5	L_1	3 g × 2 (106)	5	13.7 ± 11.9	0.24 ± 0.21	15.8 ± 4.7	0.28 ± 0.08
		2 g × 3	10	20.7 ± 11.6	0.37 ± 0.21	14.1 ± 4.5	0.25 ± 0.08
		1.5 g × 4	4	29.9 ± 3.9	0.53 ± 0.07	17.1 ± 1.4	0.30 ± 0.03
	DFX	2.5 g SC	2	7.1	0.13	11.6	0.21
6	L_1	3 g × 2 (119)	7	41.1 ± 7.0	0.82 ± 0.14	12.3 ± 1.3	0.24 ± 0.03
		1.5 g × 4	4	31.7 ± 3.9	0.63 ± 0.08	9.8 ± 2.0	0.19 ± 0.04
	DFX	3 g SC	2	12.0	0.24	8.2	0.16
8	L_1	2.5 g × 2 (85)	9	11.4 ± 4.2	0.19 ± 0.07	9.4 ± 1.8	0.17 ± 0.03
		1.25 g × 4	6	15.7 ± 7.8	0.27 ± 0.13	10.3 ± 2.8	0.18 ± 0.05
	DFX	2.5 g SC	2	35.2	0.60	5.7	0.10
9	L_1	2.5 g × 2 (105)	8	14.5 ± 7.2	0.31 ± 0.15	12.2 ± 6.0	0.26 ± 0.13
		1.25 g × 4	6	15.6 ± 1.5	0.33 ± 0.03	15.7 ± 6.3	0.33 ± 0.13
	DFX	2.5 g SC	2	12.5	0.26	ND	ND
10	L_1	2 g × 2 (107)	10	16.1 ± 6.0	0.43 ± 0.16	4.7 ± 2.8	0.13 ± 0.08
	DFX	2.5 g SC	2	13.8	0.37	4.0	0.11
11	L_1	2 g × 2 (98)	5	33.4 ± 14.3	0.82 ± 0.35	10.9 ± 8.5	0.27 ± 0.21
	DFX	2 g SC	2	51.5	1.26	26.1	0.64

Abbreviations: SC, subcutaneous; ND, not done.

10), three of whom were anti-hepatitis C virus (HCV) positive (Table 1), developed a transient fluctuating increase of serum AST shortly after starting the treatment with L_1. This eventually settled in all of the patients (Fig 5).

Serum and urinary zinc. Serum zinc levels decreased in four patients to subnormal levels (<11.5 μmol/L) (Fig 6). This decrease was accompanied by an increase (>9 μmol) in 24-hour urinary zinc excretion (UZE) in eight patients (Table 2). Two patients became symptomatic, requiring treatment with zinc (see below). UZE in the 10 patients ranged from 4.7 to 23.4 (13.1 ± 5.5) μmol/24 h (normal range, <9 μmol/L). The correlation between L_1 total dose and urine zinc was poor (P > .2). The effect of the

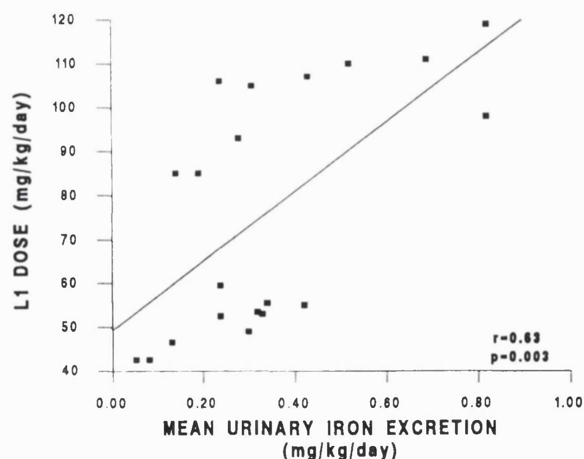

Fig 1. The correlation between L_1 dose and mean UIE.

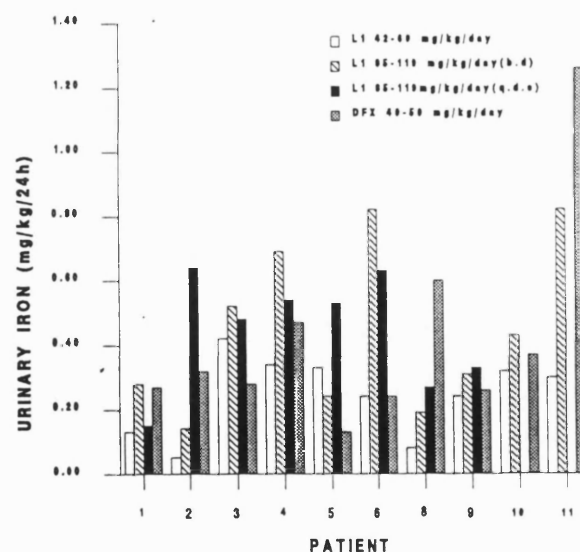

Fig 2. The effect of L_1 and DFX on UIE. (□) L_1 (42 to 60 mg/kg/d); (▧) L_1 (85 to 119 mg/kg/d; two doses daily); (■) L_1 (85 to 119 mg/kg/d; four doses daily); (▩) DFX (40 to 50 mg/kg/d).

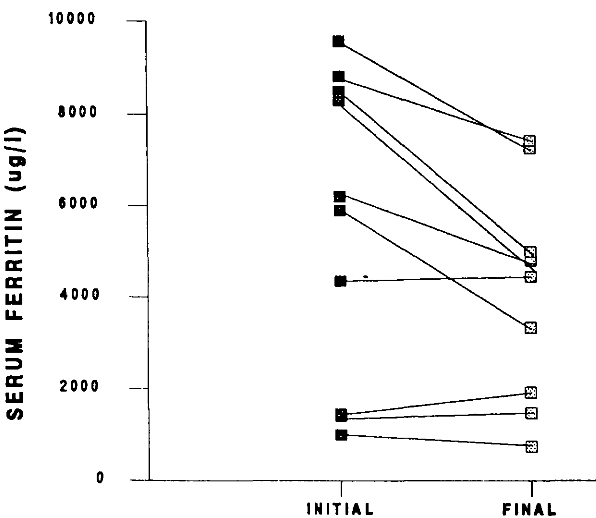

Fig 3. Patients' initial and final (7 to 13 months) serum ferritin levels.

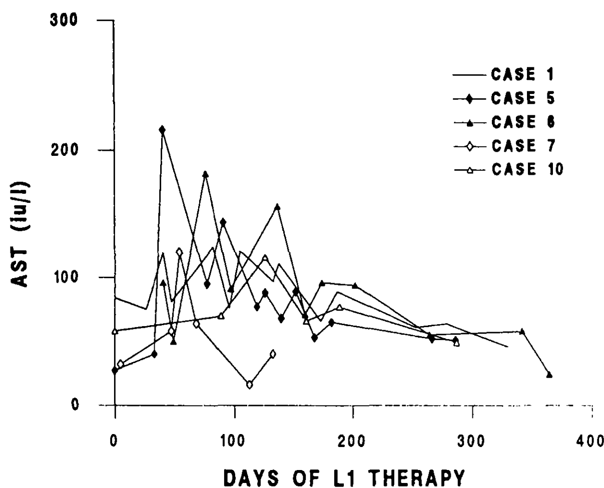

Fig 5. Transient and fluctuating increase of serum AST in five patients receiving L_1 therapy.

frequency of L_1 administration on UZE in the individual patient was similar to that observed for UIE (Table 2). It is of interest that in three patients, UZE also increased above 9 μmol when DFX was used by these patients before the trial (Table 2).

Leukocyte ascorbate levels. Seven patients (1, 3, 4, 5, 6, 9, and 10) showed a decrease in WBC ascorbate level after variable periods (3 to 5 months) from the commencement of treatment with L_1. None became symptomatic as all were restarted on vitamin C tablets 200 mg/d administered at breakfast separately from L_1. No significant change in UIE was observed in these patients with vitamin C therapy.

Gastrointestinal symptoms. The drug was generally well tolerated by the patients. However, 3 patients (3, 7, and 9) developed nausea on starting the treatment or on increasing the dose of L_1 that lasted for few days only and required no treatment. One patient complained of loose bowel motions beginning 1 week after the commencement of L_1. Stools were negative for microorganisms and the problem settled spontaneously 6 weeks later.

Musculoskeletal symptoms. Three patients (1, 6, and 9) had some form of musculoskeletal symptoms. Patient 1 complained of generalized muscular stiffness that was worse in the morning that began 4 months after starting treatment and lasted for 6 weeks before resolving spontaneously. This patient was negative for RhF and ANF throughout the trial. Patient 6 developed two episodes of generalized musculoskeletal pain with low-grade fever and epigastric pain. The first episode began 4 weeks after starting L_1 and the second 2 weeks later. Each episode lasted for 24 hours only and resolved spontaneously, leaving no residual symptoms. RhF was positive at the outset of the trial with a titre of 1/320, which decreased 6 months later to 1/80. ANF was negative in the first 8 months and has then become weakly positive with a titre of 1/40. Patient 9 developed generalized muscular pain 9 months after start-

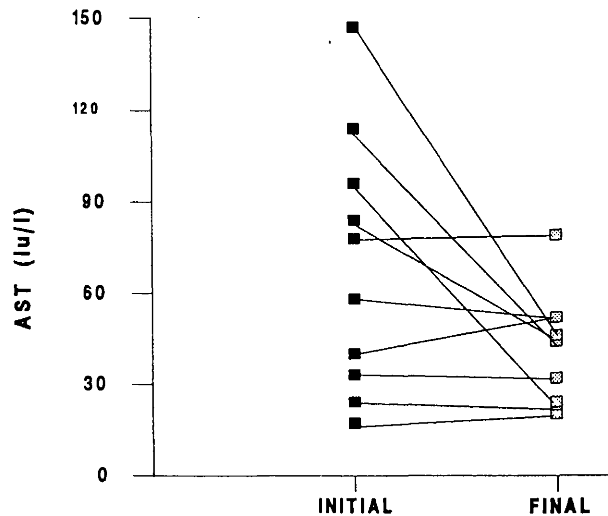

Fig 4. Initial and final (7 to 13 months) serum AST levels.

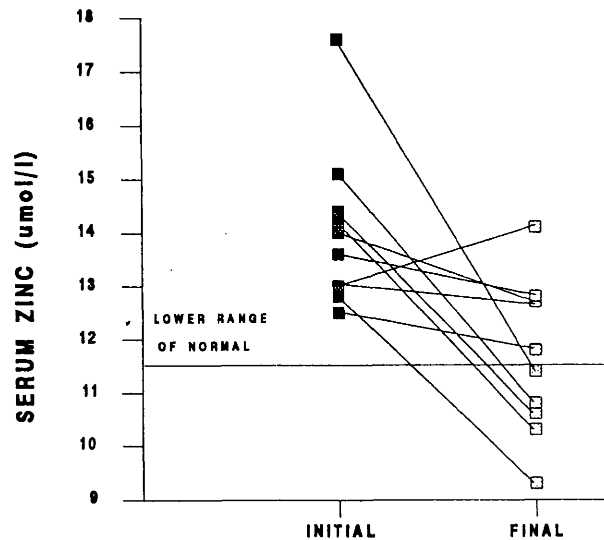

Fig 6. Initial and final (7 to 13 months) serum zinc levels.

ing L_1 treatment that became progressively worse over the next 4 weeks. When L_1 was temporarily withheld, the pain disappeared completely within 48 hours and reappeared, but to a lesser degree, when L_1 was restarted at a full dose. RhF and ANF were negative throughout the trial.

Autoantibodies. At the beginning of the trial, two patients showed positive serum RhF (titres: 1/80 and 1/320) and two patients had positive serum ANF (titres: 1/160 and 1/40). At the end of the trial, the incidence and titre of RhF was unchanged; four patients were found to have positive ANF (titres: 1/320 [one patient] and 1/40 [three patients]). There was no overall relation between the presence or absence of positive ANF or RhF tests either before or during L_1 therapy and the presence of muscle or joint symptoms. To assess the significance of these results, an unselected group of patients with TM (n = 51) were checked for the presence of these autoantibodies. Nine (17.6%) were positive for RhF, but none was ANF positive.

Dermatologic changes. Nine patients were heavily pigmented before the start of the trial. This was partly racial, but also secondary to iron overload. In seven of them, there was a substantial decline in skin pigmentation with noticeable lightening of skin color. This occurred mainly in the first 3 months of treatment and before there was any change in serum ferritin. Two patients (1 and 2) developed skin eruptions on the face and shoulders resembling folliculitis. Microbiologic tests on swabs taken from these lesions were negative. The eruptions lasted for 6 weeks in patient 1 and for 2 weeks in patient 2 and required no treatment. Four patients (3, 6, 7, and 9) complained of dry skin. This was accompanied by hair loss in patients 3 and 9 and later by dry and itchy skin patches in the latter. This was associated with low serum zinc levels in these patients (see above). They were started on Zn tablets 220 mg/d, which was associated with improvement in the skin lesions within 4 weeks.

DISCUSSION

In this trial we tested the long-term use of the iron chelating drug L_1 in high doses (up to 119 mg/kg/d) in patients with β TM. The drug was generally well tolerated with no detectable changes in renal, cardiac, respiratory, retinal, or auditory function. The UIE results are consistent with previous reports.[6,8,10] The important determinant of iron excretion was the total L_1 dose. The mean UIE at an L_1 dose of 85 to 119 mg/kg is comparable with that achieved with DFX at a dose of 40 to 50 mg/kg (0.44 ± 0.26 *v* 0.42 ± 0.32 mg/kg/24 h). However, some patients achieved iron excretion comparable with that with DFX at similar doses of the two chelators (Fig 2). We[3,4,6] and Olivieri et al[10] reported similar findings. In the latter study, the mean UIE was lower with L_1 therapy than with subcutaneous DFX (12.3 ± 6.7 *v* 18.2 ± 15.3 mg/d) at an L_1 or DFX daily dose of 50 mg/kg. When the L_1 dose was increased in five of their patients to 75 mg/kg/d, mean UIE increased from 13.8 ± 7.0 to 26.7 ± 17.8 mg/d, comparable with that with DFX (50 mg/kg/d; UIE of 24.9 ± 24.3 mg/d). The average intake of iron from blood transfusions in TM is approximately 0.5 mg/kg/d. Six of 10 patients tested showed UIE greater than this on a daily total dose of 85 to 119 mg/kg/d. Clearly, the iron excretion of the patients will depend not only on the dose and frequency of L_1 administration, but also on the iron load of the patient, and this will depend on the number of blood transfusions and previous chelation therapy.

The effect of the frequency of L_1 administration on iron excretion in our present trial was variable. An increased frequency of smaller doses with the same total daily dose caused a substantial increase in urinary excretion in three patients, but had little or an opposite effect in the other five patients tested. It remains to be determined whether more frequent L_1 dosing with the resultant lowering of serum peak levels of the drug would also lead to fewer side effects.

The percentage of patients having a decline in serum ferritin level was higher than previously reported. Kontoghiorghes et al[6] reported no overall change in serum ferritin levels over the period of their study (1 to 15 months) in 13 patients receiving various doses of L_1. Töndury et al[8] reported a decline in serum ferritin levels in three of the eight patients who received L_1 at a daily dose of 55 to 80 mg/kg for 4 to 10 months. Therefore, it is likely that the high L_1 dose used in the present trial for a relatively long period is responsible for this result. The decrease in serum ferritin levels appeared to be greater in those with the highest initial levels (Fig 3), but larger numbers of patients are needed to confirm this. There was also a significant decrease in the patients' NTBI levels. However, the final mean NTBI was still significantly higher than that estimated in a comparable group of DFX-treated patients. Further studies are therefore necessary to determine whether more prolonged chelation with L_1 will achieve NTBI values similar to those achieved by prolonged chelation with DFX.

