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Determination of phylloquinone in intravenous fat emulsions and soybean oil by high-performance liquid chromatography

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Abstract

A method for determining vitamin K_1 (VK_1) in intravenous fat emulsions (IVFE) and soybean oil (SBO) using a single-step HPLC procedure is described. The method is linear from 9.7 to 617 ng of VK_1 per gram of IVFE and from 41 to 5234 ng of VK_1 per gram of SBO (signal-to-noise ratio = 3). The results obtained by this method with four samples of IVFE indicate the existence of a variability in VK_1 content depending on the nature of the preparation (long- or medium-chain triglycerides), the producer and the production batch. This variability of the VK_1 content, also observed with the same method in SBO, is ascribable to the raw materials used to produce the different preparations.

1. Introduction

Intravenous fat emulsions (IVFE) are used as a source of energy and essential fatty acids for adults and for children and infants on total parenteral nutrition (TPN). These emulsions contain 5–20% of soybean oil, considered to have a high vitamin K_1 or phylloquinone (VK_1) content [1–5]. Two studies have shown that, under certain conditions, the VK_1 content of IVFE is amply sufficient to cover the needs of children on TPN [6,7]. However, as doctors lack specific data on the vitamin content of these emulsions, they continue to prescribe daily injections of phylloquinone to their patients on TPN. Whereas the essential role of this vitamin in blood coagulation is well known, there is no proof of its presumed functions on calcium metabolism [8–10]. Further, a recent study [11] showed that intramuscular administration of vitamin K to newborns was statistically related to the later development of childhood cancer. In order to be able to control the VK_1 intake of patients of TPN, a simple, reliable method for determining phylloquinone in IVFE is needed. To our knowledge such a method is not available to date. It is also important, at the production level, to be able to determine this vitamin in the soybean oil used to prepare IVFE.

The method for assaying phylloquinone in soybean oil proposed by Zonta and Stancher [1] is difficult to implement on a routine basis. More

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recently, Ferland and Sadowski [2] applied the method for the determination of plasma VK_1 developed by Haroon *et al.* [12] to the study of various vegetable oils. More sensitive and specific than the former [1], this latter method nonetheless includes two chromatographic steps, the second of which is coupled to a precolumn derivatization [2].

In this paper, we propose a one-step HPLC method for the determination of VK_1 in IVFE and soybean oil. The proposed method was applied to the study of VK_1 content and conservation in four samples of IVFE and three samples of soybean oil.

2. Experimental

2.1. Samples

The study was conducted on four samples of IVFE commonly used for the various medical departments in the Trousseau hospital: two IVFE samples composed of long-chain triglycerides [(i) LCT_1 containing 20% of soybean oil and (ii) LCT_2 containing 10% of soybean oil]; two IVFE samples composed of LCT and medium-chain triglycerides [(iii) MCT_1 containing 5% of soybean oil and (iv) MCT_2 containing 10% of soybean oil]; three samples of commercial soybean oil, including two from the same producer (SBO_1, 1-l clear bottle, expiry date January 1993, and SBO_2, 5-l opaque bottle, expiry date December 1992). The third sample (SBO_3, 1-l clear bottle, expiry data February 1993) came from another producer.

2.2. Reagents

Standards of vitamin $K_{1(20)}$ and vitamin $K_{1(25)}$ [13] used as internal standards (I.S.) were purchased form Hoffmann-La Roche (Neuilly-Sur-Seine, France). Stock standard solutions of the different vitamins, 1 g l^{-1} in hexane, were stored at −20°C in the dark. These standard solutions were diluted with hexane as required and stored under the same conditions.

Hexane, acetonitrile and diisopropyl ether were of Uvasol grade from Merck (Darmstadt, Germany). Absolute ethanol was obtained from Carlo Erba (Milan, Italy) and sodium perchlorate from Merck. All chemicals were used as received.

2.3. Apparatus

The HPLC system was the same as described previously for the determination of *trans*-phylloquinonemia [13], namely a reversed phase system with a 70 mm × 4.7 mm I.D. XL 3-μm octyl cartridge (Beckman, Gagny, France) combined with fluorimetric detection after post-column coulometric reduction. We are currently using a Linear Fluor LC 304 detector (Spectra-Physics, Courtaboeuf, France).

During the tests, we also used Sep-Pak silica cartridges (Waters, St. Quentin-en-Yvelines, France) to purify the fat emulsions containing VK_1 and I.S. before injection into the chromatograph.

