Menaquinone-4 Accumulation in Various Tissues after an Oral Administration of Phylloquinone in Wistar Rats

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(Received October 3, 1996)

Summary The distributions of phylloquinone (PK) and menaquinone-4 (MK-4) in various tissues were assessed after the oral administration of phylloquinone. Wistar rats were fed a vitamin-K-deficient diet for nine days, fasted for 24 h and then given phylloquinone orally at 4 mg/kg body weight. Rats were sacrificed 0, 6, 12 and 24 h after the administration, and an analysis was made of the vitamin K analogues in the plasma, liver, brain, testis, kidney and spleen. The phylloquinone concentration in plasma and the tissues reached a peak 6 h after the oral administration of phylloquinone. By contrast, the concentration of MK-4 peaked in the liver, plasma, kidney and spleen at 12 h, and in brain and testis at 24 h. This data suggests that the ingested phylloquinone was probably converted into MK-4 within the tissues themselves, rather than via hepatic metabolism. The evidence for this is that, after phylloquinone administration, (i) in each of the tissues, the MK-4 concentration increased much more slowly than that of phylloquinone, and (ii) the MK-4 concentration in the plasma and liver reached only much lower levels than those seen in other tissues.

Key Words vitamin K, phylloquinone (PK), menaquinone-4 (MK-4), metabolic conversion, rats, brain, testis, kidney, spleen, liver

Vitamin K is essential for the synthesis of clotting factors (prothrombin, factors VII, IX and X, etc.) in the liver of animals (1). Many compounds such as phylloquinone (PK, vitamin K<sub>1</sub>=VK<sub>1</sub>), menaquinones (MK-<i>n</i>, vitamin K<sub>2</sub>=VK<sub>2</sub>), and menadione (vitamin K<sub>3</sub>) and its derivatives exhibit vitamin K activity. Phylloquinone is the form of vitamin K isolated from green plants, and the MK-<i>n</i> series are synthesized by microorganisms. In Japan, phylloquinone and MK-4, which are naturally-occurring forms of vitamin K, have been used as therapeutic agents for

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vitamin-K-deficient syndromes, such as hypoprothrombinemia in newborn babies, and in antibiotic-treated patients. MK-4 is most often given by intravenous injection to children and adults, or as a syrup to babies. However, information on the physiological and pharmacological roles of vitamin K in animals is still limited because, until the 1980s, it was difficult to detect low levels of vitamin K in tissues.

It has been well known since the 1960s that both menadione and phylloquinone are converted to MK-4 in rats and chickens (2-5). On the basis of their results, these researchers hypothesized that orally ingested phylloquinone was probably converted to MK-4 via an enteral pathway; namely, via the enzymatic action of intestinal microorganisms. However, this hypothesis was not supported by our experiment using germfree and conventional mice (6). That experiment revealed that the conversion of phylloquinone to MK-4 in the liver occurred to a similar extent in germfree and conventional mice; in fact, if anything, the germfree mice converted it more efficiently than the conventional mice. In that experiment, the germfree condition was confirmed by both a microbiological culture test and by HPLC data which revealed the lack of fermentation products, such as MK analogues, in the contents of the large intestine (cecum and colon (6, 7)).

More recently, it has been suggested that dietary phylloquinone might be the source of the MK-4 seen in various tissues in rats (8, 9), though the mechanism of this conversion has not yet been established. Therefore, to get more detailed information on the kinetics of phylloquinone and MK-4 in various tissues, we attempted to follow the changes, with time, in the concentrations of phylloquinone and MK-4 in Wistar rats after the oral administration of phylloquinone.

