Kinetics of Removal and Reappearance of Non–Transferrin-Bound Plasma Iron With Deferoxamine Therapy

By J.B. Porter, R.D. Abeysinghe, L. Marshall, R.C. Hider, and S. Singh

The rapidity and duration of the response of non–transferrin-bound iron (NTBPI) to chelation therapy are largely unknown and have important implications for the design of optimal chelation regimens. Methodology was developed to measure simultaneously NTBPI, deferoxamine (DFO), and its major metabolite. NTBPI was present in all but 2 of 28 thalassaemia major (TM) patients who had received conventional subcutaneous DFO the previous night, suggesting a short duration of NTBPI clearance by DFO. The detailed kinetics of NTBPI were therefore studied in response to intravenous DFO at 50 mg/kg/27 h for 48 hours and compared in 17 regularly transfused TM and 8 untransfused thalassaemia intermedia (TI) patients to determine the influence of hypertransfusion and iron overload on NTBPI response. Before DFO infusion, NTBPI was present in all patients and was significantly higher in TI (4.52 ± 0.53 μmol/L) than TM (2.92 ± 0.03 μmol/L; $P = .03$). NTBPI values in TM correlated with transferrin saturation ($r = .6$, $P = .03$) but not with serum ferritin. Removal of NTBPI by intravenous DFO is in a biphasic manner. The initial rapid rate constant (α) was similar in TI (1.5 hour^{-1}) and TM (1.6 hour^{-1}), but the subsequent β phase was slower (0.04 hour^{-1}) in TI when compared with TM (0.4 hour^{-1}, $P = .002$). Detectable NTBPI persisted during the β phase, particularly in TI, despite an excess of plasma DFO also being present (steady state 8 μmol/L). On cessation of DFO infusion, NTBPI reappearance was rapid; the kinetics also being biphasic. The rapid initial rate constant ($\alpha = 2.5$ hour^{-1}) lasted less than 30 minutes and was approximately equal to the summation of the initial rate constant for removal of DFO (1.8 hour^{-1}) and its major metabolite (0.6 hour^{-1}). This was followed by a slower return to pretreatment levels, usually between 6 and 12 hours, which was faster in TI than in TM. This marked NTBPI lability supports the use of continuous rather than intermittent DFO in high risk patients.

THE IMPORTANCE OF non–transferrin-bound iron (NTBPI) in iron overload was first recognized by Hershko and Rachmilewitz[1] and Hershko et al.[2] Despite initial skepticism, subsequent studies confirmed the existence of NTBPI by a variety of methods[3-7] as well as demonstrating that NTBPI can generate harmful hydroxy radicals and promote lipid peroxidation.[5] NTBPI has not yet become a routine measurement in the treatment of iron overload, partly because of the technical difficulty in its reliable measurement. There also seems to be relatively weak correlations with other markers of iron overload,[3,8] and it is unclear how best to use NTBPI measurement in monitoring chelation treatment. Very little is known about the kinetics of NTBPI, particularly in response to chelation therapy, partly because it has previously been difficult to differentiate NTBPI from low molecular complexes of iron chelators such as ferioxamine (FO). Without knowledge of how rapidly NTBPI responds to chelation treatment and how quickly such a response is reversed on cessation of treatment, it is not clear how to use NTBPI most effectively as a marker of iron overload and chelation efficacy or indeed of how to plan chelation treatment so as to minimize tissue damage from this form of toxic iron.

We previously described methodology for the direct quantification of NTBPI in patients undergoing chelation therapy with deferoxamine (DFO), using nitrilotriacetic acid (NTA), ultrafiltration, and UV detection with high-performance liquid chromatography (HPLC).[7] We also recently published methodology to measure both DFO and its metabolites in such patients.[9-11] In this study, we therefore sought to examine the kinetics of removal of NTBPI by DFO in iron-overloaded patients. We aimed to establish how recent chelation treatment might influence NTBPI before DFO infusion as well as the response to DFO therapy once started. We also sought to establish the duration of the response of NTBPI to DFO therapy and how this might be influenced by the degree of iron overload or by the degree of erythropoiesis. After preliminary pilot studies, patients with iron overload were admitted to hospital for 48 hours of continuous intravenous DFO at 50 mg/kg/24 h. The rate at which NTBPI was removed by DFO and returned after DFO infusion was then compared in regularly chelated patients with thalassaemia major (TM) and relatively untreated patients with thalassaemia intermedia (TI).

MATERIALS AND METHODS

Patient Selection

All thalassaemia patients were attending the thalassaemia clinic at University College Hospital, London. Local ethical committee approval was granted for the studies. TM patients were receiving regular transfusion every 3 to 4 weeks of two to four units of blood to keep the pretransfusion hemoglobin (Hb) above 10 g/dL and were receiving DFO by subcutaneous (SC) infusion as their regular chelation therapy four to seven nights a week. (TI) patients selected for the study were not receiving regular blood transfusion or regular iron chelation therapy but had significant anemia (Table 1).

Preliminary Study in Outpatients

Serum samples were taken from 11 healthy adults not on any medication as reference controls. Adult TM patients (≥18 years), attending the outpatient department at University College Hospital and receiving conventional DFO treatment by SC infusion of 30 to 50 mg/kg DFO as a 10 to 12 hour infusion five to seven nights a week, were asked when the previous SC DFO infusion had been completed and a serum sample for NTBPI measurement was taken.

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Table 1. Clinical Details and Previous DFO Treatment for TM and TI Patients Who Received Intravenous DFO at 50 mg/kg/24 h for 48 Hours

