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ORIGINAL CONTRIBUTION

Can changes in hydrophobicity increase the bioavailability of α-tocopherol?

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Summary *Background* Bioavailability of fat-soluble vitamins from conventional oral supplements is insufficient in some conditions in which fat digestion and absorption are chronically impaired (e. g. cystic fibrosis). *Aim of the study* We used a water-soluble form of fat-soluble vitamin E (AQUANOVA® solubilisate) to create a nutritional supplement (NS) in the form of vitaminized gummi bears (with micellised water-soluble α-tocopheryl acetate (100 IU) and 400 mg crystalline vitamin C). We assessed the bioavailability of the NS in comparison to conventional preparations. *Methods* The trial consisted of three study days (d0: NS sucked; d10: NS swallowed; d20: reference products swallowed). A total of 14 subjects (6 male/8 female), aged 25.3 (22.7–35.3) years, BMI 24.3 (19.0–31.7) kg/m^2 participated in the study. They had blood samples drawn after fasting for ≥12 hours and then 1, 5, 15, 30, 60, 120, 180, 240, 300 and 320 minutes after ingesting the vitamins. HPLC and a colorimetric method were used to determine vitamin E and vitamin C, respectively. Areas under the curve ($AUC_{0-320min}$) and maximum increases in plasma concentrations (Δ concentration) were calculated to assess bioavailability. *Results* The $AUCs_{0-320min}$ of α-tocopherol from d0 were significantly larger ($p = 0.016$) when compared to d20. Moreover, the maximum increase in α-tocopherol plasma concentrations was significantly higher for d0 ($p = 0.023$) and d10 ($p = 0.002$) when compared to d20. *Conclusions* Short-term bioavailability of AQUANOVA® micellised fat-soluble vitamin E from our NS was significantly higher than from regular supplements. The NS will now be tested for its clinical efficacy in a randomized double-blind controlled intervention trial with CF patients.

Key words antioxidants – alpha-tocopherol – ascorbic acid – fat malabsorption – bioavailability – solubilised vitamins

Introduction

Fat-soluble vitamin deficiencies are frequently encountered in chronic diseases with impaired fat digestion and absorption, e. g. in patients with cholestasis [1, 2], short bowel syndrome [3] or cystic fibrosis [4, 5]. In these diseases, absorption of fat-soluble substances – including fat-soluble vitamins – is impaired due to one or more of the following factors [6]: decreased intraluminal lipid digestion and defective micelle formation (lack of bile acids, pancreatic enzymes and bicarbonate), reduced absorptive area (intestinal resections) or shortened transit time. Many researchers reported that vitamin E deficits in these patients persisted even when (fat-soluble) vitamin E was administered in what was considered suffi-

ciently high doses [1–3, 5, 7]. Besides a potential problem with treatment compliance, this observation clearly points to an insufficient bioavailability of vitamin E from the supplements prescribed to these patients.

We hypothesized that by using a supplement which contained fat-soluble vitamins in water-soluble form most of the problems mentioned above could be circumvented: Solubilisation enables fat-soluble vitamins to pass the unstirred water layer in the intestine. In contrast, conventional oral fat-soluble vitamin supplements can only be absorbed when the vitamins are sufficiently micellised during digestion.

As a proof of principle, we exemplarily studied the bioavailability of a micellised water-soluble form of vitamin E (AQUANOVA® vitamin E solubilisate). According to the specifications of the manufacturer, vitamin E product micelles are similar in size to naturally formed micelles and are furthermore stable to the low pH encountered in the stomach. They can thus directly deliver the fat-soluble vitamins packed in their core to the intestinal brush border membrane where the vitamins are absorbed. If our assumptions regarding the properties of the micellised fat-soluble vitamin E hold true, a higher proportion of solubilised vitamin E should be absorbed in a shorter period when compared to regular fat-soluble vitamin E, because absorption starts immediately after the product micelles have reached the intestine. Thus, in this present study, we have, as a first step, investigated the bioavailability of water-soluble vitamin E in comparison to commercially available fat-soluble vitamin E in healthy adult volunteers.

