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Original Research

Bioavailability of menaquinone-7 in milk formulation. Comparison of different solubilization techniques

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Abstract

Menaquinone-7 (MK-7) is a type of vitamin K of fermentative origin that plays a key role in cardiovascular and bone health as a cofactor of gamma-glutamyl carboxylase enabling the activation of several vitamin K-dependent proteins, particularly at the extrahepatic level.

Despite its proven biological efficacy and outstanding bioavailability over other dietary menaquinones, its presence in food is marginal, especially in the Western diet.

Dairy products are a major dietary source of menaquinones of minor biological relevance for human health and constitute optimal carriers due to their lipid content.

Dairy fortification with MK-7 represents an attractive formulative strategy, also for the high calcium and vitamin D content that may act synergistically in promoting vitamin K-dependent functions.

However, limited solubility of MK-7 in aqueous solution may limit its use and affect its bioavailability *in vivo*.

The present study compares the bioavailability of MK-7 in enriched 1%-fat milk either as a direct powder solution or a pre-emulsified dispersion. Bioavailability data show that formulation strategies strongly affect the bioavailability of MK-7 and that the results are greatly improved when it is prepared as an oil–water emulsion.

Keywords: bone health, dairy products, vitamin K

Introduction

Vitamin K constitutes a family of compounds with a common chemical structure, 2-methyl-1,4-naphthoquinone (**Fig. 1**).
Despite their different molecular structures, the molecules of this family share a common function as specific cofactors in the formation of γ-carboxyglutamyl (Gla) from specific glutamate residues in vitamin K-dependent proteins (VKDP) [1]. Today, 17 VKDP are known and their functions range from involvement in blood coagulation to playing a role in bone and cardiovascular health, and lately there has been a growing interest in an even wider array of functions for the molecules involving anti-inflammatory and cell cycle regulatory effects [2-5].

Approximately 60% of the dietary intake of vitamin K is represented by vitamin K1 (phylloquinone) [6] of plant origin that is abundant in green leafy vegetables [7,8]. The second main component of the vitamin K family is vitamin K2 (menaquinone), which is primarily of bacterial origin. Menaquinones can be produced either by the microflora of the digestive tract or ingested in the diet, accounting for 25% of the total vitamin K intake. Among the forms of vitamin K2, the most bioavailable form is menaquinone-7 (MK-7), found at the highest concentrations in the Japanese traditional food natto, a soybean product fermented using *Bacillus subtilis natto.* Despite its limited presence in other foods, MK-7 has shown unique characteristics in terms of bioavailability and biological effects that are far superior to other components of the vitamin K family that have attracted remarkable interest in this molecule over the last decade [9,10]. In fact, MK-7 administered in the form of natto in equimolar amounts, compared to phylloquinone administered in the form of spinach, shows a more than 10-fold peak height difference and a half-life of 56 hours compared to 7.5 hours for phylloquinone [11], and this is also higher than

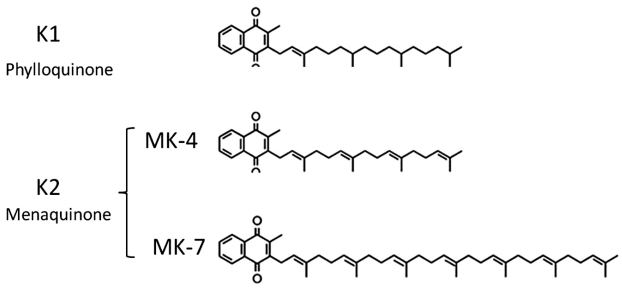

Figure 1 Vitamin K structures

what is observed for MK-4, the other menaquinone of major nutritional interest [12]. Moreover, while plasma K1 and MK-7 levels remarkably increase between 2–4 hours after a single intake, K1 levels return to basal values within 24 hours [11] and in the organism this is mainly taken up and recycled at the hepatic level where it acts as an essential cofactor of coagulative systems. On the contrary, vitamin MK-7 plasma levels remain elevated for days, enabling a consistent build-up following daily supplementation. Vitamin MK-7 in the organism is mainly present in plasma lipoproteins [13] and it therefore acts at the extrahepatic level in support of critical pathways involved in bone and cardiovascular health such as osteocalcin deposition and matrix Gla protein activation.

