Factors Influencing the Measurement of Bioavailability, Taking Calcium as a Model

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ABSTRACT For non-metabolizable supplemental nutrients, bioavailability is effectively equivalent to absorbability. Methods for measuring absorbability (balance, pharmacokinetic, tracer, urine increment, evoked physiological responses, and in vitro) are briefly characterized and their utility compared. When intrinsic labeling of a source is possible, tracer methods are generally the most accurate and precise, as well as often the least expensive. Factors influencing the measured end points of the various methods are described briefly. These include source factors such as pharmaceutical formulation, subject factors such as mucosal mass and the need status of the absorbing subject, and co-ingested factors such as other foods or food constituents. Extensive experience has shown that absorbability is difficult to predict from knowledge of the chemistry of the source, or even from the results of in vitro testing. Hence direct measurement of absorbability is essential to assure regulators and the general public that the source delivers what it promises. J. Nutr. 131: 1344S–1348S, 2001.

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Bioavailability is variously defined. For drugs and other substances that act within the body (as contrasted to within the gut), it is generally considered to be the quantity or fraction of an administered dose of a substance that gets into the circulation and then is not metabolized, complexed or excreted before it can exert its intended biological effect. With nutrients, for which metabolism is usual and appropriate and the route of administration is nearly always oral, the notion of bioavailability generally designates simply the quantity or fraction of the ingested dose that is absorbed. It is physiologically appealing to add “and utilized” to that definition, because doing so gets at the reason for taking the supplement. However, for many nutrients, utilization is a function of the nutritional status and physiological state of the subject. The same supplement will be utilized in some individuals and not in others. Hence, for the purpose of standardizing or characterizing products, utilization is probably a trap.

Closely related to bioavailability is the notion of bioequivalence. This takes at least two forms. A well-studied example of the first is found in the families of compounds in the vitamins A, D and E groups, the folic acid group and the vitamin K group. Here, equal absorption does not necessarily mean equal biological effect. This is understandable, because the compounds concerned are chemically different, even if closely related. But a second aspect of bioequivalence, less well studied but more relevant to my topic, is the effect of different pharmaceutical formulations on identical compounds. An example would be two preparations of calcium carbonate, chemically identical, but pharmaceutically different. They may not be bioequivalent precisely because they may not have equal bioavailability. This problem was pointed out in its most extreme form by Carr and Shangraw (1987) a number of years ago, and it ultimately led to the adoption by the U.S. Pharmacopeia of new disintegration and dissolution standards for calcium supplements (1999).
The balance method

The balance method—the classic of nutritional studies for the last 80 years—is, in a sense, the gold standard. It refers in this case not to total body balance, but to intestinal balance, i.e., the difference between what goes in at the mouth and what comes out in the feces. In this conceptually simple form, the method is cumbersome, imprecise, time consuming and expensive. Additionally, its endpoint is subject to the influence of bacterial action on the nutrient concerned in the colon (a problem for organic compounds, although not for minerals). A much simplified form, largely eliminating bacterial interference, is the method of intestinal lavage (Bo-Linn et al. 1984), in which the gut is first thoroughly emptied of all of its contents by drinking a large volume of an isosmotic solution, then feeding a test meal containing the nutrient for which bioavailability information is sought along with a nonabsorbable marker, then several hours later following with a second lavage and measuring the content of the test nutrient in the effluent. The method directly yields the quantity absorbed. It measures net rather than gross absorption and, thus, provides nutritionally relevant information, particularly for minerals, such as calcium, which enter the gut with digestive juices, as well as leave it in the process of absorption. Although much simpler and more accurate than the classical balance approach, this method, nevertheless, tends to be somewhat difficult in execution and is not widely used.

Serum concentration

The measurement of serum concentration of the nutrient after ingestion is based on the fact that serum concentration rises as the nutrient is introduced into the circulation during its absorption. This approach is an analog of the classical pharmacokinetic measure used for drugs, i.e., it yields an area under the curve (AUC), as well as the other traditional pharmacokinetic measures. Thus, it has the attractiveness of familiarity. Unless parallel AUC determinations are made for intravenously administered doses of the same substance, this method does not yield absolute bioavailability values and is better suited to the comparison of two (or more) preparations. It tends to be relatively expensive because of the number of analyses required and because of the time involved, for which volunteers must be compensated. It also tends to have a very low signal-to-noise ratio, particularly for minerals. This is because, in contrast to drugs, the test substance is normally present in the serum, and its level often tightly regulated. The absorptive increment tends to be a small fraction of what is already present and homeostatic forces actively damp the absorptive rise. Hence, this method exhibits limited sensitivity.

