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Short communication

Determination of vitamins A and E in infant milk formulae by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed for the determination, in one run, of retinol and α-tocopherol in infant milk formulae. The method involved saponification at room temperature and a later extraction of vitamins with *n*-hexane. The vitamins were resolved with a C_{18} reversed-phase column and they were detected by UV spectrophotometry. Linearity, precision, recovery and sensitivity were satisfactory. The main advantage of the method proposed is the simultaneous determination of both vitamins using a common extraction procedure and UV detection with a variable-wavelength spectrophotometer. © 1997 Elsevier Science B.V.

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1. Introduction

Due to the nutritional importance of liposoluble vitamins, much research has been performed to develop sensitive and selective methods for their determination in foods. Several fluorometric methods have been developed for vitamin A in milk [1–4]; however, those procedures were not considered to be reliable for infant milk formulae, because of possible interferences from other fluorescent substances. The official Association of Official Analytical Chemists (AOAC) colorimetric methods [5] to determine vitamins A and E have several disadvantages related to sample preparation, precision and specificity. High-performance liquid chromatographic (HPLC) methods are claimed to have greater specificity than colorimetric or fluorimetric procedures. Few HPLC methods have been reported to quantify both vitamins A and E in one run, since most techniques determine only one vitamin. Sample preparation for HPLC analysis can be performed according to various procedures, such as direct extraction with organic solvents [6,7], saponification followed by extraction with organic solvent [8–12], or enzymatic hydrolysis [13]. The main disadvantage of the simultaneous determination of liposoluble vitamins is the lack of sensitivity due to differences in the optimal absorption wavelength for each vitamin. In general, a compromise wavelength for the simultaneous detection of vitamins A, E and D is adopted [14]. The aim of this study was to develop and validate a rapid HPLC method for the simultaneous determination of vitamins retinol and α-tocopherol. The selectivity, precision and accuracy of the method should be sufficiently acceptable to permit quantification of these vitamins in infant milk formulae.

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2. Experimental

2.1. Equipment

The HPLC system consisted of a HP (Hewlett-Packard, CA, USA) 1050 Series quaternary pump, a HP 1050 Series degassing device, a Waters 717 autosampler with a 20-μl fixed loop injector (Waters, Milford, MA, USA) and a Series HP 1050 UV detector. Chromatographic data were acquired and analyzed with a Chemstation System HP 3365-II. The column was a Tracer Spherisorb ODS 2 C_{18} column, 25×0.46 cm, particle diameter 5 μm (Teknokroma, Barcelona, Spain) with a matching guard cartridge. Water–acetonitrile–methanol (4:1:95, v/v/v) was used as the mobile phase, working in isocratic mode. A Labo Rota S 300 (Resona Techins, Switzerland) rotatory evaporator with a Labo Term SW 200 water-bath was used for sample preparation.

2.2. Reagents and solvents

All vitamin standards were purchased from Sigma (Madrid, Spain). Standard solutions of vitamin A, all *trans*-retinol (from 1 to 10 μg/ml) and of vitamin E, α-tocopherol (from 10 to 100 μg/ml) were prepared in methanol. Ultra pure water, generated by the Milli-Q System (Millipore, Bedford, MA, USA), was used. Acetonitrile, *n*-hexane and methanol were of HPLC grade and were obtained from SDS (Barcelona, Spain). All other reagents, such as absolute ethanol, 85% potassium hydroxide and phenolphthalein (Panreac, Barcelona, Spain), ascorbic acid and butylated hydroxytoluene (BHT) (Sigma) and anhydrous sodium sulfate (Merck, Barcelona, Spain) were of analytical grade.

2.3. Sample preparation

Samples were commercial infant milk formulae (liquid and powdered). Sample preparation was based on the method described by Bognar [9] to determine vitamin A in foods, but the saponification was carried out at room temperature. Subdued light and the addition of an antioxidant (ascorbic acid) were necessary to prevent loss of liposoluble vitamins during sample saponification and extraction. A 25-g amount of sample liquid or of reconstituted powdered sample, were accurately weighed and transferred to a 250-ml amber erlenmeyer flask. Then, 0.5 g of ascorbic acid, 50 ml of absolute ethanol and 10 ml of 60% potassium hydroxide solution were added, under a stream of nitrogen. Saponification was performed overnight with slow constant stirring at room temperature. Then, the saponified mixture was transferred to a 250-ml amber separatory funnel, rinsed with 30 ml water and extracted five times (shaking for 2 min) with three fractions containing 50 ml of *n*-hexane and two fractions containing 25 ml of *n*-hexane. The combined *n*-hexane extract was washed with 50 ml fractions of water, which were added with some drops of phenolphthalein, until the aqueous layer appeared colorless. A 1-g amount of BHT was added as the antioxidant and the mixture was then passed through a Whatman No. 1 filter (containing 20 g of anhydrous sodium sulfate) and was collected into a 250-ml amber volumetric flask. The extract was concentrated by rotatory evaporation at 40°C. Finally, the evaporated residue was reconstituted with 10 ml of methanol and passed through a 0.45-μm filter before liquid chromatographic (LC) analysis. The wavelengths selected were optimum for the detection of vitamin A (323 nm) and vitamin E (292 nm) and were changed during the chromatographic run.

