*Journal of Chromatography*, 305 (1984) 61—72
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1880

# DETERMINATION OF (ENDOGENOUS) VITAMIN $K_1$ IN HUMAN PLASMA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING FLUOROMETRIC DETECTION AFTER POST-COLUMN ELECTROCHEMICAL REDUCTION

## COMPARISON WITH ULTRAVIOLET, SINGLE AND DUAL ELECTROCHEMICAL DETECTION

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(First received March 30th, 1983; revised manuscript received July 29th, 1983)

SUMMARY

A new method for the fluorometric detection of $K_1$ and $K_2$ vitamins using post-column electrochemical reduction is described. Dual electrochemical detection in the reduction/reoxidation mode for coulometric/coulometric as well as coulometric/amperometric detection appears to be more sensitive and selective towards the plasma background than simple reductive electrochemical detection, but fluorometric detection after coulometric reduction offers the best results. Combination of normal-phase chromatography and the described method is only possible if supporting electrolyte is added post-column, but leads to higher detection limits.

This highly sensitive method is applied to the determination of vitamin $K_1$ in human plasma samples.

## INTRODUCTION

Vitamin K (Fig. 1) is an essential cofactor in a microsomal enzyme system that activates the precursors of the blood-clotting factors II, VII, IX and X. In order to obtain quantitative information about the role of vitamin K in haemostasis a sensitive method of analysis is required.

Several papers describing the analysis of low concentrations of vitamin $K_1$ in milk and plasma using high-performance liquid chromatography (HPLC) with UV detection have been published recently [1—4]. However, due to the

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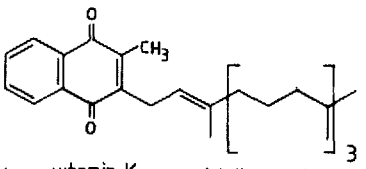
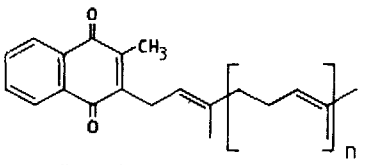
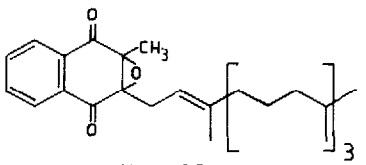


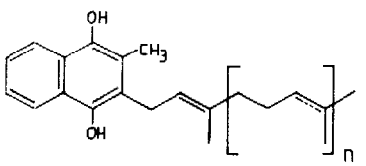

*trans*-vitamin $K_{1(20)}$ (phylloquinone)




*trans*-vitamin $K_{2(5n)}$ (menaquinone-n)




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




*trans*-vitamin K-hydroquinone

**Fig. 1. Structural formulae of the K vitamins of interest.**

unfavourable ratio of the amount of $K_1$ to the other lipids extracted from the biological material, an off-line multidimensional chromatographic system is required for the complete separation of $K_1$ from the interfering lipids. Such a multidimensional chromatography is not practical for routine analysis because two chromatographic systems are needed. Besides, eluent fractionation with extra sample handling as evaporation and second injection is necessary. Use of a more selective and sensitive detection could simplify the assay considerably.

Electrochemical detection of $K_1$ based on the amperometric reduction of the quinone has been applied to the determination of relatively high concentrations of $K_1$ in rat plasma [5, 6]. However, detection in the reductive mode appears to be non-selective towards the plasma background resulting in unfavourable limits of detection.

The selectivity of detection can be improved by reoxidizing the formed hydroquinone using a dual-electrode configuration [7]. This can be done with amperometric [8] as well as with coulometric detectors.

Although amperometric detectors generally offer low detection limits in the subnanogram range, one of the main drawbacks of dual amperometric detection is that only a small fraction of the analyte is reoxidized which results in loss of sensitivity. With coulometric detectors theoretically 100% of the analyte is converted. This method appeared to be applicable to deter-

mine $K_1$ levels after oral and intravenous administration, but endogenous levels (0.1—0.7 ng/ml [2], 0.9—7.8 ng/ml [4]) cannot be monitored. The combination of coulometric reduction with amperometric reoxidation appears to be more promising.

Generally, fluorometric detection is a sensitive and selective method. However, since the K vitamins do not possess native fluorescence, a reduction to the highly fluorescent hydroquinone is necessary. Methods based on chemical [9—11] and photochemical reduction [11] have been described. These methods require post-column addition of reagents which can cause problems concerning reproducibility in long-term routine analysis.

