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Note

Determination of K vitamins (phylloquinone and menaquinones) in umbilical cord plasma by a platinum-reduction column

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The K vitamins (VK) are required for the biosynthesis of γ-carboxyglutamic acid (Gla) in several proteins (factors II, VII, IX, and X, protein C, S, and Z, bone Gla protein, etc.). These VK-dependent proteins play crucial roles in haemostasis and calcification [1,2].

It is well known that neonatal and infantile VK deficiency causes melena neonatorum and intracranial haemorrhagic disorders. These disorders occur more frequently in breast-fed babies than bottle-fed babies, but the reason is still obscure [3].

Endogenous concentrations of VK (Fig. 1) in plasma are very low and the literature data differ [4]. It is difficult to determine plasma VK concentrations, especially those of the umbilical cord because they are much lower than those in adults.

We have previously reported the separation of K vitamins (K_1 and MK-4, 6, and 7) in placentae by thin-layer chromatography (TLC) followed by high-performance liquid chromatography (HPLC) using post-column chemical reduction and fluorimetric detection [5]. Recently, we have reported on the use of the electrochemical reduction method for the analysis of umbilical cord plasma and amniotic fluid [6,7]. Although this method is highly sensitive, it is unstable, very expensive and less efficient for prolonged use. In this paper, we describe a simple, stable, inexpensive and durable platinum-reduction column to follow the separation column. We obtained a sensitivity as high as that of electrochemical reduction [7–9].

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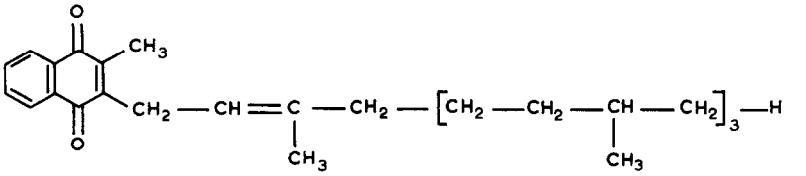

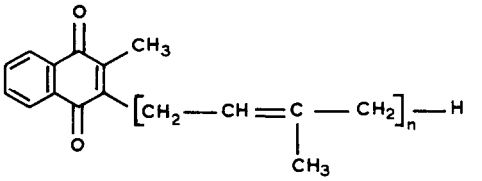

Fig. 1. Structures of the K vitamins.

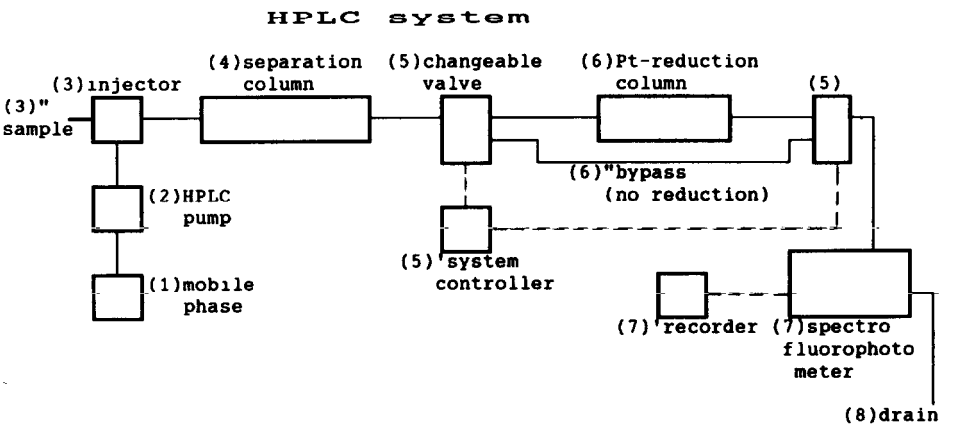

Fig. 2. Schematic representation of the chromatographic system.

EXPERIMENTAL

Chemicals

Standard vitamin K_1 was purchased from Wako (Kyoto, Japan) and standard menaquinones (MK-4, 6, and 7) were obtained from Shionogi Research Labs. (Osaka, Japan). All other chemicals were of the best grade commercially available for HPLC.

