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BIOAVAILABILITY OF PHYLLOQUINONE AND MENAQUINONES AFTER ORAL AND COLORECTAL ADMINISTRATION IN VITAMIN K-DEFICIENT RATS

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Abstract—Rats were made vitamin K-deficient by feeding them a diet devoid of vitamin K and by rigorously preventing coprophagy. After one week, circulating prothrombin concentrations were between 5 and 10% of initial values, and various amounts of phylloquinone, menaquinone-4, and menaquinone-9 were given in a single dose either subcutaneously, orally, or colorectally. The relative 'vitamin K activities' of these compounds were assessed by comparing their ability to support prothrombin synthesis after subcutaneous injection. Intestinal and colonic absorption were deduced from the difference between subcutaneous and either oral or colorectal administration of the vitamers. It is concluded that the colonic absorption of all three forms of vitamin K is extremely poor, suggesting that physiological menaquinones in the colon do not contribute substantially to vitamin K status in rats. Furthermore, the stimulation of prothrombin synthesis by menaquinone-9 lasted much longer than that by the two other K-vitamers, resulting in a substantially higher 'vitamin K activity' of menaquinone-9.

Key words: vitamin K; phylloquinone; menaquinone; γ-carboxyglutamate; blood coagulation; intestinal absorption

Vitamin K is a group name for a series of related compounds that share the ability to serve as a cofactor for the microsomal enzyme γ-glutamylcarboxylase. This enzyme is involved in the posttranslational conversion of peptide-bound glutamate residues into γ-carboxyglutamate (Gla), which occurs in a number of blood coagulation factors and bone proteins [1–3]. Natural forms of vitamin K are K_1† and menaquinones (K_2). They share a naphthoquinone ring structure, but differ in their aliphatic side chain. In phylloquinone this side chain contains 4 isoprenoid residues, one of which is unsaturated; in menaquinone the side chain consists of a variable number of isoprenoid residues, all of which are unsaturated. The common nomenclature for menaquinones is MK-*n*, where *n* represents the number of isoprenoids. The most abundant menaquinones in human food are MK-4, MK-7, MK-8, and MK-9 [4–6]. Phylloquinone becomes available exclusively via the diet (green vegetables and dairy produce), whereas menaquinones occur in food (dairy produce, meat, and fermented products) as well as in the colon, where they are produced by the intestinal flora. The extent to which the various nutritional and colonic menaquinones contribute to the biosynthesis of the various Gla-proteins is a matter of debate at this time [7–11].

On the basis of a diet including 50% (w/w) white rice to reduce intestinal menaquinone production [12] and using anal cups to prevent coprophagy [13], we have developed a protocol according to which rats can be made vitamin K-deficient in one week. Previously we used a similar technique to compare the absorption of phylloquinone and MK-4 after oral and colorectal administration [14]. In that study the bioavailability of both vitamers was assessed from the shortening of the clotting time in a standard overall blood coagulation test. A problem in this type of study is the high lipophilicity of vitamin K, which increases even with increasing side chain length. Detergents used to solubilize the K-vitamers may substantially influence their intestinal absorption. For that reason we previously used detergent-free 30% albumin in saline to bring phylloquinone and MK-4 into a water environment [14]. Higher menaquinones cannot be solubilized in this way, however.

In the present study we have used plasma prothrombin concentration as a sensitive blood coagulation marker, and have solubilized three forms of vitamin K (K_1, MK-4, MK-9) using the detergent HCO-60, which has been shown not to interfere with the colonic absorption of compounds of widely differing lipophilicity [15]. If not specified further, the term 'vitamin K' will be used hereafter to designate all three K-vitamers. 'Vitamin K activity' is defined as the ability of a compound to stimulate prothrombin synthesis after its subcutaneous injection in vitamin K-deficient rats. Intestinal and colonic absorption were deduced from the difference between subcutaneous and either oral or colorectal administration of the vitamers. In this model system we have measured (a) whether the data obtained for the bioavailability of K_1 and MK-4 were comparable to those observed when the albumin-solubilized vitamers were used, and (b) the importance of the number of isoprenoid residues in menaquinones for their vitamin K activity and intestinal absorption.

MATERIALS AND METHODS

Chemicals

K_1 and MK-4 were obtained from Sigma (St. Louis, MO), MK-9 was a kind gift from Hoffmann-La Roche

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† Abbreviations: K_1, phylloquinone; MK-4, menaquinone-4; MK-9, menaquinone-9; HCO-60, polyoxyethylene hydrogenated castor oil derivatives.

