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Determination of cholecalciferol and related substances by calcium phosphate hydroxyapatite and calcium phosphate fluoroapatite high-performance liquid chromatography

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Abstract

A qualitative and comparative study by HPLC using two new stationary phases, hydroxy- and fluoroapatites, showed the separation under optimized conditions of vitamin D_3 and its related compounds, produced by irradiation and heating of provitamin D_3.

Keywords: Stationary phases, LC; Provitamin D_3; Vitamin D_3; Tachysterol; Maleic anhydride

1. Introduction

Vitamin D_3 (cholecalciferol) is produced in vivo in cutaneous tissues by UV irradiation of provitamin D_3 (7-dehydrocholesterol). The sigmatropic and thermotropic interconversion of these and related compounds [1–3] is shown in Fig. 1. Vitamin D_3 is manufactured by irradiation of provitamin D_3 at 254 nm, followed by heating [4–6]. Tachysterol is eliminated in the process by trapping in a Diels–Alder reaction with maleic anhydride.

HPLC techniques have been applied extensively to separations of these compounds. These procedures involve silica or bonded normal-phase [7–11] or reversed-phase [12–18] materials. In this work, we compared such separations with those using two new stationary phases:

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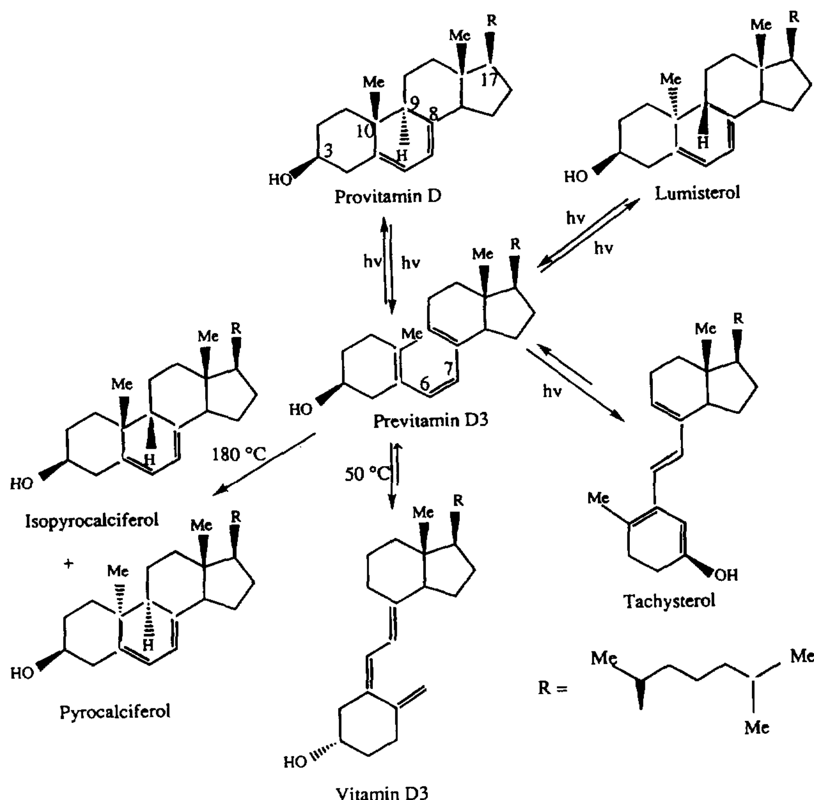

Fig. 1. Photochemical and thermal isomers of provitamin D_3.

hydroxyapatite (HAp) and fluoroapatite (FAp).

2. Experimental

2.1. Study A on reversed-phase material (optimized in-house conditions)

The following conditions were used: column, LiChrospher 100 RP-18, 5 μm (Merck 50734), 125×4 mm I.D.; mobile phase, acetonitrile–water (95:5); detection, UV at 265 nm; sensitivity, 0.002 AUFS; flow-rate, 1.2 ml/min; sample load, 20 μl (loop); room temperature; chart speed, 5 mm/min; pump, Merck L-6200A; detector, Merck L-4250 UV–Vis; integrator, Merck D-2500.

The HPLC procedure was validated with a solution of vitamin D_3 in methanol, showing linearity for five samples between 0.05 and 10 mg/l in triplicate (correlation coefficient = 0.9999), repeatibility of 5 mg/l (eight replicate samples; R.S.D. = 2.0%) and a limit of detection of 0.5 ng.

2.2. Study B on a bonded normal phase (optimized in-house conditions)

The following conditions were used: column, LiChrosorb CN, 5 μm (Merck 16028), 250×4 mm I.D.; mobile phase, *n*-heptane–chloroform (60:40); detection, UV at 265 nm; sensitivity, 0.002 AUFS; flow-rate, 1.8 ml/min; sample load, 20 μl (loop); room temperature; chart speed, 5 mm/min; pump, detector and integrator as in study A.

