# A double stable isotope technique for measuring iron absorption in infants

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A stable isotope technique has been developed which uses $^{57}$Fe and $^{58}$Fe as labels and which enables the simultaneous measurement of Fe absorption from two test meals in infants. The method was evaluated by measuring Fe absorption from a commercial whey-adjusted infant formula in nine healthy infants aged 13–25 weeks. Each infant was fed 210 ml formula, labelled with either $^{57}$Fe or $^{58}$Fe, on four consecutive mornings, in random order. The total Fe content in each feed was 2·5 mg Fe; either as 2·5 mg $^{57}$Fe, or 0·6 mg $^{58}$Fe plus 1·9 mg Fe with normal isotopic composition. Isotopic enrichment of Fe in erythrocytes was measured by thermal ionization mass spectrometry 14 d after the last administration, and Fe absorption was calculated based on isotope ratio shifts, total circulating Fe and intake of each isotope. Geometric mean absorption for the $^{57}$Fe and $^{58}$Fe labels was 6·72 and 6·58 % respectively, and the absorption of the two isotopes was not significantly different (Student's paired *t* test). By this technique, paired comparisons of Fe absorption can be obtained and systematic studies of the influence of dietary factors on Fe absorption during infancy can be conducted.

Iron: Stable isotopes: Infants: Thermal ionization mass spectrometry

Maintaining adequate Fe nutrition is a major problem early in life, and after weaning there is a high risk of Fe deficiency (Dallman *et al.* 1980). Although Fe absorption in adults is relatively well documented, very little information about Fe absorption and metabolism in infants is available (Fomon *et al.* 1989). This lack of data is largely due to the restrictive use of radioisotopes in infants because of ethical considerations and to the methodological problems involved in balance studies. The metabolic balance technique is not a useful approach for measuring Fe absorption because of the low fractional absorption of this element. Stable isotopes offer an alternative to radioisotopes and could be an ideal approach when studying mineral absorption in infants and other population groups for which radioisotopes should be restricted (Janghorbani & Ting, 1990).

Natural Fe has four stable isotopes ($^{54}$Fe, $^{56}$Fe, $^{57}$Fe, and $^{58}$Fe), two of which have a low enough natural abundance to be used as labels when measuring absorption; $^{57}$Fe (22 g/kg total Fe) and $^{58}$Fe (2·8 g/kg total Fe) (De Bièvre & Barnes, 1985). Fomon *et al.* (1988) have recently proposed a method using the incorporation of $^{58}$Fe into erythrocytes to measure Fe absorption in infants. However, they reported a relatively large inter-individual variation in Fe absorption suggesting the need for a technique where each infant acts as his

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or her own control. With only one isotope it is possible to make repeated administrations with a time lapse of at least 2 weeks between test meals. However, changes of Fe status in the rapidly growing child could influence absorption between administrations and therefore result in data which would be difficult to interpret. A double-isotope technique would thus be preferred and such a technique is routinely used in adult subjects using radioisotopes (Cook *et al.* 1972; Hallberg & Björn-Rasmussen, 1972). Using this technique, subjects with widely different Fe status can be studied since Fe absorption from two different test meals is measured simultaneously in the same subject.

The aim of the present study was to develop a double-isotope technique for measuring Fe absorption in infants using the stable isotopes $^{57}Fe$ and $^{58}Fe$. The isotopes were administered on consecutive days in identical feeds of infant formula and enrichment of erythrocyte haemoglobin was measured by thermal ionization mass spectrometry (TIMS) 14 d after administration.

## MATERIALS AND METHODS
### Preparation of labels

Elemental Fe enriched with $^{57}Fe$ (95·3%) was purchased from Medgenix Diagnostics (Ratingen, Germany). A total of 70·2 mg Fe was dissolved in 2 ml 7 M-$HNO_3$; 8·75 ml 0·5 M-$H_2SO_4$ was then added and the solution was heated to dryness at 120°. After further heating for 30 min at both 230° and 500° followed by cooling, the white powder was dissolved in 17 ml 0·2 M-$H_2SO_4$. To remove impurities the solution was filtered through a 0·5 μm Teflon filter (Millex-FH13; Millipore, Zürich, Switzerland). $Fe^{3+}$ was reduced to $Fe^{2+}$ by adding ascorbic acid (Merck, Darmstadt, Germany) at a molar ratio of 1:1 (Sevenich & Fritz, 1985) and the solution was finally diluted to 70 ml to give an Fe concentration of approximately 1 mg/ml.

The Fe concentration was measured by atomic absorption spectrophotometry (AAS; model 975; Varian Techtron, Mulgrave, Australia) and the isotopic composition by TIMS. The solution was de-aerated with $N_2$, dispensed into 5 ml Reacti-vials (Pierce, Rockford, IL, USA), and stored at 4° until administration. Each child received 5 ml of this solution (containing 5·075 mg total Fe, 4·836 mg $^{57}Fe$ and 0·122 mg $^{58}Fe$) added in two equal parts (2·5 ml/feed) to the two feeds of infant formula at the time of feeding.

Enriched $^{58}Fe$ was purchased as $Fe_2O_3$ (Oak Ridge National Laboratory, Oak Ridge, TN, USA). A quantity of 28·2 mg $Fe_2O_3$ was dissolved in 1·8 ml 10·2 M-HCl and 1 ml 14·5 M-$HNO_3$, followed by the addition of 3·5 ml 0·5 M-$H_2SO_4$. The solution was then heated as described for the $^{57}Fe$ label. The white powder was redissolved in 7·8 ml 0·2 M-$H_2SO_4$ and filtered. After addition of ascorbic acid (molar ratio 1:1) the volume was adjusted to 28 ml and the Fe concentration as well as the isotopic composition were determined as described for $^{57}Fe$.

