Journal of Chromatography, 430 (1988) 21–29
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4245

SIMULTANEOUS DETERMINATION OF VITAMIN K_1, VITAMIN K_1 2,3-EPOXIDE AND MENAQUINONE-4 IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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(First received January 25th, 1988; revised manuscript received April 7th, 1988)

SUMMARY

A highly sensitive method for measuring endogenous vitamin K_1, menaquinone-4 (which is one of the K_2 vitamins) and vitamin K_1 2,3-epoxide in human plasma was developed, based on high-performance liquid chromatography with coulometric reduction and fluorimetric detection, following extraction from plasma and purification on a Sep-Pak silica cartridge. The detection limits of vitamin K_1, menaquinone-4 and vitamin K_1 2,3-epoxide were 5, 5 and 8 pg per injection for the standard substances and 30, 30 and 50 pg/ml in human plasma, respectively.

INTRODUCTION

Vitamin K_1 2,3-epoxide (K_1-epo) is the main metabolite of vitamin K_1 (K_1) and has an important role in the vitamin K cycle in relation to clotting factor synthesis in the liver [1]. A highly sensitive method is required for the measurement of endogenous K_1-epo in biological materials because it is present in only very small amounts.

Several methods for the measurement of K_1-epo in biological materials using high-performance liquid chromatography (HPLC) based on ultraviolet [2–4] and fluorimetric detection [5, 6] have been reported. However, these methods cannot be applied to the determination of endogenous K_1-epo. Recently, the endogenous level of K_1-epo in pooled plasma from fasting subjects was found to be 15 pg/ml ($n=2$) based on HPLC combined with zinc reduction and fluorimetric detection [7], but this was only a preliminary result.

In a previous paper [8], we described a highly sensitive method for measuring

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K_1 and K_2 (MK-n) in human and animal plasma using HPLC combined with coulometric reduction and fluorimetric detection, but the method could not be applied to the determination of endogenous K_1-epo because it was not reduced under the assay conditions. In this paper, we describe a highly sensitive method for the simultaneous determination of K_1, K_1-epo and MK-4 in human plasma using an HPLC system which is a modification of the previous method [8].

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Shimadzu LC-3A reciprocating pump, a Rheodyne 7125 injection valve, a Rheodyne 7000 six-way switching valve with a Rheodyne 7001 pneumatic actuator, an Izumi FA-1 programmable controller, a Hitachi Super Bebicon, which was used to change the switching valve, a Shimadzu LC-3A reciprocating pump, a precolumn (5 cm×4.6 mm I.D.) packed with TSKgel ODS-120T (5 μm) (Tosoh, Tokyo, Japan), an analytical column (20 cm×4.6 mm I.D.) packed with TSKgel ODS-120 T (5 μm), an Environmental Sciences (ESA, Bedford, MA, U.S.A.) 5020 guard cell, an ESA 5100A Coulochem equipped with an ESA 5010 analytical cell, which consisted of two porous graphite electrodes in series [8] and was used as a reduction reactor, a Hitachi 650-10S spectrofluorimeter and a Shimadzu C-R3A recorder. Each component was tightly connected with stainless-steel tubing (1 mm I.D.) through which the mobile phase was passed.

Chemicals and materials

K_1 was purchased from Wako (Osaka, Japan). K_1-epo and MK-4 were obtained from our laboratories. All other chemicals were of analytical-reagent grade.

Blood plasma was obtained from men (22–46 years) and women (25–45 years) 12 h before and 3 h after they had a meal that contained about 15 μg of K_1 by our measurements.

A stock standard solution containing K_1, K_1-epo and MK-4 was prepared by dissolving them in ethanol at 10 μg/ml. This solution was stable for at least two months when stored in the dark. A working standard solution was obtained by diluting the stock standard solution with ethanol to the desired concentration.

