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IMPROVED METHOD FOR THE DETERMINATION OF VITAMIN K_1 EPOXIDE IN HUMAN PLASMA WITH ELECTROFLUORIMETRIC REACTION DETECTION

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SUMMARY

The use of the previously described electrofluorimetric reaction detection method for phylloquinone (vitamin K_1) was extended to the determination of vitamin K_1 epoxide in human plasma. The limit of detection for vitamin K_1 epoxide is 60 pg, corresponding to plasma concentrations of about 120 pg/ml. The isolation method involves a liquid–liquid extraction of the lipids from the plasma, followed by a clean-up with silica. Synthesized 2′,3′-dihydrovitamin K_1 is used as an internal standard. The method was applied to the study of the presence of vitamin K_1 epoxide and phylloquinone in plasma samples from healthy volunteers and from patients undergoing anticoagulant therapy.

INTRODUCTION

Phylloquinone (PK) and its main metabolite vitamin K_1 epoxide (K1-ep) (see Fig. 1) are involved in the cyclic process leading to the carboxylation of the precursers of a number of blood clotting factors. As coumarin anticoagulants inhibit the function of K1-ep reductase, the administration of coumarins leads to the accumulation of K1-ep in the liver[1]. After administration of PK to patients receiving coumarins, high concentrations of K1-ep are found in plasma[2,3].

To the best of our knowledge, the presence of K1-ep in plasma from healthy persons has not yet been established. Further, no information is available on the vitamin K status of patients treated with coumarins; it may be that in these patients only K1-ep is present in the blood. These investigations are hampered by the lack of a sensitive method for the determination of K1-ep. Most studies concerning K1-ep are performed with tritiated PK[2], which does not give information about endogenous K vitamins. High-performance liquid chromatography (HPLC) combined with UV detection is possible, but unfortunately the molar absorptivity of K1-ep is a three times smaller than that of PK, resulting in a limit of detection of about 1.5 ng. Therefore, the use of UV detection is limited to *in vitro* studies of the PK-dependent carboxylation reaction with relatively high concentrations of K1-ep[4].

HPLC combined with fluorimetric detection after a two-step chemical reduc-

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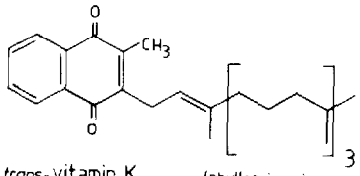
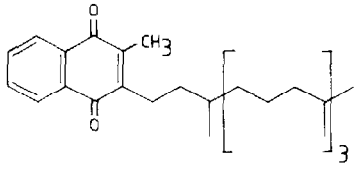
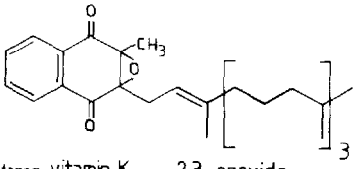


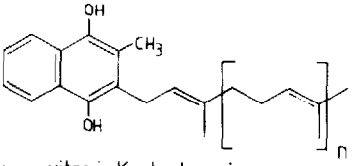
trans-vitamin $K_{1(20)}$ (phylloquinone)



2',3'-dihydrovitamin $K_{1(20)}$



trans-vitamin $K_{1(20)}$-2,3-epoxide



trans-vitamin K -hydroquinone

Fig. 1. Chemical structures of K vitamins.

tion offers sufficient selectivity towards the plasma background. However, owing to the extra band broadening in the post-column reactor, the limit of detection is still too high (3 ng) for the determination of endogenous levels[5].

Gas chromatography (GC) combined with electron-capture detection offers about the same limit of detection with good selectivity[3]. Unfortunately, the GC analysis of underivatized K vitamins is still difficult. None of the methods described so far has sufficient sensitivity for the study of endogenous levels of K1-ep. The so-called electrofluorimetric reaction detection method, combining coulometric reduction and fluorimetric detection, that we developed for the determination of K_1 and K_2 vitamins[6] appeared to be the most promising method. The reduction of K1-ep requires relatively low potentials (lower than -1 V), resulting in high background currents. As a consequence, the applied potentiostat was overloaded and the potential was not well defined. This problem has been overcome by replacement of the bipotentiostat with a simple potentiostat, allowing higher residual currents. The application of this method to the investigation of endogenous levels of K1-ep is described in this paper.