In contrast to other vitamins, vitamin K deficiency does not produce acute effects and is difficult to diagnose rapidly because a limited amount of this molecule is required to support coagulative function. Moreover, vitamin K is recycled through an enzymatic redox process defined as the vitamin K cycle [1]. However, impairments of extrahepatic vitamin K-dependent functions, mainly supported by menaquinones, are more difficult to detect and may coincide with the development of age-related diseases such as osteoporosis and atherosclerosis. In fact, epidemiological data highlight that inactive undercarboxylated vitamin K-dependent proteins, requiring vitamin K for activation, significantly increase in the general population

after 40 years of age [14]. Moreover, at the paediatric stage, a deficit in carboxylation was also observed in the critical phases of skeletal system development [15]. Taken together, all these data, together with the very low presence of menaquinones in the Western diet, highlight the importance of oral supplementation with this molecule for optimal health both in children and in adults.

Due to the lipophilic nature, lipid-rich food matrices have been identified as being potential carriers for MK-7. Menaquinone-rich foods have been developed using olive oil [16] or dairy products [17]. MK-7-enriched dairy products represent a very interesting formulation for bone health, being also a natural source of calcium. The recommended dosage is up to 180 mcg/day, and at these concentrations, MK-7 is relatively soluble, even in a low-fat matrix like 1%-fat milk; nonetheless, preparation of an oil–water emulsion may significantly improve the homogeneity of the preparation, its stability and, most importantly, bioavailability after intake.

The aim of the present study was to compare plasma levels and absorption kinetics over 8 hours after a single bolus intake of 1 mg of vitamin K-7 in 1%-fat milk directly dissolved or prepared as an oil–water emulsion.

Materials and methods

Preparation of MK-7-enriched milk

One litre of MK-7-enriched milk (5 mg/litre) was prepared using pasteurized fresh milk (1% fat) and MK-7 powder, 1000 ppm natural product from fermentation of Bacillus subtilis natto (Gnosis, Desio, Italy). MK-7 powder was either directly dissolved in the milk by preparing a concentrated solution of 200 ml dispersed with a food homogenizer (5 times/30 sec at 300 watt), which was subsequently diluted to the required concentration and further homogenized. Alternatively, an oil–water emulsion was prepared. For this purpose, MK-7 powder was dissolved in vegetable oil, 1 mg/g, and dissolved by stirring the suspension at 40°C for 10 min. The emulsion was prepared by slowly adding 5 g of vitamin-enriched oil to 1.25 g Arabic gum while vigorously mixing in a mortar to obtain a homogeneous nucleus. Subsequently, 2.5 g of water was rapidly added while vigorously mixing in the mortar to form the homogenous emulsion.

Study design

This study was conducted on eight (4M/4F) healthy subjects aged 25–47, with a mean BMI of 23 kg/m^2, not currently taking any medications or dietary supplements. Moreover, volunteers were recommended to undertake a vitamin K-low diet for one week before study entry and throughout the experiment. The protocol was approved by the university ethical committee and informed consent from all volunteers was provided in accordance with the Declaration of Helsinki. Volunteers were randomized into two groups (n=4) and tested with both formulations in two separate experimental sessions according to a crossover design with a washout phase of ten days between them. Bioavailability was evaluated after the intake of 200 ml of milk (1% fat) while fasting, containing 5 g MK-7 1000 ppm/litre either as a powder or as an oil-in-water emulsion. Blood withdrawal was carried out according to the known pharmacokinetics of menaquinones at baseline in fasting conditions, and subsequently, after 2, 4 and 8 hours. Enriched milk intake was associated with a standardized breakfast containing 15 g of fat. On the experimental day, all subjects received the same meals, and the nutrients and energy levels were adjusted according to health and nutrition guidelines. At each timepoint, blood was withdrawn into heparinated vacutainers, the plasma was separated by centrifugation at 1500×g for 10 min, aliquoted and cryopreserved at -80°C for analysis in a single batch for each subject in order to

reduce between-day experimental variability. The area under the curve (AUC) of serum MK-7 levels was taken as a measure for absorption and bioavailability.

