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Selective extraction of blood plasma exchangeable copper for isotope studies of dietary copper absorption

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Measuring mineral absorption by fecal monitoring is labor-intensive and relies on good volunteer compliance. Blood indicators of absorption could be advantageous and we have developed a method for selective extraction of recently absorbed (exchangeable) copper based on dialysis of plasma with histidine and subsequent copper extraction using Chelex resin. The potential for measuring copper absorption by transient enrichment of exchangeable copper with the stable isotope ^{65}Cu from an ingested tracer, was also investigated. This method was compared with that of the fecal monitoring technique in a human volunteer, who consumed a 6 mg dose of ^{65}Cu with inhibitors of copper absorption. Holmium was used as a non-absorbable rare-earth marker of unabsorbed tracer excretion, allowing estimation of re-secreted ^{65}Cu (44 μg d^{-1}), and hence calculation of true tracer absorption, which was only 10.8%. Monitoring plasma tracer kinetics showed potential for estimation of copper absorption without the need for fecal copper analysis.

Introduction

The use of stable isotopes in combination with fecal monitoring is an established method of accurately measuring mineral absorption.[1] Nevertheless, it involves a considerable concerted effort by volunteers and investigators alike over a period of at least 7 days, and the necessity to collect fecal samples can discourage volunteer participation or full compliance. Blood indicators of copper absorption would be advantageous but require identification and separation of the copper fraction that reflects recent gastrointestinal absorption.

When copper is absorbed, it is rapidly transported, principally to the liver, as a complex bound to amino acids, serum albumin and possibly transcuprein.[2,3] The remaining ceruloplasmin (CP) fraction in plasma, which is secreted from the liver, constitutes about 80% of plasma copper and does not exchange with the other copper containing fractions. In contrast, it is thought that copper exchanges between amino acids, albumin and transcuprein.[2] For the purposes of identifying a single copper pool that reflects gastrointestinal absorption of copper, the Cu on these 3 binding ligands are collectively referred to as the non-CP copper fraction.

In studies with rats injected intravenously with a radioisotope of copper,[4] it is clear that the biological half-life of copper bound to albumin is short, being of the order of 10 min. The biological half-life of copper bound to CP is much longer at about 48 h. This is consistent with the proposed carrier role of albumin and the exchangeable nature of the bound copper. In the case of an injected copper tracer, the transient binding of the tracer to non-CP ligands and then to CP after metabolism in the liver, is resolved in relation to time.[4] Therefore, analysis of sequential plasma copper samples over time may be sufficient to separate the binding of tracer to each fraction. However, when the tracer is administered orally, there is considerable temporal overlap of each plasma copper fraction[5] due to gastrointestinal absorption of tracer over a prolonged period. In order to measure each fraction separately, it is therefore necessary to physically separate the two copper-binding fractions.

An elegant method for extraction of CP utilises an affinity resin[6] and this technique has been applied to the separation of CP and non-CP plasma copper fractions.[7] However, the resin is not commercially available, is expensive to make and has a limited shelf-life. It is well known that certain amino acids such as histidine at sufficient concentration can remove copper from the N-terminal binding site on albumin[8] and so we investigated the use of histidine to selectively remove copper from the non-CP fraction in plasma. This copper fraction was separated from CP-bound copper by dialysis and extraction using Chelex-100 resin. The potential utility of this method was evaluated by comparing the appearance of exchangeable ^{65}Cu in plasma with fecal appearance data in a human study.

Materials and methods

Extraction of exchangeable copper

Preliminary studies showed that direct extraction of Cu^{2+} from non-CP copper in plasma using the copper-chelating resin Chelex-100 was not suitable due to contamination of the resin by protein and copper from CP. The amino acid histidine, which binds Cu^{2+}, was therefore used as a sequestration and carrier system for copper transfer across a dialysis membrane. Chelex-100 resin (200–400 mesh, Na form, Bio-Rad, Hemel Hempstead, UK) was added to the dialysate to sequester copper from the histidine complex and drive the removal of all non-CP copper from the plasma sample. Low molecular weight components of plasma were removed by dialysis with buffer only prior to dialysis with histidine containing buffer. The copper content of buffers and reagents was substantially reduced by passing the solutions through equilibrated columns of Chelex-100 resin prior to use. The recovery of copper from

