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Note

Resolution of fat-soluble vitamins in high-performance liquid chromatography with methanol-containing mobile phases

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Retention and selectivity characteristics of bonded-phase packings may vary from lot to lot and manufacturer to manufacturer. According to Snyder and Kirkland[1], the main source of non-uniformity in column performance is incomplete coverage of the particle surface by the bonded stationary phase. The sorptive behavior of residual SiOH groups is different from that of the bonded stationary phase and may lead to tailing. Even when a completely bonded column is used, the addition of methanol, acetic acid or triethylamine to the mobile phase may be required to suppress tailing of certain compounds.

In the determination of vitamin A in margarine and dry fortified breakfast cereals with non-aqueous mobile phase, high-performance liquid chromatography (HPLC) was successfully used[2,3]. However, elution of the μBondapak C_{18} column using acetonitrile–methylene chloride (700:300) mobile phase failed to resolve vitamins D_2 and D_3 adequately. A Zorbax ODS column (6 μm) and a non-aqueous solvent system proved capable of resolving these compounds. Further advantages of this method are increased solubility of samples of low polarity in the mobile phase and extended column life[4].

EXPERIMENTAL

Materials

All solvents were of high-purity grade: acetonitrile (UV grade) and methylene chloride (Burdick & Jackson Labs., Muskegon, MI, U.S.A.); methanol (HPLC grade, Fisher Scientific, Pittsburgh, PA, U.S.A.); triethylamine (Eastman, Rochester, NY, U.S.A.).

Vitamin standards

Retinol, β-carotene and retinyl palmitate (Tridom Chemicals, Hauppage, NY, U.S.A.) α-tocopheryl acetate, retinyl acetate, vitamins D_2 and D_3 (U.S. Pharmacopeia).

Column

The chromatographic column was a stainless-steel tube (25 cm × 4.6 mm I.D.) packed with 6 μm Zorbax ODS (DuPont, Wilmington, DE, U.S.A.).

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Mobile phase

The ternary mobile phase consisted of methylene chloride (0.001% triethylamine)–acetonitrile–methanol (300:700:X) where X = 0, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 or 40.0.

Instrument

The apparatus was a ALC/GPC series 200 high-performance liquid chromatograph (Waters Assoc.), equipped with a 6000 A pump, U6K loop injector, two Model M 440 absorbance detectors with wavelength-filter kit, dual-pen recorder (Houston Instruments) and reporting integrator (Hewlett-Packard).

Irradiation apparatus

The irradiation lamp (General Electric Model 15T8) was mounted vertically in a 5-gallon can, the top of which was replaced by a 3/4-in. plywood lid. Six 2-cm holes, drilled in the plywood, held six 15.0 × 1.8 cm silica test tubes (Vycor No. 7900) 13 cm from the lamp surface.

Vitamin standard solutions

The stock standard solutions of each vitamin were prepared in methylene chloride containing 0.001% triethylamine. For HPLC, standards were prepared in the mobile phase at the following concentrations, in ng/μl: retinol, 6.9; β-carotene, 8.3; retinyl palmitate, 8.8; α-tocopheryl acetate, 25.6; retinyl acetate, 2.9; vitamin D_2, 7.3; vitamin D_3, 7.4. The injection volume was 50 μl.

HPLC parameters

The instrument conditions were: wavelength, 280 and 254 nm; flow-rate, 1.0 ml/min (800 p.s.i.); sensitivity, 0.05 a.u.f.s.; chart speed, 0.05 cm/min; integrator attenuation, 64×; slope sensitivity, 0.3 mV/min.

Formation of vitamin D degradation products

(1) For 10-min UV irradiation, 3.0 ml stock standard solution (740 μg) in a Vycar test tube was diluted with 10 ml acetonitrile. Afterward, the solution was transferred to a 100-ml volumetric flask with 27 ml methylene chloride and finally made to volume with acetonitrile.

(2) For heat degradation, a 3.0-ml aliquot of stock standard solution (740 μg) and 25 ml of acetonitrile were refluxed in a water-bath at 80–90°C for 1 h. After it had cooled to room temperature, the solution was transferred to a 100-ml volumetric flask with 27 ml methylene chloride and made to volume with acetonitrile.

RESULTS AND DISCUSSION

Acetonitrile–methylene chloride (700:300) gave baseline resolution of retinyl acetate, retinol, α-tocopheryl acetate, β-carotene, and retinyl palmitate in 16 min, but resolution of vitamins D_2 and D_3 was poor and erratic (Fig. 1). The addition of 0.5 ml methanol per liter of mobile phase reduced the retention of compounds containing hydroxyl groups and improved their peak symmetry. Under these conditions, retinol and retinyl acetate are not separated, but vitamins D_2 and D_3 are completely resolved