The musculoskeletal adverse effects observed during the course of the trial were mild and transient. These effects resembled those we[7] and others[11] have previously reported. There was no relation between the presence of these symptoms and the presence of the different autoantibodies tested before or after the therapy. The changes in the liver enzyme AST were also transient and settled without discontinuation of L_1 therapy. The initial fluctuating liver enzyme pattern occurred in three of four anti-HCV–positive patients and two of seven anti-HCV–negative patients. Further studies are needed on whether HCV is important to these changes in liver enzymes during L_1 therapy. Similar changes have not previously been reported. Bartlett et al[7] reported an increase in AST in one of their patients receiving L_1. However, this was secondary to alcohol consumption and settled on abstinence.

The occurrence of agranulocytosis in one patient during the early stage of the trial highlights the potential toxicity of this drug and the need for relentless vigilance in monitoring the blood count in patients receiving L_1. We have undertaken several experiments to elucidate the role of L_1 in the pathogenesis of agranulocytosis in this patient. When compared with those of normal individuals, we have not found an increased sensitivity of the patient's bone marrow WBC progenitors to L_1 in culture.[18] These studies have suggested but not proven the presence of a weak drug-dependent antimyeloid precursor IgM antibody in the

blood of the patient during agranulocytosis or convalescence.[17] This is the second case of L_1-induced agranulocytosis to be reported. The first case was a 28-year-old woman with Blackfan-Diamond anemia who also developed agranulocytosis 6 weeks after receiving L_1 at a dose of 105 mg/kg. She had previously received the drug for 5 months, largely at the lower dose of 50 mg/kg. Also, in that case the marrow myeloid progenitors were not particularly sensitive to L_1 in vitro.[19] Therefore, the exact mechanism of L_1-induced agranulocytosis remains obscure. However, it is of interest that both were females and had been receiving L_1 at the maximum doses of about 100 mg/kg/d for 6 weeks when agranulocytosis developed and both had shown serum red blood cell antibodies. In the present case, there were the alloantibodies, anti-C, anti-D, anti-Kpa, and anti-Kell, present since early childhood. In the Blackfan-Diamond patient, there was a reversible anti-Lw autoantibody.[19]

Although L_1 is an avid iron chelator with a high binding constant, it nevertheless can bind other cations, although with a much lower affinity. No change in serum zinc levels or increase in UZE in patients receiving L_1 has been reported previously. However, in the present trial, L_1 administration was associated with a decrease in serum zinc levels in four of the patients. There was an increase in UZE in eight of the patients and symptoms attributable to zinc deficiency in two of them. These symptoms improved after zinc administration. However, the correlation between the amount of zinc excreted in urine and the severity of serum zinc decline was poor. Zinc excretion also increased when subcutaneous DFX was administered to these patients at a daily dose of 40 to 50 mg/kg before the trial and by a large group of patients (n = 42) with TM receiving subcutaneous DFX (unpublished data). However, none of the L_1-treated patients showed low serum zinc levels before the trial and only 8 of the 42 non-L_1 patients showed subnormal serum zinc levels, with none having symptoms attributable to zinc deficiency. Taken together, these observations suggest that zinc status in patients receiving L_1 or DFX may be finely balanced, dietary intake being normally sufficient to prevent deficiency, except in some patients receiving a high dose (85 to 120 mg/kg) of L_1 long term. The degree of zinc deficiency noted in patients treated here with L_1 is minor and easily corrected. It is much less severe than that encountered in patients receiving the iron chelator diethyltriamine penta-acetic acid (DTPA).[20]

In conclusion, in most patients in this study, the administration of L_1 as a dose in the region of 100 mg/kg/d induced iron excretion (>0.5 mg/kg/d) sufficient to maintain a negative iron balance and there was an overall significant decline in serum ferritin levels. The dose and regime need to be determined in each patient, as there has been substantial variation in response to different dose regimens. All 11 patients stated a clear preference for using L_1 compared with subcutaneous DFX and the patients' apparent compliance with the drug throughout the trial was excellent. As yet, only a few possible side effects have been noted. The results here show for the first time that these side effects include mild zinc deficiency in some patients and report agranulocytosis in a TM patient. As L_1 has the potential of causing agranulocytosis, it is necessary to closely monitor the blood count and, in particular, the WBC count in patients receiving it.

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Agranulocytosis in a Patient with Thalassaemia major during Treatment with the Oral Iron Chelator, 1,2-Dimethyl-3-Hydroxypyrid-4-One

Key Words
Agranulocytosis
1,2-Dimethyl-3-hydroxypyrid-4-one (L1)
GM-CSF
Iron chelation
Liquid cultures
Thalassaemia major

Abstract
Agranulocytosis developed in a 20-year-old Greek patient with β-thalassaemia major, 11 weeks after commencing chelation with the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L1) and 6 weeks after receiving the drug at a total daily dose of 105 mg/kg. The patient presented with generalised weakness, low-grade fever and sore throat. The total white cell count was 2.0×10^9/l with 0.1×10^9/l neutrophils. The patient was admitted to hospital and successfully treated with intravenous broad-spectrum antibiotics. Neutrophil count recovered 7 weeks later. A number of immunological tests were performed in an attempt to elucidate the cause of agranulocytosis. These investigations gave inconclusive evidence for the presence of a weak IgM antibody to myeloid cells exposed to L1 in this patient. Further studies are required, however, to evaluate the mechanism in any other patient who develops agranulocytosis in association with L1 therapy.

Despite the effectiveness of desferrioxamine (DFO) in the management of transfusional iron overload, its high cost and need for parenteral administration underline the necessity of developing an inexpensive, safe oral chelator. During the last few years, several potential new iron-chelating agents have been elaborated. The most promising are the α-ketohydroxypyridines, one of which, 1,2-dimethyl-3-hydroxypyrid-4-one (L1), has been evaluated in several clinical trials [1–7]. We have previously reported a patient, a woman of 28, with the Blackfan-Diamond syndrome who developed agranulocytosis after taking L1 at a dose of 105 mg/kg daily for 6 weeks, having previously received a longer course of L1 mainly at a lower dose [8]. In a subsequent trial, 11 patients, all with thalassaemia major, have taken L1 at doses up to 120 mg/kg/day. During the early stage of this trial, we have encountered agranulocytosis in one of the patients 11 weeks after starting treatment with L1 and 6 weeks after taking a total daily dose of approximately 105 mg/kg. We describe here the case history of this patient and also report the results of culture studies aimed at determining whether an immune mechanism was responsible for the agranulocytosis.

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Case History

A 20-year-old Greek female was diagnosed as having β-thalassaemia major at the age of 6 months. She commenced regular blood transfusion at the age of 9 months to maintain a haemoglobin greater than 10 g/dl and has subsequently received over 300 units of packed red cells. She began using occasional intramuscular DFO at the age of 1 year. At the age of 5 years she developed 2 episodes of haemolytic anaemia following blood transfusions when anti-D and anti-Kell antibodies were found in her blood. She did not respond to steroid therapy alone and eventually responded to azathioprine which was then withdrawn gradually. She has subsequently been transfused with D-negative, Kell-negative blood. She has used DFO subcutaneously since the age of 5, and currently was taking 2.5 g on 2–5 nights each week with poor compliance.

In September 1990, she volunteered to participate in the clinical trial of the oral chelator L1 which has the approval of the Royal Free Hospital's Ethical Committee and the DHSS. At that time, she was asymptomatic with normal physical examination. The liver and spleen were both impalpable and she had normal blood count (total white cells 4.6×10^9, granulocytes 2.6×10^9, platelets 185×10^9) and normal renal and liver function. Serum ferritin was 5,700 μg/l, serum iron 42 μmol/l, TIBC 36 μmol/l, and she had multiple red cell alloantibodies (anti-D, C, Kell and Kp^a) and diffusely positive antinuclear antibody with a titre of 1/40.

She started oral L1 therapy at a single daily dose of 3 g by mouth, increased 4 weeks later to 3 g twice daily equivalent to 105 mg/kg body weight. She was seen weekly and had her blood count checked on each visit. The white cell and platelet counts remained normal during the first 11 weeks of treatment (fig. 1).

At the beginning of the 12th week, she presented with generalised weakness, easy fatigability and a sore throat. On examination she was found to have low grade fever (38 °C) and enlarged and congested tonsils. The rest of the clinical examination was normal. Her total white cell count was 2.0×10^9/l, neutrophils 0.1×10^9/l and platelets 253×10^9/l. She had negative anti-viral antibody screen and her autoantibody status was unchanged.

L1 was discontinued and she was admitted to hospital and commenced on intravenous broad-spectrum antibiotics (vancomycin and amikacin). Bone marrow aspirate was performed next day and showed increased cellularity with erythroid hyperplasia. Megakaryocytes were plentiful with normal morphology. Myelopoiesis was markedly suppressed with absence of promyelocytes, myelocytes and mature neutrophils. In an attempt to speed up marrow recovery, a single dose (5 μg/kg) of GM-CSF (Sandoz) was given subcutaneously 3 weeks after the onset of agranulocytosis. This was immediately followed by a systemic reaction with pyrexia, rigor, shortness of breath, hypotension and peripheral cyanosis. This was successfully managed with intravenous fluid, antihistamine and hydrocortisone. It was, therefore, decided not to administer further GM-CSF.

In the subsequent days she made a progressive clinical recovery but her neutrophil count remained low ($< 0.5 \times 10^9$) for 7 weeks (fig. 1). She was then discharged from hospital and has since been asymptomatic. She has resumed chelation with subcutaneous DFO.

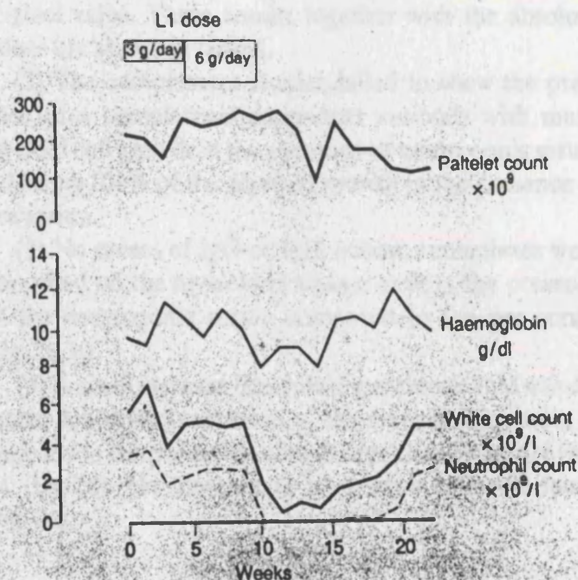

Fig. 1. Course of peripheral blood count of patient who experienced an episode of reversible agranulocytosis whilst taking L1. Blood product support is omitted for clarity.

Tests

Laboratory Investigations

L1 over 99.5% pure was synthesised at the Royal Free Hospital as previously described [9]. The purity of L1 was characterised by ^{1}H-NMR, Mass spectroscopy, HPLC, melting point, thin-layer chromatography and UV spectroscopy. Encapsulation of L1 was carried out in the Pharmacy Department of the Royal Free Hospital. Preservative-free heparinised samples of bone marrow were obtained from a normal volunteer and from the patient whose blood count had recovered to normal several months earlier.

Tests for Direct Antibody Inhibition of Myeloid Growth

Mononuclear cells were isolated using Lymphoprep centrifugation. Cells other than myeloid precursors were removed by adherence on a petri dish and immunological selection using My8 and glycophorin A antibodies [10]. The myeloid progenitors were then cultured in triplicate in 1 ml liquid cultures of Iscoves Modified Dulbeccos Medium (IMDM) with 15% fetal calf serum (FCS) and 10 ng/ml granulocyte colony stimulating factor (G-CSF) (Amgen) at a cell concentration of 1×10^6/ml. 10% heat-treated autologous serum taken prior to the neutropenic episode (pre) or serum taken during the neutropenic episode (day 2) were incorporated into the culture with and without the addition of a previously established [unpublished data] non-myelotoxic concentration of L1 (31 μ*M*). Myeloid cell growth was then measured by analyzing expression of membrane CD16, a marker of late myeloid differentiation, as previously described [11].

Table 1. Effect of the neutropenic serum and L1 on myeloid cell growth

Sample (serum)	Mean myeloid growth	
	absolute value	%
Pre	574	100
Neutropenic	715	125
Pre + L1 (31 μM)	400	70
Neutropenic + L1 (31 μM)	316	44

Mean myeloid growth is expressed as absolute number and percentage of CD16+ cells counted in 5,000 cells.

Test for Complement-Dependent Inhibition of Myeloid Growth

Tests for a complement-dependent antibody were performed by removing aliquots from each culture established as described above after 12 h incubation with 62 μM L1. The cells were centrifuged in IMDM at 1,200 rpm for 5 min, incubated with equal volumes of either pre-serum and fresh normal serum or neutropenic serum and fresh normal serum for 1 h at 37 °C. Cells were then washed in IMDM and resuspended in liquid culture with IMDM and 15% FCS and myeloid growth analysed as above.

Immunofluorescent Tests for Immune Complex/Auto-Antibody Mechanisms

The non-adherent bone marrow mononuclar cells were incubated for 12 h in IMDM and 15% FCS, washed and fixed with 1% PFA, washed again and used as target cells for equal volumes of a premixture of pre or neutropenic serum and 31 μM L1. After a 30-min incubation at 37 °C, deposition of an immune complex or antibody on the cell surface was detected using rabbit anti-FITC Fab_2 anti-human IgG and IgM as above.

Immunofluorescent Tests for Antibody to Myeloid Cells: Hapten Mechanism

Following lymphoprep centrifugation, bone marrow mononuclear cells were adhered on a petri dish for 1 h and the non-adherent bone marrow mononuclear cells were removed and incubated with 31 μM L1. At 12 h the cells were washed, fixed with 1% paraformaldehyde, washed again and incubated for 30 min at 37 °C with equal volumes of either pre or neutropenic serum. The cells were then washed again and analysed for the presence of IgG and IgM using rabbit anti-FITC Fab_2 anti-human immunoglobulin polyclonal antibodies as described previously [12].

Results

(1) The neutropenic serum enhanced growth of autologous myeloid cells to a mean value of 125% of the pre serum. The addition of L1 to the pre serum reduced mean myeloid growth to 70% of its original value (L1 concentration in culture 31 μM), whereas the addition of L1 to the neutropenic serum reduced mean growth to 44% of its original value. These results together with the absolute values are shown in table 1.

(2) The complement studies failed to show the presence of a complement-dependent antibody with mean myeloid cell growth in the presence of neutropenic serum achieving 104% of the myeloid growth in the presence of pre serum.

(3) No excess of IgG or IgM immune complexes were deposited on the myeloid precursor cells in the presence of the neutropenic serum compared to the pre serum (fig. 2a, b).

(4) A small increase in myeloid membrane IgM was detected following incubation of the neutropenic serum as opposed to the pre serum with cells primed in culture with L1 (fig. 2d). No increase in membrane IgG was found (fig. 2c).

Discussion

We describe here a second case of agranulocytosis occurring in a patient receiving L1 and the first in a patient with thalassaemia major. A brief description of this patient has been reported elsewhere [13]. The experiments described here provide inconclusive evidence that agranulocytosis in this patient may be associated with an L1-induced antibody directed against white cell precursors.

