Biochemical Pharmacology, Vol. 30, No. 21, pp. 2931–2936, 1981.
Printed in Great Britain.

0006-2952/81/212931-06 $02.00/0

VITAMIN K_1, VITAMIN K_1 EPOXIDE AND WARFARIN INTERRELATIONSHIPS IN THE DOG*

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(*Received* 20 *October* 1980; *accepted* 27 *February* 1981)

Abstract—The cyclic interconversion of vitamin K_1 and vitamin K_1 epoxide, in the presence and absence of administration of warfarin, was studied in two dogs, using tritiated vitamin K_1 and vitamin K_1 epoxide. Warfarin, at therapeutic doses, completely blocked the conversion of vitamin K_1 epoxide to vitamin K_1. Alternative routes of metabolism of vitamin K_1 epoxide appear to account for about one-third of its clearance from plasma. Warfarin did not influence the clearance of vitamin K_1 from plasma. Nonetheless, it reduced the volume of distribution of vitamin K_1 (but not of vitamin K_1 epoxide), possibly by displacing vitamin K_1 from specific binding sites in the liver. In the absence of warfarin, approximately 10 per cent of an administered dose of vitamin K_1 appeared as vitamin K_1 epoxide in plasma, whereas 45 per cent of an administered dose of vitamin K_1 epoxide appeared in plasma as vitamin K_1. Warfarin increased the former percentage to 65 per cent by inhibiting reconversion of the epoxide, as shown by the absence of vitamin K_1 in plasma when vitamin K_1 epoxide was administered in the presence of warfarin.

Warfarin and the other coumarin anticoagulants have been shown to interfere with the effect of vitamin K_1 on the post-translational γ-carboxylation of specific glutamyl residues on clotting factors II, VII, IX and X [1, 2]. The mechanism of action of warfarin is unknown, but it does not appear to be a direct antagonist of the carboxylase; its action may be related to its effect on the interconversion of vitamin K_1 and vitamin K_1 epoxide [3, 4]. *In vitro*, warfarin has been shown to block the reduction of vitamin K_1 epoxide to vitamin K_1 and also to block the dithiothreitol (DTT)-dependent formation of a reduced form of vitamin K_1 which is required both for the carboxylation of clotting factors and for the formation of vitamin K_1 epoxide [5]. Warfarin has no effect upon NADH-dependent formation of reduced vitamin K_1 except at high concentrations [6].

In vivo, in laboratory animals and in humans, administration of warfarin has been shown to cause the accumulation of vitamin K_1 epoxide in plasma and to increase the levels of certain metabolites of administered vitamin K_1 that are present in the urine of normal humans [7, 8]. The importance of the accumulation of vitamin K_1 epoxide to the pharmacologic effect of warfarin remains controversial [2]. Although a significant fraction of vitamin K_1 has been shown to be converted to the epoxide *in vitro*, the extent of *in vitro* conversion from the epoxide back to vitamin K_1, both in the presence and absence of warfarin, is uncertain. The aims of the present study were to determine the *in vivo* disposition of tritiated vitamin K_1 and vitamin K_1 epoxide before and during the administration of warfarin and to determine the fraction of each compound which refluxes into plasma in the presence and absence of warfarin. These data would then supply an estimate of the cyclic interconversion of the two compounds under physiological conditions and under the influence of therapeutic concentrations of warfarin.

METHODS

Study design. Scheme 1 provides an outline of the experimental protocol. Two conscious male mongrel dogs were studied; dog A weighed 22.7 kg and dog B weighed 26.3 kg.

The study itself lasted 30 days and consisted of three phases. During the first phase each dog received an intravenous injection of specifically labeled tritiated vitamin K_1 and vitamin K_1 epoxide (supplied by Joseph Wursch, Hoffmann LaRoche, Basel, Switzerland) on two separate occasions separated by 1 week. The doses of both vitamin K_1 and K_1 epoxide were the same, consisting of approximately 100 μCi (0.5 mg) of each given over a 30-sec interval into a foreleg vein. Doses are listed in Table 1. Serial plasma samples were obtained for 24 hr

Scheme 1. Study design

Day of study	Compound administered Dog A	Dog B
1	K*	Kep†
8	Kep	K
9	Begin Warfarin	
22	K	Kep
30	Kep	K

*K referes to an intravenous dose of tritiated vitamin K_1.
†Kep refers to an intravenous dose of tritiated vitamin K_1 epoxide.

