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Simultaneous determination of fat-soluble vitamins and provitamins in milk by microcolumn liquid chromatography

D.B. Gomis*, M.P. Fernández, M[a].D. Gutiérrez Alvarez

Departamento de Quimica Física y Analítica, Universidad de Oviedo, E-33006 Oviedo, Spain

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Abstract

The fat-soluble vitamins A, D_2, D_3, E and K_1, retinyl acetate, retinyl palmitate, tocopherol acetate, ergosterol and 7-dehydrocholesterol in milk were separated on a packed reversed-phase fused-silica microcolumn, and determined with UV detection. Conventional liquid chromatographic equipment was adapted for such purposes, providing a highly efficient and sensitive analytical system. The vitamins could be separated in gradient mode with detection limits comprised between 0.02 ng/ml for retinol and 2 ng/ml for vitamin E. All vitamins were separated in less than 17 min. Recovery studies showed good results for all solutes (89–107%) and the intra-day coefficients of variations ranged from 2 to 8%. Application of the proposed method to the quantification of fat-soluble vitamins in milk is reported.

Keywords: Vitamins, fat-soluble; Provitamins

1. Introduction

Microcolumn liquid chromatography (μLC) did not see much development in the first decade since its invention as a result of the lack of commercially available instrumentation and columns. Nevertheless, in recent years, as a consequence of the demand for new, highly efficient and sensitive analytical methods in the fields of medicine, biotechnology, the pharmaceutical industry, food technology and other areas, μLC has shown considerable progress both in instrumentation and applications. The analytical advantages of μLC (solvent economy, higher efficiency, mass sensitivity, easy coupling with other techniques and the small amounts of sample required [1,2] make this technique indispensable for the resolution of some modern analytical problems.

The purpose of this work was to separate and determine the fat-soluble vitamins in milk. These compounds are present in low concentration in milk, therefore, in addition to carrying out sample-handling procedures in order to isolate and preconcentrate them, a sensitive detection system is necessary. Unfortunately, not all of the fat-soluble vitamins can be determined directly by fluorescence or electrochemical techniques. Consequently, because of its simultaneous determination, spectrophotometric detection is the sensible choice. In general, UV detection shows a lack of sensitivity, but with μLC the solute concentration at the column outlet increases, thus making its use possible for the sensitive determination of all vitamins.

In recent years, many HPLC assays on vitamins

*Corresponding author. Fax: +34-98-510-3125.

E-mail address: dbg@sauron.quimica.uniovi.es (D.B. Gomis).

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have been published, the principles of which have been summarised [3,4]. However, methods currently available for determining physiological concentrations of vitamins in biological samples by HPLC require several preliminary steps of clean-up and, sometimes, of preconcentration because the methods do not possess sufficient sensitivity. Several excellent HPLC separations of fat-soluble vitamins have been published [5–20]. However, few report, to our knowledge, have been published on the use of HPLC for the simultaneous determination of all of these vitamins [21,22].

2. Experimental

2.1. Reagents

Methanol and tetrahydrofuran were of HPLC grade and were used as received. Ultrapure water was obtained through a Milli-Q system (Millipore, Milford, MA, USA). Analytical-reagent grade retinyl acetate, retinyl palmitate, retinol, tocopherol acetate and vitamin K_1 (Sigma, St. Louis, MO, USA), DL-α-tocopherol, cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) (Merck, Darmstadt, Germany), ergosterol (provitamin D_2) and 7-dehydrocholesterol (provitamin D_3) (Fluka, Buchs, Switzerland) were used. Butylated hydroxytoluene (BHT) was purchased from Sigma (St. Louis, MO, USA). Absolute ethanol and hexane were purchased from Romil (Loughborouh, UK) and Merck, (Darmstadt, Germany), respectively.