The enhancec myeloid cell growth in the test for direct inhibition of myeloid growth by the neutropenic serum probably reflects the presence of increased concentrations of circulating myeloid growth factors as a result of the profound peripheral neutropenia. It also suggests that no direct myelotoxic action of L1 or L1 metabolite in the serum was present. The addition of a low concentration (31 μM) of drug to these cultures caused toxicity greater in the neutropenic than pre serum. This could be explained by the formation of a drug antibody immune complex or the formation of a hapten with recognition by an antibody present in the neutropenic serum. It is, however, also possible that the increase in myeloid turnover induced in culture by the neutropenic serum may have potentiated a myelotoxic effect due to iron deprivation at the low concentration of L1 in this system.

The significance of a weak deposition of IgM found in the screen for a hapten mechanism is uncertain. The lack of IgM deposition on the cell surface in the immune complex screen suggests that this result is not just a reflection of a non-specific increase in immunoglobulin complexes in the neutropenic serum.

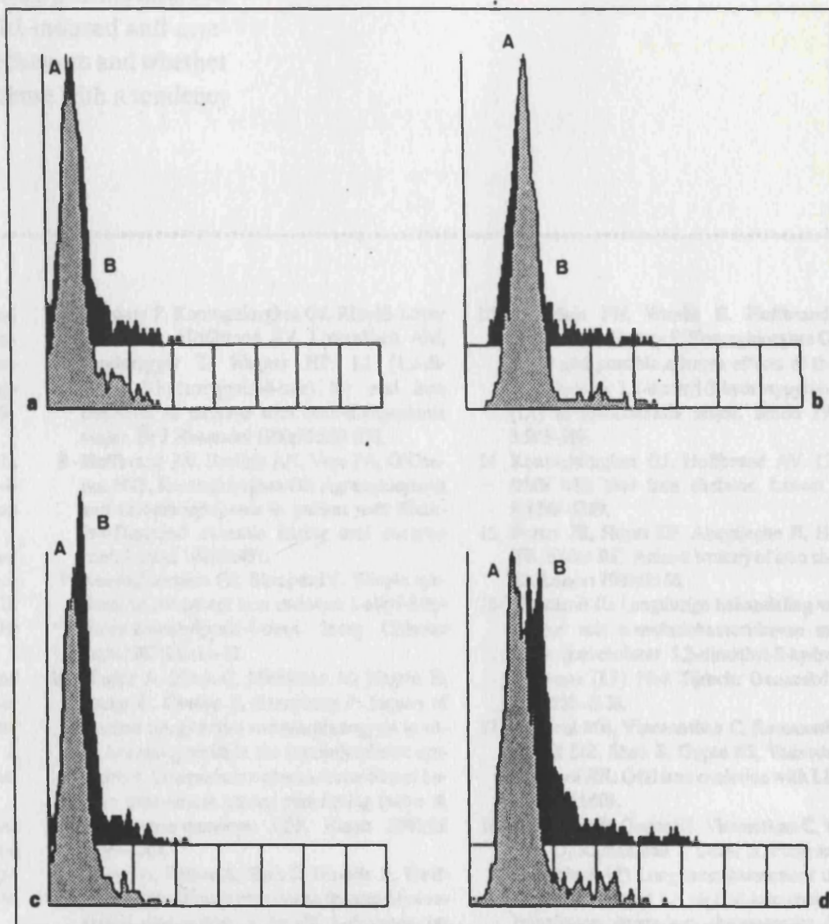

Fig. 2. Flow cytometric histograms of IgG and IgM antibody binding to myeloid progenitors/progeny detected by FITC-conjugated anti-IgG or anti-IgM (Fab) (intensity of fluorescence X-axis) against number of cells (Y-axis). A = Pre serum; B = neutropenic serum. **a** Immune complex/auto-antibody anti-IgG. **b** Immune complex/auto-antibody anti-IgM. **c** Hapten mechanism anti-IgG. **d** Hapten mechanism anti-IgM.

On separate studies to be reported elsewhere, no evidence of increased L1 toxicity as compared with other α-ketohydroxypyridines and desferrioxamine to bone marrow myeloid precursors from the patient compared with normal marrow was demonstrated when a semi-solid agar culture system was used [unpublished data].

Previous studies of long-term (0.5–1.0 year) administration of L1 at 200 mg/kg to normal rats have shown a drop in white blood cell count but no changes in the ratio of neutrophil to lymphocytes [14]. Histological examination showed mild hypocellularity of the bone marrow, affecting mainly white cell progenitors [unpublished data]. A significant mean fall in haemoglobin and white cell count has also been described in iron-overloaded and non-iron-overloaded mice treated with L1 at 200 mg/kg orally daily for 60 days [15].

No evidence of drug-related anti-neutrophil antibody was found in the previous case of L1-associated agranulocytosis [8]. Both cases of agranulocytosis were in young women after 6 weeks of L1 treatment at the highest dose level used. Other cases of idiosyncratic drug-induced agranulocytosis do occur during this period of therapy and are more frequent in females. Therefore vigilance in monitoring the blood count particularly in these weeks in patients commencing L1 therapy is essential, especially in those receiving higher doses.

Another case of transient agranulocytosis has been reported in Holland in a patient with myelodysplastic syndrome receiving L1 at a total daily dose of 3 g [16]. As the patient was also receiving other drugs at the same time, the association with L1 therapy was not clear. No other cases of agranulocytosis have been reported in clinical trials in Canada, Switzerland and India [5 7, 17, 18]. Whether the occurrence of the only two confirmed cases in London represents a real difference in incidence is uncertain. The dose of L1 used in our trials has in general been higher than that used in other studies. The patient described here had multiple red cell alloantibodies and the other developed a rare red cell auto-antibody (LW) while receiving L1 [8]. Further studies are needed to deter-

mine the true incidence of L1-induced agranulocytosis and confirm if the mechanism is due to L1-induced anti-myeloid antibodies or to a direct toxic mechanism and whether agranulocytosis is more frequent in those with a tendency to form red cell antibodies.

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SHORT REPORT

The effect of deferiprone (L_1) and desferrioxamine on myelopoiesis using a liquid culture system

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Summary. Agranulocytosis was observed in a 63-year-old patient with myelodysplasia 6 weeks after commencing chelation with the oral iron chelator deferiprone (1,2-dimethyl-3-hydroxypyrid-4-one, L_1) at a daily dose of 79 mg/kg. Using a liquid culture system no difference was observed when L_1 toxicity to normal and patient myelopoiesis was compared (IC_{50}: 150 *v* 172 μM respectively). L_1 was found to be less toxic than desferrioxamine (DFX) (IC_{50}: 150 *v* 9 μM respectively) to normal myelopoiesis. Delayed addition of iron to myeloid cultures containing an inhibitory concentration of L_1 or DFX was associated with reversal of chelator-induced inhibition of myelopoiesis up to 6 h but not after 24 h. Further studies are needed to determine the incidence and elucidate the pathogenesis of agranulocytosis associated with L_1 therapy.

Keywords: agranulocytosis, deferiprone (L_1), desferrioxamine, liquid culture, myelopoiesis.

Deferiprone (L_1) has undergone worldwide clinical trials and its efficacy found to be comparable to that of DFX. However, its use has been associated with adverse reactions, the most serious of which is agranulocytosis. Several cases of agranulocytosis have now been reported and a number of studies performed in an attempt to elucidate the mechanism(s) involved (reviewed in Al-Refaie & Hoffbrand, 1993). We have recently observed another case of L_1-induced agranulocytosis which developed in a 63-year-old male patient with myelodysplasia 6 weeks after commencing chelation with the oral iron chelator deferiprone (L_1) at a daily dose of 79 mg/kg. We report here the results of the liquid cultures performed on the patient's marrow and normal marrow, intended to elucidate the mechanism of L_1 toxicity to myelopoiesis.

MATERIALS AND METHODS

L_1 was synthesized at the Royal Free Hospital as previously described (Kontoghiorghes & Sheppard, 1987) and DFX was obtained from Ciba Geigy. Preservative-free heparinized samples of bone marrow were obtained from normal volunteers and the patient 3 months after complete recovery from agranulocytosis. The myeloid progenitors were then cultured in triplicate in 1 ml liquid cultures of Iscove's Modified Dulbeco's Medium (IMDM) with 15% fetal calf serum (FCS) and 10 ng/ml granulocyte colony-stimulating factor (G-CSF) (Amgen) at a cell concentration of 1×10^6/ml. Myeloid cell growth was then measured by analysing expression of membrane CD16, a marker of late myeloid differentiation, as previously described (Veys *et al*, 1992). Statistical significance was evaluated using the Student's *t*-test. The following four experiments were performed:

Experiment 1. Serum samples obtained from the patient before (pre) and during agranulocytosis (day 2) and during convalescence (recovery 1) and 3 months after complete recovery (recovery 2) were heat treated at 56°C for 30 min and incorporated into myeloid cultures obtained from the patient's recovery bone marrow with and without the addition of a non-myelotoxic concentration of L_1 (31 μM). These sera were used at 10% concentration and the pre and day-2 sera were also used at 40% concentration.

Experiment 2. L_1 at concentrations of 0–1000 μM was added to myeloid cultures obtained from the patient's recovery marrow and from a normal volunteer. A saturating concentration of iron (1 Fe:3 L_1) was added to parallel cultures.

Experiment 3. L_1 or DFX with or without saturating concentrations of iron (1 Fe:3 L_1, I Fe:1 DFX) was added at concentrations of 0–1000 μM to bone marrow myeloid cultures obtained from a normal volunteer.

Experiment 4. An inhibitory dose (250 μM, see below) of L_1

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Table I. Effect of autologous sera and L_1 on the patient's myeloid cell growth.

Serum (conc.)	Mean* CD16+ cells counted in 5000 cells (% of pre-serum values)	
	Control	L_1 (31 μmol/l)
Pre (10%)	977 (100)	872 (100)
Day 2 (10%)	1016 (104)	1194 (137)
Recovery 1 (10%)	884 (91)	811 (93)
Recovery 2 (10%)	872 (89)	1060 (122)
Pre (40%)	422 (100)	326 (100)
Day 2 (40%)	576 (137)	796 (244)

* Mean of triplicate cultures.

or DFX was separately incorporated into cultures of normal myeloid progenitors and saturating concentrations of iron (as above) were added to the cultures at different time intervals (0, 4, 6, 10, 24 h) after the addition of the chelator. Separate controls for each time interval were used to which similar concentrations of iron were added. Results from each time interval at the end of the incubation period were compared to control cultures.

RESULTS

When patient's recovery myeloid cultures where incubated with autologous sera (pre, day 2 or recovery 1 or 2) for 7 d, no significant inhibition of myeloid growth was observed in any of the cultures in the presence or absence of L_1 (31 μmol/l) (Table I).

No significant difference between the IC_{50} of L_1 for normal and patient's myelopoiesis was found (150 *v* 172 μM respectively). The simultaneous addition of a saturating dose of iron was associated with complete abolition of toxicity in both cultures. DFX was found to be more toxic than L_1 to myelopoiesis with an IC_{50} of 9 μM compared to 150 μM for L_1 ($P < 0.05$). The addition of a saturating concentration of iron was associated with complete suppression of inhibitory effects to myelopoiesis of both L_1 and DFX.

When saturating concentrations of iron were added at intervals of up to 6 h to cultures containing L_1 it was still capable of abolishing the inhibitory effect of L_1 on myeloid cell proliferation, whereas at 10 h L_1-induced inhibition was only partially reversible resulting in myeloid growth of only 55% of control levels. AT 24 h, L_1 inhibition was not affected by iron. Similarly, the DFX inhibitory effect on myelopoiesis was reversible when iron was added to the cultures in the first few hours. However by 6 h the addition of iron failed to completely reverse the DFX effect and by 24 h addition of iron had no effect (Fig 1).

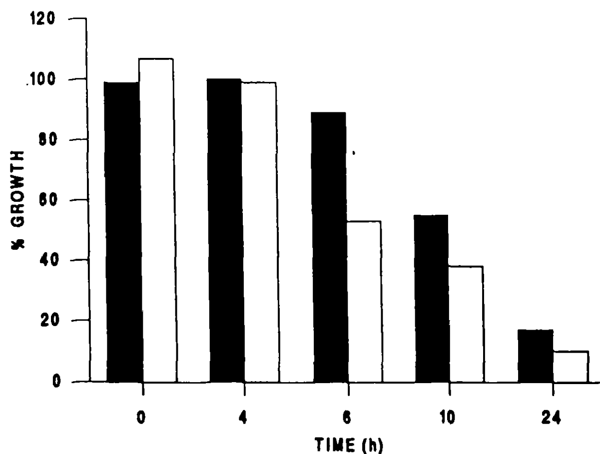

Fig 1. Effect of delayed addition of saturating concentrations of iron to normal myeloid cultures in the presence of an inhibitory concentration (250 μM) of L_1 (solid columns) or DFX (open columns).

DISCUSSION

The present studies failed to demonstrate in the patient's neutropenic or recovery sera the presence of inhibitory factors, L_1 independent or dependent, to myelopoiesis. Enhancement of myeloid growth by the neutropenic serum occurred in both the present and previous study (Al-Refaie *et al*, 1993). It is probably due to increased concentrations of myeloid growth factors in acute-phase serum as a response to neutropenia. The present results are consistent with those of Cunningham *et al* (1994) in which no significant difference was observed between L_1-induced inhibition of normal and patient (with previous agranulocytosis) myelopoiesis. Thus, in both studies, the patients' myelopoiesis was not unduly sensitive to L_1 *in vitro*.

Toxicity of L_1 to normal myelopoiesis was found to be much lower than that of DFX (IC_{50}: DFX 9 μM versus L_1 150 μM, $P < 0.05$). These results are consistent with the previous report of Cunningham *et al* (1994) who used semisolid rather than liquid myeloid cultures (IC_{50}: DFX 7·9 μM versus L_1 130 μM). In both studies the myelotoxicity of L_1 and DFX at doses up to 1000 μM was abolished by a saturating concentration of iron added at the beginning of culture, indicating that the *in vitro* toxicity of L_1 or DFX at these concentrations is due to chelation of iron in cultures. These *in vitro* observations are in contrast to *in vivo* studies in

animals which have shown that iron overload has no bearing on L_1-induced suppression of myelopoiesis (Porter *et al*, 1991; Grady *et al*, 1992). Morever, the patients developing L_1-associated agranulocytosis have all been heavily iron overloaded. Thus it seems that L_1-induced agranulocytosis *in vivo* is not a direct consequence of iron depletion.

Delayed addition of iron to myeloid cultures containing an inhibitory concentration of L_1 or DFX showed that chelator-induced inhibition of myelopoiesis *in vitro* is still reversible by iron for 6 h but not after 24 h of incubation with the chelator. The exact explanation for individual susceptibility to L_1 remains obscure, as does the mechanism by which iron deprivation leads to irreversible cell death. There is no *in vitro* or *in vivo* evidence for an immune mechanism. Although rechallenge with L_1 has not been carried out in our three cases of agranulocytosis, re-exposure of our two cases with a milder degree of neutropenia (unpublished observation) and one case of Goudsmit & Kersten (1992) has not led to a fulminant neutropenia as may be seen when a drug-dependent antibody is present. It appears, therefore, that a toxic mechanism is the more likely cause for L_1-induced myelotoxicity. At all events, constant vigil is required in patients receiving L_1, particularly during the first 2 months of therapy, and L_1 therapy should be restricted to Ethical Committee approved clinical trials. The overall incidence of agranulocytosis and its reversibility on withdrawing L_1 therapy does not imply to us that trials of this drug, which is potentially life saving for many thousands of patients, should be discontinued. Rather, further data are needed of the incidence and pathogenesis of this side-effect.