Assay procedure

A 1-ml plasma sample was placed in a brown glass centrifuge tube, then 1 ml of water, 4 ml of ethanol and 6 ml of n-hexane were added. The mixture was shaken for 5 min, followed by centrifugation at 800 g for 5 min. A 5-ml portion of the upper layer was transferred into a brown glass centrifuge tube and evaporated to dryness under reduced pressure at room temperature. After dissolving the residue in 2 ml of n-hexane by shaking for 1 min, the solution was applied to a Sep-Pak silica cartridge (Waters Assoc., Milford, MA, U.S.A.) which had previously been cleaned by successive washing with 10 ml of n-hexane–diethyl ether (96:4, v/v) and 10 ml of n-hexane. The Sep-Pak cartridge was washed with 10 ml of n-hexane and K_1, K_1-epo and MK-4 were eluted with 5 ml of n-hexane–diethyl ether

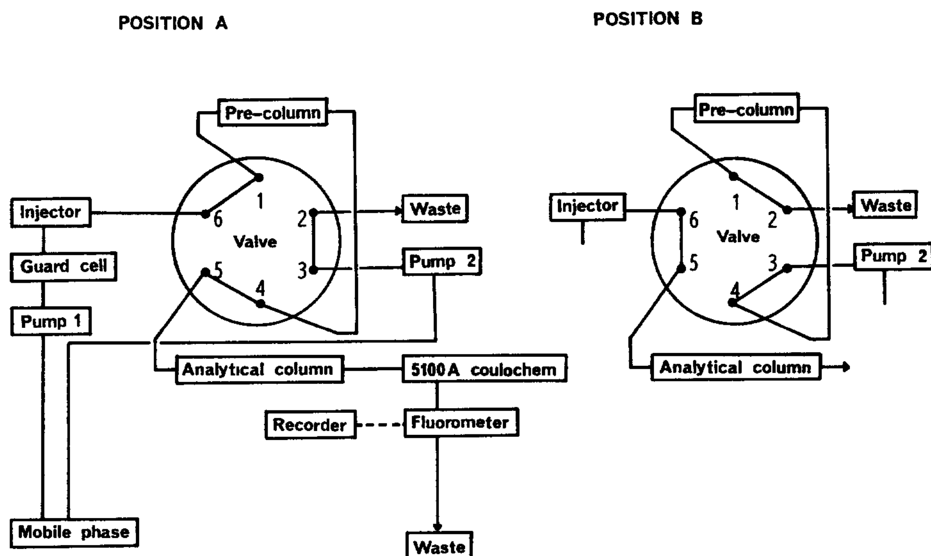


Fig. 1. Column-switching scheme.

(96:4, v/v). The eluate was transferred into a brown conical centrifuge tube and evaporated to dryness under reduced pressure at room temperature. The residue was dissolved in 200 μl of ethanol by shaking for 1 min, then the solution was filtered through a disposable 0.45-μm filter and 50 μl of the filtrate were injected into the HPLC system. At the same time, 50 μl of the working standard solution for the calibration graph were also injected into the system.

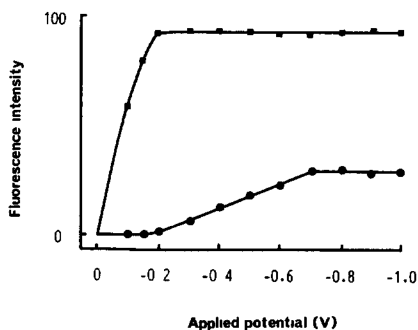
Chromatography

A sample extract was injected on to the precolumn and eluted with methanol–acetonitrile (6:4, v/v) containing 0.25% sodium perchlorate at a flow-rate of 1 ml/min for 6 min. The column-switching valve was set to position A (Fig. 1) at this time. Next, the valve was switched to position B (Fig. 1) and K_1, K_1-epo and MK-4 loaded on the top of the analytical column were separated and fed into a post-column reaction system for their reduction. Detection was performed by spectrofluorimetry at an excitation wavelength of 320 nm and an emission wavelength of 430 nm for 30 min. The precolumn was back-flushed with the mobile phase during the analytical period. The whole cycle was started again with the valve in position A, when the next sample extract was injected. K_1, K_1-epo and MK-4 concentrations in the sample extract were measured by the peak-height method and calculated from their calibration graphs. The mobile phase was deaerated by bubbling argon gas through it from 5 h before and throughout the measurements.

RESULTS AND DISCUSSION

Reduction conditions

K_1, K_1-epo and MK-4 must be reduced prior to their fluorimetric determination because they do not possess native fluorescence. K_1-epo requires a much



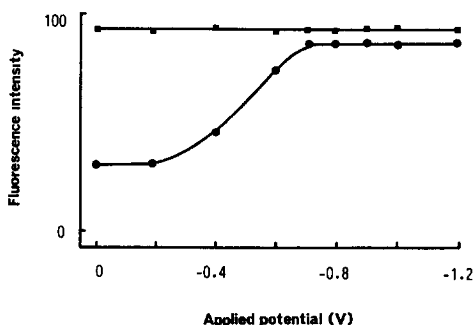


Fig. 2. Relationship between the fluorescence intensity and the potential applied to the upstream electrode with the potential of the downstream electrode constant fixed at 0 V. (■) K_1; (●) K_1-epo.