The aim of our work was to develop an on-line fluorometric detection method with sufficient selectivity towards the plasma background by applying a complete electrochemical, i.e. coulometric, reduction after a single chromatographic run. The method is applied to the determination of $K_1$ in human plasma and its capabilities are compared with single and dual electrochemical detection.

## EXPERIMENTAL

### *Apparatus*

The liquid chromatograph was constructed from commercially available and custom-made parts and consisted of a thermostatted glass eluent reservoir (293 K), a reciprocating plunger membrane pump (DMP 1515, Orlita, Giessen, F.R.G.) with a Bourdon-type damping system, a manometer, an injection device (Model U6K, Waters Assoc., Milford, MA, U.S.A.) and a thermostatted column (stainless-steel precision-bore tubing, 100 × 3.0 mm I.D.). A fixed-wavelength detector (Model 440, Waters Assoc.) operating at 254 nm, an amperometric electrochemical detection system (E.D.T., London, U.K.), a dual coulometric detection system (Model 5100-A, Coulochem, E.S.A., Bedford, MA, U.S.A.) and a fluorometric detection system of the double-monochromator type (SFM-23LC, Kontron, Zurich, Switzerland) were used for detection.

Chromatograms were recorded on flat-bed recorders (BD 8, Kipp & Zn., Delft, The Netherlands). Columns were packed by means of an air amplifier booster pump (DSTV-122, Haskel, Burbank, CA, U.S.A.) according to a procedure described elsewhere [12].

### *Chemicals and materials*

Vitamins $K_1$, $K_1$ epoxide, $K_{2(20)}$ and $K_{2(30)}$ were kindly donated by Hoffmann-La Roche (Mijdrecht, The Netherlands). Organic solvents were of analytical grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Water was purified by means of a Milli-Q Water Purification System (Millipore, Bedford, MA, U.S.A.). Hypersil-MOS 5 $\mu$m (Shandon, Cheshire, U.K.) was purchased from Ahrin (The Hague, The Netherlands).

### *Procedures*

*Chromatography* Capacity ratios were calculated from the retention times of the compounds and of an unretained compound, for which potassium

periodate was used. The theoretical plate height was calculated from the retention time and half the peak width at 0.6 of the peak height. The detection limits are based on a signal-to-noise ratio of 3.

*Removal of oxygen from the mobile phase.* Oxygen was removed from the mobile phase by continuously bubbling through nitrogen that was made oxygen-free by means of a pyrogallol solution and that was presaturated with the mobile phase. No attempts were made to remove oxygen from the samples.

*Preparation of the plasma samples.* Blood samples were taken before and after oral administration of vitamin $K_1$ to a healthy volunteer. The samples were collected in heparinized tubes and centrifuged at 1000 *g* for 5 min. The plasma samples were stored at 243°K until analysis.

*Isolation of K vitamins from plasma.* To 1 ml of plasma in a brown conical flask 50 ng of $K_{2(30)}$ were added as an internal standard. Then 1 ml of a sodium chloride solution (0.9%, w/w) was added. The proteins were denaturated by mixing with 3 ml of isopropanol. After addition of 10 ml of *n*-hexane the stoppered tubes were placed on a vertically rotating disk for 1 h at 20 rpm. After centrifugation for 10 min at 1000 *g* the upper layer was removed and evaporated at reduced pressure at ambient temperature. The residue was dissolved in 1 ml of methanol. Aliquots of 50 µl were injected onto the column. The samples were shielded from light.

## RESULTS AND DISCUSSION

### *Chromatographic phase system*

The K vitamins are strongly hydrophobic compounds only differing in carbon chain length and number of double bonds. These compounds can be separated by normal-phase as well as by reversed-phase chromatography. In this study we focussed our main attention on the reversed-phase systems because of their compatibility with electrochemical detection and reaction systems. The modifier content has to be very high (90—100%) in order to

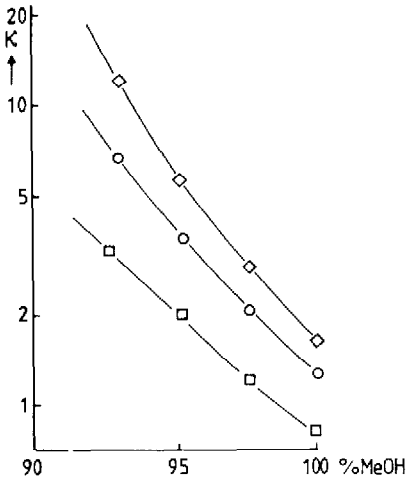



Fig. 2. Log—log plot of the relationship between the capacity ratios of vitamins $K_1$ (○), $K_{2(20)}$ (□) and $K_{2(30)}$ (◇) and the methanol content of the mobile phase.

achieve reasonably small capacity ratios and consequently short analysis times. Both methanol and acetonitrile can be used as modifier, but we prefer methanol for the reasons outlined below.