2.2. Sample preparation

Milk sample (5 ml) was transferred into a 10 ml glass tube and 5 ml of ethanol with 0.025% of butylated hydroxytoluene (to protect the vitamins against oxidation) were added and the mixture was treated for 2 min in an ultrasonic bath to disrupt the fat globule membranes (proteins and phospholipids) that encapsulate fat-soluble vitamins. The solution was then transferred into a separatory funnel and 15 ml of hexane were added, followed by vortex mixing for 5 min. The organic layer was transferred and the extraction process was repeated with 15 ml of hexane. The two organic layers were combined and transferred into another separatory funnel for washing with two portions (5 ml each one) of methanol–water (9:1). The organic upper layer was separated and passed through a 0.45 μm filter. This was then evaporated under nitrogen until dryness, the residue was reconstituted in 100 μl methanol and a 60 nl aliquot of this solution was injected into the HPLC system. For large volume injections into the microcolumn (e.g., 5 μl), greater than the volume overload (400 nl for this microcolumn), the sample can be reconstituted in 50 μl of MeOH plus 50 μl of water. In this way, the solvent strength of the sample is lower than the solvent strength of the mobile phase and the sample will be enriched at the top of the column bed [1].

2.3. Stock and working standard solutions

Individual stock standard solutions of each vitamin were prepared in ethanol containing 0.025% of BHT to provide a concentration of 1 mg/ml for all fat-soluble vitamins and provitamins.

Individual working standard solutions were prepared by appropriate dilution of the stock standard solution and filtered through a 0.22 μm membrane (Millex-HV_{13}, Millipore) before being injected into the system. All solutions were degassed with helium and stored in dark glass flasks at −20°C. By mixing suitable volumes of each stock solution the working solution was prepared to give concentrations ranging from 1 to 4 μg/ml for retinol, retinyl acetate, retinyl palmitate and vitamin D_3 and from 2 to 8 μg/ml for vitamin D_2, tocopherol, tocopherol acetate, vitamin K_1 and provitamins D_2 and D_3.

2.4. Apparatus and conditions

The experiments were carried out using a HPLC system equipped with two Kontron 422 pumps (Kontron Instruments, Milan, Italy) equipped with a split-flow system at a flow-rate of 0.1 ml min^{-1} providing a 6 μl/min flow, a 60 nl internal volume injector (C14W; Valco, Houston, TX, USA), a 500 nl internal volume injector (7520 Rheodyne, Cotati, C, USA) or a 8125 Rheodyne (Cotati, C, USA) injection valve with a 5 μl loop, a Kontron model 430 UV–VIS detector with a 90 nl, 20 mm, flow cell and a Data System 450 software program from Kontron

Instruments. The wavelengths of the detector were set at 325 nm for retinol, retinyl acetate and retinyl palmitate; 264 nm for vitamin D_2 and vitamin D_3; 280 nm for vitamin K_1, tocopherol, tocopherol acetate, ergosterol and 7-dehydrocholesterol, programmed in the gradient run.

The column used was a Hypersil C_{18} BDS (150× 0.3 mm I.D., 3 μm) (LC Packings, Amsterdam, The Netherlands). Chromatography was carried out in gradient [A=methanol–water (99:1); B=methanol–tetrahydrofuran (70:30)] mode. As a result of optimization, the gradient run conditions were programmed as follows: 0–4 min, 0% B; 4–10 min, 100% B; 10–15 min, 100% B; 15–17 min, 0% B (gradient I). For 5 μl injected volume, gradient program II was used: 0–6 min, 0% B; 6–12 min, 100% B; 10–14 min, 100% B; 14–16 min, 0% B (gradient II). A=methanol–water (99:1), B=methanol–tetrahydrofuran (70:30). Before use, the mobile phases were vacuum filtered through a 0.22-μm nylon filter (Lida, Kenosha, WI, USA) and degassed with helium. The chromatographic experiments were carried out at room temperature (20±2°C).

3. Results and discussion

Most of the published reversed-phase applications for fat-soluble vitamins utilise semi-aqueous phases, which typically consist of mixtures of methanol [24] or acetonitrile [25] and water, or non-aqueous mobile phases.