The amount of $^{58}Fe$ which had to be administered to get sufficient enrichment of erythrocytes was much less than that for $^{57}Fe$ because of the lower natural abundance of $^{58}Fe$. In order to keep the volume and the Fe concentration of the formula constant, the $^{58}Fe$ solution was diluted with Fe of natural isotopic composition. A solution of $FeSO_4.7H_2O$ (Merck) with the same Fe concentration as the $^{58}Fe$ label and a content of 0·5 mol ascorbic acid/mol Fe was prepared. Weighed amounts of both solutions were mixed, de-aerated with $N_2$ and dispensed into 5 ml vials. A total of 5·09 ml solution (containing 5·075 mg total Fe, 1·196 mg $^{58}Fe$ and 0·131 mg $^{57}Fe$) was added to the two feeds (2·545 ml/feed) of infant formula administered to each infant.

Table 1. *Sex, age, weight and Fe status of subjects on day 1 of study*

| Subject | Sex | Age (weeks) | Weight (kg) | Haemoglobin (g/l) | Serum ferritin ($\mu$g/l) |
|---------|-----|-------------|-------------|-------------------|---------------------------|
| A | M | 22 | 7·80 | 117 | 16·6 |
| B | M | 22 | 7·56 | 114 | 1·0 |
| C | F | 15 | 6·00 | 112 | 12·6 |
| D | M | 25 | 6·92 | 106 | 1·0 |
| E | F | 14 | 6·60 | 112 | 73·6 |
| F | F | 20 | 7·31 | 109 | 36·2 |
| G | M | 22 | 8·02 | 109 | 40·0 |
| H | F | 15 | 7·53 | 113 | 29·1 |
| I | M | 13 | 6·00 | 128 | 85·6 |

## Subjects

Nine infants were recruited at a local health centre in Paris. All infants were apparently healthy and living at home with their parents. The sex, age, weight and Fe status of the subjects are shown in Table 1. Two infants had little or no storage Fe as indicated by the serum ferritin measurement and three had marginally low erythrocyte haemoglobin concentrations ($< 110$ g/l). The mean age of the children was 19 weeks (range 13–25 weeks) at the time of the administrations. All children were given a commercial whey-adjusted cow's milk formula with 8 mg Fe/l (Nidina 1; Nestlé, Paris, France) for a period of at least 3 weeks before the study. Some of the children were still partly breast-fed and small amounts of solids had been introduced into the diets of the older children. The study was approved by the ethical committee of the Centre de Recherche sur les Anémies Nutritionnelles, CNAM, Paris. The purpose of the study and all procedures were explained to the parents orally and by written information, and informed consent was obtained from all parents.

## Test meal

A whey-adjusted infant formula (PreBeba; Nestlé, Frankfurt, Germany) without added Fe was used for the administrations of stable isotopes. Ascorbic acid concentration was 80 mg/l and each feed consisted of 210 ml formula. The spray-dried powder was diluted with boiled, deionized distilled water according to the instructions given by the manufacturer (14 g powder plus 90 g water gives 100 ml formula). The formula was labelled with the enriched isotopes immediately before administration.

## Study design

On the mornings of days 1–4, each infant received one formula feed labelled with either $^{57}$Fe (2·418 mg) or $^{58}$Fe (0·598 mg). Each labelled formula was given twice, either on days 1 and 3 or days 2 and 4. The formula was given after an overnight fast and no food or liquid was allowed for a period of at least 2 h following each administration. The labelled test meals were given under close supervision of the investigators at the health centre. Preweighed feeding bottles and bibs were used to estimate losses during administrations. For standardization, the meal following each labelled test meal consisted of a portion of the whey-adjusted formula normally consumed by the infants (Nidina 1) and was provided by the investigators. Venous blood samples were drawn on days 1 and 18 for analysis of stable isotope ratios in erythrocytes. Tubes without any additives were used when drawing blood.

Blood samples were frozen and stored at $-18°$ until analysis. Body weight was registered on days 1 and 18.

### Preparation of blood samples for mass spectrometric analysis

All enriched blood samples taken at day 18 were analysed in duplicate. Baseline Fe ratios were determined on blood samples drawn on day 1 for six of the nine infants.

The samples of whole blood were mineralized using a microwave digestion system (MDS-81D; CEM Corporation, Matthews, NC, USA). Approximately 400 mg thawed blood was weighed into the 120 ml Teflon digestion vessels, 5 ml concentrated $HNO_3$ was added and vessels were closed on the capping station. In order to obtain a clear, colourless digest a two-step heating procedure was required. The first step involved a 14 min power ramp from 268 W to 595 W. After 5 min cooling time the vessels were vented and 2 ml $H_2O_2$ (300 ml/l) was added, followed by further heating for 5 min at 268 W and then 5 min at 536 W. The clear digest was transferred to Teflon tubes and evaporated to dryness under filtered $N_2$. The samples were dissolved twice in 5 ml concentrated HCl and heated to dryness before finally being taken up in 6 ml 5 M-HCl.