HPLC apparatus and conditions

The HPLC system (Fig. 2) consisted of an LC-3A reciprocating pump (Shimadzu, Kyoto, Japan), a Rheodyne Model 7125 syringe-loading sample injector

TABLE I

ASSAY SEQUENCE FOR HPLC

Step No.	Procedure
1	Plasma (2 ml) + water (1 ml) + ethanol (3 ml) + hexane (5 ml)
2	Shake for 5 min; centrifuge at 1300 *g* for 5 min
3	Hexane layer (4 ml), evaporate
4*	Residue + hexane (2 ml) (+ hexane 2 ml: wash)
5	Sep-Pak silica
6	Wash with hexane (10 ml)
7	Elute with hexane–diethyl ether (96:4, v/v) (5 ml)
8	Evaporate the eluate
9	Residue + ethanol (200 μl), filtration (0.45 μm)
10	50 μl injection

* Up to this step, the sequence was repeated three times.

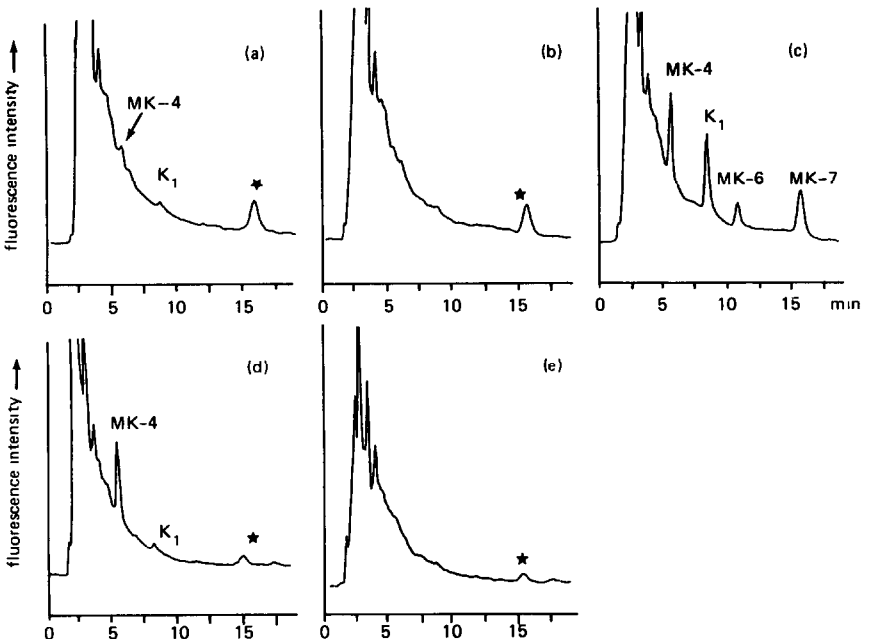

Fig. 3. Chromatograms of K vitamins in umbilical cord plasma. (a) Peaks of endogenous VK (Table II, non-treated group, No. 1). (b) Peaks of (a) did not appear without reduction. (c) Endogenous VK from (a) spiked with standards (added dose: K_1 = 600, MK-4 = 600, MK-6 = 300, MK-7 = 300 pg per injection). (d) Peaks of endogenous VK (Table II, MK-4-treated group, No. 6), high peak of MK-4. (e) Peaks of (d) did not appear without reduction. The peak marked with an asterisk, at the retention time of MK-7, appeared with and without reduction, therefore it is not MK-7.

(Cotati, CA, U.S.A.), a stainless-steel column packed with Nucleosil 5C-18 (150 mm × 4.6 mm I.D., Chemco Scientific, Kyoto, Japan), an FCV-2AH changeable valve with an SCL-6A system controller (Shimadzu), an RC-10 platinum-reduction column (10 mm × 4 mm I.D., Irica, Kyoto, Japan), an RF-540 spectrofluorophotometric detector (Shimadzu) and a DR-3 data recorder (Shimadzu).

The mobile phase was a 1:4 (v/v) mixture of ethanol and methanol containing 0.25% sodium perchlorate. The flow-rate was 1.0 ml/min and the injection volume was 50 μl. The excitation and emission wavelengths were 320 and 430 nm, respectively.

Subjects

The subjects were eight healthy new-born babies, weighing 2980–3320 g all of whom had Apgar scores of 8–10. Their mothers were 24–30 years old at term. Three mothers were administered MK-4 (vitamin K_2, 10 mg) by dripping method ca. 2 h before delivery [10] (MK-4-treated group). The remaining five subjects were not treated.

