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A Comparison of Physical Properties, Screening Procedures and a Human Efficacy Trial for Predicting the Bioavailability of Commercial Elemental Iron Powders used for Food Fortification

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Abstract: Elemental iron powders are widely used to fortify staple foods. Experimental evidence indicates that there is considerable variation in the bioavailability of different products. For some powders, it may be too low to permit a significant impact on iron status. This study was designed to evaluate possible approaches to screening commercial iron powders for predicted bioavailability, to identify products that have the potential to improve

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iron status, and to ascertain whether bioavailability is related to the method of manufacture. Nine commercial iron powders were allocated to one of five types based on the production process; carbonyl, electrolytic, hydrogen-reduced (H-reduced), carbon monoxide-reduced (CO-reduced), and other reduced. Structure by scanning electron microscopy and physical properties (pycnometric and apparent density, particle size distribution, Fisher subsieve size, and surface area) were determined on all samples. Selected samples (one or more of each type depending on the cost of the assay) were then subjected to five screening procedures that have previously been advocated for predicting bioavailability in humans - dissolution rate in 0.1 mol/L HCl, dialyzability and Caco-2 cell iron uptake, both after simulated *in vitro* gastrointestinal digestion, relative bioavailability (RBV) with respect to ferrous sulfate by the AOAC rat hemoglobin repletion method, and plasma iron tolerance tests in human volunteers. The results for particle size distribution, surface area, Fisher subsieve size, dissolution rate in 0.1 mol/L HCl, and RBV in rats were significantly correlated and consistent for powders of the same type. However, values for different powder types were significantly different. There was no correlation between either dialyzability or Caco-2 cell uptake and the predicted bioavailability estimates based on the physical properties, dissolution rates, RBV in rats, or human efficacy data. Although human plasma iron tolerance tests were in general agreement with the other measures of predicted bioavailability, they did not provide information that would have improved the precision of bioavailability estimates based on physical properties, dissolution in HCl and/or RBV in rats. Our observations indicate that the dissolution rate in 0.1 mol/L HCl under standardized conditions is highly predictive of potential bioavailability and that it would be the most practical approach to developing a reliable and sensitive screening procedure for predicting and monitoring the bioavailability of commercial elemental iron powder products.

Some, but not all, of the carbonyl and electrolytic iron powders had the highest predicted bioavailability values. The predicted bioavailability for the reduced iron products was lower and variable, with the lowest values being recorded for the carbon monoxide and other reduced iron products. Two powder types were selected for a human efficacy trial, electrolytic (because it is the iron powder type recommended by WHO) and hydrogen-reduced (because of its widespread use). Electrolytic/A131 and H-reduced/AC-325 had relative efficacies compared with ferrous sulfate monohydrate of 77% and 49%, respectively, based on the change in body iron stores in Thai women with low iron stores, who received an additional 12 mg iron per day, six days per week for 35 weeks in wheat-based snacks.

We conclude that there is significant variability in the bioavailability of the commercial iron powders that we evaluated (those used for food fortification at the time that our studies were initiated), and that bioavailability is related in part to production method. The bioavailability of some carbonyl and electrolytic iron powders may be adequate for effective food fortification. The reduced iron powders that we tested are unlikely to have an adequate impact on iron nutrition at the fortification levels currently employed, although preliminary analysis of a new H-reduced product indicates that it may be possible to improve the bioavailability of individual powders of this type of product. We did find significant differences among products in both the electrolytic and carbonyl categories. Therefore, all products should be screened rigorously.

Key words: Elemental iron, food fortification, bioavailability, screening procedure

Introduction

Iron deficiency continues to be a serious public health concern, particularly for women and children in developing countries [1]. Fortification of staple foods and other dietary products is considered to be an effective long-term strategy for combating nutritional iron deficiency [2]. Elemental iron powders are the fortificants used most widely in wheat flour and other cereal products, including infant foods [3], because the cost of iron in this form is lower and it is likely to affect the storage properties and shelf life of fortified flour, or the color and taste of the final food products. However, several investigators have questioned the adequacy of information on the bioavailability of commercial elemental iron powders [2, 4, 5].

The published experimental evidence related to the bioavailability of elemental iron powders was reviewed recently by a multidisciplinary task force [3]. Estimates of bioavailability varied markedly and the task force was, for several reasons, unable to draw definitive conclusions about currently available commercial iron powders . Comparability between different methods used for predicting

bioavailability in the published studies has not been established. Experimental powders that are unlikely to have been representative of commercial products were evaluated in many of the experiments. The tests were all carried out between 1971 and 1991 and may therefore not reflect the bioavailability of today's commercial powders. The task force recommended that a series of experiments be undertaken to evaluate the bioavailability of currently available commercial iron powders and that comparisons be made between the results obtained by different screening procedures.

The most accurate estimates of bioavailability are derived from absorption measurements in human volunteers employing isotopic tags [2]. This approach has been used to measure the bioavailability of soluble iron salts and chelates because the compound of interest can be prepared from isotopically labeled iron in a form that is identical to the commercial product. Iron powders, on the other hand, consist of small particles with variable surface morphology and internal porosity that is determined by the manufacturing process. Absorption depends on the dissolution of the iron in gastric juice, which is in turn determined by the rate and extent of oxidation of the elemental iron from the Fe^0 state [6]. The rate of oxidation is markedly influenced by physical properties, such as particle size, internal porosity, and surface area as well as surface chemistry. The rate and extent of dissolution is likely to have a biologically important effect on the amount of iron available for absorption. The validity of bioavailability estimates derived from the absorption of isotopically labeled elemental iron powders is questionable because it has not been feasible to prepare labeled products with the exact physical characteristics of their commercial counterparts [3].

It is therefore necessary to turn to animal models and indirect methods to predict the bioavailability of elemental iron powders. The AOAC rat hemoglobin repletion test [7] is a well-established method for measuring the relative bioavailability (RBV), with respect to ferrous sulfate, of iron compounds commonly used to fortify foods [2, 8–10]. With a few exceptions there is good agreement between RBV by the AOAC rat hemoglobin repletion assay and RBV based on human absorption studies employing isotopically labeled iron salts. The task force concluded that this test provided valid data on the bioavailability of commercial elemental iron powders in use at the time the studies were conducted. On the other hand the variability in the products tested, methodological protocols, and results of measurements of the physicochemical characteristics, solubility, and dialyzability made it difficult to draw definitive conclusions about bioavailability from these screening procedures [3]. Furthermore, there was no information relevant to iron powders on two other potential screening procedures, Caco-2 cell uptake, and plasma iron tolerance tests in human volunteers. Systematic comparisons between different screening methods and human absorption measurements were made in only one study that included one type of elemental iron powder, an experimental electrolytic iron preparation [10]. The investigators in this study concluded that the AOAC rat hemoglobin repletion method provided the most reliable prediction of bioavailability in human beings.

In response to the recommendations of the task force [3], SUSTAIN initiated a series of collaborative studies to evaluate the potential bioavailability of nine commercial iron powders that were used for food fortification at the time the project was initiated. The powders were classified by the method of manufacture (Table I). Specifications for three production methods (carbonyl, electrolytic, and reduced) are given in the Food Chemicals Codex (FCC) [11]. Different manufacturers use generally similar methods to produce carbonyl and electrolytic powders, although process variations exist. Reduced iron powders are manufactured by three distinct processes: hydrogen reduction (H-reduced), carbon monoxide reduction (CO-reduced), and other reduction (Atomet products). Each reduced powder type has different physical properties [3]. The selected commercial elemental iron powders were therefore assigned to one of five groups, carbonyl, electrolytic, H-reduced, CO-reduced, and other reduced, and are referred to throughout this document by manufacturing process/product name (e.g. Carbonyl/Ferronyl).

The primary objectives of the study were to compare potential screening procedures by applying them to aliquots of the same batch of each powder and to determine whether there is significant variation in the bioavailability of currently available commercial iron powders. The morphological characteristics, physical properties (pycnometric and apparent density, particle size distribution, Fisher subsieve size, and surface area), and the following screening procedures were evaluated: dissolution rate in 0.1 mol/L HCl, dialyzability and Caco-2 cell uptake, both after simulated *in vitro* gastrointestinal digestion, RBV by the rat AOAC hemoglobin repletion method, and human plasma iron tolerance tests. Cost made it necessary to limit the number of powders submitted for evaluation by the rat hemoglobin repletion and human plasma tolerance tests. However, at least one powder of each type was tested by each method.

The screening procedures predicted a threefold difference in the bioavailability of the iron powders, which was determined primarily by method of manufacture. Dissolution in dilute HCl was selected as the most promising screening test on the basis of both the scientific observations and pragmatic considerations related to cost and its potential use as a means of predicting and monitoring the bioavailability of different iron powders. Further evalua-

tion of this test was therefore carried out to provide preliminary information about its reproducibility in different laboratories and to determine optimal parameters for a standardized test protocol.

