Pharmacokinetic Characterization of Menaquinone-4 in Dogs by Sensitive HPLC Determination

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Summary A simple and sensitive assay method for a pharmacokinetic study of Menaquinone-4 in dogs was established using HPLC with fluorescence detection following extraction with organic solvent. The quantification limit of this method was 1 ng/ml of plasma. A new oily solution formulation of Menaquinone-4 was administered orally to non-fasted dogs at doses of 0.4, 4 and 40 mg/kg. The plasma concentrations reached maximum levels at 1 to 1.5 h after dosing, and then decreased slowly. AUC values up to 24 h after administration were almost dose-proportional. Menaquinone-4 was also administered to dogs in soft-capsules, for comparison with a conventional hard-capsule oral formulation and an intravenous lecithin formulation. The mean AUC for oral dosing in the soft-capsule formulation was 13.5% of that for intravenous dosing in lecithin, and was 4.6 times higher than that for oral dosing in hard-capsules. Additional dosing in fasted dogs indicated that the AUC in pre-fed dogs was about 4 times higher, suggesting that feeding before giving Menaquinone-4 raises the bioavailability. Overall Menaquinone-4 was absorbed rapidly after administration in non-fasted dogs and dose-proportional bioavailability was obtained among the doses of 0.4 to 40 mg/kg. Higher plasma concentrations were observed after administration in the soft-capsule formulation rather than in the hard-capsule formulation. These findings suggest that the soft-capsule formulation would show a good pharmacokinetic profile for elderly patients with osteoporosis.

Key Words Menaquinone-4, vitamin K, pharmacokinetics, dogs, oral, bioavailability, fluorescence detection, osteoporosis

Vitamin K is an essential nutrient and a cofactor for the carboxylation of specific glutamyl residues in the blood coagulation system (1,2). Vitamin K dependent carboxylase was discovered in liver and has been reported in other tissues (3–6). In bone, γ-carboxylated osteocalcin is involved with remodeling, and therefore vitamin K deficiency might cause related diseases such as osteoporosis (7–9).
Menaquinone-4 (vitamin K₂) is a potent coagulation cofactor even compared with Phylloquinone (vitamin K₁), for hemorrhagic diseases, which is now being developed as a prophylaxis for osteoporosis (10). In addition to the conventional hard-capsule formulation a more condensed formulation was required to improve compliance for elderly patients. A soft-capsule formulation, consisting of Menaquinone-4 in some fatty acid esters was developed in order to reduce the capsule size and to increase the absorption rate relative to the hard-capsule formulation. The relative and absolute bioavailability of those formulations were to be evaluated in dogs. However, the pharmacokinetic profile of Menaquinone-4 has not been investigated in dogs until now, although those in rodents were reported previously (11–14). Additionally, an improvement of the assay for Menaquinone-4 was required for these studies in dogs, because previous methods needed large volumes of plasma and/or complicated pre-treatment such as several steps of chromatography (15–20).

In this report, we describe a simple and sensitive assay method for measurement of Menaquinone-4 in plasma, and its use to determine the pharmacokinetic profile in dogs after oral administration of the soft-capsule formulation of Menaquinone-4.

MATERIALS AND METHODS

Materials. Menaquinone-4 and Menaquinone-6 were synthesized by Eisai Co., Ltd. (Tokyo, Japan). Propyleneglycol dicaprylate (Cefsol 228®) and glycerol monooleate (MGOL-70®) were purchased from Nikko Chemicals Co., Ltd. (Tokyo, Japan). Reagents for buffer solutions were of analytical grade and other solvents were of HPLC grade purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals. Beagle dogs (29–31 month age, 10–14 kg) were obtained from Toyo Research Animal Inc. (Shizuoka, Japan). Animals were separately housed in stainless cages and fed 220 g of food (DS, Oriental Yeast Co., Ltd.) daily and water ad libitum. Before each administration of the test compound, dogs were fasted for more than 12 h and then fed 1 h before dosing, except for animals used to evaluate the absorption under fasting conditions, which were not fed until 8 h postdose.