Fig. 3. Relationship between the fluorescence intensity and the potential applied to the downstream electrode with the potential of the upsteam electrode constant fixed at −0.8 V. (■) K_1; (●) K_1-epo.

lower negative potential than K_1 for electrochemical reduction. To optimize the applied potentials for the analytical cell, the relationship between the applied potential and the fluorescence intensity was examined by varying the applied potential. Two relationships were obtained, the first by keeping the potential of the downstream electrode constant at 0 V and varying the potential of the upstream electrode from 0 to −1.0 V (Fig. 2), and the second by keeping the potential of the upstream electrode constant at −0.8 V and varying the potential of the downstream electrode from 0 to −1.2 V (Fig. 3). It is apparent from these relationships that the maximum fluorescence intensity of K_1-epo occurred at a potential of −0.7 V at both electrodes, but the maximum intensity in Fig. 3 was about three times that in Fig. 2. Therefore, a potential had to be applied to both electrodes to determine K_1-epo, and a potential of −0.8 V was selected. On the other hand, the maximum fluorescence intensity of K_1 occurred at a potential of −0.2 V at the upstream electrode (Fig. 2) and the intensity did not increase on application of potential to the downstream electrode (Fig. 3). Hence K_1 could be completely reduced by the application of a potential of −0.2 V at one electrode. The behaviour of MK-4 was the same as that of K_1.

Next, an applied potential of −1.0 V was chosen for the guard cell, because oxidizing substances had to be effectively removed from the mobile phase. When the reduction force of the analytical cell became depressed, the cell was detached from the HPLC system and cleaned by successive washing with 5 ml/min of water for 5 min, 5 ml/min of 30% hydrogen peroxide for 10 min and 5 ml/min of water for 30 min. The cell was conserved by filling it with methanol.

Sodium perchlorate concentration

The effect of the sodium perchlorate concentration used as the electrolyte in the mobile phase on the fluorescence intensities of K_1 and K_1-epo was examined by varying the electrolyte concentration from 0 to 0.4% with both electrode potentials held constant at −0.8 V (Fig. 4). No fluorescence was obtained for K_1-epo without the electrolyte. The fluorescence intensity of K_1-epo increased with

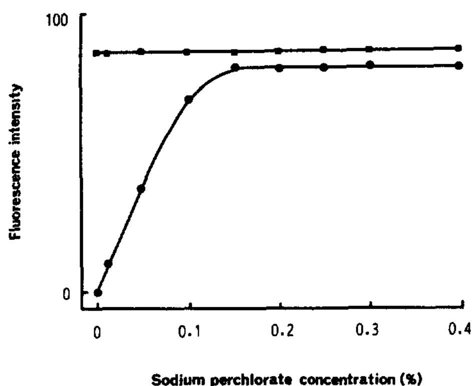


Fig. 4. Effect of sodium perchlorate concentration in the mobile phase on the fluorescence intensities of K_1 (■) and K_1-epo (●).

increasing electrolyte concentration at a low concentration (under 0.15%), with the maximum intensity being obtained at this concentration. Conversely, the maximum fluoresecence intensity of K_1 was obtained without the electrolyte. The behaviour of MK-4 was the same as that of K_1. When an electrolyte concentration of 0.20% was used, the maximum fluorescence intensity of K_1-epo was obtained at applied potentials of −0.8 V to the upstream electrode and −0.9 V to the downstream electrode. The operating manual for the analytical cell advises avoiding the use of a potential lower than −1.0 V in order to maintain the stability of the electrode materials. Therefore, an electrolyte concentration of 0.25% was chosen for the procedure.