Fe was separated from matrix elements by anion exchange chromatography, using a modified method of Fasset *et al.* (1984). Econo columns (Bio-Rad Laboratories, Glattbrugg, Switzerland), inner diameter 7 mm, were filled to a height of 70 mm with anion exchange resin (Type AG1-X8, 200–400 mesh, Cl-form, Bio-Rad). The resin was washed with 60 ml 2 M-$HNO_3$ using an Ismatec IPN 4 peristaltic pump (Ismatec SA, Zürich, Switzerland) at a flow rate of 1 ml/min and then regenerated to the chloride form with 100 ml 1 M-HCl. After addition of the sample, the column was washed with 30 ml 5 M-HCl and the Fe was eluted with 20 ml 1 M-$HNO_3$. Total Fe was determined in the Fe fractions by AAS. Samples were then evaporated to dryness in Teflon tubes under a stream of filtered $N_2$ and redissolved in 0·1 M-$HNO_3$ to give a concentration of 3 $\mu$g Fe/$\mu$l.

To check for contamination of samples during processing, chemistry blanks were measured using an [57]Fe enriched spike (95·3%). A precisely known amount of spike was put through all the procedures used for sample preparation and the change of the isotopic composition was determined by TIMS.

All acids used were purified by sub-boiling distillation in a quartz still (Kürner Analysentechnik, Rosenheim, Germany). Other chemicals were analytical grade purity, except the $H_2O_2$ solution which was suprapure. Only ultra-pure water (18 M$\Omega$) was used (Milli-Q water system; Millipore AG, Zürich, Switzerland). To minimize contamination through vessel materials, only acid-washed quartz, Teflon and polyethylene containers were used during all procedures.

### Preparation of enriched standards

A set of standards was prepared for the enriched isotopes [57]Fe and [58]Fe. A standard reference solution (Merck) with a certified Fe concentration of 1·000 g/l was diluted with 0·1 M-$HNO_3$ to give a concentration of 25 $\mu$g Fe/ml. Enriched isotope solutions were prepared by dissolving [57]Fe-enriched metal and [58]Fe-enriched $Fe_2O_3$ in 0·5 ml 14·5 M-$HNO_3$ and 2 ml 10·2 M-HCl. The concentration of the enriched isotope solutions was determined by reverse isotope dilution, using the standard reference solution. Enriched standards were prepared by weighing the diluted standard reference solution and different quantities of enriched solutions into acid-washed Teflon vials. The enrichment for each set of standards was chosen to include a range of enrichments expected in the blood samples. Isotopic ratios of enriched isotope solutions, of the natural reference solution and of enriched standards

were determined by TIMS. Isotopic enrichment of standards was then calculated using the concentration and the isotopic composition of the natural reference solution and the pure spike solutions.

*Mass spectrometric analysis*

Isotope ratios were measured with a computer-controlled thermal ionization mass spectrometer (Model THQ; Finnigan MAT, Bremen, Germany) equipped with a secondary electron multiplier (SEM), a 13-sample turret and a reference pyrometer. Re filaments of $0.04 \times 0.7$ mm, purity $99.98\%$ (Wagner Analysentechnik, Worpswede, Germany) were cleaned by out-gassing in a bakeout device (Finnigan MAT) under vacuum at $4.5$ A for 20 min to remove impurities. Samples were loaded onto filaments in a laminar flow bench (Model Gelaire twin 30; Scan AG, Basel, Switzerland). During loading the filaments were heated by a power-supply (Finnigan MAT) to evaporate the acid. Fe was analysed using single filaments and a silica gel ionization enhancement technique (Götz & Heumann, 1987) slightly modified by addition of Al which increases the ion yield (Beer & Heumann, 1992). Silica gel was made according to the procedure of Barnes *et al.* (1973) and further concentrated by reducing the volume of the suspension to one-third. Silica gel (5 $\mu$l) was loaded onto the filament, heated to dryness at $1.2$ A and 1 $\mu$l of $Al(NO_3)_3$ solution (Merck) containing 3 $\mu$g of Al was added. Sample (2 $\mu$l; 6 $\mu$g Fe) was deposited into the white silica gel layer and dried at $0.8$ A. Finally, $0.5$ $\mu$l saturated $H_3BO_3$ solution was placed on the filament and heated for 1 min at $0.8$ A, $1.2$ A and $1.5$ A.

Samples were analysed automatically under computer control. The filament was heated to $1320°$ within 30 min to obtain a beam of 3–5 V on the SEM operated at an amplification of 25 relative to the Faraday detector. Three blocks with twelve scans each across the isotope pattern were collected in the peak jumping mode. Data were analysed for outliers by a Dixon test (Dixon & Massey, 1969). Measurements which had an internal standard deviation greater than the target precision of $0.5\%$ for the $^{57}Fe:^{56}Fe$ ratio and $1\%$ for the $^{58}Fe:^{56}Fe$ ratio were discarded and analysis was repeated.

Enriched standards were measured in triplicate, baseline and enriched blood samples once. The natural isotopic composition of Fe was determined on Fe isolated from blood (Reference material A-13; Animal blood; International Atomic Energy Agency, Vienna, Austria) and on Fe from an Fe standard solution (Merck). Enriched blood samples were always measured in close conjunction with two baseline samples which were averaged to give the baseline ratio.