* This research was supported by a grant from the National Institutes of Health (GM22209). David Carlisle is a recipient of a Medical Student Research Fellowship from the Pharmaceutical Manufacturers Association Foundation. T. F. Blaschke is a Burroughs Wellcome Scholar in Clinical Pharmacology and recipient of a Research Career Development Award (GM00407) from the NIH.

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after each dose through an indwelling intravenous catheter in the foreleg opposite the site of administration.

The second phase of the study was used for warfarin pretreatment and adjustment of the dose of warfarin. Warfarin was given initially at a dose of 2 mg orally once daily and was increased 1 week later to 6 mg daily. One-stage prothrombin-time determinations were performed prior to the study and periodically after the initiation of warfarin administration. The prothrombin time during the first phase of the study was 9.4 sec in both dogs and had increased to 15.8 and 14.1 sec by the beginning of the third phase of the study.

The third phase began approximately 2 weeks after initiation of oral warfarin, which was continued at the same dose throughout the remainder of the study. During this last phase, tritiated vitamin K_1 and vitamin K_1 epoxide were again given intravenously, using the same dose and sampling as employed during the first phase. The order of administration was different for the two dogs, but was kept the same before and during warfarin administration in each dog (Scheme 1).

Measurement of [^{3}H] *vitamin* K_1 *and* [^{3}H] *vitamin* K_1 *epoxide.* Concentrations of radiolabeled vitamin K_1 and vitamin K_1 epoxide in plasma were quantitated by high pressure liquid chromatography (h.p.l.c.) using the method of Bjornsson *et al.* [9], developed in this laboratory. Standard ratios of [^{3}H]vitamin K_1 and [^{3}H]vitamin K_1 epoxide were run concurrently with each set of plasma samples. The average coefficient of variation for the standards was 4.2 per cent. The method is accurate and reproducible over a wide range of vitamin K_1/vitamin K_1 epoxide ratios (from 0.01 to 10).

Data fitting and calculations. The plasma concentration (C_p) versus time (t) data were fit to a biexponential expression of the form:

$$C_p = Ae^{(-\alpha t)} + Be^{(-\beta t)} \quad (1)$$

using the nonlinear regression program MLAB [10]. The terms A and B are the intercepts, and α and β are the exponentials of the two components of equation 1. Because of equal coefficients of variation for the assay procedure at high and low ratios, and in order not to give undue influence to large values, the data points were weighted by the inverse of the squared plasma concentration [11]. The goodness of fit of the data for the computed expression was assessed by examining the weighted residuals [11].

The plasma radioactivity–time profiles obtained were also used to calculate plasma clearance (Cl_p) according to the standard model-independent expression:

$$Cl_p = \frac{\text{Dose}}{\text{AUC}]_0^\infty} \quad (2)$$

The area under the curve from time 0 to the last data point was determined using the trapezoidal rule, and the residual area after the last data point was estimated by dividing the plasma concentration at the time of the last data point by the value of β from the best fit of the biexponential expression. The $\text{AUC}]_0^\infty$ is the sum of the two areas (trapezoidal plus residual).

The apparent volume of distribution (Vd_{area}) was calculated as follows:

$$Vd_{area} = \frac{\text{Dose}}{\beta \times \text{AUC}]_0^\infty} \quad (3)$$

The steady-state volume of distribution for a 2-compartment open model with elimination from the central compartment (Vd_{ss}) was determined using standard methods [12].

Using the calculated values for Vd_{ss} and clearance, the turnover time (t_t) and turnover rate (k_t) were calculated:

$$t_t = \frac{Vd_{ss}}{Cl_p} \quad (4)$$

$$k_t = 1/t_t \quad (5)$$

The turnover time provides an indication of the lifespan of an average molecule of material in the system, whereas the turnover rate provides an estimate of the fractional catabolism of the pool of material per unit time.

