Predicted Serum Folate Concentrations Based on In Vitro Studies and Kinetic Modeling are Consistent with Measured Folate Concentrations in Humans

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Abstract

The nutritional quality of new functional or fortified food products depends on the bioavailability of the nutrient(s) in the human body. Bioavailability is often determined in human intervention studies by measurements of plasma or serum profiles over a certain time period. These studies are time and cost consuming and often appear to lack an optimal study design, leading to follow-up intervention trials. Therefore, an alternative approach is needed that will optimize the development of new products. This study describes an approach to predict human serum concentrations after the consumption of (fortified) food products. The concept is based on the integration of in vitro results with kinetic modeling. As a case study, human serum folate concentrations were predicted after the consumption of folate-fortified milk products for 4 wk. Oral bioavailability was investigated using a step-wise approach in which luminal bioaccessibility and intestinal absorption were independently evaluated. Subsequently, these in vitro data were integrated in a kinetic mathematical (in silico) model to predict serum folate concentrations after the intake of a single dose and during long-term consumption. This approach was evaluated in comparison to a human intervention study in which folic acid–fortified milk products were tested for their effect on serum folate concentrations. A high predictive quality of this alternative in vitro/in silico approach was demonstrated. Finally, this methodology was applied to predict serum folate concentrations after intake of different fortified milk products for 4 wk, showing its benefits for the development of new nutritional products. J. Nutr. 136: 3074–3078, 2006.

Introduction

New food products entering the market are developed to increase human health and lower the risk for diseases such as cancer and cardiovascular disease. The efficacy of these functional or fortified food products depends upon the bioavailability of the active nutrients. The bioavailability of nutrients is normally determined using human intervention trials and is based on the differences in plasma or serum profiles among the intervention groups. These human studies are highly time and money consuming and often appear to lack an optimal study design. Therefore, an alternative approach is needed that cuts down development time and the cost of new products.

The serum profile of a nutrient after consumption of food products is the result of many processes in the human body. The most important ones include the release of the nutrient from the food matrix during gastrointestinal (GI)1 passage and digestion (i.e., bioaccessibility), transport through the intestinal wall and, subsequently, distribution and metabolism in, and elimination from, the body. All these successive processes can be studied in separate in vitro experiments of which the data give a prediction of the kinetic parameters of a compound in vivo. These in vitro data can be used as input for a kinetic mathematical (in silico) model to predict bioavailability and serum profiles of a compound after single or multiple oral doses.

The current article describes a case study in which the bioavailability of folate from fortified milk products was studied using in vitro methods and kinetic modeling (Fig. 1). Several folate-fortified milk products were tested in TNO’s gastrointestinal model (TIM). This in vitro dynamic gastrointestinal model simulates the human physiological conditions in the digestive tract (1). In the TIM system, the bioaccessible fractions of folate from milk products were determined (2,3). These fractions of folate are released from the food matrix in the lumen of the GI tract and are available for absorption. Subsequently, the transport of folate through the intestinal wall was examined using cultured monolayers of human colon carcinoma (Caco-2) cells (4). Monolayers of Caco-2 cells are a commonly used in vitro model to study human intestinal absorption (5,6). After absorption from the intestinal lumen, folate is distributed and metabolized in the body, a process previously described (7–9). These parameters determined in in vitro studies with the TIM system and Caco-2 cells were used in a kinetic model describing folate kinetics in the human body. This kinetic model would

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1 Abbreviations used: 5MTHF, 5-methyltetrahydrofolate; FBP, folate binding proteins; GI, gastrointestinal; P_app, apparent permeability; TIM, TNO’s gastrointestinal model; UHT, ultra high temperature.