The relation between the capacity ratios of $K_1$, $K_{2(20)}$ and $K_{2(30)}$ and the methanol content of the mobile phase is given as a double-logarithmic plot in Fig. 2.

The phase system was optimized with respect to the selectivity between the K vitamins and co-extracted plasma constituents. A methanol content of 92.5% offers the best results, while 95% can be used for higher concentrations of K vitamins, e.g. after administration of K vitamins. This phase system appeared to be stable for several months, showing sufficient efficiencies (theoretical plate number = 2500—3000 on 10-cm columns at a flow-rate of 1 ml/min) to obtain adequate detection limits.

*Electrochemical detection*

The influence of the composition of the mobile phase, the concentration of the supporting electrolyte and the pH on the reduction and reoxidation signals was investigated for the coulometric detection system. If oxygen is not removed from the mobile phase, the background current of the reductive electrode is raised to such an extent that no reduction or oxidation signals can be observed. Removal of oxygen by bubbling through nitrogen proved to be satisfactory.

The influence of the electrolyte concentration on the reduction and oxidation signals is shown in Fig. 3a and b. Without sodium perchlorate no response is obtained. At very low concentrations of electrolyte the response increases with the concentration of the electrolyte. Although the addition of 0.1% of

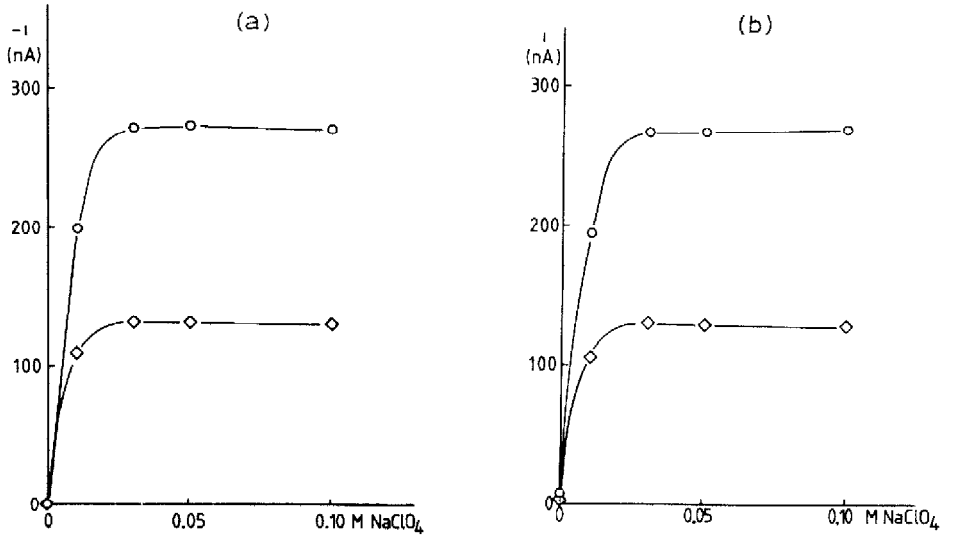



Fig. 3. (a) Influence of the concentration of $NaClO_4$ on the reduction signals. Conditions: stationary phase, Hypersil-MOS (5 μm); mobile phase, 5% water in methanol; flow-rate, 1.0 ml/min; applied potential, −400 mV. (○), $K_1$ (50 ng); (◇), $K_{2(30)}$ (35 ng). (b) Influence of the concentration of $NaClO_4$ on the oxidation signals. Conditions as for Fig. 3a, applied potential +400 mV.