**METHODS**

**Animals and diets.** Sixteen male Wistar rats (Wistar/Slc) which had been maintained under SPF conditions, 8 weeks old and weighing 195-215 g, were purchased from Nippon SLC (Hamamatsu, Japan). They were fed a commercial pellet diet (F-2, Funabashi Farms, Co., Ltd., Funabashi, Japan) under conventional conditions with wood shavings as bedding for three days while they became acclimated to our rearing conditions. Thereafter, the rats were kept in individual cages, with a stainless-steel wire floor, in a room with a temperature of 22-25°C and a 12 h light/dark cycle (lights on from 08:00 h). The rats were fed a casein protein, sucrose, vitamin K-free, oil-based, vitamin-K-deficient diet which was slightly modified from the previously published composition (10), and which contained a 1.33 μg phylloquinone/kg diet in this experiment by analysis. The composition of the vitamin K-free diet was as follows: 20 g of vitamin-free casein that had been extracted with petroleum ether for 12 h, 63 g of sucrose, 5 g of cellulose, 5 g of fat-soluble vitamin-free oil (10), 4.8 g of mineral mixture (11), 2.0 g of vitamin mixture (11) and 0.2 g of D,L-methionine. The composition of the vitamin mixture was as follows: 80,000 IU of vitamin A acetate, 5,000 IU of vitamin D₂, 300 mg of vitamin B₁, 150 mg of vitamin B₂, 120 mg of vitamin B₆·HCl, 1.25 mg of vitamin...
MK-4 Conversion from Phylloquinone

B12, 3,000 mg of ascorbic acid, 500 mg of vitamin E acetate, 5 mg of d-biotin, 50 mg of folic acid, 1,500 mg of calcium pantothenate, 250 mg of p-aminobenzoic acid, 500 mg of nicotinic acid, 5,000 mg of inositol, and 10,000 mg of choline chloride per 100 g of vitamin mixture; cellulose was added as a carrier to make up the 100 g.

The rats were given access to the diet from 19:00 each day to 10:00 the next day, with free access to drinking water. After nine days of feeding, the rats were fasted for 24 h, and then phylloquinone was administered orally at 4 mg/kg body weight. The solubilized phylloquinone in HCO-60 (hydrogenated polyoxy-ethylene castor oil) was a gift from Eisai Co., Ltd., Japan, and was dissolved in saline (0.5 ml/each administration) for oral administration. The rats were sacrificed by removing blood under diethyl ether anesthesia. Groups of four rats were sacrificed 0 (=the saline administered control group), 6, 12 and 24 h after oral administration. The liver, brain, testis, kidney and spleen were immediately removed, and stored at −20°C until analysis.

The experimental plan of this study was approved by the Animal Research-Animal Care Committee of the Faculty of Agriculture, Tohoku University. The entire experiment strictly followed the guidelines laid down by that committee, which, in their entirety, follow Japanese government legislation (enacted in 1980).

Analytical procedures. Phylloquinone and MK-n were analyzed essentially as described by Shino (12) and Hirauchi et al. (13), except that we omitted the thin-layer-chromatography step of the latter's original procedure because we found that this step seemed to cause a positive error. For the analysis of phylloquinone and MK-n in the liver, brain, testis, kidney and spleen, 1 g of wet tissue was homogenized in 2 ml of 66% 2-propanol solution in a Biotron tissue homogenizer (Type R8/11; Switzerland). Menaquinone-3 (MK-3) was used as the internal standard, 30 ng being added in 3 ml of n-hexane to each sample before homogenization in the case of liver (10 ng in 1 ml of n-hexane to each sample for all other tissues). Then 3 ml of n-hexane (5 ml of n-hexane for the non-hepatic tissues) was added and the whole mixed thoroughly for 5 min. For the analysis of phylloquinone and MK-n in plasma, 1 ml of plasma was homogenized in a solution of water:ethanol:n-hexane (1:4:6 ml), as described previously (13), with 10 ng of internal standard (i.e. MK-3 in n-hexane), and then the whole was mixed for 5 min.

