Original Article *Ann Clin Biochem* 2000; **37**: 457–466

Assessment of iron absorption from ferric trimaltol

David M Reffitt[1], Trevor J Burden[1], Paul T Seed[2], John Wood[3], Richard P H Thompson[1] and Jonathan J Powell[1]

From the [1]Gastrointestinal Laboratory, The Rayne Institute, and the [2]Department of Public Health Medicine, St Thomas' Hospital, London SE1 7EH, and the [3]Department of Chemical Pathology, Southampton General Hospital, Southampton, UK

SUMMARY. Therapeutic iron compounds have limited absorption and often have side-effects, which limits patient compliance. Iron trimaltol is a novel, stable complex, formed between ferric iron (Fe^{3+}) and maltol (3-hydroxy-2-methyl-4-pyrone), and is effective in the treatment of iron deficiency anaemia with few side-effects. However, the kinetics of iron absorption from ferric trimaltol and the reliability of normal colorimetric analysis in detecting iron absorbed from this complex have not been established. We measured increases in serum iron levels in 12 volunteers following oral challenge with four different pharmaceutical formulations of ferric trimaltol in a double-blind, cross-over, randomized study. The conventional colorimetric method for detecting serum iron was compared with thermal analyses after trichloroacetic acid (TCA) treatment of serum. Measurements of serum iron levels by TCA treatment and thermal analysis closely agreed with measurements by colorimetry. For all formulations, serum iron levels peaked at 90 min with a plateau of at least 5 h [mean (standard deviation) peak absorption 8·3% (6·3%) of ingested dose, $n = 48$]. Absorption of iron, based on peak serum values or area under the serum curve, was not different for the four formulations ($n = 12$ each) and correlated with the individual's iron status, as assessed by serum ferritin values ($r = -0{\cdot}6$; $P < 0{\cdot}001$). Normal colorimetry is suitable for analysis of serum iron levels following ingestion of ferric trimaltol. There is rapid and sustained absorption of iron from ferric trimaltol and, as with ferrous iron, uptake appears to be controlled through normal mechanisms of iron acquisition that depend upon body iron stores.

Millions of people suffer from iron deficiency anaemia owing to inadequate and/or poorly available dietary iron, which is compounded by variable obligatory losses of iron.[1] Fortification of the diet with iron is not practical, and supplements are either poorly tolerated, causing epigastric pain, nausea and constipation, or are ineffective.[2] Slow-release iron capsules have recently been investigated, but, because gastrointestinal transit times vary, bioavailability is unreliable, and such preparations may not overcome the problem of the toxicity of iron to the intestinal mucosa.[3,4]

The interaction of iron with luminal and mucosal glycoproteins, as well as poor absorption, results in residual concentrations in the small bowel and colon, which may account for the toxic side-effects. An iron complex or 'chelate', rather than a simple salt, could offer better delivery and render the metal ion less able to interact with and damage the mucosa. Although Fe^{3+} (ferric) rather than Fe^{2+} (ferrous) iron forms high-affinity complexes,[5] simple ferric salts are even less well absorbed than ferrous salts. However, some complexed ferric species are as well absorbed as ferrous compounds.[6] A complexing agent or 'ligand' has to compete with two processes in the gut lumen to bind ferric iron effectively: hydrolysis of the hydrated iron, which causes polymerization of ferric-hydroxy species, and mucus secretion, since mucus glycoproteins avidly bind trivalent metals or their hydroxy species.[7] Thus, for efficient ferric-iron supplementation, either a high-affinity ligand or a high ligand:iron ratio is needed. The latter was well studied with ferric fructose, but this is a weak complex that requires

Correspondence: Dr D M Reffitt.
E-mail: dreffitt@rayne.umds.ac.uk

impracticably large fructose:iron ratios for efficient delivery.[8] However, a high-affinity ferric complex, namely ferric trimaltol, has been synthesized and studied for both efficacy and tolerability.[9–12] In a trial of patients intolerant of ferrous sulphate, ferric trimaltol successfully treated iron deficiency anaemia with few side-effects.[9]

Nonetheless, the factors that affect the absorption of iron from ferric trimaltol are not known. The optimal pharmaceutical preparation has not been established and it is not known whether the complex is absorbed whole or whether it dissociates at the mucosa and thus undergoes normal homeostatic mechanisms of absorption. In this study, we aimed to investigate these issues by measuring accurately and precisely the increases in serum iron in volunteers following oral challenge with ferric (30 mg) trimaltol, and correlating iron absorption with body iron stores.

