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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FAT-SOLUBLE VITAMINS

## SEPARATION AND IDENTIFICATION OF VITAMINS $D_2$ AND $D_3$ AND THEIR ISOMERS IN FOOD SAMPLES IN THE PRESENCE OF VITAMIN A, VITAMIN E AND CAROTENE

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SUMMARY

    Vitamins $D_2$ and $D_3$ and their corresponding previtamins and provitamins were resolved by reversed-phase high-performance liquid chromatography using a ternary solvent system (acetonitrile–methanol–water) pumped according to a gradient elution programme. The D vitamins were also resolved in the presence of other lipid-soluble vitamins (A, E and $K_1$) and carotene. The peaks were monitored with a UV–visible variable-wavelength detector and were detected at their maximum absorbance, resulting in maximum sensitivity. Lipid-soluble vitamins and carotene were resolved in extracts obtained from oils and butter, thus permitting their identification in a single chromatographic run.

INTRODUCTION

    The analysis of D vitamins in food is difficult because the total concentration of vitamins is usually low, and that of D vitamins is very low; in addition, the concentration of interfering substances is higher than that of the vitamins. A good review on the determination of D vitamins in food was recently published by Parrish[1].

    High-performance liquid chromatography (HPLC) has been applied successfully to the determination of D vitamins and related compounds in pure standard solutions[2–5], pharmaceutical formulations[6–10], fortified foods and dry concentrates, animal feeds and fish products[8,11–20]. Hydroxylated metabolites of D vitamins have also been studied[21].

    Adsorption chromatography was found to be the best method for resolving vitamin D isomers[3,5,8–10,13–15] (and also vitamin A isomers[22]), but only reversed phases proved able to resolve vitamins $D_2$ and $D_3$. For solving this problem, one

report suggested the use of silver nitrate in the mobile phase[6], others used a mixture of methanol and water[4,7] and most recently Landen described a baseline separation of vitamins $D_2$ and $D_3$ obtained with a non-aqueous reversed phase (NARP) system[2].

This paper describes a baseline separation of vitamins $D_2$ and $D_3$ obtained using two reversed-phase columns connected in series and a mixture of three solvents (water, methanol and acetonitrile) as the eluent.

To test the column selectivity the vitamin $D_2$ and $D_3$ pair was also separated in a chromatographic run from the other lipid-soluble vitamins (A, E and $K_1$) and carotene.

The main purpose, however, was to resolve vitamins $D_2$ and $D_3$ together with their isomers, and to identify them in food samples, in order to assay only biologically active forms. As stressed elsewhere[15,23,24], in order to obtain values of vitamin D units corresponding to those found by rat bioassay, it is necessary to take into account only *cis*-vitamin D, plus previtamin D (which is only 35% as active as *cis*-vitamin D), while other isomers possess little if any antirachitic activity[1,13,25].

Therefore, in this study vitamins $D_2$ and $D_3$, the corresponding previtamins and provitamins and some other inactive isomers were resolved and identified in pure standard solutions and in samples obtained from foods such as olive oil, cod liver oil and butter.

EXPERIMENTAL

*Apparatus*

The liquid chromatographic apparatus was as previously described[22]. Two reversed-phase columns connected in series were used: (1) Perkin-Elmer (Norwalk, CT, U.S.A.) ODS-HC Sil-X-1 (25 × 0.26 cm I.D.) and (2) Supelco (Bellefonte, PA, U.S.A.) Supelcosil LC18 (15 × 0.46 cm I.D.).

A Supelco guard column dry-packed with 40-$\mu$m LC18 pellicular packing was also employed.

*Reagents and materials*

The following substances were obtained at the stated concentrations by appropriate dilution of concentrated stock standard solutions: vitamin $D_2$ cryst. (E. Merck, Darmstadt, G.F.R.), methanol solution, 10 mg per 100 ml; vitamin $D_3$ cryst. (E. Merck), methanol solution, 10 mg per 100 ml; ergosterol (provitamin $D_2$) purum, >97% by HPLC (Fluka, Buchs, Switzerland), methanol solution, 10 mg per 100 ml; 7-dehydrocholesterol (provitamin $D_3$), purum, *ca.* 98% by HPLC (Fluka), methanol solution, 10 mg per 100 ml; vitamin $K_1$ (Sigma, St. Louis, MO, U.S.A.), methanol solution, 10 mg per 100 ml; DL-$\alpha$-tocopherol (Sigma), methanol solution, 50 mg per 100 ml; all-*trans*-retinol (puriss) (Fluka), methanol solution, 1 mg per 100 ml; $\beta$-carotene (Type III, crystalline, natural from carrots; 10–20% $\alpha$-isomer, 80–90% $\beta$-isomer) (Sigma), chloroform solution, 8 mg per 100 ml.