After centrifugation for 5 min at 3,000 rpm, the hexane layer was removed and dried in a Centrifugal-Concentrator (VC-360; TAITEC Co. Ltd., Tokyo, Japan), and the residue was dissolved in 2 ml of n-hexane. A silica Sep-Pak cartridge (Waters Associates, Milford, MA, USA) was washed with 10 ml of n-hexane and 10 ml of an ethyl ether:n-hexane (4:96) solution. Then, the sample was loaded, washed with 10 ml of n-hexane and eluted with 5 ml of an ethyl ether:n-hexane (4:96) solution. The eluate, transferred into a brown glass tube, was evaporated to dryness in the Centrifugal-Concentrator, dissolved in 200 µl of ethanol and filtered through a Millipore filter (0.45 µm) for injection onto a HPLC apparatus. In the case of liver samples, further dilution (×20) was necessary to obtain adequate conditions for HPLC analysis. The phylloquinone and MK-n were quantitatively detected.
measured by injecting 10–30 μl of the remaining ethanol solution onto a reverse-phase Chemcosorb C_{18}-UH analytical HPLC column (15 cm × 4.6 mm i.d.; Chemco Scientific Co., Ltd., Osaka, Japan), and eluting with methanol:ethanol (1:1) saturated by H₂-gas bubbling for 2 h at a flow rate of 1.0 ml/min. The vitamins were detected fluorometrically by a fluorescence spectrophotometer (Hitachi, F 1000, excitation = 320 nm, emission = 430 nm) after post-column PtO₂ column reduction (12). The platinum oxide column was a gift from Dr. Mitsumasa Shino, Eisai Co. Ltd., Tsukuba, Japan. We compared the accuracy of the HPLC analysis obtained from the PtO₂ column system with one obtained using an electrochemical reduction system, and found no significant discrepancies in either the qualitative or quantitative analyses of vitamin K analogues as reported previously (14).

RESULTS

There were no apparent outward symptoms of vitamin K deficiency in the experimental rats fed a vitamin-K-deficient diet for nine days. The only vitamin K analogues detected in the plasma and in all the tissues of all the animals using this experimental system were phylloquinone and MK-4. Menaquinones with an isoprenoid unit number greater than 4 were not found as major peaks on the HPLC charts. Figure 1 shows the typical HPLC charts obtained from the vitamin K fraction of the kidney from experimental rats sacrificed before or 12 h after phylloquinone administration per os. These charts clearly show that there is not only an increase in phylloquinone, as expected, but also a considerable MK-4 increase after phylloquinone administration.

The changes in the concentrations of phylloquinone and MK-4 in various tissues after phylloquinone administration are shown in Figs. 2–7. The concentration of phylloquinone reached its peak in plasma and in all tissues some 6 h after administration. In contrast, the concentration of MK-4 peaked in the liver, plasma, kidney and spleen at 12 h, and in the brain and testis at 24 h. It is noteworthy that the concentrations of MK-4 in the liver and plasma were strikingly low compared to other tissues, and that the phylloquinone concentrations decreased more rapidly than the MK-4 concentrations in the plasma and all other tissues; the former returning to a level close to the background value within 24 h.

Among the various tissues tested, there was a marked difference in the distributions of both phylloquinone and MK-4 after the oral administration of phylloquinone. In fact, phylloquinone was incorporated efficiently into the liver and spleen, and there was also a high level in the plasma. However, it was less well incorporated into the brain, testis and kidney, though MK-4 concentrations increased greatly in these tissues. It is noteworthy that, in rats fed the vitamin-K-deficient diet for nine days, MK-4 concentrations before the administration of phylloquinone (time 0) were at relatively high levels in the brain and testis, intermediate in the kidney and spleen, and very low (not detectable) in the liver and...
Fig. 1. HPLC chromatogram of vitamin K analogues in the kidneys of mildly vitamin-K-deficient (0 h) rats, and of PK-treated rats (12 h after administration). MK-3 was used as the internal standard (IS). Unmarked peaks are not vitamin K analogue.

Fig. 2. Concentrations of PK and MK-4 in rat liver after oral administration of PK.
Fig. 3. Concentrations of PK and MK-4 in rat plasma after oral administration of PK.

