

# Bioavailability of AREDS1 micronutrients from softgel capsules and tablets: a pilot study

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**Purpose:** The benefits of antioxidant micronutrients in slowing progression to advanced stages of age-related macular degeneration (AMD) was supported by the 4/day tablet form investigated in the Age-related Eye Disease Study 1 (AREDS1) and the 2/day softgel form in the Age-related Eye Disease Study 2 (AREDS2). However, the choices of excipient, dosage form, and ingredient chemistry as well as the patient physiologies and pathologies can influence bioavailability and efficacy. The objective of the study was to explore the influence of dosage form on the bioavailability of the five primary AREDS1 and Tier-2 AREDS2 micronutrients: the metals zinc and copper,  $\beta$ -carotene, and vitamins E and C. The intent was to establish by chemical analysis the relative bioavailabilities of these five micronutrients in plasma, or serum for the metals, as well as to identify any opportunities for improvements.

**Methods:** A total of 15 healthy men (5) and women (10) were recruited for a controlled, randomized, three-arm, crossover trial of the AREDS1 micronutrients. The study investigated responses in bioabsorption to a single dose of either four tablets or two softgels at the full dose level, or one softgel at the half-dose level. The bioavailability of each micronutrient was based on the pharmacokinetic profiles established through 15 samplings for each ingredient/dosage form in plasma/serum over the course of one week.

**Results:** Bioavailability was estimated using model-independent and model-dependent procedures. A statistical advantage of the dosage form was observed in only two cases from the exaggerated effects using the half-dose softgel and for the tablet dosage form for  $\beta$ -carotene and vitamin E. An unanticipated complexity was suggested by the bimodal absorption of zinc. For these micronutrients, no disadvantage (though potential advantage) was inferred for the water-soluble components presented in a softgel formulation. Increased fractional absorption was observed for the smaller dose (one capsule versus two), but it was not sufficient to reach the level achieved by the full dose of either four tablets or two softgels. A model-dependent analysis permitted an estimation of the percentage of micronutrients absorbed, with zinc, the single most important ingredient, absorbed at about a 10% level.

**Conclusions:** The results suggest modestly contradictory requirements in the dosage form for water-soluble and lipid-soluble ingredients, as based on a goal of improved bioavailability. Comparative consistency in bioavailability was observed across dosage forms, and most nutrients between AREDS1 and AREDS2 (full dose) formulations relative to the significant variations observed within this controlled population. The results emphasize the importance of defining the requisite bioavailability of each micronutrient and the influence of the dosage form that provides it. With the recognition of global and population-specific micronutrient deficiencies, notably in the elderly populations afflicted with AMD and their significant metabolic and health consequences, establishing efficient means of supplementation are of continuing epidemiologic interest.

In the U.S.A., approximately 40% of the population regularly consumes micronutrient supplements [1], which are commonly available in vegetable oil-filled soft gelatin capsules or tablets. The bioavailability of a drug, from a softgel or a tablet, has been an important area of interest [2,3]. However, little information is available concerning the effect of dosage form—softgel versus tablet—on micronutrient bioavailability. There is special relevance in the Age-Related Eye Disease Studies 1 and 2 (AREDS1 and AREDS2, respectively), where critical vitamins and minerals are provided at

high levels in multiples of the recommended dietary intakes (RDI) or above the tolerable upper level (TUL). The National Eye Institute's (NEI) AREDS1 found that a specific high-dose formulation of antioxidants and zinc significantly reduced the risk of progression to advanced age-related macular degeneration (AMD), a particularly devastating disease for the elderly [4], and its associated vision loss [5]. The second AREDS trial, AREDS2, is another large masked placebo-controlled investigation studying patients with later, more advanced stages of AMD [6,7]. AREDS2 evaluated the effects of a combination of AREDS1 nutrients along with lutein and zeaxanthin and omega-3 fatty acids on delays in the progression of AMD. Its study design included the effects of the addition of lutein (10 mg), zeaxanthin (2 mg),

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and omega-3 fatty acids (eicosapentaenoic acid, 667 mg; docosahexaenoic acid, 333 mg) as Tier 1 in the investigation. As well, it included variants based on the nominal AREDS1 formulation of vitamin C (500 mg of ascorbic acid), vitamin E (400 IU provided dl- $\alpha$ -tocopheryl acetate),  $\beta$ -carotene (25,000 IU vitamin A), zinc (80 mg as zinc oxide), and copper (2 mg as cupric oxide) as Tier 2 of the investigation. Another key distinction between the AREDS1 and AREDS2 designs is the dosage form, which was either tablets (4/day, AREDS1) or softgels (2/day, AREDS2).

The objective of this research was to explore the consequences to bioavailability of the AREDS1 minerals, vitamins, and  $\beta$ -carotene, resulting from a change in dosage form from tablet to softgel. A third arm of the study evaluated the magnitude of a reduction in bioavailability following a reduction in the softgel dose to one-half that investigated in the AREDS1 trial. Adjunct to these goals would be any information about the pharmacokinetics of these micronutrients or their combinations, as well as insight into improved sampling intervals in support of a more thorough investigation. A young, healthy, and nearly homogenous population was selected to diminish the influence of the physiologic and pharmacological effects expected from an older and more diverse population, as well as to avoid the need for segregation in the analysis required by larger, more diverse populations, such as those studied in the AREDS trials. While unmasked, as required by the differences in dosage form, the crossover design removed population-based bias in the responses.

## METHODS

This randomized, controlled, crossover, interventional trial was designed to explore the bioavailability equivalences of micronutrients contained in tablets or soft gelatin capsules, with the latter either at comparable levels of micronutrients or at half those levels.

**Subjects:** Healthy women and men ( $n = 15$ , 18–45 years, body mass index [BMI] 21–29 kg/m<sup>2</sup>) were recruited from the general population using flyers, website listings, and local advertisements; their characteristics are listed in Table 1. One month before the study, 16 ml of screening fasting blood was collected as a check for clinical chemistries. Volunteers with any history or biochemical evidence of liver, kidney, or pancreatic disease, anemia, active bowel disease or resection, insulin-dependent diabetes, easy bruising or bleeding, bleeding disorders, hyperglyceridemia, hyperlipoproteinemia, or alcoholism were excluded from the study. Moreover, individuals taking mineral oil or medications suspected of interfering with fat-soluble vitamin absorption as well as cholesterol lowering drugs, e.g., statins, such as

lovastatin and pravastatin, and bile acid sequestrants, such as cholestyramine, were excluded. Subjects using steroids or non-steroidal anti-inflammatory drugs or antihistaminic drugs were excluded.

**Study design:** The bioavailabilities of five micronutrients (zinc, copper, vitamins C and E, and  $\beta$ -carotene) from two preparations (softgel capsule or tablet) were measured using serum or plasma response curves on three separate occasions (softgel half-dose, softgel full dose, and tablet full dose) in the presence of a standardized test meal in 15 healthy adults who had fasted overnight (>12 h). The full dose supplement provided  $\beta$ -carotene (28,640 IU), vitamin C (452 mg of ascorbic acid), vitamin E (400 IU provided as dl- $\alpha$ -tocopheryl acetate), zinc (69.6 mg as zinc oxide), and copper (1.6 mg as cupric oxide) at levels and with nutrients identical to those reported in the AREDS1 trial [5]. The controls on subject diet for the days on which the supplements were consumed are summarized in Appendix 1. The tablet and softgel supplements were commercially available, formulated by Alcon Laboratories, Inc. The supplements were given at the start of the test meal, which was consumed within 15 min. The supplements were swallowed whole and neither broken nor chewed. Neither coffee nor tea was allowed with the test meal.

During the testing week, subjects were instructed by the research dietician to consume a low fruit and vegetable diet. Subjects consumed no food following the test meal until after the 4-h blood collection. To maintain an equivalent micronutrient intake during each of the three test days, identical, standard lunches and dinners were provided to each volunteer. Each subject participated in each arm. A minimum of one month elapsed as a washout period among the three different arms (softgel half-dose, softgel full dose, tablet full dose). The order of the three arms among the subjects was randomized following an order determined by a research statistician. A masked, double-blinded study was not possible given the visual differences between softgels and tablets and the number of pills required to reach the desired dose. Between tests, the subjects resided at home and consumed their normal diet, though any micronutrient supplements were excluded.