Finally, the conclusions drawn from the bioavailability screening studies were tested by measuring the absorption of two powders (Electrolytic/A131 and H-reduced/AC-325) in a six-month efficacy trial in women with low iron stores.

It is important to note that this was an exploratory project designed in part to identify the best options for screening and monitoring the bioavailability of elemental iron powders. Investigators were therefore selected because of their expertise in performing specific screening procedures. They were supplied with masked samples and asked to provide bioavailability predictions by using the protocols considered optimal in their respective laboratories. Although the powder aliquots supplied were identical, no attempt was made to assure uniform sample preparation prior to testing in the individual laboratories because the requirements for each of the procedures vary so much. For example, direct addition of iron powders is the best approach for measuring dissolution in dilute HCl, while dialysis and Caco-2 cell uptake of iron powders require suspension in a food matrix and are customarily performed after *in vitro* simulated gastric and pancreatic digestion.

Three additional commercial powder samples were acquired after the studies of the original sample pool were under way. They were submitted to selective screening. This work is referred to and described briefly as "additional studies" and has been included because the results provide further insight into the potential variations in the bioavailability of elemental iron powders of the same type.

A significant part of the work has already been published [12–16]. Our aim in this report was to assemble both the published and unpublished information into a single manuscript that both addresses the objectives outlined above and interprets the relationships between the previously reported observations.

Research Methods

Sample Pool

Industry donors provided samples (approximately 25 kg) of nine commercial elemental iron powders that were being used for food fortification at the time the study was initiated. A 50-kg sample of bakery grade ferrous sulfate monohydrate was also acquired for use as the control in most of the screening procedures and the human efficacy trial (Table I). All samples were supplied with certificates of analysis indicating that they met FCC specifications. The date of manufacture was March 2001 or later in all cases with the exception of Electrolytic/IMP-Electrolytic (March 2000) and Other reduced/Atomet 75 (May 2000). Homogeneous distribution of the bulk materials was ensured by repeated tumbling of the original containers before partition of the powder samples into 42 aliquots of different sizes, thirty-six 30-g, five 2.3-kg, and one 1-kg sample. The aliquots were taken at multiple levels from the pooled mixed material (Seedburo Sampling Probe, Glison Co.). The 30 g aliquots were placed in sealed plastic containers, which were stored together with a desiccant in one-gallon metal containers in a temperature-controlled laboratory. The 2.3 kg and 1 kg aliquots were also placed in plastic containers and stored with a desiccant in individual one-gallon metal cans. Samples were coded and shipped to participating laboratories in a masked fashion with a desiccant.

Three additional iron powders were made available for selected testing after the original sample pool had been assembled. Two were commercial products (Carbonyl/CF

Table I: Iron Powder Sample Pool[1]

Manufacturing Process	Product Name	Company	Country
Carbonyl	Ferronyl	ISP	USA
Carbonyl	OF	BASF	Germany
Electrolytic	A131	SCM Metal Products [now North American Höganäs High Alloys]	USA
Electrolytic	IMP-Electrolytic	IMP	India
CO-reduced	MH300.29	Höganäs AB	Sweden
CO-reduced	RSI-325	Höganäs AB	Sweden
H-reduced	AC-325	North American Höganäs	USA
Other reduced	Atomet 75	QMP	Canada
Other reduced	Atomet 95SP	QMP	Canada
Ferrous Sulfate Monohydrate (USP/FCC)	$FeSO_4.1H_2O$	Crown Technology	USA

[1] Iron powders are referred to by manufacturing process/product name (e.g. Carbonyl/Ferronyl).

and Other reduced/Atomet 195SP). The third was a new H-reduced iron powder (H-reduced/Hi-Sol), which was developed by North American Höganäs as a hydrogen-reduced iron powder with improved bioavailability.

Morphological Characteristics and Physical Properties

The *surface morphologies* of all of the powders were examined under a Topcon ABT-32 Wet 3D scanning electron microscope after applying the powders to aluminum sample stubs covered with a piece of electrically conducting copper tape. The *cross-sectional morphologies* were examined on powder samples suspended in epoxy resin and then metallographically polished. A Micrometrics AccuPyc 1330 automated pycnometer, calibrated with standardized stainless steel spheres, was used for the determination of *pycnometric density* (a measure of the true density of the particles, where volume is determined by quantifying the induced pressure difference when a known volume of pressurized gas is introduced into a sample cell containing the powder. The result is not affected by the extent of the open porosity in the particles). *Apparent density* was measured with an Arnold Meter according to Metal Powder Industries Federation Standard 48. *Particle size* was measured in a Horiba LA-920 Laser Scattering Particle Size Distribution Analyzer after dispersion of the iron powders in distilled water and alconox. Fraunhofer algorithms were employed for the calculation of particle size. Particle size was also measured with a *Fisher Subsieve Sizer* that employs a gas permeability method to determine the average particle diameter of iron powders. *Surface area* was measured by a method that depends on the condensation of a monolayer of gas molecules of known size on the sample surface. The surfaces on the interiors of the particles with open porosity are included. The Brunauer, Emmet, Teller (BET) model is used most frequently to calculate surface area based on the quantity of gas condensed and resultant sample pressure. The iron powder samples were outgassed for 2 hours at 200° C at a pressure of < 0.05 mm Hg. Measurements were made with a Coulter SA3100 (5 pt BET; adsorbate, nitrogen), a Micromeritics Tristar 3000 (5 pt BET; adsorbate, nitrogen), or a Micromeritics Flowsorb 2300 (1 pt BET; adsorbate, nitrogen/helium) surface area analyzer.

Dissolution Assays

The initial dissolution assays were based on the method of Shah *et al* [17] as modified by Forbes *et al* [10]. A preliminary series of studies was undertaken to determine the effect of pH and time on the rate of dissolution. Unbuffered HCl (0.1 and 0.02 mol/L) was used for observations at pH 1 and 1.7 respectively. A citrate buffer was employed for experiments in which the pH was adjusted to 2 or 3. Dissolution was measured at timed intervals for up to 150 minutes at 37° C. The dissolution patterns varied for the different iron powders [12] and the exploratory experiments demonstrated that the best separation (data not reported) and correlation with rat RBV results (Figure 1) was obtained after 30 minutes at a solvent pH of 1.0 using unbuffered HCl (0.1 mol/L).

These preliminary experiments provided the basis for developing a standardized dissolution test protocol that was used for all subsequent assays. An accurately weighed iron powder sample (~ 50 mg) was placed in 250 mL 0.1 mol/L HCl (pH 1.0) and incubated at 37° C with constant stirring (nonmagnetic stirrer, 150 rpm). At precisely 30 minutes an aliquot sufficient to obtain ~ 20 mL solution was removed and immediately filtered through a 0.22 micrometer HCl-resistant filter with sufficient pressure to attain a flow rate of 7–15 mL/min/cm^2. A 10-mL aliquot of the filtrate was immediately diluted to 100 mL with deionized water. Iron concentrations were measured by ICP-AES (ASTM E 1479-99, American Society for Testing and Materials, http://www.techstreet.com/inf/astm.tmpl).

A collaborative study was conducted to evaluate the repeatability and reproducibility of the method by submitting 14 masked duplicate samples of seven powders to two universities and seven industrial laboratories, together with a detailed description of the method. Local equipment availability necessitated minor adjustments to the protocol in some laboratories.

It has been suggested that storage under poorly controlled environmental conditions, particularly high ambient temperature and humidity, may affect the acid solubility and absorption of iron powders. We therefore carried out a preliminary study to evaluate the effects of four different environmental variables (time, temperature, humidity, and airflow) on samples of four powders (Car-

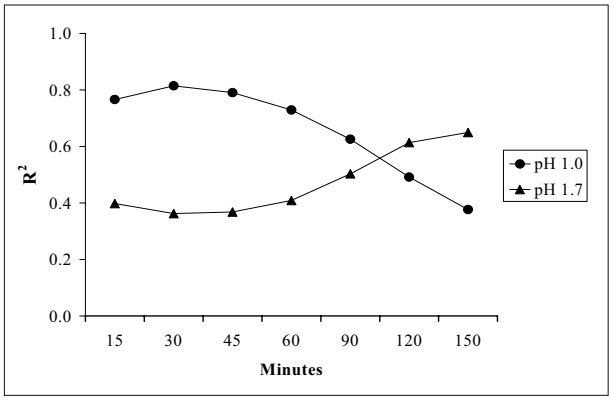

Figure 1: Predictability of RBV (rat AOAC method) by solubility at pH 1.0 and 1.7.

bonyl/Ferronyl, Electrolytic/A131, H-reduced/AC-325, and Other reduced/Atomet 95SP). Each powder was exposed to eight different environments (temperature: room temperature 22–24° C (72–75° F) or elevated temperature 35° C (95° F); relative humidity: 33% or 85%; and air flow condition: stagnant or flowing) in modified desiccator chambers. Temperature was regulated with thermocouple-controlled 100 W heater cartridges and humidity with saturated $MgCl_2$ and KCl salt solutions. Airflow was allowed to be stagnant (no air exchange) or flowing (one full air changeover every 6 hours) by running compressed air through a flow meter. Powder samples were evaluated at baseline and after 1, 2, 4, 8, 16, 32, and 64 days.