The AUC was calculated from the following equation: $\mathrm{AUC} = \sum(x_n - x_{n-1})(y_n + y_{n-1})/2$ where x_n and x_{n-1} are two adjacent values on the horizontal axis, and y_n and y_{n-1} are the corresponding values on the vertical axis.

HPLC method

Serum MK-7 was measured using HPLC with fluorescence detection after on-line, post-column reduction, which converts quinone forms of vitamin K into their fluorescent quinol forms, as described previously [16] with slight modifications. In particular, thawed plasma samples were extracted in ethanol 1:6, centrifuged at 20900×g at 4°C and 50 µl of supernatant were injected onto a HPLC (YL Instrument 9300) system associated with a fluorescence detector (Nanospace SI-2; Shiseido) and equipped with an analytical column (2.6 µm C18 100A, 100×4.6 mm; Phenomenex Kinetex) and a post-chromatographic reducing column (CQ-R 2.0×20 mm; Shiseido) since vitamin K is fluorimetrically detected in its reduced state. The mobile phase used was ethanol:water (97:3, v/v) and the flow rate was adjusted to 0.7 ml/min. The optimized detection wavelengths were 335 nm (excitation) and 430 nm (emission). External standards were used to quantify levels of plasma MK-7 and results were expressed as ng/ml.

Conclusions

Serum vitamin K levels in healthy subjects were compared after a single oral administration (1 mg) of MK-7 diluted in 1%-fat milk either as a powder or as an oil–water emulsion. The results highlighted a significant role for the methodology used with respect to vitamin bioavailability. In particular, while baseline serum levels of MK-7 were not statistically different on the experimental days (day 1=0.9±0.3; day 2=1.1±0.5 ng/ml; p=0.3), single intake of MK-7 with both formulations increased serum MK-7 levels in all subjects, which reached maximum levels at 2 hours with the emulsified formula (Cmax=23.8±5.2) and 4 hours after administration of the powder solution (Cmax=8.3±2.0). Data reported in **Fig. 2a** show the mean and SEM of MK-7 plasma levels at study entry and all subsequent experimental points for both enriched milk formulations. **Fig. 2b** shows the percentage variation in MK-7 plasma levels, indicating a highly significant difference already after 2 hours of ingestion and a significant difference at 8 hours.

