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Milk protein complexation enhances post prandial vitamin D3 absorption in rats

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This study investigated the effect of complexation with whey and casein protein, respectively, on post prandial absorption of vitamin D3. For this purpose, Sprague-Dawley rats (n = 78) were administered 840 IU vitamin D3 dissolved in ethanol and either (i) complexed with whey protein isolate (protein : vitamin ration 2 : 1), (ii) complexed with caseinate (protein : vitamin ration 2 : 1), or (iii) provided in a water solution. Serum concentrations of vitamin D3, 25-hydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 were measured before and 2, 3, 4, 5, 6, 7, 8, and 10 hours after administration of vitamin D3. Significant effects of complexation on serum concentrations of vitamin D3, 25-hydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 were demonstrated. Complexation with whey protein isolate resulted in the fastest and highest absorption of vitamin D3 while an effect of complexation with caseinate was evident but more modest and non-significant. In conclusion, the study demonstrates that complexation with milk proteins is an efficient strategy to enhance bio-accessibility of vitamin D3.

Introduction

Vitamin D is a lipophilic vitamin mainly obtained through animal-based foods. Vitamin D can also be produced in the skin from 7-dehydrocholesterol by ultraviolet (UV) irradiation, which breaks the B ring to form pre-Vitamin D.1–3 Most commonly known for its role in the regulation of calcium and phosphorus homeostasis,4 vitamin D is also involved in a plethora of other biochemical processes in various tissues, including the regulation of blood glucose and insulin release, muscle contraction, transmission of action potentials and proper immune function.5,6 Despite its recognized importance for normal physiological function, vitamin D deficiency is a rampant issue worldwide,7 often explained by insufficient dietary intake as well as lack of UV exposure needed for endogenous synthesis of the vitamin. Among the consequences of vitamin D deficiency are an increased risk of cardiovascular and infectious diseases, as well as certain types of cancers.4,6

As a strategy to combat vitamin D deficiency and its associated health risks, supplementation as well as fortification of foods such as juice, milk, dairy and cereals is commonly used. Unfortunately, fortification is often inadequate due to oxidation and degradation during storage as well as the vitamin being poorly solubilized in aqueous liquids and low-fat foods.5 Moreover, the solubility of ingested vitamin D in gastrointestinal fluids is poor, thus adding to the high frequency of low levels of circulating vitamin D.4 Due to the unstable nature of vitamin D during processing, storage and digestion, different protective strategies have thus been explored in vitro and in vivo, and complexation and encapsulation of vitamin D with various
carrier proteins has been investigated. 5,8 In many cases, milk proteins, which are known for their ability to self-assembly and form complexes with otherwise poorly soluble compounds, 9 have been used and shown to have a potential for improving stability and bio-accessibility of vitamin D. 10 Abbasi et al. (2014) showed that encapsulation with whey protein isolate increased stability of vitamin D3 during storage. 11 Diarrassouba et al. (2015) showed that entrapment in microspheres formed by a combination of β-lactoglobulin and lysozyme from egg white increased stability and apparent absorption of vitamin D3. 5 Using a Caco-2 cell model, Cohen et al. (2017) showed that re-assembled casein micelles improved in vitro bioavailability of vitamin D3. 12 Thus, former work suggests that both milk proteins derived from the whey fraction as well as casein may elicit beneficial effects on vitamin D absorption. However, to the best of our knowledge, no studies have compared the use of whey and casein proteins in strategies targeted at enhancing vitamin D bio-accessibility. Caseins are generally regarded to be slow-metabolized proteins due to prolonged gastric emptying, mainly as a result of aggregation in the gut; conversely, whey proteins are soluble in the gastrointestinal milieu and easily digested. 13 Therefore, it can be hypothesized that the digestion pattern of the respective milk proteins might affect vitamin D absorption kinetics differently.

Using a rat model, the aim of this study was to investigate if vitamin D3 complexation with whey protein and casein, respectively, elicits different postprandial responses concerning the absorption kinetics of vitamin D and resultant bioavailability of the vitamin. Rats are considered a useful model as both humans and rats metabolize and activate vitamin D through hydroxylation, and both human and rats also catabolize vitamin D metabolites mainly via C24-hydroxylation. 14 For this purpose, an acute intervention study where plasma concentrations of vitamin D3, and its metabolites 25-hydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 were determined up to 10 hours after administration of vitamin D3 was conducted.

