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FLUORIMETRIC DETERMINATION OF VITAMIN K_3 (MENADIONE SODIUM BISULFITE) IN SYNTHETIC ANIMAL FEED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A POST-COLUMN ZINC REDUCER

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for analysis of vitamin K_3 (menadione), as the water-soluble sodium bisulfite salt, in synthetic animal feed is described. The menadione sodium bisulfite (MSB) is extracted with aqueous methanol, converted to oil-soluble menadione with sodium carbonate, and partitioned into *n*-pentane. After evaporation, the menadione is dissolved in methanol and analyzed by reversed-phase HPLC. The menadione is reduced on-line to its fluorescent 1,4-dihydroxy analogue by zinc reduction prior to fluorescence determination at 325 nm excitation wavelength and 425 nm emission wavelength.

The fluorescence response was linear in the range 1 to 100 ng of menadione injected. Recovery experiments were performed on synthetic animal feed spiked at 20 and 200 mg/kg levels of MSB. Average recoveries from feed were greater than 90% with an average relative standard deviation of 5.5%. Additional confirmation of menadione in actual feed extracts was performed using capillary gas chromatography–Fourier transform infrared spectroscopy (GC–FT-IR). The HPLC–fluorescence and GC–FT-IR methods had lower limits of detection of 20 μg/kg and 2 mg/kg, respectively.

INTRODUCTION

Vitamin K, a class of closely related naphthoquinones, is essential to all human and animal diets. It plays an important role in blood coagulation as a clotting factor and in bone mineralization processes[1]. Naturally occurring K vitamins, phylloquinone (K_1) and farnoquinone (K_2) are usually available to humans from the daily intake of leafy vegetables. However, at the National Center for Toxicological Research (NCTR), laboratory-raised rats and mice, must rely upon synthetic feeds that have been fortified with vitamin premixes for their vitamin K_3 or menadione intake. Menadione is an oil-soluble vitamin, which has exhibited extreme instability in most industrial feed matrices. Therefore, the more stable, water-soluble form, menadione sodium bisulfite (MSB) or some other menadione salt, is used to give a reliable source

of vitamin K_3 in synthetic feeds. Levels found in animal diets used at the NCTR range from approximately 2 to 20 mg/kg of K_3. A fast and dependable analytical method was necessary for determining the K_3 content, in the form of MSB, of synthetic feed prior to feeding laboratory animals at the NCTR.

Several methods are described in the literature to analyse for MSB in animal feed and vitamin premixes. Many of these methods have utilized gas chromatography with flame ionization detection (GC–FID) for detection of the pyrrolytic product of MSB[2–4]. In addition various direct spectrophotometric determinations[5–8] of vitamin K_3 have been reported. These methods lack the sensitivity needed to quantitate microgram amounts of MSB in synthetic feed matrices. Most of the available extraction methods for vitamin K_3 are similar to the official European Community (EC) colorimetric method[8] in which an aqueous alcohol solution extracts the MSB from the feed matrix. The MSB is then converted to the oil-soluble menadione form in the aqueous extract and solvent-partitioned into an organic solvent (*i.e.* 1,2-dichloroethane, *n*-hexane, chloroform, etc.). Recently, a number of high-performance liquid chromatographic (HPLC) methods for vitamin K have been published using ultraviolet (UV) spectrophotometric detection[9,1] and fluorescence detection[10,11]. These UV and fluorescence detection methods utilized reversed-phase HPLC analysis of menadione and other vitamin K compounds with subsequent post-column reaction. The fluorescence detection exhibited higher sensitivity and specificity than the UV methods. Speek *et al.*[11] reported the analysis of MSB in several different types of animal diets and premixes at levels ranging from 0.1 to 362 mg/kg with a minimum detection limit (MDL) of 0.02 mg/kg. However, their method is based upon an extremely complicated post-column reaction with sodium borohydride requiring a reaction coil, a secondary pumping system, a debubbler to remove incidental hydrogen bubbles, and a special debubbler fluorescence flow cell. In 1987, Haroon *et al.*[12] reported utilizing an 'on-line' zinc metal post-column reducer consisting of a 20 × 3.9 mm I.D. steel precolumn packed with high purity 200-mesh zinc particles, for reduction of phylloquinone (K_1) and its metabolite (phylloquinone-2,3-epoxide) in plasma samples with subsequent fluorometric detection of the generated hydroquinones.

