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Iron, copper, and zinc absorption and turnover; the use of stable isotopes

Abstract This overview demonstrates the increasing use of low natural abundance stable isotopes in the investigation of mineral metabolism. There are many practical problems associated with their use and analysis and their expense has limited their application in some areas such as studies in adults. Undoubtedly we will have to assess our ideas and protocols as the practical problems and their metabolic implications become better appreciated but none the less, the use of such isotopes will certainly refine our understanding of the way the body uses elements such as zinc, copper, iron and selenium and other essential elements and will enable us to determine our dietary requirements for these nutrients and to find ways of detecting more efficiently early deficiency and toxicity states.

Key words Iron · Copper · Zinc · Absorption · Stable isotopes

Abbreviation MS mass spectrometry

Introduction

The original remit of this paper included some consideration of the recommended intakes of iron, copper and zinc in children with inborn errors of metabolism. However, other than with inborn errors affecting specifically the metabolism of these elements, there is, at the moment no good information, on the precise amounts of these trace elements which might be needed by children with other inborn errors of metabolism. However it is conceivable that in such circumstances requirements might differ from those of healthy children, even if it is only because allowance has to be made for the possibility that the absorbability of these minerals from the synthetic diets used

in the management of inborn errors of metabolism might differ from that from normal diets. Studies of mineral metabolism based on techniques using stable isotopic labels offer a means of approaching such problems [5, 17, 24].

Although the biochemical importance of trace elements such as zinc, iron, copper and selenium is well known, we still need to know more about: (1) ideal dietary intakes; (2) how the metal species and dietary food matrix influence the efficiency with which trace elements are absorbed; (3) the processes by which the elements are absorbed, distributed around the body, stored and excreted; and (4) how these metabolic processes change with disease states and adapt with incipient deficiencies and toxicities. This information would help us more reliably to detect early stages of toxicity and deficiency and to assess the physiological and dietary requirements for these elements during health and disease.

Systemic metabolism of trace elements

The systemic control of trace element distribution, biological exploitation and homeostasis is effected by exploiting their oxidation states and affinities for organic molecules to create a series of selective physicochemical compartments which together achieve specific pathways which deliver the elements to their operative sites in appropriate forms and concentrations. The accumulative specificity of these pathways is important because if each step of the metabolic sequence for the cationic trace metals is examined in isolation, numerous interactions amongst the elements can be found.

In this 'metabolic' context, the essential trace elements can be regarded as forming three groups:

1. Cationic elements such as zinc, manganese and copper. These are transferred and utilized as inorganic ions; they need specific carriers to transfer them across lipid membranes and to maintain their solubility at physiological pH within the intracellular and extracellular environment. Their homeostasis is controlled mainly by the gastrointestinal tract and liver.

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Table 1 Factors influencing the intestinal uptake and transfer of trace elements

Systemic factors
Anabolic demands
Growth in infancy and childhood
Pregnancy and lactation
Post-catabolic states
Endocrine effects
Infection and stress
Specific systemic reserves of metal
Genetic influence, inborn errors of metabolism
Nutritional status for other nutrients
Luminal and dietary
Chemical form and oxidation state of element in the diet
Presence of:
antagonistic ligands (phosphate, carbonate, tannates, polyphenols, oxalate)
facilitatory ligands (ascorbate (for iron), carboxylic acids, some sugars, amino acids, fatty acids)
competing metals
Intestinal redox state
Luminal redox state

2. Anionic elements such as molybdenum, selenium, chromium and fluoride. These have a greater ability to cross lipid membranes spontaneously and are more water soluble at physiological pH, these elements have a highly efficient gastro-intestinal uptake and transfer, and their systemic utilization and compartmentalization is effected by changes in their oxidation states. Their homeostasis is dependent upon renal excretion.

3. Other elements, such as cobalt (in vitamin B12), molybdenum and, possibly, chromium, which have many oxidation states are metabolized and used in organic complexes.

Confidence in the current estimations of human requirements for trace metals is limited by our ignorance of the precise interplay between systemic, luminal and dietary factors which affect the intestinal absorption and systemic use of trace elements (Table 1). This efficiency with which a dietary constituent is absorbed and utilised in the body is known as 'bioavailability': this term reflects the combined effect of all the factors in Table 1.

Stable isotopic labels

Stable isotopic labels provide an invaluable means for studying the metabolism of iron, zinc, copper, and selenium which constitute the main trace elements of current interest. There is also interest in manganese but unfortunately manganese is mono-isotopic and thus has no possible stable isotopic labels.