An analytical study was also carried out on a subset of serum samples from these volunteers. The accurate measurement of iron is vital in oral serum–time-curve absorption studies, since only small changes in serum iron are observed.[13] In addition, serum iron must be differentiated from contaminating haem iron arising from invisible haemolysis.[14] Under normal conditions, all absorbed iron is transferred to transferrin, so that estimates of absorption are based on the selective removal of iron from transferrin (and not from haemoglobin), which is then determined colorimetrically.[15] However, it is not known whether any of the iron from ferric trimaltol is absorbed as the complex and, if it is, whether this complex is detected using the conventional colorimetric method, because this requires the iron to be available for chromagen chelation.

In this study we have compared the conventional rapid, automated colorimetric method with a precipitation and thermal analysis method which is more time-consuming but reliably detects all serum species of iron.

METHODS

Absorption study

The absorption of iron from four formulations (see below) of ferric trimaltol was assessed in a double-blind, cross-over randomized study. Twelve healthy male volunteers aged 19–26 years were studied; all had normal liver and renal blood tests and blood haemoglobin concentrations < 13 g/dL. At between 7 a.m. and 8 a.m. after an overnight fast, an all-plastic indwelling cannula was inserted into a forearm vein and a baseline blood sample (10 mL) was taken for measurement of serum levels of iron and ferritin. A randomly assigned capsule of one of the four forms of ferric trimaltol was ingested with 100 mL of water. Subsequent blood samples were taken at 15, 30, 45, 60, 90, 120, 180, 240 and 300 min, during which time subjects remained recumbent. For each time point, an initial 10 mL of blood was discarded to minimize haemolysis, and a further 10 mL was then taken for serum iron analysis. Volunteers returned at intervals of exactly 1 week for similar study of the other three formulations.

Formulations of ferric trimaltol

Four different pharmaceutical formulations of ferric trimaltol (Adcock Ingram, Pharmaceuticals, South Africa; current manufacturers are Vitra Pharmaceuticals, Clavering, UK) were investigated (Table 1). All contained 0·54 mmol iron with similar 'inactive' ingredients (inactives), although capsule C also contained 160 mg maize starch, replacing some of the inactives (Table 1). All capsules were made of hard gelatin except for capsule D, which was cellulose starch; all were of similar colour, including 0·5 mg of the common colourant iron oxide, except for capsule B.

TABLE 1. *The four pharmaceutical formulations of ferric trimaltol*

Capsule code	Capsule type	Capsule contents*	Capsule colourant
A	Gelatin	Ferric maltol + inactives	Iron oxide 0·5 mg
B	Gelatin	Ferric maltol + inactives	Without iron oxide
C	Gelatin	Ferric maltol + inactives + maize starch	Iron oxide 0·5 mg
D	Cellulose starch	Ferric maltol + inactives	Iron oxide 0·5 mg

*The iron content of all four formulations was 0·54 mmol (30 mg iron as 23 ± 33 mg ferric trimaltol). For all formulations, inactives were lactose (155–295 mg), polyvinyl pyridine (0–10 mg), silicon dioxide (1–6 mg), magnesium stearate (2·5–3 mg) and sodium lauryl sulphate (0–2·7 mg). In capsule C, 160 mg maize starch replaced some of the lactose.