(Basel, Switzerland). The non-ionic surfactant HCO-60 (polyoxyethylene hydrogenated castor oil derivatives) was purchased from Nikko Chemicals (Tokyo, Japan).

Animals and diets

All studies were performed in male rats of the Lewis strain, which were 12 weeks old when entering the experiment. The animals were housed in individual, metabolic cages with a 12-hr light-dark cycle and controlled temperature (20 ± 2°C) and humidity (50 ± 10%). They had free access to a radiated (0.9 Mrad) vitamin K-deficient diet (Hope Farms, Woerden, The Netherlands), mixed with cooked and dried white rice in a 1:1 (w/w) ratio [12]. The contents of the commercial vitamin K-deficient food has been detailed earlier [14], and it was verified not to contain detectable amounts of phylloquinone or menaquinone. All food was powdered with a blender and mixed in a professional food processor before use. Starting at day 5 of the vitamin K-deficient regimen until the end of the experiment, the rats were equipped with anal cups to exclude coprophagy [13]. At day 7 prothrombin concentrations were observed to be between 5 and 10% of initial values, and vitamin K was administered as described below. Blood (0.5 mL) was taken by venipuncture of the tail vein in 0.05 mL of 0.14 M trisodium citrate before the experiment (=initial value), after 7 days of vitamin K deficiency, and at 6, 10, 24, and 48 hr after vitamin K administration. These time points were chosen on the basis of pilot experiments that showed that the increase of plasma prothrombin continued from 6 hr (low dose of phylloquinone) up to 24 hr (high dose of phylloquinone) following vitamin K administration. The protocol for this experiment was approved by the Experimental Animal Ethics Committee of the University of Limburg.

Vitamin K administration

Stock solutions were prepared containing 3.5 g/L HCO-60 and 1 g/L of either K_1, MK-4, or MK-9 in buffer A (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5). The K-vitamers were dissolved by sonication during five pulses of 5 sec with an amplitude of 6 μm. Solutions thus obtained were clear, homogeneous, and stable. Shortly before vitamin K administration the stock solutions were diluted five times with buffer A, leading to a final HCO-60 concentration of 0.7 g/L. Further dilutions (as required) were made with 0.7 g/L HCO-60 in buffer A. Each dilution step was followed by sonication as described above. In all cases vitamin K was administered to the rats in 0.5 mL samples, with amounts ranging between 0 and 50 nmol (as indicated). Subcutaneous administration was performed in the neck of the animals. Oral doses were given via a syringe equipped with a plastic cannula, which was protruded into the esophagus. For colorectal administration, the animals were anaesthetized with carbon dioxide and a cannula introduced via the rectum and moved up to the colon loop, where the vitamin was applied.

Blood coagulation assays

One-stage prothrombin concentration was determined with a coagulometer (KC-4, Amelung, Germany), using a commercial thromboplastin preparation (Thromborel S®) and clotting factor II-deficient plasma (both from Behringwerke AG, Marburg, Germany). Prothrombin concentrations were calculated with the aid of a reference curve from pooled normal rat plasma. The initial prothrombin level was calculated for each rat, and all later data are expressed as a percentage of the respective initial values. Each point in Figs. 1–3 represents the means ± SEM for five rats.

RESULTS

Circulating prothrombin concentrations were measured after the administration of 2–5 different dosages of either K_1, MK-4, or MK-9, and in all cases three different routes of administration were compared: subcutaneous, oral, and colorectal. The dose-response curves are given in Figs. 1–3, and it is clear that after colorectal administration the response of all three vitamers was extremely poor. Both after oral and subcutaneous administration, on the other hand, the response to K_1 and MK-4 was rapid, reaching a maximum after 6–10 hr. The response to MK-9 was slightly slower, but the curves for oral and subcutaneous administration were of a similar shape, suggesting that intestinal absorption is