Previtamin $D_2$ (pre-ergocalciferol) and previtamin $D_3$ (precholecalciferol) were obtained by refluxing isopropanol solutions (85°C, 2 h) of the corresponding vitamins[9,12]. Their identities were confirmed by UV scanning of the eluting peaks, and the spectral data thus obtained were as expected.

Experiments on the enzymatic digestion of samples were carried out using lipase (E.C. 3.1.1.3), Type VII, from *Candida cylindracea* (Sigma).

The *n*-hexane used for extractions was extra-pure (96% by GC; E. Merck). Other solvents (methanol, acetonitrile) were of HPLC grade (E. Merck).

L-(+)-Ascorbic acid (cryst., extra pure; E. Merck), potassium hydroxide pellets (GR; E. Merck), anhydrous sodium sulphate (RPE; Carlo Erba, Milan, Italy), monobasic sodium phosphate and dibasic sodium phosphate (RP; Carlo Erba), sodium chloride (AnalaR; BDH, Poole, Great Britain) and ethanol (absolute. RPE; Carlo Erba) were also employed.

*Sample preparation*

Food samples (10 g) were submitted to alkaline digestion overnight at room temperature and then extracted with *n*-hexane as previously described[26]. Minor modifications of the method were as follows: (i) magnetic stirring was used occasionally during alkaline digestion; (ii) after evaporation of the *n*-hexane extracts, the residue was dissolved in methanol, in 2- or 5-ml volumetric flasks depending on the expected concentration levels of D vitamins in the starting sample. The methanol solutions thus obtained were ready for direct injection into the chromatograph.

RESULTS AND DISCUSSION

*Nomenclature*

Vitamins $D_2$ and $D_3$ differ only in the side-chain attached at C-17, and both possess several structural isomers; their names are given in Table I, which also gives

TABLE I

COMMON NAMES OF SOME STRUCTURAL ISOMERS OF THE VITAMINS $D_2$ AND $D_3$. PERCENTAGES OF BIOLOGICAL ACTIVITY AS ANTIRACHITIC VITAMINS AND UV ABSORPTION MAXIMA

Antirachitic activities of vitamins $D_2$ and $D_3$ are considered to be the same in humans. while in avian species ergocalciferol was found to be only 1-10% as effective as cholecalciferol[1,7]. The values of UV absorption maxima were collected from a source[27] which reported data from many different authors, obtained mainly in ethanol and in a few instances in other solvents. Data reported are those found in close agreement. The variability intervals observed for some maxima are given in parentheses.

| Name | Biological activity (%)[1,13,24] | UV absorption maxima[27] |
|---|---|---|
| *Vitamin $D_2$-related compounds:* | | |
| Ergocalciferol (5.6-*cis*-vitamin $D_2$) | 100 | 208, 265 |
| 5,6-*trans*-Vitamin $D_2$ | 0–5 | 273 (272–273) |
| Pre-ergocalciferol (previtamin $D_2$) | 35 | 260 (260–263) |
| Ergosterol (provitamin $D_2$) | 0 | 262, 271 (271–275), 282 (280–285), 293 (293–296) |
| Lumisterol $D_2$ | 0 | 272(271.5–273), 280(278.5–280). 294 |
| Tachysterol $D_2$ | 0 | 280(280–281), 290, 302 |
| Isotachysterol $D_2$ | 0 | 279(277–280), 290(289–290.5), 302(302–303) |
| *Vitamin $D_3$-related compounds:* | | |
| Cholecalciferol (5,6-*cis*-vitamin $D_3$) | 100 | 213, 265 |
| 5,6-*trans*-Vitamin $D_3$ | 0–5 | – |
| Precholecalciferol (previtamin $D_3$) | 35 | 260 (260–262) |
| 7-Dehydrocholesterol (provitamin $D_3$) | 0 | 260, 270, 281(281–281.5), 293.5 |
| Lumisterol $D_3$ | 0 | 265, 270, 280 |
| Tachysterol $D_3$ | 0 | 272, 281, 289 |
| Isotachysterol $D_3$ | 0 | 280, 290, 302 |

UV absorption maxima and biological activity data. The corresponding structural formulae can be found elsewhere[1,5,25]. Compounds deriving from high-temperature cyclization (e.g., pyrocalciferols) or from over-irradiation (e.g., toxisterols) were omitted as they are not found in natural products.