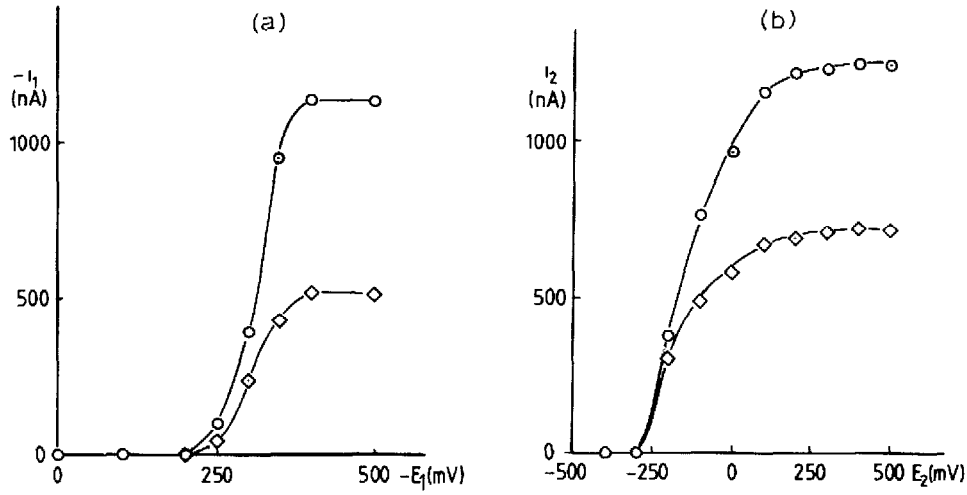



Fig. 4. (a) Relation between the reduction current and the applied potential. Conditions as for Fig. 3a, mobile phase containing 0.03 *M* $NaClO_4$. Potential applied to electrode 2, +400 mV. (b) Relation between oxidation current and potential applied to cell 2 for $K_1$ (○) and $K_{2(30)}$ (◇). Conditions: stationary phase, Hypersil-MOS (5 μm); mobile phase, 5% water in methanol, containing 0.03 *M* $NaClO_4$. Flow-rate 1.0 ml/min; potential applied to cell 1, —400 mV.

perchloric acid results in lower absolute reduction and oxidation potentials, a concentration of 0.03 *M* of sodium perchlorate appeared to be optimal with respect to the signal-to-noise ratio.

In comparison with methanol—water mixtures, the baseline noise levels with acetonitrile—water mixtures are increased, while the response is about the same resulting in inferior detection limits.

Typical relations between reduction and oxidation currents and the applied potentials are shown in Fig. 4a and b. In long-term use the relations between current and potential tend to change to a small extent, probably due to pollution of the electrodes. Regeneration appeared to be possible by changing the polarities of the potentials of the electrodes overnight. In a phase system consisting of 7.5% water in methanol with 0.03 *M* sodium perchlorate the detection limit of $K_1$ is about 250 pg in the reductive mode and about 150 pg in the reoxidation mode.

The yield of conversion is determined by injecting known amounts of the K vitamin and measuring the number of coulombs involved in the conversion. For the reduction of $K_1$ the yield of conversion to the hydroquinone amounts to at least 99.6%, which is confirmed by the signal of the second electrode, having the same potential as the first electrode (—400 mV), where no peaks were observed.

Coulometric detectors convert electrochemically active compounds with absolute half-wave potentials even higher than those of the compounds of interest, resulting in a decreased selectivity of detection in comparison with amperometric detection. Therefore we combined the coulometric cells, applied in the reduction mode, with an amperometric cell in the oxidation mode. Although with this combination the noise level appeared to be increased to a