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In vitro studies. Materials and Methods
be a cost- and time-efficient way to develop novel and fortified fortified ultra-high temperature (UHT) milk or pasteurized milk the subjects consumed unfortified control milk, folic acid–compared predicted blood folate concentrations with measured intestinal transport values rather than using a theoretical value for food products, the bioaccessible folate fractions are measured in the GI tract. In our approach, after the ingestion of individual
scribe the size of the fraction of folate that enters the blood from and metabolism in humans (7,9) use a theoretical value to de-
status.
makes it feasible to predict serum concentrations, reflecting folate
status.
Previously published kinetic models for folate distribution and metabolism in humans (7,9) use a theoretical value to describe the size of the fraction of folate that enters the blood from the GI tract. In our approach, after the ingestion of individual food products, the bioaccessible folate fractions are measured in the TIM system and are combined in the kinetic model with intestinal transport values rather than using a theoretical value for the bioavailability of folate from a broad range of food products.
The aim of this article is to evaluate and apply an integrated in vitro-in silico approach to predict human serum concentrations of folate after multiple doses over a certain time period. We compared predicted blood folate concentrations with measured folate concentrations from a human intervention study in which the subjects consumed unfortified control milk, folic acid–fortified ultra-high temperature (UHT) milk or pasteurized milk for 4 wk (10). Application of this integrated methodology would be a cost- and time-efficient way to develop novel and fortified foods with high nutritional quality.

Materials and Methods

In vitro studies. The bioaccessible fractions of folate from 10 different fortified milk products (Table 1) were determined in studies with the gastrointestinal model (TIM system) as described in earlier publications (2,3). The milk products were either fortified with synthetic folic acid or the natural folate compound 5-methyltetrahydrofolate (5MTHF). In addition, supplemental folate binding proteins (FBP) were studied as to their effect on the bioaccessibility of folate (2,3). The TIM system represents the stomach, duodenum, jejunum, and ileum (1). The pH curves, peristaltic movements, gastric emptying, intestinal transit, and gradual additions of digestive juices are computer-controlled events and comparable to human GI conditions. The jejunum and ileum compartments are both connected with semipermeable hollow fiber membranes with a cut-off of 5 kDa, which mimics the bioaccessibility of digested nutrients and water (i.e., bioaccessible fractions). The nonbioaccessible fractions are collected at the end of the ileum compartment of the TIM system and represent the undigested food that will enter the large intestine. Each fortified milk product was tested in duplicate in the in vitro gastrointestinal model to measure the bioaccessible fractions of folate from the jejunum and ileum compartments (2,3). The measured bioaccessible fractions were converted to unbound fractions of folate to be used in the kinetic model.

During GI passage of the pasteurized milk with 5MTHF, the folate fractions were completely unbound in both the jejunum and ileum (2,3,11). This value was set to 1 (Table 1). For the other milk products, part of the folate continued to be bound to FBP during GI transit and digestion. This was converted to the calculated values given in Table 1.

The rates of folic acid and 5MTHF transport across intestinal cells, expressed as apparent permeability values ($P_{\text{app}}$), were experimentally determined in an in vitro model using cultured monolayers of Caco-2 cells in Transwell plates (4). The $P_{\text{app}}$ values were determined on the basis of the appearance of folic acid or 5MTHF in the receiver compartment before 10% of the compound was transported (i.e., under sink conditions) according to the following equation:

$$P_{\text{app}} = \frac{dQ/dt}{A'C_0} \text{ (cm/s)},$$

where $dQ/dt = \text{permeability rate (mol/s)}$, $A = \text{surface area of the filter (1.1 cm}^2\text{)}$, and $C_0 = \text{initial concentration (mol/mL)}$.

The transport of folic acid was slightly higher than that of 5MTHF, i.e., $1.7 \times 10^{-6}$ cm/s and $1.4 \times 10^{-6}$ cm/s, respectively (4). It was found that the intestinal transport of folic acid and 5MTHF was dependent on the extent of binding to FBP at the luminal side of the intestinal cells (4).

Combining the results of both in vitro models, we used the (FBP–) unbound folic acid and 5MTHF values that were determined in the TIM system (Table 1) and the $P_{\text{app}}$ values of the unbound folic acid and 5MTHF determined in the Caco-2 cells as input for the kinetic model. This represents the bioaccessibility and intestinal transport, respectively (Fig. 1).

After intestinal absorption, folate is distributed over the different tissues in the human body, metabolized (especially in the liver) and partly excreted from the body. Published data on distribution, metabolism, and excretion of folate (7,8) were used as input for the kinetic model (Fig. 1).

Description of the kinetic model. A kinetic model for folate kinetics in humans was constructed in Advanced Continuous Simulation Language (ACSL, AEgis Technologies) using the kinetic parameters obtained from the in vitro studies and from the literature, as described above.