## Chromatographic conditions

In order to obtain a higher plate number, as required for better resolution between vitamins $D_2$ and $D_3$, two C-18 reversed-phase columns connected in series were used. A small guard column was also employed to protect the analytical columns from "dirty" samples.

The eluent system suggested by Landen[2] (acetonitrile–methylene chloride) was tested and resulted in fairly good resolution of vitamins $D_2$ and $D_3$; this resolution could be increased to a certain extent by adding methanol. Baseline resolution was not achieved. We then tried a solvent system consisting of acetonitrile, methanol and water which, pumped according to a gradient elution programme, permitted very good resolution of vitamins $D_2$ and $D_3$. The solvent mixture at pump A was methanol–acetonitrile (20:80), while pump B pumped distilled water. The gradient elution programme adopted was effective for resolving vitamins $D_2$ and $D_3$ and other lipid-soluble vitamins. The gradient profile is shown superimposed on Fig. 1, where the percentages of the solvent mixture pumped with pump A are indicated.

The column temperature has some influence on the resolution of D vitamins[14]. Our operating temperature was 44°C. The reduced viscosity obtained with this gentle heating permitted the use of higher flow-rates with not too great a pressure drop.

## Chromatography of standards

The vitamins $D_2$ and $D_3$ pair was injected and chromatographed together with other lipid-soluble vitamins (A, E and $K_1$) and carotene, and all peaks were resolved well. The chromatogram is shown in Fig. 1. Because the resolution was so good, wavelength programming was easy to adopt, so that each peak was detected at the wavelength of its maximum absorbance, resulting in maximum sensitivity. These chromatographic conditions can be of some use for testing pharmaceutical preparations. All vitamins were used in their free alcoholic form (whereas many papers describe the resolution of vitamin esters such as acetate and palmitate) because in food analysis, after alkaline digestion, vitamins are extracted from samples as free alcohols.

Fig. 2 shows the chromatogram of vitamins $D_2$ and $D_3$ and their corresponding provitamins (ergosterol and 7-dehydrocholesterol) and previtamins (pre-ergocalciferol and precholecalciferol). All peaks were confirmed by means of relative retention times, co-elution (when possible) with the corresponding internal standard and by using the stop-flow method for peak scanning. UV spectral data were as expected, and the spectra of the vitamin D isomers so obtained are shown in Fig. 3.

## Chromatography of food sample extracts

Food samples (see also sample preparation) were digested and extracted as previously described[26].

We should stress that alkaline digestion should be carried out at room temperature in order to prevent vitamin losses[11] and isomerization[1,9]. Some papers have
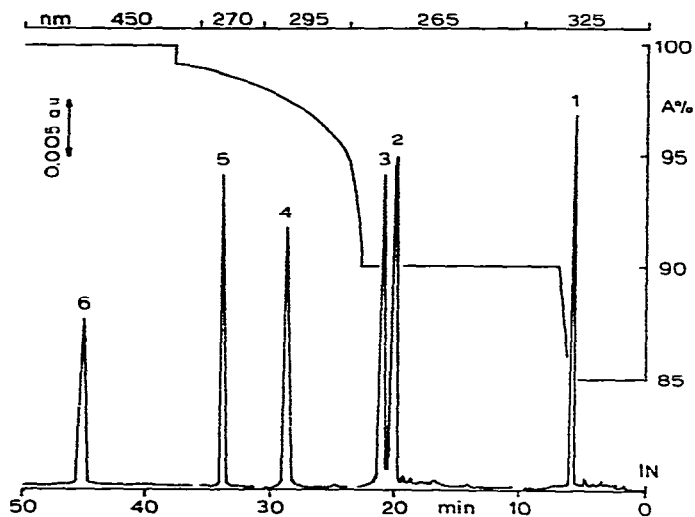
Fig. 1. Chromatogram of lipid-soluble vitamins and carotene. Peaks: 1 = vitamin A; 2 = vitamin $D_2$; 3 = vitamin $D_3$; 4 = vitamin E; 5 = vitamin $K_1$; 6 = carotene. Injection: 30 μl (5 μl of standard solution of each component). Gradient elution programme: percentage of mixture A (methanol–acetonitrile, 20:80) referred to the total volume of A + B (B is water) is reported superimposed. Wavelength programming was used and wavelength changes are reported at the top of the figure. Flow-rate: 1.5 ml/min; pressure drop ranged from 9.8 to 6.5 MPa according to the gradient programme. Temperature: 44°C. Sensitivity of the recorder: 0.05 a.u.f.s. Chart speed: 5 mm/min.
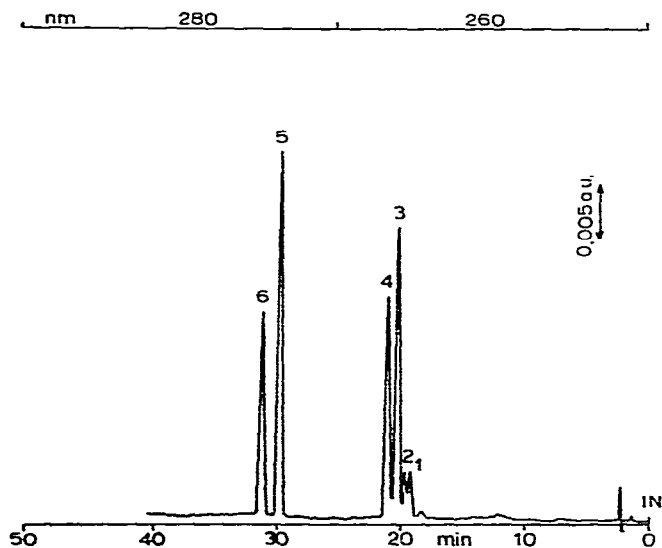