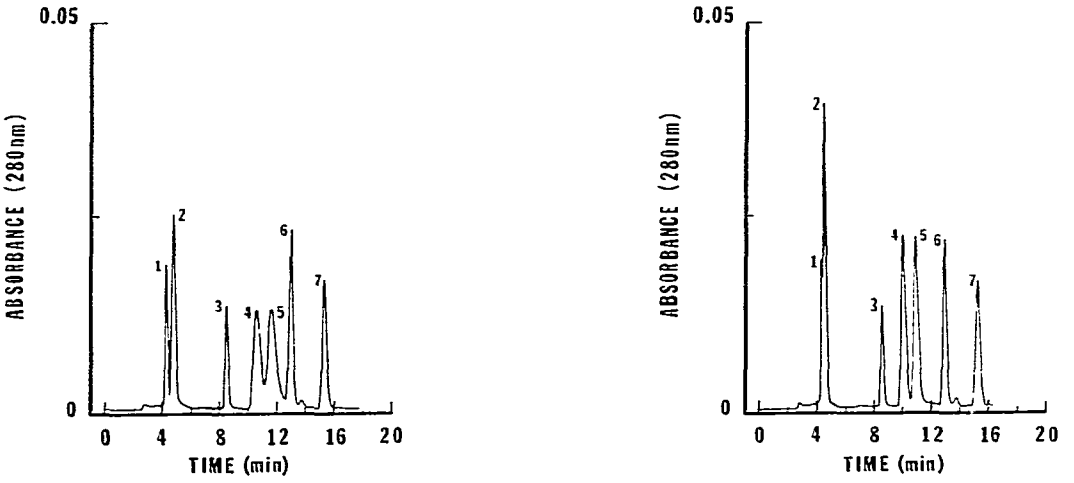

Fig. 1. Chromatogram of vitamin mixture: 1 = retinyl acetate (145 ng); 2 = retinol (345 ng); 3 = α-tocopheryl acetate (1280 ng); 4 = vitamin D_2 (365 ng); 5 = vitamin D_3 (370 ng); 6 = β-carotene (415 ng); 7 = retinyl palmitate (440 ng). Detector at 280 nm, 0.05 a.u.f.s.; mobile phase, acetonitrile–methylene chloride (700:300); flow-rate, 1 ml/min; chart speed, 0.5 cm/min; column, 250 × 4.6 mm I.D., Zorbax ODS; injection volume, 50 µl; temperature, 25°C.

Fig. 2. Chromatogram of same vitamin mixture as in Fig. 1. Experimental conditions as in Fig. 1, except 0.5 ml methanol was added per liter of mobile phase.

(Fig. 2). Change of the mobile phase composition to 2.0 ml methanol per liter produced the chromatogram shown in Fig. 3a. Retinol and retinyl acetate were not resolved, but the other fat-soluble vitamins were completely separated. Vitamins D_2 and D_3 are separated from each other and from their respective previtamins in less

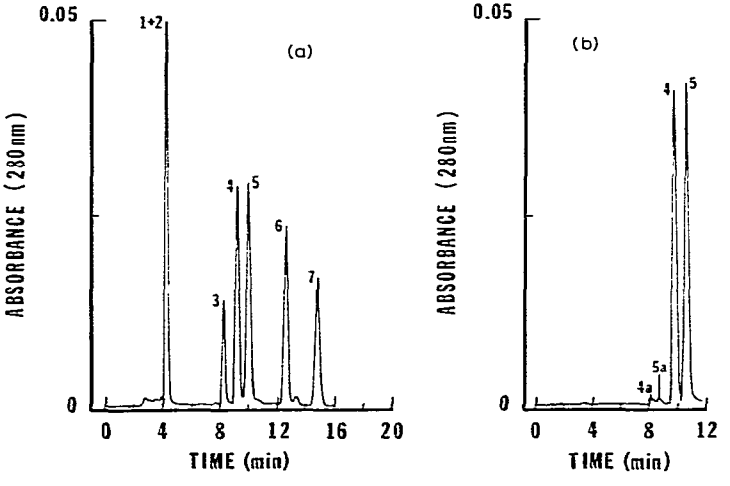

Fig. 3. (a) Chromatogram of same vitamin mixture as in Fig. 1. Experimental conditions were the same as in Fig. 1, except 2.0 ml methanol was added per liter of mobile phase. (b) Chromatogram of a mixture: 4 = vitamin D_2 (657 ng); 4a = previtamin D_2; 5 = vitamin D_3 (666 ng); 5a = previtamin D_3. Experimental conditions as in Fig. 3a, except temperature was 23°C.

