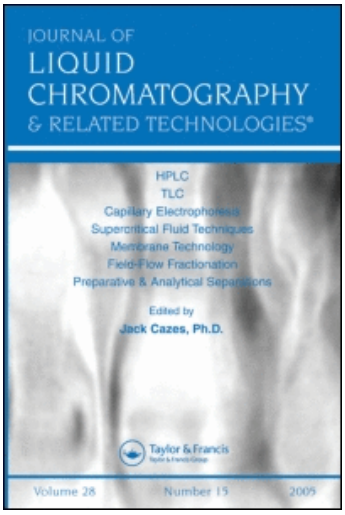

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SIMULTANEOUS SEPARATION AND DETECTION OF TEN COMMON FAT-SOLUBLE VITAMINS IN MILK

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ABSTRACT

A method for the separation of ten fat-soluble vitamins on a reversed phase C_{18} column with UV detection is described. The choice of a suitable organic solvent in conjunction with an aqueous phase composition is important in achieving a good separation of the ten common fat-soluble vitamins which include different forms of vitamins A, D, K and α-tocopherol (E). Two different mobile phases were developed for the isocratic separation of the vitamins. It was found that the separation of the ten vitamins is very sensitive to a small change in the composition of the mobile phase. The method was applied to determine the vitamins in milk sample, after sample treatment which includes saponification, drying, and reconstitution of the vitamins with the mobile phase.

INTRODUCTION

Most of the vitamin comes to us through the natural and fortified processed foods. Fish oils and egg yolks are rich natural sources of vitamins A and D. Vitamin A exists in three oxidation states. Some have different isomeric forms which are important in biochemical functions. Vitamin D is usually produced in our own tissues. Different forms of vitamin D exist upon thermal activation and non enzymatic process. Vitamin E (atocopherol) is produced by plants that enter our food supply, such as vegetable oils.

On the other hand, the fat-soluble vitamin Ks are obtained from plant, animal, and bacterial sources. The two forms of vitamin K, phylloquinones (K_1) and menaquinones (K_2), provide roughly half each of the amounts absorbed into the human body and stored in the liver. Menadione (K_3) is frequently used as an electron carrier to oxygen. Vitamin K_3 is also a cofactor of cyclic photophosphorylation.

High performance liquid chromatography (HPLC) with UV or fluorescence detection, is a common analytical method widely used in the qualitative and quantitative determination of vitamins in physiological samples and foodstuffs. These methods have been reviewed recently.[1-2] Microcolumn HPLC with multi-channel UV-VIS detection for the determination of vitamins in foodstuffs have been reported.[3-5] Specific methods for analysing individual vitamins are available with fluorimetric detection[6-7] or electrochemical methods.[8-9] Ion exchange chromatographic method for assay of vitamins is available as well.[10] Other methods, such as specific enzyme-linked ligand-sorbent assay of vitamins in human plasma and urine, have recently been reported.[11]

In addition, automated methods for the determination of a particular vitamin in human sera have been described.[12-13] These HPLC methods are good for the determination of individual vitamins or a group of vitamins. It would be more economical and time-saving to simultaneously determine as many vitamins as possible in an analysis.

In this paper, an HPLC method is described for separating ten common fat-soluble vitamins. The method was then applied to measure the vitamins in milk sample. The milk sample requires simple procedural modifications of the sample treatment process, which includes saponification, evaporation, and reconstitution of the vitamins prior to HPLC analysis.

EXPERIMENTAL

Chemicals

All-trans vitamin A (alcohol, aldehyde, acid), 13-cis vitamin A acid, D_2, D_3, E (α-tocopherol) and K_1, K_2, as well as K_3 were purchased from Sigma (MO, USA). HPLCgrade ethanol, acetonitrile, and tetrahydrofuran were obtained from Riedel-de Haen AG (Germany). Methanol and n-hexane were purchased from Mallinckrodt Specialty Chemicals (Kentucky, USA). Isopropanol was purchased from AJAX Chemicals (Australia). Other chemicals were of analytical grade.

Apparatus

Experiments were performed on a modular liquid chromatograph equipped with a Rheodyne 7126 injector fitted with a 20-μL loop. An analytical C_{18} column (particle size 5 μm, 25 x 0.46 cm) (Alltech, CA, U.S.A.) and a model UV-1 variable wavelength spectrophotometer (Rainin) with a 12-μL flow-cell attachment were used. Chromatograms were recorded with a Hewlett-Packard 3396 II integrator. A high precision solvent-delivery system SSI 300LC (Alltech, CA, U.S.A.) was used.