Dialyzability after Simulated *in vitro* Gastrointestinal Digestion

Seven powders and ferrous sulfate monohydrate drawn from the SUSTAIN sample pool were evaluated using the method of Miller *et al* [18] as modified by Hurrell *et al* [19]. Unfortified hard wheat semolina was purchased from a local supermarket (Migros, Zürich, Switzerland) and ground in a centrifugal mill using a titanium sieve (0.25 mm). The resulting flour (9.5 µg/g Fe; 0.2% phytic acid) was used for all the dialyzability tests. The test meals were prepared as follows: 450 mL of ultra pure water was added to 49 g semolina flour in a 1 L glass beaker. The mixture was heated to 90° C in a boiling water bath, under constant stirring. After cooling to room temperature, the pH of the mixture was brought to 2.0 with 1 mol/L HCl, and the total weight was adjusted to 600 g with water. Aliquots (40 g) of the acidified semolina meal were transferred to 100-mL Erlenmeyer flasks. The iron was added at this stage, as 0.5 g of a saccharose-iron premix containing 2.0 mg Fe as an elemental powder or ferrous sulfate. The pepsin (porcine pepsin, Sigma-Aldrich, St. Louis, MO, USA) digestion step was carried out over 2 hours; Spectrapor 1, 6–8 kDa dialysis membranes (Spectrum Labs, Rancho Dominguez, USA) were used for the dialysis step; the pancreatin (porcine pancreatin, Sigma-Aldrich) and bile (porcine bile, Sigma-Aldrich) solutions were added 30 minutes after placing the dialysis bags containing $NaHCO_3$ in the digest and the dialyzates were transferred quantitatively to 50-mL polyethylene bottles after a 2-hour digestion period. The iron concentrations in the dialyzates were measured by graphite furnace atomic absorption spectrometry (SpectrAA 400/GTA-96, Varian Inc., Mulgrave, Australia) using an external calibration curve, according to manufacturer's recommendations (Analytical Methods for Graphite Tube Atomizers, Varian Australia Pty Ltd, Mulgrave Australia, 1988). Each iron powder was tested in triplicate and several assays of the same iron powders and ferrous sulfate monohydrate were performed. A control meal, containing 2.0 mg of Fe as a freshly prepared ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$, Fluka Chemie, Buchs, Switzerland), was included in duplicate as a control in each assay. However the results for the elemental iron powders are expressed as a percentage of the values for ferrous sulfate monohydrate supplied from the SUSTAIN pool. The intra- and inter-assay variabilities, measured for the control meal, were 10% (n = 5) and 15% (n = 16) respectively.

Caco-2 Cell Iron Uptake after Simulated *in vitro* Gastrointestinal Digestion

Flour samples fortified with each of the nine elemental iron powders or ferrous sulfate monohydrate (target iron concentration 60 mg/kg for the low-extraction wheat flour and 100 mg/kg for the other two products) were prepared and stored in sealed plastic containers until used. The three types of flour evaluated were "low-extraction" wheat flour (Five Roses, 0.39% ash), high-extraction wheat flour (Kanabec, 0.75% ash), and nixtamalized corn flour. The detailed methods used for the wheat flour studies have been reported by Yeung *et al* [13]. Briefly, the fortified wheat flours were used to bake bread loaves in an automatic bread maker. The loaves were then freeze-dried, crushed, and stored in airtight plastic bags at –20° C. Aliquots (1.0 g) were used in the assays. Fortified corn flour was used to make tortillas that were prepared for assay in the same manner as the bread loaves. Assays were done without and with ascorbic acid added at the start of the *in vitro* digestion (200 µmol/L). After simulated *in vitro* gastrointestinal digestion with pepsin and pancreatic enzymes, sample aliquots were applied to a Caco-2 cell monolayer through a dialysis membrane and the increase in cellular ferritin used as a measure of absorption according to a previously published protocol [20]. The results reported here are a composite reanalysis of the published data from the wheat flour experiments, together with unpublished observations from the same series of experiments using fortified tortillas.

AOAC Rat Hemoglobin Repletion Assay

The RBVs of six of the iron powders (at least one of each type) were determined by a hemoglobin repletion/slope ratio method based on the AOAC rat hemoglobin repletion bioassay [7] in 220 weanling male Sprague-Dawley rats. Briefly, after a 24-day depletion period on an iron-insufficient diet (AIN-93G), the rats consumed the same diet to which one of the six elemental iron powders (Carbonyl/Ferronyl, Electrolytic/A131, Electrolytic/ IMP-Electrolytic, CO-reduced/RSI-325, H-reduced/AC-325, or Other reduced/Atomet 95SP, at ~ 12, 24, or 36 mg iron/kg di-

et) or ferrous sulfate monohydrate (at ~ 6, 12, 18, or 24 mg iron/kg diet) was added for a period of 14 days. The results of the study have been reported in detail by Swain *et al* [12]. The values reported here are based on their calculations using the model relating hemoglobin concentration to dietary iron concentration.

Plasma Iron Tolerance Tests in Human Volunteers

Six-hour plasma iron tolerance tests were performed in each of 32 healthy male blood donors aged between 35 and 61 yrs (two groups each consisting of 16 volunteers). Blood donors were chosen because their low iron stores were expected to improve absorption and therefore the sensitivity of the test [21]. The detailed methods have been reported separately [14]. Briefly, the subjects were served meals consisting of one low-extraction wheat roll fortified with either no iron, 272 mg ferrous sulfate monohydrate (89.4 mg iron), or 100 mg of one of five iron powders (Carbonyl/Ferronyl, H-reduced/AC-325, or Other reduced/Atomet 95SP in group 1 and Electrolytic/A131 or Electrolytic/IMP-Electrolytic in group 2) and 150 mL of water approximately nine weeks apart. The native iron content of each roll before fortification was 0.15 mg. The rolls were incised and the fortification iron placed inside the roll immediately before consumption. Venous blood samples were drawn prior to meal consumption and then hourly for six hours for the measurement of serum iron. The rise in plasma iron concentration was used as a measure of absorption. RBV values [ratio of iron powder area under the curve (AUC): ferrous sulfate monohydrate AUC] were calculated for each test in each subject.

Human Efficacy Trial

Samples of the two iron powder types currently used for food fortification, electrolytic and hydrogen-reduced, were selected for evaluation in a six-month efficacy trial. The detailed methods are reported separately [16]. Briefly, wheat-based snacks were fortified with sufficient ferrous sulfate monohydrate, Electrolytic/A131, or H-reduced/AC-325 (added before baking) to supply 12 mg of iron per day. Non-pregnant Thai women aged 18–50 years with low iron stores were randomly allocated to one of four groups to receive unfortified snacks or snacks fortified with one form of iron 6 days/week for 35 weeks in a double-masked intervention trial. Hemoglobin, serum ferritin, and serum transferrin receptor concentrations were measured at baseline, 20, and 35 weeks. Iron status was calculated from the serum transferrin receptor/serum ferritin ratio [22].

Additional Studies

Additional information on three commercial iron powders that were not included in the initial pool became available while the project was under way. We therefore submitted these powders to selected screening procedures and have included the data in our report because they provide further insights related to the prediction of bioavailability. A pharmaceutical carbonyl iron powder (Carbonyl/CF; BASF, Germany) was evaluated by Hoppe *et al*, [15] using their plasma iron tolerance method. We therefore measured the dissolution rate of this product. The commercial production of Other reduced/Atomet 95SP was discontinued by Quebec Metal Powders, Ltd. (QMP), Canada, and replaced by Other reduced/Atomet 195SP while our studies were being done. We therefore measured the dissolution rate and RBV by the rat hemoglobin repletion method of Other reduced/Atomet 195SP. A new reduced iron powder (H-reduced/Hi-Sol) was manufactured by North American Höganäs, USA as a result of increased awareness among manufacturers of factors that appear to affect iron bioavailability. The manufacturer supplied the particle size distribution (D_{10} = 13.54 µm, D_{50} = 30.98 µm, D_{90} = 55.40 µm) and surface area (0.56 m^2/g). We evaluated dissolution rate and RBV by the rat hemoglobin repletion method.