Fig. 4. Concentrations of PK and MK-4 in rat brain after oral administration of PK.

Fig. 5. Concentrations of PK and MK-4 in rat testis after oral administration of PK.

The subtraction of the background value (i.e. at time 0) from the area under the curve (AUC, [ng/g or ml] × h) gives the "apparent increase" for each K vitamin. The apparent increases for phylloquinone (ΔPK) and MK-4 (ΔMK-4)
Fig. 6. Concentrations of PK and MK-4 in rat kidney after oral administration of PK.

Fig. 7. Concentrations of PK and MK-4 in rat spleen after oral administration of PK.

Table 1. Ratio of $\Delta$MK-4 to $\Delta$PK in plasma and various tissues after oral administration of PK in mildly vitamin-K-deficient rats.

| Tissue     | $\Delta$MK-4 (AUC, [ng/g or ml]×h) | $\Delta$PK (AUC, [ng/g or ml]×h) | Ratio ($\Delta$MK-4/$\Delta$PK) |
|------------|----------------------------------|---------------------------------|---------------------------------|
| Plasma     | 0.126                            | 45.467                          | 0.003                           |
| Liver      | 0.105                            | 75.840                          | 0.001                           |
| Brain      | 0.094                            | 0.033                           | 2.846                           |
| Testis     | 0.059                            | 0.039                           | 1.500                           |
| Spleen     | 0.074                            | 1.758                           | 0.042                           |
| Kidney     | 0.126                            | 0.172                           | 0.733                           |

Increased MK-4 ($\Delta$MK-4) and PK ($\Delta$PK) after PK administration were represented by each area under the curve (AUC, [ng/g or ml]×h) between 0 and 24 h (Values shown are the mean from four rats).

and the ratios of $\Delta$MK-4 to $\Delta$PK in the various tissues and in the plasma were calculated, and are summarized in Table 1. By studying these data, we could identify a tissue-specific distribution and gauge the extent of the conversion of
Phyloquinone to MK-4 in each tissue. Thus, the biggest value of $\Delta P K$ and the smallest value of $\Delta MK-4$ were observed in the liver, which is clearly different from the other tissues (although the situation in the plasma is parallel to that in the liver). We could place the tissues, according to the size of the ratio $[\Delta MK-4/\Delta PK]$, into a “high” category including the brain (2.846), testis (1.500) and kidney (0.733); an “intermediate” category including the spleen (0.042); and a “very low” category including the liver (0.001). The “very low” category also included plasma (0.003).

**Discussion**

Early studies of vitamin K metabolism demonstrated that orally administered phyloquinone or menadione (vitamin K$_1$) could be converted to MK-4 in rats and chickens (3–5). Billeter et al. (5) also reported that phyloquinone given by a parenteral route was never converted to MK-4. On the basis of this observation, they put forward the hypothesis that the dealkylation of phyloquinone was the result of bacterial action in the gut rather than an indigenous enzymatic action in the tissues. However, Martius and Alvino (15) reported that phyloquinone can be converted to MK-4 in developing chick embryos that lack an intestinal microflora and, more recently, Will et al. (9) reported that phyloquinone administered by intravenous injection can be converted to MK-4 in chick liver and serum. In our previous experiment, radioactivity was detected in the MK-4 fraction of every vitamin K extract from the livers of both germfree and conventional mice after the oral administration of [8-ring-$^{14}$C]-phyloquinone (6). Actually, the conversion efficiency in germfree mice was higher than that in conventional mice, which implies that the contribution of intestinal microflora to this conversion is either nil or very low. This result led us to consider that the indigenous conversion of phyloquinone to MK-4 might proceed more efficiently in germfree tissues than in the tissues of conventional mice.