Tracer methods

The tracer methods, by contrast, are highly sensitive and reproducible, and depending upon the tracer used, can be very quick and inexpensive (DeGrazi et al. 1965, Heaney and Recker 1985, Heaney and Recker 1988). The tracers used may be either radioactive or stable (the former tending to be cheaper and easier to use). Like the balance methods, the tracer methods yield the absolute quantity absorbed, but in this instance, it is gross absorption (i.e., unidirectional flux out of the lumen and into the circulation), rather than net absorption, which is measured. This can be a nutritionally less relevant measure, but it is always a better test of the inherent absorbability of the nutrient source.

For most nutrients this approach would appear to be optimal. The method has very high sensitivity because the normal background for the tracer (particularly if radioactive) is usually very low; hence, the signal-to-noise ratio is usually very favorable. Also, homeostatic forces do not damp the rise in tracer concentration as they damp the rise in carrier.

The limitation of the method is that it requires that the source can be intrinsically labeled, i.e., every atom or molecule of the test nutrient in the ingestate must have the same probability of containing the isotopic tracer as every other atom or molecule. Extrinsic labeling of a source using a small synthetic labeled sample of the same nutrient and assuming that the labeled and unlabeled moieties will mix in the stomach cannot be relied upon unless the two methods have been shown to yield identical results for the nutrient concerned (Weaver and Heaney 1991, Heaney et al. 2000).

Urine increment

The urine increment method is based on the fact that as the serum concentration of the nutrient rises, some of the nutrient spills over into the urine. A timed urine collection, thus, represents a time integral of the serum concentration, i.e., it yields the AUC. It is a much less expensive method than the classical serum AUC method, but it is also much less precise, because it adds another layer of biological variability (variable renal clearance). Hence, it is even less sensitive than the serum method. It has, accordingly, a very low signal-to-noise ratio, and like the serum method, it does not yield absolute bioavailability values.

Target system effects

The effect of the nutrient on target systems is intuitively attractive, because methods with such endpoints get directly at the reason for taking the supplement in the first place. Their weaknesses lie in the fact that they are not easily calibrated and are often ill-suited for the testing of nutrients, because the biological response will be a function not only of the bioavailability of the product being tested, but of the need status of the individual recipient. (This approach gets at the utilization issue mentioned above in the context of defining bioavailability.) For example, the increment in serum 25-hydroxycholecalciferol that can be produced by a given oral dose of a vitamin D preparation is an inverse function both of the basal 25-hydroxycholecalciferol status and of the dose itself. A similar dependence upon need is exhibited by the hemoglobin response to oral iron, which can vary from 1 g hemoglobin/wk in patients with Fe-deficiency anemia to zero in individuals who are iron replete. Nevertheless, such methods can often be useful to complement or shed light on the results of other approaches. For example, an absorptive rise in serum calcium of as little as 5% evokes a 40–50% drop in serum parathyroid hormone (PTH), effectively amplifying the signal from the small increment in serum calcium. However, assay imprecision for PTH and other similar markers often mandates large sample sizes to obtain desired statistical power, effectively precluding sole reliance on such methods.

In vitro methods

The in vitro methods are inexpensive and attractive for that reason and are often able to identify bad pharmaceutical formulations (Carr and Shangraw 1987) before going on to
more expensive clinical tests, but they also often yield misleading information, particularly for calcium. This is because solubility of calcium salts is very poorly related to their absorbability (and, hence, the dissolution component of the standard is inappropriate), because acid is not needed for absorption, because the test conditions create artifacts in their own right, and finally because the test conditions do not mimic the conditions within the human intestinal lumen. Probably the same reservations with regard to dissolution apply to many other nutrients as well as to calcium. The underlying notion that solubilization must precede absorption, although seemingly obvious, is an effectively untested hypothesis for most nutrients. The chyme is largely a complex suspension, and dispersion of its constituents may be much more important for absorption than true dissolution, particularly because the latter usually refers to what can be measured in dilute solution in a laboratory under conditions very different from those in the gut.