The method reported here for analysis of MSB (K_3) in synthetic animal feed is a modification of several extraction methods[6,8,11] coupled with reversed-phase HPLC and an improved zinc post-column reducer and fluorescence detection. The method is simple, fast, and requires no complex derivatization reactions or specialized HPLC hardware. In addition, a confirmation procedure for the qualitative determination of menadione in synthetic feed is described using capillary gas chromatography–Fourier transform infrared spectroscopy (GC–FT-IR).

EXPERIMENTAL

Reagents

All reagents and organic solvents were analytical-reagent grade or comparable. The menadione sodium bisulfite standard (95% purity) was obtained from Sigma, St. Louis, MO, U.S.A. and the menadione standard from United States Biochemical, Cleveland, OH, U.S.A. Stock standard solutions of MSB and menadione were prepared by dissolving 50 mg in 50 ml of deionized water and methanol, respectively. The resulting 1-mg/ml MSB stock solution was diluted to 20 μg/ml and 200 μg/ml in

water–methanol (60:40, v/v) for use in HPLC analysis and MSB recovery experiments. The 1 mg/ml menadione stock solution was diluted with methanol or *n*-pentane to appropriate concentrations for HPLC–fluorescence linearity checks and GC–FT-IR assays, respectively. All standard solutions of MSB and menadione were stored in low actinic glassware at 4°C due to the long-term instability to light and ambient temperatures of these compounds in solution. The synthetic laboratory animal meals and vitamin premixes were analyzed as received. Pelletized feeds were ground using a Wiley Mill Model 4 (Arthur H. Thomas, Philadelphia, PA, U.S.A.) prior to analysis.

Extraction procedure

A 1.00-g sample of ground synthetic feed was extracted with 10 ml of water–methanol (60:40, v/v) in a 30-ml culture tube equipped with a PTFE-lined plastic cap by shaking for 30 min on a mechanical shaker. The sample was centrifuged at 1000 *g* for 10 min. A 5-ml aliquot of the clear supernatant was quantitatively transferred to a second 30-ml culture tube. After addition of 10 ml of 5% sodium carbonate diluted in deionized water and 10 ml of *n*-pentane, the tube was shaken by hand for 1 min and centrifuged at 1000 *g* for 1 min to separate the phases. The *n*-pentane (upper phase) was removed using a Pasteur pipette with care not to disturb the aqueous phase. Two additional 10-ml volumes of *n*-pentane was added to the aqueous and treated as before. The combined *n*-pentane extracts (30 ml total) were collected in a 100-ml round-bottom flask containing one borosilicate-glass boiling bead and evaporated to dryness under vacuum at room temperature maintained using a water bath. The residue was dissolved in 10 ml methanol. The finished extract containing 100 mg-equiv. of feed per ml of methanol was then ready for injection into the HPLC system.

High-performance liquid chromatographic system

A 10-μl sample volume was injected onto the 250 × 4.6 mm I.D. Supelcosil LC-18 (5-μm particle size) column using an Altex Model 210 HPLC injector connected to a Waters Model M-6000 pump. The column was eluted isocratically at ambient temperature with methanol–water (75:25, v/v) at a flow-rate of 0.9 ml/min. The effluent from the column was directed into a post-column zinc reducer consisting of a 20 mm × 2 mm I.D. stainless-steel Uptight guard precolumn with 2-μm frits (Upchurch Scientific, Oak Harbor, WA, U.S.A.) packed with powdered zinc (F&J Scientific, Monroe, CT, U.S.A.) sieved to 38-μm particle size or less. A 0.5-μm Uptight precolumn filter (Upchurch Scientific) was connected to the zinc reducer to remove any zinc particles from plugging the line to the detector. The 0.5-μm filter was then connected to the fluorescence flow-cell of a Shimadzu RF-535 fluorescence HPLC monitor operated at 325 nm excitation wavelength and 425 nm emission wavelength. Fluorescence response was recorded on a Spectra-Physics SP 4270 integrator.

