Biochemical Pharmacology, Vol. 57, pp. 559–566, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(98)00319-0

In Vivo Iron Mobilisation Evaluation of Hydroxypyridinones in ^{59}Fe-Ferritin-Loaded Rat Model

*Zu Dong Liu, Shu Li Lu and Robert C. Hider**
Department of Pharmacy, King's College London, London SW3 6LX, U.K.

ABSTRACT. Although there are a number of well-characterised animal models available for testing and comparing the efficacy of iron chelators, most are expensive to operate and are not capable of providing rapid and reproducible results. The method described herein is based on the labelling of rat liver with ^{59}Fe using rat ^{59}Fe-ferritin. This method produces highly reproducible data of the type necessary for dose–response investigations, comparison of the efficacies of different administration routes, and structure activity studies. BIOCHEM PHARMACOL **57**;5:559–566, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. iron chelators; hydroxypyridinone; ^{59}Fe-ferritin; prodrug

Transfusion-dependent patients such as those suffering from β-thalassaemia develop a fatal secondary haemosiderosis and consequently, a selective iron chelator must be used to relieve such iron overload [1]. Currently, DFO† is the only clinically useful drug which is widely available for this purpose. Unfortunately, patient compliance is poor because of the lack of oral activity and rapid plasma clearance [2, 3]. Consequently, there is an urgent need for an orally active iron-chelating agent.

The preliminary evaluation of biological activity in animal models is an essential step in the development of chelating drugs for clinical use, and a variety of such *in vitro* and *in vivo* screening procedures have been developed. *In vitro* studies based on cell systems provide a rapid screen for both activity and toxicity in which a number of compounds can be compared simultaneously; systems that have been used include both cell lines [4] and primary cell cultures [5]. Despite the advantages of cellular systems for iron chelator screening, *in vivo* animal models are essential in order to establish the disposition and distribution of the chelator, including absorption from the gastrointestinal tract. A number of methods have been developed for *in vivo* screening of iron chelators. In several studies, hypertransfused rats [6–8] or mice [9, 10] have been used. However, the iron overloading of animals by transfusion is technically demanding and time-consuming. For this reason, iron dextran has been introduced for iron loading; iron is initially taken up by the reticuloendothelial system but after equilibration, it is redistributed to parenchymal tissues [11, 12]. Longueville and Crichton [13] have developed a rat model of iron overload which exhibits biochemical, biophysical, and histological similarities to human haemochromatosis [14]. In this model, a ferrocene derivative, which delivers iron predominantly to hepatocytes, has been adopted. In contrast to the iron dextran model, which shows a rapid depletion of iron stores after cessation of iron supplementation, the hepatic iron stores in the ferrocene-loaded rat are relatively stable. Consequently, investigation of both hepatic and tissue iron mobilisation is possible using this model [14, 15].

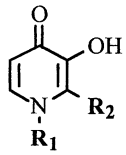
An important consideration in the design of such investigations is whether or not to use a radio-labelled iron probe. In principle, such technology circumvents many of the drawbacks associated with faecal contamination and intraluminal chelation observed with cold iron measurements and greatly simplifies the monitoring of iron excretion. A further advantage is that specific iron pools can be targeted with radio-labelled iron, allowing a more precise study of the site of action of different compounds. A range of such investigations conducted by Hershko and co-workers has demonstrated that ^{59}Fe-labelled ferritin and haemoglobin–haptoglobin complexes are predominantly taken up by hepatocytes in rats, whereas ^{59}Fe-labelled heat-damaged red blood cells can be used to selectively label reticuloendothelial cells [16]. These various modes of delivery of radio-labelled iron have been adopted in order to investigate differences in sources and routes of excretion of chelatable iron in the presence of various chelators [16–18]. Porter and co-workers selected a method based on mice overloaded with iron dextran and then subsequently labelled with ^{59}Fe-lactoferrin, allowing time for equilibration before and after administration of radio-labelled iron [11, 12]. Although time-consuming, this system is simple and reproducible. With the use of iron-free metabolic cages, urine and faeces are collected separately and both cold and

* Corresponding author: Prof. Robert C Hider, Department of Pharmacy, King's College London, Manresa Road, London SW3 6LX, U.K. Tel. 0171 333 4646; FAX 0171 937 5690; E-mail: robert.hider@kcl.ac.uk

† *Abbreviations:* DFO, desferrioxamine; and HPO, hydroxypyridinone.

Received 30 March 1998; accepted 2 August 1998.

TABLE 1. Chemical structure of selected HPO ligands



R_1	R_2	Molecular weight	Distribution coefficient at pH 7.4
CH_3	CH_3	139	0.17
CH_2CH_3	CH_2CH_3	167	1.79
CH_2CH_2OH	CH_2CH_3	183	0.22
$(CH_2)_3OH$	CH_3	183	0.13
CH_2CH_2COOH	CH_3	197	<0.001
$CH_2CH_2OCOC(CH_3)_3$	CH_2CH_3	267	14.5
$(CH_2)_3OCOC_6H_5$	CH_3	287	17.6

radiolabelled iron measured [12]. This system can also be extended to measure dose–response curves and to determine the acute toxicity by measuring the LD_{50} in the same animals [19].