Materials and methods

Ethical approval

The experiment was licensed by the Danish Animal Experimentation Inspectorate (license no 2018-15-0201-01406) according to the Danish Animal Experimentation Act and the European Union Directive 2010/63/EU.

Animals

A total of seventy-eight male Sprague-Dawley rats (248.5 ± 16.6 g) (Janvier Labs, Saint Berthevin, France) arrived at the animal facility in installments of 26 rats. The rats were group housed (3–4 rats per cage) and fed a standard chow diet (600 IU kg−1 vitamin D3) (Altromin 1324, Brogaarden, Lynge, Denmark) for one week during acclimation.

Vitamin D solutions

Four different interventions were included in the study; whey protein–vitamin D3 complex, caseinate–vitamin D3 complex, non-complexed vitamin D3 (positive control) and a negative control (no vitamin D3). In 50 mL centrifuge tubes, whey and caseinate complexes were prepared by dissolving Lacprodan DI-922A and MIPRODAN 30 Sodium Caseinate (Arla Foods Ingredients, Viby J, Denmark), respectively, in 55 mg mL−1 H2O. Crystalline vitamin D3 (DSM Nutritional Products, Basel, Switzerland) was dissolved in 99.8% ethanol (Honeywell, North Carolina, USA) (0.6 mg mL−1) and added to the centrifuge tubes in the ratio 2 : 1 (milk
protein: vitamin D) and shaken vigorously, creating a final vitamin D3 concentration of 30 μg mL⁻¹. The centrifuge tubes were covered in tin foil and left at room temperature for one hour. The positive control was prepared by dissolving vitamin D3 in 99.8% ethanol (0.6 mg mL⁻¹) which was added to a 55 mg mL⁻¹ H₂O, creating a final vitamin D3 concentration of 30 μg mL⁻¹.

**Animal intervention**

The study was conducted on three independent test days (26 rats per day) and three rats were allocated per time point. Feed was removed from the cages before initiation of the intervention while the animals had free access to water during the study. The rats were administered 0.7 mL (840 IU vitamin D3) whey-vitamin D3 complex, casein-vitamin D3 complex, positive or negative control by oral gavage. Prior to each sampling time point, intraperitoneal anesthesia (fentanyl citrate 0.35 mg mL⁻¹, fluanisone 10 mg mL⁻¹ and midazolam 5 mg mL⁻¹) was administered and blood samples were obtained through cardiac puncture at time points 0, 2, 3, 4, 5, 6, 7, 8 and 10 h. The animals were euthanized after the samples were taken. Serum was prepared by placing the blood samples at room temperature for 30 minutes, followed by centrifugation at 2000g for 10 minutes at 4 °C. The serum was transferred to centrifuge tubes and metabolism was quenched by snap freezing the serum in liquid nitrogen, after which the samples were stored at −80 °C until analysis.