Patient	Age (yr)	Sex	Diagnosis	DFO Dose Outpatient (mg/kg)	Hb (g/dL)	Fe Serum (μmol/L)	TIBC Chrom. (μmol/L)	TIBC Immuno (μmol/L)	Ferritin Serum (μg/L)	Urine Iron (mg/24 h)
1	24	f	TM	3g × 5	13.0	32.0	23.0	32.5	2,000	19.3
2	19	f	TM	1.5g × 5–6	10.4	36.0	33.0	37.5	1,750	8.3
3	30	f	TM	3g × 6	12.8	50.8	32.0	45.0	242	12.4
4	20	m	TM	3g × 6	15.5	31.0	28.0	30.0	3,225	17.7
5	30	m	TM	2g × 5	12.4	24.9	33.0	NA	6,870	8.5
6	22	f	TM	2g × 5	14.8	53.0	45.0	57.5	2,750	11.4
7	31	m	TM	3g × 5	10.8	35.7	29.0	32.5	2,050	21.9
8	32	m	TM	3g × 7	10.8	39.1	31.0	37.5	3,450	14.4
9	10	m	TM	1g × 6	10.5	43.0	22.0	35.0	2,475	19.0
10	14	m	TM	1g × 4	12.3	32.0	36.0	45.0	2,500	6.2
11	19	f	TM	4g × 4	11.8	31.1	25.0	30.0	2,450	22.7
12	19	m	TM	5g × 5	14.0	46.1	33.0	40.0	7,200	46.5
13	28	f	TM	2 × 5–6	11.0	14.3	32.0	37.5	2,510	19.9
14	16	m	TM	2.5g × 5	16.4	49.0	41.0	50.0	2,000	13.6
15	20	m	TM	2.5g × 5	11.9	38.2	35.0	40.0	1,700	6.8
16	25	f	TM	3g × 5	12.8	44.1	33.0	45.0	1,775	18.5
17	28	m	TM	2g × 7	11.8	35.4	32.0	37.5	940	10.1
Mean ± SEM	22.8 ± 1.6				12.5 ± 0.4	37.4 ± 2.4	31.9 ± 1.4	40.6 ± 2.3	2,699.2 ± 437.1	16.3 ± 2.3
18	20	f	TI	None	8.3	53.4	36.0	50.0	201	21.0
19	16	m	TI	None	8.8	40.0	17.0	32.5	1,850	31.4
20	38	f	TI	None	7.6	37.6	14.0	27.5	475	16.3
21	18	f	TI	None	8.2	39.6	26.0	40.0	380	22.0
22	23	m	TI	None	7.8	47.5	27.0	42.5	855	11.1
23	30	f	TI	Intermittent	8.0	41.6	28.0	35.0	700	8.7
24	25	m	TI	Intermittent	8.6	30.8	19.0	30.0	1,130	27.4
25	27	f	TI	None	8.4	41.0	28.0	32.5	404	18.6
Mean ± SEM	24.6 ± 2.7				8.2 ± 0.1	41.4 ± 2.5	24.2 ± 2.7	36.3 ± 2.8	749.4 ± 202.1	19.6 ± 2.9

Hb, serum iron (Fe), and TIBC samples were taken immediately before the DFO infusion. TIBC and transferrin saturation values are given by two methods: chromogenic (chrom) and immunoassay (immuno). Twenty-four–hour urinary iron values represent the mean 24-hour urinary iron (mg) over the 48 hours of DFO infusion.

Samples were also taken for serum iron, total iron-binding capacity (TIBC), and ferritin estimation. All samples were taken between 9 am and 12 noon. For the purposes of analysis, patients were divided into those who had received DFO the previous night (ie, infusion ending within last 4 hours) and those who had not taken DFO the previous evening (Fig 1).

Inpatient Study

Adult patients with TM or TI were admitted to hospital for intravenous DFO therapy. No DFO or oral ascorbate supplements were given for 72 hours before hospital admission. Intravenous cannulae were sited in each arm, and DFO was infused continuously for 48 hours, starting by between 9 am and 11 am, at a dose of 50 or 100 mg/kg/24 h in 1 L of saline. Heparinized plasma samples were taken for analysis of levels of DFO and its major metabolite and serum samples for NTBPI estimation, at predetermined time points before and after commencing and stopping the intravenous. DFO infusion. Twenty-four–hour urine samples were collected for iron estimation. Serum samples were also taken for measurement of serum iron, TIBC, and ferritin and Hb (Table 1).

Measurement of NTBPI

NTBPI was assayed in the Department of Haematology UCL using an HPLC method previously described,[7] which allows the determination of NTBPI even for patients currently receiving DFO. A number of modifications were undertaken to refine the original assay. 4-Morpholinoproprane sulphonic acid (MOPS) buffer was replaced by 5 mmol/L sodium phosphate, pH 7.0. An HPLC system free from metal parts that might come into contact with the sample was used, with nonmetallic polyetheretherketone (PEEK) tubing throughout (Waters Bio-System 625, nonmetallic gradient module with 991 Photodiode Array detector; Millipore UK Ltd, Watford, UK). NTBPI was quantitated by the injection of $FeCl_3$ standards (atomic absorption grade; Sigma, Poole, UK) made up in 80 mmol/L NTA. Standards were routinely run at 0 to 10 μmol/L, although the absorbance was linear up to 40 μmol/L. Under these conditions, the 0 μmol/L standard corresponds to 80 mmol/L NTA. The following steps were taken to standardize the assay further and to minimize iron contamination during ultrafiltration steps.

Ultrafiltration. We found that Amicon Centricon 30 microconcentrator units (Amicon Inc, Beverly, MA) were well suited for separation of the low-molecular-weight iron fraction from transferrin bound iron by ultrafiltration. Previous work performed with other ultrafiltration cones showed that 0.1 mol/L HCl liberates iron from the filtration membranes, and this procedure is therefore unsuitable as a means of depleting the units of iron. Hence, to minimize iron contamination, the membranes were prewashed by spinning 1 mL of 10 mmol/L NTA (1,000*g* for 5 minutes) followed by 1 mL of deionized water.

NTA. Disodium and trisodium salts of NTA (Sigma, Poole, UK) were prepared separately as 800 mmol/L stocks in deionized water and mixed to obtain 800 mmol/L NTA, pH 7.0. This was added to serum (for 20 minutes at room temperature before ultrafiltration) 1:10 to obtain a final NTA concentration of 80 mmol/L. As the concentration of iron in NTA has not been certified by the manufacturer, different batches may have different inherent iron contents. Therefore, a number of methods were investigated to minimize iron

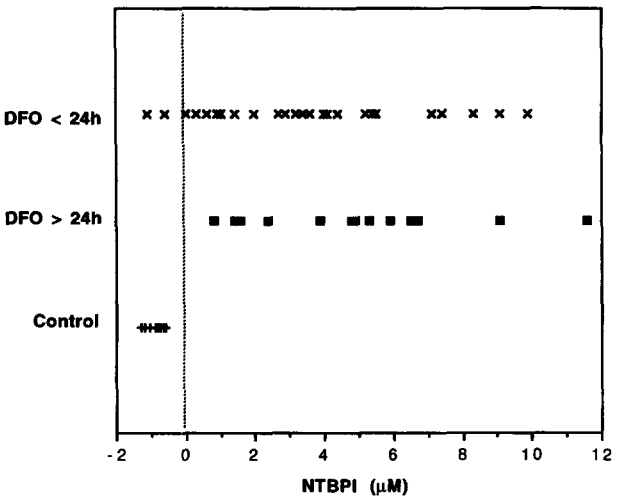

Fig 1. NTBPI was measured in outpatients with TM receiving conventional doses of DFO (30 to 50 mg/kg/24 h SC over 8 to 10 hours) and related to when treatment was last given. The results are shown for healthy controls (n = 11), TM patients having received treatment within the last 24 hours (DFO < 24 hours n = 28), and those having not received DFO for 24 hours (DFO > 24 hours, n = 13).