Initially, animal testing should be carried out using a simple reproducible system in which several compounds can be compared at the same time. Although it has been generally assumed that iron-overloaded animals are essential in order to produce a model similar to that of iron overload in man, assessment of test compounds is time consuming, and even with hypertransfusion there remain important differences, for instance the much greater proportion of iron mobilised by DFO in the stool in rodents (80–90%) [6] compared with iron-overloaded man (30–50%) [20]. Pippard and co-workers have developed a rapid assay for evaluation of iron-chelating agents in non-iron-overloaded rats using ^{59}Fe-ferritin to label hepatic parenchymal cells, followed by a challenge with test chelator 2 hrs later, at a time when iron released by lysosomal degradation of ferritin is maximally available [21]. The results suggest that ^{59}Fe is chelated while in transit within the hepatocyte, but that it becomes relatively unavailable subsequent to storage in endogenous ferritin. The "time window" of maximal availability for chelation of iron was shown to occur between the second and sixth hour after ^{59}Fe-ferritin injection. With the chelators tested, almost all the ^{59}Fe was excreted via the bile. In this study, it was concluded that animals with normal stores can be used for a rapid bioassay of iron chelators [21].

Currently, 3-hydroxypyridin-4-ones (HPO) are one of the main candidates for the development of an orally active iron chelator [22]. In order to identify promising compounds, a rapid assay for *in vivo* screening of iron chelators had to be adopted. Since the liver is the major iron storage organ under iron-overload conditions, Pippard's method in which ^{59}Fe-ferritin is used to label the liver iron pool appeared to be ideal. In this work, a modified ^{59}Fe-ferritin/rat model based on the original studies reported by Pippard *et al.* [21] has been developed for a rapid evaluation of iron-chelating agents. The influence of several key parameters has been systematically investigated, and the reliability and reproducibility of this method has been verified with various ligands.

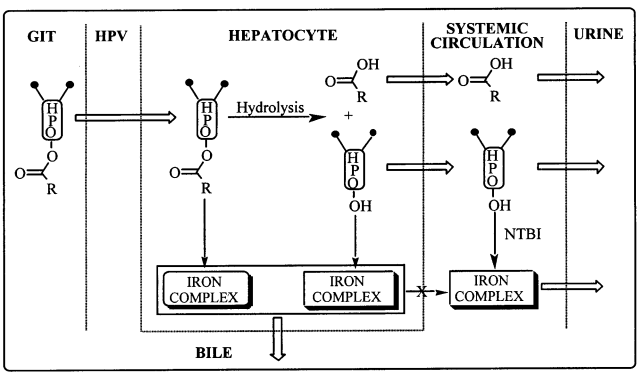
MATERIALS AND METHODS

Iron Chelators

The 3-hydroxypyridin-4-ones, CP20, CP94, CP102, CP41 and CP38 (Table 1), were synthesised according to published procedures [23]. Two ester prodrugs, namely CP117 and CP283 (Table 1), were prepared by an acid esterification method using diethyl azodicarboxylate and triphenylphosphine as reported previously [24]. These hydrophobic ester derivatives have been specifically designed to selectively deliver the drug to the target organ—the liver. Such ester prodrugs, by virtue of their lipophilicity, are expected to be rapidly absorbed from the gastrointestinal tract and then efficiently extracted by the liver during "first pass." In the hepatocyte, the ester link is cleaved to generate a much more hydrophilic chelator (Scheme 1). Such a strategy is predicted to be able to improve chelation efficacy and hence to minimise drug-induced toxicity. DFO was provided in 500 mg ampoules as a lyophilised powder (CIBA Pharmaceuticals).

Preparation of ^{59}Fe-Ferritin

Rat liver ferritin was labelled with ^{59}Fe *in vivo* based on the method described by Pippard *et al.* [21], and isolated by a method based on that originally reported by Bjorklid and



SCHEME 1. Schematic representation of the use of a 1-hydroxyalkyl ester of an HPO to enhance both the absorption from the gastrointestinal tract and the hepatic extraction of the prodrug. Subsequent intracellular hydrolysis occurs, yielding a hydrophilic chelator.

Helgeland [25]. During the preparation of ^{59}Fe-ferritin, intraperitoneal injection of iron dextran and intravenous injection of red cells were undertaken to saturate transferrin in the plasma. This ensured that the injected ^{59}Fe(III) was predominantly taken up by the liver, leading to a high specific radioactivity preparation of ^{59}Fe-ferritin.

Animal Preparation

Male rats of approximately 350–400 g were given a single intraperitoneal injection of 15 mg of iron dextran. After one week, the jugular vein was cannulated and the rats were left to recover for a 24-hr period. The following day, each rat was administered with 2 mL of packed cells/100 g. This was followed 24 hr later by intravenous injection of ferric chloride containing 200 μCi of ^{59}Fe (Amersham Life Science) from the previously implanted jugular vein cannula. The ferric chloride was diluted using 0.5 mL pH 2.5 citrate (50 mM). Five rats were used in each experiment and the total injected radioactivity was 1 mCi. The animals were killed by cervical dislocation 24 hr after injection of radiolabel and the livers were removed for ferritin isolation.