EXPERIMENTAL

Apparatus

The liquid chromatograph was constructed from commercially available and custom-made parts and consisted of a thermostated eluent reservoir (293°K), a reciprocating plunger-membrane pump (DMP 1515; Orlita, Giessen, F.R.G.), a Bourdon-type damping device, a manometer, an injection device (Model 7125; Rheodyne, Berkeley, CA, U.S.A.) and a thermostated column (stainless-steel precision-bore tubing, 100 mm × 3.0 mm I.D. × 6.35 mm O.D.). The reactor consisted of a dual electrochemical detection cell (Model 5010; ESA, Bedford, MA, U.S.A.) coupled to a polarograph (Model E44S; Bruker, Brussels, Belgium). A fluorospectrophotometer of the double monochromator type (Model RF 530; Shimadzu, Kyoto, Japan) operating at 320 and 430 nm for the excitation and emission wavelengths, respectively, was used. Chromatograms were recorded on a flat-bed recorder (BD 8; Kipp and Zonen, Delft, The Netherlands).

Semi-preparative chromatography of 2',3'-dihydrovitamin K_1 was performed with a similar pump and injector, but with a column of 250 mm × 4.5 mm I.D. × 6.35 mm O.D., using a fixed-wavelength detector (Model 440; Waters Assoc., Bedford, MA, U.S.A.) operating at 254 nm.

Columns were packed by a pressurized slurry technique with an air amplifier booster pump (DSTV 122; Haskel, CA, U.S.A.) as described elsewhere[7].

Chemicals and materials

PK and K1-ep were kindly donated by Hoffmann-La Roche (Mijdrecht, The Netherlands). 2',3'-Dihydrovitamin K_1 was prepared as described below. All organic solvents were of analytical-reagent grade (J. T. Baker, Phillipsburg, NJ, U.S.A.). Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Hypersil-MOS (5 μm) was purchased from Shandon (London, UK). Silica SI 60 (50–200 μm) was purchased from Merck (Darmstadt, F.R.G.). Silica SI 60 (8–9 μm) was prepared by grinding SI 60 with a particle size range of 63–200 μm in a rotating mortar and classifying the ground material by means of an air classifier (Alpine MZR, Augsburg, F.R.G.). The silica was activated by heating at 383°K under reduced pressure for 5 h.

Preparation of 2',3'-dihydrovitamin K-1

A 10-mg amount of PK was dissolved in 150 ml of ethanol in a 500-ml round-bottomed flask and the solution was heated to about 330°K. Oxygen-free nitrogen was passed through the solution for 5 min, 100 mg of catalyst (10% palladium on carbon) were added and hydrogen was bubbled through the solution for 1 h. After cooling to room temperature, 200 ml of *n*-hexane were added, followed by 75 ml of distilled water, and the solution was mechanically shaken for 5 min. The *n*-hexane phase was removed and was shaken with a solution of cerium(IV) sulphate for rapid re-oxidation of the hydroquinone formed. Aliquots of the *n*-hexane phase were injected on to the silica column and eluted with 1% of diisopropyl ether in *n*-hexane. The mobile phase was monitored at 254 nm and the fraction containing 2',3'-dihydrovitamin K_1 was collected and evaporated to dryness at ambient temperature using a rotary evaporator. Standard solutions of the purified 2',3'-dihydrovitamin K_1 in methanol were prepared and stored at 278°K.

Chromatography

The capacity ratios were calculated from the retention times of the K vitamins and of an unretained compound (menadione metabisulphite). Oxygen was removed from the mobile phase by bubbling through oxygen-free nitrogen pre-saturated with the mobile phase.

Preparation of the samples

Blood samples were taken by venepuncture from healthy volunteers and patients under anticoagulation therapy with fenprocoumon. Blood samples were also taken from patients receiving PK, who responded too strongly to the anticoagulation therapy. The samples were taken before and after oral or intravenous administration of PK.

