

A comparison of iron absorption from single meals and daily diets using radioFe (^{55}Fe , ^{59}Fe)

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The purpose of the present study was to compare two measures of Fe absorption, one from single meals and the other from daily diets. Ten ileostomy subjects were given the same low-fibre composite diet for all three meals each day for five consecutive days. After 3 weeks the experiment was repeated with a high-fibre diet. The morning meal constituted one-seventh of the total daily diet intake, the mid-day meal two-sevenths and the evening meal four-sevenths of the total daily diet intake. On days 4 and 5 of each diet period the morning meal was labelled with ^{55}Fe and all three meals were labelled with ^{59}Fe . The activities retained in the subjects 19 d later showed the Fe absorption from the low-fibre diet measured from the morning meals to be almost 80% greater than the average Fe absorption measured from all meals during the same 2 d. With the high-fibre diet the absorption from the morning meals was less than 50% greater than the average for all meals but the difference was not significant. We suggest that all meals of the day should be labelled with radioFe in order to avoid inflating the measures of Fe absorption.

Iron: Non-haem-iron: Ileostomy

Fe deficiency has for a long time been one of the most prevalent nutritional deficiencies in the world (Expert Scientific Working Group, 1985). Fe absorption from food is dependent on the Fe requirements of the individual and on the amount and bioavailability of dietary Fe, which is affected by various dietary factors. It is important, therefore, to have accurate and reliable methods for measuring Fe absorption from both individual foodstuffs and whole meals. The method most often used for measuring Fe absorption is the retention of a radioisotope of Fe 2–3 weeks after the ingestion of a radioisotope-labelled foodstuff, meal or diet. This method was first described by Hallberg & Björn-Rasmussen (1972) who used two radioisotopes of Fe. The method was developed primarily for measuring Fe absorption from whole diets, but later became used increasingly frequently in the study of the effects of specific inhibitors on Fe absorption from single meals. However, it has been suggested that single-meal studies overestimate the effects on Fe absorption of enhancers and inhibitors in the diet (Cook *et al.* 1991). Recently, a variation of the single-meal method has been used (Cook *et al.* 1991; Gleerup *et al.* 1995) in which one or all meals each day for a period of up to 14 d were labelled with radioFe. In these studies, where diet was varied from meal-to-meal and day-to-day, the effects of inhibitors on the absorption of radioFe were less pronounced than those observed in single-meal studies. Cook *et al.* (1991) concluded that a plausible explanation for the difference between single-meal and whole-diet results was that 'the influence of dietary inhibitors and enhancers is diluted in a normal diet by meals that have no overall effect on non-haem-Fe absorption'. While it is possible also that the design of measurements of Fe absorption (single meal *v.* diet) affect the results

and their interpretation, this cannot be decided on the basis of the results of studies in which both the circumstances and the diets were varied at the same time.

The aim of the present study was to compare Fe absorption from a morning meal (labelled with ^{55}Fe) with Fe absorption from all three meals of the day (labelled with ^{59}Fe) on two consecutive days using the same diets in all meals. The experiment was repeated with both a low-fibre (LFD) and a high-fibre (HFD) diet.

SUBJECTS AND METHODS

Subjects

Ten subjects, two females aged 34 and 51 years and eight males aged 43–65 years (mean age 54.4 years), volunteered to participate in the experiment. The subjects were given written and oral information about the aims of the study and its design. The project was approved by the Ethical Committee of the Umeå University Hospital.

The subjects had been proctocolectomized for ulcerative colitis 8–29 years before the study (mean 16.0 years). A small segment of the small intestine had been removed, mostly less than 50 mm (range < 50–150 mm). No signs of inflammation or of renal, liver or thyroid disease were found in routine laboratory tests. Their body weight at the beginning of the study ranged from 64 to 113 (mean 82.2) kg and from 64 to 111 (mean 82.0) kg at the end of the 5-week study. There were no differences in mean body weight among the different periods.