Statistical Methods

Data were tested for homogeneity of variance using Levene's test for transformation necessity. All subsequent analyses were performed on transformed data where necessary. However, raw data means and standard deviations are presented for ease of interpretation. The data related to physical properties were analyzed by the single factor analysis of variance (ANOVA) procedure. Duncan's multiple range tests were used to evaluate the significance of differences between sample means. The concordances between the various physical properties and dissolution rate were evaluated by testing the significance of linear regression models. The dissolution data for the collaborative study was analyzed in two ways. The first analysis was a single-factor ANOVA comparing all nine laboratories over seven powders; a Duncan's multiple range test at the 5% alpha level was performed to identify differences in laboratory mean dissolution responses. In the second single-factor ANOVA analysis, the IMP results were excluded because they were significantly lower than the rest. Repeatability and reproducibility were calculated for both analyses [23]. The Caco-2 data were analyzed using two single-factor ANOVA tests. The first ANOVA compared six flour/ascorbic acid combinations (low extraction flour/no AA, low extraction flour + AA, high extraction

flour/no AA, high extraction flour + AA, tortilla/no AA, and tortilla + AA), considered to be fixed effects, over all powders. The second ANOVA compared the nine powders over all flour/AA combinations. In this case the flour/AA combinations were used as replicates to compare the powders considered to be fixed effects. A Duncan's multiple range test was performed for analysis with significant F-test statistics at $p \leq 0.05$ to determine differences. Plasma tolerance data were analyzed using repeated measures ANOVA, performed separately on the two groups of volunteers.

Results

Morphological Characteristics and Physical Properties

Representative examples of the *surface and cross-sectional morphological features* of the five powder types by scanning electron microcopy are shown in Figure 2. The carbonyl powder particles were spherical and agglomerated without internal porosity on cross section; electrolytic particles were rounded elongated flakes with some open and closed internal porosity; CO-reduced powders consisted of rounded ligamental or knobby particles with some closed internal porosity; H-reduced particles were rounded and irregular with large amounts of open and closed porosity; and other reduced particles were slightly flaky with a mix of angular and rounded particles and a mix of dense, open-porosity and closed porosity structures. These observations were consistent with the known morphological features of elemental iron powders, but it was not possible to quantify them in a way that could provide meaningful information about predicted bioavailability.

With the exception of pycnometric and apparent density, there were significant differences in the physical properties and morphological characteristics of the various iron powders (Table II). The results for *pycnometric density* were all close to the theoretical value of 7.87 g cm^{-3}. The small differences observed were attributed to the presence of alloying additions, production contaminants, and surface oxides as well as closed internal porosity. Variations in *apparent density* were greater and reflected differences in particle size and irregularity, as well as open and closed internal porosity. However the variations in apparent density were also not useful for discriminating between the iron powders.

Significant differences between the iron powders could be demonstrated by using three of the measured physical properties: particle size, Fisher subsieve size, and surface area (Table II). Replicate variability was consistently low for *particle size* (CV < 2%). The reproducibility of particle size measurements was evaluated by measuring the particle size of all the powders samples in a second laboratory using a different instrument (Beckman Coulter Model LS-230 particle size distribution analyzer). The two sets of data were very closely correlated (r^2 values for D_{10}, D_{50} and D_{90}, were 0.96, 0.94 and 0.92 respectively, $p < 0.01$) with no differences in mean values between the two sets of results. Replicate variability was also low for the *Fisher subsieve size* measurements (< 3%) with one exception. The value for Electrolytic/IMP-Electrolytic was much higher (12%). The reproducibility of the subsieve measurements was tested by performing this assay on six powders (Carbony/Ferronyl, Electrolytic/A131, Electrolytic/IMP-Electrolytic, CO-reduced/RSI-325, H-reduced/AC-325, and Other reduced/Atomet 95SP) in a second laboratory. The agreement was again remarkably close ($r^2 = 0.86$, mean values 8.47 µm and 8.37 µm). *Surface area* was measured by the BET method in three laboratories (Table II). Although the variability was considerably higher, the results were consistent for all of the powders with one exception. Analyses of the Electrolytic/IMP-Electrolytic powder gave values of 0.5860, 0.4896, 0.2876, 0.2450, and 0.2360 m^2/g. The reason for the observed inconsistency was not established, but repeated measurements on different samples drawn from the original pool suggested that it could not be attributed to poor inter-laboratory reproducibility or incomplete mixing. The latter conclusion is supported by the absence of similar variations in particle size. Particle size and Fisher subsieve number were directly correlated with each other and inversely correlated with surface area ($p < 0.01$, Table VI).

Dissolution Assays

The initial series of dissolution tests was carried out in a single laboratory. The coefficient of variation was < 13% and there was a three-fold variation in percentage iron dissolution values for different powders with significant differences between powder types (Table III). Approximately 95% of the carbonyl iron powders had dissolved at 30 minutes. The two powders with the next highest dissolution rates were Electrolytic/A131 (72.5%) and Electrolytic/ IMP-Electrolytic (50.6%). Analysis of the reduced powders gave the lowest values: 37.6% for H-reduced/AC-325, 35.3% for Other reduced/Atomet-95SP, and 29.0% for CO-reduced/RSI-325. Mean dissolution rates showed significant inverse linear correlations with particle size (D_{50}, $r = -0.88$, $p < 0.01$), Fisher subsieve size ($r = -0.92$. $p < 0.01$), and a direct correlation with surface area ($r = 0.85$, $p < 0.01$, Table VI).

The dissolution test was further evaluated by an inter-

laboratory comparison. Repeatability (level of agreement for results within the same laboratory) and reproducibility (the level of agreement between different laboratories) were tested by providing duplicate masked samples of seven powders to nine independent participating laboratories (Figure 3). The coefficient of variation (CV) for repeatability was 11.3% and for reproducibility 38.7% for the nine participating laboratories. The results from one of the laboratories (IMP) were consistently and significantly lower than those from the other eight for all powders tested based on ANOVA and the Duncan's multiple range test at the 5% level. When results from IMP were removed

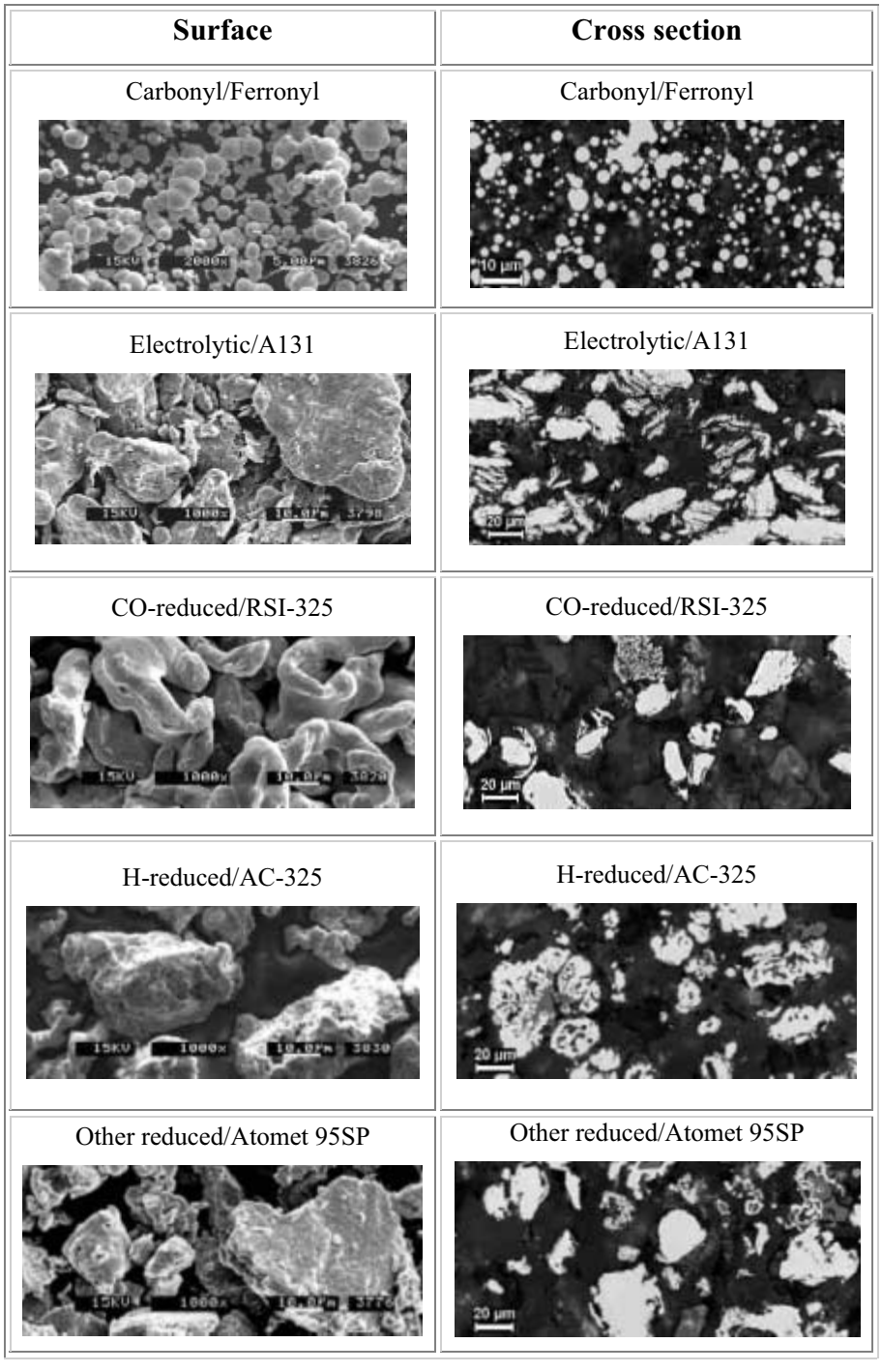

Figure 2: Surface and cross-sectional morphology of elemental iron powders.