Subjects and methods

Subjects

We conducted a bioavailability study with 14 healthy adult volunteers. Inclusion criteria were: age between 18 and 49 years; nonsmoker. Exclusion criteria were: regular intake of vitamin supplements containing the vitamins E and C during the 8 weeks preceding the study; pregnant or nursing women; disease of the oropharynx (e. g. gum bleeding, inflammations); severe liver disease (e. g. hepatitis, cirrhosis); chronic bronchopulmonary disease (e. g. asthma, cystic fibrosis); acute or chronic intestinal disease (e. g. celiac disease, Crohn's disease, diarrhea). A physician conducted a detailed anamnesis as well as a thorough physical examination before the subjects were admitted to the study.

Supplements

The study supplement consisted of vitaminised gummi bears containing α-tocopherol (100 IU/67 mg as d-α-tocopheryl acetate) as water-soluble micelles (AQUANOVA® solubilisates, Aquanova German solubilisate technologies GmbH, Darmstadt, Germany). The gummi bears were designed at our institute and produced by the University Pharmacy of the University Hospital Tuebingen. As the reference product, a commercially available supplement containing a fat-soluble form of vitamin E (100 IU α-tocopherol-acetate as softgel capsule [E-100, 100 % natural d-alpha Tocopheryl, NOW FOODS, Bloomingdale] were used. 400 mg crystalline vitamin C (Krüger, Bergisch Gladbach, Germany) was administered on all days either via the gummi bears or diluted in water together with the vitamin E capsule. Supplying this water-soluble vitamin served as a control measure: since vitamin C is absorbed via other mechanisms than the fat-soluble vitamins; no differences between the reference and study supplements should be observed.

Study design

In this bioavailability study, we wanted to assess the short-term bioavailability of the vitamins from our gummi bears as compared to vitamins from commercially available supplements and to compare different modes of application for the gummibears. The study schedule was as follows: on day 0, each subject sucked one gummi bear; on day 10, each participant swallowed one gummi bear with some water; on day 20, each participant took the reference supplements with some water. The daily routine was as follows: fasting blood samples were taken after an overnight fast (≥ 12 hours of fasting). Then the subjects received the vitamins. Further venous blood samples were taken 1, 5, 15, 30, 60, 180, 240, 300 and 320 minutes after ingestion of the vitamins. Dietary intake was restricted during the sample collection period in order to minimize vitamin and fat intake from the diet. 60 minutes after ingesting the vitamins, the subjects received breakfast which consisted of limited amounts of white wheaten toast (max. 6 pieces), 0.1 % fat cream cheese (max. 100 g), low fat pork ham (max. 2 slices), honey (ad libitum), and herbal tea (max. 2 cups) with sugar (ad libitum). After breakfast, they were not allowed to eat or drink anything but mineral water until the last sample was collected. Dietary intake was recorded and calculated from weighed dietary protocols with the nutrition software EBISpro for Windows (© J. Erhardt, University of Hohenheim). Ethical approval was received from the Ethical Committee of the Medical Faculty in Tuebingen and informed written consent obtained from all subjects.

Collection of blood samples

All subjects received a permanent venous access in the morning. It was kept open by injecting 1 ml of 0.9 % NaCl containing 100 IU heparin/ml each time after taking a sample. Before taking the subsequent sample, 2 ml of the blood/heparin/NaCl mixture was drawn into an extra syringe in order to remove the heparin block and was discarded. Blood for all analyses was drawn into lithium-heparin S-Monovettes® (Sarstedt AG & Co. Nuembrecht, Germany). The tube was shaken well, immediately placed on ice in the dark and then centrifuged at 3000 x g for 10 min at 4 °C. Plasma was separated from blood cells, aliquoted and frozen at –80 °C until analysis.