The protocol for this interventional trial was approved by the Human Investigative Review Committee of Tufts University, Tufts Medical Center. Informed consent was obtained from all subjects, and all procedures conformed to the tenets of the Declaration of Helsinki.

**Micronutrient levels:**

**Sample preparation**—The bioavailabilities of the test micronutrients were based on determining the area-under-the

TABLE 1. SUBJECT POPULATION CHARACTERIZATION.

Subject	Age, years	Sex	BMI, (kg/m <sup>2</sup> )
1	28	F	22.3
2	31	F	23.3
3	31	F	22.3
4	22	M	22.8
5	29	F	24.1
6	26	F	26.1
7	28	M	23.3
8	22	F	24.3
9	20	F	24.0
10	20	M	22.2
11	18	F	23.1
12	30	F	21.8
13	28	F	21.5
14	33	M	27.8
15	23	M	27.1
Mean	26	67% F	23.7
Sem	1		0.5

curve (AUC, see below) in plasma or serum over 168 h (t = 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12, 24, 48, and 168 h post-dose). The blood specimens were drawn into vacutainers containing EDTA for plasma and trace-free containers for serum. The blood samples used in the plasma preparation were collected and protected from light and centrifuged within 1 h for 15 min at 1,000 × g at 4 °C to separate plasma from the red blood cells. Aliquots of plasma were stored at –70 °C until analysis. The blood samples used in the serum preparation were collected in trace element-free containers, allowed to clot, and serum harvested. Aliquots of serum were stored at –70 °C until analysis.

**Analysis**—The lipid-soluble nutrients, the plasma fraction β-carotene, and the d,l-α-tocopherol were quantified using our laboratory’s published reverse-phase, gradient HPLC methodology [8]. The plasma levels of ascorbic acid were determined using the well-known 2,4 dinitrophenylhydrazine-based method [9,10]. The plasma sample was deproteinized with perchloric acid/EDTA immediately after collection [11]. The clear supernatant was frozen at –70 °C before analysis. The serum levels of zinc and copper were measured from the trace metal-free serum by atomic emission spectroscopy (Thermo Scientific iCAP 6500 ICP atomic emission spectrometer, Thermo Fisher Scientific, Waltham, MA 02,454), according to the technical and application manual (iCAP 6500 ICP atomic emission spectrometer, Thermo Scientific) [12]. The

intra- and inter-assay coefficients of variation (CVs) are 5.5% and 7.0%, respectively.

*Dietary questionnaires:* Nutrient intake (including dietary carotenoids) was assessed using the 100-item Health Habits and History Food Frequency Questionnaire [13]. Individual mean daily nutrient intakes from foods and beverages were calculated using the DietSys (version 3.7) nutrient analysis software developed specifically for that questionnaire and updated to reflect current values [14].

*Excipients:* In addition to the differentiation provided by dosage form itself, where the primary ingredients can be characterized as powders for tablets and oil fills for softgel capsules, each form consists of characteristic sets of excipients. The complete designations and descriptions of excipients and their functions in each dosage form—AREDS1 tablets, AREDS1 softgels, and AREDS2 softgels—are provided in Appendix 2.

*Data analysis-AUCs:*

**Model-independent analysis**—For the model-independent analysis, AUCs were computed using the traditional trapezoidal rule [15] for the 14 temporal intervals, as designated by the 15 samplings from each patient. The AUCs were averaged as a summary measure of vitamin and mineral bioavailability. For each subject, the AUC was calculated

**TABLE 2. MEAN ( $\pm$  STANDARD DEVIATION) OF AREAS (AUC'S) UNDER THE MODEL-INDEPENDENT CONCENTRATION CURVES OF EACH OF THE 15 SUBJECTS FOR MEASURES OF SERUM LEVELS OF B-CAROTENE AND A-TOCOPHEROL, AND PLASMA LEVELS OF VITAMIN C, ZINC AND COPPER AND FOR EACH DOSAGE FORM (MEAN OF THE AUC'S).**

Mean AUC, $\mu\text{mol/l}\cdot\text{h}$	Single Softgel (1/2 full dose)	2 Softgels (full dose)	4 Tablets
$\beta$ -Carotene	12.9 $\pm$ 11.9 <sup>a</sup>	20.6 $\pm$ 24.8	29.2 $\pm$ 17.7 <sup>a</sup>
$\alpha$ -Tocopherol	1274 $\pm$ 708 <sup>b</sup>	1827 $\pm$ 1274	1894 $\pm$ 741 <sup>b</sup>
Ascorbic Acid	2266 $\pm$ 1282	3169 $\pm$ 1383	2799 $\pm$ 1217
Zinc	746 $\pm$ 155	730 $\pm$ 216	726 $\pm$ 200
Copper	534 $\pm$ 177	608 $\pm$ 348	602 $\pm$ 263

The dosage forms with means of the areas sharing a common superscript are significantly different at  $p < 0.05$ , repeated measures ANOVA using Bonferroni adjustment for multiple comparisons.

for each of the three phases. The model-independent AUC calculations were computed with Microsoft Excel (Microsoft Office 2010; Microsoft Corporation, Redmond, WA). The mean AUCs reported in Table 2 and Table 3 were computed in two manners. The mean of the AUCs was calculated by averaging the AUCs determined separately for each subject and for each condition and micronutrient. The AUC of the means was determined by generating a composite average at each discrete sampling for each condition and micronutrient and then computing the corresponding AUC for each composite profile using the trapezoidal rule. The model-independent results are expressed as means  $\pm$  SEM.

For both model-independent methods, the plasma and serum concentrations were adjusted so that they reflected the difference from the baseline, selected to be the minimum concentration over the study. This accommodates the observation that the concentrations of analytes dropped to levels below the initial concentrations, a consequence attributed to a conversion to a restricted diet. In the model-independent analyses, the lowest concentration for each of the 225 responses was designated as the baseline for that curve.

The differences among study treatments were assessed using a repeated measures ANOVA, an analysis suitable for this small, three-arm, crossover investigation. A post-hoc Bonferroni correction was made for multiple comparisons—only three in number—to reduce the probability of a Type 1 error. SPSS, the Statistical Package for the Social Sciences, v19 for Windows 2007, was used for all statistical analyses (IBM, Armonk, NY). The level of significance was set at  $p < 0.05$ .

**Pharmacokinetic model-dependent analysis**—In this crossover study, 225 response curves were analyzed in that each subject's pharmacokinetic response was determined for five micronutrients and three dosage forms. For the evaluation of the data based on the pharmacokinetic model, 15 composite curves and the averages over all subjects for each micronutrient and dosage form were analyzed based on a simple two-compartment pharmacokinetic model, as portrayed schematically in Figure 1 [16]. These 15 composite responses, primarily three-parameter analytic functional approximations to the data, were adjusted to assess relative changes from a linear baseline by setting the concentrations at both 0 and 168 h to 0 (except for the isolated instances

**TABLE 3. AREAS (AUC'S) UNDER THE MODEL-INDEPENDENT SINGLE CONCENTRATION CURVE OBTAINED BY AVERAGING OVER ALL 15 SUBJECTS FOR EACH SAMPLING TIME, FOR EACH MICRONUTRIENT AND DOSAGE FORM (AUC'S OF THE MEAN).**

AUC of Means, $\mu\text{mol/l}\cdot\text{h}$	Single Softgel (1/2 full dose)	2 Softgels (full dose)	4 Tablets
$\beta$ -Carotene	13.2	20.7	30.4
$\alpha$ -Tocopherol	1289	1834	2035
Ascorbic Acid	2308	3180	2509
Zinc	749	730	726
Copper	539	609	603

The average percent discrepancy between the Mean of the AUC's and the AUC's of the Mean is 1.3% with the largest percentage discrepancy 7%, that for *dl*- $\alpha$ -tocopheryl acetate. The mean concentrations at each time point, corrected with the improved baseline described in the text, are provided in Appendix 3, Appendix 4, and Appendix 5.