contamination of NTA, including treatment with chelating resin or apotransferrin. Treatment of the two sodium salts of NTA with chelating resin (Chelex; Bio-Rad, Hemel Hempstead, UK) overnight at 20°C did not significantly reduce contaminating levels of iron, the concentration of iron in Chelex-treated and untreated 80 mmol/L NTA being 0.12 and 0.13 μmol/L respectively. Dialysis of NTA (800 mmol/L) at pH 7 against apotransferrin (10 mg/mL) for 2 hours at 37°C also failed to reduce iron present in NTA significantly, the concentrations of iron present in apotransferrin-treated and untreated 80 mmol/L NTA being 0.17 and 0.21 μmol/L, respectively. We therefore elected not to remove iron from the NTA but to standardize the iron concentration in each batch of 800 mmol/L NTA at 20 μmol/L (ie, 2 μmol/L final concentration in serum) by measuring the iron content by atomic absorption spectroscopy and making up the difference with exogenous atomic absorption grade $FeCl_3$. The addition of 80 mmol/L NTA to the serum of normal individuals always results in negative NTBPI values (Fig 1). These values are less than the 0 μmol/L standard (ie, 80 mmol/L NTA alone), presumably because some iron is donated from NTA to transferrin. By standardizing the iron concentration in the NTA, a greater consistency between assays for such negative values was obtained.

Assay precision. The precision of this assay has not been published previously. The interassay coefficient of variation for replicate samples analyzed on six separate days at 6 μmol/L iron was 5.7% and at 2 μmol/L iron was 28.7% (n = 6). In healthy volunteers, the interassay coefficient of variation was 16.9% (n = 6). The interassay coefficient of variation for pooled thalassaemia plasma was 38% (n = 6) at 2.1 μmol/L. The intraassay coefficient of variation for replicate samples run on the same day was 2.45% at 6 μmol/L iron and 14.6% at 2 μmol/L iron. All sequential NTBPI samples from a given inpatient were analyzed on the same day.

Pharmacokinetics. Kinetic analysis was undertaken on individual patients (Table 2) and on TM patients as a group (Fig 3) by plotting the natural log (ln) of NTBPI values against each time point.[9] This characteristically resulted in the linear resolution of the rates of appearance and disappearance of NTBPI into a fast initial phase and a subsequent slower phase (Figs 3 and 4). The initial (α) and subsequent (β) rate constants were then calculated (in $hour^{-1}$) from the slopes of each plot and the $T_{1/2}$ derived from the respective rate constants.[9]

Measurement of DFO Levels

DFO, its major metabolite, and FO were assayed on heparinized plasma samples at the Department of Pharmacy, Kings College, London, as previously described.[9-11] This method allows determination of free DFO and FO as well as the proportion of the major metabolites present in the original sample after reverse-phase HPLC analysis and simultaneous UV-vis and radioactive detection.[10,11] The major metabolite of DFO has the structure $HO_2C(CH_2)_4NOHCO(CH_2)_2CONH(CH_2)_5NOHCO(CH_2)_2CONH(CH_2)_5NOHCOMe$ and was first described by Keberle.[12] As FO has greater stability than DFO in solution,[11] DFO was stabilized within 30 minutes of sample collection. An internal prestandard (DFO E) was first added to the plasma sample. A known amount of ^{59}Fe and ^{56}Fe was then added as previously described.[9,10] The kinetics for DFO and its metabolites were analyzed as previously described using the trapezoid method to calculate area under the curve (AUC) values.[9]

Other Measurements

Serum ferritin measurement was as previously described.[13] Serum iron and total iron binding capacity (TIBC) were quantitated by the PFT-600 method (American Monitor Corporation, Indianapolis, IN).[14] Reference ranges for Fe and TIBC are 7 to 45 and 45 to 74 μmol/L, respectively. This chromogenic method is unreliable for TIBC measurement in hemolyzed samples and with high levels of other plasma pigments such as bilirubin.[15] This may explain the unusually low TIBC values (and hence high transferrin saturation) obtained in some TI patients (Table 1). TIBC was therefore measured independently by a nonchromogenic method using rate reaction immunonephelometry on a Beckman Array (Beckman Instruments UK Ltd) for transferrin determination. Calibration was achieved using an international transferrin standard (reference preparation for proteins in human serum [RPPHS]; Table 1).[16] Although there is a good correlation between TIBC by chromogenic and immunologic methods (r = .83), the unusually high saturation obtained with some TI patients is not observed using the immunoassay. Furthermore, the levels of NTBPI measured are closer to the levels predicted from the immunoassay than from the chromogenic method. Urine iron was determined by atomic absorption spectroscopy at 248.3 nm as previously described[17] on centrifuged (800g for 5 minutes) and filtered (0.45 μmol/L) samples taken from 24-hour collections.

Statistical Analysis

All values shown are the means ± SEM unless otherwise stated. Statistical differences between groups were evaluated using students (unpaired) t-test. To test for correlations between two variables, linear-regression analysis was performed and the correlation coefficient (r) calculated.

RESULTS

Preliminary Outpatient Study

Initial investigations were performed on healthy volunteers and outpatients with TM to determine whether NTBPI values were affected by the degree of iron loading or by the timing of recent SC DFO therapy. No NTBPI was present in healthy adult controls (−0.94 ± 0.08 μmol/L, n = 11). NTBPI was measured in outpatients receiving conventional doses of DFO (30 to 50 mg/kg/24 h SC over 8 to 12 hours) and related to when treatment was last given. NTBPI was present in all patients who finished treatment more than 24 hours before blood sampling (Fig 1) (4.99 ± 0.89 μmol/L,