the plasma non-CP fraction and remaining copper contamination was measured using ^{65}Cu-labelled human serum albumin instead of plasma. The ^{65}Cu recovery was found to be low (12%) and this was due to inefficient removal of copper from the Cu–His_2 complex by the suspension of ion exchange resin. While data could be corrected for this low recovery, a modification of the method that improved recovery up to 52% was subsequently introduced. This involved passing the dialysate through a 2 mL equilibrated column of Chelex-100, washing the resin and then eluting the copper using 20 mL of 2.5 M nitric acid. The amount of contaminating copper in the extracted non-CP copper from 5 mL of plasma was found to be 14.5 ± SE 0.5 ng.

Dialysis-based extraction technique for albumin-bound copper

Human plasma (5 mL) was transferred into 23 cm lengths of 1000 MWCO Spectra/Por cellulose ester dialysis membrane (NBS Biologicals, Hatfield, UK) which had been thoroughly washed with purified water. The lengths of membrane were sealed, washed with water and dialysed for 2 h against 75 mL of 175 mM ammonium phosphate buffer, pH 7.

The dialysate was then replaced with 75 mL of 175 mM ammonium phosphate buffer containing 50 mM histidine and 1 mL of Chelex-100 resin slurry, and the samples were dialysed overnight at 4 °C. The Chelex-100 resin was recovered by centrifugation, washed twice with purified water, re-suspended in 1 mL of 2.5 M nitric acid and centrifuged to yield a supernatant containing the extracted copper. The supernatants were then filtered using 0.45 μm filter units. The retentates were dried on a hot plate, ashed overnight in a muffle furnace at 480 °C and then re-dissolved in 1 mL of concentrated nitric acid. An aliquot of 0.2 mL was removed for quantitative copper analysis by atomic absorption spectrophotometry, and the remaining solution was dried on a hot plate then re-dissolved in 2.5 mL of 1 M ammonium acetate, neutralised with concentrated ammonia solution. The samples were then applied to a Chelex-100 ion exchange chromatography minicolumn to remove monovalent cations and other contaminants. The method used is described below for the digested fecal samples, but only half the volume of reagents was used. The acid-eluted copper-containing fraction was analysed for copper isotope ratios by ICP-MS (see below).

In order to measure contamination by copper during the dialysis-Chelex procedure and also to measure recovery of copper from albumin, 5 mL aliquots of a ^{65}Cu-labelled human serum albumin solution, which had been diluted 1/10 with 175 mM ammonium phosphate buffer pH 7, were dialysed and processed exactly as described for the plasma samples. Corrections were applied to the plasma data for both contamination and copper recovery.

Preparation of ^{65}Cu-labelled albumin standard

In order to remove any existing bound copper, human serum albumin (0.45 g of Fraction V, Sigma, Poole, UK) was dissolved in about 3 mL of 20 mM sodium acetate buffer pH 5 and made up to approximately 5 mL before dialysis against 3 × 1 L volumes of the same buffer for a total of 18 h. After addition of 0.134 mL of 50 mM $^{65}CuSO_4$ (99% enriched ^{65}Cu, UK Atomic Energy Authority, Harwell, UK; to give an albumin : Cu molar ratio of 1), the solution was thoroughly mixed and was made up to 10 mL with 100 mM ammonium phosphate buffer, pH 7. It was then dialysed for 18 h against 3 × 1 L changes of 50 mM ammonium phosphate buffer at pH 7. The concentration of copper in the retentate was analysed by atomic absorption spectrophotometry and the solution was diluted using 150 mM ammonium phosphate buffer, pH 7, to obtain a copper concentration of 10 μg mL^{-1}. Samples of the solution were acid digested and prepared for analysis of copper and copper isotope ratios by atomic absorption spectrophotometry and ICP-MS, respectively (see below). Aliquots (1 mL) of the remaining ^{65}Cu-labelled albumin solution were frozen and stored at −20 °C.