2.3. Study C on hydroxyapatite (HAp) phase

The following conditions were used: packed column, HAp, 10–20 μm, 250×4 mm I.D.;

mobile phase, n-heptane–ethyl acetate (85:15); detection, UV at 265 nm; sensitivity, 0.05 AUFS; flow-rate, 0.8 ml/min; sample load, 20 μl (loop); room temperature; chart speed, 2.5 mm/min; pump, Beckman Model 112; detector, Beckman Model 165.

The HPLC procedure was validated with a solution of vitamin D_3 in n-heptane, showing linearity for 15, 30, 45, 60 and 75 mg/l samples in triplicate (correlation coefficient 0.9980), repeatability of 75 mg/l (eight replicate samples; R.S.D. = 1.07%) and a limit of detection of 40 ng.

The efficiency of this packing (n) is about 40 000 theoretical plates/m [10 mg/l anthracene in n-heptane; mobile phase n-heptane–dichloromethane (90:10), flow-rate 0.8 ml/min].

2.4. Study D on fluoroapatite (FAp)

The following conditions were used: packed column, FAp, 10–20 μm, 250 × 4 mm I.D.; mobile phase, n-heptane–ethyl acetate (90:10); detection, UV at 265 nm; sensitivity, 0.05 AUFS; flow-rate, 0.8 ml/min; sample load, 20 μl (loop); room temperature; chart speed, 2.5 mm/min; pump and detector as described in study C.

The HPLC procedure was validated with a solution of vitamin D_3 in n-heptane, showing linearity for 15, 30, 45, 60 and 75 mg/l samples in triplicate (correlation coefficient = 0.9980), repeatability of 60 mg/l (six replicate samples; R.S.D. = 0.9%) and a limit of detection of 40 ng.

The efficiency of this packing (n) is about 40 000 theoretical plates/m (conditions as in study C).

2.5. Preparation of the hydroxyapatite phase (HAp)

Following the procedure given in a previous paper [19], calcium nitrate solution (0.50 M, 4 l) was used in place of the lead nitrate solution when preparing the 0.1-μm microcrystals. On the basis of both X-ray diffraction analysis and elemental analysis, it was confirmed that the HAp aggregates are pure apatite ($a = b = 9.420$ Å and $c = 6.884$ Å) with a chemical composition represented by $Ca_{10}(PO_4)_6(OH)_2$ and a Ca:P molar ratio equal to that of a stoichiometric apatite, 1.67. The total surface area (30 m^2/g) was determined by the Brunauer–Emmet–Teller (BET) method with nitrogen–helium (30:70) on a Quantasorb II apparatus (Quantachrome).

2.6. Preparation of the fluoroapatite phase (FAp) and of the column

FAp microcrystals (average particle size 0.1 μm) as starting materials were prepared by slowly dropping calcium nitrate solution (0.50 M, 4 l) into boiling ammonium phosphate solution (0.30 M, 4 l) containing ammonium fluoride (0.40 M) in basic medium over 3 h at 100°C; the precipitate was matured for 30 min. The moist precipitate was spray-dried as described previously [19]. The 10–20-μm fraction was isolated by elutriation.

On the basis of X-ray diffraction analysis and elemental analysis, the FAp aggregates are pure apatite ($a = b = 9.370$ Å and $c = 6.850$ Å) with a chemical composition represented by $Ca_{10}(PO_4)_6F_2$ and a Ca:P molar ratio close to 1.67. The total surface area (BET) was 20 m^2/g.

The spherical FAp aggregates (10–20 μm) were packed into a stainless-steel column as described previously [19], but at a pressure of 50 rather than 100 bar.

2.7. References standards and preparation of the HPLC solutions

Provitamin D_3 (purity 99.5%) and vitamin D_3 (purity 99.5%) were supplied by Hoffmann-La Roche (Basle, Switzerland) and maleic anhydride by Aldrich (Beerse, Belgium).

Tachysterol was prepared as needed by one of the following methods: study A, irradiation of provitamin D_3 (10 mg/l in methanol, in a UV-transparent Teflon tube) for 1 min at 254 nm (Hanao low-pressure mercury vapour source); study B, the same but in n-heptane rather than methanol; studies C and D, irradiation of provitamin D_3 (100 mg/l in n-heptane, in a quartz cell) for 1–20 min at 254 nm (irradiation lamp for viewing TLC plates). The irradiated solution were kept in the dark at 8°C. Owing to

the photostationary equilibrium, the resulting solutions were mixtures of tachysterol, previtamin D_3 and residual provitamin D_3.

Vitamin D_3 solutions were 8 mg/l in methanol (study A) or in *n*-heptane (study B) and 50 mg/l in n-heptane (studies C and D). Provitamin D_3 solutions were 10 mg/l in methanol (study A) or *n*-heptane (study B) and 100 mg/l in *n*-heptane (studies C and D). Tachysterol + previtamin D_3 solutions were prepared by irradiation of the above solutions of provitamin D_3 (for 1 min in studies A and B and for 1–20 min in studies C and D).

