Determination of Phytonadione in Elemental Diets by TLC–Spectrophotodensitometry

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Abstract □ The quantitative analysis of phytonadione in several elemental diet formulations at levels ranging from 90 to 150 ppb is described. After extraction with petroleum ether, the sample is cleaned up by silica gel column chromatography and separated on a TLC plate; phytonadione is determined by reflectance densitometry. Recovery data show that the method is accurate ($\bar{X}$ = 97%) and has a precision (*RSD*) of ±6%.

Keyphrases □ Phytonadione—TLC–spectrophotodensitometric analysis in elemental diet formulations □ TLC–spectrophotodensitometry—analysis, phytonadione in elemental diet formulations □ Vitamins—phytonadione, TLC–spectrophotodensitometric analysis in elemental diet formulations

Phytonadione (vitamin K_1, 2-methyl-3-phytyl-1,4-naphthoquinone) is a naturally occurring K vitamin. Its principal use in dietary or medicinal preparations is as an antihemorrhagic agent (1). Although the vitamin is usually measured by a biological assay (2), the procedure is time consuming and otherwise unsuitable for multiple analyses.

Chemical assays have been reported which employ colorimetry (3, 4), TLC (5, 6), GLC (7–9), polarography (10, 11), and liquid chromatography (12). However, for application to elemental diet formulations containing ~100 ppb of phytonadione, most of these techniques are unsuitable because of high background interference. This paper describes a chromatographic system that separates and quantitates phytonadione in two elemental diet formulations[1] at dosage levels ranging from 0.09 to 0.15 μg/g.

EXPERIMENTAL

Equipment—All measurements were made on a spectrodensitometer[2] equipped with a 200-w xenon–mercury lamp[3], a density computer[4], a strip-chart recorder[5], and a digital integrator[6]. The instrument was operated in the reflectance mode using the dual-beam system. Samples were scanned at 270 nm using a slit width of 1 mm at a scanning speed of 2.54 cm/min. The recorder was operated at a chart speed of 5.1 cm/min. The digital integrator was operated at a slope sensitivity of 0.5, a threshold of 50, and a voltage of 0.1.

Reagents and Materials—Phytonadione[7] was used without further purification. All organic solvents and reagents were reagent grade or purer. The chlorinated hydrocarbon solvents used as column eluents were specially purified by separately percolating ~5 liters of spectroanalyzed grade 1,2-dichloroethane or reagent grade carbon tetrachloride through a dual-packed column of basic alumina[8] (30 × 50 cm) in the upper layer and acidic alumina[9] (30 × 50 cm) in the lower layer.

Precoated silica gel TLC plates[10] (20 × 20 cm) were developed in a glass chamber[11] (21.5 × 8 × 23 cm) completely lined with absorbent paper[12] using toluene. Samples were spotted with a microliter pipet[13].

Column chromatography was conducted in glass columns[14] (19 × 300 mm) using silica gel[15] and 1,2-dichloroethane–carbon tetrachloride (40:60 v/v) as the solvent.

All evaporations were conducted on a steam bath under a nitrogen stream to prevent oxidative losses of phytonadione. Either 125- or 250-ml erlenmeyer flasks were used with a custom-designed evaporation adapter attached (Fig. 1). A nitrogen stream at ~325 ml/min was introduced into the flask *via* a Pasteur pipet inserted into the evaporation adapter. Four simultaneous evaporations were made possible by directing the gas stream from a nitrogen cylinder[16] into a four-port manifold positioned over the bath. Separate nitrogen streams were then directed into each flask by rubber tubing connected to the Pasteur pipets.

Procedure—The system consists of four main parts: a liquid–solid extraction of the diet to isolate the lipid fraction containing phytonadione, column chromatography to separate phytonadione from most of the neutral and polar lipids, TLC to resolve phytonadione, and measurement by reflectance spectrophotodensitometry. Since phytonadione is extremely light sensitive (14), all analytical operations were conducted under subdued light.

Standard Solutions—Separate standard solutions were prepared for each elemental diet formulation (I and II).

Standard Solution A—Phytonadione, 30 mg, was dissolved in hexane and brought to 50 ml in a volumetric flask.

Standard Solution B (for Elemental Diet I)—A 250-μl aliquot of Standard Solution A was transferred to a 50-ml volumetric flask and diluted to volume with hexane.

Standard Solutions C_1 and C_2 (for Elemental Diet II)—A 3-ml aliquot of Standard Solution A was transferred to a 50-ml volumetric flask and diluted to volume with hexane (C_1). A 1-ml aliquot of Solution C_1 was transferred to a 10-ml volumetric flask and diluted to volume with hexane (C_2).