One of many examples of these problems that could be cited is provided by an observation of Sheikh and Fordtran (1990) in which the dissolution of two calcium carbonate preparations in vitro was a function of the vigor of the agitation of the system. With mild agitation, neither substance met the disintegration and dissolution standard; with intermediate agitation, one source was completely dissolved and the other incompletely, and with vigorous agitation both were completely dissolved. The in vitro test in this case was calibrated with the intestinal lavage method, which revealed that one product was 32% absorbed and the other, 19%. It turned out that intermediate agitation yielded about the same proportion between the two dissolution values, but it would have been very difficult to predefine those conditions or to select from the three different in vitro experiments without having performed the in vivo study. A closely related issue is the tendency, with some CaCO<sub>3</sub> preparations, for the CO<sub>2</sub> released in an acid medium to adhere to the particles, thus, floating them to the top of the reaction vessel and insulating them from the acid in the solution phase. Carr and Shangraw (1987) described this phenomenon in their original publications on this topic, noting both that the effect was present with some granulations but not others and that the phenomenon itself was an artifact of the test system and not likely to represent what actually happens in the stomach (which the test conditions were designed to mimic).

Other in vitro test systems, such as the use of membranes consisting of CaCO<sub>2</sub> cells (Halleux and Schneider 1991), may be very useful for certain nutrients, particularly for screening large numbers of substances, formulations or interactions, but they have not been sufficiently calibrated against other methods to know how generally useful they might be.

**FACTORS INFLUENCING ENDPOINTS**

Factors that influence the bioavailability endpoints of the various methods include those that are unique to the nutrient source itself, those that reside in the ingesting subject and those that may reside in foods or drugs coingested with the nutrient source.

**Source factors**

Source factors include the associated ion or ligand; anti-absorbers natural to the nutrient source (such as phytic acid in most seed foods); the pharmaceutical formulation, which includes a broad range of physical and chemical factors involving the granulation, the excipients, the coatings and the hardness and disintegration potential of the final formulation; and finally the size of the ingested load.

Although important in theory, and undoubtedly so in practice for some nutrients, the associated ion or ligand plays very little role in calcium absorption. Calcium salts including CaHPO<sub>4</sub>, Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, CaCO<sub>3</sub> and calcium citrate, despite possessing aqueous solubilities ranging from 1.5 to 1800 mg/dl, exhibit essentially the same fractional absorption when tested in humans (Heaney 1990a). Much is made by supplement manufacturers of the superiority of one salt over another, but these claims are at best extrapolations from in vitro solubility data and do not reflect the results of side-by-side comparisons of absorption of the salts concerned in intact humans.

Despite their minor influence on absorbability, the anions accompanying calcium have other effects of importance in our context. For example, every 1000 mg of calcium ingested as calcium citrate leads to absorption of 4.75 g of citrate, some of which circulates in the blood and is excreted via the kidneys. Because of citrate's propensity to bind calcium ions, both the serum increment and urine increment bioavailability endpoints to some extent will be spuriously elevated and, thus, will yield erroneously high values for calcium citrate relative to other salts. The effect is seen in the comparative study of bioavailability of the carbonate and citrate salts by Heaney et al. (1999), in which the urine increment method tended to favor the citrate salt, while the simultaneously performed tracer method, unaffected by binding of calcium in the blood or urine, showed that, if anything, the carbonate salt was better absorbed.

In the opposite direction, phosphate, because of its depressant effect on renal calcium clearance, would be expected spuriously to lower bioavailability values derived from the urine increment endpoint. These anionic effects on bioavailability endpoints are probably small at the intake levels involved with supplement use and, therefore, would not be expected to produce large errors; nevertheless, they are certainly not zero. (Among the other advantages of the tracer method is its freedom from these perturbing effects of the anion on other bioavailability endpoints.)

Additionally, associated anions may play useful functional roles altogether apart from their effect (or lack thereof) on cation bioavailability or its endpoints. These depend upon the context in which they are used and upon the total nutritional status of the ingesting subject. Although not exactly relevant to my topic (which concerns bioavailability), they are never-the-less important and should not be ignored.
FAC'TORS INFLUENCING THE MEASUREMENT OF BIOAVAILABILITY

...the supplement is being taken as to merit mention.