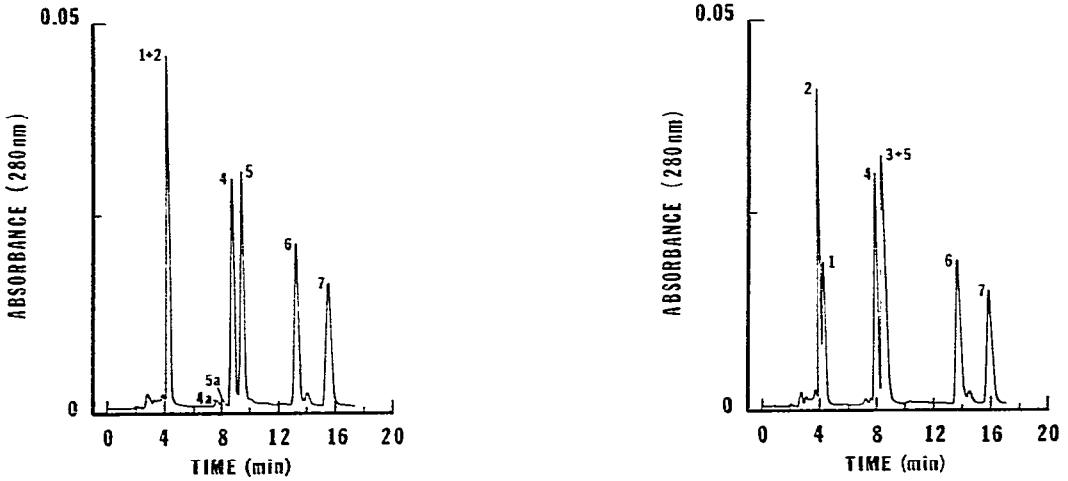

Fig. 4. Chromatogram of vitamin mixture: 1 = retinyl acetate; 2 = retinol; 4 = vitamin D_2; 4a = previtamin D_2; 5 = vitamin D_3; 5a = previtamin D_3; 6 = β-carotene; 7 = retinyl palmitate. Experimental conditions as in Fig. 1, except 10.0 ml methanol was added per liter of mobile phase.

Fig. 5. Chromatogram of vitamin mixture: 1 = retinyl acetate; 2 = retinol; 3 = α-tocopheryl acetate; 4 = vitamin D_2; 5 = vitamin D_3; 6 = β-carotene; 7 = retinyl palmitate. Experimental conditions as in Fig. 1, except 40.0 ml methanol was added per liter of mobile phase.

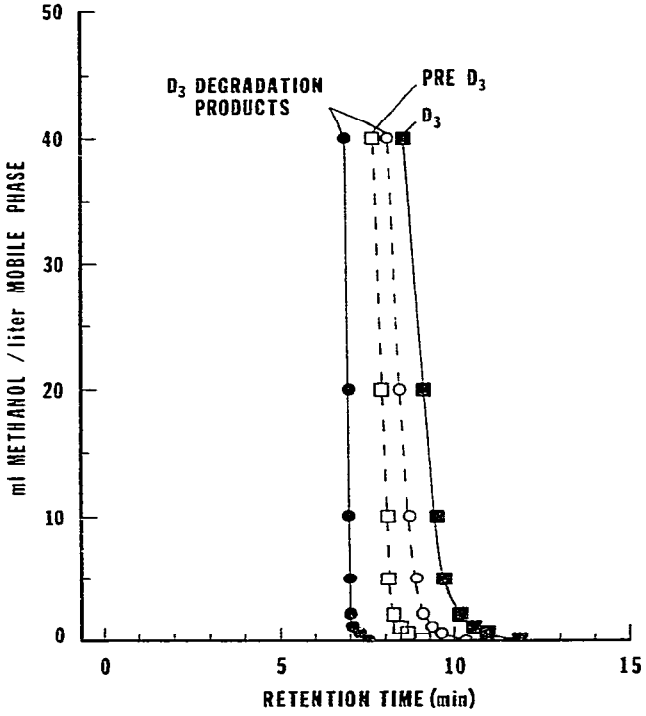

Fig. 6. Graph showing effect of added methanol on retention time of vitamin D_3, previtamin D_3, and two vitamin D_3 degradation products.

than 12 min (Fig. 3b). Fig 4 depicts the chromatogram of the same vitamin mixture without α-tocopheryl acetate, with 10 ml methanol per liter of mobile phase. Again, the retinol and retinyl acetate peaks overlap while adequate resolution of vitamin D_2, vitamin D_3 and their respective previtamins was maintained. Finally, the addition of 40 ml methanol per liter of mobile phase produced a chromatogram (Fig. 5) of the vitamin mixture in which retinol migrated ahead of retinyl acetate and vitamin D_2 ahead of α-tocopheryl acetate, which was not separated from vitamin D_3.

At each of the methanol concentrations tested, the retention time of α-tocopheryl acetate and retinyl acetate remained relatively unchanged. The retention time of β-carotene and retinyl palmitate also remained relatively constant up to 5 ml methanol per liter of mobile phase. Beyond this level these compounds had higher capacity values.

Two degradation products, formed from vitamins D_2 and D_3 by UV irradiation or by refluxing in acetonitrile in the absence of antioxidant, were resolved by this HPLC system. The overall chromatographic effect of methanol addition on the elution time and resolution of vitamin D_3, previtamin D_3 and related degradation products is illustrated by plotting retention *versus* methanol concentration in the mobile phase (Fig. 6). This plot shows a shift of each compound to shorter retention with increasing methanol concentration. A similar effect was observed for vitamin D_2, previtamin D_2, and their degradation products.

CONCLUSION

The improvements in peak profile and resolution obtained after methanol addition enhances the potential use of non-aqueous reversed-phase HPLC for vitamin D analysis in foods.

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