Partitioning value determinations for menadione

An experiment was performed to determine the partitioning (*P*) values of menadione from alcoholic solutions into organic phases. Equal volumes (5 ml) of alcohol–water (40:60, v/v) solutions containing ethanol or methanol and 4 μg/ml menadione were shaken together with several organic solvents. The organic solvents studied in the experiment included *n*-hexane, *n*-pentane, chloroform, and methylene chloride.

After shaking for 1 min, the two phases were separated by centrifugation at 500 *g* for 1 min. The organic layers were analyzed by HPLC–fluorescence as described previously for menadione content. *P* values were calculated for each of the alcohol–water solutions and organic solvents by dividing the amount of menadione (μg/ml) present in the organic phase by the initial amount (4 μg/ml) in the aqueous phase.

Recovery experiment

Triplicate 1.00-g samples of ground synthetic animal feed in 30-ml culture tubes were spiked at levels of 20 and 200 mg/kg MSB. The spiked feed samples were extracted and analyzed by HPLC–fluorescence as previously described and compared to MSB standard solutions treated identically without feed present. Triplicate 1.00-g unspiked feed samples were analyzed for use in correcting the recovery data for inherent levels of MSB in the vitamin K-fortified feed.

GC–FT-IR procedure

For confirmation of the presence of menadione in an unspiked synthetic feed extract, a 10.0-g sample of synthetic feed was extracted using ten-fold increases in the extraction reagents described previously in a 180-ml culture tube. Two 20-ml aliquots of *n*-pentane were used to extract free menadione from the aqueous sodium carbonate solution. The *n*-pentane was transferred to a 100-ml round-bottom flask and reduced to approximately 1 ml with vacuum. This concentrate was quantitatively removed to a graduated 2-ml conical vial together with a 1-ml *n*-pentane rinse of the flask. The sample was evaporated to 0.2 ml volume under a gentle stream of nitrogen. A volume of 2 μl of this concentrate was injected into a Hewlett-Packard HP 5890 gas chromatograph equipped with a splitless capillary injector operated at 220°C. A 30 m × 0.32 mm I.D. fused-silica J&W DB-1701 capillary column was programmed from 80 °C initial temperature for 0.5 min up to 250°C at 16°C/min for 15 min final hold time. The GC column was interfaced to a Digilab FTS-40 FT-IR spectrometer using a Digilab GC/C 32 module containing a 10 cm × 1.0 mm I.D. gold-coated light pipe (250°C) and liquid nitrogen-cooled mercury–cadmium–telluride (MCT) detector. Spectral searches were provided by a Digilab 3200 data station with Search 32 software and a Hewlett-Packard HP 7550A graphics plotter for plotting out the infrared data.

RESULTS AND DISCUSSION

Extraction procedure for menadione sodium bisulfite in animal feed

The extraction procedure was derived from several excellent methods[6,8,11] for MSB analysis. A water–methanol (60:40, v/v) solution is used to solubilize the MSB water-soluble salt from the feed matrix. After centrifugation to precipitate the feed particles, a 50% aliquot of the supernatant is reacted with 5% aqueous sodium carbonate which destroys the bisulfite salt releasing the free menadione, 2-methyl-1,4-naphthoquinone. In contrast to the method of Speek *et al.*[11], *n*-pentane, rather than *n*-hexane, was used as the organic solvent for partitioning the free quinone from the aqueous alcolhol–carbonate solution. This was because *n*-pentane (*P* value = 0.83) performed as well as *n*-hexane (*P* value = 0.81) as seen in Table I and the lower boiling point of *n*-pentane which allowed for faster evaporation of extracts under

TABLE I

PARTITION VALUES FOR VITAMIN K_3 (MENADIONE) BETWEEN AQUEOUS ALCOHOLIC SOLUTIONS AND VARIOUS ORGANIC SOLVENTS

Partition values (*P* values) were determined by partitioning equal 5-ml volumes of 4 μg/ml aqueous alcohol solutions of menadione with four organic solvents and subsequent HPLC analysis of the organic phase.