Table II: Physical Properties of the Elemental Iron Powders[1]

Manufacturing Process	Sample	Pycnometric Density	Apparent Density	Particle Size[1,2]			Subsieve Size[2]	Surface Area[2,3]
		gcc	gcc	D_{10}[2] (μm)	D_{50}[2] (μm)	D_{90}[2] μm	(μm)	m^2/g
Carbonyl	Ferronyl	7.700 (0.005)	2.25 (0.04)	2.71[a] (0.01)	6.78[a] (0.03)	13.08[a] (0.05)	3.4[a] (0.1)	0.3824[a] (0.0416)
Carbonyl	OF	7.645 (0.003)	2.41 (0.04)	2.52[b] (0.01)	7.52[b] (0.1)	18.37[b] (1.1)	3.3[a] (0.1)	0.5382[4b] (0.0026)
Electrolytic	A131	7.679 (0.003)	2.45 (0.02)	10.06[c] (0.07)	28.35[c] (0.23)	55.96[c] (0.81)	5.9[ab] (0.1)	0.3556[a] (0.0315)
Electrolytic	IMP-Electrolytic	7.665 (0.002)	3.14 (0.01)	11.81[d] (0.01)	25.38[d] (0.05)	52.80[d] (0.32)	9.3[c] (1.1)	0.3688[a] (0.1592)
CO-reduced	MH300.29	7.753 (0.003)	3.12 (0.01)	19.49[e] (0.04)	35.99[e] (0.05)	64.17[e] (0.05)	15.0[d] (0)	0.1100[5]
CO-reduced	RSI-325	7.764 (0.006)	2.79 (0.01)	19.66[f] (0.04)	33.24[f] (0.06)	56.81[c] (0.38)	14.3[e] (0.3)	0.088[c] (0.0055)
H-reduced	AC-325	7.545 (0.002)	2.54 (0.02)	10.99[g] (0.01)	25.18[d] (0.04)	50.02[f] (0.42)	8.0[b] (0.1)	0.2848[ad] (0.0427)
Other reduced	Atomet 75	7.725 (0.002)	2.66 (0)	18.19[h] (0.11)	36.06[e] (0.04)	63.14[e] (0.12)	13.8[f] (0.3)	0.2071[d] (0.0454)
Other reduced	Atomet 95SP	7.704 (0.003)	2.54 (0.01)	13.22[i] (0.04)	29.49[g] (0.22)	54.39[g] (1.60)	10.0[c] (0)	0.2147[d] (0.0484)

[1] Calculated particle diameters for 10th, 50th, and 90th percentile
[2] Mean (SD) values with different letters are significantly different at $p < 0.05$
[3] Mean (SD) for 2–5 measurements in 3 laboratories
[4] Based on 2 measurements
[5] Based on a single measurement, not included in ANOVA

Table III: Percentage dissolution after 30 minutes in 0.1 mol/L HCl

Sample	1	2	3	4	5	6	Mean[1] (SD)
Carbonyl/Ferronyl	95.4	96.5	93.3	94.1	95.1	94.0	94.7[a] (1.2)
Carbonyl/OF	95.9	96.5	93.2	93.7	94.3	94.5	94.7[a] (1.3)
Electrolytic/A131	66.3	79.1	66.3	80.5	75.7	67.2	72.5[b] (6.7)
Electrolytic/IMP-Electrolytic	54.2	51.3	49.0	48.7	51.6	49.0	50.6[c] (2.2)
CO-reduced/RSI-325	27.2	25.3	30.5	25.3	32.0	33.6	29.0[e] (3.6)
H-reduced/AC-325	40.6	36.9	38.1	35.8	37.7	36.4	37.6[d] (1.7)
Other reduced/Atomet-95SP	34.4	39.0	30.4	36.6	35.9	35.3	35.3[d] (2.8)

[1] Mean values with different letters are significantly different based on Duncan's multiple range test at $p < 0.05$.

from the analysis, the CVs for repeatability and reproducibility were 10.3% and 19.1%, respectively. The significant differences in dissolution rate between the various powders (after excluding the IMP results) were the same as those identified in the original single laboratory dissolution study (Figure 3).

Powders held under high humidity, particularly without air exchange, tended to clump together after 32 days of storage, making it difficult to separate sub-samples for assay. Rust deposits were visible in some powders (Electrolytic/A131, H-reduced/AC-325, and Other reduced/Atomet 95SP). There was a significant increase in mass in three powders (Carbonyl/Ferronyl, Electrolytic/A131, and H-reduced/AC-325) after 64 days under high temperature and humidity conditions, which was due to adherent water. There was no change in the dried mass after 64 days in any of the powders. Percent dissolution was measured on four samples after 64 days storage under high temperature and humidity. Despite the presence of rust there was only a small decrease in percent dissolution in all cases. Mean percent dissolution values (before environmental exposure vs. after 64 days) were 94.2, 89.8 (Carbonyl/Ferronyl), 65.5, 61.9 (Electrolytic/A131), 35.5, 28.7 (H-reduced/AC-325), and 32.4, 29.9 (Other reduced/Atomet 95SP) respectively. These preliminary observations suggest that difficulties in dispersing iron powders evenly in the fortification vehicles will be a greater problem than effects on bioavailability if powders are stored under adverse environmental conditions.

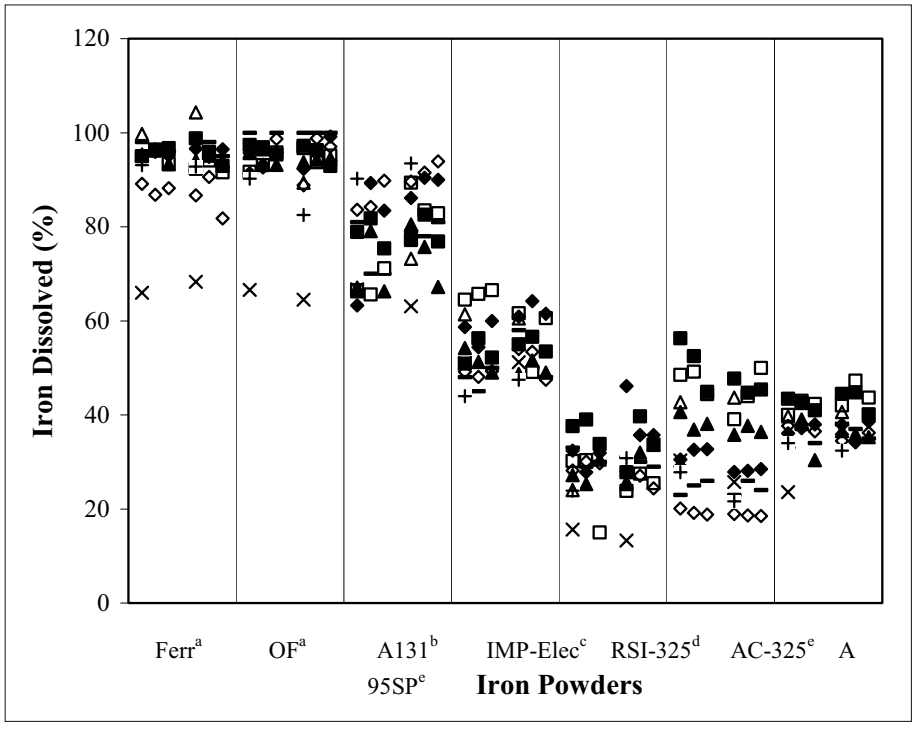

Figure 3: Percent iron dissolved based on the standard dissolution assay as tested by 9 labs: – = BASF, ▲ = SCM Metal Products Inc., ■ = Höganäs AB, □ = ISP, ◇ = Cornell, ◆ = North American Höganäs, × = IMP, + = Dr Paul Lohmann, and △ = Penn State.

[a,b,c,d,e] Dissolution rates for powders with different letters are significantly different based on Duncan's multiple range test at $p < 0.05$.