Human volunteer study

Subject. One healthy, non-smoking, female volunteer (age 36, weight 60 kg, height 1.7 m, body mass index 20.8) was recruited to take part in the study. A 10 mL blood sample was taken from the volunteer for measurement of biochemical and haematological indices (MD 8, Coulter Counter); all were within the normal range. The study protocol was explained to the volunteer and written, informed consent obtained during a visit to the Institute of Food Research (IFR) Human Nutrition Unit. The research protocol was approved by the IFR Human Research Ethics Committee and was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association.

The subject was given an oral dose of ^{65}Cu stable isotope with a standard breakfast meal, in order to measure the efficiency of copper absorption. The volunteer was taking part in a study to investigate the effect of certain dietary components on copper absorption, and so the volunteer was also administered an oral dose of ascorbic acid and iron sulfate.

Tracer dose preparation and administration. Isotopically enriched copper chloride was prepared from elemental ^{65}Cu (Europa Scientific Ltd, Crewe, UK) to give a final concentration of 0.95 mg mL^{-1} in 0.1 M HCl. The solution was divided into aliquots and stored in plastic vials at −20 °C until required.

Holmium was used as a non-absorbable rare-earth marker of unabsorbed tracer excretion.[9] The holmium oral doses were prepared by dissolving holmium chloride (Avocado Research Chemicals Ltd, Heysham, UK) in demineralised, purified water to give a final concentration of 0.510 mg mL^{-1}. A combined oral dose of ferrous sulfate and ascorbic acid (both from Sigma, Poole, UK) was prepared by dissolving both substances simultaneously in demineralised, purified water. The final nominal concentrations were 10 mg mL^{-1} and 40 mg mL^{-1} for iron and ascorbic acid, respectively. All solutions were stored in plastic vials at 20 °C until required.

The subject fasted for 12 h before receiving breakfast consisting of 70 g white bread and 17 g butter. She also consumed 6 mg of ^{65}Cu, 1 mg of holmium, 50 mg of iron (ferrous sulfate) and 200 mg of ascorbic acid in 130 g of diet cola, along with a radio-opaque marker.[9] This marker was also taken with breakfast for the next three consecutive days. The dose of ^{65}Cu is described in this paper as a tracer, but it is not a true tracer because the dose is sufficiently large to influence copper absorption and perhaps also copper metabolism.

Collection of samples. A cannula was inserted into a vein in the forearm and a blood sample (20 mL) was taken at 0, 15, 30, 60, 90, 120, 150, 180, 210, 240 and 300 min. Three other blood collections were performed at 6, 10 and 17 days post-dosing. Blood was collected into lithium heparin tubes (Sarstedt, Leicester, UK) mixed gently by inversion and centrifuged at 15,000 × *g* for 15 min. The supernatant plasma was transferred to nalgene cryogenic vials (Nalge Company, Rochester, NY, USA), snap-frozen in liquid nitrogen and stored at −80 °C. When required, samples were defrosted and 1 mL was removed for analysis of total plasma copper, and 5 mL for extraction using the dialysis-Chelex technique. Plasma for total copper analysis was prepared by dry ashing, as described above for the retentate remaining after dialysis-Chelex extraction of plasma.

The volunteer carried out a complete 12 day fecal collection post-dosing, and also collected a baseline sample on the day before the start of the study. All stools were collected in autoclavable bags (Bibby Sterilin, Stone, UK).

Preparation of fecal samples for analysis. The stool samples were weighed, autoclaved, freeze-dried and weighed again to determine water content. Each sample was ground in a mortar and pestle, sieved, homogenised in a powder mixer and then sub-sampled. The number of radio-opaque markers in each sample was recorded. Two 0.5 g replicates of each powdered fecal sample were then added to separate Savillex digestion vessels (Savillex Corporation, Minnetonka, Minnesota, USA) followed by 1.8 mL of concentrated nitric acid (Primar grade, Fisher Scientific, Loughborough, UK) and 0.2 mL hydrogen peroxide (AristaR grade, BDH, Poole, UK). The vessels were sealed, incubated overnight at room temperature, and then subjected to microwave irradiation in periods of short duration and increasing power, from 30 s at 164 W to 10 s at 855 W, until a clear solution was obtained.