Aqueous alcohol solution	*P value (1.00 = 100% extraction into organic phase)*			
	n-Pentane	*n-Hexane*	*Methylene chloride*	*Chloroform*
Water–methanol (60:40)	0.83 ± 0.02[a]	0.81 ± 0.07	1.00 ± 0.05	0.91 ± 0.05
Water–ethanol (60:40)	0.86 ± 0.05	0.85 ± 0.02	0.93 ± 0.02	0.91 ± 0.02

[a] *P* values are reported as the average of triplicate analyses (mean ± standard deviation).

vacuum at room temperature. A centrifugation step following the aqueous–organic partition has eliminated the filter paper drying step used on several methods[8,11,13]. After centrifugation, the *n*-pentane (upper) and aqueous (lower) layers are easily separated by using a Pasteur pipette to remove the menadione containing *n*-pentane. The organic extract is evaporated to dryness at reduced pressure as described elsewhere[8,11]. The residue is then dissolved in a known amout of methanol for menadione analysis by reversed-phase HPLC.

Post-column zinc reducer

Metals, such as zinc and cadmium, are well known for their ability to reduce quinones to their corresponding hydroquinones. However, only recently has a metal-filled column been developed for on-line reduction of a variety of menaquinones exhibiting vitamin K activity. Haroon *et al.*[12] used a zinc reducer to perform HPLC–fluorescence analysis of plasma samples for vitamin K_1 (phylloquinone) and K_1 epoxide. In their study, an acidic mobile phase (methanol– aqueous solution of 0.05 mol/l acetic acid, pH 3, 95:5) and one containing zinc ions (methanol–aqueous solution of 0.01 mol/l zinc chloride, 95:5) was necessary for reduction of vitamin K_1. In this study, vitamin K_3 has been shown to be reduced to its fluorescent analogue (2-methyl-1,4-dihydroxynapthalene) using methanol–water (75:25) as mobile phase coupled with an improved low volume zinc powder (< 38 μm particle size) reducer. The fluorescence response to menadione was linear in the range 1–100 ng of menadione injected. An acidic mobile phase (methanol–water, 75:25, pH 2.1 with acetic acid) was studied to detect any increase in fluorescence intensity for menadione (*i.e.* increase in reduction efficiency of the zinc metal). However, lowering the pH of the mobile phase resulted in an increase in pressure and finally plugging of the line to the detector. This may have been due to the depletion of zinc particles by acidic reaction and formation of hydrogen bubbles causing channeling and leakage of zinc particles around the frits. As shown in Fig. 1, the menadione was reduced to a fluorescent analogue in the presence of zinc metal as evidenced by the large fluorescence response with the zinc reducer in-line (chromatogram A) and without the reducer (chromatogram B). The small peak at 5.90 min in chromatogram A was due either to a small fluorescent impurity (possibly the dihydroxy reduction product) in the menadione standard or a reduction reaction of menadione with the stainless-steel tubing and column.

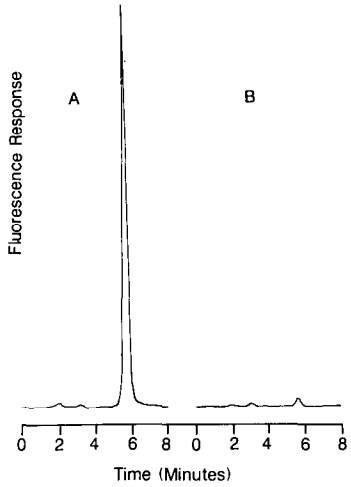

Fig. 1. HPLC–fluorescence chromatograms of 4-μg/ml menadione standard solutions (A) with and (B) without the post-column zinc reducer.

Recovery of MSB from synthetic animal feed

Triplicate samples of synthetic animal feed from a single lot were spiked at 20 and 200 mg/kg of MSB. Control animal feed samples containing only a vitamin premix were determined to contain 14.8 ± 0.9 mg/kg of MSB without correction for recovery (Table II). The percent recoveries of MSB at 20- and 200-mg/kg levels in feed of 91.2 ± 5.7 and 94.5 ± 4.4 were corrected for the control feed level. A typical HPLC–fluorescence assay of an MSB standard of 1 μg/ml in methanol (A), a control synthetic feed sample (B), and a 20-mg/kg synthetic feed spike (C) are shown in Fig. 2. The minimum detection limit for a 1-g synthetic feed sample was determined to be 20 μg/kg. Synthetic laboratory animal feed specifications for rat and mouse diets are usually 2–20 mg/kg of vitamin K_3.