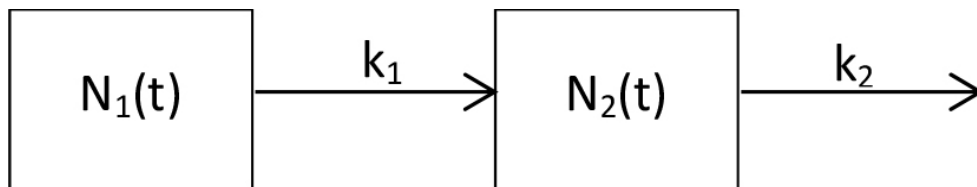


Figure 1. A simple two-reservoir pharmacokinetic model defining time-dependent amounts in each reservoir and the elimination rate constants for transport from the reservoirs. Reservoir 1 should be

understood as the gut and, in this model, undifferentiated, except in time. Reservoir 2 should be understood as the blood/serum reservoir whose concentrations were analyzed for each nutrient.  $N_1$  and  $N_2$  are considered either the time-dependent concentrations or total amounts for each nutrient, as described in the text. For  $N_2$ , these are related to the expected blood volume: about 5 l for a 73 kg adult.

discussed below). The concentrations of the data points used to generate the 15 response curves are provided in Appendix 3, Appendix 4, and Appendix 5. Those data points and analytic curves are plotted in Figure 2, Figure 3, Figure 4, and Figure 5 for  $\beta$ -carotene, dl- $\alpha$ -tocopherol, ascorbic acid, and zinc, respectively. The figure for the model-independent sets of mean responses from the copper bioavailability, as discussed below, is provided in Appendix 6.

Referring to the model in Figure 1, the  $N_i(t)$ s can be considered either the time-dependent amounts of a nutrient in a fixed volume or the time-dependent concentrations, and the  $k_i$ s in an ideal system will be time-invariant, though nutrient specific. In the basic three-parameter form, these  $k_i$ s are related through solutions to a system of coupled linear differential expressions for each ingredient and dosage form, totaling 15. Analytical curves of concentration versus

time are generated, which provide approximate fits to the data, solutions that provide the  $k_i$ s, and the pre-exponential parameters. The basic equations and some details of the analysis are provided in Appendix 7. An error-minimizing algorithm based on the deviations at the discrete sampling times provided adequate convergence between the analytic solutions and the discrete data. Reservoir 1 is representative of the gut, and Reservoir 2 of the blood plasma. The data from Appendix 3, Appendix 4, and Appendix 5 were analyzed using Microsoft Excel, and proprietary codes developed using Microsoft Visual Basic (Microsoft Office 2010; Microsoft Corporation, Redmond, WA), and MatLab® (MatLab Version R2012a(7.14.0.739); The Mathworks, Inc., Natick, MA) run in a LINUX 2.6 operating environment using an Intel Xeon X550 processor.

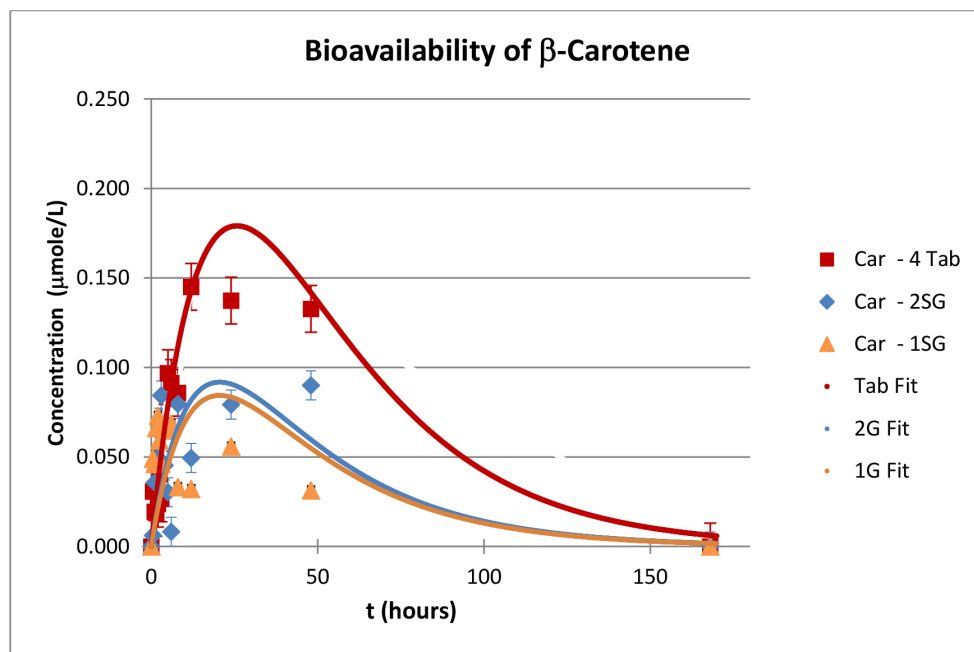


Figure 2. Experimental data (as symbols, with standard error bars) and model fits (smooth curves) regarding the bioabsorption of  $\beta$ -carotene derived from four tablets (red, 17.2 mg/day), two soft-gels capsules (blue, 17.2 mg/day), or one softgel capsule (orange, 8.6 mg/day). The data points are the average responses of the 15 subjects adjusted for any baseline drift over the course of the study. The model fits are the result of minimizing the errors between the data points and curves at the sampled times. Differences in responses are evident in

the model-independent evaluations located in Table 2 and Table 3, the model-dependent visual comparisons, and the derived AUCs and percent absorbed located in Table 4.

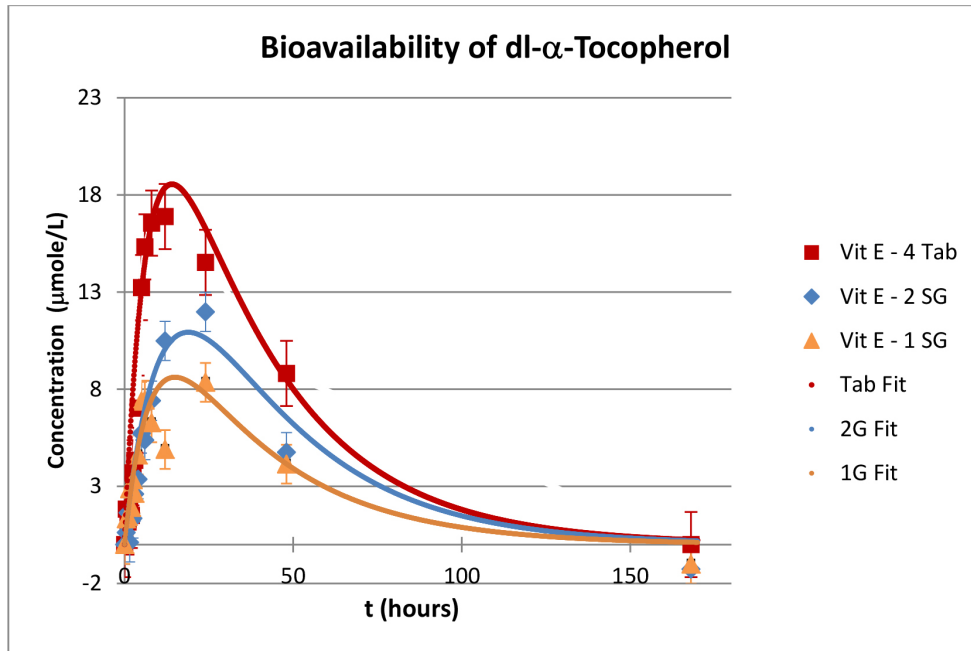


Figure 3. Experimental data (as symbols, with standard error bars) and model fits (smooth curves) regarding the bioabsorption of dl- $\alpha$ -tocopherol derived from four tablets (red, 400 mg/day), two softgels capsules (blue, 400 mg/day), or one softgel capsule (orange, 200 mg/day). The data points are the average responses of the 15 subjects adjusted for any baseline drift over the course of the study. The model fits are the result of minimizing the errors between the data points and curves at the sampled times. Differences in responses are evident in the model-independent evaluations located in Table 2 and Table 3, the model-dependent visual comparisons, and the derived AUCs and percent absorbed located in Table 4.