2.4. Method

The concentration of the standard solutions of vitamin K_1 ($SS_1 = 0.4$ mg l^{-1} for IVFE analysis and $SS_2 = 4$ mg l^{-1} for soybean oil analysis) in hexane was determined, after equilibration at room temperature, by measuring the absorbance (after suitable dilution) at 248 nm (molar absorptivitiy = 19 900 l mol^{-1} cm^{-1}). For each type of determination (IVFE or soybean oil), a calibration graph was produced by adding incremental amounts of VK_1 to a blank sample exposed to daylight to eliminate endogenous vitamin.

Preparation of blank sample

Introduce 6–7 g of IVFE or soybean oil into a 40 − ml clear glass tube, stopper the tube and place it horizontally near a window. After 48 h of exposure to daylight, the sample no longer contains any VK_1 detectable by the proposed method.

Preparation of calibration graph

Fill four 5-ml glass tubes with aliquots of standard VK_1 solution (0.10, 0.50, 1.00 and 2.00

ml of SS_1 and 0.10, 0.25, 0.50 and 1.00 ml of SS_2, depending on the circumstances) and evaporate to dryness under a stream of nitrogen. Pipette into each tube 1 g of blank sample and shake the tubes gently for 10 min in the dark. The samples thus enriched can be stored for at least 1 month in the dark.

Assay of VK_1 in IVFE and soybean oil

Pipette into a glass tube 0.10 ml of I.S. solution at the appropriate concentration (0.2 mg l^{-1} for IVFE analysis and 2 mg l^{-1} for soybean oil analysis) and evaporate under a stream of nitrogen. Pipette into the same tube about 100 mg (determine the amount accurately) of test or calibration sample and shake for 15 s. Next add 0.50 ml of 0.9% NaCl solution and 1.00 ml of ethanol. After shaking for 2 min, add 3.00 ml of hexane and agitate for 30 min at room temperature in the dark. After centrifuging at 7000 rpm for 10 min (5000 *g*), collect the supernatant in a conical tube and evaporate to dryness under a stream of nitrogen. Dissolve the dry residue (lipid extract) in 0.50 ml (5.00 ml for soybean oil analysis) of mobile phase [acetonitrile–ethanol (95:5, v/v) containing 0.005 *M* $NaClO_4$], and inject 0.05 ml into the chromatograph.

Purification of lipid extract

During the tests, we purified several lipid extracts on a Sep-Pak silica cartridge prior to injection into the chromatograph. The protocol was as follows: the lipid extract is dissolved in 1.00 ml of hexane (10.00 ml for soybean oil) and 0.10 ml is injected into a Sep-Pak cartridge prepared in advance using 10.00 ml of hexane. After passage of 10.00 ml of hexane to eliminate the less polar lipids, VK_1 and IS are eluted using 10.00 ml of hexane–diisopropyl ether (90:10, v/v). The eluent is collected in a conical tube and evaporated to dryness under a stream of nitrogen. The dry residue is carefully dissolved in 1.00 ml of hexane and the solution is evaporated to dryness under a stream of nitrogen. The final residue is dissolved in 0.05 ml of mobile phase and 0.04 ml is injected into the chromatograph.

Study of conservation

Conservation of VK_1 in IVFE (in the original packaging) was studied under three different conditions: (1) at room temperature in the dark; (2) at 4°C in the dark; and (3) at room temperature in daylight. After sampling an aliquot for an initial assay (day 0), the flask was restoppered and stored as already described. Aliquots of each sample thus stored were then taken for assaying VK_1 on days 4, 22, 40, 50 and 60.

Soybean oil conservation was studied at room temperature in the dark.

3. Results

The chromatographic profiles of an IVFE and a soybean oil sample obtained by the proposed method are shown in Fig. 1a and b, respectively. The peaks are qualitatively identical, confirming the presence of soybean oil in the IVFE samples. After 48 h of exposure to daylight, according to the operating protocol described above, we observe on the chromatogram of the soybean oil, for example, the complete resolution of the peak attributed to VK_1 (Fig. 1c).

Different criteria enabled us to identify and verify the purity of the VK_1 peak. In addition to the retention time, the disappearance caused by exposure to daylight and the effect of supplementing, the identity of the VK_1 peak and its purity were checked by analysing the hydrodynamic voltammogram and the fluorescence characteristics of the molecule [13,14].