The study took place during June and July 1987 at Aiiku Obstetrics and Gynaecology Clinic (Kobe, Japan) and was explained to all mothers who all gave their consent.

Samples

Blood samples (20 ml) from the clamped umbilical cord vein still attached to the placenta were collected in heparinized polystyrene tubes. Blood plasma was obtained by centrifugation at 1300 *g*, stored at −20°C and kept in the dark until analysis.

VK in umbilical cord plasma were extracted before HPLC analysis by the procedure outlined in Table I.

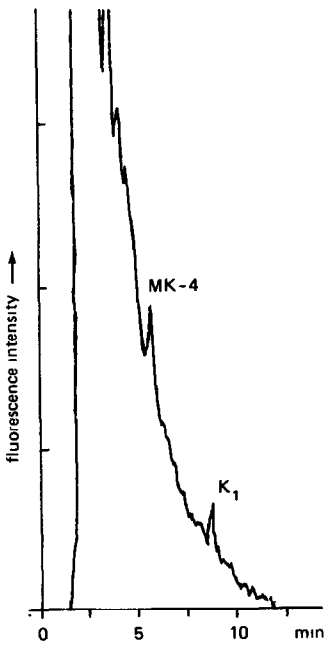

Fig. 4. Chromatogram of the second injection with a wider slit of the fluorimeter. (Table II, non-treated group, No. 1; only part of MK-4 and K_1, the baseline of the other part was outside the recorder scale.)

Methods

The fluorimetric determination of VK with a naphthoquinone structure requires their reduction to the highly fluorescent naphthohydroquinones, since they do not possess native fluorescence. VK were reduced by the platinum-reduction column and converted into VK hydroquinones, which are highly fluorescent (Fig. 3a, c and d).

The calibration curve was prepared using standard solutions of VK.

Low levels of VK were confirmed again with a wider slit of fluorimeter (Fig. 4). In the second injection, the baseline of the recorder was lower, otherwise the baseline was outside the recorder scale.

RESULTS AND DISCUSSION

Under these conditions, the detection limits of K_1 and MK-4 were 5 pg per injection, and those of MK-6 and MK-7 were 10 pg per injection at a signal-to-noise ratio of 2. The recoveries were almost 100% (1.0 ng/ml).

The peaks of endogenous and standard VK overlapped completely (Fig. 3c). VK peaks were not detected when the sample bypassed the platinum-reduction column (Fig. 3b and e), indicating that these peaks were VK.

The pump pressure and retention times of the peaks were almost the same with and without platinum reduction.

In the non-treated group, the mean concentration of vitamin K_1 (Table II) was 50 pg/ml and that of MK-4 was 40 pg/ml. Subjects in the MK-4-treated group showed values of 40 and 580 pg/ml, respectively (Table II). In umbilical cord plasma, K_1 and MK-4 were detected in most cases and MK-6 and MK-7 were not detected at all. The peak with the retention time of MK-7 appeared with and without reduction (Fig. 3b and e), indicating that the peak is not MK-7.

The peaks of VK with the platinum-reduction column in umbilical cord plasma

TABLE II

CONCENTRATIONS OF VK IN UMBILICAL CORD PLASMA

Values are in pg/ml; N.D. = not detected (< 10 pg/ml).

	No.	K_1	MK-4
Non-treated group	1	30	40
	2	N.D.	70
	3	100	N.D.
	4	20	70
	5	100	20
	Mean ± S.D.	50 ± 46.9	40 ± 30.8
MK-4-treated group	6	50	620
	7	70	220
	8	N.D.	900
	Mean ± S.D.	40 ± 36.1	580 ± 342*

*Significant difference ($0.01 > p > 0.001$) compared with the non-treated group.

were as high as those detected with the electrochemical reduction method. In our experience, the electrochemical reduction method was less efficient for ca. 100 samples and the peak heights gradually became lower, since the electrode was easily contaminated. The platinum-reduction column method gave almost the same peak heights for ca. 300 samples and the peak heights could be recovered by pumping vitamin C.

The concentration of MK-4 in the treated group was significantly higher than that in non-treated group ($0.01 > p > 0.001$, Table II). To prevent melena neonatorum, administration before delivery of MK-4 (vitamin K_2) to the mother is preferable to intramuscular injection to the neonate, which involves a certain degree of danger.

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