All samples were collected in heparinized tubes and centrifuged at 1000 *g* for 5 min. The plasma was decanted and stored at 243°K until analysis.

Isolation procedure

To 2 ml of plasma were added 50 μl of methanol containing 5 ng of the internal standard. Deproteinization was effected by mixing with 3 ml of isopropanol for 30 sec on a whirlimixer. Lipids were extracted by mixing with 10 ml of *n*-hexane for 2 min. After centrifugation for 10 min at 1000 *g*, the upper layer was removed and evaporated at ambient temperature at reduced pressure using a rotary evaporator.

Sample clean-up

A 500-mg amount of activated SI 60 (50–200 μm) was transferred into a Pasteur pipette in which a plug of cotton-wool had been inserted. A second plug was then placed on top of this small silica column. The column was conditioned by flushing through 10 ml of *n*-hexane in an upwards direction. After complete sedimentation the top plug was firmly pushed on to the silica.

The extraction residue was dissolved in 100 μl of *n*-hexane and placed on the small silica column. A fraction of the lipids was removed by eluting with 2.5 ml of 15% toluene in *n*-hexane, then the fraction containing the K vitamins was eluted with 5 ml of 3% diisopropyl ether in *n*-hexane. This fraction was evaporated to dryness, the dry residue was dissolved in 200 μl of methanol and aliquots of 20–50 μl were injected on to the analytical column.

During the isolation and clean-up procedures the K vitamins were shielded from light by using low actinic glass tubes and wrapping all other glassware in aluminium foil.

RESULTS AND DISCUSSION

Preparation of the internal standard

Under the conditions described only 4% of PK remains unchanged after 1 h. Nevertheless, removal of PK is necessary. Normal-phase HPLC can be used for this purpose, as 2′,3′-dihydrovitamin K_1 is completely separated from PK. Although in the literature[8] Raney nickel is recommended as a catalyst rather than palladium or platinum in order to avoid the formation of 5,6,7,8,2′,3′-hexahydrovitamin K_1, we did not observe the formation of this compound.

Chromatography

PK and K1-ep can be well separated in normal-phase systems consisting of a stationary phase of silica and mobile phases of *n*-hexane with isopropanol, acetonitrile or dioxane as a modifier. Unfortunately, the selectivity towards the plasma background is not sufficient for the determination of PK or K1-ep in human plasma samples. On the other hand, mobile phases with ethers as modifiers offer a much better selectivity towards the plasma background, but the resolution between PK and the K1-ep is not complete. Therefore, a simultaneous determination of PK and K1-ep in human plasma is possible only by using reversed-phase systems with selective detection.

In a reversed-phase system consisting of Hypersil-MOS as stationary phase and methanol–water (92.5:7.5) containing 0.03 *M* sodium perchlorate as the mobile phase, a good separation of PK, K1-ep and the internal standard is obtained, as shown in Fig. 2. Next to these K_1 vitamins, two K_2 vitamins, the so-called menaquinones, were injected. Although the presence of these menaquinones in human plasma has not been established so far, we accounted for a possible interference in the assay, but from Fig. 2 it can be seen that these menaquinones can be readily determined with the same system.

Fluorimetric detection of K-1 epoxide

K1-ep requires a relatively low potential for electrochemical reduction. Although the electrochemical mechanism is not completely understood, we observed