Column Standards—A 2-ml aliquot of Solution B (or C_2) was transferred onto a silica gel column and processed exactly as a sample.

Sample Extraction—Individual packets (80 g) of the elemental diet (I or II) were transferred to Büchner funnels[17] and extracted with one 75-ml portion and then two 60-ml portions of petroleum ether (bp 30–60°). The combined extracts were evaporated to dryness under nitrogen using the special assembly described. While still under nitrogen, the flask was placed in a beaker of cool water. The residue was immediately taken up in ~1 ml of hexane, which was introduced through the evaporation adapter *via* a syringe[18].

Column Chromatography—Silica gel[15] was reactivated by heating 500-g lots in a 110° oven for 4 hr, followed by cooling to room temperature in a desiccator. Columns (one for a standard plus one for each sample) were then prepared using a slurry technique with 14 g of the activated silica gel and 45 ml of column eluent. After the slurry was transferred into the column, the column was rinsed with 5–10 ml of column eluent and allowed to drain until the liquid level was ~1 mm above the adsorbent.

With the column flow stopped, the sample extract was quantitatively transferred onto the column using 1–2 ml of hexane to assist the transfer.

[1] Vivonex standard diet, unflavored (I), and Vivonex high nitrogen diet, unflavored (II), containing phytonadione at dosage levels of approximately 0.15 and 0.09 μg/g, respectively (Norwich Pharmacal Co., Norwich, N.Y.). The two products (13) are supplied as soluble powders in 80-g packets, which are dispersed in water shortly before administration.
[2] Model SD3000, Schoeffel Instrument Corp.
[3] Hanovia Lamp Division, Conrad Precision Industries.
[4] Model SDC300, Schoeffel Instrument Corp.
[5] Model Y196411-007-000-610-opt102-110, Honeywell.
[6] Model SDR304, Schoeffel Instrument Corp.
[7] USP quality, Hoffmann-La Roche.
[8] AG10, 100–200 mesh, Bio-Rad Laboratories.
[9] AG4, 100–200 mesh, Bio-Rad Laboratories.
[10] Silica gel 60, F-254, Brinkmann Instruments Co.
[11] Chromaflex developing tank, Kontes Glass Co.
[12] Saturation pads, Camag, Inc.
[13] A 100-μl Wiretrol, Drummond Scientific Co.
[14] K420530, Kontes Glass Co.
[15] Woelm 200, Waters Associates.
[16] Prepurified grade, Matheson Gas Products.
[17] Fritted glass, medium porosity, Corning Glass Co.
[18] A 10-ml glass Luer-tip syringe equipped with a 1.3-cm 27-gauge Luer-tip needle, Becton-Dickinson.

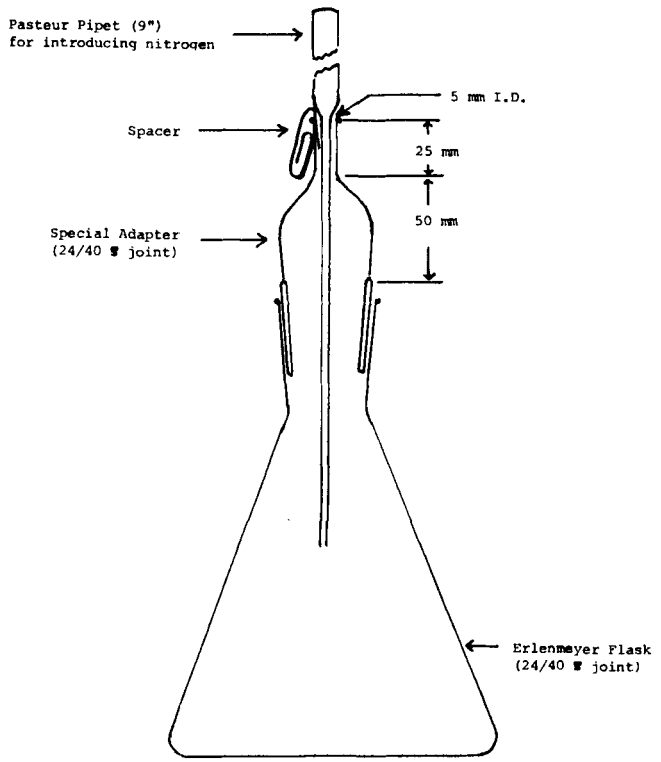

Figure 1—*Evaporation device.*

[*Note:* All quantitative transfers were performed by rinsing down the walls of the flask with a small stream of solvent (~0.5 ml) delivered from a syringe[18] and carefully transferring to the appropriate receiver. Three rinses were generally sufficient to ensure a quantitative transfer.]

