Comparison of stable isotopes and radioisotopes in the measurement of iron absorption in healthy women

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1. Stable isotope methods are being used to investigate the absorption of dietary iron. In order to be certain that this new methodology is accurate, we have compared results obtained using stable isotopes and inductively coupled plasma mass spectrometry with those determined using a radioisotope and whole body counting.

2. The stable isotope $^{54}\text{Fe}$ (2.8 mg) was given to 10 healthy non-pregnant women. Six women received the isotope in aqueous form, and four took it with a meat meal. The $^{54}\text{Fe}$ served as a carrier for $^{59}\text{Fe}$. An ampoule (200 μg) of the isotope $^{57}\text{Fe}$ or $^{58}\text{Fe}$ was then given intravenously, and in serum samples taken over the next 10 h the ratios of the stable iron isotopes were measured by inductively coupled plasma mass spectrometry and the oral iron absorption was calculated. This was then compared with the results obtained by using a whole body counter to measure (on day 0 and day 14) the γ-activity emitted by the radioisotope.

3. The mean iron absorption measured by both methods ranged from 8% to 45%. Measurement of the post-absorptive serum enrichment of the stable isotopes provided estimates of absorption from both aqueous and food iron which were similar to that yielded by whole body counting, the mean difference being $-1.5\%$ (95% confidence interval $-5.2$ to $2.1\%$). Absorption estimated by stable isotopes exhibited the same inverse relationship with the serum ferritin level (body iron stores) to that known to exist with whole body counting. Similar estimates of food iron absorption were obtained irrespective of the type of isotope used as an extrinsic label, implying that stable isotopes are as valid as radioisotopes in reflecting intrinsic food iron absorption.

4. This study validates the use of stable isotopes and post-absorption curves as a new and accurate technique in the measurement of iron absorption.

INTRODUCTION

Owing to the perceived dangers of radioisotopes, especially in studies of infants and pregnant women, stable isotope methods have been used increasingly to evaluate many aspects of trace element metabolism [1]. We have used a stable isotope method in which one isotope was given orally and the other intravenously to measure the absorption of iron in pregnant women; the use of two isotopes allows for correction of variations in iron clearance. Sample analysis was by inductively coupled plasma mass spectrometry (ICP-MS) [2]. In order to be certain that the stable isotope method yielded accurate absorption measurements we have conducted a study to validate this new method against a well-accepted radioisotope and whole body counting method [3]. Concern has been expressed that the stable isotopes may not reflect the metabolism of the non-haem iron intrinsic to food [4]; unlike radioisotopes, stable isotopes have not been validated as extrinsic labels of food iron. Therefore we have measured the absorption of food iron which had been labelled with both radioisotopes and stable isotopes and given to non-pregnant women.

MATERIALS AND METHODS

Preparation of isotopes

The stable isotopes were obtained in the form of ‘iron wire’ (Techsnabexport, London). The abundances of the different iron isotopes, as measured by the manufacturer and by ourselves, were: enriched $^{54}\text{Fe}$, $^{56}\text{Fe}$ 99.85%, $^{56}\text{Fe}$ 0.13%, $^{57}\text{Fe}$ 0.02%, $^{58}\text{Fe}$ 0%; enriched $^{57}\text{Fe}$, $^{54}\text{Fe}$ 0%, $^{56}\text{Fe}$ 0.57%, $^{57}\text{Fe}$ 0.57%, $^{58}\text{Fe}$ 3.5%; enriched $^{58}\text{Fe}$, $^{54}\text{Fe}$ 0%, $^{56}\text{Fe}$ 0.21%, $^{57}\text{Fe}$ 6.56%, $^{58}\text{Fe}$ 93.23%. The natural abundances of isotopes in elemental iron are: $^{54}\text{Fe}$, 5.8%; $^{56}\text{Fe}$, 91.72%; $^{57}\text{Fe}$, 2.2%; $^{58}\text{Fe}$, 0.28% [5]. The
radioisotope $^{59}$Fe as ferric citrate was obtained from Amersham International (Amersham, Bucks, U.K.).

$^{54}$Fe for oral use was mixed with 0.5 mol/l H$_2$SO$_4$ (ratio 10 mg/ml) and heated to 50°C until dissolved. Ascorbic acid (at a final concentration of 3 mg/ml) and deaerated, deionized water were added, giving a final iron concentration of 2.83 mg in 5 ml. The solutions were sterilized by filtration into ampoules and were sealed under nitrogen. $^{57}$Fe and $^{58}$Fe for intravenous use were made up similarly, except that 10 mg of iron was mixed with 3 ml of 0.5 mol/l H$_2$SO$_4$ and the final Fe concentrations were 200 μg in 2 ml. The final pH of the intravenous solution was 1.7 to ensure stability during storage, but was diluted in saline immediately before injection.