The kinetic model describes the residence time of the food in each individual segment of the upper GI tract (stomach, duodenum, jejunum, and ileum) in relation to gastric emptying and intestinal transit time of a meal with a glass of milk. This is in line with the transit time that was simulated in the TIM system for the milk products. The rate of absorption of folate from the individual intestinal segments over time was mathematically described in the kinetic model by combining the data from the TIM and Caco-2 cell experiments, the residence time of the food in each individual segment, and the biological data about the absorptive area of each intestinal segment (12). Absorptive area of the segments was calculated by multiplying the segment length with the estimated segment circumference. The lengths of the individual human intestinal segments are 25, 260, 395 cm, respectively (12). The absorptive area of the small intestine is enlarged by the folding of the mucosa, i.e., a factor 3 for duodenum and jejunum and a factor 2 for ileum. The resulting total absorptive areas for duodenum, jejunum, and ileum, as used in the kinetic model, were 12, 122, 124 decimeter (dm)$^2$, respectively, which is consistent with earlier published values for total absorptive areas of small intestine (12).

<table>
<thead>
<tr>
<th>Milk products</th>
<th>Jejunum</th>
<th>Ileum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pasteurized milk + 5MTHF</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Pasteurized milk + 5MTHF + FBP</td>
<td>0.95</td>
<td>0.96</td>
</tr>
<tr>
<td>Pasteurized milk + folic acid</td>
<td>0.61</td>
<td>0.82</td>
</tr>
<tr>
<td>Pasteurized milk + folic acid + FBP</td>
<td>0.57</td>
<td>0.37</td>
</tr>
<tr>
<td>UHT milk + folic acid</td>
<td>0.87</td>
<td>0.95</td>
</tr>
<tr>
<td>UHT milk + folic acid + 5MTHF</td>
<td>0.60</td>
<td>0.42</td>
</tr>
<tr>
<td>Yogurt + folic acid</td>
<td>0.86</td>
<td>1.0</td>
</tr>
<tr>
<td>Yogurt + folic acid + 5MTHF</td>
<td>0.33</td>
<td>0.21</td>
</tr>
<tr>
<td>Yogurt + 5MTHF</td>
<td>0.78</td>
<td>1.0</td>
</tr>
<tr>
<td>Yogurt + 5MTHF + FBP</td>
<td>0.50</td>
<td>0.35</td>
</tr>
</tbody>
</table>

1 The fraction of unbound folate in jejunum, determined in in vitro studies, was also used as the input value for the fraction of unbound folate in the duodenum using the kinetic model.
The rate of absorption of folate was determined by first calculating the transfer coefficient of folate (SMTHF or folic acid) across the intestinal wall for each individual intestinal segment (Eq. 1) and subsequently, the transport rate of the bioaccessible fraction from the intestinal lumen (Eq. 2). The kinetic model consists of separate mathematical equations for the duodenum, jejunum, and ileum segments. Total input rate of folate from the small intestine to the blood (serum compartment, Fig. 1) was calculated by summarizing the transport rates of folate across the individual intestinal segments (Eq. 3).

\[
K_{Si} = P_{app} \times A_{Si}, \quad \text{(Eq. 1)}
\]

where \(K_{Si}\) is the transfer coefficient of folate across the absorptive area (L/h) of the intestinal segment, \(P_{app}\) is the apparent permeability value (dm/h) derived from Caco-2 studies, and \(A_{Si}\) is the absorptive surface area (dm\(^2\)) of the individual intestinal segment (duodenal, jejunum or ileum).

\[
R_{WSi} = K_{Si} \times S_{i} \times F_{i}, \quad \text{(Eq. 2)}
\]

where \(R_{WSi}\) is the rate of transfer of folate (nmol \cdot L\(^{-1}\) \cdot h\(^{-1}\)) across the wall of the intestinal segment, \(K_{Si}\) is the transfer coefficient of folate (from Eq. 1), \(S_{i}\) is the luminal concentration of folate in the intestinal segment (nmol/L), and \(F_{i}\) is the fraction unbound in the intestinal segment.

\[
R_{INP} = \sum R_{WSi}, \quad \text{(Eq. 3)}
\]

where \(R_{INP}\) is the total input rate from intestinal lumen to blood and \(\sum R_{WSi}\) refers to the sum of the folate transfer (nmol \cdot L\(^{-1}\) \cdot h\(^{-1}\) ) from the individual segments.