For quantitative analysis of copper and holmium, 1 mL of the acid digests was diluted to 10 mL using purified water. The remaining digest solution was used for isotope ratio analysis after separation of divalent cations, including copper, from monovalent cations and other contaminants, using Chelex-100 ion exchange chromatography. The mini-columns (Econo-columns, Bio-Rad, Hemel Hempstead, UK) used, containing 2 mL of Chelex-100 resin (200–400 mesh, Na form, Bio-Rad), were washed with 20 mL of 2.5 M nitric acid, 20 mL of 2 M ammonium hydroxide and finally, 20 mL of purified water before use.

The sample digests were dried on a hot plate, the residue re-dissolved in 5 mL of 1 M ammonium acetate and the pH adjusted to 8 using concentrated ammonia solution (AristaR Grade, BDH, Poole, UK). The samples were transferred to polypropylene test-tubes and centrifuged at 4,000 rpm for 10 min. The supernatants were then applied to the bed surface of the Chelex-100 columns followed by 20 mL of 1 M ammonium acetate and 15 mL of purified water to wash out any remaining sample. The divalent metals including Cu were eluted from the Chelex-100 columns using 20 mL of 2.5 M nitric acid. These solutions were then analysed by ICP-MS for copper isotope ratios.

Copper and holmium analysis

Quantitative analysis of copper by atomic absorption spectrophotometry (AAS). Samples were quantitatively analysed for copper using a Thermo-Unicam Solaar 969 atomic absorption spectrophotometer, which had been calibrated with standards prepared from 1,000 mg L^{-1} SpectrosoL (BDH, Poole, UK) stock solution. Measurements were made using transient height injection mode and three replicate injections for each sample.[10] Standards were checked every 20 samples during analysis of batches and contamination was evaluated by analysis of sample blanks. The accuracy of copper quantification using the microwave acid digestion technique combined with atomic absorption spectrophotometry was evaluated using the standard reference material GBW 07605 Tea (Laboratory of the Government Chemist, UK).

Quantitative analysis of holmium by ICP-MS. Holmium was quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using a VG PQ2+ instrument (VG Elemental, Winsford, UK), equipped with a quartz torch, a Meinhard glass expansion nebuliser (GEG) and a Scott-type water-cooled double-pass spray chamber, cooled to 4 °C. Typical operating conditions were as follows: rf power = 1350 W, nebuliser argon flow rate = 0.8 L min^{-1}, cool argon flow rate = 14 L min^{-1} and auxiliary argon flow rate = 0.7 L min^{-1}. Samples were introduced by natural aspiration for improved stability and a peristaltic pump (Gilson minipuls 3, Gilson Medical Electronics, Middleton, WI) was used to drain the spray chamber with the flow rate set at 0.8 mL min^{-1}, a sample uptake time of 2 min and a wash time of 3 min. Blank values (for background subtraction) were acquired once, whereas samples and standards were acquired 3 times, all for 60 s. A range of calibration standards were prepared by serial dilution using a 500 ng mL^{-1} holmium stock solution, prepared from a holmium atomic absorption standard solution (Aldrich, Poole, UK). Bismuth (Aldrich atomic absorption standard solution), at a concentration of 100 ng mL^{-1}, was used as an internal standard to correct for drift in sensitivity during a series of analyses. The software used for data acquisition and computation was VG Elemental PQ Vision Issue 4.30. Data were collected using an *m*/*z* scan range of 110.0–225.0 u with a dwell time of 320 μs using 19 channels per u and 5 points per peak.

Analysis of copper isotope ratios by ICP-MS. Isotope ratios were measured by ICP-MS using the instrument, operating conditions and sample introduction described above. Typical blank values (for background subtraction) were acquired once, whereas samples and standards were acquired 5 times, all for 60 s. Data were collected using an *m*/*z* scan range of 60–68 u with a dwell time of 320 μs using 19 channels per u, and 3 points per peak. Isotope ratios obtained using copper atomic absorption standards (Aldrich) were used to correct for instrument mass bias.