Table 2. Kinetics of NTBPI Removal for TM and TI Patients

Patient	NTBPI Pre-DFO (μmol/L)	NTBPI 4 Hours (μmol/L)	NTBPI Nadir (μmol/L)	NTBPI 48 Hours (μmol/L)	NTBPI Elimination α (/h)	NTBPI Elimination $T_{1/2}$ (h)	NTBPI Elimination β (/h)	NTBPI Elimination $T_{1/2}$ (h)	DFO AUC/48 (μmol/L/h)	DFO Met AUC/48 (μmol/L/h)
1	3.89	−0.28	−0.83	−0.48	0.70	0.98	Absent		10.08	4.41
2	3.40	0.37	0.11	0.62	1.68	0.41	0.20	3.47	11.70	24.30
3	2.54	−1.07	−1.07	1.00	2.25	0.30	1.44	0.48	13.60	7.30
4	1.49	1.22	−0.40	1.05	1.04	0.66	0.11	6.08	NA	NA
5	5.65	0.26	−0.88	1.03	1.52	0.45	0.45	1.54	4.14	7.13
6	3.38	−0.11	−0.39	2.85	2.04	0.33	0.51	1.36	6.73	3.30
7	2.35	0.40	−1.20	−0.70	0.85	0.81	0.50	1.39	8.81	4.39
8	2.50	0.42	−0.72	0.64	2.23	0.31	0.40	1.73	15.50	6.10
9	4.62	0.40	0.20	1.10	1.52	0.45	0.31	2.24	NA	NA
10	0.62	−0.81	−0.81	1.36	2.20	0.31	0.36	1.93	96.46	54.62
11	5.51	0.76	0.76	0.93	1.41	0.48	0.20	3.47	5.04	1.40
12	2.19	−0.38	−0.38	−0.29	0.79	0.86	0.82	0.85	9.25	3.06
13	−0.35	−0.83	−0.83	−0.28	0.21	3.28	Absent		3.25	7.25
14	4.24	1.10	−0.49	1.20	1.24	0.55	0.14	4.95	26.30	6.26
15	3.25	0.20	−0.44	0.20	1.92	0.36	Absent		27.60	12.63
16	2.64	−0.24	−1.07	−0.25	1.64	0.42	0.65	1.07	13.66	2.00
17	1.74	−1.13	−1.90	−0.78	3.57	0.19	0.52	1.33	2.09	3.44
Mean ± SE	2.92 ± 0.41	0.02 ± 0.18	−0.61 ± 0.16	0.54 ± 0.24	1.58 ± 0.20	0.66 ± 0.18	0.47 ± 0.09	2.28 ± 0.44	16.95 ± 6.00	9.84 ± 3.51
18	6.76	1.00	−0.63	1.82	1.21	0.56	0.02	40.76	7.00	5.38
19	2.66	1.30	0.90	1.37	0.56	1.23	0.03	26.65	7.27	2.31
20	4.17	1.20	−0.87	−0.87	0.85	0.80	0.08	8.25	11.95	8.10
21	2.89	0.50	−1.11	−1.00	1.25	0.55	0.01	53.31	NA	NA
22	6.40	1.20	0.44	0.94	1.24	0.55	0.02	32.38	NA	NA
23	4.50	1.80	1.20	1.40	0.38	1.82	0.01	57.75	NA	NA
24	4.94	4.20	−1.31	0.82	5.83	0.12	0.05	13.00	8.81	2.20
25	3.82	0.73	−1.05	−1.05	0.98	0.70	0.13	5.30	10.37	11.37
Mean ± SE	4.52 ± 0.56	1.49 ± 0.44	−0.30 ± 0.38	0.43 ± 0.45	1.54 ± 0.62	0.79 ± 0.62	0.04 ± 0.02	29.68 ± 7.09	9.08 ± 0.79	5.87 ± 1.48

NTBPI values are shown immediately before starting DFO and after 4 and 48 hours of intravenous DFO infusion. The lowest NTBPI values obtained are also shown (nadir). The rate constants α and β are shown for the first and second component of NTBPI removal, together with the concomitant $T_{1/2}$ values. AUC values for DFO and the major DFO metabolite (DFO Met) are also shown.

Abbreviation: NA, results are not available.

n = 13). For patients treated the previous night with SC DFO, NTBPI was present in all but two patients (Fig 1) (3.48 ± 0.56 μmol/L, n = 28), suggesting that either the DFO infusion had failed to clear the NTBPI or that NTBPI had returned rapidly after stopping treatment. The detailed inpatient studies described below were therefore undertaken to determine the relationship between the kinetics of DFO infusion and NTBPI removal and reappearance.

NTBPI Before DFO Infusion With Inpatient Study

NTBPI was present in all but 1 patient (no. 13) with TI or TM admitted for intravenous DFO infusion and having not received DFO for at least 72 hours. NTBPI values were significantly higher in TI (4.52 ± 0.53) than in TM (2.92 ± 0.29) ($P = .026$) (Table 2). For TM but not TI patients, there was a weak linear correlation between NTBPI and transferrin saturation, whether the TIBC was derived from chromogenic determinations ($r = .61$, $P = .016$) or by immunoassay ($r = .64$, $P = .008$). There was no correlation between pretreatment values of NTBPI (Table 2) and serum ferritin, urinary iron excretion on DFO, or serum iron (Table 1).

Kinetics of Removal of NTBPI by Intravenous DFO Infusion in TM

An example of the relationship between a 48-hour intravenous DFO infusion at 50 mg/kg/24h is shown in Fig 2. Kinetic analysis was undertaken on individual patients (Table 2) and on TM patients as a group (Fig 3, inset) as described in Materials and Methods. After starting the intravenous DFO infusion, NTBPI was cleared rapidly, being absent by a mean of 5 hours (Fig 3). Kinetic analysis (Fig 3, inset) shows that removal of NTBPI is in a biphasic manner from which the respective rate constant α and β and the $T_{1/2}$ values were calculated (see Materials and Methods). An initial rapid removal of NTBPI is seen (α) (k = 1.23, $T_{1/2}$ = 0.56 hours) followed by a slower (β) phase (k = 0.57, $T_{1/2}$ = 1.2 hours).

Table 2 shows the kinetic data for NTBPI removal for each patient in the study. The mean rate constants and $T_{1/2}$ values for α (1.6 ± 0.21/h, $T_{1/2}$ = 0.6 ± 0.21 hours) and β phases (0.44 ± 0.05/h, $T_{1/2}$ = 1.9 ± 0.3 hours) are in broad agreement with values obtained from the combined data in Fig 3. In three patients, a clear β phase was not discernible on the data available (marked absent). Patients with high pretreatment NTBPI values had higher NTBPI values at 4 hours ($r = .51$, $P = .038$), but by 48 hours of DFO infusion, no correlation remained. Nadir values were generally obtained at approximately 8 hours. The subsequent small increase observed at 48 hours may in part reflect diurnal variation in NTBPI, as serum iron is known to peak at approximately 9 am and trough at 9 pm.

Comparison of NTBPI Removal Kinetics in TI and TM

Unlike the TM patients who took DFO SC regularly until 72 hours before the study, it can be seen in Table 1 that the TI patients were not receiving regular DFO therapy. Furthermore, TI patients were not receiving regular blood transfusions, and this is reflected by the lower Hb values. We wished to establish whether these two factors might influence NTBPI and the rate at which this was removed by DFO.

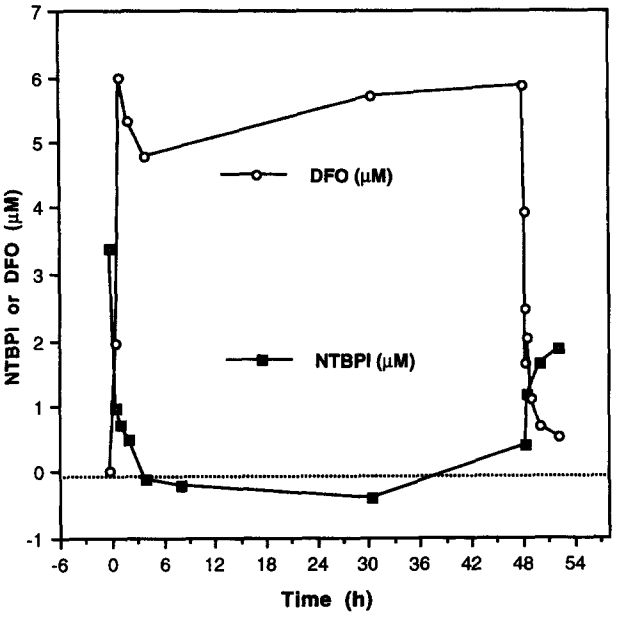

Fig 2. The effect of DFO infusion at 50 mg/kg/24 h intravenous on NTBPI is shown in a single patient with TM both on starting the DFO infusion and on stopping the infusion at 48 hours.