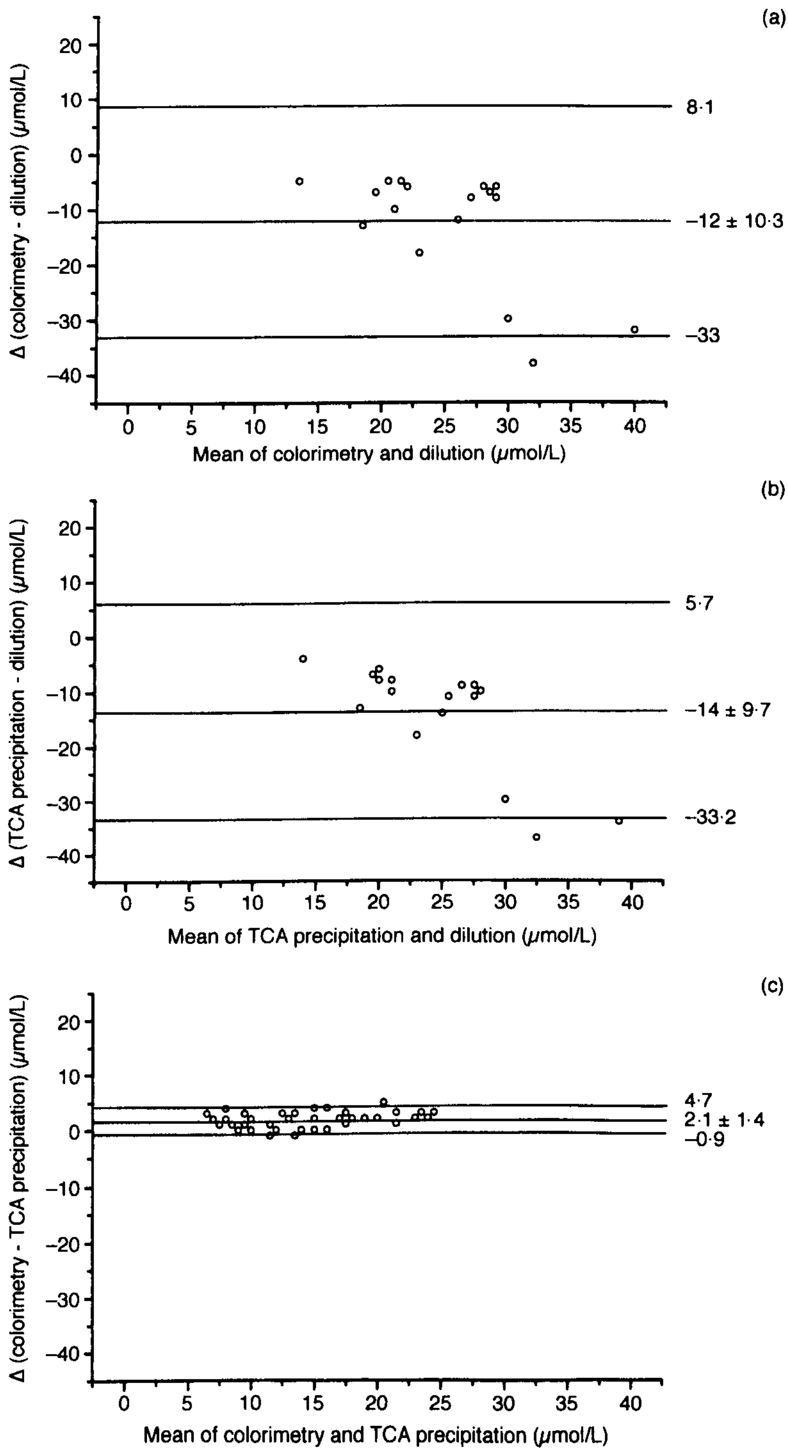

FIGURE 1. *Difference plots using the method of Bland and Altman[30] to compare the individual serum iron concentrations obtained after each method of preparation.*