**Vitamin D analyses**

Vitamin D₃, 25-hydroxyvitamin D₃ and 24,25-dihydroxyvitamin D₃ were quantitatively analyzed using a multiple reaction monitoring (MRM) based mass spectrometry method. Prior to analysis of the samples, the following sample preparation was conducted. A heavy-labelled stable isotope internal standard (IS) solution was made from a 1 : 1 : 1 mix of vitamin D₃ (6, 19, 19 – d₃) (Merck, Darmstadt, Germany), 25-hydroxyvitamin D₃ (6, 19, 19 – d₃) (Merck, Darmstadt, Germany) and (24R), 24, 25-dihydroxyvitamin D₃ (26, 26, 27, 27 – d₆) (Merck, Darmstadt, Germany) in 100% methanol. Twenty-five μL IS solution (0.1 μg mL⁻¹) was added to 100 μL serum sample, standard reference sample (NIST SRM 1950, Sigma Aldrich, St Louis, Missouri, USA) or blank in a Hybrid SPE Plus 96-wellplate (Supelco, Sigma Aldrich, St Louis, Missouri, USA) for solid phase extraction according to Burild et al. 2014. The SPE was chosen to avoid interference from phospholipids for quantitative analysis of vitamin D₃. Unfortunately, it did not enable us to analyze for 1,25-dihydroxyvitamin D₃, but allowed binding of vitamin D₃, 25-hydroxyvitamin D₃, and 24,25-dihydroxyvitamin D₃. The solution was equilibrated for 15 min while gently shaking. To precipitate protein, 300 μL ice-cold 1% formic acid in acetonitrile was added to each well. Vacuum was applied and the flow-through was collected. Then, 0.5 mL 1% formic acid in acetonitrile was added to each well still under vacuum and flow-through was collected into the same tube. Total eluates was evaporated to dryness in a vacuum concentrator. Samples were derivatized by adding 100 μL 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (Merck, Darmstadt, Germany) in acetonitrile (0.75 mg mL⁻¹). The reaction was conducted for 2 hours at room temperature and in dark before analyzed by LC-MS analysis. Vitamin D₃ analysis was performed on a 8050 triple quadrupole (QQQ) mass spectrometer (Shimadzu, Kyoto, Japan) coupled to a Nexera X2 LC system (Shimadzu, Kyoto, Japan). The LC system was equipped with a Luna Omega C18 column (100 x 2.1 mm., 1.6 μm, 100 Å) (Phenomenex, Torrance, CA, USA) kept at 40 °C. Solvent A was 5% methanol and 0.1% formic acid in MilliQ water. Solvent B was 0.1% formic acid in methanol. The gradient was t[min]/B [%]; 0/60, 8/100, 10/100, 11/60 and 13/60. The flow was 0.3 mL min⁻¹ and the
Injection volume was 1 µL. The following MRM transitions were used; vitamin D3 (m/z: 560 → 298, 280 (quantifier ion and qualifier ion, respectively); IS 563 → 301, 283), 25-hydroxyvitamin D3 (m/z: 558 → 298, 280; IS 561 → 301, 283) and 24,25-dihydroxyvitamin D3 (m/z: 574 → 298, 280; IS 580 → 298, 280). Parent ions were obtained from Kassim et al. 2016,16 expect for 25-hydroxyvitamin D3 IS. The fragment ions were automatically generated in the LCMS solution 5.97 SP1 software. Quantification was obtained from external calibration curves (1–100 ng mL−1) containing the same IS mix as described for the sample preparation. The standard curve was derivatized as described for the sample preparation but was analyzed directly (no SPE well-plates).

Statistical analyses

Reported results are expressed as means and standard deviation (SD) for the serum concentrations of vitamin D3 metabolites and its metabolites. Univariate statistical analysis was performed using Minitab 18 (Minitab Ltd, Coventry, United Kingdom). Normal probability plots were investigated to control for normal distribution of the data, with the alpha-level defined as α = 0.05. Area under the curve (AUC) from 0 h to 10 h after treatment were calculated for each treatment groups, and means were compared using one-way analysis of variance (ANOVA). Multiple pairwise comparisons between treatment means were performed using Tukey’s Honestly Significant Difference (HSD) test. A two-way factorial ANOVA with treatment and time as independent variables and vitamin D3 metabolite concentrations as the dependent variables was performed, with Tukey’s HSD testing for significant differences between time and treatment means.

Results

In total, 78 rats were included in the study. The sample from one rat was excluded due to a laboratory error, and results from analyses of 77 samples are therefore reported. On all 77 samples, vitamin D3, 25-hydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 were analyzed (Fig. 1 and ESI Table 1†). On samples from the negative control group, vitamin D3 serum concentrations were below the limit of quantification (LOQ). Serum concentrations of vitamin D3 were found to increase with increasing time after administration for all three interventions with vitamin D3 (Fig. 1A). Compared to non-complexed and caseinate complexed, serum vitamin D3 concentrations were higher across the different time points in rats that received the vitamin D3 complexed with whey protein (P = 0.001). 25-hydroxyvitamin D3 was also found to increase with increasing time after administration for all three interventions with vitamin D3 in a pattern that resembled the pattern seen for vitamin D3 (Fig. 1B). Also, serum concentrations of 25-hydroxyvitamin D3 was found to be significantly higher (P = 0.001) for the rats that received vitamin D3 complexed with whey protein isolate compared with rats receiving the non-complexed vitamin D3. Serum concentrations of 25-hydroxyvitamin D3 was markedly lower in the negative control group as compared with groups receiving vitamin D3 (Fig. 1B). Serum concentrations of 24,25-dihydroxyvitamin D3 were more constant throughout the 10 h period and only a minor increase was observed over time (Fig. 1C). Even though less pronounced than for vitamin D and 25-hydroxyvitamin D3, serum concentrations of 24,25-dihydroxyvitamin D3 were significantly higher (P = 0.023) for the rats that received vitamin D3 complexed with whey protein compared with rats receiving the non-complexed vitamin D3 and the negative control group (Fig. 1C).