Study design and diets

This comparative study of two measures of Fe absorption was performed within the framework of a larger study on the effects of the distribution of food intake during the day on mineral and sterol metabolism. The subjects were studied on an out-patient basis. During the first of two experimental periods, with five successive days for each period, they were given an LFD and during the second they were given an HFD. In each meal a nutritionally sufficient amount of Ca was available (< 200 mg), which has been shown previously to inhibit Fe absorption measured with radioFe (Hallberg *et al.* 1991). The two study periods were separated by 16 d. On days 1 and 2 of each 5 d experimental-diet period the subjects ate the experimental diet in their own homes according to explicit instructions. On days 3, 4 and 5 the subjects were admitted to the Research Ward and stayed overnight in a nearby hotel.

The LFD and HFD diets were the same except for the bread (Table 1). The bread in the LFD consisted of wheat-endosperm bread (70% extraction rate) and wheat crispbread (Wasabröd AB, Sweden), while the bread in the HFD was rye-bran bread and wholegrain rye crispbread (Wasabröd AB, Sweden). Each of the breads and crispbreads was baked in one batch.

The wheat-flour-based bread was supplemented with 2.37 mg Fe/l soft bread as $\text{Fe}_2\text{SO}_4 \cdot 7 \text{H}_2\text{O}$ in 100 ml 0.01 M-HCl added to the dough providing a total of 5.5 mg Fe per portion of bread. (One portion in the 8500 kJ energy level (see below) corresponds to two and one-third pieces of wheat-endosperm bread.)

The subjects completed 3 d dietary recalls before the start of the experiment. Based on their energy intakes for the 3 d dietary recall and the recommendations of the Food and Agriculture Organization/World Health Organization/United Nations University (1985) they were given test diets with one of three energy levels: 8500, 10600 and 12700 kJ/d for both LFD and HFD. These energy levels corresponded to 1.0, 1.25 or 1.5 portions of the diet. The diet (Table 1) was consumed by each subject in three meals each day. The breakfast meal consisted of one-seventh of the total daily energy intake, lunch of two-sevenths and dinner four-sevenths of the total energy intake during the day. The Swedish

Table 1. *The menus for the two test diets (one-seventh of a single daily portion; g)*

Item	Diet period 1	Diet period 2
Low-fat milk (0.5% fat)	100.0	100.0
Cheese (28% fat)	5.0	5.0
Margarine (Delikatess; 15% linoleic acid)	5.0	5.0
Smoked whitefish	20.0	20.0
Mashed potatoes, dehydrated	6.0	6.0
Tomato	10.0	10.0
Cucumber	10.0	10.0
Cottage cheese (4% fat)	10.0	10.0
Coffee (freeze-dried)	1.0	1.0
Sugar	2.0	2.0
Milk chocolate	3.6	3.6
Soft bread*		
Wheat-flour-based	20.4 (one-third of a piece)	—
Whole-rye-grain-based	—	25.8 (half a piece)
Crispbread		
Wheat-flour-based (Veteknäcke)	13.2 (one piece)	—
Whole-rye-grain-based (Husman)	—	13.0 (one piece)

* One piece of wheat-flour-based bread (61.1 g) contained (g): wheat flour 37, margarine 4, sugar 4, low-fat milk powder 4, yeast 2. One piece of rye-bran-based bread (51.5 g) contained (g): wheat flour 15, rye bran 18, margarine 3, sugar 3, low-fat milk powder 2, yeast 2. The wheat-flour-based bread was supplemented with 2.37 mg Fe/l soft bread added as $\text{Fe}_2\text{SO}_4 \cdot 7 \text{H}_2\text{O}$ in 100 ml 0.01 M-HCl to the dough, providing a total of 5.5 mg Fe per portion of bread.

Food Tables (Swedish National Food Administration, 1988) with a nutritional calculating program, Mats den flexible 3.0, were used to calculate the nutrient, fibre and energy contents of the test diets except for those in rye-bran bread and the wheat and rye crispbreads, for which values obtained by analysis were used (Table 2). The Fe and Ca contents of the diets were determined in our laboratory using an atomic absorption spectrophotometer (see p. 284).

During days 4 and 5 of each of the two experimental periods the subjects were given the breakfast meals labelled with ^{58}Fe and all three meals each day labelled with ^{59}Fe . The radio-Fe solutions were pipetted onto one piece of bread for each labelled meal and allowed to dry before being served. Both radionuclides were obtained as the chloride in 0.1 M-HCl (Amersham International Plc, Amersham, Bucks.) and diluted with deionized water (1:10, v/v). The amount of radioFe was related to the Fe content of the foods in order to obtain the same specific activity in each meal.