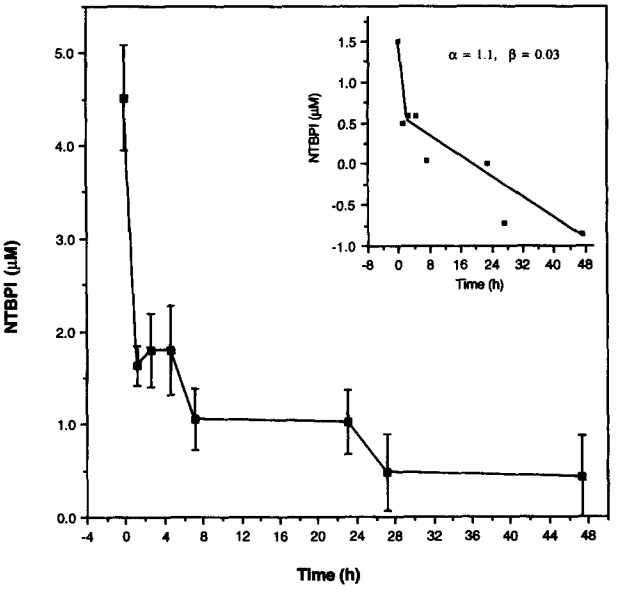

Fig 4. For inpatients with TI, the removal of NTBPI with DFO 50mg/kg/24h intravenous is shown. Each point represents the mean and SEM NTBPI serum concentration for the respective time interval (n = 8). Kinetic analysis of ln NTBPI against time is shown in the inset together with the rate constants for the initial α phase and subsequent β phase of NTBPI removal.

The initial rate constant (α) for NTBPI removal does not differ significantly between TI and TM but lasted for less than 30 minutes in contrast to the 2 hours for TM (Figs 3 and 4). The calculated α constants for NTBPI removal in TI broadly agree whether ln values for all TI patients are analyzed together (α = 1.1, Fig 4 inset) or individually (1.5 ± 0.32, Table 2). The second phase of NTBPI removal (β rate constant) is significantly slower for TI (0.04 ± 0.01) than TM (0.47 ± 0.09) (P = .002, Table 2) and is of longer duration (Fig 4). This difference is also reflected by the lower NTBPI at 4 hours in TM (0.02 ± 0.18) than in TI (1.49 ± 0.44, P = .001) (Table 2).

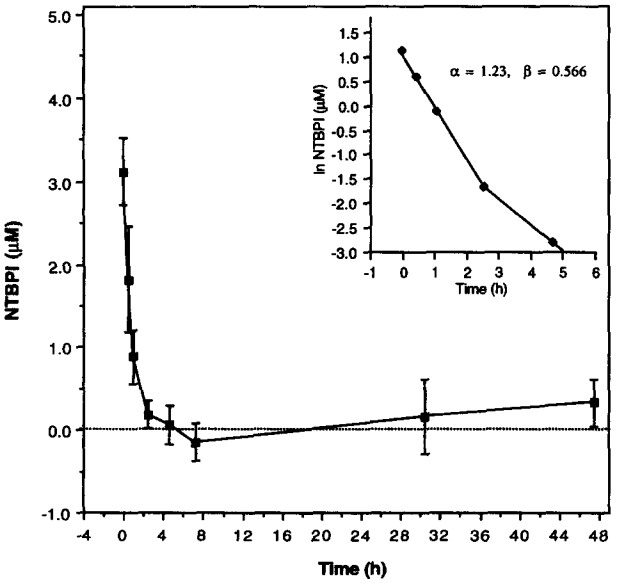

Fig 3. For inpatients with TM, the removal of NTBPI by DFO at 50 mg/kg/24 h intravenous is shown as a function of time. Each point represents the mean and SEM NTBPI serum concentration in μmol/L (n = 17). Kinetic analysis of ln NTBPI against time is shown in the inset together with the rate constants for the initial α phase and subsequent β phase of NTBPI removal.

Relationship of Plasma DFO and NTBPI Removal

The steady-state DFO concentrations were estimated in the patients shown in Table 2, from the AUC divided by the duration of infusion (48 hours). It can be seen that steady-state DFO values range from 2 to 96 μmol/L (mean 18.2 ± 2.09 μmol/L). It is clear from Table 2 that NTBPI persists in many patients in the presence of excess DFO, suggesting that (some) NTBPI species are only slowly chelated or are relatively resistant to DFO chelation. Consistent with this, there is no correlation between steady-state DFO values and the rate of NTBPI removal or values at 48 hours (Table 2).

When DFO was given at twice the infusion rate (100 mg/kg/24 h) using an otherwise identical protocol to four TM patients (two of whom had also been studied at 50 mg/kg/24h), there seemed to be no marked effect on the rate of NTBPI removal, as reflected by similar rate constants for the α (1.26 ± 0.45) or β (0.07 ± 0.03) phase.

Kinetics of NTBPI Return on Cessation of DFO Infusion in TM

On cessation of DFO infusion, there was a rapid return of NTBPI with a fast initial component (α = 2.32 ± 0.31/h) lasting approximately 30 minutes and a slower increase (β = 0.03 ± 0.007/h) (Table 3) generally reaching pretreatment