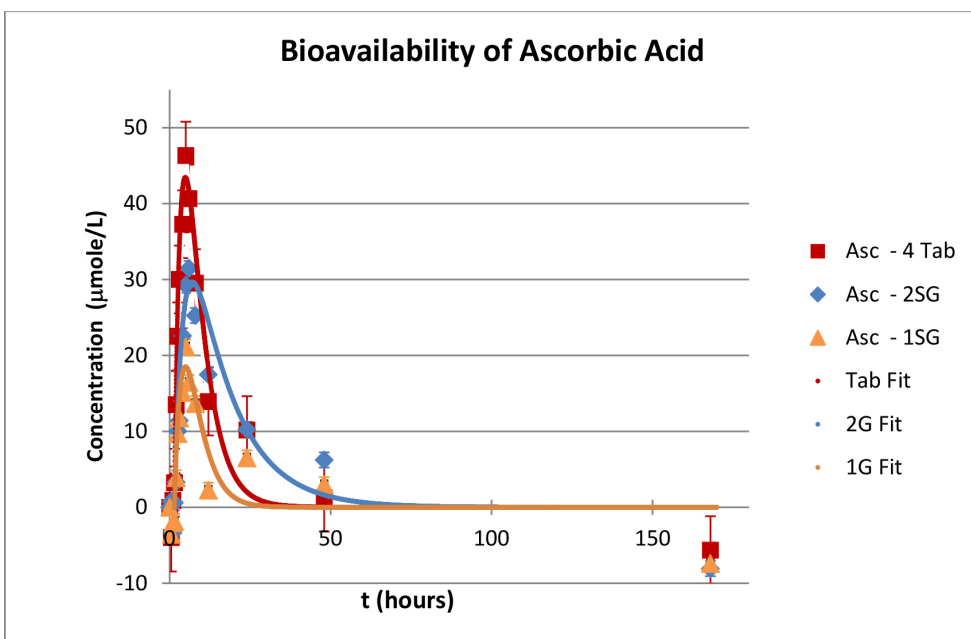


Figure 4. Experimental data (as symbols, with standard error bars) and model fits (smooth curves) regarding the bioabsorption for ascorbic acid derived from four (red, 452 mg/day), two softgels capsules (blue, 452 mg/day), or one softgel capsule (orange, 226 mg/day). The data points are the average responses of the 15 subjects adjusted for any baseline drift over the course of the study. The model fits are the result of minimizing the errors between the data points and curves at the sampled times. Differences in responses are evident in

the model-independent evaluations located in Table 2 and Table 3, the model-dependent visual comparisons, and the derived AUCs and percent absorbed located in Table 4.

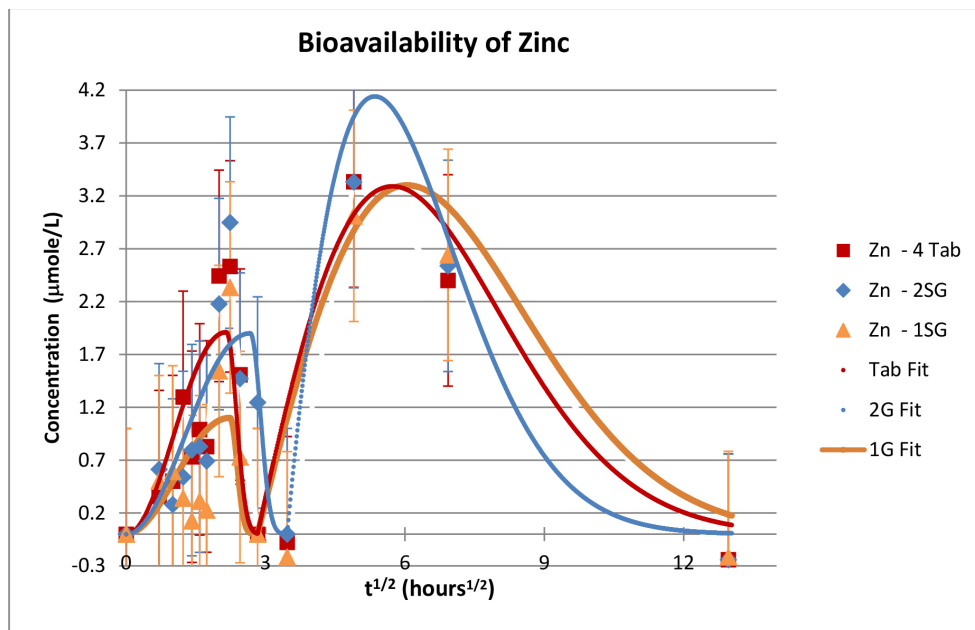


Figure 5. Experimental data (as symbols, with standard error bars) and model fits (smooth curves) regarding the bioabsorption for zinc provided as zinc oxide derived from four tablets (red, 69.6 mg/day), two softgel capsules (blue, 69.6 mg/day), or one softgel capsule (orange, 34.8 mg/day). The data points are the average responses of the 15 subjects adjusted for any baseline drift over the course of the study. The model fits are the result of minimizing the errors between the data points and curves at the sampled times. Differences in responses are evident in the model-independent evaluations located in Table 2 and Table 3, the model-dependent visual comparisons, and

the derived AUCs and percent absorbed located in Table 4. Note the abscissa for this figure is  $t^{1/2}$ , so that the detail at the short time can be displayed.

## RESULTS

The bioavailabilities of the fat- and water-soluble nutrients in the AREDS formulations were assessed initially using the AUCs ( $\mu\text{mol}/1^*\text{h}$ ) from the model-independent data of the 15 nutrient/dosage form responses and provided in Table 2 and Table 3. The expected linearity of the AUC responses, that is the independence of one nutrient from another, was reported for the model-independent analyses compared in Table 2 and Table 3. The average percentage discrepancy is about 1.3%, with the largest percentage discrepancy being 7% for dl- $\alpha$ -tocopheryl acetate for the tablet response. This strong consistency supports the fidelity of the composite responses and their utilization in the pharmacokinetic analysis, which can be presumed to be representative of this population. The nutrients were sufficiently diluted in the gut contents that no interference in nutrient absorption was observed, consistent with the equivalence between the two methods of computing the AUC's (AUC's of the mean versus mean of the AUC's). Detailed characterizations from the pharmacokinetic analysis, including kinetic constants, peak absorbencies ( $t_{\text{max}}$ s), AUCs (integrals), total amounts absorbed ( $N_1(0)$ s), percentage absorbed, parameters derived from the data found in the tables in Appendix 3, Appendix 4, and Appendix 5, are provided in Table 4.

*$\beta$ -Carotene:* Both model-independent results found in Table 2 and Table 3 and the model-dependent analyses found in Table 4 and provided pictorially in Figure 2 indicate the possibility

of improved bioavailability for  $\beta$ -carotene from the tablet formulation. An inference from the model suggests there may be little advantage of the full dose over that of a single softgel capsule. The AUC for the  $\beta$ -carotene contained in the tablet was significantly higher than the softgel only for the softgel single dose ( $p < 0.05$ ; Table 2, Table 3). The differences in time for the maximum in absorbance (peak time,  $t_{\text{max}}$ 's from Table 4) indicate the distinctness in the peak times of each of the nutrients from each other, with nutrient identity weighing more heavily than the dosage form in controlling the peak times. For  $\beta$ -carotene, the peak time of the softgel may be somewhat sooner than the tablet (Table 4); however, caution in these assessments is suggested by the caveats mentioned below.