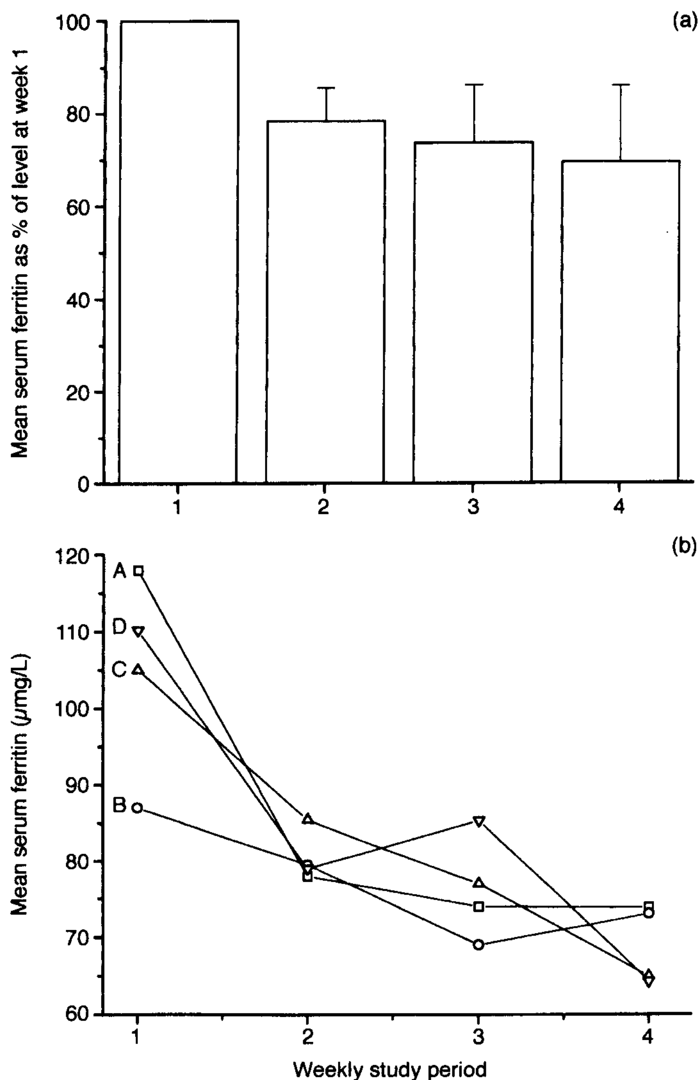

FIGURE 2. *(a) Mean (standard deviation) serum ferritin levels of volunteers over the 4-week study period, as a percentage of levels at week 1; the fall in ferritin levels is due to repeated venesection. (b) Mean serum ferritin levels for volunteers for four formulations (A to D) at each week (n=3 at each point). There was no bias between preparations in the fall in ferritin levels.*

Analytical study

Thirty-seven of the collected serum samples were randomly selected for analysis by colorimetry and thermal analysis using inductively coupled plasma optical emission spectrometry (ICPOES)[14] after protein precipitation. Seventeen of these samples with no visible haemolysis were also analysed by ICPOES following dilution (i.e. without the protein precipitation used to remove haem iron).

Sample preparation

Serum samples were prepared by protein precipitation using a modified[16] method of the International Committee for Standardization in Haematology.[17] Briefly, 100 mL 5% (w/v) of trichloroacetic acetic acid (TCA 'AnalaR', Merck, Poole, UK) was prepared in ultrapure water (UHP; Elga, High Wycombe, UK). Then 750 mg of an immobilized solid-phase hydroxypyridone iron chelator[18] was added, mixed by

shaking and left to stand for 24 h. The chelator was then removed by centrifugation and the TCA solution transferred to an acid-washed polypropylene container. The removal of iron from the TCA solution was confirmed by ICPOES (1·45 μmol/L iron pre-chelator; 0·016 μmol/L post-chelator). To new 10-mL polypropylene tubes (Elkay, Basingstoke, UK), were added 2 mL of the low-iron TCA solution and 0·5 mL of serum; the contents of the tubes were mixed by vortexing, left to stand for 20 min and then re-vortexed and centrifuged. Supernatants were pipetted into fresh 10-mL polypropylene tubes and analysed by ICPOES. Five sample blanks were prepared with 0·5 mL of UHP water in place of the serum sample using the above procedure and were found to contain 0·13 ± 0·014 μmol/L iron. Sample-based standards[17] were prepared by spiking aliquots of pooled supernatant samples with increasing concentrations of iron.

Iron chelation and colorimetry

Colorimetric measurements were carried out using a Unimate-5 *in vitro* diagnostics reagent system for the quantitative determination of iron in serum (Roche, Welwyn Garden City, UK). Briefly, Fe^{3+} was released from transferrin by denaturation with guanidine hydrochloride, then reduced to Fe^{2+} with ascorbic acid and complexed with Ferrozine for colorimetric measurement at 560 nm (Beckman CX7 Analyser; Kingsmead, High Wycombe, UK).

Dilution and ICPOES

Aliquots of serum (0·5 mL) were placed in new polypropylene tubes, diluted with 2 mL of 0·22 mol/L ultrapure nitric acid (PA Plus, Riedel de Häen, Seelze, Germany) and analysed by ICPOES. Five sample blanks were prepared as above using 0·5 mL of UHP water in place of the serum and were found to contain < 0·018 μmol/L iron. Sample-based standards were again prepared by spiking aliquots of the pooled supernatant samples with different concentrations of iron.

ICPOES

ICPOES analysis was carried out using a Jobin-Yvon JY24 spectrometer (Instruments SA, Longjumeau, France) equipped with a microconcentric nebulizer (MCN; Cetac Technologies, Omaha NB, USA) and Scott-type double-pass spray chamber.[18] The analytical wavelength used for iron was 259·94 nm with an aqueous detection limit of 0·018 μmol/L.

Comparison of stock iron standard solutions

Two different stock iron standard solutions were used for analyses. Acidic (0·5 mol/L HNO_3) iron (0·018 mol/L) nitrate (Spectrosol; Merck, Poole, UK) is conventionally used for ICPOES measurements, whereas iron (0·16 mol/L) ammonium ferrous sulphate hydrate is used for the colorimetric method. Two sets of standards (in 0·22 mol/L nitric acid) of equal concentration were prepared from each of the bulk iron solutions and compared by analysis using ICPOES.