Table 3. Kinetics of NTBPI Return After Stopping the DFO Infusion

Patient	NTBPI 1 Hour Post (μmol/L)	NTBPI 4 Hours Post (μmol/L)	NTBPI 12 Hours Post (μmol/L)	NTBPI Return Rate Constant		Elimination Rate Constant DFO		Elimination Rate Constant
				α (/h)	β (/h)	α (/h)	β (/h)	DFO Met (/h)
1	1.77	−0.57	1.30	4.16	0.040	3.55	0.19	0.48
2	3.10	3.19	NA	2.35	NA	0.35	0.45	0.45
3	1.10	1.67	1.70	1.17	0.022	2.22	0.13	0.39
4	3.80	5.05	3.80	2.14	0.082	NA	NA	NA
5	1.30	4.09	8.70	0.645	0.088	0.58	NA	0.55
6	2.04	1.85	2.80	1.15	0.011	3.46	0.35	0.77
7	−0.30	0.03	−0.20	0.47	−0.004	2.10	0.24	0.46
8	2.50	3.80	3.30	3.75	0.008	0.74	0.13	0.81
9	2.70	3.05	NA	1.9	0.014	NA	NA	NA
10	3.26	3.88	2.35	1.36	0.015	4.07	0.05	0.23
11	2.12	2.58	2.50	2.3	0.000	2.31	0.28	0.21
12	2.20	4.00	NA	1.75	NA	2.31	0.36	2.80
13	0.73	−0.21	NA	NA	NA	1.50	0.25	0.47
14	3.65	3.10	3.65	3.61	0.002	1.31	0.19	0.54
15	1.70	1.83	2.60	3.33	0.025	2.86	0.18	0.52
16	1.30	1.45	1.30	4.2	0.018	0.96	0.11	0.28
17	0.63	1.74	2.10	2.84	0.034	2.55	0.12	0.31
Mean ± SE	1.83 ± 0.28	2.40 ± 0.40	2.91 ± 0.58	2.32 ± 0.34	0.025 ± 0.008	2.06 ± 0.32	0.22 ± 0.03	0.62 ± 0.17
18	2.45	2.11	7.70	1.53	0.015	1.66	0.08	0.53
19	2.80	2.66	3.10	2.85	0.053	1.77	0.08	0.77
20	2.40	3.76	4.30	3.75	0.053	2.75	0.38	0.40
21	−0.04	0.20	0.70	4.44	0.091	NA	NA	NA
22	4.01	4.60	3.90	3.75	0.062	NA	NA	NA
23	3.30	4.30	4.30	1.53	0.016	NA	NA	NA
24	6.30	9.90	NA	5.71	0.090	2.50	0.24	NA
25	−0.76	−0.55	NA	1.28	0.120	2.20	0.40	0.54
Mean ± SE	2.56 ± 0.84	3.37 ± 1.44	4.00 ± 0.85	3.11 ± 0.71	0.062 ± 0.014	2.18 ± 0.23	0.23 ± 0.08	0.56 ± 0.08

NTBPI values represent serum samples taken 1, 4, and 12 hours after stopping the DFO infusion. The rate constants for the initial (α) and subsequent (β) return of NTBPI are shown as well as the rate constants (α and β) for removal of DFO and the major metabolite of DFO (DFO met).

NTBPI values by 12 hours (Table 3). These values are equivalent to doubling times of 0.29 and 27 hours respectively.

We have previously described that DFO elimination approximates to a two-compartment model with a rapid α phase and a slower β phase,[9] and the results in this study for DFO are consistent with these previous findings. The initial rate constant (α) for NTBPI return (2.3 hour^{-1}) is approximately equal to the summation of the initial rate constants for removal of DFO (1.8/h) and its major metabolite (0.6 hour^{-1}) (Table 3). The subsequent rate constant for NTBPI increase (0.03) beginning after 30 minutes to 1 hour is considerably slower than either the slow β phase of DFO elimination or that of the main metabolite of DFO.

Comparison of Kinetics of NTBPI Return in TI with TM

The initial mean rate constant (α) for return of NTBPI in TI patients is 3.11 ± 0.71 hour^{-1} was faster than with for TM 2.32 ± 0.34 hour^{-1}, although this does not reach statistical significance. The subsequent rate constant (β) in TI is 0.062 ± 0.013 hour^{-1}, which is significantly faster than with TM (0.03 ± 0.007 hour^{-1} (P = .015) (Table 3). These differences are also demonstrated in Fig 5, where the kinetics of NTBPI return are shown as the mean ln of NTBPI values for each time point plotted against time in TM and TI patients. These differences in NTBPI returns cannot be explained by differences in DFO elimination between TM and TI (Table 3).

Effect of vitamin C on NTBPI while receiving DFO. All patients in this study received no vitamin C supplements for 72 hours before hospital admission. Oral vitamin C is often given as a supplement to patients receiving iron chelation with the expectation of increasing iron excretion.[18] It has been unclear whether this practice is safe, however, as it has been claimed that high doses of vitamin C (0.6 to 2 g daily) are associated with cardiac damage.[19] To determine whether there was a significant effect of vitamin C on NTBPI while DFO infusion was in progress, we elected to prescribe vitamin C at current recommended doses (200 mg orally) once steady-state concentrations of NTBPI and DFO had been reached during a continuous intravenous infusion of DFO at 100 mg/kg/24 h. On two occasions within a 24-hour period, the addition of vitamin C (200 mg orally) had no effect on measurable NTBPI levels either immediately or over the subsequent 6 hours (not shown).

DISCUSSION

This study shows for the first time the kinetic response of NTBPI to DFO chelation therapy. It has not been established how rapidly NTBPI is removed by DFO and how previous

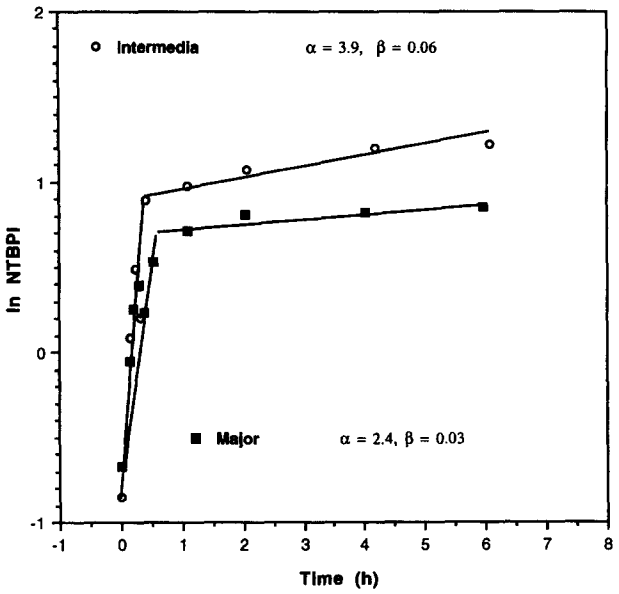

Fig 5. The kinetics of return of NTBPI after stopping intravenous DFO after 28 hour infusion at 50 mg/kg/24 h are compared for TI and TM. The ln of mean NTBPI values (μmol/L) for each patient group are plotted against the time after the DFO infusion was stopped. The rate constants for the α and β phases of NTBPI return are shown for TI and TM patients.

chelation treatment, iron overload status, or transfusion regimens affect this rate. How quickly NTBPI returns after stopping treatment and what determines the rate of recovery to pretreatment levels was also unclear before this study. The findings we report above provide important clues as to the nature of NTBPI in iron overload as well as how DFO might best be administered to minimize toxic NTBPI species.