Blood samples, haemoglobin and ferritin determinations

Before and after each study period venous blood samples were withdrawn in the morning after an overnight fast to determine the blood haemoglobin and plasma ferritin concentrations. During day 3 of each of the periods blood samples were taken every hour during the day for metabolic measurements (not presented here). The same amount of blood was taken during the LDF and HDF periods. The haemoglobin concentration was determined spectrophotometrically at 540 nm using the cyanide method (International Committee for Standardization in Human Blood, 1965). One blood sample was allowed to stand for at least 15 min. It was then centrifuged and a plasma sample was taken in a glass tube and frozen at -70° . The ferritin concentrations were determined in the thawed plasma samples on the same occasion at the end of the experiment by the Clinical Laboratory of the University Hospital with an enzyme-linked immunosorbent assay using immunological

Table 2. *Composition of the test diets (amount per d for subjects given one portion)*†*

	Low-fibre diet	High-fibre diet
Protein (g)	99.4	105.5
Fat (g)	76.1	71.7
Carbohydrate (g)‡	234.9	235.6
Dietary fibre (g)	12.4	41.1
Total energy (kJ)*	8498	8450
From protein (%)	19.9	21.2
From fat (%)	33.1	31.4
From carbohydrate (%)	47.0	47.4
Fe (mg)	11.8	10.2
Ca (g)	1.47	1.44

* Other subjects were given either 1.25 or 1.50 times these amounts depending on which was closest to their ordinary energy intake.

† All values except Fe and Ca were calculated using the menus in Table 1 and data from Svenska Livsmedelstabellen. The values for Fe and Ca were determined on freeze-dried samples of the diets using flame atomic absorption spectrometry in our laboratory. The dietary fibre in wheat flour and rye bran was determined by an enzymic method as described by Asp *et al.* (1983).

‡ Excluding dietary fibre.

reagents obtained from DAKO (Copenhagen, Denmark) and calibrated against World Health Organization Standards 80/578. During the experiment a total of 525 ml blood was taken from each person for haemoglobin and plasma ferritin determinations and ^{55}Fe and ^{59}Fe activity measurements as well as other measurements pertinent to the main body of the study.

Determination of dietary iron, calcium, inositol phosphates and fibre contents

Six samples of 1 g each of the freeze-dried homogenized daily diets were taken in borosilicate tubes and ashed for 2 d at 550°. The ash was dissolved in 1 ml 3 M-HCl and evaporated to dryness in an oven. The ash was dissolved again in 0.5 ml 3 M-HCl, left to stand overnight and diluted with 2 ml water. The samples were further diluted with 0.6 M-HCl as necessary for determination of the Fe concentrations. For the determination of Ca concentrations, La_2O_3 in 0.6 M-HCl was added to a final concentration of 10 g La/l. Both Fe and Ca concentrations were determined using a Varian Model AA-6DB atomic absorption spectrophotometer with an air-acetylene flame. Known amounts of Fe in 0.6 M-HCl and Ca in 0.6 M-HCl containing 10 g La/l were used as references. All HCl used was Supra-pure® (Merck).

Dietary fibre was analysed using the enzymic method of Asp *et al.* (1983).

Measurement of iron absorption

Whole-body activity measurements

Before the first diet period, possible background activity at the gamma energies of ^{59}Fe in each subject was measured using the whole-body counter at the University Hospital in Umeå, Sweden. The counter is mounted in a chair geometry with an APGC detector with a relative efficiency of 36%. At the time of the whole-body measurement, a blood sample was withdrawn to determine the activities of ^{55}Fe and ^{59}Fe . At 19 d after the administration

of a radioFe-labelled test diet the whole-body and blood activity measurements were repeated and the activity from the previous measurement was subtracted as background. $^{55}\text{Fe}:^{59}\text{Fe}$ in the blood sample was multiplied by the whole-body retention of ^{59}Fe to calculate the whole-body retention of ^{55}Fe .