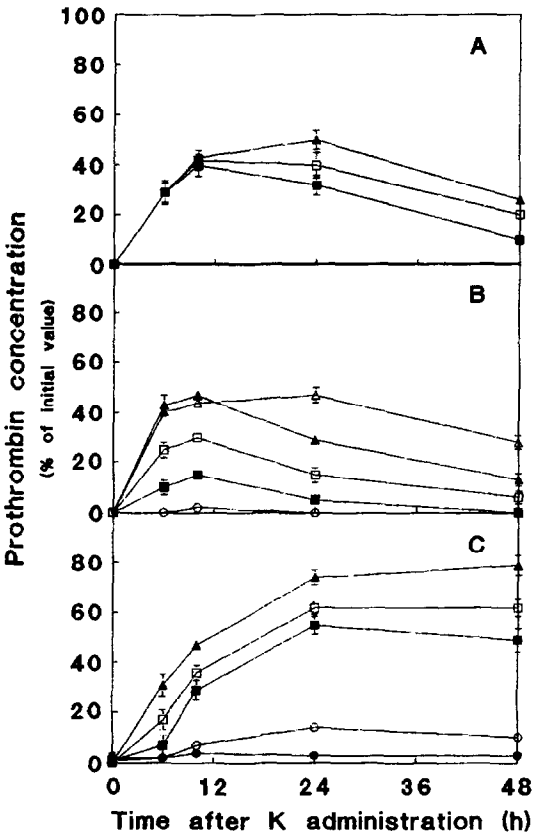

Fig. 1. Effect of subcutaneous administration of vitamin K on circulating prothrombin concentrations in vitamin K-deficient rats. Vitamin K was administered as a detergent-solubilized preparation in a single dose. Each point represents the means of five different rats ± SEM. If no error bar is shown, the SEM falls within the symbol. Blank values (solvent without vitamin K) are subtracted (A) phylloquinone (K_1); (B) menaquinone-4 (MK-4); (C) menaquinone-9 (MK-9). The following amounts were given: 0.2 nmol (●), 1 nmol (○), 5 nmol (■), 10 nmol (□), 25 nmol (▲), and 50 nmol (△).

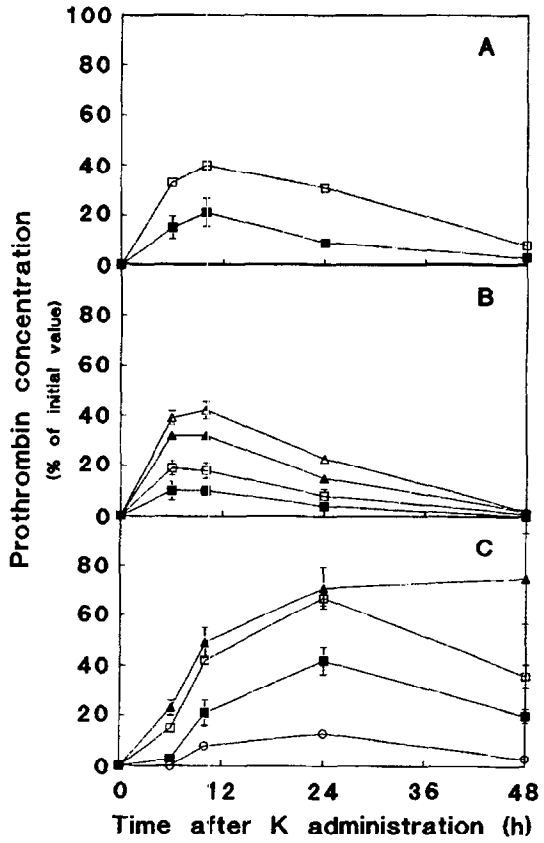

Fig. 2. Effect of oral administration of vitamin K on circulating prothrombin concentrations in vitamin K-deficient rats. Symbols are as in Fig. 1.

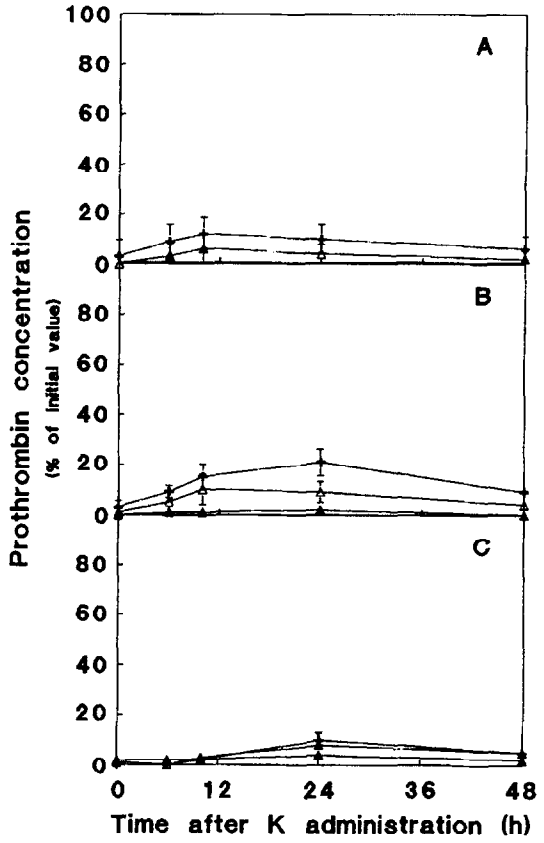

Fig. 3. Effect of colorectal administration of vitamin K on circulating prothrombin concentrations in vitamin K-deficient rats. Symbols are as in Fig. 1. The greatest amount of vitamin K applied was 100 nmol (+).

not the rate-limiting step in the transport of the K-vitamers to the liver. Remarkably, the duration of the response to MK-9 was much longer than expected on the basis of previous experiments with K_1 and MK-4. Even after 48 hours, prothrombin concentrations were still high in most cases; since the animals were killed at this time point, all calculations given below will result in an underestimation of the true vitamin K activity of MK-9.