Purification of ^{59}Fe-Ferritin

Rat livers were homogenised in 4 vol. (w/v) of 0.25 M acetate buffer (pH 4.8) in a homogeniser. The homogenate was then heated for 10 min with stirring in a water bath at 75°. After cooling to 4°, the coagulated proteins were removed by centrifugation at 2500 *g* for 15 min. The "heat supernatant" which contains tissue ferritin was precipitated by half saturation with ammonium sulfate and left to stand for 30 min in an ice-bath. The precipitate was isolated by centrifugation at 3500 *g* for 15 min at 4° and redissolved in 0.25 M acetate buffer (pH 4.8). Undissolved precipitate was removed by passing through a PD-10 column (Sephadex® G-25M, Pharmacia Biotech) and the collected liquid was supercentrifugated at 4° for 2 hr at 100,000 *g*. The final precipitate was dissolved in 0.05 M phosphate buffer (pH 7.4) and stored at 4°. The purity of ^{59}Fe-ferritin was compared with the standard ferritin using electrophoresis (Fig. 1). The radioactivity of ^{59}Fe-ferritin was measured using a gamma counter (1282 Compugamma CS, LKB Wallac). The ferritin iron concentration was determined using a colorimetric method [26]. The total radioactivity and iron concentration of four different batches of ^{59}Fe-labelled ferritin are given in Table 2.

Chemical Iron Determination Using a Colorimetric Method

One hundred μL of HCl (10M) was dispensed into two small plastic vials, one of which contained 50 μL of ^{59}Fe-ferritin solution; the other sample was treated as blank. The samples were incubated at 37° overnight in order to hydrolyse ferritin and release iron. The volume of each sample made up to 10 mL with deionised water from which 1.7 mL of solution was transferred to another vial. To each sample, 300 μL of 800 mM nitrilotriacetic acid was added and the sample allowed to stand for 30 min at room temperature. The sample was then ultrafiltrated by using a micro-filter (Centricon-30) and centrifuged at 4000 *g* for 60 min at 25°. The filtrate (1 mL) was pipetted into a container to which 250 μL of 120 mM thioglycollic acid and 250 μL of 60 mM bathophenanthroline disulfonic acid, disodium salt was added. The absorbance of each sample was monitored at 537 nm after 30 min. A six-point standard calibration curve was constructed in the same manner, using iron concentrations in the range of 2.5–50 μM in 0.1 M HCl in order to quantify the iron content in ferritin.

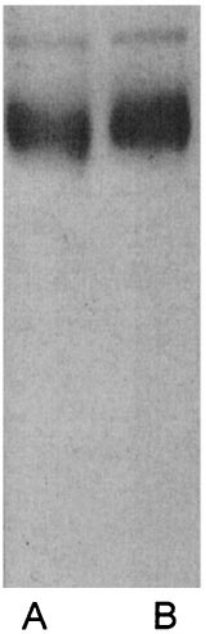


FIG. 1. Electrophoretogram of ferritin: (A) isolated ^{59}Fe-labelled rat ferritin; (B) standard rat ferritin purchased from Sigma Chemical Co.

Animal Model Studies

The ^{59}Fe-ferritin was injected into a tail vein under anaesthesia using a mixture of fentanyl and fluanisone (Hypnorm®) and midazolam (Hypnovel®) at doses of 2.5, 0.08, and 5 mg/kg, respectively. At the end of each experiment, the rats were killed by cervical dislocation, and the organ distribution of ^{59}Fe was measured using gamma counting (1282 Compugamma CS, LKB Wallac). Different doses of ferritin iron (5, 10, 20, and 40 μg) and different time periods (20, 40, 60, 90, 120, and 360 min) post ^{59}Fe-ferritin injection were investigated in order to establish the ^{59}Fe-ferritin clearance profile in rats. Different separation periods between the administration of ^{59}Fe-ferritin and the chelator, namely 1 and 12 hr, were also investigated.

Bile Iron Excretion Assay Using Normal Rats

Normal fasted Wistar rats weighing approximately 250–300 g were anaesthetised and the common bile duct cannulated

TABLE 2. Radioactivity and iron concentration of rat ^{59}Fe-ferritin

Batch	^{59}Iron (III) chloride			^{59}Fe-ferritin		
	Specific activity (mCi/mg)	Total activity (mCi)	Iron conc. (μg/mL)	Total activity (mCi)	Specific activity (μCi/μg)	Iron conc. (μg/mL)
1	52.09	1.0	148	0.101	0.071	580
2	11.49	1.0	87	0.107	0.084	254
3	3.13	1.0	320	0.105	0.082	512
4	8.55	1.0	117	0.099	0.065	256

with fine bore polythene tubing (Portex Ltd.). Chelators were administered orally by gavage. Control rats were administered with an equivalent volume of water. Bile samples were collected over a 12-hr period while the rats were maintained under light anaesthesia using a mixture of fentanyl and fluanisone (Hypnorm®) at doses of 2.5 and 0.08 mg/kg. Iron concentration in the bile was measured using the colorimetric method described above.