Blood activity measurements

Analyses of ^{55}Fe and ^{59}Fe in blood were made using the method of Eakins & Brown (1966) as described by Bothwell *et al.* (1979) and modified as follows:

(1) In the original method blood samples are wet-ashed with H_2SO_4 , HNO_3 and HClO_4 , while in the present study blood samples were dry-ashed. Samples of 10–20 ml blood containing no more than 10 mg Fe, as determined from haemoglobin content, were placed in 50 ml borosilicate-glass beakers. The blood was dried for 48 h starting at 80° and increasing slowly to 120° . The temperature was then raised very slowly to and maintained at 550° in order to ash the blood samples. The total ashing time was 48 h. The samples were allowed to cool and the ash was dissolved in 5 ml concentrated HCl and 1 ml concentrated H_2SO_4 . The solution was heated on a hot plate in a fume hood until white fumes were produced and then maintained at this temperature for another 10 min. The samples were then allowed to cool, 10 ml concentrated HCl was added and the solution was heated without boiling until approximately 5 ml remained. The solution was then filtered through a sterile filter (Sterile Acrodisc $0.2\ \mu\text{m}$; Gelman Sciences, Ann Arbor, MI, USA) into a 18×150 mm siliconized borosilicate glass tube using a 1 ml disposable syringe. The beaker, syringe and filter were rinsed five times with 1 ml 3 M-HCl. The necessity for this filtration procedure is as yet untested.

(2) Step 2.20 (stirring of the precipitate). After mixing the precipitate with water in the tube, it was allowed to stand overnight. This step allowed colloidal particles to precipitate before centrifugation.

(3) Insta-Fluor[®] liquid scintillation cocktail (Packard Instrument Company, Downers Grove, IL, USA) was used instead of Aquasol[®]. The activities of ^{55}Fe and ^{59}Fe in blood samples thus prepared were measured using a liquid-scintillation counter (LKB Wallac 1214 Turku, Finland).

Method error and precision

In order to ascertain the precision of the dry-ashing method for the determination of ^{55}Fe and ^{59}Fe activities in blood and the possible systematic error in the method, three 10 ml blood samples were taken from each subject at the time of the measurement of the absorption of the reference doses 3 weeks after their administration. Duplicate samples were prepared on two separate occasions using the dry-ashing method developed in the present study, while the third sample was wet-ashed at the National Veterinary Institute, Uppsala, Sweden with H_2SO_4 , HNO_3 and HClO_4 according to the original method (Frank, 1976). The ^{55}Fe and ^{59}Fe activities in all three samples were then measured on the same occasion according to step 3 in the method described previously. There was no statistically-significant systematic difference in the value obtained for the duplicate samples which were dry-ashed on two separate occasions for either radioisotope or for the ratio of their activities ($t\ 0.64\text{--}1.35$, $P > 0.05$). The precision of the measurement of ^{55}Fe was 2.3%, for ^{59}Fe it was 1.6% and for $^{55}\text{Fe}:^{59}\text{Fe}$ it was 1.5%. The values for the duplicate dry-ashed samples were then combined into a mean value and these means were tested against the values obtained with the wet-ashed samples for the same subject and radioisotope or ratio. The values for the ^{55}Fe and ^{59}Fe activities and their ratio obtained with the two methods were very closely and significantly correlated with each other ($r > 0.98$, $P < 0.001$). The values obtained from the wet-ashed samples were on average slightly higher (^{55}Fe 1.0 (SE

0.91%) and ^{59}Fe 2.0 (SE 0.80) %) than the corresponding values for the dry-ashed samples. While the mean difference between the values obtained for the ^{59}Fe activity with the two methods was significant (t 2.91, $P = 0.017$), the mean difference for the ^{55}Fe activity and $^{55}\text{Fe} : ^{59}\text{Fe}$ were not (t 1.38 and 1.96 respectively, $P > 0.05$).