Subjects
Ten healthy non-pregnant women between the ages of 39 and 43 years volunteered for the study. They had no history of any medical illness, and none was taking iron supplementation; all were non-smokers. All women had completed their families, and used a permanent method of contraception, either tubal ligation or vasectomy, hence the reason for asking them to volunteer for the radioisotope studies. All subjects gave their informed consent, and the study had the approval of the ethics committee of the Newcastle Regional Health Authority.

Administration of isotopes and labelling of food
For 3 days before and on the day of the study, each subject followed a diet plan to provide a daily intake of 13 mg of iron (on average 4 mg from meat). The diet plan was formulated by the Dietetic Department of the Royal Victoria Infirmary, Newcastle, with the food iron content determined from McCance and Widdowson’s food composition tables [6]. After an overnight fast, the subjects attended the Medical Physics Department of the Newcastle General Hospital. The subject’s height and weight was measured, and the serum was frozen until assay.

Isotope analysis
One hour after the administration of the radioisotope $^{55}$Fe, and in the interval between the 60 and 75 min serum samples, the subject was scanned on the whole body counter to determine the 100% administration level (after subtraction of background). Fourteen days later each subject returned to the Medical Physics Department and the oral absorption was calculated as the ratio of the 14 day retention to the 100% level, corrected for radioactive decay [3].

The stable isotopes of iron in each serum sample were measured by ICP-MS with appropriate quality control [2]. The isotope ratios were expressed by the ratio of $^{57}$Fe or $^{58}$Fe (the intravenously administered isotopes) to $^{56}$Fe, and also of $^{54}$Fe (the oral isotope) to $^{56}$Fe. For example, the basal $^{57}$Fe/$^{56}$Fe is 0.024 and 15 min after injection it might be 0.100.
Methods for iron absorption measurement

an enrichment of 0.076. The serum enrichments in
isotope ratios were plotted against time [10], and
from the area under these curves (AUC) the amount
of the orally absorbed isotope was calculated by the
formula as previously described [2]:

\[
\text{Oral absorption} = \frac{dose_{(v,i)} \times AUC_{(oral)}}{AUC_{(i,v)} \times dose_{(oral)}}
\]

The mean coefficient of variation in the measure-
ment of the \(^{54}\text{Fe}/^{56}\text{Fe}\) was 1.7\%, of the \(^{57}\text{Fe}/^{56}\text{Fe}\) was 2.2\%, and of the \(^{58}\text{Fe}/^{56}\text{Fe}\) was 4.6\%.

Other ferrokinetic aspects reflected in the curves
include the time \((T_{\text{max}})\) taken from isotope adminis-
tration to reach maximal oral isotope enrichment in
serum, and the serum half-time \((t_{1/2})\) of injected iron
isotope in the first 2 h after injection.

Statistics

Results are given as the mean and SD or mean
and 95\% confidence interval. \(T_{\text{max}}\) and \(t_{1/2}\) were
analysed using the log and inverse values, respec-
tively. Agreement between the two analytical methods
was tested as described by Bland and Altman [11].
Log transformation was used for absorption results,
but the differences, reflecting measurement error
rather than subject variation, did not increase as
absorption increased and so were not transformed.
Linear correlation was calculated between the log
absorption estimates and the log serum ferritin
levels [7].

RESULTS

Stable isotope measurement and absorption curve
analysis

Fig. 1 shows the isotope ratio enrichments of the
post-absorption serum curves for two subjects given
aqueous iron. The importance of using two isotopes
is demonstrated. Subject no. 4 had a higher overall
oral enrichment than subject no. 5 (oral AUC: 52.9
versus 36.1), but with similar initial intravenous
enrichments at 15 min (subject no. 4, 0.0780 subject
no. 5, 0.0743) and differing levels of iron clearance
\((t_{1/2}; \text{ subject no. 4, 119 min; subject no. 5, 58 min})\)
that gave different intravenous AUC (subject no. 4,
13.8; subject no. 5, 7.8) so that the oral absorption
of subject no. 4 was less than that of subject no. 5
(29.8\% versus 34.5\%). Mean \(t_{1/2}\) in all subjects was
92 min (95\% confidence interval 75–119 min). The
\(T_{\text{max}}\) was significantly longer in those subjects given
iron with food than in those given only aqueous iron
(mean difference 80 min, 95\% confidence inter-
val 31–149 min, \(P<0.01\)).