On setting up the ICP-MS each day, the analytical precision and the stability of the mass bias was checked over a period of 1 h by repeated analysis of a 500 μg L^{-1} Cu standard. Precision values of < 0.5% RSD were considered acceptable. Following analysis of a blank for background correction, samples were analysed in batches of 4 with analysis of a 500 μg L^{-1} Cu standard between each batch to correct for instrument mass bias.

For the plasma sample extracts, an MCN 100 micro-concentric nebuliser (CETAC Technologies, Omaha, Nebraska, USA) was used with unassisted aspiration and the spray chamber was drained with the help of a peristaltic pump. Each sample and standard was acquired 10 times and the nebuliser gas flow rate was set at 1.0 L min^{-1}.

Method for calculating recovery of Cu tracer. Isotope ratios were firstly corrected for fractionation by reference to the published isotope ratio for $^{63}Cu/^{65}Cu$. The correction is based on the measured ratio for the copper standard solution. Calculation of the tracer recovered in each sample was made as described elsewhere.[11]

Results

Analysis of tracer copper in plasma using the dialysis-Chelex method

^{65}Cu enrichment of whole plasma was compared to that of the non-CP fraction. The non-CP fraction copper was rapidly and significantly enriched with ^{65}Cu up to 30 min after consumption of the tracer dose (Fig. 1). Further enrichment was observed up to 210 min but thereafter decreased. The isotope ratio of CP copper on the other hand was completely unaffected by ingestion of the tracer over the initial 2 h period, (Fig. 1).

Although enrichment of whole plasma copper with ^{65}Cu could be detected following ingestion of the tracer dose, it was considerably lower than that found in the non-CP fraction. This was due to isotope dilution by CP-bound copper, which probably constitutes over 80% of total plasma copper.

Calculation of tracer in the plasma, non-CP and CP fractions yielded the profiles shown in Fig. 2. Importantly for validating the dialysis-based extraction method, independent analysis of tracer in plasma total copper and non-CP fraction copper gave very similar results during the initial 120 min period when no tracer was bound to CP (Fig. 2). As CP-bound tracer increased (120–300 min), so the non-CP fraction tracer decreased, and the sum of the two was close to the value obtained for tracer in the total plasma copper.

At 6, 10 and 17 days after ingestion of the tracer, the proportion of tracer found in the non-CP fraction was 7.9%, 7.6% and 4.9%, respectively, the remainder being associated with CP.

Validation of isotope and elemental analysis of fecal copper and holmium

As regards the accuracy of quantitative analysis for copper, values obtained for the standard reference material GBW 07605 Tea (17.5 ± *s* 0.2 μg g^{-1}) were well within the specified limits (17.3 ± 1 μg g^{-1}), and fecal samples spiked with varying amounts of copper gave a mean copper recovery of 99.8% ± *s* 9.2. Standard reference materials were not available for holmium but the recovery of this rare earth in all fecal collections was 103.7% of that administered to the volunteer. The internal standard selected for holmium analysis by ICP-MS, namely bismuth, was an important factor contributing to the acquisition of accurate data for this element. All raw data for isotope ratios of copper from fecal samples were corrected according to the difference between the empirical and published value (2.235; VG Elemental, Winsford, UK) for the natural copper isotope ratio.

The precision for quantitative analysis of copper by AAS was 1.2% RSD whereas that for isotope ratio analysis by ICP-MS was 0.4%. Hence the error on the mass of labelled copper in the fecal samples after 5 days was about 10%. Inter-replicate variation was 1.2% RSD for quantitative copper analysis whereas that for isotope ratio analysis was 0.4% RSD. The analytical and inter-replicate precision for holmium analysis was 1.2% and 1.9% RSD, respectively.

Fecal excretion of holmium and copper tracer

The excretion of holmium and copper tracer was found to be coincident, such that the relationship between them yielded an RSD of 0.9996. When presented as the amount of tracer retention within the body over time, there was a rapid decrease over the first few days when the unabsorbed tracer was excreted in the feces (Fig. 3). However, a residual amount of tracer remained and was gradually excreted over the following days at a relatively constant rate. This represented tracer which had been absorbed and was subsequently transferred back to the intestinal lumen through secretion and/or sloughing of mucosal cells.