The amount of folate that enters the blood over time is distributed between blood and the various organs in the human body, represented in the kinetic model by a fast and a slow folate compartment (Fig. 1). These compartments are connected with rate constants \(k_1\) to \(k_4\) (h\(^{-1}\)) to describe the distribution between these compartments and the elimination rate constant \(k_5\) (h\(^{-1}\)), which describes the elimination of folate from the human body (urinary excretion). The rate constants reflect the half-life in the serum compartment and in the fast and slow compartments, which were estimated to be 2 h, 7 d, and 100 d, respectively. These estimated values were based on serum folate concentrations measured in human studies in which subjects received single or repeated folate doses (7,8). The values of the rate constants \(k_1\), \(k_2\), \(k_3\), \(k_4\), and \(k_5\) as used in the kinetic model, were 0.29, 0.017, 0.055, 5.7 \times 10^{-5}\, h\(^{-1}\) and 4.2 \times 10^{-4}\, h\(^{-1}\), respectively.

The kinetic model was initially fit to correctly describe baseline serum folate concentrations (9 nmol/L) in humans after consumption of ~200 \(\mu\)g/d of folate, which is the mean daily folate intake of the Dutch population (13). The bioavailability of folate from different meals was found to range between 50 and 90% (14–16). In the kinetic model, the bioavailability of folate from the daily meals (without the fortified milk products) responsible for the baseline serum folate concentrations was set to a mean of 70% and was distributed evenly over the day. The serum baseline concentration was described by fitting the size and interaction of the compartments to this baseline concentration of 9 nmol/L at a mean intake of 200 \(\mu\)g/d of folate via the meals.

**Evaluation of the kinetic model in comparison to a human trial.** After fitting the kinetic model to baseline concentrations of folate, the response in serum folate concentrations at additional daily consumption of folic acid–fortified milk products was predicted and compared with the results of a human intervention study. In the intervention study, the volunteers consumed a strictly controlled daily (folate-deficient) diet containing 140 \(\mu\)g of folate in combination with either control milk or folic acid–fortified UHT milk or pasteurized milk, resulting in additional daily intakes of 20, 201, and 233 \(\mu\)g of folate, respectively (10). The serum folate concentrations were analyzed at the start and at the end of a 4-wk intervention period.

The control and folic acid–fortified milk products used in the human trial were the same as those tested in the TIM system (2). The bioaccessible fractions of folic acid (Table 1) were incorporated in the kinetic model together with the intestinal transport rate (\(P_{app}\) value) across the Caco-2 cells (4).

**Application of the kinetic model for various fortified milk products.** Finally, the in vitro/in silico approach was used to predict folate serum concentrations when different types of milk products, fortified with folic acid or SMTHF with and without FBP supplementation, were consumed for 4 wk in combination with a daily basic diet containing 200 \(\mu\)g of folate.

**Results**

**Evaluation of the kinetic model in comparison to a human trial.** Serum folate concentrations were predicted with the in vitro/in silico methodology after daily consumption of unfortified milk, or folic acid-fortified milk in combination with a low folate diet for 4 wk (Fig. 2). The serum folate concentrations increased directly after a single consumption of the fortified milk product, after which it leveled off during the day until the next (daily) event of consumption. After each day of consuming one of the fortified milk products with the diet, the baseline serum concentration slightly increased until a new steady-state level of serum folate was reached (lines b and c in Fig. 2). The consumption of unfortified milk in combination with the low folate diet was predicted to result in a daily decrease of serum folate concentration (line a in Fig. 2). It was also predicted that, after 4 wk of daily consumption of the fortified milk products in combination with a low folate diet, the serum folate concentrations would increase from 9.2 nmol/L to 14.6 nmol/L and 15.3 nmol/L for UHT milk (201 \(\mu\)g of folic acid/d) and pasteurized milk (233 \(\mu\)g of folic acid/d), respectively (Fig. 3). For the consumption of unfortified milk (20 \(\mu\)g of folic acid/d) combined with a low folate diet, a decrease of serum folate concentration from 9.2 nmol/L to 7.8 nmol/L in 4 wk was predicted.