Data analysis and statistics

For the analytical study, results are expressed as mean (standard deviation, SD). Difference plots[19] using the method of Bland and Altman[20] were used to compare iron concentrations by protein precipitation with dilution, colorimetry with dilution, and protein precipitation with colorimetry. Briefly, the differences (y-axis) of pairs of iron concentration values for each serum sample were plotted against their means (x-axis) for colorimetry versus dilution, protein precipitation versus dilution and colorimetry versus protein precipitation. The line of perfect agreement is $y=0$ and the extent of variation around this line indicates the extent of indeterminate (random) error. A significant deviation from $y=0$ indicates some determinate error between the two methods. Clear differences in iron concentrations were obtained when samples were analysed after preparation by colorimetry or protein precipitation compared to dilution. Results from protein precipitation versus colorimetry show good agreement (mean difference of 2·0 ± 1·4 μmol/L and limits of agreement of −0·9 to 4·7 μmol/L).

As a result of the weekly venesection, serum ferritin levels progressively fell each week and were compared using a paired t-test. For each volunteer, on each of the four occasions, serum iron values were plotted against time, and the peak serum iron (peak serum iron minus baseline level at $t=0$), or the mean rise in serum iron (from the area under the plasma curve), was calculated. The mean area under the curve was calculated as the mean increase in serum iron concentration between 0 and 6 h, as each subject was studied over the same time period. In addition, because of the different ferritin levels of each volunteer at each week, iron values were corrected using the following equation:

$$\log A_c = \log A_0 + \log F_0 - \log 40$$

where A_c is the ferritin-corrected mean or corrected peak serum iron value, and is derived from A_0, which is the observed mean or peak value, and F_0 is the observed ferritin value.[21] Mean rise and peak serum iron were compared for the four formulations using analysis of variance (ANOVA). Finally, the effect of body iron status, measured as ferritin values, on the absorption of iron from the different formulations was assessed using both between-subject regression based on subject means and ANOVA for studying within-subject regression.

Ethical approval for the study was received from St Thomas' Hospital Local Research Ethics Committee.

RESULTS

Analytical study

The concentrations of the iron standards prepared using ammonium ferrous sulphate (used in colorimetry) and the ferric nitrate solution used in ICPOES differed by 3%. All results were adjusted accordingly, although this small difference had negligible impact on comparisons.

Difference plots for iron are shown in Fig. 1. Comparisons of analyses from protein precipitation versus dilution (Fig. 1a) and colorimetry versus dilution (Fig. 1b) emphasize that, even in the absence of visible haemolysis, when samples were analysed without prior removal of haem-iron large variations in iron concentrations were obtained. Results from protein precipitation versus colorimetry showed good agreement (Fig. 1c), with a small and non-significant negative bias for analysis by protein precipitation and ICPOES compared to chelation and colorimetry (mean difference $2{\cdot}0 \pm 1{\cdot}4\,\mu$mol/L; limits of agreement $-0{\cdot}9$ to $4{\cdot}7\,\mu$mol/L). Colorimetry was therefore used to analyse serum from the absorption study.

Absorption data and analysis

The serum ferritin values of the volunteers decreased significantly over the 4-week period (Fig. 2a), but there were no differences in the rate of decrease of these ferritin values between the individual formulations, even when adjusted for differences between the subjects (Fig. 2b).

Plasma absorption curves showed that, for all formulations, serum iron values reached near maximum at 90 min but then slowly increased and/or reached a plateau by 5 h (Fig. 3). The apparent absorption of iron based on mean or peak values was not different for the four formulations either with or without ferritin correction (Fig. 4). Assuming plasma volumes of 2·5 L, the apparent minimum absorption of