The quantity of administered $^{58}Fe$ and $^{57}Fe$ (mg) incorporated into erythrocytes ($Fe_{(inc)}$) on day 18 was calculated in a manner similar to that used by Fomon *et al.* (1988):

$$^{57}Fe_{(inc)} = \frac{R^t 57:56 - R^o 57:56}{R^o 57:56} \times Fe_{(circ)} \times A_{57}, \tag{1}$$

$$^{58}Fe_{(inc)} = \frac{R^t 58:56 - R^o 58:56}{R^o 58:56} \times Fe_{(circ)} \times A_{58}, \tag{2}$$

where $R^t 57:56$ and $R^t 58:56$ are the enriched isotope ratios at time $t$ after administration of dose (day 18); $R^o 57:56$ and $R^o 58:56$ are the measured baseline ratios (day 1); $Fe_{(circ)}$ is the quantity of total circulating Fe (mg) at day 18; $A_{57}$ and $A_{58}$ are the measured natural abundances of $^{57}Fe$ ($0.0214$) and $^{58}Fe$ ($0.00287$) expressed as weight fractions.

The quantity of total circulating Fe (mg) was estimated as:

$$Fe_{(circ)} = BVol \times Hb \times 3.47, \tag{3}$$

where BVol is the blood volume (ml) assumed to be 65 ml/kg (Fomon *et al.* 1988), Hb is the haemoglobin concentration in g/ml, and 3·47 is equivalent to the concentration of Fe in Hb (mg/g).

The calculation is somewhat complicated by the fact that the $^{58}$Fe label contained a small amount of $^{57}$Fe and the $^{57}$Fe label contained a small amount of $^{58}$Fe. Absorption of $^{57}$Fe was calculated assuming that the contribution of absorbed $^{57}$Fe from the $^{58}$Fe label was negligible. Furthermore, 90% of absorbed Fe was assumed to be incorporated into erythrocytes (Rios *et al.* 1975):

$$^{57}\text{Fe}_{\text{(absorbed)}} \% = \frac{^{57}\text{Fe}_{\text{(inc)}}}{^{57}\text{Fe}_{\text{(dose)}}} \times \frac{100}{90} \times 100, \tag{4}$$

where $^{57}$Fe$_{\text{(inc)}}$ and $^{57}$Fe$_{\text{(dose)}}$ are given in mg. The amount of $^{58}$Fe coming from the $^{57}$Fe label and incorporated into erythrocytes was then calculated assuming identical absorption for the $^{58}$Fe and the $^{57}$Fe present in the $^{57}$Fe label:

$$^{58}\text{Fe}_{\text{(inc)}}** = {}^{58}\text{Fe}* \times \frac{^{57}\text{Fe}_{\text{(absorbed)}} \%}{100} \times \frac{90}{100}, \tag{5}$$

where $^{58}$Fe$_{\text{(inc)}}$** is the amount of $^{58}$Fe incorporated (mg) into erythrocytes (from $^{57}$Fe label) and $^{58}$Fe* is the amount of $^{58}$Fe administered with $^{57}$Fe label (mg).

The amount of incorporated $^{58}$Fe which came from the $^{57}$Fe label was then subtracted from the total amount of incorporated $^{58}$Fe:

$$^{58}\text{Fe}_{\text{(inc/corr)}} = \text{total } ^{58}\text{Fe}_{\text{(inc)}} - {}^{58}\text{Fe}_{\text{(inc)}}**. \tag{6}$$

$^{58}$Fe absorption was thereafter calculated as for $^{57}$Fe:

$$^{58}\text{Fe}_{\text{(absorbed)}} \% = \frac{^{58}\text{Fe}_{\text{(inc/corr)}}}{^{58}\text{Fe}_{\text{(dose)}}} \times \frac{100}{90} \times 100, \tag{7}$$

where $^{58}$Fe$_{\text{(inc/corr)}}$ and $^{58}$Fe$_{\text{(dose)}}$ are given in mg. Student's paired $t$ test was used to evaluate whether there was a significant difference for the Fe absorption obtained, using the two isotopes.

## RESULTS

### Mass spectrometric measurements

The results for mass spectrometric analysis of the enriched standards are given in Table 2. The average difference between the expected and measured ratios for $^{57}$Fe and $^{58}$Fe was within one standard deviation for ten repeated measurements of the natural ratio. This indicated that there was no systematic error in the mass spectrometric measurements.

An external precision of 0·6 and 1·1 % was obtained for Fe from the Merck standard for the isotope ratios $^{57}$Fe:$^{56}$Fe and $^{58}$Fe:$^{56}$Fe ($n$ 10). Fe isolated from animal blood was measured with an external precision of 0·7 and 1·6 % ($n$ 7). Careful control of the amount of silica gel on the filament and of the filament temperature was found to be essential to achieve high reproducibility. No fractionation correction was applied to the measured ratios as they were sufficiently stable and close to the reference natural values. The natural isotope abundances used for the absorption calculations were derived from isotope ratios

Table 2. *Isotopic ratios of enriched standards with different degrees of enrichment; expected values compared with measured ratios and the difference between the two values*

| | | Isotopic ratio | | |
| | Enrichment (%) | Expected | Measured | Difference (%) |
|---|---|---|---|---|
| | | $^{57}Fe:^{56}Fe$ | | |
| Fe enriched | 0 | | 0·022 87 | |
| with $^{57}Fe$ | 3·05 | 0·023 57 | 0·023 49 | −0·34 |
| | 4·99 | 0·024 01 | 0·024 08 | +0·29 |
| | 9·98 | 0·025 15 | 0·025 18 | +0·12 |
| | 36·98 | 0·031 33 | 0·031 48 | +0·48 |
| | Mean | | | +0·14 |
| | | $^{58}Fe:^{56}Fe$ | | |
| Fe enriched | 0 | | 0·003 005 | |
| with $^{58}Fe$ | 10·12 | 0·003 309 | 0·003 333 | +0·73 |
| | 29·95 | 0·003 905 | 0·003 913 | +0·20 |
| | 44·36 | 0·004 338 | 0·004 329 | −0·21 |
| | 299·7 | 0·012 007 | 0·011 947 | −0·50 |
| | Mean | | | +0·06 |