Apparent absorption, which does not account for endogenous excretion, was calculated as 7% by subtracting the total amount of tracer recovered in the feces from that which was ingested by the volunteer. True absorption was estimated as 10.8% by calculating the amount of tracer re-excreted per day and correcting the apparent absorption accordingly. The rate of re-excretion of tracer was estimated to be 0.044 mg d^{-1}.

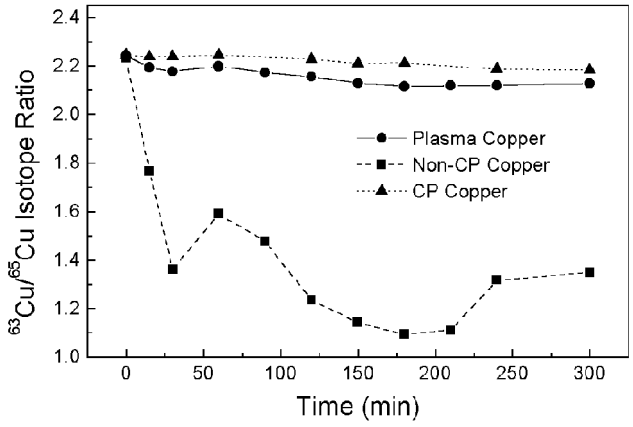

Fig. 1 Enrichment of total plasma copper, non-ceruloplasmin (non-CP) copper and ceruloplasmin (CP)-bound copper with ^{65}Cu from a tracer ingested by a volunteer with a breakfast meal.

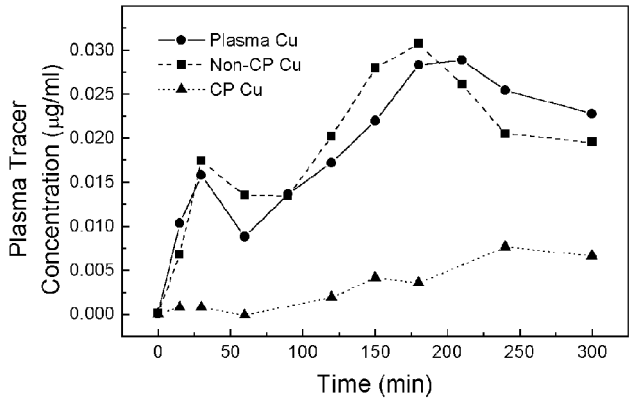

Fig. 2 Concentration of ^{65}Cu tracer in total plasma copper, non-ceruloplasmin (non-CP) copper and ceruloplasmin (CP)-bound copper of a volunteer following ingestion of a ^{65}Cu tracer with a breakfast meal.

Discussion

The dialysis-based extraction method was found to be suitable for the separation of protein-bound non-CP copper from CP-bound copper, without cross-contamination, so that both copper fractions can be independently studied. It has the advantage of being rapid and inexpensive, with all materials being commercially available. Molecular ion interference of ^{63}Cu analysis resulting from contamination of non-CP fraction copper with sodium was avoided by using ammonium phosphate rather than sodium phosphate buffers.

When applied to the plasma samples, the resulting method yielded data showing rapid and strong enrichment of ^{65}Cu in non-CP fraction copper but, initially, no enrichment in CP-bound copper. This result is consistent with the established view of copper metabolism in which absorbed copper binds first to the non-CP fraction binding ligands and is only later bound to

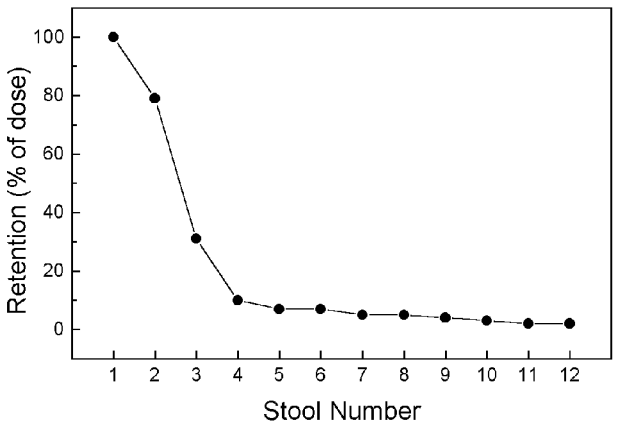

Fig. 3 Retention of the ^{65}Cu tracer at sequential stool collections after ingestion of the tracer.