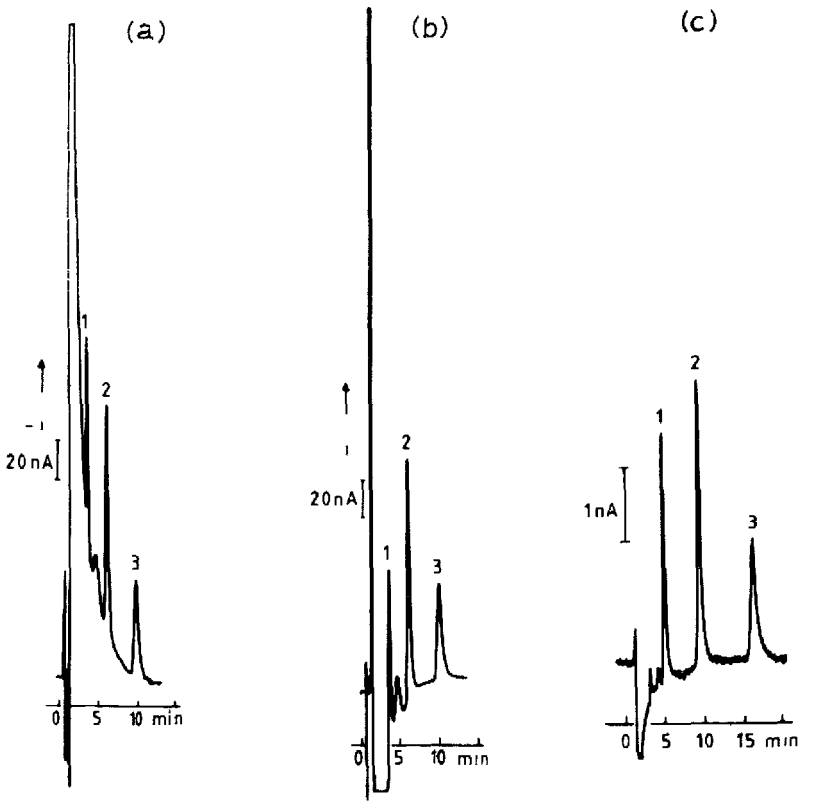



Fig. 5. Comparison of the discrimination power of detection by coulometric reduction (a), coulometric reoxidation (b) and amperometric reoxidation (c). Conditions: stationary phase, Hypersil-MOS (5 $\mu$m); mobile phase, 5% water in methanol, containing 0.03 *M* $NaClO_4$, flow-rate, 1.0 ml/min. Applied potentials: (a) —400 mV, (b) +400 mV, (c) cells 1 and 2 —400 mV, amperometric cell +300 mV vs. Ag/AgCl. 1 = $K_{2(20)}$ (50 ng), 2 = $K_1$ (50 ng), 3 = $K_{2(30)}$ (35 ng).

small extent resulting in a detection limit of 280 pg, this combination has to be preferred to the coulometric detection because of the better selectivity of detection when determining $K_1$ in plasma, as is shown in Fig. 5a—c.

*Fluorometric detection*

The influence of parameters such as solvent type, modifier content, sodium perchlorate concentration, addition of perchloric acid, solvent flow-rate and the choice of excitation and emission wavelengths, was investigated with the UV detector placed between the analytical column and the electrochemical detector, now used as a post-column reactor. Comparison of the UV signals and the fluorescence signals makes it possible to observe the net changes in fluorescence yield.

The absence of oxygen is essential for the fluorometric detection of K vitamins. If oxygen is present, either the K vitamins are not reduced and consequently no fluorescence is observed, or the fluorescence is completely quenched. The influence of the flow-rate on the fluorescence signal was very small, which means that the K vitamins are reduced quantitatively at the normally used flow-rates (0.4—1.5 ml/min).

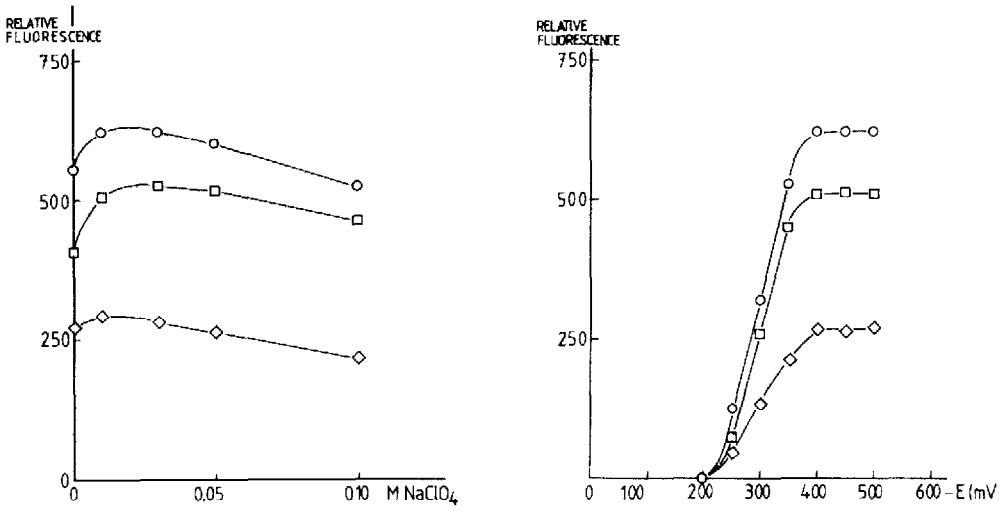



Fig. 6. Influence of the concentration of $NaClO_4$ in the mobile phase on the fluorescence signals of $K_1$ (○), $K_{2(20)}$ (□) and $K_{2(30)}$ (◇). Conditions: stationary phase Hypersil-MOS (5 μm); mobile phase 7.5% water in methanol; flow-rate, 1.0 ml/min; applied potentials —400 mV. $\lambda_{ex}$ = 320 nm, $\lambda_{em}$ = 420 nm.