In our case, due to microcolumn used, semi-aqueous or pure solvents, such as methanol, are inadvisable as the mobile phase due to the high value of the capacity factor for retinyl palmitate ($K'>14$) such as we have demonstrated in previous studies [23]. The larger K' observed for the more lipophilic vitamins may be reduced by the addition of a stronger solvent such as THF. Consequently, gradient program I was used. Fig. 1A shows that it is feasible to achieve a good resolution for all vitamins in a reasonable run time, less than 17 min.

3.1. Maximum injection volume

The maximum sample volume that can be injected into a microcolumn without loss in column efficiency is proportional to the column length and the square of the internal diameter of the column, and is inversely proportional to the square root of the column plate number. For the column used in this work, we calculated a maximum sample volume of

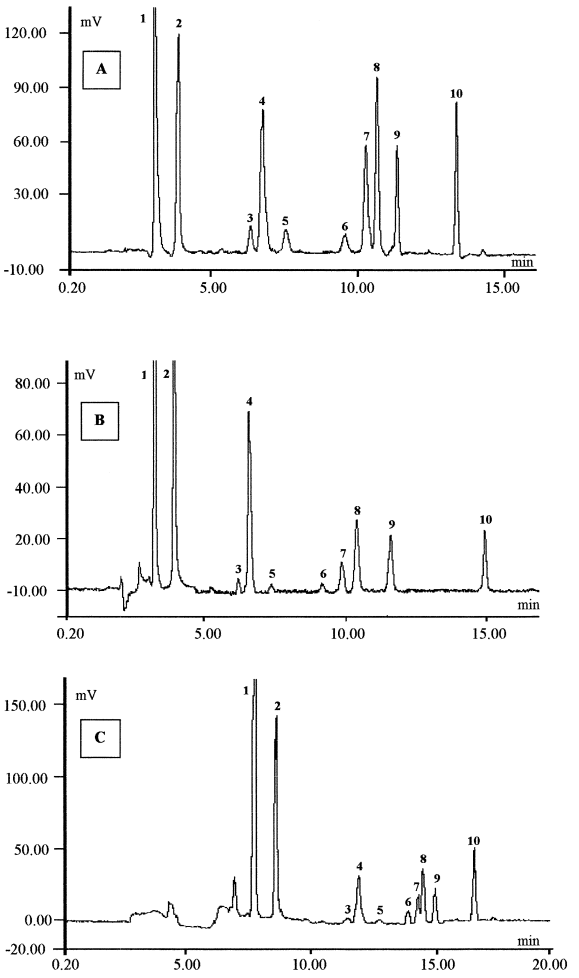

Fig. 1. Separation of fat-soluble vitamins by CLC. (A) Injected volume 60 nl; concentrations are 50 μg/ml in methanol for all vitamins; gradient I and detection conditions as in the text. (B) Injected volume 500 nl; concentrations are 1 μg/ml for all vitamins in methanol–water (50:50); gradient I and detection conditions as in the text. (C) Injected volume 5 μl; concentrations between 0.1 and 0.25 μg/ml in methanol–water (50:50); gradient II and detection conditions as in the text. Column, 150×0.30 mm I.D., fused silica, 3 μm Hypersil C_{18} BDS. Flow-rate, 6 μl/min. 1, retinol; 2, retinyl acetate; 3, vitamin D_2; 4, vitamin D_3; 5, vitamin E; 6, provitamin D_2; 7, provitamin D_3; 8, tocopherol acetate; 9, vitamin K_1; 10, retinyl palmitate.

25 nl. Nevertheless it is possible to inject much larger sample volumes than this, without a loss in column efficiency, resulting in a substantial increase in sensitivity. In fact, if the sample is injected in methanol, it is possible to inject to 400 nl of sample. The chromatogram shown in Fig. 1A has been obtained for a volume injected of 60 nl. As a consequence of column overload, when the injected volume is larger than 400 nl, different kinds of band distortion become evident (broadening, tailing and even double peaks could appear).