Fig. 2. Chromatogram of vitamins $D_2$ and $D_3$ and their isomers. Peaks: 1 = previtamin $D_2$; 2 = previtamin $D_3$; 3 = vitamin $D_2$; 4 = vitamin $D_3$; 5 = provitamin $D_2$; 6 = provitamin $D_3$. Wavelengths: 260 and 280 nm. Other conditions as in Fig. 1.
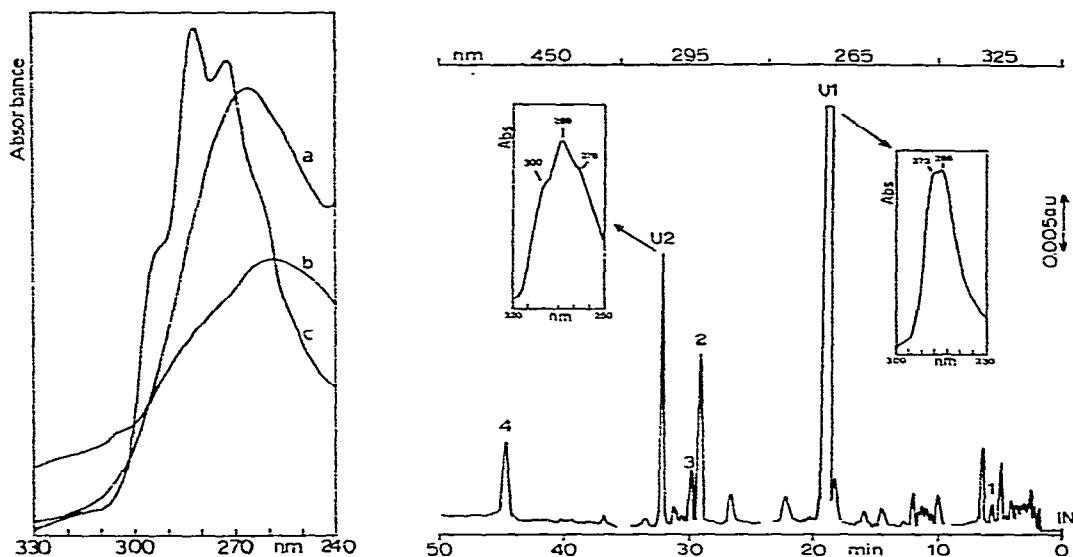
Fig. 3. Absorption spectra of vitamin $D_3$ and its isomers. Spectra are those of the eluting peaks and were recorded by means of the stop-flow method and UV scanning. Spectra: (a) vitamin $D_3$; (b) previtamin $D_3$; (c) provitamin $D_3$.

Fig. 4. Chromatogram of the extract obtained from an olive oil sample. Peaks: 1 = vitamin A; U1 = unknown U1; 2 = vitamin E; 3 = provitamin $D_2$; U2 = unknown U2; 4 = carotene. Injection: 50 $\mu$l. Wavelength programme is reported at the top of the figure. Other conditions as in Fig. 1.

reported experiments on enzymatic digestion of samples, prior to extraction. Trypsin digestion was found to be suitable for pharmaceutical preparations of multivitamin tablets[9] while lipase was used for digesting milk- and soy-based infant formulae[20]. In the latter instance hydrolysis was not complete enough to free the alcohols, so that acetate and palmitate esters of vitamins were also detected. In complex food matrices, the presence of ester derivatives of fat-soluble vitamins complicates the interpretation of results. We applied enzymatic lipase hydrolysis to a sample of cod liver oil, but interpretation of the resulting chromatogram was difficult owing to the presence of many peaks of mixed esters. However, enzymatic hydrolysis still remains interesting, and further tests will be probably carried out, because a complete conversion of vitamins into free alcohols obtained in this way will permit the analysis of vitamin K, which is otherwise destroyed by alkaline digestion.