The carbonate, lactate and citrate salts provide metabolizable anions, leaving a relative cation excess that may be useful in protecting bone from a predominantly acid-ash diet (Barzel 1995). In contrast, the phosphate salts of calcium provide the second component of the mineral phase of bone. Although it is commonly considered that the phosphorous content of the U.S. diet is high, this is not universally true and very often specifically not true in the elderly who may be receiving bone-strengthening drug therapies along with supplemental calcium. Without adequate attention to meeting the phosphate requirements for bone building, therapeutic success in these patients may be limited.

Such functionality, residing in the anion, may enhance the efficacy of a calcium supplement, if not its bioavailability as usually construed.

In contrast to anion effects, pharmaceutical formulation can make a very large difference in bioavailability. This topic has been well studied for drugs but largely ignored in the supplement industry. Figure 1 presents an example of two formulations of the same batch of labeled calcium carbonate, studied by the tracer method. Preparation A represents the salt loosely packed into gelatin capsules, and preparation B represents an experimental tablet formulation. As is evident in this case, the experimental formulation resulted in a reduction in absorbability amounting to ~40%, relative to the gelatin capsule dosage form. To my knowledge, the effect of various tableting formulations on bioavailability of the nutrient has not been well studied. I have chosen what may be a relatively extreme example. Nevertheless, it illustrates the importance that formulation can have.

Specifically this issue has not been systematically examined for marketed calcium supplements. A recent study by Heller et al. (1999) comparing two formulations, one for calcium carbonate and the other for calcium citrate, reported better absorbability for the citrate formulation, using the serum increment method. The authors incorrectly generalized their findings to the parent salts, whereas the most that they could legitimately conclude is that the formulations tested exhibited differing absorbabilities. It has been my personal experience that the pure salt is often somewhat better absorbed than the pharmaceutical mixture. It would seem that more formal, systematic investigation of this formulation question would be in order for supplements generally.

Another source factor that may influence bioavailability is the presence of anti-absorbers in the source itself. For example, absorption of calcium added to wheat bran products may well be countered by the anti-absorptive effect of the phytate in the bran. The same is true with respect to native calcium in sources such as beans, which typically possess enough oxalate and phytate to complex all of the bean calcium. Curiously, however, despite the relatively high level of these anti-absorbers, the interference found with common beans is only one half of what would be predicted from the chemical composition of the bean, and, with soybeans, there is even less interference from the anti-absorbers. A different example is afforded by the experience reported by investigators at Procter & Gamble, at the time that they were developing calcium citrate malate for addition to fruit juices (Mehnsho et al. 1989). They found good absorbability for calcium from this complex salt in orange and grapefruit juices, but, surprisingly, poor absorbability in lemon juice. A conclusion to be drawn from these various discordances between expectation and reality is that bioavailability cannot reliably be predicted from knowl-edge of the chemical content of a source but must be directly tested.

A final source factor of importance is the size of the ingested load. This may make relatively little difference for certain nutrients but can be very important for others. For example, the absorption fraction for calcium varies inversely as the logarithm of the load size (Heaney 1990b). A consequence is that distributing the nutrient intake over the course of the day can be calculated to improve absorbability by as much as 80% relative to the same intake ingested as a single bolus.

Subject factors

Subject factors have limited relevance to the pharmaceutical or supplement manufacturer because they are uncontrollable. However, knowledge of subject factors is important in interpreting, for example, age-related changes in apparent bioavailability, as well as in reconciling results from seemingly disparate studies. They may also be relevant in formulating niche products, targeted, for example, to the elderly or to pregnant women, etc.

One of the more important of these subject factors is mucosal mass. Although this variable cannot currently be measured in intact humans, its effect on absorptive performance is a well-demonstrated phenomenon in experimental animals and is seen for essentially all nutrients, both poorly and well absorbed. Also important are intestinal transit times and the rate of gastric emptying (Barger-Lux et al. 1995), especially for certain poorly absorbed nutrients such as calcium. Another factor, well understood if not often acknowledged, is the up- and down-regulation of absorption by physiological controls because of the experience of the subject with the nutrient concerned. For example, absorption fraction for calcium will tend to be lower for individuals on high calcium intakes than for those on low. One consequence is that the absorption fraction observed at a single-meal test in an individual with a low calcium intake cannot be extrapolated to what would happen in the same individual taking the supplement regularly (under which circumstances absorption may be down-regulated). A related factor, also generally well recognized for nutrients, is the nutritional status of the subject being tested with respect to the nutrient concerned, noted earlier. Thus, absorption of calcium and iron will be greater in individuals who are deficient in these minerals than in individuals who are replete.