*dl- $\alpha$ -Tocopherol:* Both analyses indicate that the absorbance of dl- $\alpha$ -tocopherol, like  $\beta$ -carotene—the other lipophilic AREDS1 nutrient—is higher from a tablet formulation than from a softgel. Both of these micronutrients appear to require 3–4 times longer to be fully absorbed than the highly water-soluble vitamin C, which is absorbed in the stomach and upper small intestine. Again, like  $\beta$ -carotene, a statistical difference was shown only for the half-dose. However, the model-dependent results shown in Figure 3 suggest there may be little delay at the beginning of the absorption of both of these lipophilic nutrients, and the fraction of vitamin E absorbed, which is provided at more than ten times the RDI, still appears to be high and reliably greater than the other

**TABLE 4. MODEL-DERIVED PARAMETERS, DESCRIBED IN THE TEXT, CHARACTERIZING THE PHARMACOKINETICS OF THE FIVE AREDS NUTRIENTS IN THE TWO DOSAGE FORMS, TABLETS AND SOFTGELS, AND FOR THE TWO DIFFERENT AMOUNTS FROM THE LATTER, TWO VERSUS ONE SOFTGEL. THESE PARAMETERS ARE DERIVED FROM FITS TO THE AVERAGED RESPONSES OVER ALL 15 SUBJECTS FOR EACH SAMPLING TIME, DOSAGE FORM, AND NUTRIENT, DATA PROVIDED IN APPENDIX 3, APPENDIX 4, AND APPENDIX 5. THESE PARAMETERS WERE USED TO GENERATE THE CURVES APPEARING IN FIGURE 2, FIGURE 3, FIGURE 4, FIGURE 5 AND APPENDIX 8.**

Formulation	$\beta$ -Carotene	$\alpha$ -Tocopherol	Ascorbic Acid	Copper	Zinc
<b>k1 (h<sup>-1</sup>)</b>					
4 Tablets	0.030	0.030	0.191	0.022	0.210, 0.040
2 Softgels	0.030	0.030	0.077	0.025	0.140, 0.060
1 Softgel	0.030	0.030	0.252	0.028	0.200, 0.035
<b>k2 (h<sup>-1</sup>)</b>					
4 Tablets	0.049	0.139	0.429	0.056	0.220, 0.040
2 Softgels	0.073	0.085	0.347	0.042	0.140, 0.060
1 Softgel	0.074	0.126	0.331	0.051	0.200, 0.035
<b>tmax (h)</b>					
4 Tablets	26	14	3.4	30	4.7, 33
2 Softgels	21	19	7.1	33	7.1, 29
1 Softgel	21	15	5.0	29	5, 37
<b>Integral (<math>\mu\text{mol/l}\cdot\text{h}</math>)</b>					
4 Tablets	12.7	935	405	180	220
2 Softgels	5.6	636	576	158	183
1 Softgel	5.2	446	163	131	248
<b>N1(0; <math>\mu\text{mol}</math>)</b>					
4 Tablets	0.64	131	186	11	14
2 Softgels	0.42	55	205	7	16
1 Softgel	0.38	57	58	7	12
<b>% Absorbed</b>					
4 Tablets	10%	76%	36%	206%	7%
2 Softgels	6%	32%	39%	134%	8%
1 Softgel	12%	65%	22%	267%	11%

AREDS1 nutrients (Table 4). These results indicate good fidelity between data points and model fit, presumably attributable in part to the high level of supplementation, second only to vitamin C.

*Vitamin C:* Even though the amount of vitamin C provided in the AREDS compositions is greater than any other nutrient, nominally 500 mg, the fraction absorbed is considerable. As well, there is agreement from both model-independent and model-dependent analyses that bioavailability is modestly—though not significantly in this small study—improved using the softgel dosage form, as illustrated in Table 2, Table 3, and Table 4. An additional subtlety emerged from the model-dependent analysis: there appeared to be a delay in the absorption of vitamin C for about 1.5 h ( $t_0$  designated in Figure 4) that was not necessary for either of the lipophilic nutrients (Figure 2, Figure 3).

*Zinc and copper:* The model-independent plots of the data did not reveal any significantly anomalous behaviors of the metal salts, except perhaps a larger baseline drift than had been observed for the previous three nutrients, as indicated for copper in Appendix 6. Nevertheless, the amounts of copper indicated in both the model-independent and model-dependent (Appendix 8 for the most accurate dosage form: tablets) results are clearly anomalously high, since the ratio of copper to zinc in the administered AREDS formulations is about 2%, while the ratio of AUCs is as high as 80%.

The observations for the zinc profile, first seen in the model-independent data in Appendix 9, reflect an unusual bimodal pattern of absorption, showing a substantial and sustained reduced serum level between two regions of increased absorption. When using the standard 0–168 baseline correction, this phenomenon was observed as a negative



absorption. By adjusting the baselines for these two regions, separately meaningful quantitations could be achieved. The results of the analysis of the pattern are presented in both Figure 5 and Table 4. There are two  $t_{max}$ s for all dosage forms, and both the larger and smaller occur at nearly consistent times across the dosage forms. The total absorbed amounts, from both portions of the profile, indicate about 9% of the zinc may be absorbed. The appearance of bimodal absorption is illustrated more prominently in Figure 5, where the serum levels are plotted versus the square root of time, which expands the axis at the early values.

## DISCUSSION

The objective of this research was to evaluate the bioavailability of the AREDS ingredients—the antioxidant nutrients  $\beta$ -carotene, vitamin C, vitamin E, zinc, and copper—from two different dosage forms: tablet and softgel. To date, considerations of the bioavailabilities of drugs in either softgel or tablet forms has been investigated [2,3]; however, such a consideration has not been made for these nutrients. The AREDS1 trial confirmed the clinical benefits of a delay in the progression of AMD by a high potency combination of these nutrients. As the NEI sought to evaluate the additional clinical benefits of omega-3 fatty acids and xanthophylls in the AREDS2 trial, there was also an effort to restrict a burdensome number of required doses, which might influence compliance. The advantage of a softgel dosage form was a reduction in the total volume of micronutrients plus excipients.

In the present study, with the results displayed in Figure 2, Figure 3, Figure 4, and Figure 5, we found that dosage forms can influence the bioavailability of some nutrients, and that the effect is not equivalent across the spectrum of their physical chemical properties. For example, the bioavailabilities of mineral salts and vitamin C appear to be equivalent or slightly improved when delivered from the lipophilic vegetable oil matrix of the softgel. Conversely, the lipophilic nutrients  $\beta$ -carotene and vitamin E appear to have improved bioavailabilities when they are delivered from a readily water-soluble matrix, such as that used in tablets, which can contain rich amounts of water-soluble excipients, including dicalcium carbonate and microcrystalline cellulose. From a different perspective, while having a lipophilic matrix that improves the solubility and dispersibility of a nutrient in the softgel core simplifies compounding, the results suggest—though not significantly in the study—that bioavailability may be diminished for these highly potent formulations.

Excluding vitamin C, smaller doses of nutrients do appear to increase the percentage of nutrient absorbed.

However, a modification of dosage form, surprisingly, does not seem to affect the location of maximum absorption along the gastrointestinal tract. The water-soluble nutrients—both vitamin C and mineral salts—initially appear to be absorbed quite rapidly. In the case of mineral salts, most notably zinc, a delay appears before a second phase of absorption occurs later in the nutrients' transit along the gut. The most lipophilic nutrient,  $\beta$ -carotene, appears to be more slowly absorbed, but that may also reflect the time needed to generate an emulsified or protein-bound form compatible with transport into the serum, thereby influencing bioavailability. The reduced bioavailability of  $\beta$ -carotene and vitamin E in softgel form may also be related to its competition with the vegetable oil-based vehicle, present in excess, for emulsification by chylomicrons integral to absorption. This is in contrast to the normal expectation of the impact of oil-based delivery in the presence of an emulsifier [17].

Many subtleties can influence bioavailability and will need to be factored into any optimization. For example, the increased bioavailability of  $\beta$ -carotene in the tablet formulation may in part reflect the influence of the use of a water-soluble gelatin beadlets as carriers rather than simple dissolutions/suspensions in the vegetable oil used in the softgels [18]. Investigations of the benefits of carotenoids indicate the interplay of genetics, auto regulation, and dietary intake, all of which contribute to complexity [19,20].

*Inferences from the model:* An inference from the percent absorbed reported in Table 4 is the mostly modest impact of dosage form on the Tier 2 arms of the AREDS2 trial, for which the dosage form was two softgels, not the original four tablets. Nutrient levels reported in accordance with the U.S. Dietary Supplement Health and Education Act of 1994 (DSHEA) of the five ingredients in AREDS1 [5] are listed in Appendix 10, and are compared with those in AREDS2 in Appendix 10 and Appendix 11. Taking into consideration the differences in the observed bioavailabilities of the two dosage forms, the potential differences in percentages of micronutrient bioavailabilities provided in AREDS2 softgels relative to those in the AREDS1 tablets have been assessed and are provided in Table 5. There, the smaller increments of bioavailable  $\beta$ -carotene, which may have influenced the clinical results, suggest that removing  $\beta$ -carotene would not be significantly detrimental. Alternatively, its substitution with lutein and zeaxanthin did offer a visible benefit [21]. In general, appreciable changes in maximum blood levels were observed and are reported in Appendix 12. While there is some uncertainty regarding the bioavailability of zinc, as discussed, increased bioavailability appears possible, as suggested at the half-dose—a single softgel—level.