Formulation. Two new and essentially identical formulations (one prepared in a soft-capsule and the other prepared as a oily solution), and two commercially available formulations (a hard-capsule for oral use and an injectable lecithin formulation) were used in this study. The two new formulations and the hard-capsule formulation were administered orally, while intravenous administration was performed with the lecithin preparation. These formulations are summarized below.

a) New formulations: Type 1 (oily solution, SC1): Menaquinone-4 was dissolved in Cefsol 228/MGOL-70 (40:55) at concentrations of 0.4, 4, and 40 mg/80 mg. The solution was administered in a gelatin capsule.
Type 2 (soft-capsule, SC2): One capsule contains 45 mg of Menaquinone-4 dissolved in 180 mg of Cefsol 228/MGOL-70 (75:105). This formulation was prepared for a clinical study on Menaquinone-4.

b) Hard-capsule formulation (HC): This conventional-type formulation is supplied commercially as Kaytwo® by Eisai Co., Ltd., and contains 5 mg of Menaquinone-4 in a capsule.

c) Lecithin formulation (LF): Menaquinone-4 was dissolved in purified soybean lecithin at a concentration of 5 mg/ml. This formulation is supplied commercially as Kaytwo N® by Eisai Co., Ltd. for intravenous injection.

Administration. Menaquinone-4 (SC1) was administered orally to three groups of four dogs at doses of 0.4, 4, and 40 mg/kg, to investigate the linearity of pharmacokinetics of Menaquinone-4. A second study was performed, with a randomized cross-over design, in three groups of three dogs using the these formulations: LF, SC2, and HC, at a dose of 4 mg/kg, to estimate the absolute and relative bioavailabilities of the last two formulations. Additionally, Menaquinone-4 (SC2) was administered orally to three fasted dogs at a dose of 4 mg/kg, to evaluate the effect of feeding prior to dosing.

Blood sampling and treatment. Samples of 1.5 ml of blood were collected from a cephalic vein, before and up to 24 h after dosing. Additional blood sampling was performed in control dogs, which had not received any formulation of Menaquinone-4, to evaluate the circulating levels of endogenous Menaquinone-4. Blood samples were centrifuged and 200 µl of plasma were collected. The plasma samples were stored in amber glass tubes at -20°C until analysis.

Determination of Menaquinone-4 in dog plasma. a) High-performance liquid chromatography (HPLC): The apparatus used was one of LC-9A and LC-10A autosampling and solvent delivery systems (Shimadzu, Kyoto, Japan) equipped with an SPD-10A UV detector (270 nm) (Shimadzu) and 821-FP fluorescence spectrophotometer (Ex. 254 nm, Em. 430 nm) (JASCO, Tokyo, Japan). Samples were analyzed on an ODS type column, Wakosil-II 5C18-HG (5 µm, 250 mm × 4.6 mm i.d.) (Wako Pure Chemical Industries, Ltd.), at ambient temperature. Additionally, a reducing column (50 mm × 4 mm i.d.) was used after the analytical column, to convert Menaquinone-4 to the corresponding hydroquinone for fluorescence detection, according to Shino’s method (20).

b) Extraction: Menaquinone-6 (50 ng for fluorescence detection method or 5 µg for UV detection method) was added to 200 µl of plasma as an internal standard. After adding 1 ml of 0.2 M phosphate buffer (pH 8.0), the samples were extracted twice with 4 ml of isopropyl alcohol/n-hexane (8:92) and the organic layers were transferred to another amber glass tube. The solvent was then evaporated under nitrogen at 40°C and the residue was dissolved in 0.2 ml of the HPLC mobile phase. An aliquot was injected on to the HPLC system and Menaquinone-4 was measured by UV absorption and/or fluorescence detection. The quantification range of Menaquinone-4 for UV detection in dog plasma was 50 ng to 100 µg/ml, while that for fluorescence detection was 1 ng to 2.5 µg/ml.
Calculation of pharmacokinetic parameters. Pharmacokinetic parameters in this study were calculated by model independent methods, except for those in the intravenous injection study, as follows.

$C_{\text{max}}$ and $T_{\text{max}}$ for oral doses were determined directly from the measured concentrations. AUC was calculated by the trapezoidal method using plasma concentrations up to 10h ($\text{AUC}(0-10h)$) and 24h ($\text{AUC}(0-24h)$) after dosing. Concentrations below the limit of quantification were treated as zero for the calculation of AUC. Bioavailability was estimated by comparing the AUCs for the oral and intravenous doses.

Half-life for intravenous injection was obtained from the plasma concentrations up to 6h after dosing, using a nonlinear least-squares method (21).