The area under the curve (AUC) of vitamin D3 metabolites was calculated (Fig. 2). AUC for serum vitamin D3 was significantly higher for the rats that received vitamin D3 complexed with whey protein compared with
rats receiving the non-complexed vitamin (Fig. 2A). AUC for serum vitamin D3 was also higher for the rats that received vitamin D3 complexed with caseinate compared with rats receiving the non-complexed vitamin D3, however, the difference was not significant (Fig. 2A). Even though non-significant, AUCs for 25-hydroxyvitamin D3 and 24,25-dihydroxyvitamin D3 showed same trends with higher values for rats that received vitamin D3 complexed with whey protein isolate, slightly lower values for rats that vitamin D3 complexed with caseinate while lowest values were seen for rats receiving the non-complexed vitamin.

Discussion

Vitamin D insufficiency is a global and severe challenge, and supplement strategies to improve vitamin D status are generally recognized as an efficient way of overcoming vitamin D insufficiency. As a result, an interest in optimizing supplementation strategies have risen. Grossmann and co-workers initiated work to elucidate how the different vehicles may influence the bioavailability of vitamin D with focus on supplements across three categories of vehicles: powders, lipids, and ethanol.17 Later a variety of carrier oil types as well as encapsulation and complexation strategies for improving stability of vitamin D has been reported.18,19 Nevertheless, the majority of studies has only examined a single complexation strategy, and thorough in vivo examinations of bio-accessibility are sparse. In the present study we compared complexation with two different milk-derived protein fractions; a whey protein isolate and caseinate, respectively. The simple complexation was carried out under similar conditions, making a direct comparison of viability possible. Conducting acute studies in a rat model, our results revealed that serum concentrations of vitamin D3 were significantly increased when vitamin D3 was administered as a whey protein isolate complex as compared with non-complexed vitamin D3 (Fig. 1A). This finding reveals that bio-accessibility of vitamin D3 can be significantly improved through complexation with whey protein isolate, probably because the complexation improves solubility of vitamin D3 in the gastrointestinal tract and protects against degradation in the acidic environment in the stomach. Considering the properties of whey protein and caseinate, where whey protein exerts high solubility in the gastrointestinal tract while casein can be expected to aggregate in the stomach, the present data indicate that solubility in the gastrointestinal tract is of utmost importance for bio-accessibility of vitamin D3. The majority of vitamin D from food is vitamin D3 (cholecalciferol), which is biologically inactive.