Larger volume injections into the microcolumn without loss in efficiency can be accomplished by enrichment techniques [26,27]. Thus, if the sample is dissolved in a solvent whose elution strength is lower than the mobile phase elution strength, in our case with more water content, then the solutes will be enriched at the top of the column without penetrating into the column bed and the sensitivity of the analysis is substantially increased. Obviously, sample solubility and recovery in the solvent must be guaranteed. As can be seen in Fig. 1B and 1C, up to 5 μl of sample dissolved in a mixture of MeOH–H_2O 50:50 (v/v) may be injected. As the column works at a constant mobile phase velocity, the retention time increases according to the elution strength of the sample matrix, since in the initial sections of the column, the solutes are eluted by a solvent with a lower elution strength than the mobile phase. In the same way, the solute retention is affected by the volume of the injected sample whose composition differs from the composition of the mobile phase. For all compounds, the experimental changes of reduced retention volumes (V'_R) with the injected volume (V_s), fit reasonably well with theoretical values calculated from the following relationship:$V'_R = V_S\ (K_S - K)/K_S + kV_M$ [4]; where V_M is the dead volume and K_S and K are the solute capacity factors in the solvent of the sample and in the mobile phase, respectively. For example, V'_R for retinol calculated from this equation was 41.2 μl (V_M=2.6 μl, K_s=26.5, K=2.9, V_s=5 μl) and V_R determined from chromatogram 1.C. was 46.5 μl. In order to reduce the time of analysis, gradient program II was used. The optimum flow rate was 6 μl/min. The time constant of the detector must be less than 0.1 s according to theoretical predictions [28].

Table 1
Detection limits of fat-soluble vitamins in milk determined by 60 nl, 500 nl and 5 μl injected

Vitamin	Detection limit (ng/ml)		
	60 nl injected[a]	500 nl injected[b]	5 μl injected[b]
Vitamin A	1	0.1	0.02
Retinyl acetate	2	0.2	0.03
Vitamin D_2	46	12	2
Vitamin D_3	5	1	0.2
Vitamin E	32	15	2
Tocopherol acetate	38	12	2
Provitamin D_2	9	4	1
Provitamin D_3	7	2	0.4
Vitamin K_1	16	5	0.4
Retinyl palmitate	7	1	0.1

[a] Solvent MeOH.
[b] Solvent MeOH/H_2O (50:50).

3.2. Limits of detection

Table 1 shown the detection limits in milk, based on a signal-to-noise of 3:1, obtained for different injected volumes in the chromatographic system. It can be observed that the limits obtained for 5 μl injected are much lower than those provided when the solvent elution strength of the sample and mobile phase are the same. This result is significant for the analysis of samples such as milk, in which vitamins occur in small amounts.

3.3. Quantification and recovery

Quantification of the vitamins was achieved by using the external standard method owing to the difficulty encountered selecting an adequate internal standard for all vitamins and because it was necessary to switch the detection wavelengths in order to select the absorption maximum for each vitamin. Calibration plots were generated by repeated injections of 5 μl of standard solutions of vitamins at different concentrations. The calibration graphs constructed daily from peak areas versus vitamin concentrations were linear (r>0.999) from the determination limit up to at least 100 ng/ml for vitamins A and retinyl acetate, and up to at least 200 ng/ml for vitamins D_2, D_3, E, K_1, retinyl palmitate, tocopherol acetate, ergosterol and 7-dehydrocholesterol.

To study the accuracy of the method, recovery

studies were performed. Known amounts of each vitamin were added to a variety of samples and the resulting spiked samples were subjected to the entire analytical sequence. Each solute was spiked at three different concentrations and recoveries were calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. All analyses were carried out in triplicate on different days. The results for a milk sample are given in Table 2. Typical recoveries ranging from 89 to 107% for vitamins at all spiking levels were obtained. The coefficients of variation were generally less than 4% ($n=3$). These results indicate that the method exhibits an adequate degree of accuracy for the analysis of these solutes.