In addition, the amount of vitamin K_1 epoxide (Kep) derived from administered vitamin K_1 that refluxed into plasma (K → Kep) and the amount of vitamin K_1 (K) derived from administered vitamin K_1 epoxide (Kep → K) were calculated from the following expressions:

$$\text{K} \rightarrow \text{Kep} = \frac{\text{AUC Kep after i.v. K}}{\text{AUC Kep after i.v. Kep}} \cdot \frac{\text{Dose i.v. K (dpm)}}{\text{Dose i.v. Kep (dpm)}} \quad (6)$$

$$\text{Kep} \rightarrow \text{K} = \frac{\text{AUC K after i.v. Kep}}{\text{AUC K after i.v. K}} \cdot \frac{\text{Dose i.v. Kep (dpm)}}{\text{Dose i.v. K (dpm)}} \quad (7)$$

RESULTS

Figure 1 shows the plasma radioactivity versus time profiles of vitamin K_1 and vitamin K_1 epoxide for dog A during the first and third phases of the study. Panels A and C represent phase I, before warfarin administration. Panel A shows the disappearance of vitamin K_1 epoxide and the appearance and disapperance of vitamin K_1 following the administration of tritiated vitamin K_1 epoxide. Panel C shows the plasma concentrations of both compounds following the administration of tritiated vitamin K_1. Note that the plasma concentrations of vitamin K_1 epoxide following vitamin K_1 administration (panel C) are lower than the plasma concentrations of vitamin K_1 after vitamin K_1 epoxide administration (panel A).

Panels B and D illustrate the effect of warfarin on the plasma radioactivity versus time profiles. In both panels B and D, the terminal half-life of radioactive vitamin K_1 epoxide in plasma is substantially prolonged. Panel D shows that the terminal half-life of vitamin K_1 was shorter than that observed before warfarin administration (panel C). Panel B shows that no vitamin K_1 appeared in plasma following

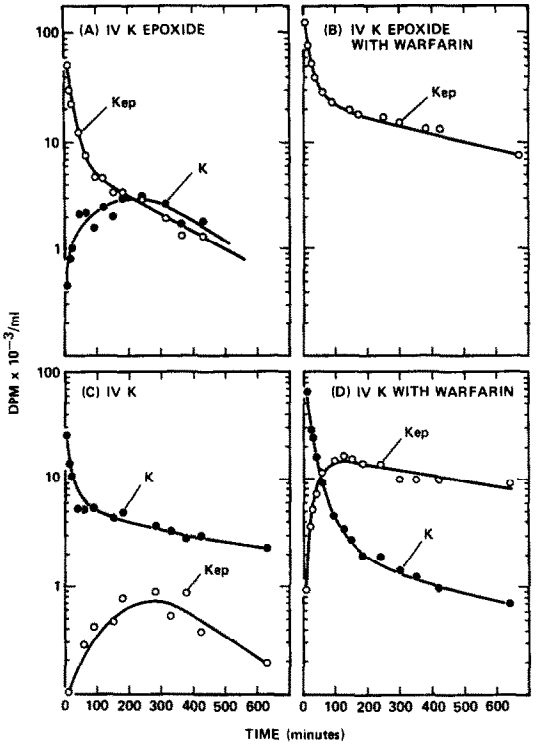

Fig. 1. Plasma concentratation versus time profiles for vitamin K_1 and vitamin K_1 epoxide in dog A. Panels A and C were obtained in the absence of warfarin, while panels B and D were obtained during administration of warfarin. K (●) refers to vitamin K_1, while Kep (○) refers to vitamin K_1 epoxide. The compound administered intravenously is shown at the top of each panel. No vitamin K_1 was detected after administration of vitamin K_1 epoxide in the presence of warfarin (panel B). See text for discussion.

vitamin K_1 epoxide administration in the presence of warfarin. Although only dog A is shown in the figure, these findings were consistent in both dogs studied.