Table 3. *Measured isotopic abundances of Fe isotopes in enriched labels*

| Isotopic abundance (A) | $^{57}Fe$ label (atomic %) | $^{58}Fe$ label* (atomic %) |
|---|---|---|
| $A_{54}$ | — | 4·331 |
| $A_{56}$ | 2·335 | 70·652 |
| $A_{57}$ | 95·30 | 2·529 |
| $A_{58}$ | 2·37 | 22·488 |
| Atomic mass (Da) | 56·939 | 56·323 |

* $^{58}Fe$ was diluted as described on page 412.

measured in animal blood. The following values were obtained (atomic %): $^{54}Fe$, 5·83; $^{56}Fe$, 91·79; $^{57}Fe$, 2·10; $^{58}Fe$, 0·277. The isotope abundances determined for the $^{57}Fe$ and the $^{58}Fe$ labels are given in Table 3.

### Fe absorption measurements

The intake of each isotope (Table 4) was calculated from the amounts of ingested formula. Of the offered volume of formula, 73–99 % of that labelled with $^{57}Fe$ and 83–99 % of that labelled with $^{58}Fe$ was consumed by the infants. The amount of $^{57}Fe$ ingested with the $^{58}Fe$ label represented 2·4–3·6 % of the $^{57}Fe$ ingested with the $^{57}Fe$ label. The $^{58}Fe$ ingested with the $^{57}Fe$ label represented 7·8–11·4 % of the $^{58}Fe$ in the $^{58}Fe$ label. As it was not possible to correct for cross-contaminations for both isotopes, the correction was made for the $^{58}Fe$ isotope, as the contribution of $^{58}Fe$ from the $^{57}Fe$ label represented a larger fraction than *vice versa*.

The Fe isotope ratios measured after incorporation of the absorbed label into the erythrocytes are shown in Table 5. The mean value of the analysis of blood from six infants

Table 4. *Intakes of $^{57}Fe$ and $^{58}Fe$, calculated from the amount of ingested formula for each infant*

| | Total $^{57}Fe$ ingested from | | Total $^{58}Fe$ ingested from | |
| | $^{57}Fe$ labelled meal (mg) | $^{58}Fe$ labelled meal (mg) | $^{58}Fe$ labelled meal (mg) | $^{57}Fe$ labelled meal (mg) |
| Subject | | | | |
|---|---|---|---|---|
| A | 4·468 | 0·109 | 0·992 | 0·113 |
| B | 4·602 | 0·124 | 1·130 | 0·117 |
| C | 4·766 | 0·129 | 1·181 | 0·120 |
| D | 4·724 | 0·125 | 1·137 | 0·120 |
| E | 4·754 | 0·130 | 1·181 | 0·120 |
| F | 4·765 | 0·126 | 1·152 | 0·121 |
| G | 4·765 | 0·128 | 1·166 | 0·121 |
| H | 4·351 | 0·127 | 1·159 | 0·110 |
| I | 3·542 | 0·127 | 1·161 | 0·090 |

Table 5. *Fe isotope ratios measured in erythrocytes of infants 2 weeks after administration of $^{57}Fe$ and $^{58}Fe$ labels in the diet\**

(Mean values and standard deviations from thirty-three measurements of duplicate samples)

| | $^{57}Fe:^{56}Fe$ | | $^{58}Fe:^{56}Fe$ | |
| Subject | Mean | SD | Mean | SD |
|---|---|---|---|---|
| A | 0·024346 | 0·000061 | 0·003399 | 0·000014 |
|   | 0·024095 | 0·000061 | 0·003348 | 0·000018 |
| B | 0·025000 | 0·000050 | 0·003575 | 0·000012 |
|   | 0·024865 | 0·000030 | 0·003545 | 0·000010 |
| C | 0·024549 | 0·000050 | 0·003351 | 0·000006 |
|   | 0·024599 | 0·000055 | 0·003354 | 0·000016 |
| D | 0·024803 | 0·000058 | 0·003671 | 0·000012 |
|   | 0·025134 | 0·000052 | 0·003739 | 0·000014 |
| E | 0·022938 | 0·000048 | 0·003080 | 0·000008 |
|   | 0·023089 | 0·000052 | 0·003089 | 0·000012 |
| F | 0·022715 | 0·000130 | 0·003090 | 0·000020 |
|   | 0·022892 | 0·000085 | 0·003110 | 0·000011 |
| G | 0·023682 | 0·000097 | 0·003230 | 0·000012 |
|   | 0·023942 | 0·000050 | 0·003285 | 0·000008 |
| H | 0·025146 | 0·000038 | 0·003744 | 0·000009 |
|   | 0·025276 | 0·000064 | 0·003785 | 0·000012 |
| I | 0·023654 | 0·000054 | 0·003296 | 0·000007 |
|   | 0·023930 | 0·000095 | 0·003331 | 0·000018 |

* Mean base line ratios for six infants on day 1 were 0·02224 (SD 0·00011) for $^{57}Fe:^{56}Fe$ and 0·002923 (SD 0·000037) for $^{58}Fe:^{56}Fe$.

on day 1 was used as a baseline value for all subjects. External precision obtained was 0·5 % for the $^{57}Fe:^{56}Fe$ ratio and 1·3 % for the $^{58}Fe:^{56}Fe$ ratio ($n$ 6). Consequently, the smallest enrichment that could be resolved was 1·5 % for the $^{57}Fe:^{56}Fe$ ratio and 3·9 % for the $^{58}Fe:^{56}Fe$ ratio respectively ($3 \times SD$). Enriched blood samples were analysed in duplicate. Average difference between the duplicates was 0·7 % for $^{57}Fe:^{56}Fe$ and 1·0 % for $^{58}Fe:^{56}Fe$, with ranges of 0·4–1·3 and 0·1–1·8 % respectively.

