Chromatographic Analysis of Vitamin K_1; Application to Infant Formula Products

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Vitamin K_1 was extracted from infant formula products by a liquid–liquid partition with a mixture of petroleum–diethyl ether (1 : 1 v/v) and aqueous ethanol. Preliminary separation of vitamin K_1 from the neutral and polar lipids was achieved with an 8% water deactivated alumina column developed by a stepwise elution of 0 to 12% benzene in petroleum ether. Reflectance densitometry at 275 nm was used to quantify the vitamin K_1 after analytical separation by thin-layer chromatography using silica gel G and a two-step development with carbon tetrachloride and benzene. The recovery and precision of the assay was studied with labeled (^{14}C) and unlabeled vitamin K_1. Satisfactory recovery (90%) and reproducibility were achieved in these studies.

Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone; phylloquinone), the antihemorrhagic vitamin, occurs primarily in green plants. The classical procedure for the measurement of this vitamin is a curative chick bioassay which reflects the influence of dietary intake of vitamin K on the clotting time (Almquist, 1947; Dam and Glavind, 1938). However, use of this bioassay for routine measurement of the vitamin in food products is not only time consuming, but the results are also quite variable. Matschiner *et al.* (1967) overcame some of these difficulties by purifying the vitamin K_1 extracted from animal and plant sources by column and thin-layer chromatography prior to quantification by an improved chick bioassay procedure (Matschiner and Doisy, 1966).

Several investigators have used various physicochemical methods to measure vitamin K_1 and other quinones occurring in plants and natural products. Bishop and King (1962) separated and quantified the vitamin from a bacterial lipid extract using alumina column chromatography and uv spectroscopy. Egger (1965) measured the vitamin present in green leaves by column and thin-layer chromatography, followed by visual comparison with known standards of authentic quinones. Nair and Turner (1963) and Libby *et al.* (1967) reported the separation of vitamin K_1 from other fat-soluble vitamins by gas–liquid chromatography. Also, Dialameh and Olson (1969) have outlined a gas–liquid chromatographic technique for quantitative determination of vitamins E, K_1, and homologs of vitamin K_2 following a systematic procedure of chromatographic purification.

This report describes a chromatographic system for the isolation of vitamin K_1 applied to infant formula products, and its subsequent quantification by reflectance densitometry.

EXPERIMENTAL SECTION

Basically, the system consists of four major steps: liquid–liquid extraction of the fat and fat-soluble vitamins from the formula; preparative column chromatographic separation of vitamin K_1 from the neutral and polar lipids; thin-layer chromatographic resolution of the vitamin; and quantification by reflectance densitometry.

All organic solvents used were Nanograde Quality (Mallinckrodt Chemical Works, St. Louis, Mo.). Since photodecomposition of vitamin K may be possible, the entire procedure was conducted under conditions of subdued indirect lighting.

Vitamin K Extraction. All samples were prepared in quadruplicate. Sufficient liquid formula (usually 15 ml) containing approximately 1 g of oil was transferred to a 250-ml polypropylene screw-cap centrifuge bottle, diluted with an equal volume of distilled deionized water, agitated until thoroughly mixed, and an equal volume (formula plus water) of 95% ethanol was added to precipitate the protein. The fat was extracted by vigorously shaking this mixture first with 50 ml of diethyl ether and then with 50 ml of petroleum ether (bp 30–60°). The phases were separated by centrifugation ($1000 \times g$) for 5 min and the epiphase containing the vitamin K_1 was transferred to a 1-l. round-bottomed flask. The extraction was repeated, the extracts were pooled, and the solvents were removed under reduced pressure at 60°. A clear oil residue which contained the vitamin K_1 was obtained.

Column Chromatography. One-hundred grams of neutral alumina (AG7, 100–200 mesh, Bio-Rad Lab., Richmond, Calif.) was slurried with petroleum ether in a 250-ml polypropylene screw-cap centrifuge bottle. Eight milliliters of distilled, deionized water was added, the container was vigorously shaken, and the mixture was allowed to equilibrate for 1 hr. The slurry was transferred by pipet (delivery tip removed) into a 35×2.5 cm o.d. glass column equipped with a fixed sintered glass frit (medium) and Teflon stopcock (Corning Glass Works, Corning, N. Y.). Fifty milliliters of petroleum ether was placed in the column, the stopcock was opened, the slurry was added, and the solvent level was adjusted to 1 mm above the adsorbent surface. The columns were packed either just prior to use or the night before.

The lipid extract, dissolved in 5 ml of petroleum ether, was carefully transferred with a Pasteur pipet to the adsorbent surface and the stopcock was opened to allow the column to flow. When the solvent level was 1 mm above the adsorbent, the column flow was stopped and 10 ml of petroleum ether was carefully added to prevent disturbing the adsorbent surface by subsequent solvent additions. A gravity-fed solvent reservoir was used to maintain constant head pressure. One-hundred milliliters of each eluent (0, 2, 4, 6, 8, 10, and 12% benzene in petroleum ether) was used to develop the column at a flow rate of 1.5 ml/min.