3. Results and discussion

Typical chromatograms of samples were relatively simple (Fig. 1), showing high resolution and certain

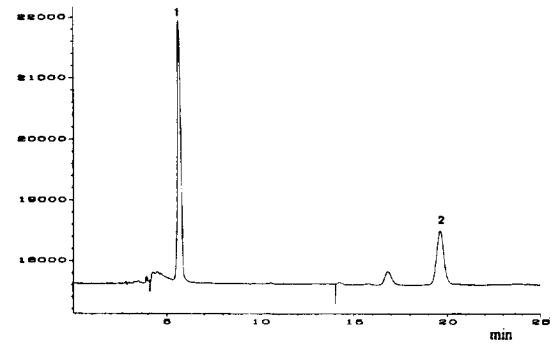

Fig. 1. Chromatogram of vitamins A and E in infant milk formulae. Peak identities: retinol (1) and α-tocopherol (2).

Table 1
Precision of the HPLC method for determination of vitamins A and E in infant milk formulae

	Mean (SD)[a] (μg/ml)	R.S.D. (%)	R.S.D.H. (%)[b]
Vitamin A	3.78 (0.12)	3.27	6.5–8.7
Vitamin E	23.22 (0.52)	2.25	4.9–6.6

[a]Mean (standard deviation).
[b]R.S.D.H.=Relative standard deviation according to Horwitz's formula for intra-laboratory studies.

identification of vitamins A and E within 20 min. Vitamins were identified on the basis of retention time by comparison with standard solutions. Relative standard deviations (R.S.D.s) of retention times ranged from 0.11 to 0.53%. The method proposed is specific for the determination of retinol and α-tocopherol. Other tocopherols, such as δ-tocopherol or γ-tocopherol eluted separately from α-tocopherol.

The calibration curves were prepared with standard solutions of each vitamin at levels similar to those contained in the milk formulae. The method was linear between 1 and 10 μg/ml for vitamin A, and between 10 and 100 μg/ml for vitamin E. Linearity was verified by analysis of variance of the regression. A correlation coefficient r value >0.9988 was obtained for both vitamins ($p<0.001$) and the coefficients of determination (r^2) were greater than 99.7%.

To study the precision of the method, eight determinations of vitamins in a milk sample were performed using the same reagents and apparatus. The R.S.D. values obtained for each vitamin (Table 1) were always satisfactory, according to Horwitz's formula for intra-laboratory studies [15]. Recovery was tested by the standard addition procedure using two addition levels for each vitamin (Table 2). Eight determinations were carried out for each addition level. The accuracy of the method did not depend on vitamin content in the sample on the basis of Cochran's test. Average recoveries were 97.51±1.45%) for vitamin A and 85.98±1.68%) for vitamin E.

To check the sensitivity of the method under the working conditions proposed, the detection limit (DL) and the determination limit (DtL) were studied. Two criteria were applied. The first was the repeated analysis of a blank according to the formula of Long and Winefordner [16], which is the most common method used for chromatographic procedures. The blank was ultra pure water that was subjected to saponification, extraction with hexane, evaporation and LC analysis as described above. The second approach was based on the analysis of the lowest concentration standard solution that can be detected [17]. The sensitivity results obtained according to both criteria were similar for both vitamins. The DL for vitamin A was 0.01 μg/ml and that for vitamin E was 0.30 μg/ml, while the DtL was 0.02 μg/ml for vitamin A and 0.40 μg/ml for vitamin E.

In addition, vitamins D_2 and D_3 can also be determined by the method proposed, in samples in which the vitamin D_2 content is greater than 0.03 μg/ml and the content of vitamin D_3 is greater than 0.04 μg/ml. Infant milk formulae contain only trace amounts of these vitamins, and cannot therefore be accurately quantified with the method proposed. Precision and recovery in samples spiked with vitamins D_2 and D_3 were satisfactory, showing an R.S.D. value of 1.67% for vitamin D_2 and of 1.05%

Table 2
Recoveries of the HPLC method for determination of vitamins A and E in infant milk formulae

	Initial content (μg/ml)	Content after addition		Cochran's test C^c_{exp}	Recovery (%) Mean (SD)[d]
		Level I[a] (μg/ml)	Level II[b]		
Vitamin A	3.83	5.82 (0.15)	7.73 (0.29)	0.388	97.51 (1.45)
Vitamin E	23.66	28.59 (0.33)	42.47 (0.93)	0.469	85.98 (1.68)

[a]2 μg for vitamin A and 10 μg for vitamin E.
[b]4 μg for vitamin A and 25 μg for vitamin E.
[c]$C_{tab\ (7,\ 2,\ 0.05)}$=0.8332, C_{tab} and C_{exp} are tabulated and experimental Cochran's C values, respectively.
[d]Mean (standard deviation).

for vitamin D_3, with a recovery of 75% for both vitamins.

The chromatographic method proposed allows rapid and complete resolution of vitamins A and E in a single chromatographic run. The sensitivity for both vitamins achieved by means of detection at specific absorption wavelengths for each vitamin is higher than that of other methods described. According to the results of the reliability study, the method proposed was precise and accurate and showed appropriate sensitivity for application to the determination of vitamins A and E in infant milk formulae.

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