Total cholesterol in plasma

Total cholesterol in plasma was determined using the ABX Diagnostics Cholesterol kit (REF.A11A00051, Axon Lab AG, Stuttgart, Germany) for Cobas Mira S (Roche, Grenzach-Wyhlen, Germany) according to the manufacturer's instructions.

Vitamin C in plasma

Vitamin C in plasma was analyzed using the method by Ihara and colleagues [8, 9]. The method was adapted at our institute to be used with a Cobas Mira S (Roche, Grenzach-Wyhlen, Germany). The details have been published previously [5].

Briefly, a 200 µL plasma aliquot was stabilised with 40 % m-phosphoric acid solution directly after separating the plasma from blood cells. The mixture was vortexed, incubated at room temperature and then frozen at –80 °C until analysis. For determination of the plasma vitamin C concentration, the mixture was centrifuged and the supernatant incubated with potassium phosphate buffer, 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy free radical and o-diphenylamine. The formation of the coloured product was measured at 340 nm and compared to the concentration of a known standard (56.6 µmol vitamin C/L).

Vitamin E in plasma

Plasma α-tocopherol was determined using an isocratic HPLC method. The details have recently been published [5]. Briefly, α-tocopherol was extracted from plasma with a 1:1 mixture (v/v) of ethanol and 1-butanol containing 5 mg BHT/ml and 8 µmol Tocol/L. The mobile phase consisted of 82 % acetonitrile, 15 % dioxane and 3 % methanol [containing 100 mM ammonium acetate and 0.1 % triethylamine as related to the amount of methanol used]. The vitamin was detected with a fluorescence detector. For quantification, the internationally certified NIST standards (standard reference material® 968c, National Institute of Standards and Technology, Gaithersburg, MD, USA) were used.

Assessment of bioavailability

In order to compare the bioavailability of vitamins from gummi bears with the one of the reference supplements, the $AUCs_{0–320\ min}$ were calculated with Origin® 7G SR1 v7.0303 (B303) software (OriginLab Corporation, Northampton, MA, USA).

Statistical analysis

Statistical analyses were performed with SPSS for Windows 9.0. As the data were not normally distributed (Shapiro Wilk: $p < 0.5$), non-parametric tests were used. Results are presented as median with minimum-maximum in parentheses. Differences within each group, i. e. day 0 vs. day 10 vs. day 20, were identified for each vitamin with Friedman test. The results of Friedman test are displayed in the tables as "p of group". A result was considered significant if $p < 0.05$. Wilcoxon signed rank test was applied to inquire further if there were significant differences between day 0 and day 20, day 10 and day 20 or day 0 and day 10, respectively.

Results

Subjects

Six healthy adult males and eight healthy adult females aged 25.3 (22.7–35.3) years with a BMI of 24.3 (19.0–31.7) kg/m² were recruited for the study. One male subject was not available on day 20 for personal reasons. The subjects' baseline plasma cholesterol and vitamin concentrations are listed in Table 1. There were no sig-

Table 1 Baseline plasma vitamin and cholesterol concentrations[1]

	Day 0 n = 14	Day 10 n = 14	Day 20 n = 13	p[2]
α-Tocopherol (µmol/L)	24.2 (15.2–43.5)	20.9 (15.1–35.9)	23.0 (18.1–39.4)	NS
Vitamin C (µmol/L)	69.5 (43.5–106.6)	68.7 (48.1–101.4)	62.3 (48.9–99.7)	NS
Cholesterol (mmol/L)	4.8 (3.1–7.0)	4.6 (3.6–6.2)	4.7 (3.7–6.3)	NS

[1] All data as median (minimum-maximum); [2] Friedman test; *NS* not significant

nificant differences between the baseline values of the three study days for any of these variables.