Fig. 2 shows the chromatogram obtained, after subtracting the blank run, from a milk sample treated according to the procedure mentioned earlier in sample preparation. As can be seen, the injection of 5 μl causes a broadening of the chromatographic peaks, above all of the last ones, as a consequence of column overloading due to the presence of the matrix compounds. In these cases, if a suitable autoinjector or loops are available, it would be advisable to inject lower volumes, e.g. 3 μl.

The precision of the method was investigated using both standards and samples. Five repeated injections of the standard over a number of different days gave a relative standard deviation (RSD) of less than 5.3%. The RSDs for the sample were determined using four different milk samples and by analysing each sample in triplicate, and were always less than 8.3%.

Table 2
Recovery studies on fat-soluble vitamins added to the various extracted milk samples (5 μl injected)

Vitamin	Concentration in milk (μg/l)	Concentration added (μg/l)	Recovery (%) (RSD[a])
Vitamin A	1.4	1.0	89±3
		2.0	91±3
		3.0	92±3
Retinyl acetate	5.4	20.0	100±1
		34.9	101±1
		49.9	97±2
Vitamin D_2	7.2	4.0	94±2
		8.0	104±2
		12.0	102±3
Vitamin D_3	8.8	4.0	107±3
		8.0	93±3
		12.0	92±4
Vitamin E	6.0	3.0	104±1
		6.0	100±2
		9.0	97±2
Tocopherol acetate	430	200	102±2
		300	96±2
		400	98±3
Provitamin D_2	–	5.0	95±4
		10.0	92±4
		15.0	94±4
Provitamin D_3	11.2	5.0	94±2
		10.0	90±3
		15.0	94±2
Vitamin K_1	8.6	4.0	94±4
		8.0	93±4
		12.0	94±3
Retinyl palmitate	13.6	6.0	103±3
		12.0	97±2
		18.0	99±2

[a] $n=3$.

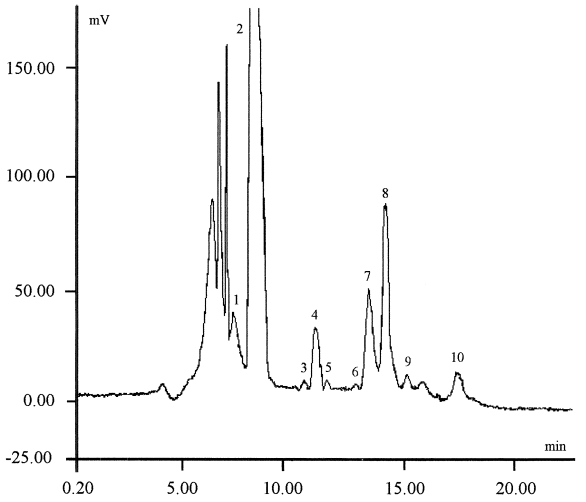

Fig. 2. Chromatogram obtained from a commercial sterilized milk sample. Chromatographic conditions as in Fig. 1C 1, retinol; 2, retinyl acetate; 3, vitamin D_2; 4, vitamin D_3; 5, vitamin E; 6, provitamin D_2; 7, provitamin D_3; 8, tocopherol acetate; 9, vitamin K_1; 10, retinyl palmitate. For vitamin concentrations see Table 2.

4. Conclusion

The combined use of the capillary liquid chromatography separation technique with conventional UV detection allows the simultaneous and sensitive determination of trace amounts of fat-soluble vitamins in complex matrices such as milk with minimum sample availability and more sensitivity. In fact, the detection limits obtained with a conventional LC method for fat soluble vitamins are of the order of μg/ml (e.g. 5.9 μg/ml for retinol) whereas for the μLC, the LOD are of the order of ng/ml (e.g. 0.02 ng/ml for retinol).

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