RESULTS

Extraction and separation on HPLC

Various organic solvents were evaluated to optimize the extraction of Menaquinone-4 from dog plasma. Although only about half the added Menaquinone-4 was recovered using n-hexane (conventional extraction solvent for vitamin K families), two extractions with isopropyl alcohol/n-hexane (8:92) from buffered plasma (pH 8.0) gave a quantitative recovery (more than 90% for both Menaquinone-4 and Menaquinone-6), with no interfering peak on the HPLC chromatograms. Additional separation of Menaquinone-4 from endogenous compounds was achieved by using EtOH/MeOH/H$_2$O (50:47:3) as a mobile phase for the fluorescence detection. For UV detection, the use of 0.1M Tris buffer (pH 8.0) instead of H$_2$O in the mobile phase was better for separating Menaquinone-4 from interfering peaks. The overall procedure is shown in Fig. 1.

Calibration curves

Several calibration curves were prepared for a variety of plasma concentrations of Menaquinone-4. Three sets of calibration curves confirmed that the assay method with fluorescence detection was well validated ranging from 1ng to 2.5 $\mu$g/ml. Typical chromatograms are shown in Fig. 2. The calibration curves showed good linearity in each assay ($r \geq 0.99994$), and the accuracy of each concentration in these calibrations was within $\pm$5.9%. Furthermore, the intra- and inter-assay variation (CV values) of the standard samples were within 5.0 and 3.0%, respectively. Similar results were obtained for the method with UV detection, ranging from 50ng to 100 $\mu$g/ml (accuracy was within $\pm$8.0% and CV values for intra- and inter-assay were 4.1 and 4.3%, respectively).

Dose-relationship of the plasma concentration

The concentrations of Menaquinone-4 in dog plasma after oral administration of the SC1 formulation at doses of 0.4, 4, and 40mg/kg are shown in Fig. 3. The pharmacokinetic parameters, calculated by model independent analysis, are shown...
PHARMACOKINETICS OF MENAQUINONE-4 IN DOGS

Plasma 200 µl

| Internal standard (Menaquinone-6, 50 ng or 5 µg) |
| 0.2 M Phosphate buffer (pH 8.0) 1 ml |
| Isopropyl alcohol/n-hexane (8:92) 4 ml |

Extract

Organic layer  Aqueous layer

| Isopropyl alcohol/n-hexane (8:92) 4 ml |

Extract

Organic layer  Aqueous layer

Evaporate

EtOH/MeOH/H₂O (50:47:3) 200 µl

HPLC

Fig. 1. Procedure for determination of Menaquinone-4 in dog plasma.

(a)  (b)  (c)

Fig. 2. HPLC chromatograms of Menaquinone-4 with fluorescence detection (Ex. 254 nm, Em. 430 nm). (a) Blank plasma, (b) plasma containing 10 ng/ml of Menaquinone-4, (c) 8 h plasma after oral dosing of SC1 (4 mg/kg), following extraction and determination as described in the text. MK-4, Menaquinone-4; IS, internal standard.
Fig. 3. Plasma concentrations of Menaquinone-4 after oral administration of SC1 to dogs at doses of 0.4, 4, and 40 mg/kg. Samples were measured by HPLC with fluorescence detection. Each point represents the M±SEM of 4 animals. □, 0.4 mg/kg; ○, 4 mg/kg; Δ, 40 mg/kg.

in Table 1.

None of the plasma samples collected from the control group at any time contained measurable concentrations of Menaquinone-4.

The concentration of Menaquinone-4 reached maximum levels at 1 to 1.5 h after administration of each dose. Although the $C_{\text{max}}$ values of these doses varied to some extent between the individual animals, the variations in the AUC values up to 24 h were comparatively small, with M±SEM values of 0.23±0.01, 3.81±0.47, and 25.79±5.43 µg·h/ml at 0.4, 4, and 40 mg/kg, respectively. These results show an almost dose-proportional relationship for the pharmacokinetic profile of Menaquinone-4 in dogs. From 12 h onwards, the plasma concentrations appeared to decline slowly, but the number of samples collected was too few to define the elimination half-life.

Table 1. Pharmacokinetic parameters of Menaquinone-4 after oral administration of SC1 to dogs at doses of 0.4, 4, and 40 mg/kg.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>$T_{\text{max}}$ (h)</th>
<th>$C_{\text{max}}$ (µg/ml)</th>
<th>AUC (0–24 h) (µg·h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4</td>
<td>1.3±0.3</td>
<td>0.14±0.01</td>
<td>0.23±0.01</td>
</tr>
<tr>
<td>4</td>
<td>1.1±0.1</td>
<td>3.11±0.51</td>
<td>3.81±0.47</td>
</tr>
<tr>
<td>40</td>
<td>1.4±0.2</td>
<td>12.32±3.20</td>
<td>25.79±5.43</td>
</tr>
</tbody>
</table>

Samples were measured by HPLC with fluorescence detection. Each value represents the M±SEM of 4 animals.
Administration route and formulation

The plasma concentrations of Menaquinone-4 in dogs after intravenous administration of lecithin formulation (LF) at a dose of 4 mg/kg are shown in Fig. 4. The corresponding pharmacokinetic parameters after oral administration of SC2 and HC are shown in Table 2. The concentrations and parameters of HC group are expressed by the mean of eight animals because one of nine animals vomited the capsule 1 h after administration.