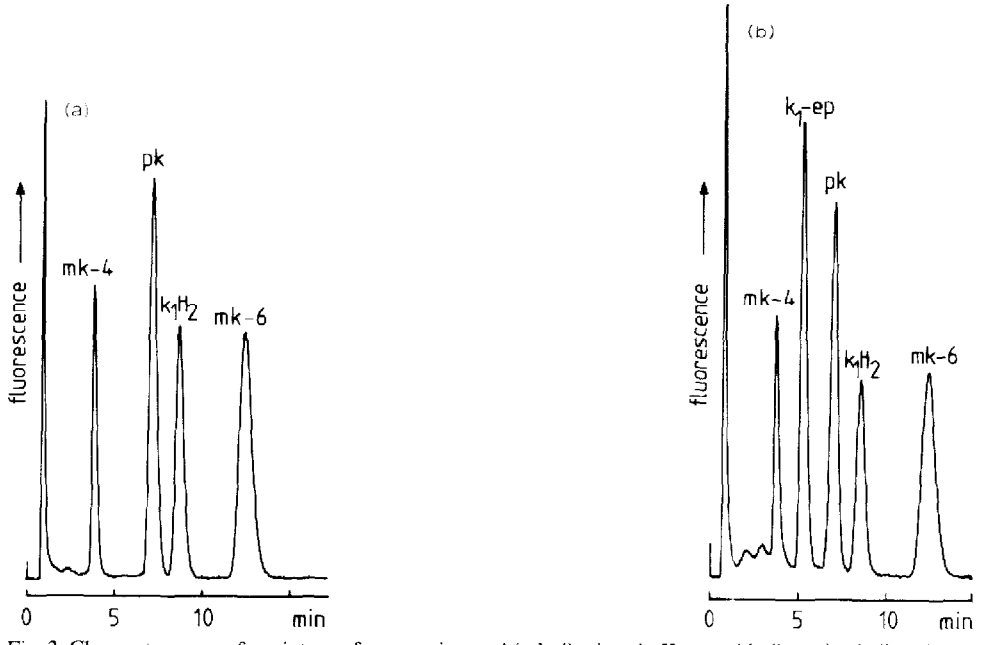


Fig. 2. Chromatograms of a mixture of menaquinone-4 (mk-4), vitamin K_1 epoxide (k_1-ep), phylloquinone (pk), 2',3'-dihydrovitamin K_1 (k_1H_2) and menaquinone-6 (mk-6) Experimental conditions: stationary phase, Hypersil-MOS (5 μm); mobile phase, methanol–water (92.5:7.5) containing 0.03 *M* sodium perchlorate; flow-rate, 0.6 ml/min. Applied potentials: (a) −400 mV and (b) −1800 mV.

that the products isolated after electrochemical reduction of PK and K1-ep show the same chromatographic behaviour. After re-oxidation with cerium(IV) sulphate, both products result in a single chromatographic peak with a capacity ratio corresponding to PK. As the final products are apparently identical, the sensitivity of the fluorimetric detection after coulometric reduction will be about the same. The relationship between the fluorescence signal and the potential applied to the coulometric cells for PK and K1-ep are shown in Fig. 3. For K1-ep the maximum signal is obtained at −1750 mV. From Fig. 3 it can also be seen that at this potential the peak height of PK is about 20% lower than at −400 mV, probably owing to some adsorption on the electrode surface. The peak area remains the same, indicating a reversible adsorption process. As a result of this phenomenon, the limit of detection for PK is higher than at −400 mV. In fact, with a mobile phase consisting of methanol–water (92.5:7.5) with 0.03 *M* sodium perchlorate, the limits of detection for both compounds are about 60 pg. Electrochemical detection in the reduction or re-oxidation mode[6] at these low potentials does not offer sufficient selectivity for the determination of low levels of K1-ep. The selectivity towards the background of the fluorimetric detection is hardly influenced by the potential, as is shown in Fig. 2. Although the manufacturer advises avoidance of the use of potentials lower than −1000 mV in order to maintain selectivity for electrochemical detection and stability of the electrode material, we observed no deterioration of the electrode performance during our experiments, provided that that the electrodes were regenerated overnight by applying a positive potential of 500 mV.