Column and mobile phase

The separation of K_1, K_1-epo and MK-4 in human plasma using the reversed-phase system was examined by using three kinds of reversed-phase columns [Nucleosil C_{18} (5 μm), 25 cm×4.6 mm I.D. (Macherey–Nagel, Duren, F.R.G.), LiChrosorb RP-8 (7 μm), 25 cm×4.6 mm I.D. (Merck, Darmstadt, F.R.G.) and TSKgel ODS-120T (5 μm), 25 cm×4.6 mm I.D. (Tosoh)] and five kinds of mobile phases (methanol–water, ethanol–water, acetonitrile–water, methanol–acetonitrile and ethanol–acetonitrile) containing 0.25% sodium perchlorate. Good separations of K_1, K_1-epo and MK-4 were obtained on both Nucleosil C_{18} and TSKgel ODS-120T with methanol–acetonitrile (6:4, v/v), but the resolution and the symmetry of the peaks of K_1, K_1-epo and MK-4 in the chromatogram obtained using the TSKgel ODS-120T system were slightly better than that of the Nucleosil C_{18} system. Therefore, TSKgel ODS-120T with methanol–acetonitrile (6:4, v/v) was used in the procedure.

Removal of oxygen

Oxygen in the mobile phase greatly affects the fluorimetric detection of K_1, K_1-epo and MK-4. If it is present, it interferes with their reduction and their fluorescence intensities are markedly depressed. To remove oxygen from the mobile phase, the effect of argon gas on the fluorescence intensities of K_1 and K_1-epo

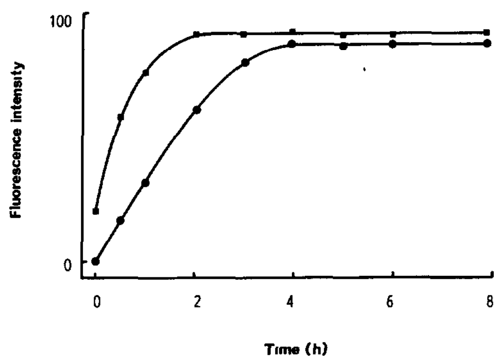


Fig. 5. Effect of the time of passage of argon gas through the mobile phase with the flow-rate fixed constant at 300 ml/min on the fluorescence intensities of K_1 (■) and K_1-epo (●).

was examined by varying the time argon gas was passed through the mobile phase from 0 to 8 h with the flow-rate fixed at 300 ml/min (Fig. 5). No fluorescence was obtained for K_1-epo without argon gas. The fluorescence intensity of K_1-epo increased as the time of passage was increased at short times (under 4 h), with the maximum intensity being obtained at 4 h. Conversely, some fluorescence was obained for K_1 without argon gas and the fluorescence intensity became maximal at a time of passage of 2 h which was shorter than that for K_1-epo. When the flow-rate was 100 or 200 ml/min, the maximum intensity of K_1-epo was obtained for times of passage of 9 and 6 h, respectively. The behaviour of MK-4 was the same as that of K_1. A flow-rate of 300 ml/min was adopted because any higher rate was liable to cause a change in the components of the mobile phase.

Measurement was started after a flow time of 5 h. Oxygen in air also interfered with the reduction of K_1-epo. When PTFE tubing was used instead of stainless-steel tubing to connect the components of the HPLC system, oxygen passed through the PTFE wall and mixed with the mobile phase. Consequently, no fluorescence was observed for K_1-epo, even if argon gas was passed through the mobile phase. Therefore, stainless-steel tubing had to be used for the reduction of K_1-epo.

Column switching

When the isocratic method was used for the simultaneous determination of K_1, K_1-epo and MK-4 in human plasma, the main problem was that there were other K vitamins and biological substances with retention times longer than those of K_1, K_1-epo and MK-4 [8]. As this could make the process time-consuming, a column-switching technique was used to overcome the problem.

Detection limit

Under the conditions described above, the detection limits of K_1, K_1-epo and MK-4 were 5, 8 and 5 pg per injection for the standard substances and 30, 50 and 30 pg/ml in human plasma, respectively.

Application to human plasma

The method was applied to the determination of K_1, K_1-epo and MK-4 in human plasma taken from healthy men and women 12 h before and 3 h after they had a meal which contained about 15 μg of K_1 (Table I). Fig. 6 shows the chromatograms obtained for plasma from the fasting and non-fasting man No. 4 in Table I. In both instances, the peaks of endogenous K_1, K_1-epo and MK-4 can be clearly seen, demonstrating that the method can be applied to their determination in plasma without any problems.