Fig. 7 Relation between the fluorescence signals and the potentials applied to the electrodes for $K_1$ (○), $K_{2(20)}$ (□) and $K_{2(30)}$ (◇). Chromatographic conditions as for Fig. 6, mobile phase containing 0.03 *M* $NaClO_4$

The optimal wavelengths appeared to be 320 nm and 420 nm for excitation and emission, respectively.

The sodium perchlorate concentration in the mobile phase hardly influences the fluorescence as is shown in Fig. 6. Even without supporting electrolyte in the mobile phase a considerable fluorescence is obtained. At higher perchlorate concentrations the capacity ratio is increased resulting in lower maximal outlet concentrations and consequently lower fluorescence signals. In Fig. 7 the relation between the fluorescence and the potential applied to the working electrodes of the coulometric cells is given. For $K_1$ and $K_2$ the reduction is complete with both cells operating at —400 mV.

Upon the addition of 0.1% perchloric acid complete reduction takes place at lower absolute potentials (—200 mV). However, the fluorescence signal is decreased to nearly 50%. Use of acetonitrile instead of methanol, which has the advantage of a lower pressure drop over the column and a slightly better efficiency, resulted in an increase of the noise level and so in a detection limit of about 150 pg. Using a mobile phase consisting of 5% water in methanol with 0.03 *M* of sodium perchlorate, the detection limit of $K_1$ is about 25 pg, and with 7.5% water about 50 pg, which is superior to UV detection (100 and 150 pg, respectively). Besides, the selectivity of this detection mode is much better than for UV detection, as is demonstrated in Fig. 8a and b.

Reduction of $K_1$ epoxide requires a much lower potential than reduction of $K_1$ and $K_2$ vitamins. With potentials as low as —1 V fluorescence was observed. Further lowering of the potentials results in an increased fluorescence. However, complete reduction could not be accomplished since at these

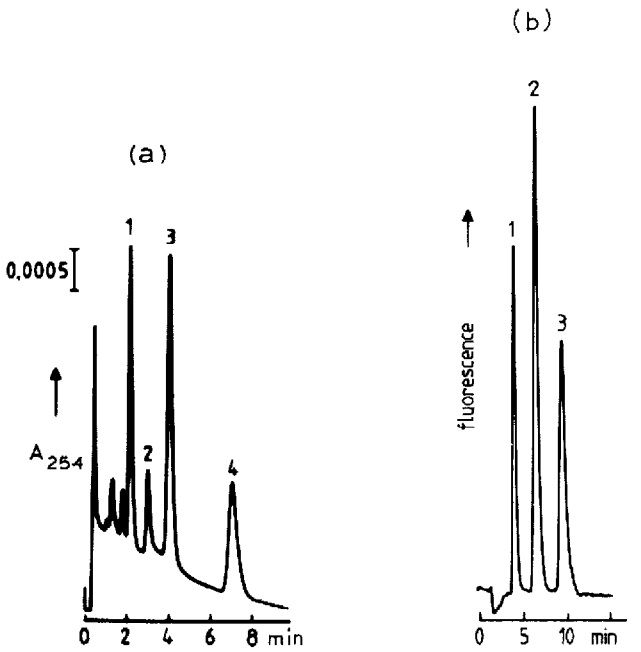



Fig. 8. Comparison of sensitivity and selectivity of UV detection at 254 nm (a) and fluorescence detection (b). Conditions as for Fig. 7. (a) 1 = $K_{2(20)}$ (50 ng), 2 = $K_1$ epoxide (50 ng), 3 = $K_1$ (50 ng), 4 = $K_{2(30)}$ (35 ng). (b) 1 = $K_{2(20)}$ (10 ng), 2 = $K_1$ (10 ng), 3 = $K_{2(30)}$ (7 ng).

potentials the electrochemical reaction system was electronically overloaded, meaning that the selected potential cannot be realized. The problem might be overcome by using a potentiostat with a higher capacity. The present method is less suited for the determination of small concentrations of $K_1$ epoxide.

*Fluorometric detection after normal-phase chromatography*

Since fluorometric detection of K vitamins after reversed-phase chromatography is possible even when no supporting electrolyte is added to the mobile phase, we also investigated the possibility of coulometric reduction and fluorometric detection in normal-phase systems.