**TABLE 5. PROJECTED AREDS2 TIER 2 SOFTGEL PERCENTAGES RELATIVE TO LEVELS PROVIDED IN AREDS1 TABLETS, PROJECTIONS ADJUSTED BASED ON BOTH DIFFERENCES IN AMOUNTS OF INGREDIENTS INCORPORATED INTO THE DOSAGE FORMS AND RESPONSES FROM THE DIFFERENCES IN RESPONSES FOR IDENTICAL AMOUNTS BUT DIFFERENT DOSAGE FORMS OBSERVED IN THIS PILOT STUDY.**

Nutrient	AREDS2-1	AREDS2-2	AREDS2-3	AREDS2-4
$\beta$ -Carotene	35%	35%	0%	0%
dl- $\alpha$ -Tocopheryl Acetate	68%	68%	68%	68%
Ascorbic Acid	128%	128%	128%	128%
Zinc	86%	30%	86%	30%
Copper	NA	NA	NA	NA

As evident from Figure 2, Figure 3, Figure 4, and Figure 5, as well as the parameters evaluated in Table 4, the model-derived kinetic constants for each nutrient changed less significantly between dosage forms than those parameters between nutrients. This effect is mirrored in the consistencies of the  $t_{\max}$ s for a given nutrient between dosage forms. The inference, anticipated in the study design, is that the dosage form would have little significant impact on the bioavailability and efficacy of the AREDS nutrients. This appears to be borne out for the water-soluble nutrients, where bioavailability, if anything, is increased for the softgels; however, some caution is needed regarding the levels of the lipid-soluble nutrients, vitamin A as  $\beta$ -carotene and vitamin E. To the extent that these antioxidants, or other lipophilic antioxidants, such as lutein and zeaxanthin, which may be substituted for them, can be shown as integral to overall efficacy, the impact of dosage form should be explored [18].

Within the caveats discussed below, the consistent pattern of the bimodal absorption of zinc across dosage forms is indicative of more than a singular mechanism of absorption. It is known that with each pH unit reduction away from seven, salts of both copper and zinc increase in solubility by about an order of magnitude [22–24]. Only a marginal effect on bioavailability is observed with changes in counterions, and this may reflect not a pH but a kinetic effect [25]. The smaller fraction appears to be absorbed in the stomach, presumably related to the bolus associated with the ingested meal, as well as the time required for acidification. Bimodal absorption was evident from the change in the serum level, which decreased below the baseline at about 8 h, and this was attributed to a diurnal or migrational variation. Bimodal or oscillatory patterns of absorption, which have been reported previously for zinc [26,27] and other di-cations [28,29], could arise through several mechanisms. Variations in absorption along the length of the intestine [30] could result from a specialization of enterocytes involving changes in the density of transport proteins, and it can be under hormonal control. Ions can compete for absorption, or they can be influenced by complexation [23,31]. Another important influence, as

suggested above, is the longitudinal variation in pH along the intestine and in the rates of proton generation at the intestinal wall [32–34]. Longitudinal changes in the gut pH are converted into temporal variations in ion bioavailability by peristaltic migration of the gut contents. As well, the role of the absorption of nutrients in the lower part of the intestine is not unprecedented [35]. These observations support the merit of further investigation.

While the  $N_i(0)$ s, reported in Table 4, can be computed in several ways, they should be proportional to the integrals: the model's AUCs (Appendix 7). The departure from this expectation is a measure and consequence of the level of errors in even the most average bioavailability data. However, none of these modifications suggests an explanation for the anomalous results for copper, perhaps observed most inexplicably in Table 4. However, these were not obviously detectable in the bioavailability profile (Appendix 8), but they were certainly suggested by even the unprocessed, model-independent serum concentrations. The high levels were not quite as evident, since the serum levels of copper and zinc are not so different [36–38]; however, the changes from the baseline are comparable. This cannot be explained by the ingested levels, where zinc is 40 times that of copper, nor can it be attributed to differences in pH/solubility profiles since these are similar. It is possible that copper can be derived from low levels in the diet, or that its absorption can be under dynamic control [39], a topic for future research. However, from the face value of these results, since the fraction absorbed appears greater than 100%, the conclusion is that such an insignificant amount of nutrient supplementation as that provided for copper cannot be assessed meaningfully.

Another implication of the results for AREDS2 is that the effect of the non-metal antioxidants may be somewhat modified from AREDS1. If vitamin C carries sway, then the AREDS2 antioxidants may be more effective than in AREDS1; however, if the lipophilic antioxidants are more important, then the AREDS2 antioxidants may be somewhat less effective than in AREDS1. More importantly, the

patient-to-patient variability in absorption and bioavailability in either of these dosage forms will contribute to irregular and uncertain benefits across the AMD population. Additionally, for the carotenoids to benefit advanced AMD patients, the selection of an improved dosage form may be critical to providing an appropriate amount of these relatively costly nutrients adequate to assure consistent benefit.

*Caveats and recommendations:* There are several obvious limitations of this study, including its small size, the absence of a predetermined baseline with patients on a controlled diet, the use of a young, healthy, unmedicated population, and an investigation of responses to an acute, non-chronic intervention. Notable, also, is that this is a single study with a single population, so the results and inferences are in serious need of further confirmation. Conversely, there are some obvious strengths, including its crossover design with all patients tested for all formulations, its extended duration that allowed the observation of the apparent bimodal absorption of at least zinc and possibly copper, and the selection of subjects less likely to exaggerate variables in responses.

Inferred are at least four areas of application. The approach has revealed the possibility for improving dosage forms. The benefits of softgels for hydrophilic nutrients needs to be balanced with improvements for lipophilic nutrients by modifying either the excipient matrix or the nutrient carrier. A clarification of the mechanisms controlling the bioregulation of minerals should follow an improved assessment of the baseline response to a controlled diet with no micronutrient supplementation. Our work suggests this should be nearly dosage-form independent. Third, improved sampling paradigms, based on our absorption profiles, should contribute to a better definition of bioavailability and enhanced correspondence with the pharmacokinetic model, an improvement clearly important for minerals and vitamin C. Lastly, a determination of the correspondence of acute and chronic responses would assist in identifying the simplest means to assure consistent benefits for a heterogeneous population.

Since the chronic dosage levels are multiples of the recommended levels for these nutrients, and since some nutrients can be autoregulated, the physiologic responses and influences of the potential toxicities on patterns of bioavailability should be investigated further. As an example, the potential adverse effects from multivitamins, notably zinc, or from high-level vitamin E supplement use [40,41], though possibly overestimated, nonetheless need to be factored into considerations of bioavailability. Given that the doses of these nutrients are well above the RDIs [42], approaches to providing increased efficacy and bioavailability at lower doses are likely to be medically important.

While the methodology offers promise, especially in its potential for assessing absolute bioabsorption, its fidelity to human physiologic responses needs further reassurance and justification. More thorough preclinical investigations would elucidate consistencies with, or contradictions to, the rudimentary model used here, and they would uncover refinements that may parallel human physiologic responses more closely. Because of the surprising response observed for copper, use of radio-tagged copper,  $^{64}\text{Cu}$ , would seem to be justified to provide a more sensitive assessment of its bioavailability, and in particular, whether copper shares the diurnal cyclic response suggested of zinc. Because of the saturability of response and dose-dependent mechanisms of elimination, a more thorough investigation of dose response, especially for vitamin C and possibly for zinc or copper, would be insightful.

*Conclusions:* The study, although limited by its scope as a pilot investigation, suggests levels of change in micronutrients can be expected to accompany interventions with the recommended clinically tested supplementation. The peaks in the levels of the four most important micronutrients— $\beta$ -carotene, vitamins C and E, and zinc—change in amounts ranging between 50–100% of the unsupplemented base values (Appendix 12). Important patient-to-patient variations in responses are likely to be one detractor from the benefits observed in the large AREDS1 and AREDS2 clinical trials, suggesting that for some patients it could be important to assure that the administered levels achieved a substantial incremental increase from the baseline. The study suggests, depending on dosage form, the most likely opportunities for improvements in bioavailability, as well as methods for evaluating those approaches. Finally, the results indicate that, despite serious differences between the formulations provided in the two large clinical trials, few differences in the clinical responses should be expected based on the Tier-2 portion of the AREDS2 trial. These inferences can be justified only by confirmation of these findings and compliance with the caveats provided in the discussion.