Table 6. *Amounts of $^{57}Fe$ and $^{58}Fe$ incorporated into erythrocytes on day 18 and calculated fractional absorption of the isotopes*

| Subject | Incorporated label (mg) | | Absorbed label (% of dose) | |
| --- | --- | --- | --- | --- |
| | $^{57}Fe*$ | $^{58}Fe†$ | $^{57}Fe$ | $^{58}Fe$ |
| A | 0·3489 | 0·0783 | 8·68 | 8·77 |
| B | 0·4968 | 0·1133 | 12·00 | 11·14 |
| C | 0·3572 | 0·0624 | 8·33 | 5·87 |
| D | 0·4460 | 0·1265 | 10·49 | 12·21 |
| E | 0·1153 | 0·0267 | 2·70 | 2·51 |
| F | 0·0786 | 0·0309 | 1·83 | 2·98 |
| G | 0·2703 | 0·0574 | 6·30 | 5·47 |
| H | 0·5218 | 0·1454 | 13·33 | 13·94 |
| I | 0·2343 | 0·0601 | 7·35 | 5·75 |
| Mean‡ | | | 6·72 | 6·58 |
| +1 SD | | | 13·20 | 12·04 |
| −1 SD | | | 3·42 | 3·59 |

\* $^{57}Fe_{(inc)}$ not corrected for $^{57}Fe$ from $^{58}Fe$ label. For derivation, see p. 415.
† $^{58}Fe_{(inc)}$ corrected for $^{58}Fe$ from $^{57}Fe$ label. For derivation, see p. 416.
‡ Geometric mean.

The incorporated amounts of $^{57}Fe$ and $^{58}Fe$ on day 18 and the calculated fractional absorption of the administered labels are presented in Table 6. The mean of the two independent isotope ratio determinations was used in the calculations. The absorption of the $^{57}Fe$ label was in the range 1·83–13·33 % with a geometric mean of 6·72 %. For $^{58}Fe$, absorption values of 2·51–13·94 % were found, with a geometric mean of 6·58 %. No statistically significant difference was found between the absorption values of the two isotopes (Student's paired *t* test).

## DISCUSSION

The results from the present study demonstrate that it is possible to use a double stable isotope technique with $^{57}Fe$ and $^{58}Fe$ as labels to measure Fe absorption in infants. The mean geometric Fe absorption found in this study, 6·71 and 6·58 % for $^{57}Fe$ and $^{58}Fe$ respectively (range 1·83–13·94 %), is similar to earlier published results of Fe absorption by infants from an infant formula containing ascorbic acid.

Rios *et al.* (1975) measured Fe absorption in infants 4–7 months old from two infant formulas based on cow's milk and one based on soya bean containing 16–18 mg Fe (as $FeSO_4$)/l. Radioactive Fe ($^{55}Fe$) was used to label the feeds (120 ml) and Fe absorption was determined by measuring the radioactivity in the circulating erythrocytes 14 d after administration. No significant difference was observed in Fe absorption from the different formulas; geometric mean absorption was 3·9 and 3·4 % from the milk formula and 5·4 % from the soya-bean formula. A wide variation in Fe absorption was observed between different infants; 0·7–23·1 % of the dose was absorbed.

In the study by Stekel *et al.* (1986) using the same radioisotope technique, Fe absorption from several different $FeSO_4$-fortified cow's milk-based formulas (10–19 mg Fe/l) was measured in children aged 5–18 months. The geometric mean Fe absorption varied between 2·9 and 5·1 % for formulas without ascorbic acid, while the addition of ascorbic acid (100–800 mg/l) increased the mean absorption of Fe to 5·9–11·3 %. No individual Fe

absorption values are given in the article, but the range of Fe absorption for each group clearly shows the relatively large variation in Fe absorption between subjects.

These data taken together thus clearly demonstrate the need for a double-isotope method making it possible to use each infant as his or her own control when identifying dietary factors influencing Fe absorption.

*Analytical methodology*

External precision for measured Fe isotope ratios from standards and samples was in the range 0·5–0·7% for $^{57}$Fe:$^{56}$Fe and 1·1–1·6% for $^{58}$Fe:$^{56}$Fe. This precision was significantly better than the values published by Götz & Heumann (1988) who obtained an external precision of 1·2% for $^{57}$Fe:$^{56}$Fe and 5·3% for $^{58}$Fe:$^{56}$Fe with a quadrupole mass spectrometer, similar to the one we used, while Eagles *et al.* (1985) reported an external precision of 1·7% for the $^{58}$Fe:$^{56}$Fe ratio using fast atom bombardment mass spectrometry (FAB-MS). Fomon *et al.* (1988) measured Fe absorption in infants with $^{58}$Fe as a single label using inductively coupled plasma mass spectrometry (ICP-MS). These authors reported an external precision of 0·3–1·6% for the $^{57}$Fe:$^{58}$Fe ratio which was chosen because it gave a better precision than the $^{58}$Fe:$^{56}$Fe ratio. Using our technique, this ratio was measured with an external precision of 0·6%.