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Deferiprone-associated myelotoxicity

Al-Refaie FN, Wonke B, Hoffbrand AV. Deferiprone-associated myelotoxicity.
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Abstract: Agranulocytosis developed in a 63-year-old patient with myelodysplasia 6 weeks after commencing treatment with the oral iron chelator deferiprone (L_1, 1,2-dimethyl-3-hydroxypyrid-4-one, CP20) at a daily dose of 79 mg/kg. This was the 3rd case of agranulocytosis (neutrophils 0×10^9/l) in clinical trials of L_1 at the Royal Free Hospital. The neutrophil count recovered 7 days after stopping L_1 and commencing G-CSF at a dose of 300 µg daily. Three other patients with milder degrees of neutropenia (neutrophils $< 1.5 \times 10^9$/l) have also been observed in our trials. The case histories of these 4 patients are described here; other reported cases of neutropenia or agranulocytosis are reviewed. Based on worldwide long-term clinical trials the incidence of agranulocytosis is about 1.6% and of neutropenia 2%.

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Key words: deferiprone – L1 – myelotoxicity – agranulocytosis – neutropenia

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Case reports

(Case 1)

A 63-yr-old caucasian male was generally well until June 1989 when he developed a deep vein thrombosis complicated by pulmonary embolism. At this time he was found to have a haemoglobin of 9.5 g/dl, the rest of the blood count being normal. A bone marrow aspirate showed increased megakaryocytes, changes consistent with myelodysplasia and red cell aplasia. He became transfusion-dependent, requiring approximately 4 units of packed red cells every 4 wk. In April 1990 he was iron overloaded with a serum ferritin 5000 µg/l and commenced subcutaneous desferrioxamine (DFX) 2 g/d for 4–5 nights per wk. In February 1991 he developed reactions to DFX with fevers (39–40°C) and malaise. He was, therefore, offered the oral iron chelator L_1 in a clinical trial approved by the Royal Free Hospital's Ethics Committee and commenced L_1 therapy in November 1992. There was generalised pigmentation of the skin with enlarged liver (3 cm) and spleen (4 cm). The blood count was: haemoglobin 11.1 g/dl (post-transfusion), white cells 8.0×10^9/l, neutrophils 5.1×10^9/l, platelets 441×10^9/l; renal and liver function were normal apart from raised AST (63 U/l) and ALT (132 U/l) levels. Serum ferritin was 3700 µg/l, serum iron 49 µmol/l and TIBC 48 µmol/l. Tests for hepatitis C antibody, ANA and dsDNA antibody were negative, RhF weakly positive (1.80). Multigated acquisition (MUGA) scan revealed a resting left ventricular ejection fraction (LVEF) at the lower limit of normal (52%) with some deterioration on cold stress to 46%, consistent with a mild cardiomyopathy. He was given L_1 at an oral daily dose of 79 mg/kg. Both the white cell and platelet counts remained normal on weekly testing during the first 6 wk. At the beginning of the 7th wk he presented with malaise and sore throat. He was febrile (temperature 38.5°C) with signs of lower zone consolidation of left lung. The total white cell count was 1.9×10^9/l with no granulocytes in the blood film (Fig. 1); platelets 319×10^9/l. Chest X-ray showed collapse of the left base with a left basal pleural effusion. The bone marrow was hypocellular with markedly decreased erythropoiesis and absent myelopoiesis. The autoantibody status was unchanged. L_1 was discontinued and he was admitted

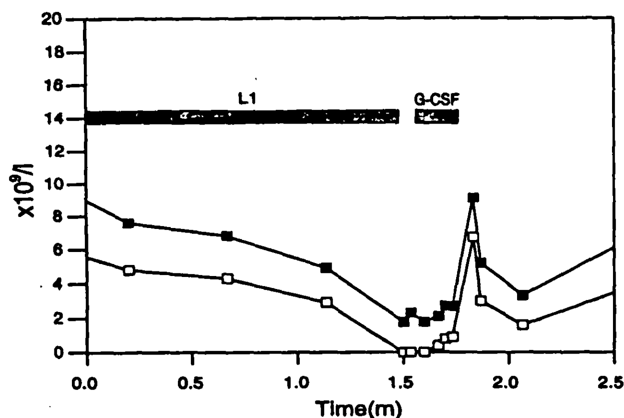

Fig. 1. The course of total white cell count (—■—) and absolute neutrophil count (—□—) in a 63-year-old patient with myelodysplasia who developed agranulocytosis while receiving L_1.

to hospital and given broad-spectrum antibiotics (vancomycin and ceftazidime). As he was still pyrexial 3 d later, intravenous metronidazole and acyclovir were added and he was also commenced on G-CSF, 300 μg daily. Next day he developed atrial fibrillation and was, therefore, commenced on oral digoxin. A day later he developed pleuritic chest pain and central cyanosis with an O_2 saturation of 88% and a ventilation/perfusion scan suggesting bilateral pulmonary embolism. He was given O_2 therapy and intravenous heparin by infusion. As he was still febrile his antibiotics were changed to imipenem, metronidazole and liposomal amphotericin. Four days later he became afebrile with an O_2 saturation of 97% on air and improved chest signs. At this time his neutrophil count was 0.4×10^9/l.

Liposomal amphotericin was stopped and 4 d later imipenem, metronidazole and G-CSF were also stopped and he was converted to warfarin for anticoagulation. His total white cell count was 4.3×10^9/l, neutrophils 2.3×10^9/l (Fig. 1) and platelets 213×10^9/l. He started daily subcutaneous DTPA infusion for iron chelation with zinc supplementation. Two months later he was admitted with intractable heart failure. He deteriorated and died 3 d later from irreversible cardiac arrest. A postmortem examination was consistent with cardiomyopathy due to iron overload as the cause of death.

(Case 2)

A 23-yr-old female patient with thalassaemia major started L_1 (90 mg/kg/d) therapy in June 1992. She had previously used DFX (50 mg/kg/d) with poor compliance. Her total white cell count was 5.9×10^9/l, neutrophils 2.7×10^9/l, serum ferritin 3770 μg/l, serum iron 34 μmol/l and TIBC 40 μmol/l. She was not splenectomized and was anti-HCV negative. In August 1993 she became neutropenic with a total white cell count of 2.7×10^9/l (neutrophils 0.3×10^9/l) but was asymptomatic. L_1 was withdrawn and 2 wk later her neutrophils were 2.5×10^9/l. In September 1993 (neutrophils 3.2×10^9/l) L_1 (40 mg/kg/d) was re-introduced, but 4 wk later her neutrophils dropped to 0.4×10^9/l. L_1 was therefore stopped and her neutrophil count returned to normal 2 wk later.

(Case 3)

A 20-yr-old male patient with thalassaemia major had been chelated with DFX 40–50 mg/kg 4–5 nights per wk. He was splenectomized at the age of 12 yr and was anti-HCV negative. At the beginning of 1992 he became allergic to DFX with urticarial skin reaction and he therefore commenced L_1 (97 mg/kg/d) in May 1992. His white cell count was 12.8×10^9/l, neutrophil count 2.6×10^9/l, serum ferritin 2168 μg/l, serum iron 31 μmol/l and TIBC 36 μmol/l. In July 1993 his neutrophil count fell to 1.8×10^9/l and the L_1 dose was reduced to 86 mg/kg/d. In December 1993 his neutrophil count fell to 0.6×10^9/l. He was asymptomatic and the neutrophil count rose to 4.6×10^9/l at 2 wk after stopping L_1. In February 1994, when the neutrophil count was 2.8×10^9/l, L_1 (43 mg/kg/d) was re-administered but the neutrophil count fell again to 1.2×10^9/l 4 wk later and reversed to normal 2 wk after the discontinuation of L_1.

(Case 4)

A 24-yr-old, male patient with thalassaemia major started L_1 therapy (103 mg/kg/d) in May 1992. He had previously received DFX (50 mg/kg/d) for iron chelation but with poor compliance. His white cell count prior to L_1 therapy was 7.1×10^9/l, neutrophils 3.0×10^9/l, serum ferritin 3350 μg/l, serum iron 33 μmol/l and TIBC 42 μmol/l. He was not splenectomized and was anti-HCV positive with abnormal liver function. In April 1993 the neutrophil count fell to 1.4×10^9/l. L_1 was discontinued and the neutrophil count recovered 2 wk later to 2.3×10^9/l. In June 1993 when the neutrophil count was 3.9×10^9/l L_1 (58 mg/kg/d) was restarted but the neutrophil count fell again to 1.2×10^9/l 4 wk later and rose back to normal 2 wk after the withdrawal of L_1.

Discussion

We report here the 3rd patient in our clinical trials of the oral iron chelator L_1 to have developed agranulocytosis. All 3 cases (Blackfan-Diamond anaemia (1), thalassaemia major (2) and the present case of myelodysplasia) developed agranulocytosis 6 wk after commencing L_1 therapy. Three patients with thalassaemia major who developed less severe degrees of neutropenia (neutrophils $< 1.5 \times 10^9$/l) while receiving L_1 are also described (Table 1). All 4 patients recovered within 2 wk of stopping L_1 therapy but neutropenia recurred 4 wk after re-treating the 3 cases with milder neutropenia and again reversed within 2 wk of stopping L_1.

Agranulocytosis occurring 10 months after staring L_1 at a dose of 75 mg/kg has also been reported in a 10-yr-old girl in Bombay with thalassaemia major. This patient developed an infected necrotic lesion over the thigh which healed soon after complete recovery from neutropenia 1 wk after stopping L_1 therapy and commencing G-CSF (3, 4) (Table 1). The child had previously received L_1 for 17 months with no fall in neutrophil count (Agarwal, personal communication). In Amsterdam, a 40-yr-old patient with myelodysplasia developed neutropenia 1 yr after commencing L_1 at a dose of 50 mg/kg/d. This re-

Table 1. Clinical details of 9 patients developing agranulocytosis or neutropenia during L_1 therapy

Age/Sex	Diagnosis	ANC at diagnosis	Dose of L_1 (mg/kg/day) at diagnosis	Duration of L_1 therapy (months)	Time to recovery (weeks)	Centre (reference)
28/F	BDA	0×10^9/l	105	1.5	3	London[1]
20/F	TM	0×10^9/l	105	1.5	7	London[2]
63/M	MDS	0×10^9/l	79	1.5	1	London (current paper, case 1)
23/F	TM	0.3×10^9/l	90	14	2	London (current paper, case 2)
20/M	TM	0.6×10^9/l	86	19	2	London (current paper, case 3)
24/M	TM	1.4×10^9/l	103	12	2	London (current paper, case 4)
10/F	TM	0×10^9/l	75	10	<1	Bombay[3,4]
16/F	TM	0.2×10^9/l	75	21	<1	Bombay (Agarwal, personal communication)
40/M	MDS	0.3×10^9/l	50	12	<1	Amsterdam[5]

ANC=absolute neutrophil count, BDA=Blakfan-Diamond anaemia, TM=thalassaemia major, MDS=myelodysplastic syndrome.

versed 3 d after stopping L_1 but neutropenia recurred 1 yr after retreating the patient with the same dose of L_1 and was again rapidly reversible within 5 d of stopping L_1 (5) (Table 1). Another case of neutropenia has also been observed in Bombay in a 16-yr-old patient with thalassaemia major occurring 21 months after commencing therapy with L_1 at a dose of 75 mg/kg/d. This patient presented with high fevers and responded rapidly to treatment with ciprofloxacin and became afebrile by d 4. The neutrophil count normalized by d 6. The patient has been retreated with L_1 for the last 3 months with no fall in neutrophil count (Agarwal, personal communication).

These observations suggest that there are two patterns of myelotoxicity associated with L_1 therapy. The first is agranulocytosis (neutrophils 0×10^9/l) usually occurring about 6 wk from the commencement of L_1 therapy in patients receiving a relatively high dose of the drug (Table 1). The second may be less severe neutropenia (neutrophils $<1.5 \times 10^9$/l), occurring at any time during L_1 therapy (Table 1). Reintroduction of L_1 appears to cause neutropenia to recur, but at the same pace as for the original development of neutropenia. This pattern is more in favour of a toxic than immune mechanism for L_1-induced agranulocytosis. In none of our 3 patients with agranulocytosis was there any *in vitro* or *in vivo* evidence for an immune mechanism (1, 2). Culture of bone marrow myeloid progenitors from the last 2 of our 3 patients with agranulocytosis, however, has provided no evidence for an increased sensitivity to L_1 of patient myelopoiesis compared to that of normal marrow (6, 7). Therefore the cause of this individual susceptibility to L_1 remains obscure. Based on worldwide long-term clinical trials in 250 patients (3, 5, 8–14) the incidence of agranulocytosis is about 1.6% and of neutropenia 2%. It is not clear why the incidence of agranulocytosis and of neutropenia has been particularly high in our own studies, except that we have tended to use high doses of L_1 (up to 100 mg/kg/d) and others have used mainly lower doses (10, 13). It remains to be seen whether using L_1 at a lower dose would lead to a fall in the incidence of agranulocytosis. At all events, patients receiving L_1 must continue to be subjected to regular monitoring of their blood counts, particularly during the first 2 months of therapy.

Note added in proof: Since the submission of this manuscript for publication, 2 additional cases of thalassaemia major with L_1-associated myelotoxicity have been reported by N. Olivieri (Toronto) at the Conference on New Trends in Therapy for Hemoglobinopathies and Thalassaemia, Paris, France, September 1994. One patient developed agranulocytosis, the other milder neutropenia while receiving L_1. Therefore, the overall incidence of agranulocytosis is now 2% and less severe degree of neutropenia 2.4%.

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Zinc concentration in patients with iron overload receiving oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one or desferrioxamine

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Abstract

Aims—**To determine the changes in serum zinc concentration and the extent of urinary zinc excretion in patients with iron overload receiving the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L_1) or desferrioxamine (DFX), and to correlate these results with blood glucose concentration.**

Methods—**Serum zinc and ferritin concentrations, urinary zinc and iron excretion were regularly assayed in 39 patients and the glucose tolerance test (GTT) was performed in each patient. Patients were segregated according to their GTT into normal, diabetic, and those with an abnormal GTT. The mean of L_1- or DFX associated urinary zinc excretion for each group was determined and compared with the other two groups and with normal value. L_1 associated urinary zinc excretion was also compared with L_1 dose, serum ferritin values, and urinary iron excretion.**

Results—**Both DFX and L_1 were associated with a significantly increased urinary zinc excretion (15·1 (7·3) µmol/24 hours, 11·1 (6·0) µmol/24 hours, respectively) compared with normal subjects. In patients receiving DFX this increase only occurred in patients with diabetes mellitus. Both diabetic and non-diabetic patients receiving L_1 treatment excreted more zinc than normal. Diabetic patients receiving L_1 or DFX excreted more zinc than non-diabetics receiving the same treatment. No correlation was found between urinary zinc excretion and L_1 dose or patients' serum ferritin concentrations. In seven patients receiving long term L_1 treatment a fall in serum zinc was observed from an initial 13·6 (1·6) µmol/l to a final 9·6 (0·8) µmol/l. In one patient this was associated with symptoms of dry skin and itchy skin patches requiring treatment with oral zinc sulphate.**

Conclusions—**In contrast to DFX, L_1 treatment is associated with increased zinc loss. This, however, is modest and does not lead in most patients to subnormal serum zinc concentrations. In a few patients whose negative zinc balance may give rise to symptoms, zinc supplementation rapidly corrects the deficit.**

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Zinc is an essential trace metal for the normal function of many enzymes involved in cell division and DNA and protein synthesis in mankind.[1] Zinc deficiency is associated with several clinical manifestations, such as growth retardation, delayed wound healing, skin changes, hypogonadism, glucose intolerance, anaemia and abnormal leucocyte function.[2] Patients with diabetes mellitus and particularly those with insulin-dependent diabetes mellitus (IDDM) are at risk of developing zinc deficiency.[3 4] Although these patients excrete more zinc in their urine than normal,[3 5 6] serum zinc concentrations may be normal, increased, or decreased.[7] Furthermore, only a few patients with diabetes mellitus develop clinical manifestations of zinc deficiency.