Since adsorbent characteristics vary, one column was used to provide a visual control of the elution of the vitamin. This was prepared by adding 1 mg of vitamin K_1 (diluted in hexane, Calbiochem, Los Angeles, Calif.) to one of the lipid extract samples. The elution pattern of the vitamin was monitored by evaporating the fractions and resuspending each in 0.1 ml of hexane. A yellow color in the resuspended fractions indicated vitamin K.

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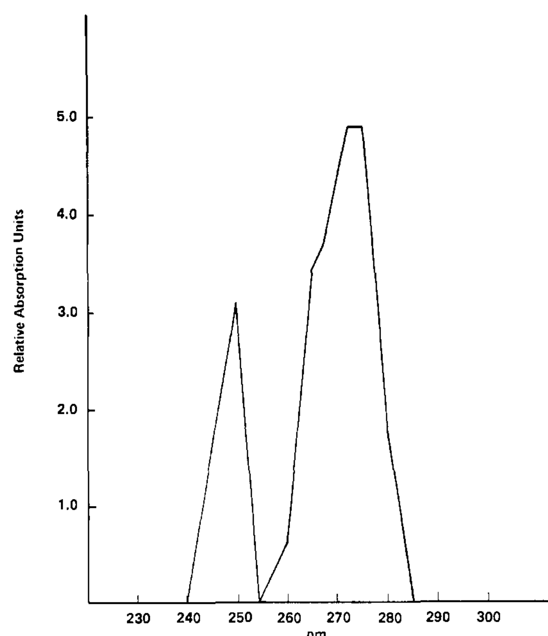

Figure 1. Absorption spectra of vitamin K_1 as determined by reflectance densitometry

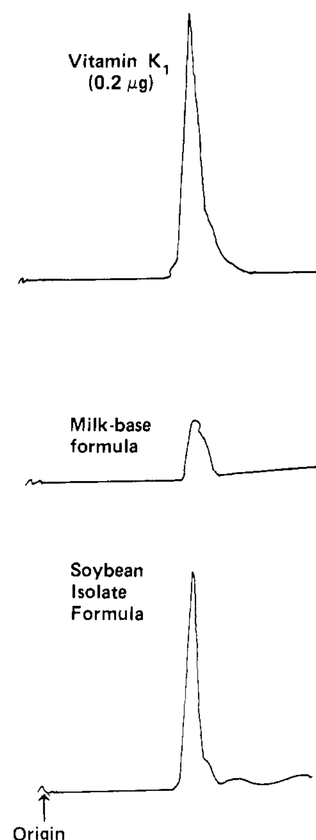

Figure 2. Reflectance densitometric recording (275 nm) of vitamin K_1 standard and vitamin extracted from infant formulas

The 0 through 6% benzene eluates were collected as a pool and discarded. Twenty-five-milliliter fractions were collected as the columns were developed with the 8, 10, and 12% benzene solvents. The same fractions from the sample columns plus two fractions on either side of the visibly eluted vitamin were pooled and the solvents were evaporated.

Thin-Layer Chromatography. Fifty μl of hexane were added to the pooled eluate and 2 μl transferred with a 5 μl syringe to a 0.25 mm thick "Adsorbosil 5" Prekote 20 × 20 cm thin-layer plate (Applied Science Lab., Inc., State College, Pa.) divided into lanes 1 cm in width. Vitamin K_1 standards were prepared in hexane so that an application of 2 μl transferred 0.05, 0.2, 0.3, or 0.4 μg to the plate surface. After spotting, the plate was developed in an equilibrated, paper-lined chamber containing carbon tetrachloride and developed to a height of 15 cm past the origin. The chromatogram was removed, air-dried at room temperature for 15 min and developed a second time in benzene in the same direction to 15 cm past the origin.

Reflectance Densitometry. After air-drying at room temperature, each lane of the chromatogram was scanned with a Chromatogram Spectrophotometer equipped with a scanning table and reflectance head (Model PMQ II, Carl Zeiss, Oberkochen, West Germany) at 275 nm, slit width 0.9 cm at a speed of 30 cm/min and chart recorder speed of 0.5 in/min. The area under the vitamin K_1 peak was calculated by triangulation and a standard curve was plotted as the log weight of the vitamin against the square root of the area. The amount of the vitamin in the sample was determined from this standard curve.

RESULTS AND DISCUSSION

The absorption maximum was found to be between 272 and 275 nm (Figure 1). At a wavelength of 275 nm a linear response was found for vitamin K_1 levels of 0.05 to 0.40 μg spotted on the tlc plate. A reflectance densitometric scan of a thin-layer chromatogram (Figure 2) indicates the resolution found with this system and the latitude of response to various levels of the vitamin found in infant formulas. This system appears to have the sensitivity to determine the level of vitamin K_1 found in most infant formulas.