At the beginning of the intervention, the serum folate concentration measured in the control group consuming unfortified pasteurized milk during the study was 9.3 ± 2.1 nmol/L (mean ± SD) (10). After 4 wk of strictly controlled consumption of a low folate diet and the milk products, the serum folate concentrations were measured again. In the control group, the mean serum folate concentration decreased to 7.5 ± 1.8 nmol/L, whereas the mean concentrations in the folate fortified UHT milk and pasteurized milk groups increased from 8.6 ± 3.3 nmol/L and 9.1 ± 2.4 nmol/L before the study to 14.1 ± 5.3 nmol/L and 13.9 ± 3.9 nmol/L after 4 wk of intervention (10). A comparison of the measured (in vivo) serum folate concentrations of the intervention study with those of the predicted (in silico) serum concentrations indicates that the concentrations were accurately predicted.

**Figure 2** In silico prediction of serum folate concentrations during 4-wk consumption of a low folate diet (140 \(\mu\)g of folate/d) combined with a) unfortified pasteurized milk (20 \(\mu\)g of folate/d); b) folic acid–fortified UHT milk (201 \(\mu\)g of folate/d); or c) folic acid–fortified pasteurized milk (233 \(\mu\)g of folate/d).
Figure 3 Serum folate concentrations of control subjects before (t = 0) and after 4-wk consumption of unfortified pasteurized milk and of subjects who daily consumed folic acid–fortified milk products for 4 wk (10) and concentrations predicted with in vitro/in silico methodology. Data are measured means ± SD, n = 18 or the predicted mean.

Application of the kinetic model for various fortified milk products. After the evaluation of the kinetic model, we used it to predict serum folate concentrations for the daily consumption of various fortified dairy products (200 μg folic acid or 5mTHF/d) with or without an equimolar amount of FBP, in combination with a diet containing 200 μg of folic acid. The folate bioaccessibility data of the fortified dairy products were based on the results of duplicate experiments in the TIM system (2,3) (Table 1). After 4 wk, the serum folate concentrations were predicted to increase from a baseline serum concentration of 9.2 nmol/L to 15.6 nmol/L for yogurt fortified with folic acid and FBP and to 17.4 nmol/L for UHT milk fortified with folic acid and yogurt fortified with folic acid. This corresponds to a 70–89% increase in steady-state serum folate concentrations (Fig. 4).

Discussion

The bioavailability of folate is normally investigated in human studies where bioavailability is measured from 1 or 2 test products relative to a control product (10,15,17,18). Besides the time it takes for preliminary work and obtaining ethical approval, these studies last a couple of weeks or even months in which human volunteers must strictly follow the diet in order to identify an effect of the test product. A number of studies do not report a significant serum folate response. Several reasons have been suggested, including the limited number of volunteers in the intervention groups, variations among human volunteers, or low amounts of folate in the test product. Many studies should be repeated by enlarging the group sizes, increasing folate content in the test product, or decreasing folate content in the control meals to decrease the baseline concentration of the control group. Such an approach for human intervention studies, in this case, for folate bioavailability, is time and cost consuming. Therefore, an alternative approach is needed. Our study presents an alternative approach by combining in vitro and in silico methods. This approach uses a mathematical model that integrates data on folate release and intestinal absorption determined in vitro studies with data on the kinetic behavior of folate in the human body to predict human serum concentrations after the ingestion of a specific food product.

The in vitro/in silico approach was evaluated by comparing the predicted serum folate concentrations with those determined in a human intervention study with the same control and fortified products. In this in vivo study, the subjects received unfortified milk (20 μg folate/d) or folic acid–fortified milk (201–233 μg folate/d) in combination with a strictly controlled folate-deficient diet (140 μg of dietary folate/d) (10). After 4 wk of intervention, the folate response in serum concentrations was determined in the human volunteers. The serum concentrations in subjects consuming unfortified pasteurized milk for 4 wk decreased from 9.3 ± 2.1 to 7.5 ± 1.8 nmol/L (10). The decrease in serum folate concentration was a result of the lower dietary folate intake (140 μg) from meals and drinks during the study than from intake before the study [~200 μg, corresponding to the mean daily folate intake of the Dutch population (13)]. The decline in serum folate was correctly predicted by the kinetic model (Fig. 3). The serum concentrations of the human volunteers consuming folate-fortified UHT or pasteurized milk for 4 wk increased from 8.6 ± 3.3 nmol/L and 9.1 ± 2.4 nmol/L prior to the study to 14.1 ± 5.3 nmol/L and 13.9 ± 3.9 nmol/L after the intervention (10). These serum folate concentrations are 85–88% higher compared with the baseline concentration of 7.5 nmol/L after intervention in which the subjects received fortified milk products combined with a folate-deficient diet. These increased serum concentrations appeared to be correctly predicted by the kinetic model using the data from the in vitro studies with the gastrointestinal model and Caco-2 cells on bioaccessibility and intestinal transport (Fig. 3). Therefore, the kinetic in vitro/in silico approach can be used to predict accurately the serum folate concentrations after short- or long-term consumption of folate-rich food products.