Table 1 summarizes the data derived from the plasma radioactivity profiles. Beta is the slope of the second exponential component obtained from the weighted least-squares fit of the plasma profiles to equation I. Cl_p, Vd_{ss} Vd_{area}, $t_{\frac{1}{2}}$ and k_t are the derived parameters obtained according to equations 2–5. The areas under the curves for both the administered compound and the derived compound [AUC(K) and AUC(Kep)] are also shown in the table.

Table 2 shows the fraction of vitamin K_1 epoxide derived from administered vitamin K_1 that appeared in plasma and the fraction of vitamin K_1 derived from administered vitamin K_1 epoxide which refluxed into plasma. These results were obtained by using equations 6 and 7.

DISCUSSION

Comparison of vitamin K_1 and vitamin K_1 epoxide without warfarin. A substantial difference was observed in the turnover times for vitamin K_1 and vitamin K_1 epoxide. The turnover times for vitamin K_1 epoxide in the two animals were only 28 and 17 per cent of the corresponding values for vitamin K_1. The two compounds, however, showed virtually identical clearance rates from plasma. Thus, the difference between the turnover time of vitamin K_1 and that of its epoxide can be attributed to a large difference in the steady-state volume of distribution of the two compounds, as shown in Table 1.

The mechanism responsible for the difference in extent of distribution into tissues is unclear; studies by Wiss and Gloor [13] have shown that when radiolabeled vitamin K_1 is administered to rats, most distributes to the liver (78 per cent by 2 hr after administration) and it is likely, therefore, that differences in hepatic concentrations of vitamin K_1 and vitamin K_1 epoxide are responsible for the differences in Vd_{ss}. Changes in hepatic concentrations probably also explain the differences in the Vd_{ss} observed for vitamin K_1 after warfarin administration (see below).

Effect of warfarin on vitamin K_1 and vitamin K_1 epoxide. As shown in Fig. 2, warfarin is thought to act at, at least, two sites in the vitamin K_1/vitamin K_1 epoxide cycle. It prevents the enzymatic conversion of vitamin K_1 epoxide to vitamin K_1 and also prevents the dithiothreitol-dependent reduction of vitamin K_1 to the hydroquinone [6]. At higher concentrations warfarin also has been shown to inhibit, *in vitro*, the formation of vitamin K_1 epoxide from the reduced form of the vitamin [14]. As yet, evidence that carboxylation and epoxidation are coupled is indirect and controversial [5, 15].

The clearance of vitamin K_1 was essentially unaffected by the presence of warfarin. The Vd_{ss} of vitamin K_1, however, was reduced by 67 per cent. The net effect of the change in the Vd_{ss} was a substantial decrease (72 per cent) in turnover time and increase in turnover rate. Whether the observed decrease in the Vd_{ss} and the increase in turnover rate of vitamin K_1 are directly related to the mechanism of action of warfarin is uncertain, although the net effect of both changes is a decrease in the hepatic content of vitamin K_1. The decrease in Vd_{ss} could be due to displacement of vitamin K_1 from binding sites in the liver by warfarin. This possibility is consistent with the observation that warfarin and vitamin K_1 compete for a specific binding site in the membranous portion of the hepatic endoplasmic reticulum [16].

In contrast to vitamin K_1, vitamin K_1 epoxide showed no change in Vd_{ss} and a decreased clearance from plasma in the presence of warfarin. The result was a 3- to 5-fold increase in the turnover time of vitamin K_1 epoxide during warfarin administration. The mechanism of the change in clearance was presumably an inhibition of vitamin K_1 epoxide reductase by warfarin (Fig. 2).