Reference doses

The day after the third whole-body measurement the subjects received reference doses of both ^{59}Fe and ^{55}Fe on two consecutive mornings after a 12 h overnight fast. Fe^{2+} (3 mg) labelled with both radioisotopes was administered as FeSO_4 together with 30 mg ascorbic acid in 10 ml 0.01 M-HCl. The subjects drank the solutions directly from the vials and the vials were then rinsed three times with water which was also consumed. No food or drink was allowed for 3 h after the dose was given. At 19 d after the reference dose was given the whole-body and blood sample measurements were repeated. Each subject received a total of 273 kBq ^{59}Fe and 390 kBq ^{55}Fe in the test diets and 25 kBq ^{59}Fe and 39 kBq ^{55}Fe in the reference doses.

Statistical analysis

Because the distribution of the plasma ferritin concentration was skewed, the data are presented as geometric means and their standard errors and the statistical tests on mean ferritin concentrations were performed using the logarithms of the ferritin values. The differences among time-points for ferritin and blood haemoglobin concentrations were tested using a one-way ANOVA for repeated measurements within subjects (Snedecor & Cochran, 1980). If the one-way analysis was significant, differences between haemoglobin and plasma ferritin were tested using a paired t test. Differences between measures of Fe absorption (^{55}Fe and ^{59}Fe) were also tested using a paired t test. Pearson product-moment correlation coefficients were calculated for retention of ^{55}Fe and ^{59}Fe . In each of the statistical tests we chose to reject the null hypothesis at the 5% level ($P \leq 0.05$).

RESULTS

The blood haemoglobin and plasma ferritin concentrations were measured as indices of Fe status (Table 3). While the haemoglobin appeared to have decreased slightly during the latter half of the experiment, the decrease was not significant. The plasma ferritin concentration decreased significantly with the duration of the experiment and the amount of blood taken as samples.

The retention of radioFe from the LFD single meal given in the morning on two consecutive days gave on average 78% higher absorption values compared with those obtained for the retention of radioFe from the same diet served at every meal on both days (Table 4). The values for the absorption of Fe obtained from the two measurements were significantly correlated (r 0.77; $P = 0.009$; n 10).

During the second study period the same design was used and the same subjects were given an HFD. With the HFD the retention from the single morning meal was 48% higher than that from the 2 d diet. This measure was not significant nor was it significantly different from the increase with the LFD. Again the two measures of Fe absorption correlated well with each other (r 0.89; $P = 0.001$; n 10).

The absorption of ^{55}Fe and ^{59}Fe given together as reference doses on two consecutive days at the end of the study was essentially identical (absorption ratio 1.00 (SE 0.001)).

Fe absorption from either of the diets or from the reference doses was not significantly correlated with the plasma ferritin concentration. No significant correlation was found within individuals for the absorption of Fe from the two diets whether they were measured from the morning meals or all meals on two consecutive days.

Table 3. Values for iron status variables 3 d before the beginning and 1 d after the end of each of the low- and high-fibre diet periods*

(Means with their standard errors, except geometric means with their standard errors for ferritin)

	Low-fibre diet				High-fibre diet				Statistical analysis: ANOVA		
	Before		After		Before		After				
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	F	df	P
Haemoglobin (g/l)	146.7	2.30	149.0	3.24	143.3	2.32	142.1	3.09	2.88	3	0.055
Ferritin ($\mu\text{g/l}$)	60.3 ^a	1.29	49.0 ^b	1.30	42.6 ^{b,c}	1.32	36.3 ^c	1.32	17.99	3	< 0.001

^{a, b, c} Means with unlike letter superscript letters were significantly different ($P \leq 0.05$).

* For details of diets and procedures, see Tables 1 and 2 and pp. 282–286.

Table 4. Individual results of iron absorption (% retention of radioFe) for the low- and high-fibre diets and the reference dose*

(Means and with their standard errors)