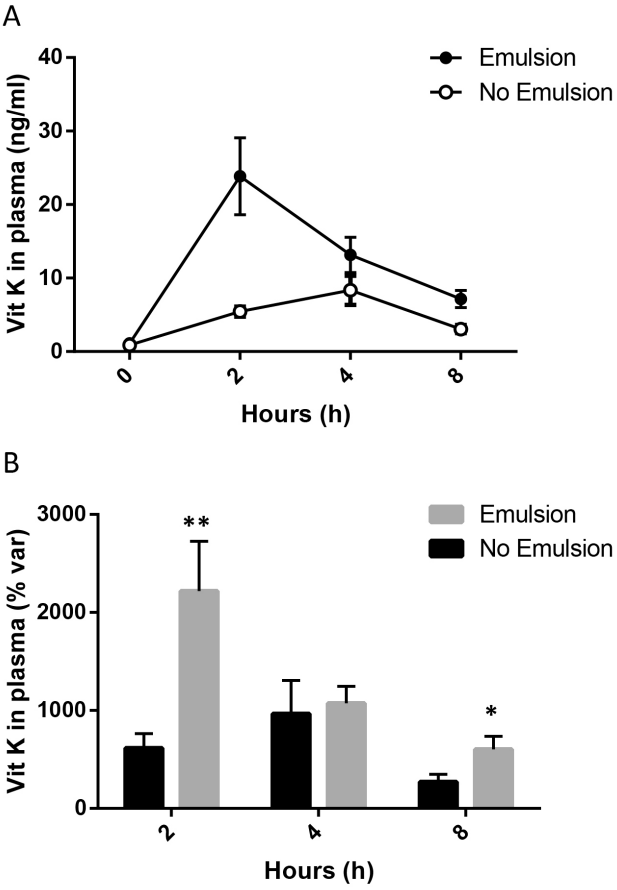

Figure 2 Pharmacokinetics of MK-7 following consumption of 1 mg MK-7 either dissolved in 1%-fat milk as a powder or as an oil–water emulsion. (A) MK-7 plasma levels expressed as ng/ml at baseline, 2, 4 and 8 hours. Data are represented as the mean±SEM (n=8). (B) Percentage variation of plasma levels compared to baseline values. Data are represented as the mean+SEM (n=8). The significance of variations was calculated using a paired Student's t test comparing the percentage variation at each timepoint. *$p<0.05$; **$p<0.01$

The comparative analysis of plasma levels showed significantly higher levels at both 2 and 8 hours after the oral intake of milk formula using the emulsified vitamin formula. Accordingly, AUC analysis showed a highly significant increase in the bioavailability of MK-7 over the 8 hours of observation using emulsified formula compared to a standard powder suspension (**Fig. 3**).

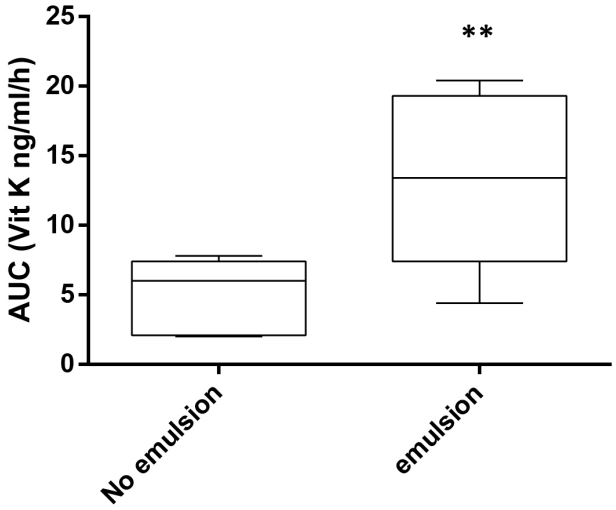

Figure 3 Distribution of AUC values expressed as ng/ml plasma/hr over 8 hours of observation. Data are shown as a box plot where values in the box are representative of 50% of the data and the lower and upper segments are representative of the lower and upper quartiles. The significance of variations was calculated using a paired Student's t test. ***p<0.01*

Dairy products represent a natural source of menaquinones, in particular, those that are fat-rich such as cheese. Therefore, they represent ideal carriers for these vitamins. Nonetheless, menaquinones are primarily present in the forms of MK-9, MK-10 and MK-11, probably derived from the microbiota of the highly specialized ruminant digestive system, which are not associated with strong evidence of a biological function in humans. On the contrary, MK-7, considered the most bioavailable and bioactive form of menaquinone, is detectable in trace amounts [18]. Dairy product supplementation with MK-7 represents a meaningful means of fortification. In fact, recent studies have shown that the consumption of dairy products fortified with individual menaquinone forms results in these being absorbed, and they may have greater bioactivity than menaquinones delivered in supplement form [19,20], also due to the natural synergistic presence of calcium and vitamin D3. However, low-fat dairy products that increasingly appeal to health-conscious consumers might decrease solubility of the vitamin. In this respect, the present data suggest that for an optimal dispersion of menaquinone in a 1%-fat milk formulation, accurate methodologies of emulsification should be used in order to guarantee optimal bioavailability of the vitamin to the formula consumer. Moreover, such techniques are also likely to guarantee an improved homogeneity and stability of the enriched formula.

Conflict of Interest

The authors have no conflict of interest to disclose.

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