Chromatographic Conditions

The eluent was monitored at 250 nm for UV detection. The flowrate was set at 1.0 mL/min. The isocratic reversed phase HPLC separation of vitamins was carried out using a mobile phase that contains ethanol and methanol (20: 80, v/v). The separation could also be achieved with another mobile phase which is composed of ethanol and water (95: 5,v/v).

Extraction of Vitamin Ds

A milk sample (50 mL) was mixed with 30 mL of ethanolic potassium hydroxide (10: 30, v/v). Ethanolic potassium hydroxide solution was prepared by the previous method.[14] The mixture was saponified at 80°C for 20 min based on a previous procedure.[15] The vitamins from the saponified mixture were 2x extracted with 10 mL of n-hexane. The n-hexane extract was evaporated to dryness. The dried extract was reconstituted with 1.0 mL of the mobile phase. An aliquot (5 μL) was injected onto HPLC for analysis.

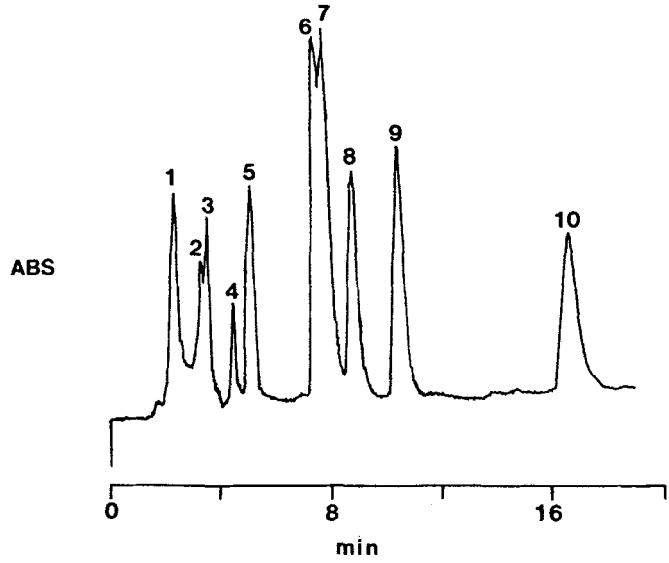

Figure 1. Chromatogram of vitamin standards. See text for experimental details. Peaks: 1, K_3 (20 ng); 2, A cis-acid (50 ng); 3, A all-trans acid (55 ng); 4, A alcohol (50 ng); 5, A aldehyde (35 ng); 6, D (80 ng); 7 D1 (80 ng); 8, E (180 ng); 9, K (70 ng); 10, K (70 ng). Eluent: ethanol and methanol (20: 80, v/v).

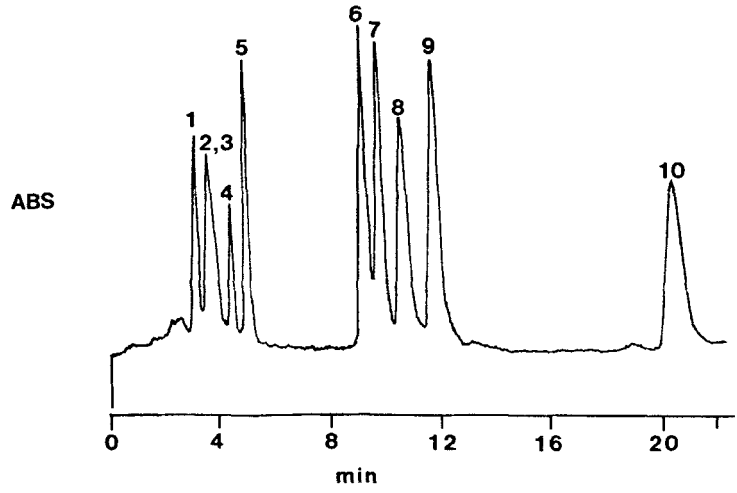

Figure 2. Chromatogram of vitamin standards. See Figure 1 for experimental conditions and labels. Eluent: ethanol and water (95: 5, v/v).