Stable isotope and radioisotope comparison

The measured absorption by each method after
the administration of aqueous and food iron is

shown in Table 1. Use of food or aqueous iron did
not reveal any significant bias in the differences, and
by combining the absorption measurements of both
the food and aqueous iron, the mean difference
between the two methods of measurement was
\(-1.5\%\) with a 95\% confidence interval of \(-5.2\) to
2.1\%. By plotting the mean difference between the
absorption measurements against the average of
these measurements for each subject (Fig. 2), it was
seen that the 'limits of agreement' between absorp-
tion measurements (mean \(\pm 2\) SD) lie between an
underestimate of 11.7\% and an overestimate of
8.6\%. These 'limits of agreement' would be experi-
mentally acceptable considering the wide range of iron absorption in normal individuals [11].

**Haematology and correlation with absorption estimates**

All patients had Hb concentrations greater than 12 g/100 ml, and all had a mean corpuscular volume greater than 85 fl. However, there were two subjects (nos. 4 and 6) with serum ferritin levels of 12 ng/ml or less, who also had elevated levels of ZPP (>3.0 µg of ZPP/g of Hb). There was a significant correlation between the log absorption estimates of both stable isotope and radioisotope methods and the log of the serum ferritin level ($r=0.78; P<0.05$ and $r=0.86; P<0.05$) (Fig. 3).

**DISCUSSION**

Whole body counting of orally absorbed radioactive iron is accepted as the reference method for iron absorption [12]. Use of the incorporation of oral and intravenous tracers into erythrocytes has been shown to have a close correlation with whole body counting [13, 14] and the use of only oral tracer data gave a poorer correlation because of the uncertainty of blood volumes and radio-iron utilisation in new erythrocytes [14]. The use of a double radioisotope technique with post-absorption serum measurements had the best correlation with whole body counting [14, 15], although debate has existed as to whether the post-absorptive serum measurement of therapeutic (>30 mg) doses of iron accurately reflects oral iron absorption [14, 16–19]. In order for the results of our studies investigating the absorption of iron during pregnancy to be accepted as accurate, a direct validation of the stable isotope methodology had to be undertaken. We found that the measurement of the post-absorptive serum enrichment of two iron stable isotopes provided estimates of iron absorption both from aqueous and food iron that were similar to that yielded by whole body counting. Furthermore, absorption estimated by stable isotopes exhibited the same inverse relationship with the serum ferritin level (body iron stores) to that known to exist with whole body counting [20–22]. By chance, a wide range of absorption was encountered in the study, and the relationship between the two methods held true across the entire range.

There has been debate over the use of stable isotopes as extrinsic labels of food iron. When radioisotopes are used as extrinsic labels, very little carrier iron (about 10 ng) is used to label the intrinsic pool of food iron, and while we used a similar method of labelling with a stable isotope to that described using a radioisotope [8], approximately 3 mg of stable isotope or 50% of the total iron content of the meal was used as a label. Theoretically, at least, this might be less exchangeable with the common pool of food iron [4, 23]. Whilst the production of food intrinsically labelled with $^{54}$Fe is possible [23], it is likely to prove expensive and was not possible within the time course or financial constraints of this study. Therefore, in an effort to test the reliability of the extrinsic labelling method, both stable isotopes and radioisotopes were used as extrinsic labels by addition to the same meal and the absorption estimates were compared. Ferric iron, the chemical form of the radioisotope, is less absorbable than ferrous iron (the form of the stable isotope) at high doses [24, 25]. However, only small doses of both forms were administered, and the
reducing effect of the ascorbic acid contained in the orange juice eliminated a potential difference in absorption due to differing valencies of the iron associated with each tracer.

When used as an extrinsic label of food iron, $T_{\text{max}}$ was significantly longer in the food iron absorption profiles than in those of aqueous iron. This suggested that the stable isotope mixed with the meal and was not absorbed in the manner of an aqueous iron dose. Furthermore, similar estimates of food iron absorption were obtained irrespective of the type of isotope used as an extrinsic label. This confirms that the stable isotope mixed and exchanged with the intrinsic food iron in a similar extent to the radioisotope; thus by inference, extrinsic stable isotopes are as valid as extrinsic radioisotope tracers [26] in reflecting intrinsic food iron absorption. Cook et al. [27] stated that the ratio of absorption derived from extrinsically and intrinsically labelled food was close to unity when the extrinsic tag was mixed with carrier iron of similar dose to our $^{54}$Fe dose.

The age-old obstetric dilemma regarding the need for the routine prescribing of iron supplements during pregnancy can only be solved when a safe, accurate and economically feasible method of measuring food iron absorption during pregnancy has been developed. The results of this study suggest that such a method is now available, and its application is under investigation.

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