General Test Procedure of Iron Chelators on ^{59}Fe-Ferritin/Rat Model

Hepatocytes of normal fasted rats were labelled with ^{59}Fe by administration of ^{59}Fe-ferritin from the tail vein. One hour later, each rat was orally administered with a chelator. Control rats were administered with an equivalent volume of water. The rats were placed in individual metabolic cages and urine and faeces collected. Rats were allowed free access to food one hr after oral administration of chelator. There was no restriction of water throughout the study period. The investigation was terminated 24 hr after the ^{59}Fe-ferritin administration; the rats were killed by cervical dislocation, the liver and gastrointestinal tract (including its contents and the faeces) being removed for gamma counting. The calculations for "Iron mobilisation," "Efficacy" and "Total recovery" are represented by equations 1, 2, and 3.

$$\text{Iron Mobilisation (\%)} = \frac{^{59}\text{Fe} - \text{Activity}_{(\text{Gut \& Faeces})}}{^{59}\text{Fe} - \text{Activity}_{(\text{Gut \& Faeces})} + {}^{59}\text{Fe} - \text{Activity}_{(\text{Liver})}} \times 100\% \quad (1)$$

$$\text{Efficacy (\%)} = \text{Iron Mobilisation (\%)} - \text{Control (\%)} \quad (2)$$

$$\text{Total Recovery (\%)} = \frac{^{59}\text{Fe} - \text{Activity}_{(\text{Gut \& Faeces})} + {}^{59}\text{Fe} - \text{Activity}_{(\text{Liver})}}{^{59}\text{Fe} - \text{Activity}_{(\text{Dose})}} \times 100\% \quad (3)$$

RESULTS

Selection of Conditions for In Vivo *Assay*

In order to achieve optimal ^{59}Fe-ferritin labelling of the hepatocyte, different doses of ferritin were investigated. One hour after i.v. administration of ^{59}Fe-ferritin, the rats were killed, and the radioactivity in the liver, heart, blood, and gut was measured (Table 3). With doses of ferritin iron of 5, 10, 20, and 40 μg, the amounts remaining in circulation at 1 hr were 0, 3.7, 37, and 50.8% respectively.

^{59}Fe-ferritin distribution studies were conducted using a 10 μg ferritin iron dose. The study was terminated at different time periods (20, 40, 60, 90, 120, and 360 min) after the injection of ^{59}Fe-ferritin and the distribution of injected ^{59}Fe was monitored (Table 4). The extraction of ^{59}Fe-ferritin by the liver when administered at a 10 μg ferritin iron dose is summarised in Fig. 2. The results demonstrate that injected ferritin at this dose is rapidly cleared by the liver and that after 60 min the majority of the label was located in this organ (87 ± 1.8%).

In order to select a suitable period between administration of ^{59}Fe-ferritin and chelator, two different time intervals between administration of ^{59}Fe-ferritin and chelator were compared, namely 1 and 12 hr. Two ligands, CP20 and CP94, were adopted for this study. Clearly, a separation

TABLE 3. ^{59}Fe-ferritin distribution studies with different doses of ferritin iron

Dose of ferritin iron (μg)	Percent of injected ^{59}Fe				
	Total recovery	Liver	Blood	Gut and contents	Heart
5 μg	95.6 ± 2.8	95.4 ± 3	0	0.2 ± 0.19	0
10 μg	91.4 ± 1.6	87 ± 1.8	3.7 ± 0.77	0.6 ± 0.16	0.04 ± 0.0
20 μg	96 ± 4.2	56.8 ± 7.5	37 ± 9.1	1.85 ± 0.4	0.36 ± 0.1
40 μg	98.1 ± 2	44.7 ± 2.5	50.8 ± 4.1	2.0 ± 0.2	0.6 ± 0.11

The experiment was terminated 1 hr after the infection of ^{59}Fe-ferritin (mean ± SD, N = 4).

TABLE 4. ^{59}Fe-ferritin distribution studies with time

Time (min)	Total recovery	Percent of injected ^{59}Fe Liver	Blood	Gut and contents	Heart
20 min	98 ± 1.5	48 ± 6.2	48.9 ± 5.2	0.31 ± 0.1	0.96 ± 0.3
40 min	97 ± 3.2	72.3 ± 1.1	23.7 ± 3.7	0.51 ± 0.1	0.47 ± 0.1
60 min	91.4 ± 1.6	87 ± 1.8	3.7 ± 0.77	0.6 ± 0.16	0.04 ± 0.0
90 min	93.4 ± 2	89.7 ± 3.5	2.46 ± 1.3	1.1 ± 0.31	0.14 ± 0.1
120 min	93 ± 1.48	90.2 ± 1.9	1.39 ± 0.3	1.35 ± 0.4	0.1 ± 0.03
360 min	91.3 ± 71.0	89.1 ± 1.1	0.51 ± 0.2	1.67 ± 0.4	0.01 ± 0.0

The injected ferritin iron was 10 μg (mean ± SD, N = 4).

of 1 hr between ferritin and chelator injections gave higher gut content and faeces counts for both chelators (Fig. 3). The increase in gut content and faeces ^{59}Fe levels, which resulted from biliary excretion, was reflected by a commensurate decrease in liver ^{59}Fe levels.