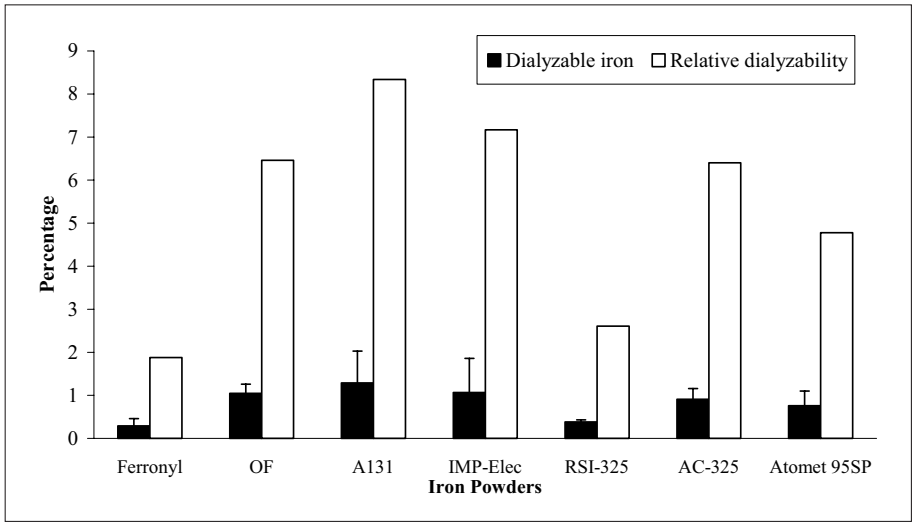

Figure 4: Dialyzable iron (% of dose, 1 SD) and relative dialyzability with respect to ferrous sulfate monohydrate (%).

Dialyzability after Simulated *in vitro* Gastrointestinal Digestion

Percentage dialyzable iron was low for all of the elemental iron powders (Figure 4) with considerable replicate variability (CV 13%–74%). The results for the two carbonyl-iron samples differed more than three-fold. Relative dialyzability with respect to ferrous sulfate monohydrate was not correlated with either RBV by rat hemoglobin repletion or dissolution in 0.1 mol/L HCl ($p > 0.1$).

Caco-2 Cell Iron Uptake after Simulated *in vitro* Gastrointestinal Digestion

The detailed results for four of the six food matrices (bread made from low- and high-extraction wheat flour without and with ascorbic acid) have been published recently [13]. We carried out a composite analysis of the four published studies and the fifth and sixth unpublished experiments that employed nixtamalized corn flour without and with ascorbic acid (Figure 5). Ferritin formation and therefore

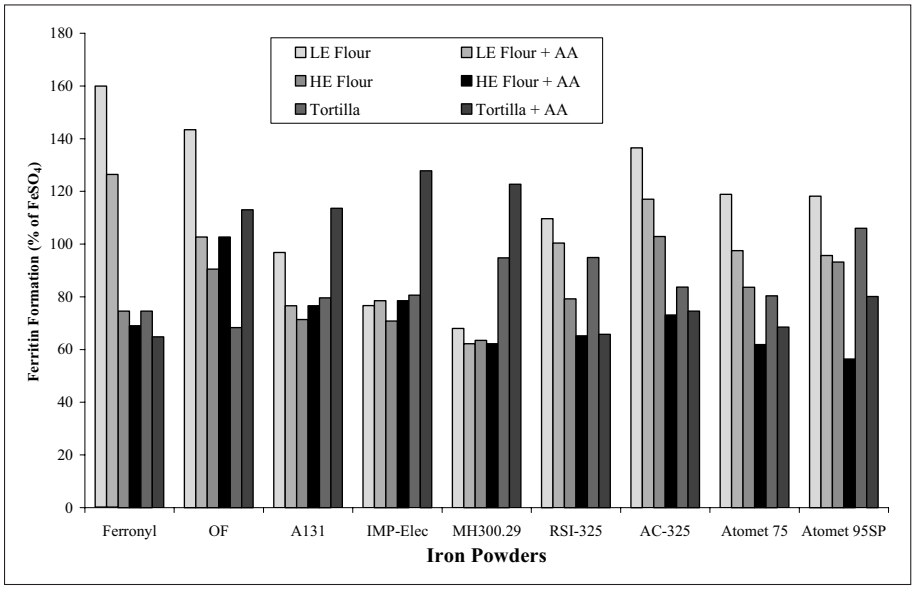

Figure 5: Iron bioavailability assessed by Caco-2 cell ferritin formation in low-extraction wheat flour (LE), high-extraction wheat flour (HE), and tortillas without and with ascorbic acid (AA).[1,2]

[1] The values are the ratio of ferritin formation between the iron powder and ferrous sulfate.
[2] Data from Yeung *et al* [13].

putative iron uptake for some of the iron powders was comparable to or better than ferrous sulfate monohydrate in certain food matrices. Moreover relative uptake compared with ferrous sulfate monohydrate was not consistent for the various iron powders in the different food matrices. Two single-factor analyses of variance demonstrated that there were statistically significant differences in ferritin formation from the different simulated meal matrices ($F = 11.78$, $p < 0.0001$), and also between iron powders ($F = 2.73$, $p = 0.0063$). Relative mean ferritin levels were significantly higher for low-extraction wheat flour without ascorbic acid and significantly lower for high-extraction wheat flour with ascorbic acid when compared with all of the other matrices. A difference in relative ferritin formation was recorded for only one powder. The value for the CO-reduced powder MH300.29 was significantly lower than those for the other 8 powders tested. Caco-2 cell iron uptake was not correlated with either dissolution in 0.1 mol/L HCl or RBV by rat hemoglobin repletion ($p > 0.1$).

AOAC Rat Hemoglobin Repletion Assay

As reported by Swain *et al* [12], the RBV of all the iron powders was significantly less than that of ferrous sulfate monohydrate ($p < 0.05$, Table IV). There was a three-fold variation between the different powders. Swain *et al* also demonstrated that surface area and dissolution rate were highly predictive of RBV. The observations reported here ($r^2 = 0.81$ for surface area and 0.86 for dissolution rate, $p < 0.01$) confirm their conclusions and also demonstrate significant inverse linear correlations with particle size (D_{50}, $r^2 = 0.64$, $p < 0.01$) and Fisher subsieve size ($r^2 = 0.86$, $p < 0.01$).

Table IV: Relative bioavailability (RBV) of elemental iron powders in the AOAC rat hemoglobin repletion assay

Iron Powder	RBV[1]
Ferrous Sulfate Monohydrate	100[a]
Carbonyl/Ferronyl	64 (62–67)[b]
Electrolytic/A131	54 (50–58)[c]
Electrolytic/IMP-Electrolytic	46 (43–50)[c,d]
CO-reduced/RSI-325	21 (17–25)[e]
H-reduced/AC-325	42 (37–46)[d]
Other reduced/Atomet-95SP	24 (20–28)[e]

[1] RBV (mean 95% CI) relative to $FeSO_4 \cdot H_2O$. Values with different letters are significantly different ($p < 0.05$). Data from Swain *et al* [12].

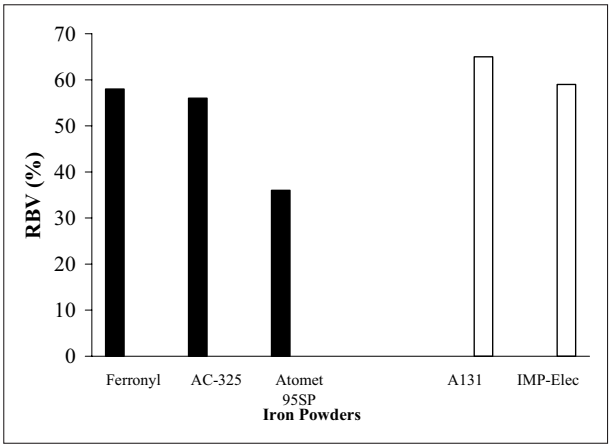

Figure 6: Relative bioavailability values (RBV) with respect to ferrous sulfate monohydrate for elemental iron powders measured as the AUC for the serum iron increase during six hours after consumption of a fortified wheat roll.[1,2]

[1] Solid columns: Group 1, open columns: Group 2
[2] Data from Hoppe *et al* [14].

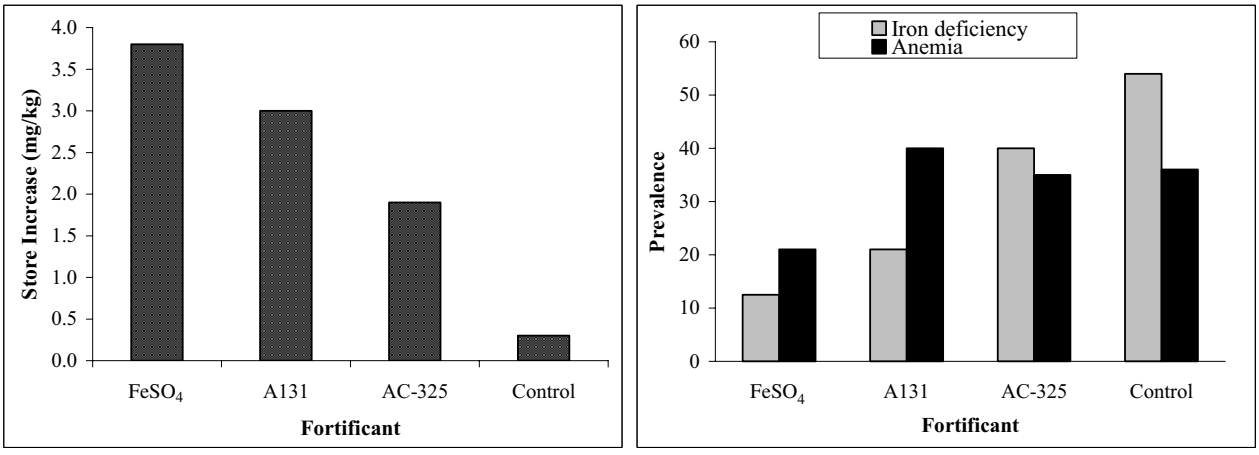

Figure 7: Human efficacy trial[1,2]

[1] Ordinate values are increased in iron stores from baseline at 35 weeks (mg/kg) and prevalence of iron deficiency and anemia respectively at 35 weeks (%).
[2] Data from Zimmermann *et al* [16].