Although several mechanisms for hyperzincuria in diabetic patients have been suggested, such as a non-osmotic process mediated by glucose and changes in gastrointestinal absorption of zinc,[3 8] the exact mechanism remains obscure. The low incidence of zinc deficiency among patients with hyperzincuria is probably due to an adequate intake or compensatory increased absorption of zinc. Furthermore, the estimation of serum zinc has its technical and interpretive limitations,[9] so patients with normal serum zinc concentrations can be zinc deficient. On the other hand, a subnormal serum zinc concentration is suggestive, but alone not diagnostic, of zinc deficiency.[2]

Patients with thalassaemia major not receiving regular chelation treatment or blood transfusions also have serum zinc values below normal, and they have increased urinary zinc excretion (Cavdar A, paper presented to 6th meeting of the Mediterranean Blood Club, Milan, Italy, 1991). It is not clear, however, whether these findings are due to diabetes mellitus in these patients.

The effect of iron chelation treatment on trace metals in patients with iron overload depends on the affinity of the chelator to these metals. DFX has now been used for many years with no report, as far as we are aware, of an associated zinc deficiency. This contrasts with the well known severe zinc loss associated with the iron chelator diethyltriamine penta-acetic acid (DTPA),[10 11] necessitating substantial oral supplements of zinc.

The oral iron chelator L_1 has now been given to many patients worldwide—for over three years in some centres.[12] None of the earlier short and long term trials reported a change in serum zinc concentrations or increased urinary zinc excretion. Recently,

however, we found increased urinary zinc excretion in eight patients receiving regular chelation treatment with L_1 for up to one year and subnormal serum zinc values in four associated with symptoms of dry, itchy, skin patches which resolved with zinc supplementation in two patients.[13]

In our current long term trial of L_1 treatment in patients with iron overload we have monitored zinc values closely and correlated them with the presence of diabetes mellitus or more subtle biochemical abnormalities of glucose metabolism. A preliminary abstract of this work has been published.[14]

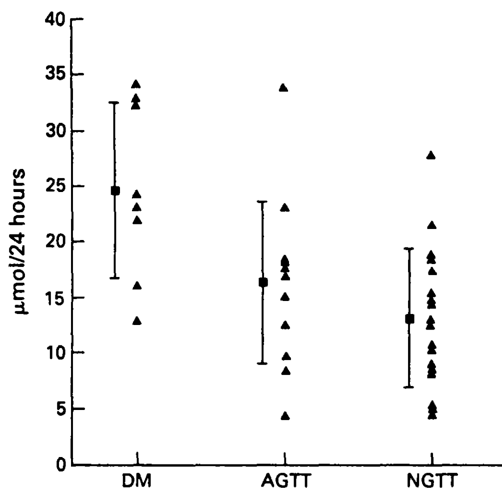

Figure 1 Urinary zinc excretion in 39 patients with iron overload receiving L_1 segregated into three groups: diabetes mellitus (DM, n = 8), abnormal glucose tolerance test (AGTT, n = 13), and normal glucose tolerance test (NGTT, n = 18). X (SD) for each group is shown.

Methods

DFX was obtained from Ciba Geigy and L_1 was synthesised, as described before.[15] Serum ferritin was estimated using an enzyme linked immunosorbent assay (ELISA) technique.[16] Urinary iron and zinc and serum zinc were measured using atomic absorption spectrophotometry.[17] Oral glucose tolerance tests were performed by administering 75 g of glucose after overnight fasting and sampling blood every 30 minutes for two hours.

Significance was evaluated using Student's *t* test. Data were expressed as mean (SD).

This study had the approval of the Ethical Committee of the Royal Free Hospital.

Thirty nine patients (24 males, 15 females) were studied. Their ages ranged from 13 to 60(27·1 (11·0)years). Initial serum ferritin ranged between 733 and 9060 μg/l (3551 (2123) μg/l). Thirty one patients had β thalassaemia major, two sickle cell disease, two congenital sideroblastic anaemia, one myelodysplastic syndrome, one pyruvate kinase deficiency, one haemoglobin E/β-thalassaemia and one sickle/β-thalassaemia. Serum zinc was assayed initially and two monthly thereafter. Two to four 24 hour urine collections were obtained from each patient while receiving subcutaneous infusion of DFX at an approximate dose of 50 mg/kg/day, and four or more collections of urine were obtained during L_1 therapy (50–100 mg/kg/day). These urine samples were analysed for both the total iron and zinc contents. Normal values for serum zinc concentration and 24 hour urinary zinc excretion are 11·5–17·0 μmol/l and 4·5–9·0 μmol/24 hours, respectively, in our laboratory.

Results

Twenty four hour urinary zinc excretion in 39 patients receiving L_1 treatment was 15·1 (7·3) μmol (range, 4·4–34·2 μmol), significantly higher than that associated with DFX treatment (11·1 (6·0) μmol; range 2·6–26·5; p = 0·01), and both were significantly higher than the normal range for urinary zinc excretion ($p < 0·001$, p = 0·04, respectively). There was a significant correlation between L_1 and DFX associated urinary zinc excretion (r = 0·74; $p < 0·001$). Different regimens of L_1 administration (twice or four times a day) showed no significant difference in their effect on urinary zinc excretion in the 19 patients studied nor did the co-administration of vitamin C. Nor did taking L_1 with food or fasting significantly alter urinary zinc excretion. No correlation was found between urinary zinc excretion and L_1 dose (p = 0·11) or urine iron excretion (p = 0·1) or between urinary zinc excretion and serum ferritin values (p = 0·92).

Urinary zinc excretion was significantly higher in patients with diabetes mellitus receiving L_1 (24·6 (7·9), n = 8) than patients with a normal glucose tolerance test (13·1 (6·2), n = 18; p = 0·0006) or those without diabetes mellitus but with an abnormal glucose tolerance test (16·3 (7·3), n = 13; p = 0·02). No significant difference was observed between the latter two groups of patients (p = 0·2) (fig 1). Comparable results were observed with DFX. Patients with diabetes mellitus receiving DFX (n = 7) excreted

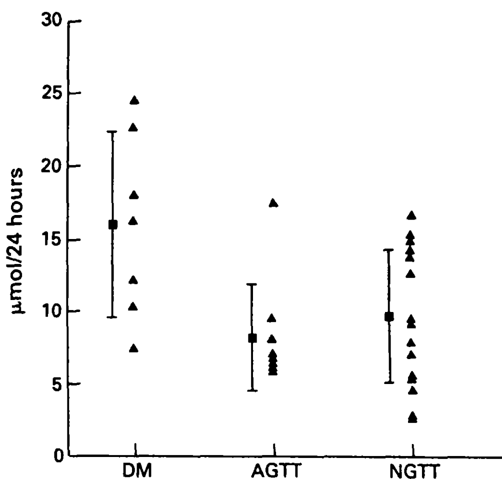

Figure 2 Urinary zinc excretion in 33 patients with iron overload receiving DFX segregated into three groups: diabetes mellitus (DM, n = 7), abnormal glucose tolerance test (AGTT, n = 9), and normal glucose tolerance test (NGTT, n = 17). X (SD) for each group is shown.

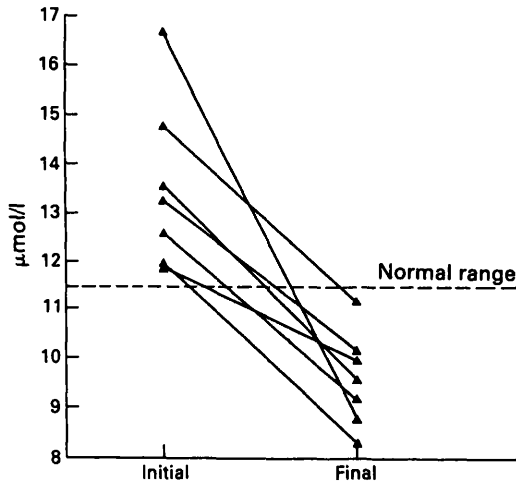

Figure 3 Fall in serum zinc concentrations in seven of 35 patients receiving long term L_1 treatment at a dose of 50–100 mg/kg/day.

more zinc than non-diabetics (n = 17) or those with abnormal glucose tolerance test (n = 9) (16 (6·4) *v* 9·7 (4·6) (p = 0·01), 8·2 (3·7) (p = 0·008), respectively) (fig 2). Again no significant difference was found between the latter two groups of patients (p = 0·59).

There were significant differences between the urinary zinc excretion of patients with diabetes mellitus or an abnormal glucose tolerance test receiving L_1 treatment and corresponding patients receiving DFX (p = 0·04 and 0·03, respectively), but no significant difference was observed between normal glucose tolerance test patients receiving L_1 and those receiving DFX (p = 0·59). However, when the paired *t* test was used to compare the excretion of zinc in the individual patients in the latter two groups, the difference was significant (p = 0·003). All three groups of patients receiving L_1 treatment had significantly increased urinary zinc excretion compared with normal (diabetes mellitus: p = 0·0009; abnormal glucose tolerance test: p = 0·0036; normal glucose tolerance test: p = 0·01). Among patients receiving DFX only those with diabetes mellitus had significantly increased urinary zinc excretion compared with normal (p = 0·03).

In seven of 35 patients receiving long term L_1 treatment serum zinc concentrations fell over a period of six to 12 months from a mean initial value of 13·6 (1·7) μmol/l (11·9–16·7 μmol) to a mean final concentration of 9·6 (1·0) μmol/l (8·3–11·2 μmol) (fig 3). The urinary zinc excretion in these patients was increased at 20·2 (9·4) μmol/24 hours (range 4·4–32·3 μmol/24 hours). This was associated in one patient with symptoms of dry skin and itchy skin patches which rapidly resolved on treatment with zinc sulphate (220 mg/day).

Discussion

In this study we confirm our previous observation that zinc excretion in the urine is increased in patients receiving L_1 treatment. This was significantly higher than the zinc excretion found in patients receiving DFX, although this was also significantly increased compared with normal. Neither the L_1 dose nor iron load of the patients correlated significantly with urinary zinc excretion. The significant correlation observed between L_1 and DFX associated urinary zinc excretion suggests that individual susceptibility for increased zinc excretion is the same with both chelators.

Patients with transfusion dependent refractory anaemias are at risk of developing diabetes mellitus as a result of iron overload. As patients with diabetes mellitus excrete more zinc in their urine than normal subjects it was essential to assess the urinary zinc excretion of patients receiving iron chelation treatment in relation to their blood glucose values. When patients receiving L_1 or DFX were segregated according to their glucose tolerance into normal, diabetes mellitus, and those with abnormal glucose tolerance test, patients with diabetes mellitus excreted significantly more zinc than the others. All three groups of patients receiving L_1 treatment excreted raised amounts of zinc compared with normal. Although the urinary zinc excretion in diabetics receiving L_1 is higher than the mean (18·4 μmol/24 hours) of previously reported values for urinary zinc excretion in patients with IDDM not receiving chelation treatment (21·4 (9·5)[5]; 18·3 (4·1)[6]; 15·4 (5·5)[18]), the difference was not significant (p = 0·06). This may have been due to the small number of patients with diabetes mellitus receiving L_1 treatment studied here. On the other hand, patients receiving DFX with normal or abnormal glucose tolerance tests did not excrete increased amounts of zinc, and in diabetics DFX was not associated with increased urinary zinc excretion compared with diabetics not receiving chelation treatment (p = 0·64). The increase in urinary zinc excretion in patients receiving DFX compared with normal seems to be mainly, if not entirely, due to the presence of diabetes mellitus in some of them.

The lack of a significant difference between the mean urinary zinc excretion of patients treated with L_1 and DFX with a normal glucose tolerance test (13·1 (6·2) *v* 9·7 (4·6); p = 0·59) may also have been due to the small number of patients studied as the difference was significant (p = 0·003) when the paired *t* test was used.

A fall in serum zinc to subnormal values was observed in seven of 35 patients with symptoms of zinc deficiency, necessitating zinc supplementation in one. The incidence of subnormal serum zinc values encountered in our study is comparable with that reported in 20 patients with diabetes mellitus (25%).[3]

In summary, patients with iron overload receiving DFX do not excrete more zinc than normal unless they have diabetes mellitus when their increased zinc excretion is comparable with diabetics not receiving DFX. Patients receiving L_1 treatment excrete more zinc than similar patients receiving DFX or normal subjects. The overall increase in zinc

loss accompanying L_1 treatment is modest and in most patients is presumably balanced by increased absorption of dietary zinc. In a few patients negative zinc balance leads to zinc deficiency. Fortunately, this is easily corrected with zinc supplementation.

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Changes in transferrin saturation after treatment with the oral iron chelator deferiprone in patients with iron overload

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Abstract

Aims—To evaluate the changes in transferrin saturation in patients with iron overload following the oral administration of the iron chelator deferiprone; to assess the correlation between the degree of transferrin desaturation, the deferiprone dose, and urinary iron excretion.

Methods—Serum samples were obtained from 16 patients with iron overload at different time intervals following the oral administration of deferiprone (50 mg/kg). These samples were analysed using 6 M urea/polyacrylamide gel electrophoresis (UPAGE). This method is able to resolve serum transferrin into four different forms (free iron, two forms of monoferric, and diferric). The deferiprone concentration in these samples was estimated using high pressure liquid chromatography (HPLC). Zero time samples (t_0) from 10 patients were incubated with 150 µM deferiprone or normal saline either at room temperature or at 37°C for 30 minutes and 24 hours, and also at −20°C for six weeks. Samples were then analysed using UPAGE.

Results—A maximum decrease in transferrin saturation from (mean (SD)) 93·0 (10·6)% to 54·5 (17·2)% was observed 72·5 (50·0) minutes after deferiprone administration and in most of the patients coincided with peak deferiprone concentration. This was associated with a maximum rise in the percentage of iron free transferrin (apotransferrin) from 2·9 (7·0)% to 27·3 (17·8)%. The total amount of iron estimated to be removed from transferrin constituted 21·3 (20·2)% of the 24 hour urinary iron excretion measured during the study. When deferiprone (150 µmol/l) was incubated in vitro with t_0 samples from 10 patients for 30 minutes and 24 hours at room temperature, 37°C, and at −20°C for six weeks, deferiprone was more efficient at removing iron from transferrin at 37°C, with maximum transferrin desaturation accomplished within 30 minutes compared with 24 hours at room temperature.

Conclusions—The results confirm that deferiprone can remove iron from transferrin when administered orally to patients with iron overload and that transferrin bound iron may, therefore, be a significant source of the iron chelated by deferiprone in vivo.