The exact chemical nature of NTBPI in human plasma in iron overload has been the subject of considerable debate. Evidence for low-molecular-weight NTBPI species has been produced both in the absence of iron overload[20,21] and in iron-overloaded patients.[22,23] In iron overload, iron-citrate complexes may predominate,[23] although other low-molecular-weight species have been described.[21] Higher molecular weight forms of NTBPI, bound to plasma proteins, are also likely to exist as the iron content of plasma ultrafiltrates is only 50% to 70% of that measured by the bleomycin assay in whole plasma or serum.[23] Plasma ferritin may contribute to this higher molecular weight pool,[24] and complexes of iron with other plasma proteins such as albumin (Hider, personal communication) may be important.

Because the chemical nature of NTBPI species in thalassaemia is unclear, the clinical relevance of the iron species used to study NTBPI kinetics in animal models remains uncertain. Nevertheless, such studies have shown that low-molecular-weight ferric and ferrous citrate complexes are cleared rapidly in perfused rat liver by an efficient saturable "first pass" process with K_m values of 14 to 22 μmol/L.[25] This process appears to require the reduction of ferric to ferrous iron, although this step is not rate-limiting. The kinetics of higher molecular weight forms of NTBPI are presently unknown.

In this study, the biphasic removal of NTBPI with DFO chelation fits with a two-compartment model. One explanation for this finding is of a rapidly chelatable NTBPI component and a more slowly chelatable component, possibly of higher molecular weight. However, the in vitro removal of iron from NTBPI citrate complexes (possibly the predominant low-molecular-weight species of NTBPI) by DFO is relatively slow in human plasma, with little or no removal by 40 minutes.[23] Furthermore, animal studies show that iron-citrate complexes are cleared rapidly by first pass through the liver.[25] Therefore, the initial rate of NTBPI fall may represent its clearance by the liver once infusion of DFO begins to inhibit the formation of new NTBPI species (formed by iron release from the reticuloendothelial system). The slower second phase of fall, particularly in the TI patients, would then be explained by a slower hepatic clearance of other NTBPI species (presumably of higher molecular weight). Poor treatment compliance with DFO, or lack of it in the TI patients, may increase the proportion of slowly chelated, higher molecular weight NTBPI species. Whether differences in iron loading between TI and TM could also modulate the rates of liver NTBPI clearance is unclear as there are contradictory findings between the effect of iron loading in whole isolated rat liver (no effect)[26] and the cultured human liver tumor HepG2 cell line (increased uptake).[27]

A further model that may explain the kinetics of NTBPI removal is that the rapid phase is primarily dependent on the plasma DFO concentration (ie, plasma chelation of NTBPI) but that the subsequent slower phase represents chelation of intracellular iron leading to less release from within reticuloendothelial cells at later time periods. It is known that DFO enters cells relatively slowly, and the rate of the slow phase of NTBPI removal is compatible with this slower rate. This model could also explain the slower second phase in TI compared with TM because the turnover of iron in the former would be greater than in TM. If this model is correct, then the concentration of NTBPI achieved at the end of phase 1 should be inversely proportional to the plasma DFO concentration. Analysis of the data in Table 2 fails to show such a correlation, suggesting that the initial rate of NTBPI removal is not solely dependent on plasma DFO concentrations.

On cessation of DFO infusion, the initial rates of return of NTBPI appear to be largely determined by the rate of clearance of DFO and its major iron chelating metabolite. It is likely that this is a causal relationship, the levels of NTBPI at any time point during the fast phase primarily reflecting plasma DFO concentrations. We have previously shown that DFO elimination corresponds to a two-compartment model with an initial rapid elimination and a slower second phase.[9] Unlike the initial α phase of NTBPI reaccumulation, the slower second β phase reaccumulation to pretreatment levels is significantly slower than the rate of DFO elimination (Table 3). This suggests that the second phase of NTBPI reaccumulation is largely independent of DFO kinetics. During this second phase, NTBPI levels are likely to reflect the balance between the rate of formation with release of intracellular iron into the NTBPI compartment and the rate of clearance of NTBPI by the tissues, predominantly hepatocytes. We

hypothesize that this slower phase of accumulation may correspond to the formation of higher molecular weight NTBPI species that are in equilibrium with lower molecular weight forms.

The difference in reappearance of NTBPI in TI and TM is of interest. The TM patients have significantly higher ferritin values and are regularly transfused and chelated unlike TI patients (Table 1). Because TI patients are not hypertransfused, the degree of ineffective erythropoiesis and hemolysis is greater, as is the plasma iron turnover.[28] If transferrin is already saturated, then NTBPI from these sources will be formed at a greater rate in TI patients than TM. The finding that NTBPI reaccumulates faster in TI than TM (Fig 5, Table 3) suggests that the formation of NTBPI from red cell breakdown is a relatively important contributor to NTBPI turnover and that absolute levels of iron overload less important. The higher pretreatment levels of NTBPI (Table 1) therefore may also reflect increased rates of NTBPI formation from red cell breakdown. The levels obtained presumably reflect an equilibrium between formation and clearance in NTBPI, which judging by the time taken to reach pretreatment values (Figs 3 and 4), occurs within a few hours of stopping DFO. However, the nature of the NTBPI species may change with time "off treatment" with the gradual accumulation of higher molecular weight forms. This would explain the slower response of the second phase of NTBPI clearance in TI than TM.

This study has implications for the design of chelation strategies. The broad aims of chelation therapy in iron overload are twofold: first, to promote negative iron balance and second, to reduce the harmful effects of iron while or until iron overload is removed. Although intermittent high dosage treatment with DFO may be effective at promoting negative iron balance, this study shows that on cessation of DFO, intravenous NTBPI returns rapidly. Even at higher doses of 100 mg/kg, which are above standard recommended doses for prolonged use if DFO toxicity is to be avoided,[13] the rate of NTBPI reappearance is rapid, reaching pretreatment levels with 6 hours of cessation of DFO. Thus, for patients who have serious complications such as cardiac dysfunction, this study suggests that it will be more beneficial to give continuous rather than intermittent chelation therapy. This is supported by experimental evidence that NTBPI species are rapidly taken up by myocytes causing lipid peroxidation.[29]

This study also suggests that the patients with TI may benefit from regular chelation therapy even when the serum ferritin is less than 1,000 μg/L (Table 1), if damage from NTBPI is to be minimized. On the other hand, previous studies have suggested that thalassaemia patients with low ferritin values may be at risk from DFO toxicity.[13,30] To minimize NTBPI-mediated damage, relatively frequent daily treatment with low doses of DFO (10 to 20 mg/kg SC) may be a more logical approach to TI treatment than intermittent administration of doses used to treat TM (40 to 50 mg/kg). Unfortunately, although continuous low-dose DFO treatment is logical in TI, problems with compliance may make this impractical in many patients. However, with emerging new oral chelators[31] or new preparations of DFO,[32] the design of treatment regimens to minimize the duration of tissue exposure to NTBPI may become increasingly realistic.