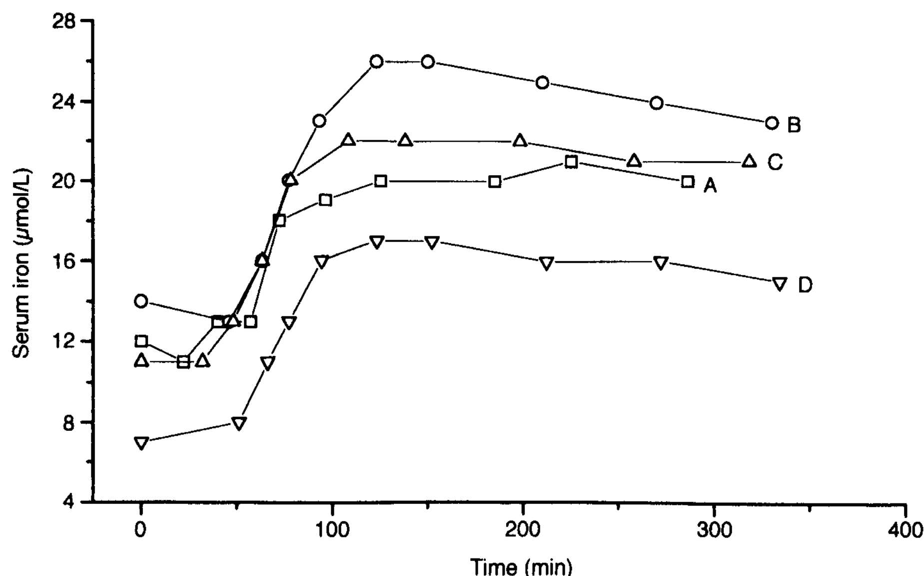

FIGURE 3. *Typical serum iron absorption curves from one volunteer following ingestion of four formulations (A to D) of ferric (30 mg) trimaltol. No differences were observed in absorption between different preparations.*