Techniques for controlling the bioavailability of the seemingly most critical ingredient, zinc, for which sources of oscillatory or multimodal absorption require further clarification, might offer advances in efficacy and, by limiting dosage size, potentials for improved compliance. The value of zinc for increasing superoxide dismutase activity and countering the depleted levels of enzyme in aging cataractous lenses is suggestive of at least one mechanism reflecting its benefits in the AREDS trials [43]. If the intention is to utilize only one dosage form, and acknowledging that excipient loads are in general lighter for softgels, then the primary concern will be

for the bioavailability of the lipid-soluble nutrients. Different types of microencapsulation may offer advantages.

From a more global perspective, there appears to be great promise for technologies that can improve the reliability and consistency of the bioavailability of orally administered nutrient supplements. With continued advances in automation, means of improving assessments of the impact of nutrition should be more readily discernible [44,45]. Through an awareness of the consequences of the dietary deficiencies of zinc, the value of improving bioavailability may transcend elderly populations having incipient ocular diseases [46–48].

#### **APPENDIX 1.**

To access the data, click or select the words “[Appendix 1.](#)” Standardized diet.

#### **APPENDIX 2.**

To access the data, click or select the words “[Appendix 2.](#)” Excipients used in the Softgel and Table formulations. The AREDS1 excipients are those used in the dosage forms investigated in this study; the AREDS2 excipients are those used in the dosage forms in that Trial, here for reference. For tablets, oil-based nutrients were provided as gelatin-coated beadlets dispersible in a mixture of solid actives, flow controlling powders such as microcrystalline cellulose and dicalcium phosphate, lubricants like magnesium stearate that contribute to proper flow rheology during blending and compression, and antioxidants or stabilizers to assure stability of actives during manufacture and storage. A number of minor ingredients that originate in preparations of the raw material actives often are reported as excipients. Polymers and colorants provide an exterior region that contributes to the esthetics of the tablet presentation. For softgel capsules, similarly there are two separate regions, a liquid, or at least flowable, core and an enclosing capsule. The core is composed of actives, oils, viscoelastic and suspending agents sufficient to assure uniformity in composition during manufacture, minor ingredients accompanying the actives, and like the tablets antioxidants and stabilizers to assure stability. The polymer capsule is routinely comprised primarily of a biopolymer, which in these formulations is gelatin to which plasticizers and other minor ingredients are added to assure compatibility with the core, proper flow, and adherence during fusion of the capsule halves at the time of manufacture. The levels of excipients are proprietary.

#### **APPENDIX 3.**

To access the data, click or select the words “[Appendix 3.](#)” Mean, normalized serum levels ( $\mu\text{mol/l}$ ), as described in the text, for  $\beta$ -carotene and dl- $\alpha$ -tocopheryl acetate for the three dosage forms as a function of sampling time.

#### **APPENDIX 4.**

To access the data, click or select the words “[Appendix 4.](#)” Mean, normalized serum levels ( $\mu\text{mol/l}$ ), as described in the text, for ascorbic acid and copper for the three dosage forms as a function of sampling time.

#### **APPENDIX 5.**

To access the data, click or select the words “[Appendix 5.](#)” Mean, normalized serum levels ( $\mu\text{mol/l}$  for zinc), as described in the text, for the three dosage forms as a function of sampling time.

#### **APPENDIX 6.**

To access the data, click or select the words “[Appendix 6.](#)” Chart of model-independent serum concentrations of copper as a function of the time course following supplementation.

#### **APPENDIX 7.**

To access the data, click or select the words “[Appendix 7.](#)” Pharmacokinetic Model.

#### **APPENDIX 8.**

To access the data, click or select the words “[Appendix 8.](#)” Experimental data (as symbols, with standard error bars) and model fits (smooth curves) to the bioabsorption for copper (1.6 mg/day) derived from four tablets. The data points are the average responses of the 15 subjects adjusted for any baseline drift over the course of the study. The model fits are the result of minimizing the errors between the data points and curves at the sampled times. These data and the model fit for the response to four tablets are the best data for copper, but as indicated in the text have greater uncertainty than the results for the other four nutrients. Copper was supplemented at the lowest level of the five micronutrients.

#### **APPENDIX 9.**

To access the data, click or select the words “[Appendix 9.](#)” Chart of model-independent serum concentrations of zinc as a function of the time course following supplementation.

**APPENDIX 10.**

To access the data, click or select the words “[Appendix 10.](#)” Labelled and actual amounts of the micronutrients in the AREDS1 and full strength AREDS2 formulations. The actual amounts are the levels in the preparations guaranteed at shelf life.

**APPENDIX 11.**

To access the data, click or select the words “[Appendix 11.](#)” Amounts of the AREDS1 micronutrients in the alternate AREDS2 Tier2 formulations, mg/day.

**APPENDIX 12.**

To access the data, click or select the words “[Appendix 12.](#)” Mean maximum change in nutrient concentrations (mol/l) in plasma or serum. For this population the change was between 50% and 100% of the lowest value observed, except for copper for which no meaningful change was observed.

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**REFERENCES**

- Ervin RB, Wright JD, Kennedy-Stephenson J. Use of dietary supplements in the United States. *Vital and Health Statistics*. 1999; xxx:i-iii. [PMID: 10464471].
- Melia CD, Davis S. Review article: mechanisms of drug release from tablets and capsules. I: disintegration. *Aliment Pharmacol Ther* 1989; 3:223-32. [PMID: 2520618].
- Melia CD, Davis S. Review article: Mechanisms of drug release from tablets and capsules. 2. Dissolution. *Aliment Pharmacol Ther* 1989; 3:513-25. [PMID: 2518865].
- Taylor A. Introduction to the issue regarding research regarding age related macular degeneration. *Mol Aspects Med* 2012; 33:291-4. [PMID: 22542402].
- Group A-REDSR. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. *Arch Ophthalmol* 2001; 119:1417-1436. [PMID: 11594942].
- Ferris FL, Group A-REDSR. A simplified severity scale for age-related macular degeneration: AREDS Report No. 18. *Arch Ophthalmol* 2005; 123:1570-1574. [PMID: 16286620].
- Danis RP, Domalpally A, Chew EY, Clemons TE, Armstrong J, SanGiovanni JP, Ferris FL. Methods and reproducibility of grading optimized digital color fundus photographs in the age-related eye disease study 2 (AREDS2 report number 2). *Invest Ophthalmol Vis Sci* 2013; 54:4548-54. [PMID: 23620429].
- Johnson EJ, Hammond BR, Yeum K-J, Qin J, Wang XD, Castaneda C, Snodderly DM, Russell RM. Relation among serum and tissue concentrations of lutein and zeaxanthin and macular pigment density. *Am J Clin Nutr* 2000; 71:1555-62. [PMID: 10837298].
- Roe JH, Kuether CA. A color reaction for dehydroascorbic acid useful in the determination of vitamin C. *Science* 1942; 95:77-[PMID: 17791074].
- Roe J, Kuether C. Determination of vitamin C in blood and urine by means of the compound of 2, 4-dinitrophenylhydrazine with dehydroascorbic acid. *J Biol Chem* 1943; 147:339-406.
- Lee W, Hamernyik P, Hutchinson M, Raisys VA, Labbe R. Ascorbic acid in lymphocytes: cell preparation and liquid-chromatographic assay. *Clin Chem* 1982; 28:2165-9. [PMID: 7127749].
- Spence B, Cassap M. Environmental Series. Thermo Fisher Scientific Application Note 2008.
- Block G, Hartman AM, Dresser CM, Carroll MD, Gannon J, Gardner L. A data-based approach to diet questionnaire design and testing. *Am J Epidemiol* 1986; 124:453-69. [PMID: 3740045].
- Block G, Coyle L, Hartman A, Scoppa S. HHHQ-DIETSYS analysis software, version 3.0. Bethesda, MD: National Cancer Institute 1993.
- Lindfield GR, Penny JE. Numerical Methods: Using MATLAB: Academic Press; 2012.
- Gibaldi M, Perrier D. Noncompartmental analysis based on statistical moment theory. *Pharmacokinetics* 1982; 2:409-17.
- Unlu NZ, Bohn T, Clinton SK, Schwartz SJ. Carotenoid absorption from salad and salsa by humans is enhanced by the addition of avocado or avocado oil. *J Nutr* 2005; 135:431-6. [PMID: 15735074].
- Bierer TL, Merchen NR, Erdman JW Jr. Comparative absorption and transport of five common carotenoids in preruminant calves. *J Nutr* 1995; 125:1569-77. [PMID: 7782912].