Dietary energy and nutrient intake

The respective data for fat, cholesterol, vitamin E and vitamin C intake are shown in Table 2. Except for cholesterol, dietary intake of all nutrients was significantly lower on day 0 when compared to day 10 and 20. Overall, the dietary intake of all relevant vitamins was very low when compared to the doses given with the supplements (vitamin E: 0.6–3 % of supplemental dose, vitamin C: 0.2–0.4 % of supplemental dose).

Bioavailability of vitamins from study and reference supplements

Vitamin plasma concentrations tended to fall below the fasting plasma concentration at subsequent samplings, although all participants had already been fasting for at least 12 h before the baseline value was assessed. We therefore evaluated the area under the plasma concentration-time curve using the lowest point in the plasma concentration-time curve as the baseline value. The AUC was calculated above this baseline and onwards from the respective point in time, when the lowest point of the plasma concentration was reached. Table 3 gives an overview of the $AUCs_{0-320\ min}$ of all vitamins at days 0, 10 and 20.

There was a significant difference between the α-tocopherol $AUCs_{0-320min}$ of day 0, day 10 and day 20 which was located between day 0 and day 20. However, "p of group" was not significant for vitamin C.

In order to assess the magnitude of change in plasma concentrations after ingestion of the jelly beans and reference supplements, the difference between maximum and minimum plasma concentrations was calculated for each of the study days (Table 4).

There was a significant group difference between Δ plasma α-tocopherol concentration of day 0, day 10 and day 20. The difference was located between day 0 and day 20 as well as between day 10 and day 20. For vitamin C, Δ plasma concentration was – as expected – not significantly different between the three study days and was always around 40 µmol/L.

Discussion

The results of our bioavailability study point to a higher short-term bioavailability of our micellised (i. e. water-soluble) versus a non-micellised form of vitamin E in healthy adult volunteers both with regard to AUCs and with regard to maximum increases in vitamin plasma

Table 2 Dietary intake of fat, cholesterol, vitamin E and vitamin C[1]

	Day 0 n = 14	Day 10 n = 14	Day 20 n = 13	p[2]
Fat (g)	6.1 (2.7–7.0)	7.7 (3.6–11.0)	8.5 (3.7–10.3)	p of group = 0.002 d 0 vs. d 10: p = 0.002 d 0 vs. d 20: p = 0.004
Cholesterol (mg)	26.4 (12.9–28.9)	27.2 (14.1–28.6)	26.9 (13.2–27.5)	NS
Vitamin E-equivalents (mg)[3]	1.05 (0.40–1.20)	1.30 (0.40–2.00)	1.50 (0.60–1.90)	p of group = 0.001 d 0 vs. d 10: p = 0.003 d 0 vs. d 20: p = 0.003
Vitamin C (mg)	1.15 (0.80–1.50)	1.25 (1.00–1.60)	1.10 (0.90–1.60)	p of group = 0.029 d 0 vs. d 10: p = 0.007 d 0 vs. d 20: p = 0.046

[1] All data as median (minimum-maximum); [2] Friedman test for differences within the whole group; Wilcoxon signed rank test for sub-testing of pairs; *NS* not significant; [3] One vitamin E equivalent = 1 mg RRR-α-tocopherol

Table 3 $AUCs_{0-320min}$ of α-tocopherol and vitamin C at days 0, 10 and 20[1]

	AUC day 0 n = 14	AUC day 10 n = 14	AUC day 20 n = 13	p[2]
α-Tocopherol [(µmol/L)*h)]	20.1 (10.9–58.1)	20.7 (9.7–39.5)	13.5 (6.8–30.6)	p of group = 0.023 d0 vs. d20: p = 0.016
Vitamin C [(µmol/L)*h)]	145.0 (77.4–196.6)	131.2 (47.7–206.4)	118.0 (53.3–220.6)	NS

[1] All data as median (minimum-maximum); [2] Friedman test for differences within the whole group; Wilcoxon signed rank test for sub-testing of pairs; *NS* not significant