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Keywords: Deferiprone, transferrin, gel electrophoresis.

The oral iron chelator deferiprone (L_1,1,2-dimethyl-3-hydroxypyrid-4-one, CP20) has now been given to a large number of patients with iron overload in many centres worldwide and shown to have an efficacy comparable to that of desferrioxamine (DFX).[1] The use of both chelators is associated with a wide variation between patients, and even from day to day in individual patients, in the amount of iron chelated and excreted in urine. The exact body pools of iron available for chelation by deferiprone or DFX have not been established. However, transferrin has been suggested as one source of iron available for chelation by deferiprone but not by DFX.[2,3]

Transferrin has a molecular weight of 80 000 and two binding sites for iron, one at each of its two terminals. In normal individuals transferrin is only one third saturated, whereas in patients with iron overload it is usually completely saturated. Transferrin saturation is tested routinely by estimating the difference between the total iron content of serum before and after the addition of a saturating concentration of iron. Although this method is adequate for most purposes, it can be inaccurate. Other forms of iron such as non-transferrin bound or ferritin iron can cause over estimation of transferrin saturation.[4,5] Therefore, if minor changes in transferrin saturation are to be estimated, such as those caused by an iron chelator, a more sensitive method is required. Furthermore, the presence of an excess of the chelator in a serum sample might interfere with the routine measurement of transferrin saturation.

Mackey and Seal[6] showed that partially saturated human transferrin separates into four bands on urea/polyacrylamide gel electrophoresis (UPAGE). The slowest and fastest represent the iron free transferrin (apotransferrin) and diferric transferrin (transferrin-Fe_2), respectively, whereas the two intermediate bands represent the C and N terminal monoferric forms.[7]

In contrast to DFX, deferiprone at concentrations similar to those observed in plasma of patients receiving deferiprone treatment can

[illegible]ent characteristics.

[illegible]	Age(years)/ Sex	Diagnosis	Serum ferritin (μg/l)	TIBC (μmol/l)	Transferrin saturation(%)*	UIE (mg/ 24 hours)
[illegible]	21/F	BTM	8130	33	78·8	15·8
[illegible]	23/F	BTM	7400	NA	NA	NA
[illegible]	13/M	CSA	3131	39	100	30
[illegible]	60/F	SCD	4006	36	100	8·7
[illegible]	81/M	MDS	5650	36	83	12·5
[illegible]	15/M	SCD	5950	66	36	8·1
[illegible]	46/M	CSA	2055	38	81·6	17·3
[illegible]	26/M	BTM	9060	48	79	11
[illegible]	17/M	PKD	3050	36	94·4	32·7
10	22/M	BTM	3350	NA	NA	NA
11	23/M	BTM	3520	30	100	16·5
12	27/F	BTM	3980	33	100	11·5
13	30/F	BTM	3850	54	100	5·2
14	43/F	ASA	1285	36	83·3	9·8
15	15/M	BTM	1320	27	100	3·1
16	31/F	BTM	4000	47	100	NA

BTM = β-thalassaemia major; CSA = congenital sideroblastic anaemia; SCD = sickle cell disease; MDS = myelodysplastic syndrome; PKD = pyruvate kinase deficiency; ASA = acquired sideroblastic anaemia; UIE = urinary iron excretion.
* Measured by routine laboratory technique.

remove an appreciable amount of iron from transferrin in vitro.[2] Evans *et al*[3] recently observed a progressive fall in transferrin saturation after the administration of deferiprone to a patient with iron overload. In this study we have explored the interaction between deferiprone and transferrin both in vivo in a larger group of patients and in vitro. The results help to establish the degree of transferrin desaturation in vivo following administration of deferiprone and its relation to the amount of iron excreted in urine.

Methods

Deferiprone was synthesised at the Royal Free Hospital as described previously.[8] Acrylamide bisacrylamide (19:1), as a ready-made soluction of 40% (w/v), and rivanol were obtained from Sigma (Poole, Dorset, UK). This study had the approval of the Ethical Committee of the Royal Free Hospital. Blood samples were obtained from 16 patients with iron overload (table) at different time intervals (zero, 10, 20, 30, 45, 60, 75, 90, 120, 180, 240, and 300 minutes) following the oral administration of deferiprone (50 mg/kg), separated within 30 minutes of collection and stored at −20°C until analysis. Serum samples were thawed within four weeks of collection and immediately analysed using UPAGE, as described by Williams *et al.*[9] Serum samples were also obtained from normal volunteers and used as controls with each run of UPAGE.

For analysis, samples were treated with rivanol as described previously[10] and applied to the gel. After staining and destaining, gels were scanned using a laser densitometer (Molecular Dynamics, Sunnyvale, California, USA). The deferiprone concentration in these samples was estimated using high pressure liquid chromatography (HPLC), as described before.[11]

A further experiment was performed: zero time samples (t_0) from 10 patients were incubated with 150 μM deferiprone or normal saline, either at room temperature or at 37°C for 30 minutes and 24 hours, and also at −20°C for six weeks. This concentration of deferiprone was chosen to be comparable to the mean of peak deferiprone concentration observed in the

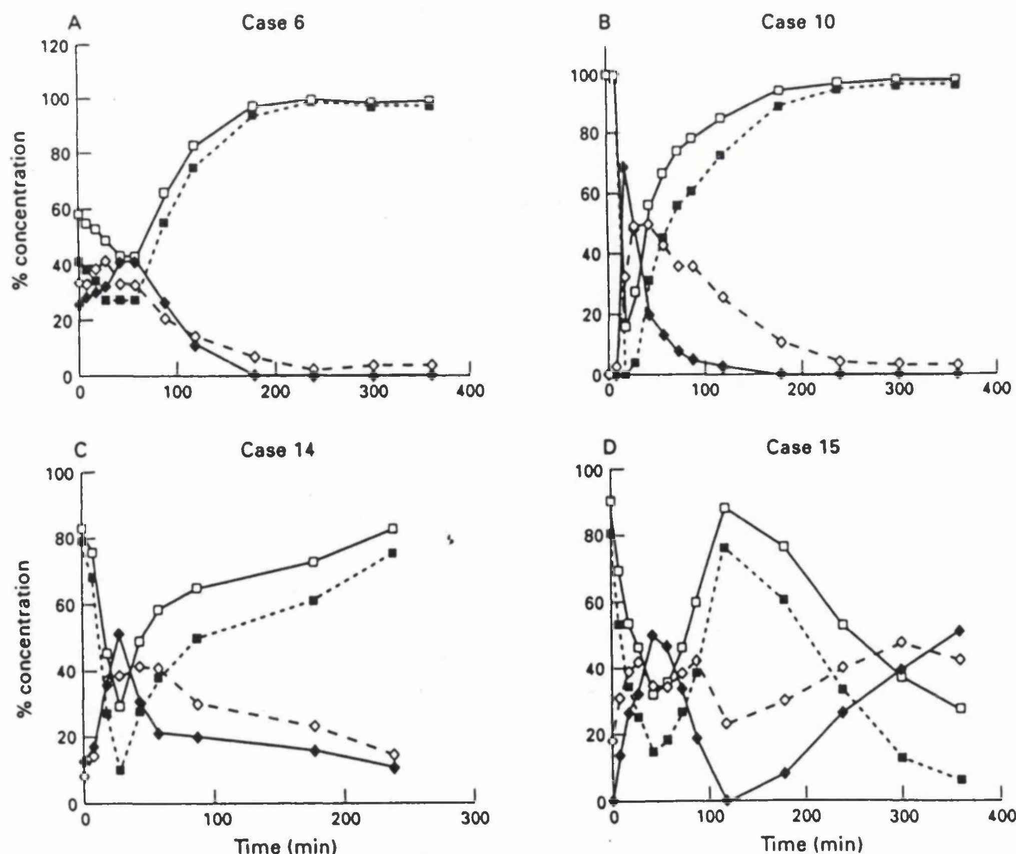

Figure 1 Changes in serum transferrin saturation (□) and in the percentages of apotransferrin (◆), transferrin-Fe(C) (◇), and transferrin-Fe₂ (■) in four cases (cases 6, 10, 14, and 15) after the oral administration of deferiprone (50 mg/kg).

patients' sera. Samples were then treated with rivanol and analysed using UPAGE as above.

Serum iron and the total iron binding capacity (TIBC) were measured by routine laboratory techniques.[12 13] Urinary iron was measured using atomic absorption spectrophotometry. Serum ferritin was estimated by an enzyme linked immunosorbent assay (ELISA) technique.[14] Statistical significance was assessed using Student's t test.

Results

Using the UPAGE method, three bands were observed in patients' sera following deferiprone administration. These represent iron free transferrin (apotransferrin), C terminal monoferric transferrin (transferrin-Fe(C)), and diferric transferrin (transferrin-Fe_2) in order of increasing mobility.[7] Adding an excess of iron to the sera caused complete disappearance of the first two bands and an increase in the density of the third band.

Deferiprone was capable of removing iron from transferrin (fig 1). The mean transferrin saturation at t_0 in patients' sera ranged between 57·8 and 100% (mean (SD) 93·0 (10·6)), compared with between 12·7 and 20·5% (16·4 (3·0)) for normal volunteers (n = 10). The correlation between the transferrin saturation measured by UPAGE and that obtained by using the routine laboratory method (88·3 (17·6)) was significant (r = 0·83, p = 0·0003). Following deferiprone administration, there was a progressive fall in the degree of transferrin saturation and the percentage of transferrin-Fe_2 and the appearance or rise in the percentage of apotransferrin and transferrin-Fe(C) (fig 2). The lowest transferrin saturation observed following deferiprone administration was 54·5 (17·2)% (range 16·0–74·7%), occurring 72·5 (50·0) minutes (10–180 minutes) after deferiprone administration. Transferrin desaturation (difference between t_0 and the lowest values) was 39·5 (17·4)% (14·8–84·0%). In six patients transferrin saturation returned to the t_0 value after 1·6–6 hours (4·5 (2·0 hours)) of deferiprone administration whereas in the rest it returned to 86·0–98·0% of the t_0 value after six hours of follow up. In case 6 transferrin saturation rose to 170% of the t_0 value at six hours (fig 1A) and in case 15 transferrin desaturation was biphasic (fig 1D). Deferiprone concentrations observed at the time of the lowest transferrin saturation ranged between 57 and 310 µmol/l (128·4 (69·6)). In 11 patients the lowest transferrin saturation coincided with the peak deferiprone concentration. There was a significant correlation between maximum transferrin desaturation and the simultaneous deferiprone concentration (r = 0·56, p = 0·02; fig 3). No significant correlation was found between the degree of transferrin desaturation and the serum ferritin concentration (p = 0·25) or 24 hour urinary iron excretion (p = 0·64). The amount of iron removed from transferrin

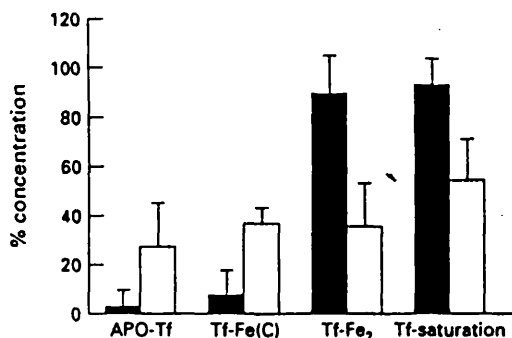

Figure 2 The percentages of the various forms of transferrin at zero time (t_0(■)) and at the time of maximum change (□) in 16 patients with iron overload following the oral administration of deferiprone (50 mg/kg). Data expressed as mean (SD).

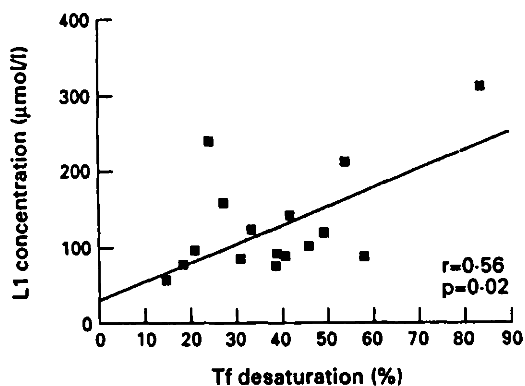

Figure 3 The correlation between the maximum transferrin (Tf) desaturation (the difference between t_0 and the lowest values) and the associated deferiprone (L1) concentration in 16 patients following the oral administration of deferiprone (50 mg/kg).

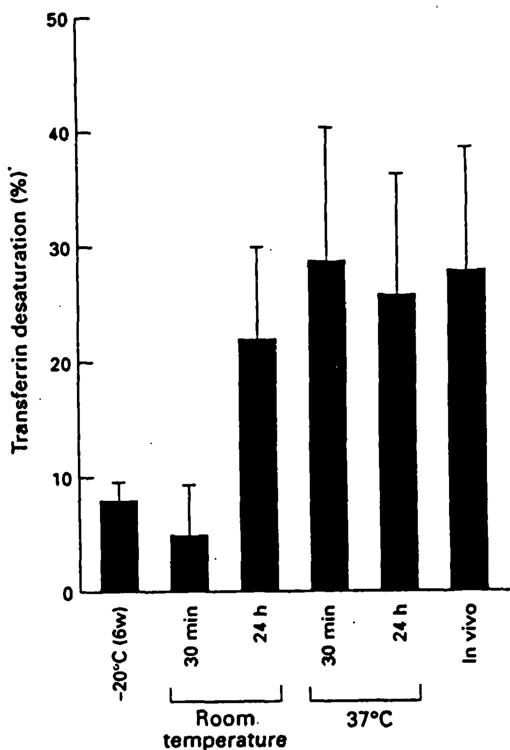

Figure 4 Transferrin desaturation in 10 patients with iron overload following the incubation of their zero time (t_0) samples with deferiprone (150 µmol/l) for 30 minutes and 24 hours at room temperature and 37°C and for six weeks at −20°C compared with the maximum in vivo desaturation of transferrin (In vivo) observed in their serum samples following the oral administration of deferiprone (50 mg/kg). Data expressed mean (SD).

in vivo was 13·8 (5·1) μmol/l (6·7–22.1 μmol/l), estimated by multiplying the TIBC value by maximum transferrin desaturation. The total amount of iron chelated from transferrin by deferiprone was approximately estimated by multiplying the amount of iron removed from transferrin by each patient's predicted plasma volume (weight(kg) × 70(ml/kg) × (1-PCV) (packed cell volume)) and was 35·0 (15·3) μmol (13·9–67·1 μmol). Urinary iron excretion measured during the study was 250·3 (156·4) μmol (55·4–583·9 μmol). No significant correlation was found between urinary iron excretion and the total amount of iron removed from transferrin ($p = 0·9$).

Incubation of t_0 serum samples ($n = 10$) with 150 μM transferrin for 30 minutes at room temperature caused a small fall in transferrin saturation (4·8 (4·4)%); when incubation was continued for 24 hours this fall was more pronounced (21·7 (8·2)%, $p = 0·004$). By contrast, at 37°C a significant drop in transferrin saturation was observed at 30 minutes (28·6 (11·7)%, $p = 0·04$), comparable to that achieved in 24 hours at room temperature ($p = 0·31$), but this fall did not significantly increase after 24 hours of incubation (25·6 (10·5)%, $p = 0·68$). Only a small fall in transferrin saturation was observed when the samples were incubated at -20°C for six weeks (7·8 (1·7)%) (fig 4). In this experiment a fourth band was observed in the UPAGE gels, corresponding to transferrin-Fe(N), after incubating the samples with transferrin at room temperature and at 37°C for 30 minutes and 24 hours. No similar band was found after six weeks of incubation at −20°C. The percentages of this form of transferrin were 10·8 (4·9), 29·3 (7·4), 25·1 (1·6), and 26·7 (1·7), respectively. These changes were associated with a rise in the transferrin-Fe(C) concentration of 10·8 (4·9), 4·9 (3·0), 8·2 (3·3), and 8·2 (5·3)%, respectively.