It is noteworthy that, although warfarin substantially reduced the clearance of vitamin K_1 epoxide from plasma, the clearance in the presence of warfarin was still 30 per cent of the value without warfarin. At warfarin concentrations that are therapeutic *in vivo*, there was complete inhibition of the reductase *in vitro*. Therefore, these results suggest that under normal circumstances approximately one-third of the epoxide is not reconverted to vitamin K_1, but is eliminated by other pathways (Fig. 2). By

Table 1. Pharmacokinetic parameters for vitamin K_1 and vitamin K_1 epoxide*

		AUC(K)† (dpm/ml·min × 10^{-6})	AUC(Kep)‡ (dpm/ml·min × 10^{-6})	β (min^{-1})	$T_{\frac{1}{2}\beta}$ (min)	Cl_p (ml/min)	Vd_{area} (ml × 10^{-4})	Vd_{ss} (ml × 10^{-4})	t_t (min)	k_t (min^{-1})	Dose§ (dpm × 10^{-8})
Without warfarin											
K‖	Dog A	4.12	0.350	0.0016	433	62	3.87	3.72	602	0.0017	2.54
	Dog B	4.20	0.498	0.0015	456	71	4.71	4.29	601	0.0017	3.00
Kep¶	Dog A	2.12	2.86	0.0039	176	80	2.03	1.34	168	0.0060	2.29
	Dog B	1.75	6.04	0.0039	178	53	1.36	0.55	103	0.0097	3.20
With warfarin											
K	Dog A	3.37	12.6	0.0028	248	68	2.46	1.18	172	0.0058	2.31
	Dog B	3.60	10.2	0.0025	273	79	3.13	1.42	179	0.0056	2.86
Kep	Dog A	0	17.0	0.0018	386	17	0.98	0.89	507	0.0020	2.97
	Dog B	0	13.6	0.0020	355	22	1.13	1.07	489	0.0020	2.97

* Symbols and calculations are summarized in Methods.
† $AUC]^{\infty}_0$ for vitamin K_1.
‡ $AUC]^{\infty}_0$ for vitamin K_1 epoxide.
§ Administered doses of vitamin K_1 or vitamin K_1 epoxide.
‖ Values after intravenous administration of radiolabeled vitamin K_1.
¶ Values after intravenous administration of radiolabeled vitamin K_1 epoxide.

Table 2. Vitamin K_1–vitamin K_1 epoxide interconversion

	% Interconversion			
	K → Kep* (without warfarin)	K → Kep (with warfarin)	Kep → K† (without warfarin)	Kep → K (with warfarin)
Dog A	14	58	47	0
Dog B	8	73	44	0
Average	11	65	45	0

* K → Kep is the percentage of vitamin K_1 that was converted to vitamin K_1 epoxide *and* which refluxed to plasma.

† Kep → K is the percentage of vitamin K_1 epoxide that was converted to vitamin K_1 *and* which refluxed to plasma.