Table 4 Increase in plasma vitamin concentrations (c_{max}–c_{min}) within 320 min of ingesting the study and reference supplements[1]

	Δc day 0 n = 14	Δc day 10 n = 14	Δc day 20 n = 13	p[2]
α-tocopherol (μmol/L)	9.6 (4.5–19.9)	8.6 (4.4–15.6)	6.1 (2.1–10.2)	p of group: p = 0.025 d 0 vs. d 20: p = 0.023 d 10 vs. d 20: p = 0.002
Vitamin C (μmol/L)	41.36 (18.4–53.25)	40.06 (20.53–59.45)	36.08 (27.55–52.64)	NS

[1] all data as median (minimum-maximum); [2] Friedman test for differences within the whole group; Wilcoxon signed rank test for sub-testing of pairs; *NS* not significant

concentrations. This supports our hypothesis that absorption can be enhanced and bioavailability be improved when fat-soluble vitamins are administered in the form of water-soluble micelles instead of oily solutions.

There are a lot of bioavailability trials and intervention studies in the literature, in which the bioavailability of water-miscible (i. e. dispersible in water, but not water-soluble as our own preparation) vitamin E has been compared with regular fat-soluble forms of vitamin E [4, 10–13]. The results of these studies can, however, not be directly compared with our own observations, because the properties of water-*miscible emulsified* vitamin E differ from the ones of *micellised* water-*soluble* vitamin E from AQUANOVA® in the following ways: The particles in an emulsion are larger than in micellar solutions (> 500 nm vs. < 50 nm) and are thus – other than micelles – unable to directly diffuse between the microvilli of the brush border membrane. Furthermore, emulsions can become unstable when pH changes occur and as a consequence, the particles disaggregate [14]. In contrast, the micelles in the solubilisate are stable from pH 7 to pH 1 according to the manufacturer. The area of application of emulsified fat-soluble substances seems therefore more limited than that of micellised substances. Moreover, when looking at the highly controversial results observed in the above mentioned studies, the usefulness and the chemical properties of a specific vitamin emulsion apparently depend largely on the emulsifier(s) employed.

Only one more truly water-soluble form of vitamin E called d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) could be identified in the literature. So far, it has only been clinically tested in patients with chronic or congenital cholestasis. The results were, however, very convincing: Sokol et al. [2] as well as Traber et al. [1, 15] reported that vitamin E deficits in patients with chronic or congenital cholestasis could be corrected by administration of TPGS, yet not by administration of dl-α-tocopherol, dl-α-tocopheryl acetate or tocopheryl acetate emulsified with medium chain triglycerides and polysorbate 80.

Based on these observations, TPGS would have been an interesting candidate for our own study as well. Instead of TPGS, we chose to use vitamin E solubilisate, because the technology employed for the manufacturing of this solubilisate can easily be applied to solubilise other fat-soluble substances (e. g. vitamins A, D and K, carotenoids, coenzyme Q_{10}) as well. As patients with fat malassimilation often suffer from fat-soluble vitamin deficiencies other than vitamin E, we deemed it best to have a water-soluble form of these substances at our disposal as well.

Conclusion

Based on the evidence from our own bioavailability study as well as from the studies discussed above, it seems overall justified to conclude that absorption of vitamin E from micellised water-soluble preparations is more efficient when compared to fat-soluble preparations – especially in conditions where fat digestion and absorption is impaired. It is furthermore conceivable that a similar improvement in bioavailability can be achieved when solubilising other fat-soluble vitamins. Fat-soluble vitamin deficiencies in patients with fat malassimilation can thus presumably be corrected more efficiently by using water-soluble instead of fat-soluble preparations.

Final remarks

In contrast to the preparations used so far, our vitaminised gummi bears stand out through their attractive sensory properties which will certainly help to improve long-term compliance, especially in younger patients. The gummi bears are presently being tested for their clinical benefits in a randomised, double-blind and controlled intervention trial with CF patients.

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