RESULTS AND DISCUSSION

A representative chromatogram for the isocratic separation of the common fat soluble vitamins, with the mobile phase containing ethanol and methanol (20: 80, v/v), is shown in Figure 1. The second mobile phase containing ethanol and water (95: 5, v/v) was also developed for the isocratic separation of the vitamins (Figure 2). The mobile phase composition was studied to improve resolution between peaks. The isomeric separation of the all-trans-, and 13-cis-, retinoic acid, was not achieved with ethanol-water as the mobile phase. On the other hand, the ethanol-methanol solvent system allows the separation of structurally similar vitamin As and a qualitative detection of the two retinoic acids, which are peaks number 2 and 3 in Figures 1 and 2. The resolution between these two vitamin peaks markedly decreased as the methanol concentration of the mobile phase slightly increased. Likewise, the resolution between vitamins D_2 and D_3 also decreased with the elevation of methanol concentration. The two vitamin Ds structurally differ from each other by an extra double bond in vitamin D_2. Hence, the chromatographic behavior of these two compounds are very similar.

The mobile phase which contains ethanol and water (95: 5, v/v) could separate vitamins D_2 and D_3. However, the overall run time takes longer (> 20 min) and coelution of the isomeric vitamins A occurs with this ethanol-water solvent system as the mobile phase. In contrast, the ethanol-methanol solvent system takes about 16 min to complete the elution. Neither the change of the flow rate nor the elution strength of the ethanol-water mobile phase could resolve the isomeric peaks.

The composition of the two different mobile phases described in this study has to be carefully prepared to allow resolution between isomeric vitamins A, as well as vitamin D_2 and D_3. The two mobile phases are complimentary to each other in terms of elution time and the resolution capability. The disadvantage of using acetonitrile instead of methanol in the mobile phase is longer elution time and similar selectivity results.

The determination of fat-soluble vitamins presents detection problems.[16] They show differing UV absorbance maxima. Their absorbance spectra are often affected by interferring compounds that show similar spectral characteristics. The absorbance spectra of the ten fat-soluble vitamins were initially studied using a Waters diode-array UV detector (Model 990, MA, USA) connected to the HPLC system. The results are shown in Figure 3 (A and B).