Different ligands will have different pharmacokinetic profiles, which in turn leads to different iron excretion time profiles. In order to select an optimum collecting period, bile cannulation studies of normal rats were undertaken with several HPOs, namely CP102, CP41, CP117 and CP283. The iron concentration in bile was measured using the bathophenanthroline disulfonic acid, disodium salt colorimetric method. The peak of iron excretion associated with both CP102 and CP41 was achieved within a 4-hr period (Fig. 4, a and b). In contrast, with the two prodrugs CP117 & CP283, peak iron excretion was found to be more delayed, 9 and 6 hr respectively (Fig. 4, c and d).

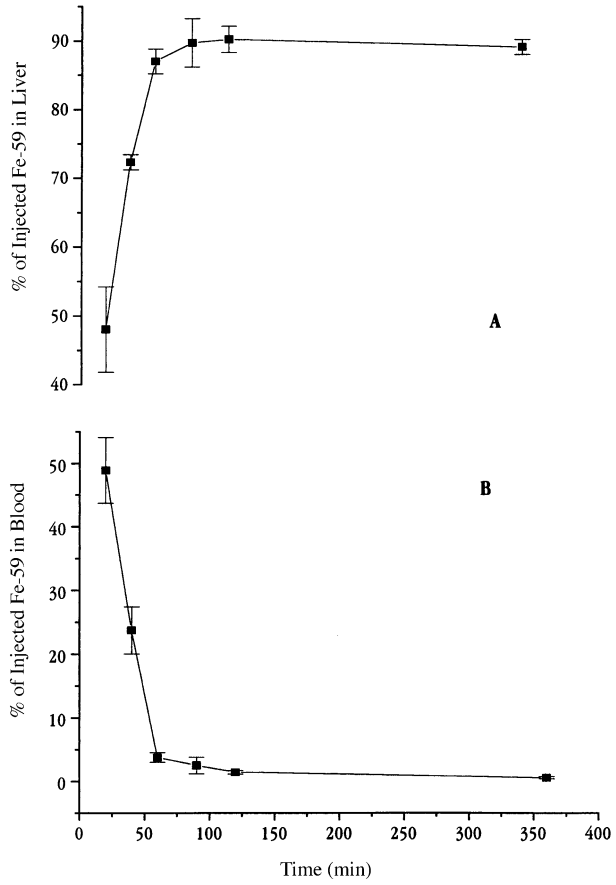


FIG. 2. The distribution of injected ^{59}Fe-ferritin (10 μg) with time: (A) percent of injected ^{59}Fe in liver; (B) percent of injected ^{59}Fe in blood. Values are expressed as means ± SD (N = 4).

Iron Mobilisation Studies with Iron(III) Chelators

The iron mobilisation efficacies of DFO and several HPOs were compared using the ^{59}Fe-ferritin-loaded rat model outlined above. HPO chelator solutions were prepared in water to give a final dose volume of 1 mL. The DFO dose was 40 mg/kg, which corresponds to the same iron binding capacity as the 450 μmol/kg dose adopted for HPOs. Solutions of DFO were prepared in 0.9% sodium chloride to give a final volume of 0.5 mL; i.p. injection of DFO was given 2 hr after ^{59}Fe-ferritin administration. With all studies, ^{59}Fe was not detected in the urine and less than 5% of injected ^{59}Fe dose was detected in tail. The results are presented in Table 5.

DISCUSSION

In order to develop chelating drugs for clinical use, a rapid animal assay was adopted to evaluate novel iron-chelating agents such as hydroxypyridinones. In this model, hepatic cells of non-iron-overloaded rats were labelled using ^{59}Fe-ferritin. Rat ^{59}Fe-ferritin is cleared almost exclusively by rat hepatocytes [16] and in the present studies, the extraction of ^{59}Fe-ferritin by the liver was found to be dose-dependent (Table 3). Further detailed ^{59}Fe-ferritin distribution studies demonstrated that injected ferritin is rapidly cleared by the liver (Fig. 2). With 10 μg doses of ferritin iron, the majority of the label was located in the liver (87 ± 1.8%) at 1 hr after the ^{59}Fe-ferritin injection. Thus, in agreement with Pippard *et al.* [21], the iron pool of the liver is labelled efficiently using this method. In subsequent studies, a dose of 10 μg of ferritin iron was used, since it is cleared rapidly and provides sufficient ^{59}Fe to monitor chelator-induced iron excretion. Considering the half-life (44 days) associated with ^{59}Fe, the ^{59}Fe-labelled ferritin can be used for periods up to six weeks.