Discussion

The results obtained following deferiprone administration showed that deferiprone is effective in removing an appreciable amount of iron from transferrin in most of the patients studied. These data are comparable to previously reported results.[3] The degree of transferrin desaturation was dependent on the deferiprone concentration as there was a significant correlation between maximum transferrin desaturation and the associated deferiprone concentration.

The significant correlation between transferrin desaturation and the deferiprone concentration may suggest that these changes could have occurred in vitro after obtaining the serum samples rather than in vivo. We therefore carried out experiments to establish the speed of iron removal from transferrin by deferiprone at different temperatures. The results indicate that this process is much faster at 37°C than at room temperature or −20°C, being accomplished within 30 minutes (fig 4). There was no significant difference between the degree of transferrin desaturation achieved in vitro after 30 minutes or 24 hours at 37°C or 24 hours at room termperature and maximum transferrin desaturation observed in vivo (27·7 (10·8); fig 4). These results suggest that the observed changes in transferrin saturation are more likely to have occurred in vivo than to be purely caused by the interaction between deferiprone and transferrin in the stored serum sample. Furthermore, changes in transferrin saturation observed in cases 6 and 15 (figs 1A and D) suggest that transferrin saturation is not entirely dependent on deferiprone concentration and other in vivo factors may influence the interaction between transferrin and deferiprone. The absence of transferrin-Fe(N) from the serum samples is consistent with previous reports.[15 16] Williams and Moreton[15] found that storage of serum samples at −15°C for several days causes redistribution of iron between transferrin-Fe(C) and transferrin-Fe(N), resulting in the disappearance of the latter from serum but with no effect on overall transferrin saturation. Therefore, it was not possible to examine, in vivo, the distribution of iron between the four different forms of transferrin following administration of deferiprone. However, a fourth band representing transferrin-Fe(N) was observed in vitro after incubating serum samples taken from 10 patients with deferiprone. The changes in the concentration of this form of transferrin during incubation with deferiprone were more marked than the concurrent changes in the concentration of transferrin-Fe(C). In keeping with previous reports[2 3] this suggests that deferiprone preferentially binds the iron atom present in the C terminal site of diferric transferrin. Following deferiprone administration, transferrin saturation usually returned to the t_0 values within six hours whereas in case 6 transferrin saturation continued to increase, reaching 170% of its t_0 value at six hours (fig 1A). As this patient's t_0 transferrin saturation was relatively low (54%), it is possible that deferiprone at an initial high concentration caused a fall in transferrin saturation whereas at a later stage, when the deferiprone concentration fell, it participated in the transport of iron to transferrin. Previous studies have shown that deferiprone given to a normal volunteer can cause a progressive increase in transferrin saturation.[3] The cause of the biphasic transferrin desaturation observed in case 15 (fig 1D) is unclear.

The degree of the iron load had no bearing on the degree of transferrin desaturation, as is evident from the lack of a significant correlation between the latter and the serum ferritin concentration. Assuming that all the iron removed from transferrin by deferiprone was excreted in urine in 24 hours, it was found that this comprised 21·3 (20·2)% (5·8–67·1%) of total urinary iron excretion. This calculation does not allow for possible flux of iron from tissue to transferrin when free deferiprone is present in plasma.

These data suggest, therefore, that transferrin is a source of iron available for chelation by deferiprone. Its rapid resaturation within six hours of deferiprone administration indicates that it could act efficiently in recruiting more iron for chelation should further doses of deferi-

prone be given. However, it is not the sole source of chelatable iron. Non-transferrin bound iron may be another form readily available for chelation. Its contribution to the total urinary iron excretion has yet to be evaluated. Other sources of iron such as tissue iron stores presumably also contribute either directly or indirectly through an intermediate vehicle such as transferrin to the iron chelated by deferiprone.

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Pharmacokinetics of the oral iron chelator deferiprone (L_1) in patients with iron overload

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Summary. Single oral dose pharmacokinetics of the iron chelator deferiprone (L_1) were studied in 24 patients with chronic iron overload and correlated with 24 h urinary iron excretion (UIE) and creatinine clearance. Absorption of L_1 was rapid with a $t_{1/2}$ of 22·2 ± 17·7 (mean ± SD) min. The elimination half-life (el$t_{1/2}$) of the drug was 91·1 ± 33·1 min and of its metabolite, L_1-glucuronide (L_1G) 147·7± 52·0 min. Creatinine clearance of the patients correlated significantly with the elimination $t_{1/2}$ of L_1G ($r = -0.79$, $P = 0.002$). There was also a significant correlation between 24 h UIE in the 14 patients studied and L_1 versus time area under the curve (AUC) ($P = 0.007$). The total amount of L_1 recovered in urine in 24 h comprised 77·9 ± 13·3% of the L_1 dose. L_1 efficiency (the 24 h UIE divided by the amount of iron the oral dose of L_1 is capable of binding) in the 14 patients was 3·8 ± 1·9%. These data show for the first time that the urinary elimination of L_1G is influenced by the renal function of the patient. Although no significant accumulation of L_1 and L_1G will occur in most of the patients if L_1 is given more than once daily, in some patients with impaired renal function, L_1G may accumulate.

Keywords: deferiprone, L_1, L_1-glucuronide, iron overload, pharmacokinetics.

The oral iron chelator deferiprone (1,2-dimethyl-3-hydroxypyrid-4-one, L_1, CP20) has now been evaluated in several clinical trials worldwide (Bartlett *et al*, 1990; Töndury *et al*, 1990; Al-Refaie *et al*, 1992; Agarwal *et al*, 1992; Olivieri *et al*, 1992; Al-Refaie & Hoffbrand, 1993). Its efficacy has been shown to be comparable to that of desferrioxamine (DFX) with generally an excellent compliance resulting in a progressive fall in serum ferritin in most patients. Its use, however, has been associated with some adverse reactions, the most important of which is agranulocytosis. Any new drug such as L_1 requires a detailed pharmacokinetic profile in order to assist in designing the optimum regimen for drug administration. Furthermore, drug pharmacokinetics help to evaluate the effect of various parameters such as bioavailability, speed of absorption, metabolism, and elimination on the efficacy and possible side-effects of the drug. Such studies are also important to assess the pharmacokinetics of drug metabolites and their possible effects on the efficacy and toxicity of the drug. Only a few small studies on L_1 pharmacokinetics have now been published (Kontoghiorghes *et al*, 1990a, b; Olivieri *et al*, 1990; Matsui *et al*, 1991), but no similar reports on the pharmacokinetics of L_1G have so far emerged. In none of the previous studies has the relation between the pharmacokinetics of L_1 or L_1G and the renal function of the patients been determined. In this study we have examined the pharmacokinetics of both L_1 and L_1-glucuronide in 24 patients with iron overload. Correlations between various pharmacokinetic parameters and L_1 efficacy and renal function have been examined.

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PATIENTS

This study had the approval of the Ethical Committee of the Royal Free Hospital. 24 patients were included; their clinical details are summarized in Table I. L_1 was withheld for 24 h prior to the study in the 17 patients who had already received L_1 (50–100 mg/kg/d) for durations ranging from 4 to 32 (16·9 ± 10·3) weeks (Table I). L_1 (approximately 50 mg/kg) was given orally after an overnight fast. Blood samples were collected at regular intervals (0, 10, 20, 30, 45, 60, 75, 90, 120, 180, 240, 300, 360 min) and serum was immediately separated and stored at −20°C until the time of analysis. 24 h urine collections were obtained simultaneously from 14 patients; in eight of them urine was collected at intervals (2, 4, 6, 12, 24 h), whereas the other six produced one single 24 h collection.

Table I. Clinical details of 24 patients with iron overload.

Case	Age (yr)/ sex	Diagnosis	Duration on L_1 prior to study (weeks)	Serum ferritin (μg/l)	Transferrin saturation (%)	Serum creatinine (μmol/l)	Creatinine clearance (ml/min)	Serum AST (iu)	UIE (mg/24 h)
AA	21/F	BTM	0	8130	95	76	54	31	16·0
AD	23/F	BTM	10	7400	83	82	ND	61	ND
AE	22/M	BTM	32	2127	97	52	ND	15	ND
AM	13/M	CSA	0	3131	95	62	76	23	30·3
BJ	60/F	SS	0	4000	100	130	35	33	9·2
CH	35/M	BTM	22	2922	90	46	ND	239	ND
CSo	29/M	BTM	30	2108	85	60	99	26	ND
CSt	81/M	MDS	11	5650	83	168	24	42	12·5
DI	15/M	SS	9	5950	36	78	90	58	8·1
EM	26/M	BTM	9	2393	87	78	ND	92	ND
FM	25/M	BTM	32	1365	63	72	ND	20	ND
FV	20/M	BTM	28	4122	100	58	ND	128	ND
GZ	21/M	BTM	16	7875	100	66	ND	69	ND
JT	46/M	CSA	12	2055	82	66	89	35	17·3
LL	26/M	BTM	4	9060	79	88	96	104	11
MH	17/M	PKD	0	3050	94	73	128	55	32·7
MM	22/M	BTM	28	3350	100	65	ND	49	ND
MS	23/M	BTM	8	3520	100	82	114	56	16·5
PD	27/F	BTM	25	3980	100	64	57	41	ND
SA	30/F	BTM	6	3850	100	61	100	49	8·9
SB	43/F	MDS	0	1285	83	78	78	36	9·8
SG	15/M	BTM	0	1320	100	57	124	22	4·7
TR	51/M	PKD	0	3131	96	92	87	28	21·2
TT	31/F	BTM	6	4000	88	80	84	68	7·3
Mean ± SD	30·1 ± 16·0		16·9 ± 10·3*	3991 ± 2236	89·0 ± 14·7	76·4 ± 25·7	83·4 ± 29·3	57·5 ± 47·5	14·7 ± 8·4

ND = not done, AST = aspartate transaminase, UIE = urine iron excretion, BTM = β-thalassaemia major, CSA = congenital sideroblastic anaemia, SS = sickle cell disease, MDS = myelodysplastic syndrome, PKD = pyruvate kinase deficiency.

* Mean ± SD of durations >0.

MATERIALS AND METHODS

L_1 was synthesized at the Royal Free Hospital as described previously (Kontoghiorghes & Sheppard, 1987). L_1 and L_1-glucuronide (L_1G) concentrations were estimated in serum samples and urine using a previously described high-pressure liquid-chromatography (HPLC) technique (Goddard & Kontoghiorghes, 1990) with the following modification: Amicon Centrifree filters were used instead of perchloric acid for protein removal from the serum samples. When serum samples were analysed using either Amicon filters or perchloric acid no significant difference was found (data not shown). Iron in 24 h urine samples was estimated using atomic absorption spectrophotometry. Serum ferritin was assayed by an ELISA technique (Flowers *et al*, 1986). UIE was assayed using atomic absorption spectrophotometry. Transferrin saturation, serum creatinine levels and creatinine clearance were measured using routine laboratory techniques. $t_{1/2}$ of absorption and elimination (ab$t_{1/2}$, el$t_{1/2}$), areas under the curve (AUC) of L_1 and L_1G concentrations versus time and $t_{1/2}$ of L_1G appearance (app$t_{1/2}$) in the blood were calculated using a computer program specially designed for pharmacokinetic analysis (Johnston & Woollard, 1983). Data are expressed as range and mean ± SD.

RESULTS

All patients but one had undetectable serum levels of L_1 or L_1G in their baseline serum samples. The exception, a man of 81 with myelodysplastic syndrome, was found to have a high baseline serum level of L_1G (346 μmol/l) and a maximum concentration (C_{max}) of 698 μmol/l observed 4 h following L_1 administration (Fig 1a). He had commenced L_1 therapy 11 weeks earlier and his last dose was taken 24 h before the study. His L_1G pharmacokinetic results are therefore not included in the statistical analysis of the data obtained from other 23 patients. The details of the L_1 and L_1G pharmacokinetics are given in Table II and the changes in serum concentration of L_1 and L_1G versus time in four of the patients are shown in Fig 1.

When the pharmacokinetics of L_1 and L_1G in patients who had taken L_1 ($n = 17$) prior to the study were compared with the pharmacokinetics of L_1 and L_1G in those who had not taken L_1 ($n = 7$), no significant differences were observed ($P > 0.05$). AUC of L_1 and L_1G beyond the 6 h period of the study (6 h–∞) comprised 9·9 ± 8·5 (0·4–29·5)% and 24·4 ± 15·1 (6·8–56·3)% respectively of the total AUC (0–∞). The AUC of L_1 and L_1G beyond 12 h (12 h–∞) were found to comprise 1·3 ± 2·1 (0–6·4)% and 5·3 ± 6·0 (0·5–23·5)% respectively of the total AUC.

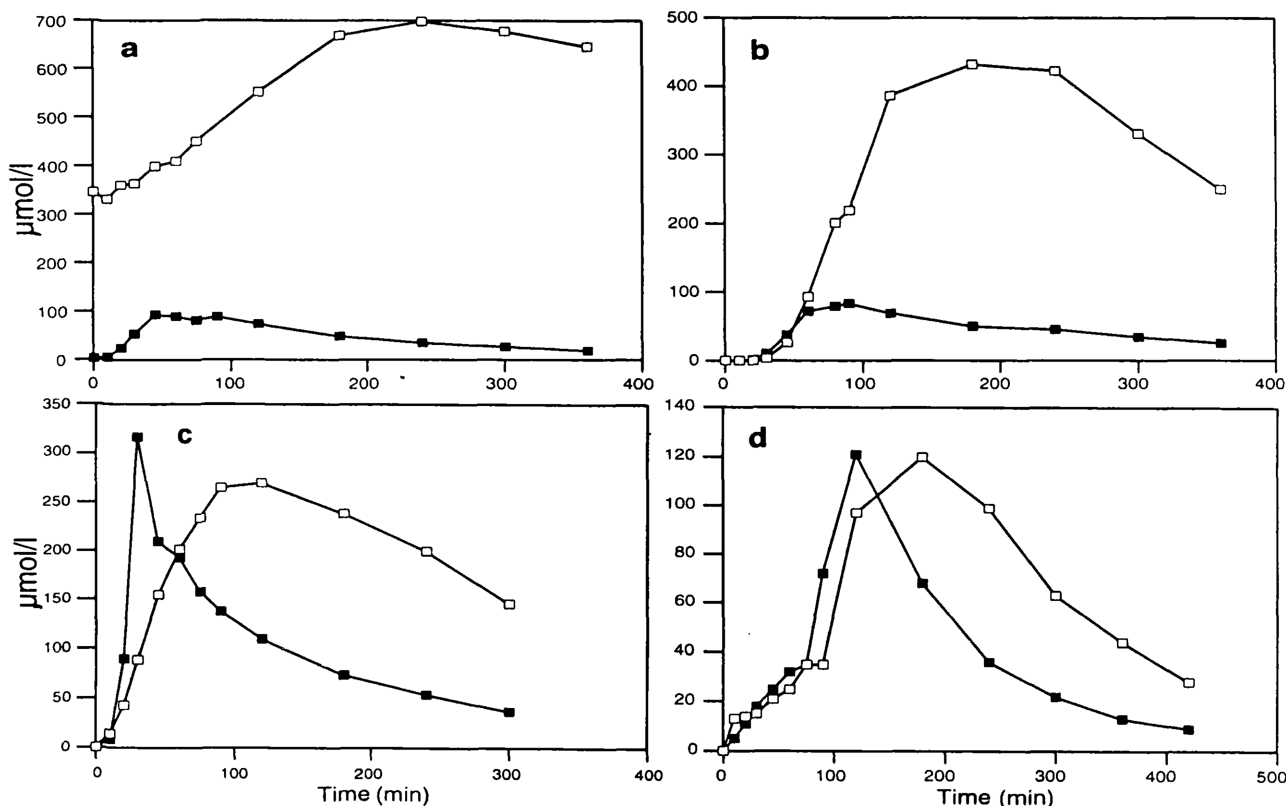

Fig 1. Pharmacokinetic profile of L_1 (■) and its glucuronide (□) in the serum of four patients with iron overload following L_1 administration at an oral dose of approximately 50 mg/kg. (a) and (b) (cases CS and BJ respectively) show delayed elimination of L_1G resulting in high serum levels of L_1G. Both patients have impaired renal function. (c) (case PD) shows the typical profile seen in most patients with fast absorption of L_1 and fast elimination of both L_1 and L_1G. (d) (case TT) shows an example of the delayed absorption of L_1, seen in four patients.