Isolation procedure

Isopropanol proved to be the most effective alcohol in penetrating the lipo-

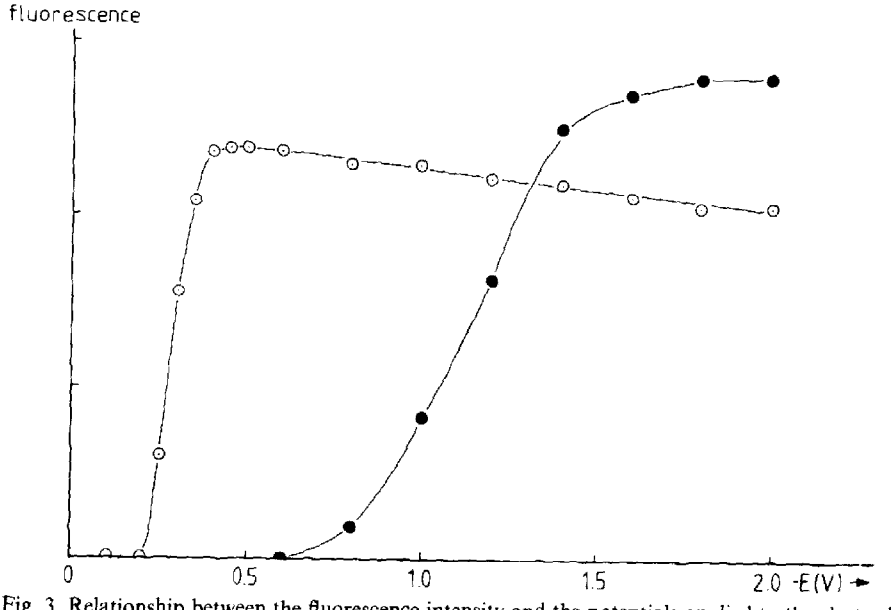


Fig. 3. Relationship between the fluorescence intensity and the potentials applied to the electrodes of the electrochemical cell for phylloquinone (○) and vitamin K-1 epoxide (●).

proteins, enabling quantitative extraction of the lipids. Extraction procedures involving methanol and chloroform or dichloromethane with methanol offer equally high recoveries and reproducibilities, but have some practical drawbacks such as emulsification, which is difficult to break, problems with respect to complete removal of the lower layer, the necessity for drying and the problematic evaporation.

In contrast, the extraction procedure described is much easier to carry out in routine analysis. A minor drawback to the use of isopropanol is its solubility in the upper phase, which implies co-extraction of some of the more polar compounds. Analysis of the extracts by normal-phase HPLC without further clean-up leads to rapid deterioration of the efficiency of the column owing to irreversible adsorption of these polar compounds on the silica surface. This problem does not exist when using reversed-phase systems. Another disadvantage of this extraction procedure is the necessity for evaporation of the *n*-hexane–isopropanol layer prior to clean-up with silica. For all of the K vitamins described in this paper the recovery of the extraction is about 98% at a level of 10 ng/ml.

Clean-up

The clean-up procedure is based on the method of Barron and Hanahan[9], which is designed for the separation of neutral lipids from bovine liver tissue. The liquid solid extraction step can be based on the use of Sephadex LH-20, (de)activated alumina, silica, zeolite or Florisil and XAD-2. The best results with respect to recovery and clean-up are obtained with activated silica or 7% deactivated Florisil. As silica is cheaper and easier to handle than Florisil, it is to be preferred for column chromatography.

2′,3′-Dihydrovitamin K_1 is a better internal standard than menaquinone-6 (MK-6), which we used previously[6], because the solubility properties are similar to those of PK and K1-ep. Nevertheless, it should be noted that with extremely fatty plasma samples problems concerning the solubility arise, indicating that further improvement of the sample clean-up would be beneficial for general application of the method.

Quantification

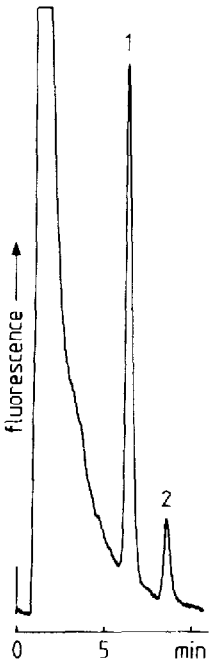
The linearity and precision of the method were determined by assaying samples of human plasma spiked with known amounts of K1-ep corresponding to concentrations of 1–1000 ng/ml. Peak-area ratios of K1-ep to the internal standard were plotted against the amount of K1-ep added to the plasma. The linearity, characterized by the correlation coefficient, was 0.99995.

The coefficient of variation (C.V.) for the determination of K1-ep in plasma within 1 day was 2.4% ($n = 5$), and that from day to day was 3.7% ($n = 10$).