Pippard and co-workers investigated the time relation-

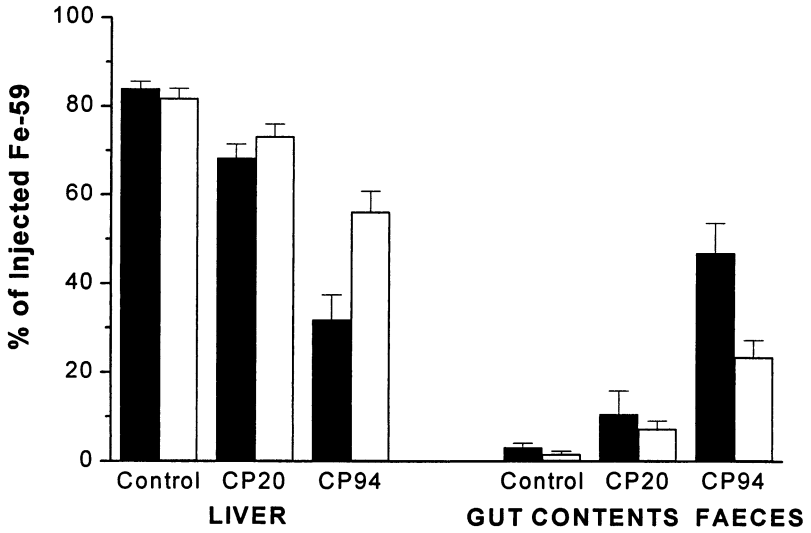


FIG. 3. Timing of the availability of ^{59}Fe injected as ^{59}Fe-ferritin to orally administered chelators: (■) 1 hr between administration of ^{59}Fe-ferritin and chelator; (□) 12 hr between administration of ^{59}Fe-ferritin and chelator. The chelator dose was 450 μmol/kg and the experiment was terminated 24 hr after the administration of chelators. Values are expressed as means ± SD (N = 5).

ship between ^{59}Fe-ferritin and chelator administration. The results suggested that a separation of 1–2 hr between ferritin and chelator injections provided maximal availability of the ^{59}Fe label to the administered chelators [21]. In that assay, test chelators such as DFO were given by intravenous (i.v.) or by intramuscular (i.m.) injection 2 hr after the administration of ^{59}Fe-ferritin. Previous pharmacokinetic studies conducted with orally administered HPOs in the rat indicated that the maximum plasma concentration of the ligand would be achieved at 0.5–1.5 hr after oral adminis-

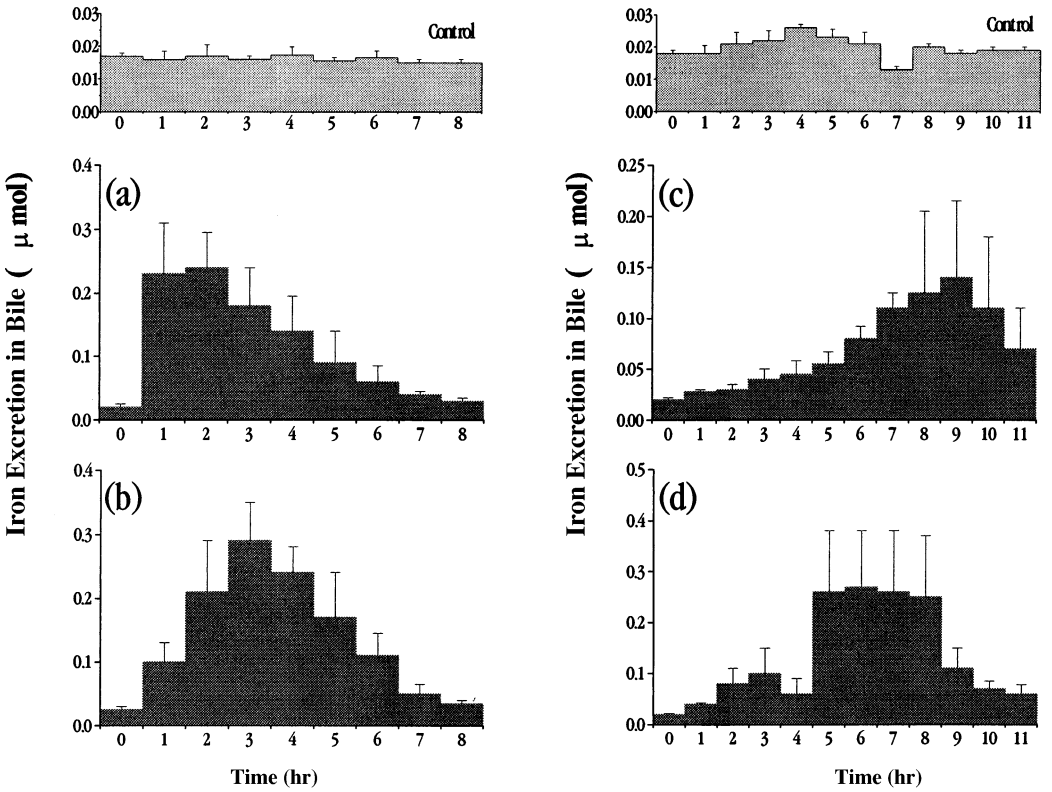


FIG. 4. Iron excretion studies using the bile duct cannulation model in normal rats: (a) CP102 (N = 3); (b) CP41 (N = 3); (c) CP117 (N = 3); (d) CP283 (N = 4); control (N = 3). All chelators were given orally; the chelator doses were 450 μmol/kg. The iron concentration in bile was measured using the bathophenanthroline sulfonic acid, disodium salt colorimetric method. Values are expressed as means ± SD (N = 4).