Plasma Iron Tolerance Tests in Human Volunteers

Five iron powders were evaluated in two groups of volunteers (Figure 6). The detailed results have been reported [14, 15]. The mean absorption values for all the iron powders were significantly less than that for ferrous sulfate monohydrate (RBV 36% to 65%, $p < 0.05$, Figure 6). The RBV for Other reduced/Atomet 95SP was significantly lower than the values for Carbonyl/Ferronyl and H-reduced/AC-325 ($p < 0.05$); there was no difference between the latter two. The RBV values for Electrolytic/A131 and Electrolytic/IMP-Electrolytic in Group 2 were not different and comparable to those for Carbonyl and H-reduced iron powders.

Human Efficacy Trial

The results of the human efficacy trial have been reported [16]. Based on the calculated change in body iron stores, all of the iron fortificants improved iron status. However there were significant differences in the quantitative increases in iron stores in the three intervention groups (calculated body iron stores increased from 1.5 to 5.3 mg/kg, 1.5 to 4.4 mg/kg, 1.3 to 3.2 mg/kg, and 1.0 to 1.4 mg/kg in the ferrous sulfate monohydrate, Electrolytic/A131, H-reduced/AC-325, and control groups respectively, Figure 7). There was no significant change in body iron stores in the control group. After 35 weeks the prevalence of iron deficiency (serum ferritin < 15 µg/L or serum transferrin receptor > 8.5 mg/L) decreased from 49% to 13%, 44% to 21%, and 48% to 40% in the respective fortified groups,

Table V: Results of the additional studies

Powder type	Particle Size (µm) D_{10}	D_{50}	D_{90}	Surface Area (m^2/g)	Dissolution % (SD)	Rat RBV % (CI)	Human RBV[1, 2] %
Powders included in additional studies							
Carbonyl/CF	3.27	6.78	13.08		50.7 (3.2)		37
Other reduced/Atomet 195SP					16.8	10 (4-17)	
H-reduced/Hi-Sol	13.54	30.98	55.40	0.560	74.3 (8.4)	40 (33-47)	50
Powders from SUSTAIN sample pool							
Carbonyl/Ferronyl						54 (47-62)	
Carbonyl/OF						65 (58-73)	
Electrolytic/A131						42 (36-49)	
H-reduced AC-325						28 (22-35)	

[1] Data from Hoppe *et al* [15]
[2] Relative bioavailability values (RBV) with respect to ferrous sulfate monohydrate for elemental iron powders measured as the AUC for the serum iron increase during six hours after consumption of a fortified wheat roll.

and from 61% to 54% in the control unfortified group (Figure 7). The impact on the prevalence of both iron deficiency and anemia was only significant for ferrous sulfate monohydrate. Electrolytic/A131 fortification had a significant impact on the prevalence of iron deficiency, but not anemia. The prevalence of iron deficiency and anemia in individuals receiving H-reduced/AC-325 snacks was not different from the control group at the end of the study. Based on the changes in body iron store for all subjects over the 35-week period, the relative efficacies for Electrolytic/A131 and H-reduced/AC-325 with respect to ferrous sulfate monohydrate were calculated to be 77% and 49% respectively.

Additional studies

The results of the additional dissolution studies (Table V) support the predictive validity of the method. Percentage dissolution for the H-reduced/Hi-Sol product was better than for H-reduced/AC-325 in keeping with higher RBV in the second rat experiment, while the value for Other reduced/Atomet 195SP was only about 50% of Other reduced/Atomet 95SP in keeping with the rat RBV of 10%. The dissolution rate for Carbonyl/CF was about 50% of that for the two carbonyl-iron products in the original pool. The validity of this finding is supported by the plasma iron tolerance result reported by Hoppe *et al* [15]. Although the two forms of iron were not studied in the same group of subjects, the RBV was considerably lower for Carbonyl/CF (37%, Table V) than for Carbonyl/Ferronyl (58%, Figure 6).

The second RBV experiment using the rat hemoglobin repletion model was done to evaluate two newer iron powders that were not included in the original pool, H-reduced/Hi-Sol and Other Reduced/Atomet 195SP. It also provided the opportunity to evaluate the repeatability of the test. For this reason three powders that were evaluated in the original RBV test (Carbonyl/Ferronyl, Electrolytic/A131, and H-reduced/AC-325) were included. A fourth powder from the SUSTAIN sample pool that had not been tested previously (Carbonyl/OF) was also tested. Although the values of the three powders tested in both experiments were modestly lower in the second test (the ratios of the RBVs were between 0.7 and 0.8), carbonyl iron again had the highest RBV; values were lower for electrolytic and hydrogen-reduced iron (Table V). The previously untested carbonyl-iron powder (Carbonyl/OF) was not different from Carbonyl/Ferronyl, but the bioavailability of the new H-reduced product (H-reduced/Hi-Sol) was higher than that of H-reduced/AC-325 and not significantly different from that of Electrolytic/A131 iron. On the other hand the RBV of the new other reduced product (Other reduced/Atomet 195SP) was less than half that of Other reduced/Atomet 95SP (10% vs. 24%).

Discussion

The observations reported here indicate that there are significant differences among commercial iron powders that predict variations in the rate of dissolution in the dietary nonheme iron pool, which will have nutritionally important effects on bioavailability.

Evaluation of physical properties and *in vitro* methods for predicting bioavailability

The *scanning electron microscopy* results were in accord with the known morphology of the commercial products and revealed the expected differences in surface characteristics as well as closed and open (to the particle surface) porosity. These properties are likely to be related to bioavailability, but we are not aware of a method for quantifying them in a way that could be employed for predicting bioavailability.

There were significant differences among the types of iron powder with respect to three physical properties (particle size, subsieve size, and surface area), as well as the dissolution rate in 0.1 mol/L HCl. The results for these parameters were correlated with each other (Table VI). *Par-*

Table VI: Pearson correlation coefficients for comparisons of physical properties, dissolution rate in 0.1mol/L HCl and RBV by the rat AOAC method

	Subsieve size	Pycnometric density	Apparent density	Surface area	Dissolution rate	RBV
Particle size (D_{50})	0.90[1]	0.40	0.62	–0.83[1]	–0.88[1]	–0.80[1]
Subsieve size		0.56	0.71[2]	–0.91[1]	–0.92[1]	–0.93[1]
Pycnometric density			0.32	–0.56	–0.06	–0.30
Apparent density				–0.50	–0.57	–0.39
Surface area					0.85[1]	0.90[1]
Dissolution rate						0.93[1]

[1] $p < 0.01$
[2] $p < 0.05$

ticle size is unquestionably an important determinant of solubility and bioavailability [2, 3]. All powders met the criteria given in the FCC specifications. Although it remains essential to ensure that iron powders used for food fortification meet the FCC particle size specifications, particle size alone is inadequate as a single criterion that could be applied to all elemental iron powders to ensure adequate bioavailability for the following reasons. It is significantly affected by production method. Carbonyl-iron particles are always much smaller than those of other powders, yet bioavailability may not be very different. Particle shape influences size estimates because the algorithms in sizing instruments assume that the particles are spherical. It would therefore be necessary to define a specific particle size criterion for each powder type.

Surface area is also a potentially useful criterion for categorizing iron powders with respect to predicted bioavailability. However, the procedure is time-consuming and expensive. Moreover, difficulty was encountered in measuring the surface area of one electrolytic iron powder (Electrolytic/IMP-Electrolytic) in this study. The reason for the wide variation in replicate assays was not established. However, Swain *et al* [12] have also observed an unexplained difference in the surface area in samples of the other commercial electrolytic iron powder (Electrolytic/A131) produced by the same manufacturer to identical specifications six years apart. One plausible explanation is the tendency for these flat particles to stack, reducing the surface area available for gas adherence in the BET method.