By purifying the lipid extract on a Sep-Pak silica cartridge, we were able to eliminate the unidentified peaks of k' higher than those of the I.S. and to shorten the run time (Fig. 1d). This treatment also affords a gain in detection sensitivity of *ca.* 10%, ascribable to the elimination during purification of the interfering substances that co-eluted with the two vitamins during the HPLC step. The interferences probably occur at the level of the electroreduction of the two vitamins by competition with the active sites of the working electrode. In fact, injection into the chromatograph of *ca.* 30 samples not purified on the Sep-Pak cartridge led to gradual passivation

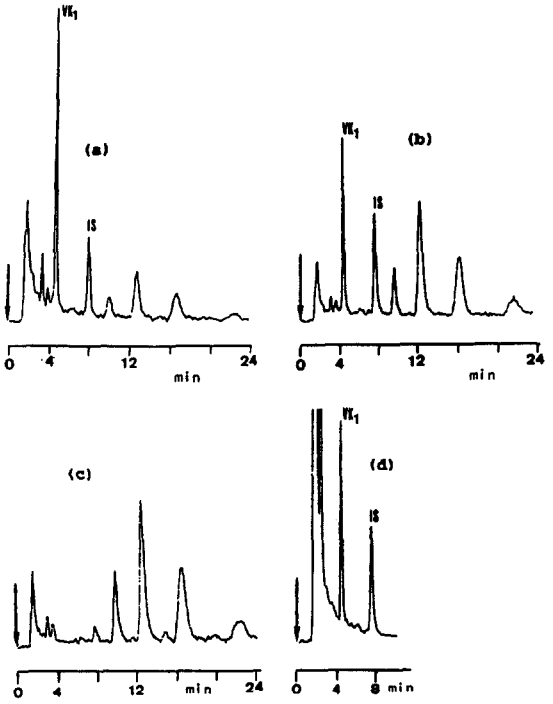

Fig. 1. Chromatograms of (a) an extract of IVFE (MCT) and of an extract of soybean oil (b) before and (c) after 48 h of exposure to daylight and (d) after Sep-Pak silica cartridge purification. Column, 70 mm × 4.6 mm I.D. XL 3-μm octyl cartridge; mobile phase, acetonitrile–ethanol (95:5, v/v), containing 0.005*M* sodium perchlorate; flow-rate, 0.80 ml min^{-1}. Electrochemical reduction followed by fluorescence detection [13]. Unmarked peaks are unidentified.

of the working electrode, which resulted in a decrease in the sensitivity of the detection system. Applying a positive potential (+0.9 V) for a few minutes regenerates the electrode surface and leads to recovery of the initial sensitivity after desorption of the material adsorbed. We therefore decided to retain the direct injection method, leaving aside the long and costly purification of the lipid fractions on Sep-Pak cartridges. Lastly, prior lipase treatment of the IVFE or soybean oil samples, as proposed by Zonta and Stancher [1], makes no noticeable difference in terms of the chromatographic profiles.

The linearity of the proposed method was studied using a range of concentrations obtained through successive dilutions with blank sample of a sample of IVFE with an initial VK_1 content of 617 ng g^{-1}. The method is linear from 9.7 to 617 ng g^{-1} of VK_1 [$y = 0.85x + 7.5$, $r = 0.998$, $n = 6$, where y is the area ratio: VK_1/I.S. and x is the sample VK_1 concentration (ng g^{-1})]. For soybean oil, the linearity was studied using a range of concentrations obtained by supplementing an oil sample, followed by successive dilutions with blank sample. The method proved linear from 41 to 5234 ng of VK_1 per gram of oil ($y = 0.77x + 3.6$, $r = 0.996$, $n = 7$).

The detection limit of the method is 2 ng of VK_1 per gram of IVFE and 5 ng per gram of soybean oil (signal-to-noise ratio = 3).

The accuracy of the method is shown in Table 1. The relative standard deviation (R.S.D.) is satisfactory for both the upper and lower values.

The recoveries of VK_1 and the I.S. were determined after supplementing a blank sample, by comparing their peak areas with those obtained after direct injection into the chromato-

Table 1
Precision of the method

Sample	Within-run ($n = 5$)		Between-run ($n = 3$)	
	Mean (ng g^{-1})	R.S.D. (%)	Mean (ng g^{-1})	R.S.D. (%)
MCT_1	46	3.5	44	7.1
MCT_2	216	–	–	–
LCT_1	617	2.0	618	4.0
LCT_2	220	–	–	–
SBO_1	1080	1.7	1020	5.2
SBO_2	3080	0.9	3130	3.2
SBO_3	1080	–	–	–