The flow rate was adjusted to 1.5 ml/min, and the sample was consolidated onto the column head. The column was eluted with 120 ml of 1,2-dichloroethane–carbon tetrachloride (40:60 v/v). The eluate was collected in a 125-ml erlenmeyer flask containing 1 ml of a citric acid solution, prepared at 300 μg/ml by dissolving ~15 mg of citric acid monohydrate in ~5 ml of methanol and diluting to 50 ml with column eluent.

The column eluate was evaporated to dryness under nitrogen, the residue was taken up in ~1 ml of hexane (as described), and the solution was transferred quantitatively to a 2-ml volumetric flask using a small funnel.

TLC—TLC plates were divided into 20 1-cm tracks using a scoring device[19]. A score line was made perpendicular to the tracks exactly 13 cm above the origin. The plates were activated by heating in an oven at 105° for 2 hr and stored in a desiccator.

Aliquots (100 μl) of samples, column standards[20], and one plate standard[21], containing similar quantities of phytonadione (Elemental Diet I, 600 ng; Elemental Diet II, 360 ng), were spotted in duplicate in alternate tracks of a TLC plate under a stream of air. The plate was developed in toluene to a 13-cm score line and dried in a hot air stream[22]. A routine visual inspection of the plate under shortwave UV light was made to verify the position of the phytonadione (R_f ~0.40) and to evaluate the resolution.

Reflectance Spectrophotodensitometry—The spectrodensitometer[2] was adjusted for reflectance mode operation, as previously described, and the TLC plate was placed in the plate frame. The beam was then centered over the developed column standard spot (R_f ~0.40), and the pen was adjusted to ~90% of full-scale deflection using the range and vernier (gain) controls. Each phytonadione spot on the plate was then scanned in the direction of development. Area estimates were obtained for each peak from the digitized data. The results were then calculated from the average values obtained based on the column standard.

[19] Model SDA320, Schoeffel Instrument Co.

[20] A solution of phytonadione (in hexane) applied to the column and processed through the method.

[21] A solution of phytonadione (in hexane) spotted directly on the plate and processed through the method.

[22] Heat gun, model HG751, Master Appliance Corp., Racine, Wis.

Table I—Recovery of Phytonadione from Synthetic Samples of Elemental Diet I

Percent of Theory[a] (Approximation)	Phytonadione Added, μg/80 g[b]	Found, μg/80 g[c]	Recovery, %
80	9.82	9.86	100.4
	9.82	9.34	95.1
	9.82	10.02	102.0
	9.82	10.36	105.5
100	12.29	12.86	104.6
	12.29	11.31	92.0
	12.29	11.81	96.1
	12.29	11.92	97.0
120	14.76	12.92	87.5
	14.76	13.59	92.1
	14.76	13.34	90.4
	14.76	15.29	103.6
			$\bar{X}$ = 97.2
			SD = 6.0
			RSD = ±6.2

[a] The theoretical level of phytonadione in Elemental Diet I is about 0.15 μg/g or 12 μg/80 g. [b] Phytonadione was added to the diet by blending a dry Elemental Diet I concentrate into the remaining placebo (an Elemental Diet I blank) in a twin-shell blender. [c] Each determination was based on duplicate aliquots of samples and standards being spotted on the plate.

RESULTS AND DISCUSSION

The absorption maximum of phytonadione was 270 nm. A spectrophotodensitometric response study at this wavelength showed a linear relationship for plate loads ranging from 40 to 1000 ng of phytonadione. An area ratio technique was used to minimize day-to-day fluctuations.

Sample Extraction—Phytonadione was extracted efficiently from the (powdered) elemental diet with petroleum ether. Studies showed that the direct extraction of the dry diet gave less background interference than did a partitioning extraction of the diet from an aqueous solution.

Column Chromatography—Attempts were made to separate the vitamin from the diet excipients on both silica gel and alumina columns. While neither approach gave a complete separation, the silica gel column provided sufficient cleanup for use in conjunction with a final TLC separation.

Because of the relatively high lipid levels in the diet extract, 14 g of silica gel was required per column. A slurry-packing technique was pre-