Fisher subsieve size is a third potential method for predicting bioavailability that we have identified. It was possible to distinguish between the different powder types by using this method; however, powder manufacturers no longer commonly use this technique. Moreover the assays are affected by both particle size and surface area. The possibility that measurements on electrolytic iron will present problems similar to those encountered with estimates of surface area is suggested by higher replicate variability for subsieve size estimates for the Electrolytic/IMP-Electrolytic iron.

The two more complicated *in vitro* screening procedures, dialysis and Caco-2 cell uptake, were not satisfactory for predicting bioavailability for different reasons. The replicate variability of the results obtained by the measurement of *dialyzable iron* was considerably higher than that for dissolution in 0.1 mol/L HCl. In addition the results for the two carbonyl-iron powders (Carbonyl/Ferronyl and Carbonyl/OF) that had similar predicted bioavailability values based on the dissolution rate in dilute HCl and RBV by rat hemoglobin repletion showed a three-fold difference in dialyzability. *Caco-2 cell uptake* may provide valuable information about food factors that affect iron bioavailability [20] although enhancers and inhibitors of iron absorption appear to have a greater effect in the Caco-2 cell model than they do on human iron absorption [24]. The ANOVA that we carried out using all the experiments conducted by Yeung *et al* (both the bread-based [13] and tortilla meals) demonstrated significant matrix effects for two of the meals (significantly higher relative absorption from the iron powders with respect to ferrous sulfate monohydrate in the low-extraction wheat flour bread without ascorbic acid, and significantly lower relative absorption in high-extraction wheat flour bread when ascorbic acid was added). These matrix effects may have overwhelmed smaller differences in potential absorption of the various iron powders. The relative absorption of an elemental iron powder was consistently different from other powders (across all food matrices) in only one case – lower for the CO-reduced/MH300.29. This powder would be expected to have low bioavailability because of the method of manufacture (CO reduction). However it was not evaluated in our other screening procedures because the large particle size, large Fisher subsieve size, and small surface area (Table II) indicated that it was likely to be the least bioavailable powder in our sample pool. We concluded that the sensitivity of the Caco-2 method, employing the protocol described by Yueng *et al* [13] was inadequate for predicting the potential bioavailability of elemental iron powders.

Our observations suggest that the *dissolution rate* in 0.1 mol/L HCl, under carefully standardized conditions, may prove to be the best basis for developing a reliable *in vitro* screening test to monitor the predicted bioavailability of elemental iron powders. It was possible to distinguish between different powder types, replicate reproducibility was satisfactory for all the powders tested, and there was a close correlation with RBV by the AOAC rat hemoglobin repletion method. The dissolution rates of the two powders used in our human efficacy trial also predicted the outcome of that trial. Finally, we demonstrated an adequate level of replicate reproducibility when the test was performed in nine different laboratories. The reproducibility of the results reported by the independent laboratories is very encouraging. Significant systematic inter-laboratory variation was observed in the results from only one laboratory, but protocol refinement or the provision of calibration standards would be likely to correct this problem. Finally, the approach is attractive from the practical point of view. A single criterion could be used for all powder types, and the apparatus required is inexpensive and already available in most laboratories. Alternative iron assay methods could be substituted for ICP-AES.

Evaluation of *in vivo* screening for predicting bioavailability

At the present time, *RBV measured by the AOAC rat hemoglobin repletion method* is considered to be the most reliable biological screening method for predicting the bioavailability of fortification iron compounds if human absorption tests are not obtainable [3, 10]. Less iron was absorbed from the elemental iron powder than from ferrous sulfate monohydrate in all of our rat hemoglobin repletion assays. There was a three-fold variation in the RBV of the six powders tested in the initial experiment (Table IV). The highest values were obtained for the carbonyl and electrolytic iron powders. The additional RBV experiment confirmed the initial findings, although the absolute values were lower (approximately 70 to 80% of the original value for the three powders tested in both experiments).

The *plasma iron tolerance* method has been shown to provide useful information about the bioavailability of iron salts that are absorbed rapidly [25–29]. The results for the iron powders reported here are generally consistent with the conclusions drawn from their characterization based on the physical properties and dissolution rates, the rat RBVs, and the results of the human efficacy trial. However the sensitivity for distinguishing between powder types was lower than that of the dissolution test and the AOAC rat hemoglobin repletion assay. We conclude that the use of this relatively expensive and time-consuming screening procedure is not justified for evaluating the bioavailability of elemental iron powders.

In summary, while it is possible that modifications in the protocols used (food matrix, etc.) would improve the precision of some of the screening procedures, our observations suggest that a well-standardized dissolution assay will prove to be the most accurate and precise screening procedure for determining and monitoring the potential bioavailability of elemental iron powders. Its predictive value was confirmed by both the AOAC hemoglobin repletion test and the human efficacy trial.

Predicted bioavailability of commercial iron powders

Our results demonstrate that there are substantial differences in the predicted bioavailability of the commercial iron powders that were available to us at the time the study was initiated and that bioavailability is related in part to the method of manufacture. Taken together, the screening tests indicate that some carbonyl and electrolytic iron powders have the highest potential bioavailability, although iron absorption was always lower than from ferrous sulfate monohydrate. Carbonyl iron is of limited value for food fortification because of its higher cost and the limited production of food-grade material. Electrolytic iron is the preferred elemental iron powder in the recommendations for wheat or maize flour fortification published by SUSTAIN [30], the Pan American Health Organization (PAHO) [31], and the World Health Organization (WHO) [32]. The WHO "Guidelines on Food Fortification with Micronutrients for the Control of Micronutrient Malnutrition" [32] advocate the use of electrolytic iron at twice the iron concentration recommended for ferrous sulfate if an iron powder is the preferred fortificant. This recommendation is supported by the results of the human efficacy trial in which the overall relative efficacy, calculated from the change in estimated iron status, was 77% [16]. These results suggest that Electrolytic/A131 would be a satisfactory substitute for ferrous sulfate if added at twice the concentration. However it is important to note that the electrolytic iron was substantially less effective than ferrous sulfate in correcting iron deficiency in the most vulnerable women. The prevalence rates for iron deficiency and anemia were 13% and 21%, and 21% and 40% for ferrous sulfate and electrolytic iron, respectively, at the end of the trial (Figure 7). Further research is needed to determine whether the addition level of twice that for ferrous sulfate is sufficient to meet the requirements of this group.

Other iron powders are not recommended by WHO [32]. The RBV of 49% that we obtained for H-reduced iron in the efficacy trial might be considered marginally adequate. However it is important to note that the impact on the prevalence of iron deficiency was not statistically significant. At the end of the trial 40% of the women receiving snacks fortified with H-reduced/AC-325 were iron-deficient compared with 54% in the control group given unfortified snacks. The prevalence rates for anemia were essentially the same (35% and 36% respectively). On the other hand, the prevalence rates for iron deficiency and anemia were reduced to 13% and 21%, respectively, in the positive control group receiving ferrous sulfate. We therefore recommend that the commercial reduced-iron powders available to us at the time this project was initiated not be selected for food fortification.

We conclude on a note of caution. Although our studies indicate that some carbonyl and electrolytic products will be more bioavailable than other iron powders and potentially efficacious for food fortification, they also provide provisional evidence of variability in bioavailability among powders of the same type. One carbonyl-iron powder (Carbonyl/CF) was significantly less soluble than the other carbonyl products. The plasma iron tolerance tests also predicted lower bioavailability. This finding could provide an explanation for the lower bioavailability of carbonyl iron reported by Hallberg *et al*, [5] which is at variance with other reports based on observations in animal models [3] and human volunteers [33]. It is also worth not-

ing that both the dissolution and RBV in the rat model of Electrolytic/IMP-Electrolytic was lower than Electrolytic/A131 in our studies. On the other hand our preliminary analyses of the new Höganäs product (H-Reduced/Hi-Sol) suggest that it may be possible to manufacture an H-reduced iron powder with adequate bioavailability (comparable to Electrolytic/A131 in the dissolution screen and rat hemoglobin repletion assay), although the commercial feasibility of introducing this product into the market has not been established.

Since a product's designation (e.g. electrolytic or carbonyl) may not assure its adequate absorption, it will be necessary to screen each product. We plan to refine the dissolution procedure and anticipate that it will fulfill this role in the future. As an interim measure, powders could be screened using the provisional dissolution method that we have described and the product(s) with the highest dissolution rates evaluated by the rat AOAC hemoglobin repletion method. However the inter-assay variability that we observed in the two hemoglobin repletion tests demonstrates that it would be necessary to include a reference powder in all such assays. As an interim measure the SUSTAIN pool Electrolytic/A131 could be employed as the reference standard because its properties and bioavailability in human volunteers have been established. Powders would be considered to have acceptable potential bioavailability if RBV by the rat hemoglobin repletion test was equal to or better than that for the SUSTAIN electrolytic/A131. However, the quantity of electrolytic/A131 remaining in the SUSTAIN pool is limited. It will therefore be necessary to prepare, characterize, and maintain a suitable iron powder standard for future tests.

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