TABLE 5. Iron mobilisation efficacy studies of DFO and selected HPOs in the ^{59}Fe-ferritin-loaded rat model (mean ± SD)

Chelator	Dose	Iron mobilisation (%)	Efficacy (%)	Total recovery (%)	
Control	—	3.87 ± 1.0	—	78.7 ± 6.5	n = 5
DFO (oral)	40 mg/kg	4.23 ± 1.67	0.36	86.7 ± 1.5	n = 5
DFO (i.p.)	40 mg/kg	28.8 ± 3.5	24.9	85.1 ± 3.5	n = 6
CP20	450 μmol/kg	13.4 ± 5.2	9.5	78.8 ± 4.5	n = 8
CP94	450 μmol/kg	59.7 ± 10.9	55.8	78.9 ± 5.0	n = 8
CP102	450 μmol/kg	16.8 ± 2.7	12.9	83.8 ± 2.9	n = 5
CP41	450 μmol/kg	29.9 ± 3.8	26.0	77.2 ± 1.8	n = 8
CP38	450 μmol/kg	17.1 ± 1.7	13.2	76.9 ± 1.7	n = 5
CP117	450 μmol/kg	23.0 ± 7.3	19.1	80.8 ± 3.7	n = 5
CP283	450 μmol/kg	36.4 ± 6.5	32.5	81.4 ± 5.5	n = 8

tration [27]. Based on this information, test HPO compounds were administered 1 hr after the administration of ^{59}Fe-ferritin. The study reported by Pippard *et al.* [21] was terminated 4 hr after the chelator injection, by which time the chelating activity of the test ligands was estimated to be complete. However, with HPO chelators, especially the prodrugs, excretion of iron occurs over a longer time period. The bile iron excretion assay using normal rats confirmed that the peak of iron excretion varies with different ligands (Fig. 4). Therefore, collections of excreted iron were made over a 24-hr period.

In order to test this system, the iron mobilisation efficacy of DFO and several HPOs has been compared on this model. Clearly, the total recovery was constant (Table 5), indicating the reliability of the system. DFO is active in this system when given by i.p. injection, whereas orally applied DFO is inactive. In this test system, CP20 and CP102 were found to be less effective than DFO, whereas CP41 was found to possess a similar efficacy to that of DFO. In contrast, CP94 was much more effective. CP117, which is the pivaloyl ester prodrug of CP102, was found to increase the efficacy of CP102 from 16.8 to 23%. A moderately enhanced iron excretion was also produced with the benzoyl ester prodrug; for instance, CP283 provided 36.4% iron excretion compared with 29.9% for CP41. The mean values together with the SD indicate that the errors associated with this assay are acceptable for comparative purposes.

In summary, a rapid assay for *in vivo* screening of orally active iron chelators has been developed. The influence of several key parameters has been systematically investigated, and both the reliability and reproducibility of this method have been verified with various chelators. This method facilitates the rapid investigation of structure–activity relationships with such compounds.

The research described in this manuscript was supported by Apotex Research Inc. Canada. Z. D. Liu would like to thank the KC Wong Education Foundation (Hong Kong) and the Committee of Vice-Chancellors and Principals of the Universities of the United Kingdom for the joint scholarships which enabled him to carry out the studies. The authors also would like to thank Professor M. J. Pippard, University of Dundee for critically helpful advice.