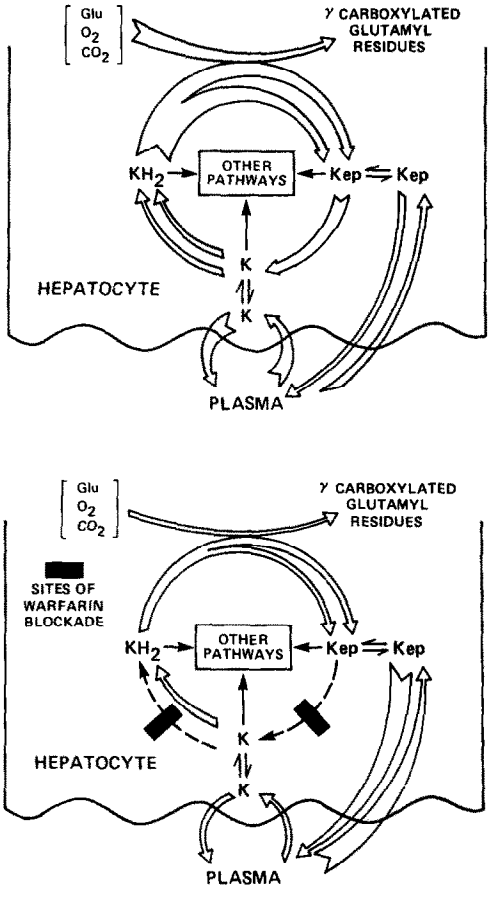

Fig. 2. Schematic diagram of interrelationships of warfarin, vitamin K_1, and vitamin K_1 epoxide. The top panel illustrates the proposed coupling of epoxidation and γ-carboxylation with the hydroquinone (KH_2) intermediate. Although the cyclic interconversion of vitamin K_1 and vitamin K_1 epoxide occurred intracellularly, there was some reflux of both compounds into plasma (see also panels A and C of Fig. 1). The lower panel illustrates the sites of action of warfarin on the reductase and on the DTT-dependent (but not the NADPH-dependent) reduction of vitamin K_1 to the hydroquinone. The change in size of the arrows represents the effect of blockade of these pathways by warfarin on the plasma reflux of vitamin K_1 and vitamin K_1 epoxide.

inference, they also suggest that two-thirds of the epoxide formed *in vivo* is reconverted to vitamin K_1 in the cycle depicted in Fig. 2. The amount of vitamin K_1 which appeared in plasma is a somewhat smaller fraction (approximately 45 per cent) of the administered dose.

Interconversion of vitamin K_1 and vitamin K_1 epoxide. The top panel of Fig. 2 schematically illustrates the linkage between the cyclic interconversion of vitamin K_1 and vitamin K_1 epoxide and the γ-carboxylation of precursor coagulation factors [2, 4, 6]. The interconversion occurs intracellularly, almost exclusively in the liver; data obtained from measurements of concentrations of radioactivity in plasma are indirect methods of examining these intracellular events. When radiolabeled vitamin K_1 or vitamin K_1 epoxide are administered intravenously the plasma disappearance profile of the *administered* compound can be used to determine clearance, distribution and turnover, even when there is intracellular cyclic interconversion. Plasma concentrations of the *derived* compound (e.g. vitamin K_1 epoxide after vitamin K_1 or vitamin K_1 after administration of vitamin K_1 epoxide), however, reflect only that fraction of the *derived* compound which refluxes into plasma. Assuming that the plasma disposition of either vitamin K_1 or K_1 epoxide was the same when it was administered directly intravenously or derived from hepatic conversion, equations 5 and 6 can be used to estimate the fraction of administered compound which was converted to derived compound *and* which passes through the plasma. These derived values, in the presence and absence of warfarin, are shown in Table 2. When vitamin K_1 was administered in the absence of warfarin, only about 11 per cent of the compound was detected in plasma as the K_1 epoxide metabolite. In the presence of warfarin, which blocks the reconversion of the epoxide to vitamin K_1, the amount of K_1 epoxide derived from vitamin K_1 which passes through plasma increased to about 65 per cent. It is likely, therefore, that even in the absence of warfarin a *minimum* of 65 per cent of vitamin K_1 was converted to its epoxide metabolite. Examining the plasma levels of vitamin K_1 after intravenous administration of the epoxide in the absence of warfarin, 45 per cent of the administered dose of the epoxide was seen in plasma as vitamin K_1. Again, this represents the minimum frac-

tion which was reconverted in the absence of warfarin. As expected, no vitamin K_1 was detected in plasma after administration of vitamin K_1 epoxide in the presence of therapeutic concentrations of warfarin.

These results provide comparative data in the absence of warfarin about the disposition of vitamin K_1 and its epoxide metabolite in the dog, which reveal a difference in tissue distribution but not in clearance. Half-life, a hybrid rate constant, was shorter for the epoxide, but this was due to the smaller volume of distribution. Warfarin caused a decrease in the volume of distribution of vitamin K_1 but little, if any, change in the distribution of vitamin K_1 epoxide. Conversely, the clearance of vitamin K_1 was unaffected by warfarin, whereas the clearance of the epoxide was reduced by two-thirds. Interestingly, the change in the volume of distribution of vitamin K_1 during warfarin treatment was not observed in our previous studies in man [17], suggesting the possibility of a species difference in the effect of warfarin on vitamin K_1 disposition.

Acknowledgements—We would like to thank Sarah Swezey and George Snidow for technical assistance, and Linda Halloran for preparation of the manuscript.

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