The liver is responsible for hydroxylation and conversion of vitamin D3 into the biologically active form, which is 25-hydroxyvitamin D3. 25-Hydroxyvitamin D3 is the major circulating form of vitamin D, and its concentration in serum is considered as one of the most reliable biomarkers of vitamin D status.20 Consequently, for a true evaluation of bioavailability of vitamin D3, it is of importance to examine serum concentrations of 25-hydroxyvitamin D3. In the present study, we demonstrated that serum concentrations of both vitamin D3 and 25-hydroxyvitamin D3 were increased with the whey protein isolate complexation. This finding corroborates that the complexation strategy secured high bioactivity of vitamin D3. In fact, a close association in the circulating levels of vitamin D3 and 25-hydroxyvitamin D3 was identified as the two vitamin D compounds exhibited same time progression. The findings are supported by a recent intervention study reported on rats that received vitamin D3 doses between 100 IU kg−1 d−1 and 1600 IU kg−1 d−1, which also showed a close association in the circulating levels of vitamin D3 and 25-hydroxyvitamin D3.21 Thus, apparently, hydroxylation of vitamin D3 in the liver was rapid and immediately triggered, and thereby not a limiting step for obtaining the biologically active form, possibly mediated through stimulation of CYP3A11 activity.21 To the best of our knowledge, no work has yet identified the exact relationship between circulating levels of vitamin D3 and 25-hydroxyvitamin D3 after acute intake.
25-Hydroxyvitamin D3 can be further hydroxylated in the kidneys to become the steroid hormones 24,25-dihydroxyvitamin D3 or 1,25-dihydroxyvitamin D3. 1,25-Dihydroxyvitamin D3 receives considerable attention because of its role in regulating calcium homeostasis along with parathyroid hormone (PTH). While attracting less attention, 24,25-dihydroxyvitamin D3 has recently been in focus in relation to a proposed role in modulating of breast cancer tumors via an estrogen receptor dependent mechanism. Furthermore, 24,25-dihydroxyvitamin D3 has attracted attention for its possible role in bone fracture repair. In the present study we also analyzed serum concentrations of 24,25-dihydroxyvitamin D3. To the best of our knowledge, the present study is the first to study the acute post prandial association between serum levels of 25-hydroxyvitamin D3 and 24,25-dihydroxyvitamin D3, respectively. We found the serum concentration of 24,25-dihydroxyvitamin D3 to be relatively stable irrespective of vitamin D supplementation. The fact that an increase 25-hydroxyvitamin D3 was not accompanied by an increase in 24,25-dihydroxyvitamin D3 probably reflects that a steady-state had not been reached, probably because of a lag in activation of the 24-hydroxylase CYP24A1 enzyme triggering the hydroxylation in the kidneys. A human study that investigated the 24,25-dihydroxyvitamin D3 : 25-hydroxyvitamin D3 ratio when vitamin D was either administered as a single bolus or for 4 weeks also found that the ratio differed between single bolus and daily administration, supporting a lag in steady-state conditions. Another human study of 16 weeks reported that in humans where a circulating level of 25-hydroxyvitamin D3 were 16.6 ng mL$^{-1}$, serum concentrations of 24,25-dihydroxyvitamin D3 were in average approx. 1.3 ng mL$^{-1}$. When the subjects then were supplemented with vitamin D (2400 IU) per day for 16 weeks, associations between serum levels of 25-hydroxyvitamin D3 and serum levels of 24,25-dihydroxyvitamin D3, respectively, strengthened. While 25-hydroxyvitamin D3 increased up to a factor 2, serum levels of 24,25-dihydroxyvitamin D3 increased by a factor 4.25 which also corroborates a lag in establishment of steady state and that ratio is dependent on the dose of vitamin D3 administered.

Recently, an alternative pathway for vitamin D activation has been identified, namely 20-hydroxylation of vitamin D by CYP11A1. Thus, further studies investigating relationships between vitamin D3, and 20-hydroxyvitamin D3 could be of interest. Furthermore, future studies including analysis of the epimer of 25-hydroxyvitamin D3 may also provide further understanding of the full vitamin D metabolism.

**Conclusions**

In conclusion, using an in vivo rat model, the study demonstrated that complexation with milk proteins is an efficient strategy to enhance bio-accessibility of vitamin D3. Both complexation with whey protein isolate and caseinate, respectively, enhanced bio-accessibility of vitamin D3, but complexation with whey protein isolate was found to be more potent than complexation with caseinate. Analysis of serum concentration of vitamin D3, 25-hydroxyvitamin D3 as well as 24,25-dihydroxyvitamin D3 revealed immediate hydroxylation to 25-hydroxyvitamin D3 in the liver whereas serum levels of 24,25-dihydroxyvitamin D3 indicated a lag in activation of hydroxylation in the kidneys.

**Conflicts of interest**

There are no conflicts to declare.
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References


Fig. 1. Post prandial change in serum concentration (A) vitamin D3, (B) 25-hydroxyvitamin D3 and (C) 24,25-dihydroxyvitamin D3 in rats after administration of 840 IU vitamin D3 (time 0) that were either complexed with whey protein isolate (WPI) (n = 24), caseinate (n = 24) or non-complexed (Vit D3) (n = 23). For negative controls (Control) (n = 6), vitamin D3 < LOQ. Letters “a”, “b”, “c” and “d” indicate significant differences.
Fig. 2. Area under curve (AUC) for (A) vitamin D₃, (B) 25-hydroxyvitamin D₃ and (C) 24,25-dihydroxyvitamin D₃ in rats after administration of 840 IU vitamin D₃ (time 0) that were either complexed with whey protein isolate (WPI) (n = 24), caseinate (n = 24) or non-complexed (Vit D₃) (n = 23).