The recovery of a known amount of vitamin K_1 from an infant formula was investigated using both labeled (^{14}C) and unlabeled vitamin K_1. Labeled vitamin K_1 (0.2 μg in ethanol) was added to four 15-ml aliquots of an infant formula prior to extraction. Aliquots of each sample were transferred to counting vials containing 10 ml of Permablend (Packard Instrument Co., Inc., Downers Grove, Ill.). Radioactivity was measured using a liquid scintillation counter (Tri-Carb Model 3375, Packard Instrument Co., Inc.) operated at optimal counting conditions for ^{14}C. The recoveries, expressed as a percentage of total radioactivity added to each aliquot, averaged 95 ± 5% (standard deviation) after the liquid–liquid extraction of the oil residue and 91 ± 3% (standard deviation) following column chromatography.

Data for the recovery of unlabeled vitamin K_1 are shown in Figure 3. Graded levels of the vitamin were added to an infant formula known to be low in vitamin K_1. The three levels of the vitamin (1.0, 2.5, and 5.0 μg/g of oil) were added as an ethanolic solution prior to extraction of the liquid infant formula. A linear recovery of the added vitamin was obtained and subtraction of the base value (1.46 μg/g of oil in the infant formula) yielded a linear recovery with a zero intercept.

The reproducibility of this assay was evaluated using four different infant formulas (Table I). Good reproducibility was obtained with an average coefficient of variation of 10%. A similar coefficient of variation was found in the labeled vitamin study.

The levels of vitamin K in these formulas (Table I) were lower than those previously found by chick bioassay (Gold-

Figure 3. Recovery of graded levels of vitamin K_1 added to infant formula

Table I. Reproducibility of the Measurement of Vitamin K in Infant Formulas

Infant formula	Protein source	Repli-cates	Vitamin K content, μg/qt[a]	Coefficient of variation, %
A	Cows' milk	3	53 ± 2[b]	4
B	Cows' milk	3	18 ± 2	14
C	Soybean isolate	4	74 ± 3	4
D	Soybean isolate	4	94 ± 11	12

[a] Normal dilution (20 kcal/fluid oz). [b] Standard deviation.

man and Deposito, 1966) but were of the same order of magnitude.

To determine if different oils would greatly affect the column elution pattern of vitamin K_1, 500 μg of the vitamin was added to 1-g samples of corn, soy, oleo, or coconut oil suspended in petroleum ether and applied to the column. The data (Table II) indicate that the elution of vitamin K_1 from the alumina absorbent was not affected by the four oils investigated; therefore, it appears that the various types of fats found in infant formulas will not limit use of the assay. However, these data do not eliminate the need of the visual control column recommended in the section above on column chromatography.

A further study was made to determine the purity and chemical form of the vitamin isolated from infant formula products. Pure vitamin K_1 and that isolated from an infant formula were chromatographed on both paraffin and $AgNO_3$-impregnated silica gel G plates as described by Matschiner and Amelotti (1968). After development in a sandwich-type development chamber (Brinkmann Instruments, Inc., Westbury, N. Y.), the location of the vitamin was determined by reflectance densitometry. Vitamin K isolated from the infant formula was identical to the phylloquinone standard in both chromatographic conditions. These data represent further support that the compound extracted and separated from infant formula lipids was phylloquinone.

Table II. The Effect of Different Oils on the Elution of Vitamin K_1 from the Alumina Column

Fraction no.	% benzene	Soy oil	Corn oil	Coconut oil	Oleo oil
		Percent of vitamin K_1 applied to column[a]			
1	6	...	...	...	...
2	6	3	...	2	...
3	8	3	3	6	5
4	8	23	26	23	10
5	8	29	28	29	29
6	8	17	16	15	20
7	10	15	12	14	17
8	10	4	10	7	15
9	10	...	...	...	...
10	10	...	...	...	...
Total		94%	95%	96%	96%

[a] Average of duplicate determinations.

Other chromatographic techniques were investigated in the development of this assay procedure. Silicic acid columns as described by Matschiner and Amelotti (1968) were used for the preparative separation of vitamin K_1 from other lipids; however, lipids extracted from formulas containing soybean oil were poorly resolved. Although the resolution of the vitamin was improved by a linear gradient elution, other lipids still interfered. The more involved reverse-phase column chromatographic method of Matschiner and Taggart (1967) obtained the desired resolution prior to thin-layer chromatography.

Basic and neutral alumina adsorbents also were studied utilizing a stepwise elution. Basic alumina was found to promote isomerization of the vitamin, but neutral alumina clearly separated the vitamin from the other lipids, thus providing the simpler column procedure described above.

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