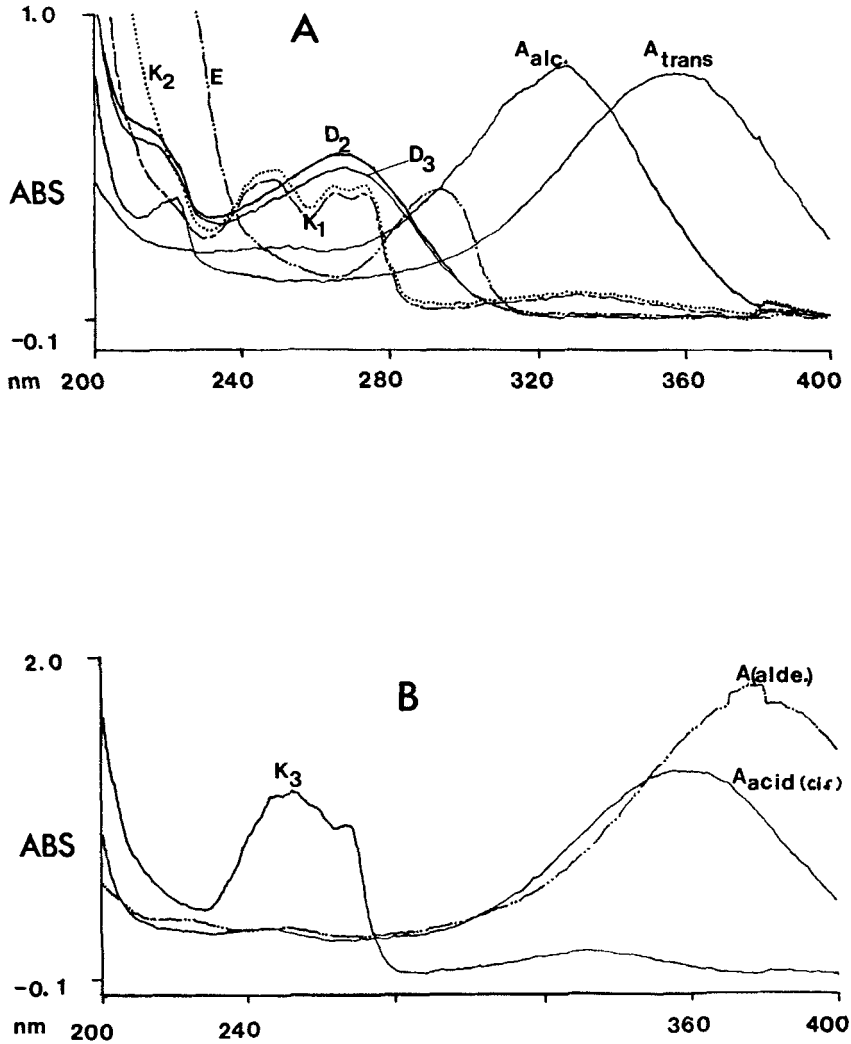

Figure 3. Ultraviolet Absorption Spectra of vitamins. See text for experimental conditions.

The spectra were obtained with the vitamin standards and presented in two Figures for clarity. The diode-array detection provides both the qualitative and quantitative information. The composition of analyses under each peak can be established by comparing the spectra taken through the peak elution. A

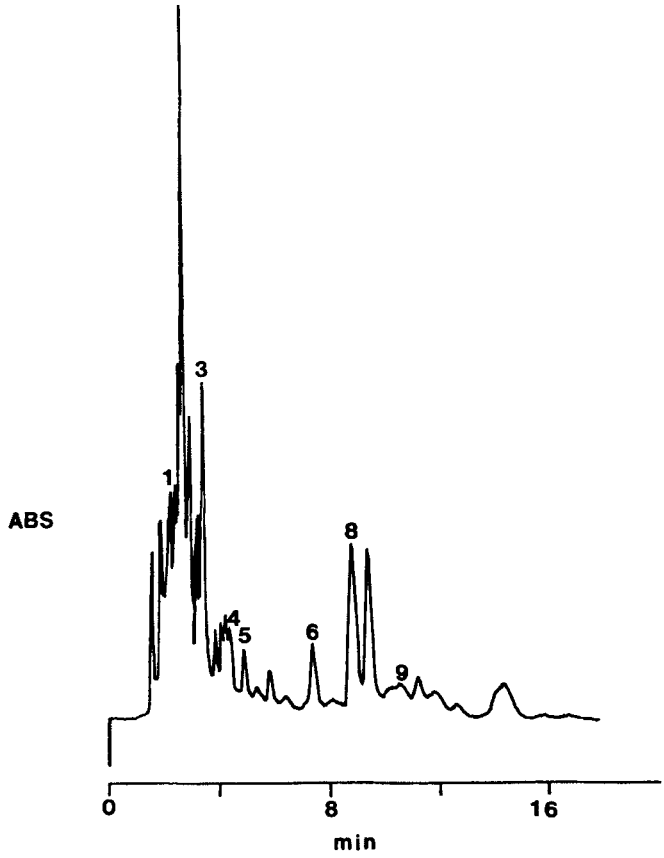

Figure 4. Chromatogram of vitamins from a milk sample. See Figure 1 for experimental conditions and labels.

relative strong absorbance common to the ten vitamins, appears around 250 nm at which no interference from the milk sample was detected. For a more specific vitamin analysis, the spectral data provide useful information for establishing the absorbance maximum under the described experimental conditions.

The application of the method to milk samples was performed. The chromatogram of vitamins from a milk sample is shown in Figure 4. The detection of vitamin Ks was not achieved by the described procedures. Hence, the milk sample was spiked with vitamin K standards prior to the sample treatment. However, vitamin K_3 was not stable enough in the extraction

process. The extraction procedure was also studied using different extraction solvents after saponification. The saponification of milk sample to hydrolyze fats is necessary before extraction. n-Hexane is an effective extraction solvent for vitamins A, D, and E. Although the detection limits of the vitamins are in the range of a few nanograms at three times of the background noise level, a large volume of milk sample is required for the detection of the fat-soluble vitamins. The correlation parameters of linearity of the line graphs are close to unity in the range of nanomolar concentration.

In conclusion, the isocratic separation method for analysing the ten vitamins is simple and efficient. The wavelength can be changed for the detection of a particular vitamin of interest to minimize interferences from matrix in biological and food samples. The method is useful for other applications.

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