The integration of results from in vitro studies and kinetic modeling was subsequently used to determine what combination of dairy product, type of folate fortification, and FBP supplementation will lead to the highest increase in serum folate response after a 4-wk daily consumption. For simulations with the kinetic model, the results on bioaccessibility of folic acid and 5mTHF with and without FBP were used that were obtained from in vitro studies with the gastrointestinal model (2,3). The in silico simulations indicated that consumption of UHT milk or yogurt fortified with folic acid leads to the greatest increase (89%) in serum folate concentration. Due to processing of these dairy products, endogenous FBP was no longer present in UHT milk products.
milk and yogurt (19). Thus, in these products, the bioavailability of folic acid was not decreased as a result of its binding to FBP. The addition of FBP to yogurt fortified with folic acid led to a lesser rise (70%) in serum folate concentrations. FBP also decreased the bioavailability of folic acid from pasteurized and UHT-treated milk. These results confirmed that supplementing yogurt, UHT milk, and pasteurized milk with folic acid is an efficient strategy to enhance the folate status of humans. However, based on our results, we recommend that FBP not be added to folic acid-fortified dairy products because doing so will lower the bioavailability of folic acid and thus counteract the effect of fortification.

5-MTHF as a supplement has a few practical limitations, such as a lower stability than folic acid without the presence of FBP (20). Nevertheless, the kinetic model also predicts that the daily intake of 5-MTHF-fortified milk products will significantly increase the folate serum concentrations of humans. The rise in serum concentrations after ingesting 5-MTHF-fortified pasteurized milk (84%) and yogurt (83%) is comparable to those of folic acid–fortified pasteurized milk (86%) and yogurt (89%). A similar increase in serum folate concentrations after administration of folic acid or 5-MTHF was also demonstrated in a long-term human intervention study (21). After 24 wk of the daily consumption of 208 μg 5-MTHF, 416 μg 5-MTHF, or 400 μg folic acid, an increase of 13.3 nmol/L, 22.2 nmol/L, and 20.3 nmol/L in plasma folate concentrations was measured, respectively (21). Simulations with the kinetic model predicted an increase in serum folate concentration of 12.3 nmol/L, 22.3 nmol/L, and 22.8 nmol/L, respectively. It should be noted that our model does not distinguish among the individual folate compounds with regard to their distribution in and elimination from the human body. This could be a limitation of the model insofar as previous work demonstrated a difference in kinetic behavior and distribution of folic acid and reduced folates (16). In addition, the model could be extended by a detailed mathematical description of folate metabolism in the human body (9,22).

FBP supplementation to stabilize 5-MTHF in fortified pasteurized milk did not negatively affect the increase of the serum folate concentrations (Fig. 4). However, the FBP supplementation of 5-MTHF-fortified yogurt inhibited the increase of the serum folate concentrations. The prediction for the effect of FBP on the bioavailability of folate from 5-MTHF-fortified yogurt agrees with the results from a recently performed in vivo study in which the effect of additional FBP on the bioavailability of 5-MTHF from fermented milk was studied (23).

The present study shows that serum folate concentrations in humans can be correctly predicted by means of an in vitro/in silico approach. This combined methodology can be efficiently integrated to develop novel foods with high nutritional quality, to use as a test strategy for identifying the critical steps in bioavailability of nutrients, and to predict the short- and long-term effects of various exposure scenarios (dose, frequency, and duration) in humans. The in vitro/in silico methodology can support an optimal and efficient design for a human trial and avoids the need to perform multiple human intervention studies.

### Literature Cited

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