The general advantages and disadvantages of the use of low abundance isotopic markers or tracers of the lighter

elements which have been reviewed by Koletzko (this volume) apply equally to inorganic elements. But there are some further considerations. First is the assumption that any isotopic tracers exchange freely with, and behave identically to the natural elemental pool (element being traced) being investigated: one cannot always be confident of this with the trace metals because by virtue of their chemical nature they do not necessarily equilibrate effectively, and, additionally, the nature of their homeostatic control might limit effective mixing. These points apply as much to radiotracers as to stable isotopic tracers. However with radiotracers, truly trace amounts of label can be used and this provides a little more tolerance and sensitivity with which to explore systemic metabolism, and the relative ease of quantitative detection in biological tissues and fluids, and whole body counters, and imaging techniques, the rates of accumulation and loss from the body, and their distribution in the body can be monitored. In the case of trace elements truly trace levels cannot be achieved even with the least abundant stable isotope. This creates a significant disadvantages in that it is usually impossible to use such tracers in sufficiently small amounts to avoid altering the mass and thus the metabolism of the pool being investigated.

One cannot assay the inorganic stable isotopes easily and whatever analytical technique is used it usually involves pretreatment to isolate from interfering matrices and other elements. The analytical equipment is expensive as are the isotopes themselves. Furthermore commercial availability and purity of isotopes are often unreliable, and it is always prudent to analyse the new supplies of isotopes to confirm their declared enrichment and purity.

The isotopic composition of iron, copper zinc and selenium are shown in Table 2. It can be seen that low abundance isotopes suitable for tracers are available for iron (Fe-54, Fe-57, Fe-58), zinc (Zn-67, Zn-70) and selenium (Se-74) but not for copper. Unfortunately the high abundance of the least abundant copper isotope, severely limits its use and our understanding of the metabolism and bioavailability of copper. Representative costs are shown in Table 3 – not surprisingly the least naturally abundant isotopes are particularly expensive. All these factors have to be considered in designing studies [5, 17, 24].

Table 2 Isotopic composition of iron, copper, zinc and selenium

	Atomic weight	Natural abundance		Atomic weight	Natural abundance
Copper	63	69.2	Iron	54	5.8
	65	30.8		56	91.8
				57	2.2
				58	0.3
Zinc	64	48.63	Selenium	74	0.88
	66	27.90		76	9.0
	67	4.10		77	7.7
	68	18.75		78	23.5
	70	0.62		80	49.6
			82	9.4	

Table 3 Representative costs of stable isotopes

Element	Isotope	Enrichment (atom %)	Cost (£/mg)
Iron	54	95–97	10–20
	57	80–97	10–28
	58	65–93	100–300
Copper	63	99	< 2
	65	91–99	2–5
Zinc	67	76–94	12–28
	68	37–99	2–6
	70	65–88	80–300
Selenium	74	31–99	60–520
	76	74–99	4–25
	77	68–94	15–20
	82	73–99	14–35

The practical problems is planning investigations are therefore: cost-effectiveness, consideration of whether the label being used is physiologically relevant with respect to the amount being used, its form or species, its ability to equilibrate with the native pool of the element; and the analytical method's precision and accuracy of the data and their suitability for computation.

Analytical methods

The earliest study using a stable isotope label involved neutron activation analysis of Fe-58. Neutron activation analysis has the obvious disadvantage of needing a nuclear reactor and the use of stable isotopes with irradiation daughter products with suitable decay characteristics for determination. This technique has a variable sensitivity and precision (1%–10%) which, with its lack of versatility, disadvantaged it compared with the mass spectrometric techniques which have been increasingly used since 1978 [24]. Of these thermal ionisation mass spectrometry (MS) is widely accepted as giving the best precision and sensitivity, however for some types of investigation inductively coupled plasma MS and fast atom bombardment

Table 4 Methods for mass spectrometric stable isotope analysis of metals

Method	Sensitivity (µg)	Relative precision
Thermal ionisation MS		
magnetic sector	1–10	< 0.1%
quadrupole	1–10	0.1%–1%
Inductively coupled plasma MS		
quadrupole	1–20	0.3%–1%
Fast atom bombardment MS	1–10	0.3%–2%
Electron ionisation MS		
Gas chromatography MS	< 1	0.1%–10%

are equally valuable and have practical advantages in terms of time and cost. Thermal ionisation MS is most appropriate for analysis of small samples such as plasma and blood components when high sensitivity and precision are paramount, whereas inductively coupled plasma MS is useful for dietary and faecal analysis in which larger amounts of isotopes are usually present and for multi-elemental isotope comparison and ratio-determination analyses. Additionally inductively coupled plasma MS can provide a faster throughput of samples than fast atom bombardment and thermal ionisation MS. Gas chromatography MS and electron ionisation MS have found most use with determination of Se, but it is possible to analyse organic chelates of the other elements [5] (Table 4).