Figs. 4–6 show the chromatograms obtained from samples of olive oil, cod liver oil and butter, respectively.

With respect to the chromatogram of pure standards, the wavelength change to 270 nm used for detecting vitamin $K_1$ was omitted in the chromatography of food samples because, as stated above, vitamin K is destroyed by alkaline digestion. In these latter chromatograms, many peaks from the unsaponifiable matter are detected and obviously the resulting "fingerprint" is strongly dependent on the detection wavelength used. In both oil sample chromatograms, a large unknown peak (U1) is eluted slightly earlier than the D vitamins. Its absorbance spectrum also has a shape similar to that of the D vitamins, but possesses two maxima, at 265 and at 272–273
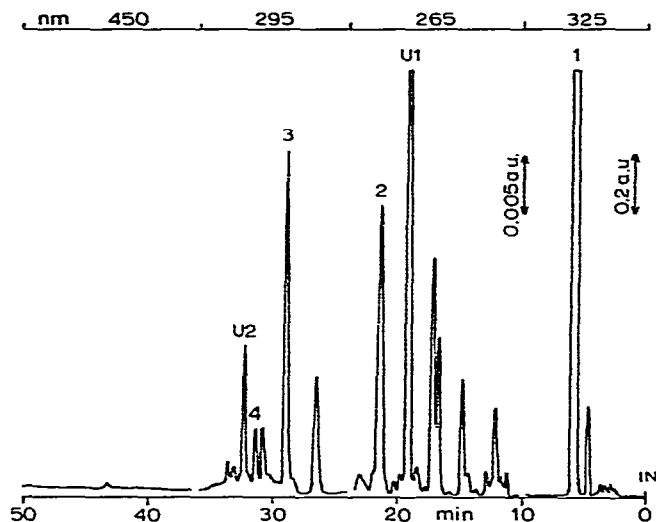
Fig. 5. Chromatogram of the extract obtained from a cod liver oil sample. Peaks: 1 = vitamin A; U1 = unknown U1; 2 = vitamin $D_3$; 3 = vitamin E; 4 = provitamin $D_3$; U2 = unknown U2. Other conditions as in Fig. 4.

nm (see Fig. 4). Another unknown peak (U2) eluting after the two D provitamins was found in all three food samples, and its absorbance spectrum, resembling those of vitamin D isomers, is reported in Fig. 4.

Cod liver oil, although a dietetic product rather than a proper food, was chosen as a sample because it illustrates the usefulness of the method: vitamin $D_3$ and provitamin $D_3$ as well as vitamin A and vitamin E are easily detected in a single chroma-
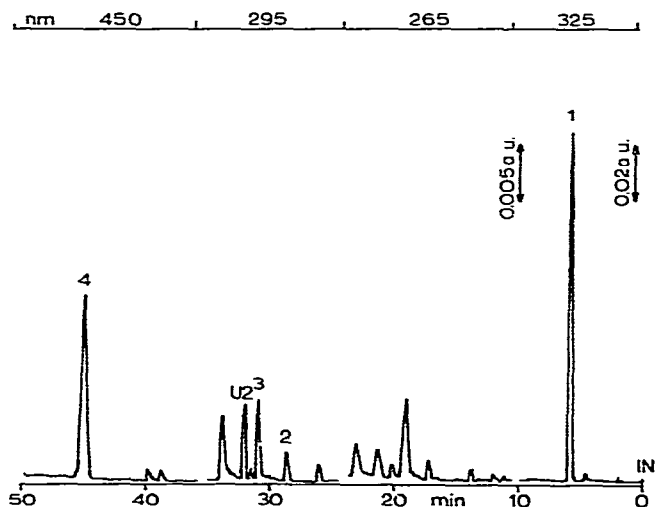


Fig. 6. Chromatogram of the extract obtained from a butter sample. Peaks: 1 = vitamin A; 2 = vitamin E; 3 = provitamin $D_3$; U2 = unknown U2; 4 = carotene. Other conditions as in Fig. 4.

tographic run. In butter, together with vitamin A, carotene and vitamin E, provitamin $D_3$ was also positively identified.

Recovery studies on lipid-soluble vitamins, especially D vitamins, from various food samples are in progress, with the aim of achieving results for the quantitation of analyte levels by HPLC that are in close agreement with those obtained by the more reliable but more time-consuming biological assay.

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