References

1. Pippard MJ, Callender ST, Letsky EA and Weatherall DJ, Prevention of iron loading in transfusion-dependent thalassaemia. *Lancet* **i:** 1178–1180, 1978.
2. Summers MR, Jacobs A, Tudway D, Perera P and Ricketts C, Studies in desferrioxamine and ferrioxamine metabolism in normal and iron-loaded subjects. *Br J Haematol* **42:** 547–555, 1979.
3. Pippard MJ, Callender ST and Weatherall DJ, Intensive iron-chelation therapy with desferrioxamine in iron-loading anaemias. *Clin Sci Mol Med* **54:** 99–106, 1978.
4. White GP, Jacobs A, Grady RW and Cerami A, The use of Chang cells cultured *in vitro* to evaluate potential iron chelating drugs. *Br J Haematol* **33:** 487–494, 1976.
5. Porter JB, Gyparaki M and Burke LC, Iron mobilisation from hepatocyte monolayer cultures by chelators: the importance of membrane permeability and the iron binding constant. *Blood* **72:** 1497–1503, 1988.
6. Graziano JH, Grady RW and Cerami A, The identification of 2,3-hydroxybenzoic acid as a potentially useful iron-chelating drug. *J Pharmacol Exp Ther* **190:** 570–575, 1974.
7. Grady RW, Graziano JH, Akers HA and Cerami A, The development of new iron chelating drugs. *J Pharmacol Exp Ther* **196:** 478–485, 1976.
8. Grady RW, Graziano JH, White GP, Jacobs A and Cerami A, The development of new iron chelating drugs. *J Pharmacol Exp Ther* **205:** 757–765, 1978.
9. Gralla EJ, Efforts to develop a bioassay system for detecting compounds which actively deplete iron stores. In: *Proceedings of the Symposium on the Development of Iron Chelators for Clinical Use* (Eds. Anderson WF and Hille M), pp. 229–245. DHEW Publication, Bethesda, MD, 1975.
10. Pitt CG, Gupta G, Estes NE, Rosenkrantz H, Metterville JJ, Crumbliss AL, Palmer AR, Nordquest KW, Sprinkle Hardy KA, Whitcomb DR, Byers BR, Arceneaux JEL, Gaines CG and Sciortino CV, Selection and evaluation of new chelating agents for the treatment of iron overload. *J Pharmacol Exp Ther* **208:** 12–18, 1979.
11. Gyparaki M, Hider RC, Huehns ER and Porter JB, Hydroxypyridinone iron chelators; *in vitro* and *in vivo* evaluation. In: *Thalassaemia Today, the Mediterranean Experience* (Eds. Sirshia G and Zanella A), pp. 521–526. Maggiore Policlinico Di Milano, Milan, 1987.
12. Gyparaki M, Porter JB, Burke LC, Huehns ER and Hider RC, *In vivo* evaluation of hydroxypyridinone iron chelators in a mouse model. *Acta Haematol* **78:** 217–221, 1987.
13. Longueville A and Crichton RR, An animal model of iron overload and its application to study hepatic ferritin iron

mobilisation by chelators. *Biochem Pharmacol* **35:** 3669–3678, 1986.
14. Ward RJ, Florence AL, Baldwin D, Abiaka C, Roland F, Ramsey MH, Dickson DPE, Peters TJ and Crichton RR, Biochemical and biophysical investigations of the ferrocene-loaded rat. An animal model of primary haemochromatosis. *Eur J Biochem* **202:** 405–410, 1991.
15. Florence A, Ward RJ, Peters TJ and Crichton RR, Studies of *in vivo* iron mobilization by chelators in the ferrocene-loaded rat. *Biochem Pharmacol* **44:** 1023–1027, 1992.
16. Hershko C, Cook JD and Finch CA, Storage iron kinetics III. Study of desferrioxamine action by selective radioiron labels of RE and parenchymal cell. *J Lab Clin Med* **81:** 876–886, 1973.
17. Hershko C, A study of the chelating agent diethylenetriaminepentacetic acid using selective radioiron probes of reticuloendothelial and parenchymal iron stores. *J Lab Clin Med* **85:** 913–921, 1975.
18. Hershko C, Determinants of faecal and urinary iron excretion in rats. *Blood* **51:** 415–423, 1978.
19. Huehns ER, Porter JB and Hider RC, Selection of hydroxypyridin-4-ones for the treatment of iron overload using *in vitro* and *in vivo* models. *Haematology* **12:** 593–600, 1988.
20. Waxman HS and Brown EB, Clinical usefulness of iron chelating agents. *Prog Hematol* **6:** 338–373, 1969.
21. Pippard MJ, Johnson DK and Finch CA, A rapid assay for evaluation of iron chelating agents in rats. *Blood* **58:** 685–692, 1981.
22. Tilbrook GS and Hider RC, Iron chelators for clinical use. In: *Metal Ions in Biological Systems. Vol. 35. Iron Transport and Storage in Microorganisms, Plants, and Animals* (Eds. Sigel A and Sigel H), pp. 691–730. Marcel Dekker, New York, 1998.
23. Dobbin PS, Hider RC, Hall AD, Taylor PD, Sarpong P, Porter JB, Xiao G and van der Helm D, Synthesis, physicochemical properties, and biological evaluation of *N*-substituted 2-alkyl-3-hydroxy-4(1*H*)-pyridinones: Orally active iron chelators with clinical potential. *J Med Chem* **36:** 2448–2458, 1993.
24. Rai BL, Liu ZD and Hider RC, Synthesis, physicochemical properties, and biological evaluation of hydrophobic esters of hydroxypyridin-4-ones: Design of orally active chelators with clinical potential. *Eur J Med Chem* (in press, 1999).
25. Bjorklid E and Helgeland L, Sex difference in the ferritin content of rat liver. *Biochim Biophys Acta* **221:** 583–592, 1970.
26. Zhang D, Okada S, Kawabata T and Yasada T, An improved simple colorimetric method for quantitation of non-transferrin-bound iron in serum. *Biochem Mol Biol Int* **35**(3): 635–641, 1995.
27. Choudhury R, *Metabolism and Pharmacokinetics of 1-Hydroxyalkyl-3-hydroxypyridin-4-one Chelating Agents*. Ph.D. Thesis. King's College, London, 1995.