Intrinsic and extrinsic labelling

Extrinsic labelling is achieved by adding the label to the pool being studied. Thus, with a foodstuff the tag is added and allowed time in which to equilibrate with the various pools in that food. Equilibration might be more efficient and rapid if the pool is in solution. Equilibration with the dietary pool is much more limited with dry foods, but none the less, protocols use used in which the isotope is added to a meal or foodstuff immediately before it is eaten: it is assumed, or hoped, that adequate mixing and equilibration of the tracer and trace pools will occur within the intestinal lumen. One should not be too confident of this, and this approach, as mentioned earlier, raises concern about the identity and representivity of the pools labelled and about perturbations which the label may induce in the size and behaviour of the various pools. (For a fuller discussion of this issue and for unreferenced statements see references [5, 17].

These problems can be avoided by biologically incorporating the label in the foodstuff. Thus isotopes can be supplied to plants by hydroponic cultivation or, less physiologically, injections into their leaves, or to animals orally or parenterally. This is an expensive approach because the incorporation of the isotope an edible part of the animal or plant is often quite low; e.g. that of Zn-68 and Zn-70 into chicken muscle was 2%–3% and of Zn-70 into soy bean seeds was 18% [12].

Fortunately, several studies have shown that an acceptable similarity between extrinsic and intrinsic tags can be achieved for copper [13] and for zinc [6], but the choice needs to be reviewed in the context of the study aims, the type of foodstuff and the desired tolerance of any inconsistencies which might arise from the labelling strategy [7, 17].

For example the absorption by infants of extrinsic and intrinsic Zn labels from infant formulas was similar [6, 19]. However a 10%–20% lower absorption of Zn from extrinsically labelled chicken compared with an intrinsic label has been described. Bigger differences noted between the absorption of extrinsic and intrinsic Se labels probably represent differences in the metabolism of the labels (selenite and selenomethionine) used. Selenite would

be handled as an anion whereas selenomethionine would use pathways taken by methionine [3, 20]. On the basis of our understanding of the absorption of inorganic iron and organic (haem) iron similar differences would be exposed if such contrasting labels of this element were used. These last two examples illustrate the importance of selecting appropriate chemical forms of labels for metabolic studies. However, for most studies involving inorganic species it would seem that extrinsic labels might prove satisfactory providing adequate time is allowed for full exchange with the native element and as long as one remembers the possibility that this equilibration might not be complete.

Experimental approaches

These can be summarised as the metabolic balance and faecal monitoring techniques, plasma appearance and tissue incorporation or retention, and a kinetic analysis based on monitoring the alimentary, circulating and occasionally the urinary concentrations of oral or intravenous labels [17, 24].

Metabolic balance or faecal monitoring

A traditional approach used to estimate the apparent or net absorption of a trace element such as Zn, Cu, Se, and Fe has been to measure, over a period of time (usually 3–7 days), its intake in the diet and its loss in faeces over an appropriate corresponding period, and to determine the difference. Strictly speaking this is the luminal disappearance of the element. The disappearance of the element between the mouth and anus does not necessarily mean that the substance has been truly absorbed into the body – it may have been retained in the intestinal mucosa. Furthermore the gut itself may deliver the element into its lumen via biliary, pancreatic and mucosal secretions or by mucosal cell loss. This entry of endogenous element into the luminal and, ultimately the faecal pool, mixes the two sources, and consequently gives a lower overall net absorption value for the exogenous material. This difficulty can to a large extent be overcome by using an isotopic label of the exogenous element and determining its fractional luminal disappearance between the dietary and faecal pools. By measuring the total size of these pools the relative contributions to the faecal pool of exogenous and endogenous elements can be calculated and their changes with physiological states or different dietary intakes can be calculated, as has been done for zinc and copper [22, 23, 25].

It is also possible to label the endogenous pool of an element, usually by giving the isotope intravenously and using the appearance of this tracer in the stools as an indicator of the faecal loss from this compartment [9].

The metabolic balance approach can be refined further by measuring the urinary appearance of tracers: this is certainly needed for selenium, which is absorbed quite efficiently and which is then excreted via the kidneys.

Examples of the analysis of data are given in the various references cited.