REFERENCES

1. Hershko C, Rachmilewitz EA: Non transferrin plasma iron in patients with transfusional iron overload, in Crichton RR (ed): Proteins of Iron Storage and Transport in Biochemistry and Medicine. Amersham, The Netherlands, North Holland, 1975, p 427
2. Hershko C, Graham G, Bates GW, Rachmilewitz EA: Non-specific serum iron in thalassaemia: An abnormal serum specific iron fraction of potential toxicity. Br J Haematol 40:255, 1978
3. Anuwatanakulchai M, Pootrakul P, Thuvassethakul P, Wasi P: Non transferrin plasma iron in B thalassaemia/hb E and haemoglobin H disease. Scand J Haematol 32:153, 1984
4. Batey RG, Petit JE, Nicholas AW, Sherlock S, Hoffbrand AV: Hepatic iron clearance from serum in treated hemochromatosis. Gastroenterology 75:856, 1978
5. Gutteridge JMC, Rowley DA, Griffiths E, Haliwell B: Low molecular weight iron complexes and oxygen radical reactions in idiopathic haemochromatosis. Clin Sci 68:463, 1985
6. Wagstaff M, Peters SW, Jones BM, Jacobs A: Free iron and iron toxicity in iron overload. Br J Haematol 61:566, 1985
7. Singh S, Hider RC, Porter JB: A direct method for quantification of non-transferrin bound iron (NTBPI) Anal Biochem 186:320, 1990
8. Al Refaie FN, Wickens DG, Wonke B, Kontoghiorghes GK, Hoffbrand AV: Efficacy and possible adverse effects of the oral iron chelator. 1,2-Dimethyl-3-hydroxypyrid-4-one (L1) in thalassaemia major. Br J Haematol 82:431, 1992
9. Lee P, Mohammed N, Marshall L, Abeysinghe RD, Hider RC, Porter JB, Singh S: Intravenous infusion pharmacokinetics of desferrioxamine in thalassaemic patients. Drug Metab Dis 21:640, 1993
10. Singh S, Hider RC, Porter JB: Separation and identification of desferrioxamine and its metabolites by high performance liquid chromatography and fast atomic bombardment mass spectrometry: Choice of complexing agent and application to biological fluids. Anal Biochem 187:212, 1990
11. Singh S, Mohammed N, Ackerman R, Porter JB, Hider RC: Quantification of desferrioxamine and its iron chelating metabolites by high performance liquid chromatography and simultaneous ultraviolet-visible/radioactive detection. Anal Biochem 203:106, 1992
12. Keberle H: The biochemistry of desferrioxamine and its relation to iron metabolism. Ann NY Acad Sci 119:758, 1964
13. Porter JB, Jaswon MS, Huehns ER, East CA, Hazell JWP: Desferrioxamine ototoxicity: Evaluation of risk factors in thalassaemic patients and guidelines for safe dosage. Br J Haematol 73:402, 1989
14. Higgins T: Novel chromogen for serum iron determinations. Clin Chem 27:1619, 1981
15. Young DS, Pestaner LC, Gibberman V: Effects of drugs on clinical laboratory tests. Clin Chem 21:321D, 1975
16. Whicher JT, Ritchie RF, Johnson AM, Baudner S, Bienvenu J, Blirup-Jensen S, Carlstrom A, Dati F, Ward AM, Svendsen PJ: New international reference preparation for proteins in human serum (RPPHS). Clin Chem 40:934, 1994
17. Zettner A, Mansbach L: Application of atomic absorption spectroscopy in the determination of iron in urine. Am J Clin Pathol 44:517, 1965
18. Wapnick AA, Lynch SR, Charlton RW, Septel HC, Bothwell TH: The effect of ascorbic acid deficiency on desferrioxamine-induced urinary iron excretion. Br J Haematol 17:563, 1969
19. Nienhuis AW, Peterson DT, Henry W: Chelation Therapy in Chronic Iron Overload, in Zaino EC, Roberts RH (eds): Miami, FL, Symposia Specialists, 1977, p 1
20. Lau S, Sarkar B: Comparative studies of manganese(II), nickel(II), zinc(II), copper(II), cadmium(II) and iron(III) binding

components in human cord and adult sera. Can J Biochem Cell Biol 62:449, 1984

21. Stojkovski S, Groumakos W, Sarkar B: Iron(III)-binding polypeptide in human cord and adult serum: Isolation, purification and partial characterisation. Biochim Biophys Acta 1137:155, 1992

22. Hershko C: Non-transferrin plasma iron. Br J Haematol 66:149, 1987

23. Grootveld M, Bell JD, Halliwell B, Aruoma OI, Bomford A, Sadler PJ: Non-transferrin-bound iron in plasma and serum from patients with idiopathic hemochromatosis. J Biol Chem 264:4417, 1989

24. Pootrakul P, Josephson B, Huebers HA, Finch CA: Quantitation of ferritin iron in plasma, and explanation of non transferrin iron. Blood 71:1120, 1978

25. Brissot P, Wright TL, Ma W-L, Weisiger RA: Efficient clearance of non-transferrin-bound iron by rat liver. J Clin Invest 76:1463, 1985

26. Wright TL, Brissot P, Ma W-L, Weisiger RA. Characterisation of NTBPI clearance by rat liver. J Biol Chem 261:10909, 1986

27. Randell EW, Parkes JG, Olivieri NF, Templeton DM: Uptake of non-transferrin iron by both reductive processes is modulated by intracellular iron. J Biol Chem 23:16026, 1994

28. Pootrakul P, Kitcharoen K, Yansukon P, Wasi P, Fuchareon S, Charoenlarp P, Brittenham G, Pippard MJ, Finch CA: Effect of erythroid hyperplasia on iron balance. Blood 71:1124, 1988

29. Link G, Pinson A, Hershko C: Heart cells in culture: A model of myocardial iron overload and chelation. J Lab Clin Med 107:147, 1985

30. Olivieri NF, Buncie JR, Chew E, Gallanti T, Harrison RV, Keenan N, Logan W, Michell D, Ricci G, Skarf B, Taylor M, Freedman MH: Visual and auditory neurotoxicity in patients receiving subcutaneous desferrioxamine infusions. N Eng J Med 314:869, 1988

31. Hider RC, Epemolu O, Singh S, Porter JB: Iron chelator design. Adv Exp Med Biol 356:343, 1994

32. Hedlund BE, Hallaway PE, Hanson GJ, Kling PJ, Berger AE, Dragsten PR: A novel high molecular weight iron chelator produces prolonged high plasma chelator levels in humans, Beuzard Y, Lubin B, Rosa J (eds): in Sickle Cell Disease and Thalassaemia: New Trends in Therapy. Colloque INSERM 234, 1995, p 383

1996 88: 705-713

Kinetics of removal and reappearance of non-transferrin-bound plasma iron with deferoxamine therapy

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