Subject no.	Fe absorption								
	Low-fibre diet			High-fibre diet			Reference dose (3 mg Fe)		
	⁵⁵ Fe	⁵⁹ Fe	⁵⁵ Fe: ⁵⁹ Fe	⁵⁵ Fe	⁵⁹ Fe	⁵⁵ Fe: ⁵⁹ Fe	⁵⁵ Fe	⁵⁹ Fe	⁵⁵ Fe: ⁵⁹ Fe
1	1.05	1.04	1.01	2.00	0.78	2.58	46.63	46.07	1.01
2	3.85	1.25	3.07	2.16	2.92	0.74	43.36	42.81	1.01
3	4.62	1.93	2.40	3.95	2.37	1.67	43.71	44.01	0.99
4	2.68	1.16	2.31	2.99	0.99	3.02	58.77	58.07	1.01
5	1.85	1.05	1.76	0.66	0.90	0.73	64.22	64.30	1.00
6	4.67	3.57	1.31	4.50	3.70	1.22	46.89	48.23	0.97
7	2.40	1.69	1.42	3.11	2.76	1.13	28.82	28.51	1.01
8	0.98	0.78	1.26	2.31	2.24	1.03	37.37	37.04	1.01
9	4.25	2.47	1.72	3.11	2.89	1.08	89.02	89.71	0.99
10	2.35	1.52	1.54	9.73	6.23	1.56	82.57	81.86	1.01
Mean	2.87 ^a	1.65 ^b	1.78	3.45	2.58	1.47	54.13	54.06	1.00
SE	0.44	0.27	0.2	0.48	0.51	0.24	6.15	6.18	0.004

^{a, b} Means with unlike superscript letters in the low-fibre-diet period were significantly different ($P < 0.01$).

* For details of diets and procedures, see Tables 1 and 2 and pp. 282–286.

DISCUSSION

Measurement of the absorption and retention of radioisotopes of Fe from single meals has been for some time the method of choice for the assessment of the effects of factors which enhance or inhibit Fe absorption. In the present study the single-meal design was compared with a modified design in which all three meals of the same diet as that consumed as a single meal were labelled with a radioisotope of Fe during two consecutive days. The absorption values obtained for the LFD with the single-meal technique (apparent absorption 2.87%) were on average almost 80% higher than those obtained for the same diet with the modified technique (apparent absorption 1.65%). The Fe absorption from the HFD measured with the single meal technique was less than 50% higher than that measured with the modified

technique and the difference was not significant. As the absorption of the reference doses of ^{55}Fe and ^{59}Fe was almost identical, there is no reason to believe that a method error was responsible for these systematic differences in Fe absorption from single meals and whole-day diets. The differences between the ratio of the two absorption measurements from the LFD and the HFD (1.78 v. 1.48) was not significantly different. As the present study was made within the framework of a larger study which was not designed primarily to compare the LFD and HFD, no crossover was used. The Fe status of the subjects, as reflected in the plasma ferritin concentration, decreased between the LFD and HFD periods mainly due to blood sampling. This as well as other unnoticed changes may have occurred between the test periods but it is unlikely that the change in Fe status is the main cause of the comparatively low absorption ratio during the HFD period.

There may be several explanations which either separately or combined may explain the differences observed between the results of the single-meal design and the all-day design. One of them may be that the single meal was given after a 12 h period of fasting (overnight), compared with 4 h without food before the lunch and dinner meals. This difference in the length of the fasting period may have caused greater absorption from the breakfast meal than the average for the same diet throughout the whole day. This would appear to contradict the results of Bjorn-Rasmussen *et al.* (1974) who found a higher absorption of Fe from lunch and dinner compared with that of a breakfast meal. The meals in that study, however, were not identical. The lunch and dinner contained fish and meat, which are known to stimulate Fe absorption, and the breakfast contained more phytic acid, which is known to inhibit Fe absorption.

A second factor which may explain the differences in results is that the single meal given in the morning represented only one-seventh of the total daily intake and, therefore, only one-seventh of the total Fe intake. In the present study the ratios of Fe, Ca, phytic acid and fibre to one another were equal in all meals in order to avoid the possibility that differences in absorption between the single meal and all-day design could be caused by differences in relative proportions of enhancers and inhibitors among meals, as in the studies of Glerup *et al.* (1995) and Cook *et al.* (1991). As the intake of Fe in the morning was comparatively low, this may have given rise to a higher Fe absorption from the morning meal.

On the basis of the results of the present study, we recommend that radioFe-labelled test diets should be given at all meals during the test day(s) and not as single meals in the morning after an overnight fast as the values obtained with the single-meal technique may be inflated. In some situations the single-meal technique may exaggerate the effects of dietary factors on Fe absorption as suggested by Cook *et al.* (1991). Therefore, we recommend that the results of single-meal studies as a measure of Fe absorption should be interpreted with caution.

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