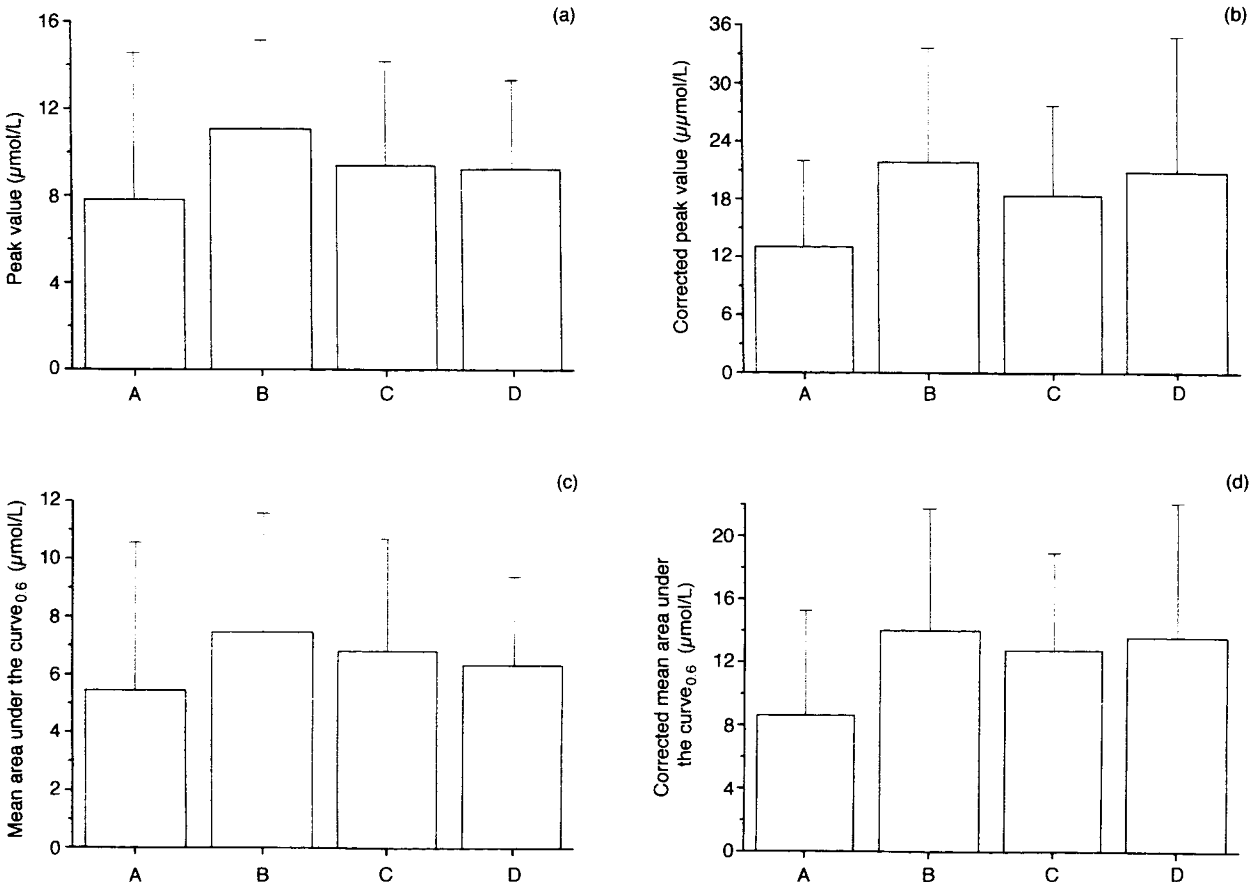

FIGURE 4. *(a) Mean (standard deviation, SD) serum iron following ingestion of four formulations of ferric trimaltol (A to D; see Table 1); peak absorption (peak level of serum iron minus baseline level at t = 0). (b) As for (a), but peak values corrected for differing iron status of volunteers, as outlined in the methods section. (c) Mean (SD) serum iron following ingestion of four formulations (A to D) of ferric trimaltol, shown as mean area under serum curve between 0 and 6 h as outlined in the methods section. (d) As for (c), but values of mean area under serum curve corrected for differing iron status of volunteers, as outlined in the methods section.*