The concentration of Menaquinone-4 appeared to decrease biphasically after intravenous injection of LF with $t_{1/2}$ values of $0.143 \pm 0.007$ and $1.395 \pm 0.045$ h, respectively. Although the latter probably does not represent the terminal elimination half-life (as the samples were analyzed by the UV detection method, and the levels could only be measured up to 6 h after administration), it was considered sufficiently reliable to project the AUC value for the LF dose up to 10 h after administration, for an estimation of the bioavailabilities of the oral formulations.

After oral administration of SC2, there were large variations in time-concentration profiles among the individual animals. Three representative patterns

![Graph showing plasma concentration of Menaquinone-4 in dogs after intravenous administration of LF at a dose of 4 mg/kg. Samples were measured by HPLC with UV detection. Each point represents the M±SEM of 9 animals.](image)

![Table 2. Pharmacokinetic parameters of Menaquinone-4 in dogs after oral administration of SC2 and HC at a dose of 4 mg/kg in the manner of a randomized cross-over.](image)

<table>
<thead>
<tr>
<th></th>
<th>$T_{\text{max}}$ (h)</th>
<th>$C_{\text{max}}$ (µg/ml)</th>
<th>AUC (0–10 h) (µg·h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC2</td>
<td>$4.9 \pm 1.3$</td>
<td>$2.05 \pm 0.91$</td>
<td>$2.90 \pm 0.80$</td>
</tr>
<tr>
<td>HC</td>
<td>$5.2 \pm 1.5$</td>
<td>$0.38 \pm 0.14$</td>
<td>$0.64 \pm 0.16$</td>
</tr>
</tbody>
</table>

Samples were measured by HPLC with UV detection.

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Table 3. Pharmacokinetic parameters of Menaquinone-4 after oral administration of SC2 to fasted and non-fasted dogs at a dose of 4 mg/kg.

<table>
<thead>
<tr>
<th></th>
<th>$T_{\text{max}}$ (h)</th>
<th>$C_{\text{max}}$ ($\mu$g/ml)</th>
<th>AUC (0–10h) ($\mu$g·h/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-fasted</td>
<td>4.9±1.3</td>
<td>2.05±0.91</td>
<td>2.90±0.80</td>
</tr>
<tr>
<td>Fasted</td>
<td>2.0±0.0</td>
<td>0.39±0.10</td>
<td>0.75±0.20</td>
</tr>
</tbody>
</table>

Samples were measured by HPLC with fluorescence (fasted dogs) and UV (non-fasted) detection. Each value represents the M ± SEM of 3 fasted and 9 non-fasted animals.

were observed: a) rapid absorption type showing $T_{\text{max}}$ at 1 to 3 h, b) slow absorption type with $T_{\text{max}}$ of 8 to 10 h, c) long-term absorption type. As a consequence, the mean AUC up to 10 h was 13.5% of that for intravenous administration of LF.

Lower concentrations after oral administration of HC were observed relative to those of SC2. The time-concentration profile was, however, similar to that of SC2 showing both rapid and late absorption patterns. The mean AUC of SC2 was 4.6 times higher than that of HC, indicating that the bioavailability of Menaquinone-4 after dosing of SC2 was higher.

Effect of feeding on bioavailability

In all the experiments above, an appropriate amount of food was given to animals prior to dosing. To evaluate the effect of feeding on absorption, Menaquinone-4 (SC2) was administered to dogs that had been fasted for more than 12 h. The pharmacokinetic parameters for fasted and non-fasted dogs are shown in Table 3. Only rapid absorption was observed in the fasted dogs, with $T_{\text{max}}$ at 2 h after dosing. Concentrations of Menaquinone-4 were, however, reduced markedly at each time point, and the $C_{\text{max}}$ and AUC for the fasted dogs were 5.2 and 3.9 times lower than those for the pre-fed animals. These results suggest that feeding prior to dosing facilitates the intestinal absorption of Menaquinone-4 in dogs.

DISCUSSION

A sensitive assay method to measure Menaquinone-4 in dog plasma was established and used for a characterization of pharmacokinetics in dogs.