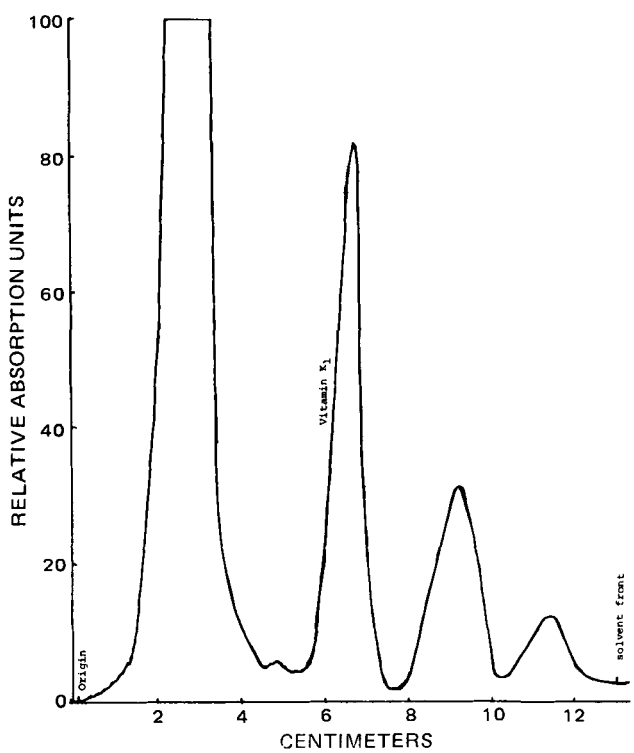

Figure 2—*Reflectance densitometric scan (270 mm) of Elemental Diet I.*

Table II—Assay of Elemental Diets I and II

Elemental Diet	Sample, g	Phytonadione, μg/g Found[b]	Theory, %
I[a]	79.1	0.128	83.5
	80.0	0.154	100.2
	79.9	0.135	87.7
	79.8	0.128	83.6
	80.7	0.136	88.3
	80.0	0.142	92.4
		$\bar{X}$ =	89.3
		SD =	6.3
		RSD =	±7.1
II[c]	80.9	0.094	102.2
	80.5	0.106	115.2
	82.0	0.090	97.8
	81.7	0.090	97.8
	82.7	0.098	106.3
		$\bar{X}$ =	103.9
		SD =	7.3
		RSD =	±7.0

[a] The phytonadione level in Elemental Diet I is about 0.15 μg/g. [b] Each determination was based on duplicate aliquots of samples and standards being spotted on the plate. [c] The phytonadione level in Elemental Diet II is about 0.09 μg/g.

ferred over a dry-packing procedure, since it gave more homogeneous packings and yielded more reproducible recoveries.

Several elution procedures were investigated during method development. A successive elution approach, using a relatively nonpolar eluent followed by a more polar eluent mixture, failed to give the desired separation. The chosen system employs an eluent of 1,2-dichloroethane–carbon tetrachloride (40:60 v/v), which elutes the vitamin prior to the main lipid fraction. All grades of chlorinated hydrocarbons should be purified, as indicated, to remove any interfering impurities.

Citric acid (300 μg) was added to each receiver prior to elution to protect phytonadione against oxidation during the subsequent evaporation step. This method was required to protect the standard, since the sample solution was amply protected by antioxidants in the formulation.

TLC–Spectrophotodensitometry—Phytonadione was separated effectively from diet excipients by the final TLC procedure. A typical scan of an Elemental Diet I sample is shown in Fig. 2. Peaks other than phytonadione have not been identified.

Separate standard solutions were required for each elemental diet formulation to provide equivalent phytonadione plate loads. The plate standard was run as a check on column standard loss. If column standard loss exceeded 20%, the entire run was arbitrarily rejected because of uncertainties in either the sample or standard.

Method Performance—Method accuracy was estimated by processing 12 synthetic samples of Elemental Diet I, prepared at 80, 100, and 120% of the theoretical level[1], by the final method. Recovery data (Table I) showed an ungrouped mean value of 97.2%. Elemental Diet I blanks (placebo) processed through the method showed no peak for phytonadione.

Method precision was estimated by assaying regular production samples of Elemental Diets I and II. These data (Table II) showed an average percent label of 89.3 and 103.9 for the respective diets, with an estimated precision (*RSD*) of ±7%.

Table III—Recovery Data: Comparison of Synthetic Samples of Elemental Diet I[a] Prepared before and after Aging the Placebo 1 Month at 50°

	Recovery, %	
	Before Aging	After Aging
Elemental Diet I placebo	0	0
Elemental Diet I samples	92.6	97.7
	105.7	98.2
	94.8	86.7
	100.7	96.0
	102.7	94.5
$\bar{X}$ =	99.3	94.6
SD =	5.5	4.7
RSD =	±5.5	±4.9

[a] All synthetic samples were prepared by spiking the Elemental Diet I placebo with phytonadione (in hexane) immediately before assaying.

Comparisons also were made by assaying synthetic samples prepared from both fresh and aged (1 month at 50°) placebos. These results (Table III) showed mean recovery values of 99.3 and 94.5% for the fresh and aged diets, respectively. Comparison of the means by a *t*-test showed no significant difference at the 95% confidence limits. Thus, there are no adverse effects emanating from placebo artifacts formed during storage.

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