The vitamin K activity of the various K-vitamers were calculated from the dose-response curves as the Area Under Curve (*AUC*), and is expressed per nmol vitamin K given to the rats. In Table 1 the results are given for 5 and 10 nmol of the three K-vitamers after subcutaneous and oral administration, and for 50 and 100 nmol of vitamin K after colorectal administration. If expressed per nmol of vitamin K, the highest biological effect was obtained at the lowest doses. For all vitamers the efficacy of subcutaneous injection was approximately twofold higher than the values obtained after a similar oral dose, indicating that roughly half of the vitamin had adsorbed in the intestines. This value is an upper estimate, based on the assumption that 100% of the subcutaneously administered vitamin K will reach the circulation. From Table 1 it is also clear that K_1 was 2–3 times as effective as MK-4, but that its efficacy was 40–60% that of MK-9. The bioavailability of colorectally applied MK-9, a typical product of the intestinal flora, was less than 0.2% of that following oral administration, suggesting that the direct colonic absorption of this product is negligible.

DISCUSSION

In this paper we have demonstrated that the colonic absorption of three abundant forms of vitamin K is extremely poor. If compared with earlier reports [14], the technique for application of the solubilized vitamers in the colon has been improved by executing all manipulations while the animals were under carbon dioxide anaesthesia. This resulted in a complete release of muscle tension, so that injury of the colonic epithelium (with occasional bleeding) was prevented. A second improvement was the rigorous prevention of coprophagy by equipping the rats with anal cups throughout the experiment. The fact that the bioavailability of colonic vitamin K decreased from the previously reported 30–50% [14]) to less than 0.2–2% of the values obtained after oral ingestion of the vitamin shows that epithelial damage and unexpected coprophagy are factors that may lead to substantial overestimation of the physiological importance of this pathway. If adequate precautions are taken, the absorption of the various K-vitamers is negligible. In nontreated rats the total amount of colonic menaquinones was reported to be 3–6 μg, most of which are MK-7, MK-8, and MK-9 [11, 16]. It should be realized,

Table 1. Biological effect of various forms of vitamin K as tested in vitamin K-deficient rats

Route of administration	Amount (nmol)	Apparent vitamin K-activity (AUC, mm²/nmol)		
		K_1	MK-4	MK-9
Subcutaneous	5	334 ± 39	80 ± 9	534 ± 59
Oral	5	134 ± 15	64 ± 6	352 ± 41
Colorectal	50	5 ± 1	10 ± 2	7 ± 2
Subcutaneous	10	212 ± 26	104 ± 14	329 ± 31
Oral	10	163 ± 17	56 ± 8	298 ± 26
Colorectal	100	6 ± 1.5	10 ± 3	4 ± 1

The total effect of each vitamer was calculated from the appearance and disappearance of plasma prothrombin for 48 hr following a single dose of purified detergent-solubilized vitamin K. AUC is the area under the dose-response curve, the data are given ± SEM. For further details see text.

however, that most of these menaquinones are not available for absorption because they are tightly bound to insoluble material (bacteria, membrane remnants, etc. [6]). In our colonic absorption experiment up to 100 nmol (80 μg) was used, but even at this extremely high dose of well-solubilized menaquinone, the biosynthesis of prothrombin was hardly affected. It must be expected, then, that the direct absorption of menaquinones produced by bacteria in the gut hardly contributes to the vitamin K status in the rat.

The three routes through which vitamin K may enter the human circulation are (a) absorption in the small intestine (from the diet), (b) absorption in the colon (from the intestinal flora), or (c) by subcutaneous injection. In our experimental design we have tried to mimic these three routes as closely as possible. The response to intravenous injection may be quicker than that to any of the three routes mentioned above [17], but in humans intravenous injection is not recommended because of the risk of anaphylactic shock. Hence, we have excluded this route from our experiments. Furthermore, it was decided to follow the circulating prothrombin concentrations prospectively in each individual rat, so that the total effect of a single dose of vitamin K could be measured. Obviously this choice implies that hepatic vitamin K levels cannot be determined simultaneously. In a recent paper, however, Akiyama *et al.* showed that at 1 and 3 hr after oral administration of various menaquinones their hepatic concentration was increased [18]. This is consistent with our data showing increasing plasma prothrombin concentrations during the first hours following vitamin K administration.