Determination of K1-ep in human plasma samples

Fig. 4 shows the chromatogram of an extract of plasma from a patient sampled 14 h after oral administration of 10 mg of PK. The concentrations of both K1-ep and PK are relatively high, demonstrating that the method can be applied to the determination of plasma levels after oral administration of PK without any problems.

However, after administration of only coumarin anticoagulants, *i.e.*, without administration of PK, the plasma levels of K1-ep are expected to be very low, which



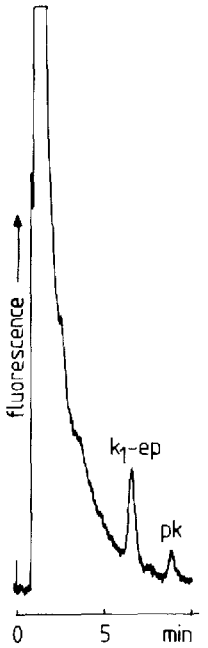


Fig. 4. Chromatogram of an extract of a human plasma sample, taken 14 h after oral administration of 10 mg of phylloquinone to a patient under anticoagulation therapy with fenprocoumon. 1 = Vitamin K_1 epoxide (9.6 ng/ml); 2 = phylloquinone (1.7 ng/ml). Conditions as in Fig. 2b.

Fig. 5. Extract of plasma of a patient under anticoagulation therapy with fenprocoumon. Peaks indicated are vitamin K_1 epoxide (k_1-ep; 0.92 ng/ml) and phylloquinone (pk; 0.28 ng/ml). Conditions as in Fig. 2b.

is confirmed by Fig. 5, which shows that endogenous K1-ep can also be determined with the described method. To the best of our knowledge, the presence of endogenous K1-ep in plasma samples has not been established before. In ten patients we found concentrations of K1-ep ranging from 150 pg/ml to 1.2 ng/ml. Next to K1-ep we observed that a small amount of PK was present in the plasma of these ten patients, probably originating from food uptake or from a certain type of depot in the body.

As expected, K1-ep is not detectable in the plasma of healthy volunteers, implying that the concentration of K1-ep is lower than 120 pg/ml. So far we must conclude that the endogenous level of K1-ep will be even lower than this level. We improved the detectability of K1-ep in order to determine extremely low concentrations by handling larger sample volumes, while the sample clean-up was modified in such a way that semi-preparative normal-phase HPLC was used. Even with a plasma volume of 10 ml no K1-ep could be detected in the plasma samples assayed.

CONCLUSIONS

The assay of PK by means of reversed-phase HPLC with electrofluorimetric reaction detection has been extended to K1-ep. The limit of detection is 60 pg corresponding to a plasma concentration of about 120 pg/ml. With the described

method, the presence of endogenous K1-ep in plasma samples of patients treated with fenprocoumon has been demonstrated. K1-ep could not be detected in plasma obtained from healthy volunteers.

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REFERENCES

1 J. T. Matschiner, R. G. Bell, J. M. Amelotti and T. E. Knauer, *Biochim. Biophys. Acta*, 201 (1970) 309–315.
2 T. D. Bjornsson, S. E. Swezey, P. J. Meffin and T. F. Blaschke, *Thromb. Haemostas.*, 39 (1978) 466–473.
3 H. Bechtold and E. Jähnchen, *J. Chromatogr.*, 164 (1979) 85–90.
4 M. De Metz, B. A. M. Soute, H. C. Hemker, R. Fokkens, J. Lugtenburg and C. Vermeer, *J. Biol. Chem.*, 257 (1982) 5326–5329.
5 O. Hiroshima, K. Abe, S. Ikenoya, M. Ohmae and K. Kawabe, *Yakugaku Zasshi*, 99 (1979) 1007–1014.
6 J. P. Langenberg and U. R. Tjaden, *J. Chromatogr.*, 305 (1984) 61–72.
7 U. R. Tjaden, M. T. H. A. Meeles, C. P. Thys and M. van der Kaay, *J. Chromatogr.*, 181 (1980) 227–235.
8 M. Tishler, L. F. Fieser and N. L. Wendler, *J. Amer. Chem. Soc.*, 62 (1940) 2866–2871.
9 E. J. Barron and D. J. Hanahan, *J. Biol. Chem.*, 231 (1958) 493–503.