Until now several methods to measure vitamin K families in plasma have been reported using HPLC with fluorescence detection following reduction of quinone moiety to corresponding hydroquinones (15–20). Most of these methods, however, require large volumes of plasma and/or complicated pre-treatments to obtain high sensitivity. Therefore only limited number of samples could be collected, so the time-concentration profiles of vitamin K could not be fully defined. Recently, Shino improved the previous methods in which the reduction was performed conveniently with $\text{H}_2$ gas saturated mobile phase and a reduction column, and plasma concentrations of vitamin K families in several species including humans.

were measured (20). However, large volume (1 ml) of plasma samples and several steps of chromatography such as silica gel column chromatography and alumina column chromatography as the pre-treatment were still required in this method. We have now further modified this method to reduce (a) the amount of plasma to 200 μl and (b) the pre-treatment required prior to analysis on HPLC. These advantages were achieved by improving the HPLC conditions, which could reduce the analysis time of one sample and could separate Menaquinone-4 from endogenous compounds, and by the use of isopropyl alcohol/n-hexane (8:92) as extraction solvent (following bufferizing at pH 8.0) in place of n-hexane which has usually been used in previous reports (15–17, 19). This assay method of Menaquinone-4 was sufficiently simple and sensitive to make it possible to evaluate time-concentration profiles of Menaquinone-4 in dog plasma for 24 h after oral dosing.

Endogenous Menaquinone-4 could not be detected in any of the pre-dose and control samples. This observation was consistent with previous reports that the concentrations of Menaquinone-4 in dog plasma are 0.34 ng/ml (19) or below 0.1 ng/ml (20). Although only 200 μl of plasma was available in the present study, we confirmed at least that the circulating levels of Menaquinone-4 in dogs are below 1 ng/ml all day long. The actual levels will be determined by a more complicated assay method which requires a larger sample volume.

The concentrations of Menaquinone-4 in plasma rapidly reached maximum levels at 1 to 1.5 h after oral administration of Menaquinone-4 to dogs in solution (SC1) at doses of 0.4, 4, and 40 mg/kg and then decreased at comparable rates for all three doses. The pharmacokinetic profiles of Menaquinone-4 after oral administration was, therefore, considered to be independent of the dose administered, within this dose range. Furthermore, it is supposed that the absorption and metabolism of Menaquinone-4 was not saturated because a linear relationship was observed between the doses and AUC values.

In previous studies (22–28), vitamin K families including Phylloquinone and Menaquinones were reported to be absorbed via the lymphatic system, with the involvement of bile acids except Menadione (vitamin K₃) which could be absorbed via portal vein without bile acids. However, the limited absorption observed in this study even under fasting conditions suggested the existence of additional absorption mechanisms for Menaquinone-4, such as via the portal vein. This assumption was supported by the recent study (29) that Menaquinone-4 was partially absorbed via portal vein in an in situ model of rats. However, further investigations of the absorption route and rate will be required to clarify this question.

The time-concentration profile of Menaquinone-4 was rather more variable after oral administration as the SC2 and HC formulations. This might be due to different release rate from the two capsule formulations in the gastrointestinal tract of individual dogs. Several different types of time-concentration profiles were, therefore, observed in the SC2 groups. However, the variation of AUC values was not so large and was independent of the formulation, so that a valid comparison of relative bioavailabilities was obtained. The value for SC2 was 4.6 times higher than
that of HC. These findings suggested that the bioavailability of Menaquinone-4 following oral dosing of SC was superior to that of HC in dogs.

In fasted dogs, only the rapid absorption was seen in all animals, and time-concentration profiles showed a simple pattern. However, the $C_{\text{max}}$ and AUC values were markedly lower than those in non-fasted dogs. As previous studies have demonstrated that the bile is required for the efficient absorption of Phylloquinone (3–5), it can be supposed that the small amount of bile limited the absorption of Menaquinone-4 in fasted dogs. Consequently, oral administration after feeding is recommended to raise the bioavailability of Menaquinone-4.

In conclusion, the development of an improved assay method for Menaquinone-4 has enabled us to study the pharmacokinetics of the drug in dogs. Menaquinone-4 was absorbed rapidly after oral administration in oily solution. Feeding the dogs prior to dosing raised the bioavailability, and the dose-proportional bioavailability was observed with the dose-range of 0.4 to 40 mg/kg. The plasma concentrations were also higher after administration of the soft-capsule formulation, rather than the conventional hard-capsule formulation. These findings suggest that the soft-capsule formulation would show a good pharmacokinetic profile for elderly patients with osteoporosis.

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REFERENCES