19. Meyers KJ, Mares JA, Igo RP, Truitt B, Liu Z, Millen AE, Klein M, Johnson EJ, Engelman CD, Karki CK. Genetic Evidence for Role of Carotenoids in Age-Related Macular Degeneration in the Carotenoids in Age-Related Eye Disease Study (CAREDS). *Invest Ophthalmol Vis Sci* 2014; 55:587-99. [PMID: 24346170].
20. Meyers KJ, Johnson EJ, Bernstein PS, Iyengar SK, Engelman CD, Karki CK, Liu Z, Igo RP, Truitt B, Klein ML. Genetic determinants of macular pigments in women of the Carotenoids in Age-Related Eye Disease Study. *Invest Ophthalmol Vis Sci* 2013; 54:2333-45. [PMID: 23404124].
21. Group A-REDSR. Lutein+ Zeaxanthin and Omega-3 Fatty Acids for Age-Related Macular Degeneration: The Age-Related Eye Disease Study 2 (AREDS2) Randomized Clinical Trial. *JAMA* 2013; 309:2005-2015. .
22. Bénézeth P, Palmer DA, Wesolowski DJ, Xiao C. New measurements of the solubility of zinc oxide from 150 to 350 C. *J Solution Chem* 2002; 31:947-73. .
23. Palmer DA, Bénézeth P, Simonson J, Petrova A. The transport and chemistry of copper in power plants as determined by laboratory experiments. Oak Ridge National Library. 2000.
24. Palmer DA, Bénézeth P, Simonson J. Solubility of copper oxides in water and steam. 14th International Conference on the Properties of Water and Steam in Kyoto; 2004. p. 491-6.
25. Prasad A, Beck F, Nowak J. Comparison of absorption of five zinc preparations in humans using oral zinc tolerance test. *J Trace Elem Exp Med* 1993; 6:109-15. .
26. Markowitz ME, Rosen JF, Mizruchi M. Circadian variations in serum zinc (Zn) concentrations: correlation with blood ionized calcium, serum total calcium and phosphate in humans. *Am J Clin Nutr* 1985; 41:689-96. [PMID: 3984922].
27. Néve J, Hanocq M, Peretz A, Khalil FA, Pelen F. Absorption and metabolism of oral zinc gluconate in humans in fasting state, during, and after a meal. *Biol Trace Elem Res* 1992; 32:201-12. [PMID: 1375057].
28. Markowitz M, Rotkin L, Rosen JF. Circadian rhythms of blood minerals in humans. *Science* 1981; 213:672-4. [PMID: 7256269].
29. Ishida M, Seino Y, Yamaoka K, Tanaka Y, Satomura K, Kurose Y, Yabunchi H. The circadian rhythms of blood ionized calcium in humans. *Scand J Clin Lab Invest* 1983; 165:83-6. [PMID: 6578582].
30. Emes JH, Arthur D. The site of zinc absorption in the rat small intestine. *Exp Biol Med* 1975; 148:86-8. [PMID: 1129277].
31. Overall N, Macfarlane N, Sedgwick R. The effects of water hardness upon the uptake, accumulation and excretion of zinc in the brown trout, *Salmo trutta* L. *J Fish Biol* 1989; 35:881-92. .
32. Evans DF, Pye G, Bramley R, Clark A, Dyson T, Hardcastle J. Measurement of gastrointestinal pH profiles in normal ambulant human subjects. *Gut* 1988; 29:1035-41. [PMID: 3410329].
33. Watson BW, Meldrum S, Riddle H, Brown R, Sladen G. pH profile of gut as measured by radiotelemetry capsule. *BMJ* 1972; 2:104-6. [PMID: 5018285].
34. Lucas M. Determination of acid surface pH in vivo in rat proximal jejunum. *Gut* 1983; 24:734-9. [PMID: 6873735].
35. Mahé S, Huneau J, Marteau P, Thuillier F, Tome D. Gastroileal nitrogen and electrolyte movements after bovine milk ingestion in humans. *Am J Clin Nutr* 1992; 56:410-6. [PMID: 1636619].
36. Markowitz H, Gubler C, Mahoney J, Cartwright G, Wintrobe M. Studies on copper metabolism. XIV. Copper, ceruloplasmin and oxidase activity in sera of normal human subjects, pregnant women, and patients with infection, hepatolenticular degeneration and the nephrotic syndrome. *J Clin Invest* 1955; 34:1498-1508. [PMID: 13263429].
37. Soinio M, Marniemi J, Laakso M, Pyörälä K, Lehto S, Rönnemaa T. Serum zinc level and coronary heart disease events in patients with type 2 diabetes. *Diabetes Care* 2007; 30:523-8. [PMID: 17327315].
38. Breskin MW, Worthington-Roberts BS, Knopp RH, Brown Z, Plovie B, Mottet NK, Mills JL. First trimester serum zinc concentrations in human pregnancy. *Am J Clin Nutr* 1983; 38:943-53. [PMID: 6650451].
39. Turnlund JR, Keyes WR, Anderson HL, Acord LL. Copper absorption and retention in young men at three levels of dietary copper by use of the stable isotope <sup>65</sup>Cu. *Am J Clin Nutr* 1989; 49:870-8. [PMID: 2718922].
40. Klein EA, Thompson IM, Tangen CM, Crowley JJ, Lucia MS, Goodman PJ, Minasian LM, Ford LG, Parnes HL, Gaziano JM. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA* 2011; 306:1549-56. [PMID: 21990298].
41. Mursu J, Robien K, Harnack LJ, Park K, Jacobs DR. Dietary supplements and mortality rate in older women: the Iowa Women's Health Study. *Arch Intern Med* 2011; 171:1625-33. [PMID: 21987192].
42. Antioxidants IoMPoD. Compounds R. Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids: A Report of the Panel on Dietary Antioxidants and Related Compounds, Subcommittees on Upper Reference Levels of Nutrients and of Interpretation and Use of Dietary Reference Intakes, and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition Board, Institute of Medicine: National Academies Press; 2000.
43. Rajkumar S, Vasavada AR, Praveen MR, Rajendran A, Reddy GB, Tripathi H, Ganatra DA, Arora A, Patel AR. Exploration of molecular factors impairing superoxide dismutase (SOD) isoforms activity in human senile cataractous lenses. *Invest Ophthalmol Vis Sc* 2013; 54:6224-6233.
44. Kankanahalli S, Burlina PM, Wolfson Y, Freund DE, Bressler NM. Automated classification of severity of age-related macular degeneration from fundus photographs. *Invest Ophthalmol Vis Sci* 2013; 54:1789-96. [PMID: 23361512].

45. Garg S, Jani PD, Kshirsagar AV, King B, Chaum E. Telemedicine and Retinal Imaging for Improving Diabetic Retinopathy Evaluation. *Arch Intern Med* 2012; 172:1678-80. [PMID: 23026969].
46. Meydani SN, Barnett JB, Dallal GE, Fine BC, Jacques PF, Leka LS, Hamer DH. Serum zinc and pneumonia in nursing home elderly. *Am J Clin Nutr* 2007; 86:1167-73. [PMID: 17921398].
47. Prasad AS. Discovery of human zinc deficiency: its impact on human health and disease. *Adv Nutr* 2013; 4:176-90. [PMID: 23493534].
48. Abrams SA. Zinc for preterm infants: who needs it and how much is needed? *Am J Clin Nutr* 2013; 98:1373-4. [PMID: 24132977].

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