TABLE II

RESULTS OF ANALYSIS OF SYNTHETIC ANIMAL FEED SPIKED WITH VITAMIN K_3 (AS MENADIONE SODIUM BISULFITE) USING HLPC–FLUORESCENCE

All results are mean ($\bar{x}$), standard deviation (S.D.), and relative standard deviation (R.S.D.) of triplicate 1.00-g synthetic feed samples from a single lot of feed.

Amount added (mg/kg)	*Amount found (mg/kg)*	*Amount recovered (%)*	
		$\bar{x}$ ± *S.D.* [a]	*R.S.D.*
Control	14.8 ± 0.9	–	–
20	33.0 ± 1.1	91.2 ± 5.7	6.3
200	203.8 ± 8.7	94.5 ± 4.4	4.7

[a] Recoveries are corrected for the menadione sodium bisulfite level found in the control feed samples.

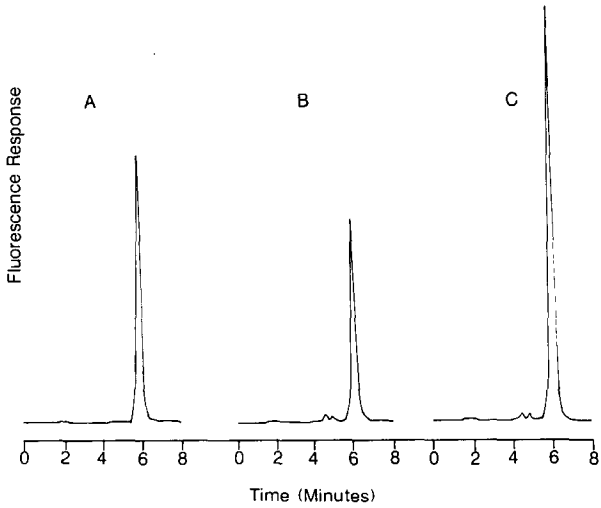

Fig. 2. Typical HPLC–fluorescence chromatograms of a (A) 1-μg/ml MSB standard, (B) 1.00-g control synthetic animal feed, and (C) 1.00-g synthetic animal feed sample spiked with MSB at the 20-ppm level.

Vitamin K_3 (MSB) analysis of synthetic animal feeds and vitamin premixes

Several synthetic feed lots and feed premixes were analyzed. The vitamin K_3 contents in mg/kg are reported in Table III. Vitamin K_3 in unspiked synthetic feed samples ranged from 1.90 to 20.7 mg/kg with premixes containing high levels (approx. 5%, w/w). As shown in Table III, vitamin-fortified meals and pellets stored for

TABLE III

RESULTS OBTAINED FROM HPLC–FLUORESCENCE ANALYSES FOR VITAMIN K_3 IN SYNTHETIC FEED AND VITAMIN PREMIXES

NA = Not available.

Type of sample analyzed	*Manufacturer*	*No. of days stored prior to sampling*	*Vitamin K_3 found (mg/kg)*
Meal, fortified[a]	A	2	9.67
Meal, fortified	A	280	1.90
Meal, fortified	B	2	20.7
Meal, fortified	B	125	14.8
Pellets, unfortified[b]	B	2	6.71
Pellets, unfortified	C	10	1.97
Pellets. fortified	C	16	5.89
Pellets, fortified	C	219	2.13
Vitamin feed premix[c]	D	NA	$55.4 \cdot 10^3$
Vitamin feed premix	D	NA	$57.3 \cdot 10^3$

[a] Meal or pellets contain vitamin premix.
[b] Pellets contains no vitamin premix.
[c] Vitamin premix, high in vitamin K_3 content and were diluted 1:5000 with methanol prior to HPLC analysis.