Tissue incorporation

This approach is of most relevance to the investigation of bioavailability and is of use if the element of interest is incorporated efficiently in a specific tissue which is easy to sample. Thus it is particularly valuable for studies of the bioavailability of iron – an isotope of iron can be given orally and its subsequent incorporation into newly synthesised haemoglobin can be measured by taking a blood sample, usually 14 days later [8, 11]. An important refinement of this approach exploits the availability of more than one low abundance tracers for iron [14]. A standard solution and dose of iron containing a second iron isotope can be used to correct for the considerable inter-individual variation in the initial uptake of iron and enables the precise effect of different foodstuffs to be ascertained. This technique is particularly useful for infant populations in which there are considerable problems with kinetic analysis and the use of oral and simultaneous intravenous administration of isotopes.

Recent investigations in the intestinal uptake and transfer of iron during pregnancy demonstrate the potential benefits of using stable isotopes for longitudinal studies in situations in which the use of radioisotopes would now be unacceptable [1]. One study using radio isotopes has shown that the absorption of non-haem iron from a standard meal was 3.1% in non pregnant women and, 0.8% in early pregnancy, then 4.5% and 13.9% at 24 and 36 weeks gestation respectively [21]. This impressive adaptation has now been confirmed using stable isotopic markers which demonstrated absorption from a solution containing 5.23 mg inorganic iron of 7.6% (range 1%–22%) at 12 weeks gestation, 21.1% (9%–58%) at 24 weeks, 37.4 (18%–56%) at 36 weeks and 26.3% (range 8%–54%) 12 weeks after delivery [27]. The practical relevance of these findings has subsequently been demonstrated in a longitudinal study of 12 normal pregnant women in whom the geometric mean absorption of a stable isotopic marker of iron as an extrinsic label of a test meal increased from 7.7% at 12 weeks gestation to 36% and 66% at 24 and 36 weeks respectively, and a decrease to 11% at 16–24 weeks post-partum [2]. Iron absorption in this instance was measured by plasma appearance/red cell incorporation of the element.

Metabolic modelling and kinetic analysis

If an isotope is given orally its appearance in the plasma can be plotted against time by taking several samples (over 4–6 h) for analysis. The resultant curve and its shape is the product of the rate of entry of the isotope from the intestine into the plasma and of the rate of disappearance of the isotope caused by its uptake into the body tissues. The latter process can be also be assessed either

independently or simultaneously by giving a label intravenously. The two curves can then be assessed by deconvolution analysis using standard compartmental and kinetic modelling analysis to provide specific information about the size and variation of the different metabolic pools during various physiological and disease states and during systematic adaptation to different intakes of the element under investigation [4, 26].

This approach assumes that the oral and intravenous labels are metabolised similarly. This may not be the case because orally administered elements may be bound to different carriers in the vascular compartment than those with which the intravenous isotope binds and because they are more likely to be subjected to first pass hepatic processing than those given directly into the systemic circulation. The implications of these metabolic differences and their relevance to specific elements are not yet fully assessed. Even so, this is a potentially powerful technique for studying changes in the metabolism of elements, particularly zinc [16] and it has recently been extended by the exploitation of the observation that the urinary ratios of enriched isotopes match those of plasma 24 h after their administration. This phenomenon which was first applied to studies of the metabolism of calcium is now being applied to zinc and enables longer term and less invasive studies.

Detailed studies of zinc kinetics in man have been carried out using radio isotope ^{65}Zn in man using multiple compartments [26]. More recently stable isotopes have been used [16]. Fewer compartments can be incorporated in the models based on intravenous ^{70}Zn labels, given the limitations of our current models it is probably non-productive to develop models involving more than four systemic compartments, indeed two might be enough for the analysis of some studies [10].

I have mentioned already the limitations on studies of copper metabolism which are imposed by the absence of any suitable low abundance stable isotopic tracer. One way of getting around this problem has been used to investigate copper metabolism in copper deprived rats: all exogenous dietary copper was provided as the more abundant isotope ^{63}Cu and the subsequent rate of decline in the abundance of the endogenous ^{65}Cu was monitored over 8 weeks in tissues and plasma collected at autopsy to provide a measure of copper turnover in the labelled physiological and kinetic compartments [15].

This ingenious approach is probably not a tracer technique. Single dose studies of copper turnover and analysis have been used for sheep, cattle and rats with the analytical models varying in complexity [for references see 18]. More recently a multiple dose approach has been applied to human studies. ^{65}Cu tracer was given intravenously, and orally (over an extended period of 2 days to ensure an adequate plasma pool enrichment without the perturbation of metabolism which would have been encountered had the dose required been given as a single dose) to volunteers on varying intakes of dietary copper, and the total mass of the copper plasma pool and the isotopic abundance were monitored and the data analysed to generate a

compartmental model of copper metabolism which could be used to monitor homeostatic adaptation to low and high copper intakes [18].

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