The recoveries of K_1, K_1-epo and MK-4 from human plasma were determined by extracting plasma to which 0.53–5.27 ng/ml K_1 ($n=12$), 0.50–5.03 ng/ml K_1-epo ($n=12$) and 0.45–4.48 ng/ml MK-4 ($n=12$) had been added. The values obtained for K_1, K_1-epo and MK-4 were 91, 90 and 92%, respectively. In Table I, no significant difference was found in the concentrations of K_1, K_1-epo and MK-4 between men and women, nor was any difference found in the concentrations of K_1-epo and MK-4 between fasting and non-fasting men and women. However, the K_1 concentrations between fasting and non-fasting male subjects differed significantly ($p<0.05$), indicating that the K_1 in the meal affected the K_1 concentration in plasma. This difference in K_1 concentrations for fasting and non-

TABLE I

ENDOGENOUS CONCENTRATIONS OF VITAMIN K_1, VITAMIN K_1 2,3-EPOXIDE AND MENAQUINONE-4 IN HUMAN PLASMA TAKEN FROM FASTING AND NON-FASTING SUBJECTS

Fasting and non-fasting indicate plasma taken, respectively, at 12 h before and 3 h after a meal containing about 15 μg of vitamin K_1.

Subject	K_1 (ng/ml)		K_1-epo (ng/ml)		MK-4 (ng/ml)	
	Fasting	Non-fasting	Fasting	Non-fasting	Fasting	Non-fasting
Men						
1	1.10	1.54	0.11	0.12	0.06	0.08
2	1.02	1.44	0.12	0.13	0.07	0.11
3	0.83	1.34	0.12	0.13	0.05	0.09
4	0.82	1.59	0.17	0.23	0.06	0.08
5	0.60	0.71	0.17	0.12	0.06	0.05
6	0.69	2.00	0.09	0.15	0.05	0.16
7	0.21	0.47	0.05	0.13	0.13	0.06
Mean	0.75	1.30	0.12	0.14	0.07	0.09
Women						
1	1.95	3.66	0.10	0.20	0.07	0.18
2	0.54	0.86	0.05	0.06	0.05	0.07
3	0.60	0.78	0.09	0.11	0.04	0.10
4	0.63	0.92	0.12	0.12	0.07	0.10
5	0.84	1.32	0.06	0.07	0.07	0.08
6	0.24	0.57	0.05	0.08	0.03	0.06
7	0.58	1.00	0.08	0.11	N.D.*	0.06
Mean	0.76	1.30	0.08	0.11	0.05	0.09

*The amount of menaquinone-4 was less than 30 pg/ml.

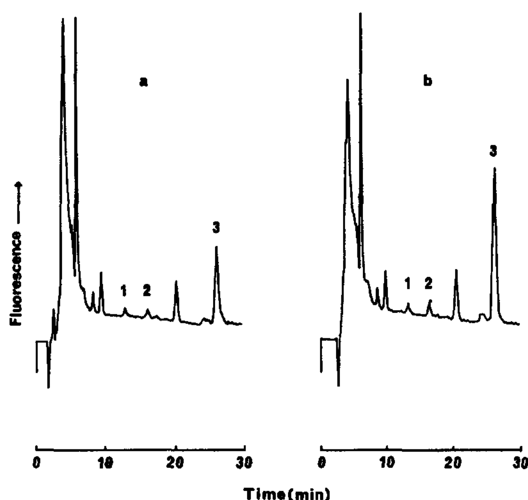


Fig. 6. Reversed-phase high-performance liquid chromatograms. (a) Extract of 1 ml of human plasma taken 12 h before a meal. (b) Extract of 1 ml of human plasma taken 3 h after a meal. Peaks: 1 = MK-4; 2 = K_1-epo; 3 = K_1.

fasting female subjects was also significant ($p < 0.05$) when the value of subject No. 1, which seemed to be abnormal, was omitted.

MK-4 epoxide was not detectable in human plasma, suggesting that its concentration was lower than 50 pg/ml. The detection limit of the standard MK-4 epoxide was the same as that of the standard K_1-epo. The K_1 values seem comparable to most recently reported values obtained using electrochemical [9] or fluorescence detection [7, 8, 10] and we have clearly shown the difference between fasting and non-fasting levels. The MK-4 values were probably the first to have been measured and are interesting in that respect.

CONCLUSIONS

The method described here shows good sensitivity for the detection of K_1, K_1-epo and MK-4 and can be used to measure their endogenous levels in human plasma. The detection limits for the standard substances are less than 10 pg compared with 25 pg for the zinc reduction method [11], which is the lowest among the reported methods. If MK-4 epoxide is present at levels above 50 pg/ml in human plasma, it also can be determined by the present method.

ACKNOWLEDGEMENTS

The authors are grateful to Drs. M. Narisada and M. Ohtani and Mr. F. Watanabe of our laboratories for providing the menaquinones.

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