125–280 days were much lower in K_3 content than those stored 2–16 days indicating the instability of menadione, even as the more stable bisulfite salt, in synthetic animal feed stored for long periods. For determining if feed samples meet vitamin K_3 specifications, it will be advantageous for analyses to be performed as soon as possible after receiving the samples.

GC–FT-IR confirmation of menadione in synthetic feed

To confirm the presence of menadione in a synthetic feed extract, a 10.0-g sample of a typical synthetic animal feed was analyzed by capillary GC–FT-IR. Gram-Schmidt chromatograms are shown in Fig. 3 of a 0.5-mg/ml menadione standard (A) and a 10.0-g synthetic feed sample in 200 μl *n*-pentane (B). The minimum detection limit for menadione using the GC–FT-IR method was determined to be 2 mg/kg for a 2-μl splitless injection of the pentane–feed extract. Following a computer spectral-library search, an infrared spectral match was found for the animal feed peak at 6.48 min retention (IR trace A) with the 3250 compound EPALIB 8 library spectrum number 1343, menadione (IR trace B) as shown in Fig. 4.

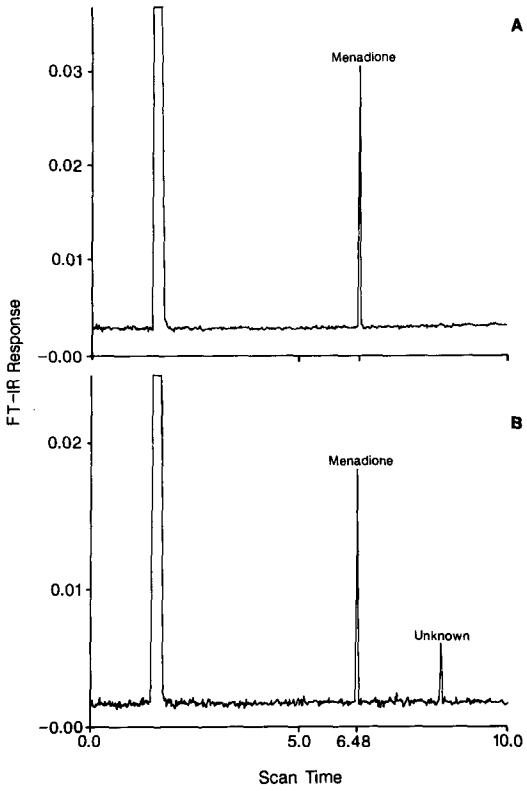

Fig. 3. DB-1701 capillary GC–FT-IR Gram-Schmidt chromatograms of 2-μl splitless injections of (A) a 0.5-mg/ml menadione standard in *n*-pentane and (B) a 10.0-g animal feed in 200 μl of *n*-pentane. Menadione is the peak at 6.48 min scan time.

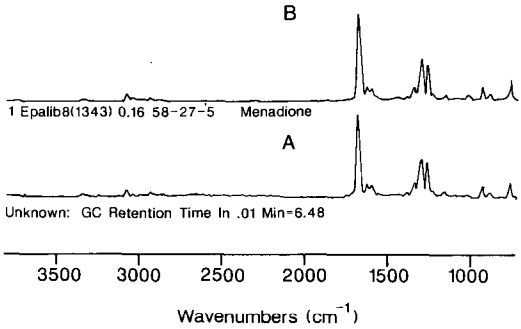

Fig. 4. Results of the FT-IR spectral-library search showing an FT-IR trace (A) of the 6.48 min peak for a 10.0-g synthetic animal feed sample and the FT-IR trace (B) for compound No. 1343, menadione, from the 3250 compound EPALIB 8 vapor phase library.

CONCLUSIONS

The improved zinc reducer reported here has been shown to be a very effective on-line method for reduction of menadione to its fluorescent analogue, 2-methyl-1,4-hydroxynaphthalene. Using this technique, a fast and sensitive HPLC method for analysis of vitamin K_3, as menadione sodium bisulfite, in synthetic animal feed has been described. The GC–FT-IR method has produced a good confirmatory analysis in addition to the specificity of the fluorescence method.

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