Coingested factors

Important coingested factors include anti-absorbers in other foods ingested at the same meal. Thus, as has been well described, phytic acid in whole grain cereals may interfere with iron and zinc absorption, and wheat bran, with calcium absorption (Weaver et al. 1991). On the other side of this issue, some substances enhance absorption, as seen in the effect of ascorbic acid on iron absorption. Further, there is the enhancing effect of the meal itself (Heaney et al. 1989). The effect is probably a composite of prolonged gastric emptying from a meal source (as contrasted with dumping that may occur with a supplement tablet taken on an empty stomach), as well as interactions between food macromolecules and calcium particles in ways that enhance the presentation of the calcium to the absorptive surface. Once again, although the effect is well established for several nutrients, the precise mechanism remains unclear. Finally, there is competition for limited absorptive transport capacity with other chemically similar substances, for example, the well-studied competition...
between calcium and strontium, as well as the very large (and beneficial) interference by calcium with lead absorption.

COMMENT

Behind a concern for bioavailability of nutritional supplements is a desire to make certain that constituents or properties of the supplement are not standing between our patients and/or the public and the benefit we both expect will flow from our products. Therefore, such a concern is a kind of quality assurance issue, and measurement of bioavailability serves to demonstrate that the product does at least a part of what it purports to do. As such, every responsible supplement manufacturer and food fortifier ought to assume the burden of demonstrating that the respective product exhibits appropriate bioavailability.

But there is a second bioavailability issue, particularly for poorly absorbed nutrients such as calcium, and that is the pursuit of a kind of holy grail of enhanced bioavailability. This quest stands behind both the usually exaggerated marketing claims of superior performance for one salt or one formulation relative to another, as well as the search within the industry for additives that might enhance the absorption of calcium, thereby conferring, it is assumed, a market advantage on the product concerned. In general, this emphasis seems inappropriate and misdirected from both cost benefit and nutritional considerations.

Take, for example, a preparation that is absorbed at 30% efficiency, but is inexpensive, and another that is absorbed at 40% efficiency, but costs twice as much. Nine pills per week of the less expensive product actually delivers about the same amount of calcium into the circulation as seven pills per week of the more expensive one, but at 40% less cost. Only when products exhibit very large differences in absorbability or are priced about the same will the cost-benefit analysis reveal that the better absorbed product is actually a better bargain. In the final analysis, the simplest (and cheapest) way to absorb more calcium is to ingest more calcium.

Also, nutritionally, there seems very little advantage to improving absorbability, because unabsorbed calcium exhibits valuable functionality in its own right. Calcium remaining in the food residue forms complexes with harmful substances left over from digestion, such as oxalic acid, unabsorbed fatty acids and bile acids. This complexation is the mechanism by which high calcium diets reduce the risk of kidney stones and colon cancer. Incidently, the latter protection provides another illustration of the functional role of the accompanying anion. Calcium phosphate has been shown to be more efficacious at inhibiting colon cancer in animal models than the same amount of calcium as the carbonate (Lupton 1997).]

Theoretically, sources with high intrinsic absorbability, ingested at low load sizes, could meet the body’s skeletal needs for calcium, but they would leave unmet the detoxification function that unabsorbed calcium serves within the intestinal lumen itself. In brief, there is little or no nutritional advantage to ingesting one’s calcium in a form with absorbability higher than that of natural calcium sources.

Many factors can influence both the actual absorbability of nutrient sources and the endpoints by which it is measured. With respect to actual bioavailability, the formulation by which calcium is added to the diet, either pharmaceutical or food, may be the most important controllable factor and also the one producing the greatest effect. At the existing state of understanding of the chemistry of the chyme and of the mechanisms of the absorption process, predicting bioavailability is chancy, and there is today no substitute for direct bioavailability testing. Finally, ultimate bioavailability of a nutrient source can only be known when testing is performed under fully adapted conditions. This latter point is not applicable to the demonstration of product quality or bioequivalence, but it is important for the understanding of the impact of supplement use on the nutritional status of a population.

LITERATURE CITED


U.S. Pharmacopeial Convention Inc., Rockville, MD.