In the study by Fomon *et al.* (1988) the coefficient of variation (CV) for $^{58}$Fe absorption measurement was determined by analysis of three blood samples taken at 4, 8 and 12 weeks after administration of the label, assuming no change in the isotopic composition of Fe in the erythrocytes during this period of time. The resulting CV was between 1·0 and 30·5% depending on the amount of label absorbed. The smallest error was found for the highest Fe absorption (16·0%) and the largest error for the lowest absorption (3·3%.). In their study it was also shown that measured errors agreed well with the expected errors calculated based on the uncertainties in the determination of the baseline and enriched isotope ratios. Using their approach and the variation of our isotope ratio measurements we obtained CV of 5·2 and 5·6% ($^{57}$Fe absorption 13·3% and $^{58}$Fe absorption 13·9%) respectively. For the lowest measured absorption of 1·83% for $^{57}$Fe and 2·51% for $^{58}$Fe we obtained coefficients of variation of 30·4 and 23·3% respectively. These values are thus in the same order of magnitude as the values reported by Fomon *et al.* (1988). Furthermore, the values reflect the capability of ICP-MS and TIMS using a quadrupole mass analyser to measure the $^{57}$Fe:$^{58}$Fe ratio and the $^{58}$Fe:$^{56}$Fe ratio with approximately the same precision. However, if ICP-MS were to be used in conjunction with the double-isotope technique, the $^{58}$Fe would need to be measured relative to either $^{54}$Fe or $^{56}$Fe as the reference isotope, and it is not certain whether the ICP-MS technique could measure these ratios with sufficient precision when using commercially available instruments.

As isotopically pure labels are not available, the measured absorption values had to be corrected for cross-contamination of Fe isotopes. The correction was done for $^{58}$Fe. No correction was made for $^{57}$Fe absorbed from the meal labelled with $^{58}$Fe which resulted in a slight overestimate of the $^{57}$Fe absorption (equation 4). This can be further demonstrated by making the hypothetical calculations for a study in which the first meal is labelled with 5 mg $^{57}$Fe and the second meal with a $^{58}$Fe label containing 0·1 mg $^{57}$Fe. Assuming that the Fe absorption from the two meals is identical at 8%, 0·4 mg of $^{57}$Fe will be absorbed from meal 1 and 0·008 mg from meal 2. Absorption from meal 1, measured from total $^{57}$Fe incorporated into haemoglobin, will be 8·16 rather than 8%, resulting in an error of 2%. If the absorption of Fe from meal 2 labelled with $^{58}$Fe was 16%, and that from meal 1 remained at 8%, then the measured absorption of the $^{57}$Fe label would be 8·32%, giving an error of 4%. In the present study a correction was made for the $^{58}$Fe present in the $^{57}$Fe tracer. However, as the uncorrected $^{57}$Fe absorption was used to make this correction, a

small error was again introduced. This error is usually less than 1% and negligible compared with the much larger errors associated with the mass spectrometric measurement itself.

The precision of the absorption measurements and the lowest absorption that can be detected are determined by the precision of the mass spectrometric measurements. To obtain a better precision with the thermal ionization quadrupole mass spectrometer the ionization efficiency for Fe must be increased so that higher ion currents are obtained and the Faraday detector can be used. At the present time, however, no other technique is known to give higher ion yields than the mixture of silica gel and Al already used in the present study. With a high sensitivity magnetic-sector mass spectrometer equipped with a multicollector system for simultaneous detection of isotopes, Fe isotope ratios can be measured with a precision approximately two to five times better than with a quadruple instrument (Turnlund & Keyes, 1990). The lowest Fe absorption possible to measure can be calculated using the smallest enrichments, relative to the baseline ratio, which can be resolved. In our study the detection limit for shifts of the $^{57}Fe:^{56}Fe$ ratio was 1·5% and for the $^{58}Fe:^{56}Fe$ ratio 3·9%. In an infant weighing 7 kg (with 173·5 mg circulating Fe) the resulting detection limit for $^{57}Fe$ absorption would be 1·2% and for $^{58}Fe$ 1·8%. By improving the precision of the isotope ratio measurements to 0·1% for $^{57}Fe:^{56}Fe$ and 0·2% for $^{58}Fe:^{56}Fe$, by using a magnetic sector mass spectrometer, the detection limits for Fe absorption could be lowered to 0·24 and 0·27% respectively.

Improved precision of the isotope ratio measurements also increases the precision of the absorption measurement. This is especially important for studies where very low absorption values are to be expected. For example, for the lowest measured absorptions of 1·83% ($^{57}Fe$) and 2·51% ($^{58}Fe$) the calculated CV were 30·4 and 23·3% respectively, while with the improved precision these values would be reduced to 9·2% for $^{57}Fe$ and 5·3% for $^{58}Fe$. Another possible way to increase the precision of the double-isotope technique would be to give more label in order to increase the isotope ratio shifts. The present level of label, 5 mg $^{57}Fe$ and 1·2 mg $^{58}Fe$, was given over two meals each consisting of 210 ml formula. The two options available when trying to increase the isotope ratio shift would be either to increase the number of labelled meals or to increase the amount of isotope per meal. Neither of these possibilities was practically feasible since the amount of added Fe per meal (2·5 mg) was dictated by the normal Fe concentration of infant formula, and increasing the number of meals would have resulted in a more laborious study design. Furthermore, stable isotopes of Fe are expensive, approximately 30 Swiss francs per mg $^{57}Fe$ and 350 Swiss francs per mg $^{58}Fe$, and any increase of the dose would further increase the cost of the studies. Total cost of isotopes for a study including ten infants is approximately 5700 Swiss francs.