We did not observe differences in response time after either oral or subcutaneous administration of the K-vitamers. This suggests that the intestinal absorption after oral ingestion is rapid. In all cases the effect of MK-9 was slower than that of the other K-vitamers, but its biological half-life was substantially longer. From the curves shown in Fig. 1 it seems that, if measured over longer periods (e.g., 1 or 2 weeks) the total activity of MK-9 will be at least two-fold higher than that suggested by the data given in Table 1. From our data it appears that the relative molar activity of the three vitamers is: MK-9 > phylloquinone > MK-4. Similar data were reported by Matschiner and Taggart, who administered the vitamers by intracardial injection in partly vitamin K-deficient rats [19]. On the basis of these data it is to be expected that the relative importance of long-chain menaquinones in food is larger than generally assumed. Our observation that both long- and short-chain menaquinones are readily absorbed after oral ingestion is consistent with data recently published by Conly and Stein, who showed that, if taken orally, long-chain menaquinones are absorbed and counteract the effect of vitamin K antagonists [7, 20]. Our data do not support their conclusion that menaquinones synthesized by the intestinal flora are directly absorbed in the bowel. On the other hand, it is well known that—at least in the human—bacteria also inhabit to some extent the terminal ileum, and the possibility exists that some bacterially synthesized menaquinones may be absorbed from this region by a bile-salt mediated pathway [6].

The data obtained after subcutaneous and oral administration of detergent-solubilized K_1 and MK-4 were comparable to those obtained with the albumin-solubilized vitamers [14]. Since solubilization in HCO-60 was quick, practical, and even applicable for very lipophilic compounds, this detergent is the solvent of choice for experiments such as those described in this paper. From Table 1 it seems that the gastrointestinal absorption of vitamin K is 50–75% that after subcutaneous administration, irrespective of the side chain length. This is remarkable, because there is a substantial difference in lipophilicity between long- and short-chain menaquinones. A clear difference between the short-chain and the long-chain vitamers was that the response to a single dose of MK-9 lasted significantly longer than that to a dose of K_1 or MK-4. This is consistent with the observation that the hepatic turnover rate of phylloquinone is three times higher than that of MK-9 [21]. As a consequence, the apparent vitamin K activity of MK-9—notably after oral administration—was much higher than that of MK-4. It is to be expected that this effect will be even more pronounced if the response-curve is measured over a longer time period.

Recently Akiyama *et al.* used the warfarin-treated rat as a model to study the effectiveness of various menaquinones [18]. A major discrepancy between their result and ours is that they found MK-9 to be substantially less active than MK-4. An explanation for this discrepancy may be found by comparing the different model systems used. Our results were obtained in vitamin K-deficient animals, which means that the enzymes of the vitamin K cycle were operational. Under these

conditions vitamin K is substantially recycled via the dithiol-dependent enzyme vitamin K-epoxide reductase [22]. Akiyama *et al.*, however, used warfarin-treated animals for their experiments, which means that in their system the epoxide reductase was blocked, and vitamin K could only be converted into the active metabolite via an NADH-dependent enzyme, which is unable to complete the recycling of the vitamin [23]. The author's conclusion, namely, that MK-4 is more active than MK-9, is only valid for the reduction of vitamin K via the NADH-dependent pathway, but not for the efficiency with which the vitamin is recycled via the natural dithiol-dependent system. We want to put forward the hypothesis that the NADH-dependent and the dithiol-dependent reductases after substantially with respect to their ability to reduce MK-4 and MK-9. This point is subject to further *in vitro* research.

Since nutritional menaquinones are generally dissolved in the lipid fraction of products such as dairy produce and meat [4, 5], these K-vitamers are likely to be incorporated into well-absorbable micelles and chylomicrons through the action of bile salts. The efficacy with which phylloquinone is extracted from the thylakoid membranes of green vegetables in the gastrointestinal tract, on the other hand, may be variable and far more incomplete. It is our opinion, therefore, that the physiological importance of nutritional menaquinones is presently underestimated. To be able to assess the relative contribution of both phylloquinone and menaquinones to human vitamin K status, it is important that concentrations of the various forms of vitamin K in food, as well as their respective bioavailability, be determined in human volunteers.

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