Creatinine clearance correlated significantly with L_1G-el$t_{1/2}$ ($r = -0{\cdot}79$, $P = 0{\cdot}002$, Fig 2) but not with L_1-el$t_{1/2}$ ($r = 0{\cdot}44$, $P = 0{\cdot}087$). No significant correlations were observed between the serum AST levels of the patients and AUC of L_1G and the half-life of L_1G appearance (L_1G-app$t_{1/2}$) in the serum ($P > 0{\cdot}05$).

Twenty-four hour UIE during the L_1 clearance study in the 14 patients studied ranged from 4·7 to 32·7 (14·7 ± 8·4) mg. The amounts of L_1 and L_1G excreted in urine over the same period were 329 ± 326 (62–1191) mg and 1927 ± 423 (1167–2516) mg respectively. Hydrolysis of L_1G in 24 h urine collections resulted in a total urine L_1 of 2257 ± 383 (1635–3000) mg. The total amount of L_1 recovered in the urine over the first 24 h therefore comprised 77·9 ± 13·3 (46·7–100)% of L_1 dose. When the 24 h UIE

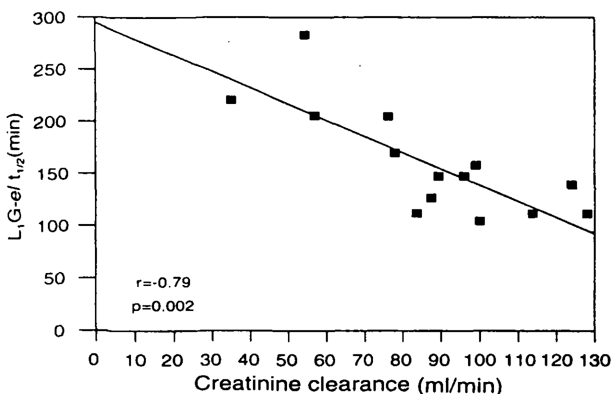

Fig 2. The correlation between creatinine clearance and elimination half-life of L_1G (L_1G-el$t_{1/2}$) in 14 patients with iron overload.

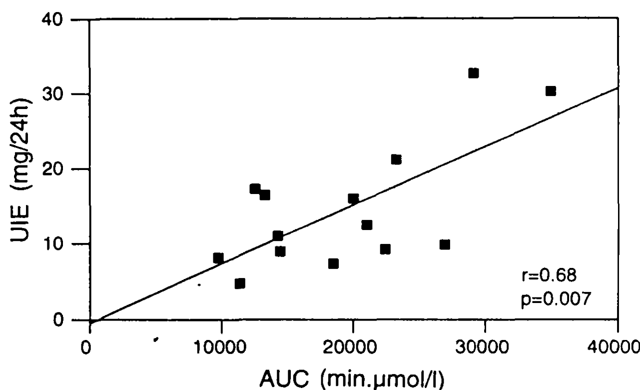

Fig 3. The correlation between urine iron excretion (UIE) and area under the curve of L_1 serum concentrations versus time (AUC) in 14 patients with iron overload.

Table II. Pharmacokinetics of deferiprone (L_1) and its metabolite L_1-glucuronide (L_1G) in 24 patients with iron overload.

Case	L_1-ab$t_{1/2}$ (min)	L_1-el$t_{1/2}$ (min)	L_1-C_{max} (μmol/l)	L_1-T_{max} (min)	L_1-$AUC_{0-\infty}$ (min.μmol/l)	L_1G-app$t_{1/2}$ (min)	L_1G-el$t_{1/2}$ (min)	L_1G-C_{max} (μmol/l)	L_1G-T_{max} (min)	L_1G-$AUC_{0-\infty}$ (min.μmol/l)
AA	51·8	101·1	99	90	20005	55·4	283·1	150	240	79133
AD	41·2	138·5	85	120	24361	53·6	212·1	146	240	66876
AE	26·6	55·5	163	60	11248	30·2	106·4	133	120	28662
AM	5·3	91·8	240	45	34880	51·9	205·1	90	240	40250
BJ	12·5	165·1	83	90	22357	44·4	220·8	432	180	185077
CH	6·3	83·8	189	30	24211	30·2	90·0	113	90	26615
CSo	68·5	82·8	222	120	28518	81·4	153·6	140	240	48981
CSt	18·2	134·9	94	45	20977	–†	–†	–†	–†	–†
DI	15·6	79·0	73	60	9737	ND	ND	ND	ND	ND
EM	–*	80·0	356	20	43968	39·3	99·9	225	120	60305
FM	–*	58·0	241	20	14524	21·3	93·0	173	90	34241
FV	–*	57·5	128	30	21898	23·6	140·6	121	120	34266
GZ	4·5	56·5	104	30	26384	21·7	184·2	119	120	39054
JT	–*	64·7	143	20	12548	36·6	147·5	187	120	63057
LL	25·4	89·1	101	75	14246	45·3	147·6	167	180	52246
MH	24·3	166·5	96	90	29072	29·2	111·4	135	120	33326
MH	–*	52·9	310	20	19616	18·2	86·3	147	90	26784
MS	15·8	82·6	119	60	13272	33·3	111·3	159	120	39392
PD	14·6	95·4	317	30	35259	26·8	205·6	270	120	102691
SA	20·0	91·1	87	75	14450	49·4	104·2	134	180	37356
SB	8·1	94·4	212	30	26889	29·5	169·8	193	120	63196
SG	13·0	59·3	112	60	11364	31·1	139·4	120	120	33629
TR	4·1	126·8	116	45	23205	41·4	126·6	137	180	42107
TT	45·0	78·9	121	120	18465	61·8	111·8	120	180	31739
$X \pm SD$	22·2 ± 17·7	91·1 ± 33·1	158·8 ± 82·9	57·7 ± 33·2	21727 ± 8625	38·9 ± 15·5	147·7 ± 52·0	164·1 ± 72·0	151·4 ± 51·9	53136 ± 35093

$t_{1/2}$ = half-life, ab = absorption, el = elimination, app = appearance, C_{max} = maximum concentration, T_{max} = time to maximum concentration, AUC = area under the serum concentration versus time curve, ND = not done.

* Not enough points to calculate ab$t_{1/2}$.

† Results omitted because of high baseline values.

Table III. Comparison between the pharmacokinetics of L_1 observed in this study with those previously reported in two major studies.

Study	No. of patients	$abt_{1/2}$ (min)	$elt_{1/2}$ (min)	L_1 recovery (%)	L_1 efficiency (%)
Current study	24	$22{\cdot}2 \pm 17{\cdot}7$	$91{\cdot}1 \pm 33{\cdot}1$	$77{\cdot}9 \pm 13{\cdot}3$	$3{\cdot}8 \pm 1{\cdot}9$
Kontoghiorghes *et al* (1990b)	7	$7{\cdot}1 \pm 11{\cdot}3$	$74{\cdot}3 \pm 28{\cdot}7$	$90{\cdot}5 \pm 8{\cdot}2$	$6{\cdot}8 \pm 5{\cdot}6$
Matsui *et al* (1991)	14	NA	$159{\cdot}6 \pm 20{\cdot}5$	NA	NA

$t_{1/2}$ = half-life, ab = absorption, el = elimination, NA = not available.

was divided by the amount of iron the oral dose of L_1 was capable of binding, the L_1 efficiency was found to be $3{\cdot}8 \pm 1{\cdot}9$ ($1{\cdot}4$–$7{\cdot}5$)%.

UIE in the 14 patients was found to correlate significantly with L_1-AUC ($r = 0{\cdot}68$, $P = 0{\cdot}007$, Fig 3), but not with L_1-C_{max} or L_1-$elt_{1/2}$ nor with L_1G-C_{max}, L_1G-$appt_{1/2}$ or L_1G-AUC ($P > 0{\cdot}05$).

Analysis of the different fractions of urine collected in eight patients over a period of 24 h showed that $75{\cdot}4 \pm 20{\cdot}7$% of L_1, $68{\cdot}4 \pm 16{\cdot}5$% of L_1G and $70{\cdot}7 \pm 13{\cdot}0$% of iron excreted in 24 h following L_1 ingestion were excreted in the first 6 h following L_1 administration.

DISCUSSION

The results here confirm that L_1 absorption is rapid in most patients (Figs 1a–c). Some patients, however, showed a delayed absorption despite taking L_1 on an empty stomach (Fig 1d). The mean absorption $t_{1/2}$ observed in this study is higher than that reported by Kontoghiorghes *et al* (1990b) in six iron-overloaded patients and one normal volunteer ($7{\cdot}1 \pm 11{\cdot}3$ min) (Table III). The speed with which L_1 is absorbed suggests that it occurs through the upper part of the gastrointestinal tract, but whether from the stomach or duodenum or both remains to be established.

L_1 elimination occurred mainly via the kidneys with an average of >75% of the drug recovered in the urine in the first 24 h, mainly as L_1G. The mean elimination $t_{1/2}$ in this study is higher than that reported by Kontoghiorghes *et al* (1990b) ($74{\cdot}3 \pm 28{\cdot}7$ min) but lower than the one reported by Matsui *et al* (1991) ($159{\cdot}6 \pm 20{\cdot}5$ min) (Table III). The amount of total L_1 recovered in urine showed that about 20% of the ingested dose remained unaccounted for at 24 h (Table III). This could be due to faecal excretion due to incomplete absorption of the drug or its re-excretion into the gastrointestinal tract as a free form or complexed with iron. Reports on faecal L_1 excretion are contradictory. Kontoghiorghes *et al* (1990a) reported no increase in iron excretion and no evidence of L_1 in the stools of two patients with iron overload given L_1 at an oral dose of 50 mg/kg. On the other hand, Olivieri *et al* (1990) and Collins *et al* (1994) reported an increase in faecal iron excretion in 10 patients with iron overload following the oral administration of L_1 accounting for up to 33% of the total iron excretion. Although in the latter study no attempt was made to measure faecal L_1, their data suggest that L_1 is excreted in stool at least in some patients following L_1 ingestion.

In a few patients studied here, 24 h urine L_1 and L_1G accounted for 100% of the oral dose of L_1, indicating that the main, and possibly only, route of L_1 metabolism in man is glucuronidation. L_1G elimination in the urine was found to be slower than that of L_1 (Fig 1, Table I). This is probably due to the difference in size and physiochemical properties of the two molecules.

The chelation efficiency of L_1 at 24 h in this study ($3{\cdot}8 \pm 1{\cdot}9$%) is lower than that reported by Kontoghiorghes *et al* (1990b) ($6{\cdot}8 \pm 5{\cdot}6$%) (Table III) and higher than reported previously in animal studies. Bergeron *et al* (1992) reported an L_1 chelation efficiency in iron-overloaded monkeys of $2{\cdot}1$%, whereas Venkataram & Rahman (1990) found the chelation efficiency of L_1 in iron-overloaded rats to be $1{\cdot}3$%, which is not significantly different from that found in normal rats ($1{\cdot}2$%) (Bergeron *et al*, 1992).

The lack of significant difference between the pharmacokinetics of L_1 and L_1G in patients who had received L_1 prior to the study and those who had not, implies that L_1 does not induce its own metabolism as has previously been suggested (Matsui *et al*, 1991).

Because L_1G cannot bind iron, it seemed possible that the speed and degree of L_1 glucuronidation would inversely correlate with L_1 efficacy as reflected by UIE. However, no such correlation was found between UIE and C_{max}, $appt_{1/2}$ or AUC of L_1G.

In the present study we found that about 70% of the L_1, L_1G and iron excreted in the first 24 h was excreted in the urine within 6 h of L_1 ingestion. In a smaller group of patients, Kontoghiorghes (1990) reported that 85–90% of L_1 and L_1G were eliminated in the first 6 h of L_1 ingestion.

The $elt_{1/2}$ of L_1 was in the range of 1–3 h, and this suggests that the L_1 dose has to be given every few hours if continuous levels of L_1 in the serum are essential to achieve higher efficacy. However, data from our current clinical trials show no overall difference in UIE whether the total daily dose of L_1 is divided into two or four (Al-Refaie *et al*, 1994).

As L_1 is given at a maximum dose of about 50 mg/kg twice daily, it was necessary to assess whether this dose would lead to accumulation of L_1 or L_1G. The mean AUC of both L_1 and L_1G after the first 12 h of L_1 administration constituted only a small fraction ($1{\cdot}3 \pm 2{\cdot}1$% and $5{\cdot}3 \pm 6{\cdot}0$%, respectively) of the total AUC, suggesting that, in most of the patients, no

significant accumulation of L_1 or L_1G occurs. However, the range of AUC after first 12 h for L_1G was wide (0·5–23·5%), implying that in a minority of patients L_1G accumulation is possible (Figs 1a and 1b). The present study has shown that the elimination of L_1G is influenced by the renal function of the patient as there is a significant correlation between the speed of the elimination of L_1G and the creatinine clearance of the patients. The lack of a similar correlation between L_1-el$t_{1/2}$ and creatinine clearance may be due to the difference in size of the two compounds.

Severe gastrointestinal tract symptoms, especially nausea and anorexia, developed in the patient (CS) who was found to have high baseline levels of L_1G associated with previous L_1 therapy and in whom L_1G reached a very high level after a single dose (Fig 1a). This patient, the oldest studied, was also found to have impaired renal function (Table I) which may explain the accumulation of L_1G. When this patient was kept off the drug for a few days his serum L_1G fell to undetectable levels and his tolerance to L_1 improved only to deteriorate after a few days of L_1 therapy. Whether there is a true association between high L_1G levels and gastrointestinal symptoms remains to be established in other patients. A mild degree of nausea was also observed in a few more patients during long-term L_1 therapy (Al-Refaie *et al*, 1994), but with no correlation to L_1G clearance.

In summary, L_1 is rapidly absorbed and eliminated mainly as L_1G but also as free L_1 and L_1-iron complex in urine. The L_1 efficiency in this study was found to be better than that reported previously in several animal models. The fast absorption and elimination of L_1 suggests that more than one dose of L_1 can be administered daily without significant accumulation of the drug. L_1G may, however, accumulate if high doses of L_1 are given repeatedly to patients with impaired renal function.

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