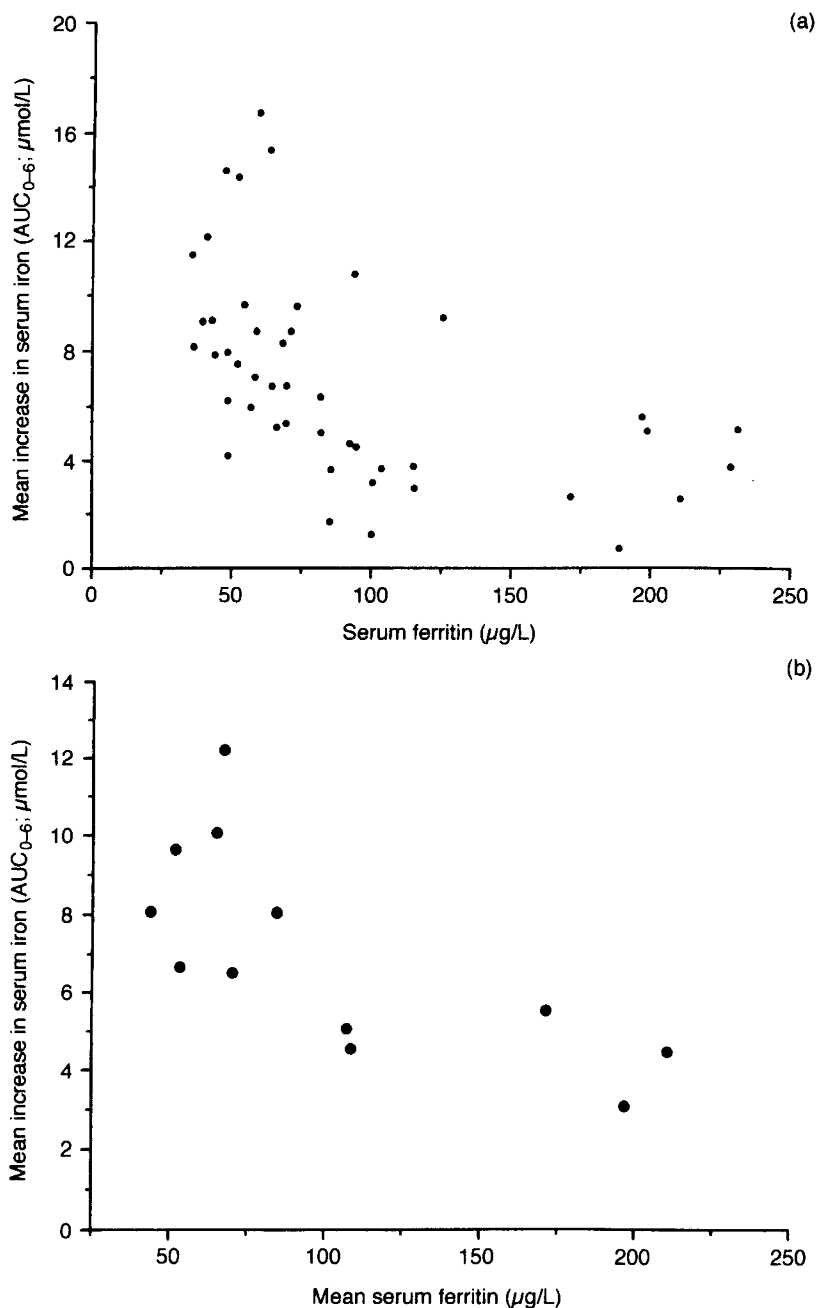

FIGURE 5. *(a) Correlation of areas under the serum iron curves (AUC_{0-6}) with serum ferritin levels for the 48 separate absorption studies. (b) Correlation of subject mean AUC_{0-6} values against their mean serum ferritin levels. Both AUC and ferritin means are based on absorption studies on four occasions for each subject. Analysis of variance (ANOVA) for within-subject regression, shown in (a), or regression between subject based on subject means, shown in (b), are inversely correlated* ($r = -0{\cdot}6$, $P<0{\cdot}001$, *and* $r = -0{\cdot}8$, $P<0{\cdot}001$, *respectively*).

iron into blood from the four formulations in the 12 volunteers, calculated from mean (SD) peak absorption, was 8·63% (6·3%), which is similar to that measured using whole body counting.[6]) However, throughout the study, the absorption of iron from ferric trimaltol clearly correlated with the iron status of the individual, based on their concomitant serum ferritin levels ($r = -0{\cdot}6$; $P < 0{\cdot}001$; Fig. 5).

DISCUSSION

The determination of serum iron by simple aqueous dilution without prior removal of haem iron is well established to be unsuitable, since contaminating haemoglobin iron is measured, and we confirm this finding even when the samples show no visible haemolysis. We therefore compared two analytical methods that distinguish between transferrin-bound iron and haem iron in volunteers, and studied serum iron levels after ingestion of ferric trimaltol (30 mg iron). Measurement of serum iron by precipitation and ICPOES showed a small insignificant negative bias compared with chelation and colorimetry. Previous studies[13,22] showed that, for serum samples with high levels of ferritin (<2000 μg/L[13] and <1200 μg/L[22]), iron levels were overestimated when prepared by protein precipitation rather than iron chelation; however, in both studies, methods were in agreement when levels were normal, as in our volunteers (35·5–230·9 μg/L). It is probable that differences were due to the precipitation method, which may not have been sufficient to liberate all the iron from transferrin. However, differences were small and, since colorimetry did not underestimate iron levels, this rapid and cheap method was used for iron analysis in the serum of volunteers or patients following ingestion of ferric trimaltol.

Maltol (3-hydroxy-2-methyl-4-pyrone) is a naturally occurring sugar derivative that is a product of caramelization and a common flavour-enhancing additive for food. This pyrone strongly chelates iron, thus rendering the metal soluble and potentially available to the mucosa at the physiological pH of the intestinal tract.[9–12] Our recent studies have shown that ferric trimaltol can be used successfully, even in patients intolerant for ferrous sulphate, in the correction of iron deficiency anaemia.[9] The complex is palatable, leads to few side-effects and provides iron in a form that is at least as well absorbed as that in ferrous sulphate.[6]

Iron absorption can be influenced by gender, recent food intake, age, physical activity and diet.[23–25] Hence, to minimize these variables, recumbent, young, fasting male volunteers were investigated at the same time each week. In addition, the co-ingestion of dietary agents frequently inhibits iron absorption.[23] Inhibitors of iron absorption are other metal ions, including iron itself, and non-absorbable dietary agents such as phytate and polyphenols.[24,25] However, in this study, low-dose iron oxide, maize starch and cellulose starch, which are typically used in pharmaceutical formulations, had no significant effect on the apparent absorption of iron from ferric trimaltol. In contrast, body stores of iron, as judged from the serum ferritin levels, had a strong influence on the absorption of iron, such that absorption increased sharply at ferritin levels below 100 μg/L. This is well known for dietary and ferrous preparations[26] but has not been shown previously for ferric iron. Our data suggest that iron from ferric trimaltol readily dissociates at the intestinal mucosa, because (1) the absorption of iron from this complex is controlled through normal iron acquisition mechanisms, and (2) all iron absorbed into serum from this complex is available for chelation in the colorimetric assay.

In conclusion, our study shows that iron is absorbed in a rapid and sustained manner from ferric trimaltol and further supports the use of ferric trimaltol as a suitable agent for oral iron therapy since its absorption is normally regulated by body iron stores.

Acknowledgements

We thank Adcock Ingram Pharmaceuticals for their help and support and Dr Michael Stockham for his advice, the Wellcome Trust for provision of the ICPOES facility, and the Special Trustees of St Thomas' Hospital for their continuing support.

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Accepted for publication 24 November 1999