A normal-phase system consisting of LiChrosorb Si 60 as stationary phase and 1% diisopropyl ether in *n*-hexane as the mobile phase combines good selectivities and sufficient retention to minimize peak broadening and consequently to optimize detection limits. However, such a system does not allow the direct use of the coulometric reaction system. It is not possible to dissolve sufficient supporting electrolyte, e.g. lithium perchlorate, to apply well-defined potentials. Therefore an electrolyte solution consisting of 0.1 *M* sodium perchlorate in methanol with 10% ethanol was added post-column to the mobile phase in a ratio of 7:3 according to a method used for the amperometric detection of *cis*- and *trans*-$K_1$ in a normal-phase system [6].

Although fluorometric detection appeared to be possible, the detection limits were much higher than with reversed-phase systems. The detection limit of $K_1$ amounted to about 5 ng, which, compared with 25 pg for reversed-phase

chromatography, cannot be explained only by the dilution due to the addition of supporting electrolyte solution. In fact the quantum efficiency for $K_1$ is much smaller in an apolar medium such as hexane than in a polar medium such as methanol, which was demonstrated by reducing a solution of $K_1$ with lithium boron hydride and measuring the fluorescence with a spectrofluorometer.

*Isolation of $K_1$ from plasma*

$K_1$ can be isolated from plasma by liquid—liquid extraction using an alcohol to liberate the lipids from the lipoprotein complex, followed by extraction with an apolar solvent such as *n*-hexane, diethyl ether or dichloromethane. The described method appeared to be the easiest and most reliable of all possible methods to extract $K_1$ from plasma.

The residue after evaporation has to be redissolved in at least 1 ml of methanol, since it is not possible to dissolve the lipids completely in smaller volumes. In the latter case lipid droplets will be formed in which a part of the amount of $K_1$ will dissolve preferentially. The recovery of $K_1$ when 1 ml of methanol is used, is 98%, while with 0.2 ml of methanol the recovery amounts to only about 30%.

These problems do not exist when using the described normal-phase system. The residue can be dissolved completely in 0.2 ml of *n*-hexane. Unfortunately the sensitivity and selectivity of the normal-phase system with UV detection are not adequate for accurate determination of the low endogenous levels.

The recovery of $K_1$ isolated from plasma was determined by extracting plasma samples spiked with amounts of $K_1$ in the range 1—1000 ng/ml. The peak areas were compared with those obtained by direct injection of the same amounts of $K_1$ dissolved in methanol or hexane. The recovery appeared to be 98.0 ± 2.2%.

*Quantification*

The linearity and precision of the determination of $K_1$ by HPLC with fluorometric detection, preceded by liquid—liquid extraction, were investigated by determining human plasma samples spiked with known amounts of $K_1$ varying from 1 to 1000 ng/ml.

The linearity, characterized by the correlation coefficient, amounts to 0.99998.

Because of the presence of endogenous $K_1$ in human plasma, the blank value was determined by the method of standard addition. The within-day and day-to-day coefficients of variation amount to 2.1% ($n$ = 5) and 3.2% ($n$ = 10), respectively.

*Application to plasma samples*

The developed method has been applied to the determination of $K_1$ in human plasma. Fig. 9 shows the chromatogram of an extract of a plasma sample taken from a healthy fasting volunteer. It is clearly demonstrated that the endogenous concentration corresponding to 2.3 ng/ml can be determined, even without any clean-up. It should be noted that, due to the large volume used to redissolve the sample, the injected amount is only 115 pg.