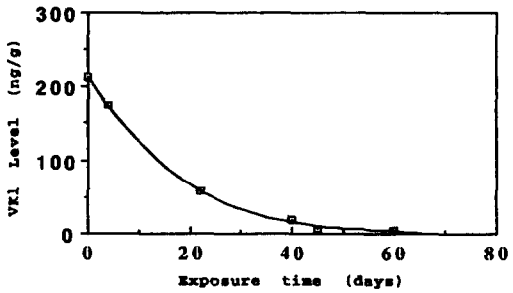

Fig. 2. Photodegradation of vitamin K_1 in a 250-ml flask of IVFE (LCT) on exposure to daylight. □ = VK_1 level (ng g^{-1}).

graph of pure solutions of the two vitamins. The recovery of VK_1 was 75–85% for IVFE ($n = 3$) and 85–91% for soybean oil ($n = 3$). Under the same conditions, the recovery of the I.S. was 65–70% for IVFE ($n = 3$) and 69–76% for soybean oil ($n = 3$).

The recovery of the method, calculated in relation to the I.S., after supplementing two samples of IVFE and two samples of soybean oil, exceeded 92% in all instances.

Fig. 2 shows the effect of daylight on the VK_1 content of a 250-ml flask of LCT. Under these conditions, there is considerable deterioration of the vitamin. However, its half-life remains relatively long (14 days). Also, the decrease in the concentration of this vitamin after 24 h of exposure remains less than 5%. Consequently, the deterioration of the vitamin during a 12–24-h infusion is negligible. Conversely, when kept in the dark, VK_1 remained stable at the two storage temperatures studied. Lastly, in the dark and at room temperature, phylloquinone remained stable for over 6 months (length of the study) in the two samples, SBO_1 and SBO_2, studied.

4. Discussion

To assay VK_1 in soybean oils, Zonta and Stancher [1] recommended, in order to eliminate interfering lipids, a 15-h enzymatic digestion step, followed by two chromatographic steps, the second by HPLC combined with UV detection. According to Lambert and De Leenheer [14], "Due to the complexity of the matrices and the problems in the detection, the quantification of endogenous vitamin $K_{1(20)}$ levels in humans, as well as the analysis of vitamin $K_{1(20)}$ in food samples, remains an analytical challenge". However, when a sample has a relatively high VK_1 content (several tens of nanograms of VK_1 per gram of sample), our results show that use of a sufficiently sensitive detection mode makes it possible to simplify sample purification. In their method, which features a low detection sensitivity, they used 15 g of sample [1]. By decreasing this amount to 0.25–1 g, Ferland and Sadowski [2] succeeded in adapting the method for the determination of phylloquinonemia developed by Haroon *et al.* [12] to the determination of VK_1 in vegetable oils. However, the latter method consists of two chromatographic steps, including chemical reduction of the VK_1 molecule to make it fluorescent. Now, in the reduced form, VK_1 is highly unstable and difficult to handle [14]. The use of a detection mode as sensitive as fluorescence for VK_1 makes it possible to work with a sample of a few milligrams, thereby considerably decreasing the amount of lipids to be treated. Under these conditions, the enzymatic digestion step becomes useless. Likewise a single HPLC step is sufficient to separate and assay VK_1 in soybean oils and *a fortiori* in IVFE containing only 10–20% oil. This is what is proposed in our method in which the final amount of oil injected into the chromatograph is *ca.* 1 mg.

The results obtained with the various IVFE samples indicate the existence of a variability stemming from the origin and nature of the sample and also from the production batch studied (Table 1). Without specifying the number of samples analysed, Goulet *et al.* [6] reported VK_1 concentrations of 208 ± 34 ng ml^{-1} for the IVFE they used in their clinical study. Although they also did not specify their assay methodology, especially as regards the amount of sample and its preparation, their findings tend to confirm the origin-related variability of the emulsions observed in our study.

As regards soybean oils, the results we obtained (Table 1) agree with those of Zonta and

Stancher (121–3330 ng g^{-1}) [1]. They are lower than those obtained by Schneider *et al.* (4500–6300 ng g^{-1}) [3], while those obtained by Ferland and Sadowski [2] are even lower (1400–2000 ng g^{-1}). Zonta and Stancher attributed [1] these differences to the industrial production method and/or to the raw materials used. Our results for both the soybean oils and the IVFE tend to confirm the raw materials assumption. According to Ferland and Sadowski [2], the differences do not stem from the raw materials, but rather from the assay method. This is surprising as they asserted in another paper [15] that climate, soil and the conditions of growth can affect the VK_1 content of greenery. The influence of such factors on the phylloquinone content of soybeans is under study.

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