From the data presented here, it would appear that the feeding of a vitamin-K-deficient diet for nine days was sufficient to minimize the phyloquinone levels in plasma and in all the tissues examined, but not the MK-4 levels (except in the liver and plasma) (see data at time 0, Figs. 2–7). This led us to speculate that (i) phyloquinone might be an easily metabolizable and transiently effective type of vitamin K in most tissues, though only slight in the liver, and (ii) in contrast, MK-4 could serve as a means of storage and have a longer-lasting effect, especially in the testis and brain, as indicated previously (8). The evidence in favor of these ideas is: firstly, that a basal high-level accumulation of MK-4 was clearly observed in the brain and testis even at time 0 (Figs. 3 and 4), at which time the dietary vitamin K supply had been completely stopped for nine days; and secondly, that the circulating level of MK-4 (Fig. 3) remained high longer than that of phyloquinone. This phenomenon also led us to wonder whether vitamin K might have a physiological role inside the tissues of the mammalian body in addition to its role as a coenzyme in the formation of Gla-proteins in the liver and bone.

*J. Nutr. Sci. Vitaminol.*
There have been many studies relating to the conversion of phylloquinone to MK-4 in experimental animals. However, most of these studies have focused on the vitamin K metabolism in the liver or plasma, probably because the liver, where clotting factors (prothrombin, factors VII, IX and X, etc.) are synthesized, has been thought to be a major target tissue for vitamin K. A breakthrough observation was reported by Miura et al. (16), who suggested that the conversion of phylloquinone to MK-4 might also occur in rat brain, though they did not use an isotope-labeling method. This speculation, and the finding (Thijssen and Drittij-Reijnders (8), Will et al. (9) and this paper) that MK-4 is distributed more to other tissues than to the liver, suggests that the conversion of phylloquinone to MK-4 might take place in other tissues rather than in the liver.

Indeed, the low levels of hepatic and circulating MK-4 (Figs. 2 and 3), even after phylloquinone was given orally (this paper, and Thijssen and Drittij-Reijnders (8)), suggest that the liver might not be an active organ for the formation of MK-4 from phylloquinone or menadione. Moreover, it has been reported that MK-4 is conjugated or decomposed into much lower molecules in the liver (17). In non-hepatic tissues, however, relatively high levels of MK-4 were actually observed, which suggests that MK-4 could be formed by an enzymatic or other mechanism inside the tissues. Thijssen and Drittij-Reijnders (8, 18) already proposed a similar idea, though the HPLC separation seemed to be less than perfect. Moreover, there is a recent experimental result which suggests a local synthesis of protein S in the Leydig cells of the human testis; this may be functionally important as part of a local anticoagulation mechanism (19).

A recent experiment by our group revealed that radioactivity originating from orally administered 1',2' (side chain)-3H-phylloquinone was not detected in parallel with the increase in unlabeled phylloquinone in rats (Komai et al., paper submitted). This implies that the isoprenoid side chain of the MK-4 found in various tissues may originate mainly from other molecules such as geranyl-geranyl compounds inside the tissues. However, detailed studies on the conversion mechanism are still required.

In conclusion, this study indicates that orally administered phylloquinone, which is absorbed through the intestine and distributed to most or all tissues (20), is efficiently converted to MK-4 in the brain, testis, kidney and spleen (but only very slightly in the liver) of Wistar rats. Our results show, for the first time, that the conversion occurs efficiently over time in the testis and spleen of the rat. This study has also revealed an abundant MK-4 distribution and storage in various tissues in vitamin-K-deficient rats after the oral administration of phylloquinone. The reason for the high levels of accumulated MK-4 in the testis, brain, kidney and spleen of the control rats needs to be clarified before we can understand the true physiological role of MK-4.

We would like to thank Dr. Mitsumasa Shino, Eisai Co. Ltd., Tsukuba, Japan, for the gift of a platinum oxide column, and Mr. Yukio Kitajima, Eisai Co. Ltd., Tokyo, Japan, for Vol. 43, No. 1, 1997
the gift of standard substances of the phylloquinone and menaquinone series for HPLC and for distribution of the tissues.

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