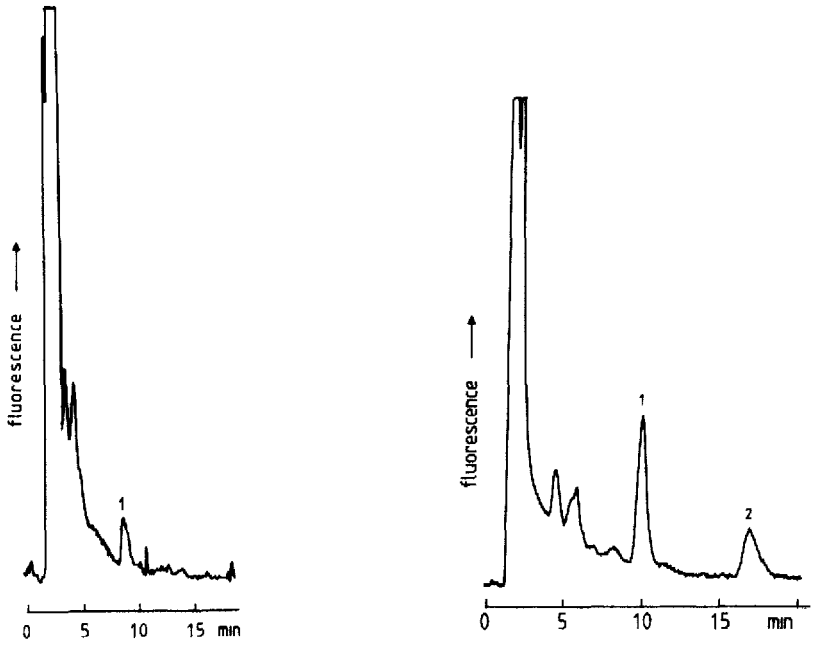



Fig. 9. Chromatogram of an extract of plasma of a healthy volunteer. 1 = phylloquinone ($K_1$). Conditions: see Fig. 8.

Fig 10. Chromatogram of an extract of plasma of a healthy volunteer after oral administration of 10 mg of phylloquinone. Blood sample was taken 6 h after administration. 1 = Phylloquinone ($K_1$), 2 = $K_{2(30)}$. Conditions: see Fig. 8, except for flow-rate = 0.9 ml/min.

Fig. 10 shows a chromatogram of an extract of a plasma sample taken from a healthy volunteer, 6 h after oral administration of 10 mg of $K_1$.

The method is directly applicable to the determination of $K_1$ in human plasma after administration. The determination of endogenous levels is also possible, although a further clean-up of the extract by fractionating the plasma lipids will increase the reliability of the method, especially when small changes in the endogenous level have to be observed.

The method is being applied to a pharmacokinetic study of $K_1$ and to the determination of $K_1$ in vegetables.

## CONCLUSIONS

A relatively simple method based on improvement of the detection for the determination of low concentrations of K vitamins in biological specimens is described. Post-column coulometric reduction combined with either fluorometric detection or amperometric reoxidation allows the determination of endogenous levels of vitamin $K_1$. Fluorometric detection has the lowest detection limit (25 pg), coulometric reoxidation shows a better detection limit (150 pg) than amperometric reoxidation (280 pg), but the selectivity of the latter mode is more favourable.

Combination of normal-phase chromatography with the described method is possible but more complicated and is inferior to reversed-phase chromatography.

Further research will be devoted to the optimization of the sample pretreatment in order to increase the recovery, and will be reported in due course.

ACKNOWLEDGEMENTS

This study is supported by the Dutch Thrombosis Foundation. The authors gratefully thank Kipp & Zn., Delft, The Netherlands, for the loan of the coulometric detection system.

REFERENCES

1 Y. Haroon, M.J. Shearer, S. Rahim, W.G. Gunn, G. McEnery and P. Barkhan, J. Nutr., 112 (1982) 1105—1117.
2 M.J. Shearer, S. Rahim, P. Barkhan and L. Stimmler, Lancet, 8296 (1982) 460—461.
3 M.F. Lefevere, A.P. de Leenheer and A.E. Claeys, J. Chromatogr., 186 (1979) 749—762.
4 M.F. Lefevere, A.P. de Leenheer and A.E. Claeys, Int J Vit. Nutr. Res., 51 (1981) 179—180.
5 S. Ikenoya, K. Abe, T. Tsuda, Y. Yamano, O. Hiroshima, M. Ohmae and K. Kawabe, Chem. Pharm. Bull., 27 (1979) 1237—1244.
6 O. Hiroshima, S. Ikenoya, M. Ohmae and K. Kawabe, Chem. Pharm. Bull., 29 (1981) 451—455.
7 D.A. Roston, R.E. Shoup and P.T. Kissinger, Anal. Chem., 54 (1982) 1417A—1434A.
8 J.C. Hoogvliet, F. Elferink, C.J. van der Poel and W.P. van Bennekom, Anal. Chim. Acta, in press.
9 K. Abe, O. Hiroshima, K. Ishibashi, M Ohmae, K. Kawabe and G. Katsui, Yakugaku Zasshi, 99 (1979) 192—200.
10 O. Hiroshima, K. Abe, S. Ikenoya, M. Ohmae and K. Kawabe, Yakugaku Zasshi, 99 (1979) 1007—1013.
11 M.F. Levefere, R.W. Frei, A.H.M.T. Scholten and U.A.Th. Brinkman, Chromatographia, 15 (1982) 459—467.
12 U.R. Tjaden, M.T.H.A. Meeles, C.P. Thys and M. van der Kaay, J. Chromatogr., 181 (1980) 227—241.