CP when secreted from the liver.[2,3] The accuracy of the method for measuring non-CP fraction tracer was confirmed by independent analysis of tracer in total plasma copper at early time points when the non-CP fraction was the only significant ligand binding copper. Hence, for evaluation of copper absorption within 2 h of tracer administration, it may be more practicable to measure enrichment of total plasma copper with ^{65}Cu. Indeed Lyon and coworkers[5] measured ^{65}Cu enrichment in total plasma copper after oral administration of 3 mg ^{65}Cu and found that the plasma appearance of the tracer peaked at approximately 2 h. However, after reaching a minimum level at approximately 6 h, there was a secondary increase over a period of several days, indicating the appearance of tracer bound to CP. A similar result was obtained in the present work and the majority of tracer remaining in the plasma at 6, 10 and 17 days was bound to CP. Therefore, for longer term (>120 min) studies of copper absorption, non-CP fraction and CP-bound copper must be separated. In the case of a large extrinsic dose of tracer, as used in the present study, significant amounts of tracer were bound to the plasma non-CP fraction within 30 min of its ingestion. This indicates significant absorption of copper from the stomach, which concurs with previously published observations.[12]

As an established technique for measuring absorption,[13], fecal monitoring for unabsorbed tracer provided an independent way of evaluating results obtained with the dialysis extraction method. Tracer detected after the passage of all holmium was clearly derived from endogenous excretion. Values of 7% and 10.8% for apparent and true absorption, respectively, were lower than might have been expected from an unsupplemented diet and much lower than other published values of $>50\%$.[14] This is probably due in part to the ingestion of copper absorption inhibitors.[15–17] However, in addition, the ingested tracer dose (6 mg) added significantly to the overall dietary intake of copper and an intake over 6 mg d^{-1} is predicted to reduce absorption to $<20\%$.[18] It seems likely that saturation of copper transport systems in the gut mucosa, and perhaps other homeostatic mechanisms, prevented excessive copper absorption from this large dose.

In comparison with the fecal copper data, our initial evaluation of the plasma copper data using a SAAM II modeling system (data not shown), suggests that an accurate estimate of Cu absorption could be calculated from enrichment of non-CP copper with ^{65}Cu. The reproducibility of this encouraging result will be tested in a larger on-going human study. Regarding previous attempts at modeling Cu absorption from plasma data, only one study,[19] separated the non-CP bound from the total labelled copper. This is essential if any estimate of absorption is to be made since the newly absorbed copper is not carried by ceruloplasmin. Buckley and coworkers,[19] gave the labelled copper as an infusion to try and ascertain whether the total labelled copper in the plasma could be separated into CP and non-CP. They used a two-compartment model to fit their data. Another attempt to model human copper metabolism was described by Scott and Turnlund.[20] This is a much more complicated model with five compartments, two delay components and two routes of excretion. They did not attempt to separate the CP from the non-CP bound copper in their plasma samples.

There is also considerable potential for application of the dialysis extraction method in the study of liver copper kinetics and copper status. In the current study, sampling times up to 5 h after tracer ingestion were selected to observe the transient enrichment of non-CP fraction copper with ^{65}Cu. Plasma samples taken at longer time-points after fecal excretion of all unabsorbed tracer confirmed that most of the absorbed tracer was bound to CP rather than the non-CP fraction. Monitoring the period between 5 h and a few days is likely to yield considerable additional information concerning the rate of uptake and secretion of copper by the liver. Since hepatic copper reserves constitute about 10% of the body copper content and the liver is a key organ in the homeostatic regulation of this metal, information thus gleaned about the kinetics of hepatic copper turnover could be very useful in evaluating status.

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