*Study design*

The design of the present study, using two administrations of each labelled test meal, was chosen to make it possible to keep the Fe concentration in the formula at the commercial level used in the US (12 mg/l) and to avoid the practical problems involved in administrations of large volumes of formula to young children. A volume of 210 ml of labelled formula was given at one feed on each of 4 consecutive days. An infant can consume this volume within a short time thus making the procedure of administration as convenient as possible for the mother and child. If a lower total Fe concentration were to be used in the formulas studied it would be necessary to give a larger volume per feed or to include a third administration of each test meal which would result in a more complicated study design when non-hospitalized infants are studied.

With the present double-isotope study design only two blood samples (approximately

1 ml each) are needed to determine Fe absorption from two different test meals. This is an advantage compared with repeated administrations of labelled feeds with 14 d in between when a total of three blood samples would have to be drawn. In studies of infants it is a major concern to keep the number of blood samples, as well as the blood volume drawn, as low as possible.

Each infant's blood volume was calculated at the time of the second blood sample by multiplying body weight (kg) by a factor (65 ml/kg). This factor has been used previously by Fomon et al. (1988) for calculations of blood volume in infants. The original data were reported by Bratteby (1968); in this study of erythro-kinetics the blood volume of six infants (113–138 d old) varied between 62·9 and 68·6 ml/kg body weight, resulting in a mean value of 65 ml/kg. In older children, or children with specific physiological conditions, another factor would be necessary. Direct measurement of blood volume is difficult in non-hospitalized infants due to ethical and practical considerations. Using our double stable isotope technique the measurement of Fe absorption from two test meals can be made with a very short time lapse between administrations and changes in blood volume will have a minimal influence on the results.

In our study, 90 % of the absorbed Fe was assumed to be incorporated into erythrocytes 14 d after intake. This value has previously been used by Rios et al. (1975). Very little information is available concerning the percentage of absorbed Fe that is incorporated into erythrocytes. Garby et al. (1963) found that about 85 % of an injected dose of $^{59}$Fe was present in erythrocytes 9–14 d post-injection in three infants 81 to 129 d old. No data are available for older infants and we therefore agree with Fomon et al. (1988) that there is no reason to doubt the assumption by Rios et al. that 90 % of absorbed iron is incorporated into erythrocytes.

As discussed above, the total amount of the Fe labels administered was 5 mg $^{57}$Fe and 1·2 mg $^{58}$Fe. These rather high amounts of isotopes are necessary to give the required precision for measuring the isotope shifts in infants with widely different capacities for Fe absorption. All test meals studied will consequently contain an added quantity of Fe which is not negligible. This is not a major concern when investigating the absorption of fortification Fe, but could lead to difficulties in the interpretation of Fe absorption from home-made weaning foods that are not normally Fe-fortified (e.g. vegetable purée, scrambled eggs, etc.). The increase of total Fe by the addition of stable isotopes is therefore a limitation compared with studies using radioactive isotopes where the total content of Fe is not increased by the addition of the label. The measurement of native food Fe absorption is much more complicated when using a stable isotope technique and would require incorporation of the stable isotope(s) in vivo into the food followed by administration over several meals or several days. The use of $^{58}$Fe as a label followed by analysis using a magnetic sector mass spectrometer would present the best possibility for such a study as with its higher precision it would permit the use of a smaller quantity of isotope over a short time period. However, in studies of infants a safe method which does not involve any risks associated with radiation is a necessity. The limitations of the stable isotope technique must therefore be carefully considered when designing studies of Fe absorption and the increased Fe content taken into account when interpreting the results.

The stable isotope technique for the study of Fe absorption from foods by measuring enrichment of erythrocytes is limited to studies in infants because of the considerable amounts of isotopes that need to be administered when studying adults. To enrich the haemoglobin in a 70 kg adult to the same extent as a 7 kg infant would require about ten times more isotope to be administered. These amounts of Fe are of such a magnitude compared with the normal level of Fe in diets consumed by healthy adults that they have to be regarded as Fe-supplements and not food Fe-fortification if given over only 1 d. It

would theoretically be possible to spread the intake of the labelled test meals over a longer period of time (several days or weeks) to overcome this problem, but the cost of the stable isotopes would be excessively high, approximately 57000 Swiss francs for ten adult subjects.

A further improvement of the double-isotope technique would be to include a reference dose of a third isotope of Fe, i.e. $^{54}$Fe. The inclusion of a reference dose would provide information which would make it possible to standardize the individual values of Fe absorption, e.g. to 40 % absorption of the reference dose for comparisons between different groups of subjects. This approach is used to compare Fe absorption using radioactive Fe in different studies in adults (Cook *et al.* 1972).

In conclusion, a double-isotope technique to measure Fe absorption in infants using stable isotopes has been developed. Two isotopes, $^{57}$Fe and $^{58}$Fe, were used to label test meals given on consecutive days and Fe absorption was calculated based on the incorporation of $^{57}$Fe and $^{58}$Fe in erythrocytes 14 d after administration. Isotope ratios were measured by TIMS. By this technique paired comparisons of Fe absorption from two different test meals can be obtained and studies of dietary factors such as phytic acid, ascorbic acid and Ca which influence Fe absorption in infants can be made.

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