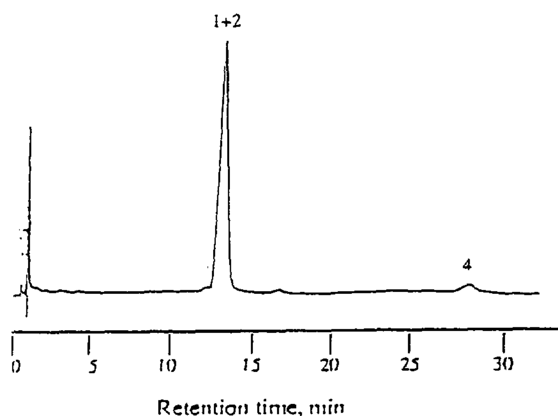

Fig. 2. Chromatogram of provitamin D_3 irradiated for 1 min (study A). UV detection at 265 nm. Peaks: 1 = previtamin D_3; 2 = tachysterol; 4 = residual provitamin D_3.

3. Results

Retention times obtained under the various conditions are given in Table 1.

3.1. Conditions A

Irradiation of the provitamin D_3 (peak 4) solution for 1 min effected its transformation into 1 and 2, shown as a single peak (t_R = 13.5 min) on the chromatogram (Fig. 2); 1 min represented the optimum time for irradiation (Fig. 3).

Heating the vitamin D_3 (3) solution (t_R = 15 min) for 12 h at 60°C effected its transformation into previtamin D_3 (1) (t_R = 13.5 min).

Irradiation of the provitamin D_3 solution, followed by heating, produced two peaks, **1** + **2** and **3** (Fig. 4a). Addition of maleic anhydride to this solution resulted in a significant decrease in the peak at 13.5 min (Fig. 3b), explained by the formation of an adduct between tachysterol and anhydride [20]. This demonstrates the co-elution of tachysterol and previtamin D_3 in this previously described HPLC procedure.

3.2. Conditions B

Irradiation of the provitamin D_3 solution produced three peaks: previtamin D_3 (1), tachysterol (2) and residual provitamin D_3 (4). Addition of vitamin D_3 to the resulting solution resulted in an increase in the peak at 8.0 min (Fig. 5); this shows that tachysterol cannot be separated from vitamin D_3 under these conditions using normal-phase HPLC.

3.3. Conditions C

Irradiation of the provitamin D_3 solution at 254 nm at room temperature for 10 min produced three peaks (Fig. 6): previtamin D_3 (1), tachysterol (2) and residual provitamin D_3 (4).

Table 1
Retention times (t_R, min) of the various compounds obtained by HPLC under different conditions

Conditions	Provitamin D_3 (peak 4)	Previtamin D_3 (peak 1)	Tachysterol (peak 2)	Vitamin D_3 (peak 3)
A	28	13.5	13.5	15
B	12	5.5	8	8
C	13.2	7.2	10.4	11.6
D	10.8	6.8	8.6	9.4

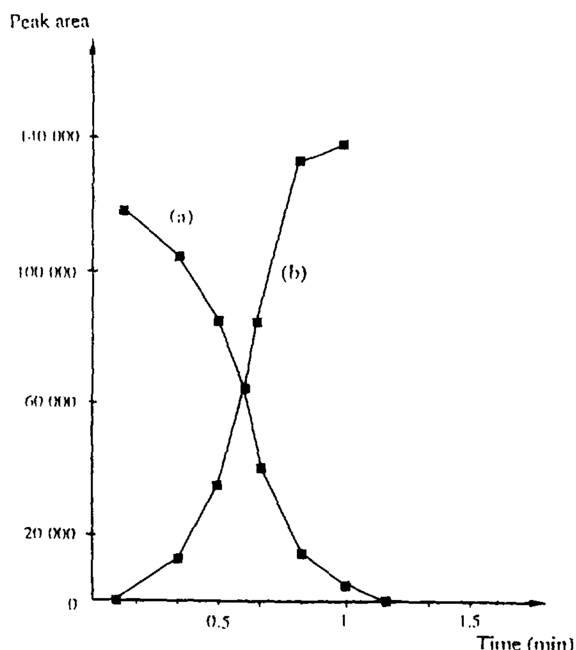

Fig. 3. Irradiation of provitamin D_3. (a) Surface of the provitamin D_3 peak; (b) surface of the tachysterol + previtamin D_3 peak.

Previtamin D_3 was identified by the HPLC analysis of a vitamin D_3 solution (t_R = 11.6 min) when heated for 5 h at 80°C (Fig. 7).

Heating for 5 h at 70°C the provitamin D_3 solution previously irradiated for 10 min at 254 nm gave a mixture of previtamin D_3, tachysterol, vitamin D_3 and residual provitamin. Addition of maleic anhydride to this solution resulted in a decrease in the tachysterol peak area (Fig. 8).

Under these operating conditions with HAp stationary phase, vitamin D_3 and related products are better separated than by using normal or reversed-phase silica-based materials. The determination of vitamin D_3 (60 mg/l) with various amounts of provitamin D_3 (6, 12 and 18 mg/l) gave an accuracy of 96%.

3.4. Conditions D

Irradiation for 5 min of a provitamin D_3 solution at 254 nm, without heating, produced three peaks. Previtamin D_3 was identified as

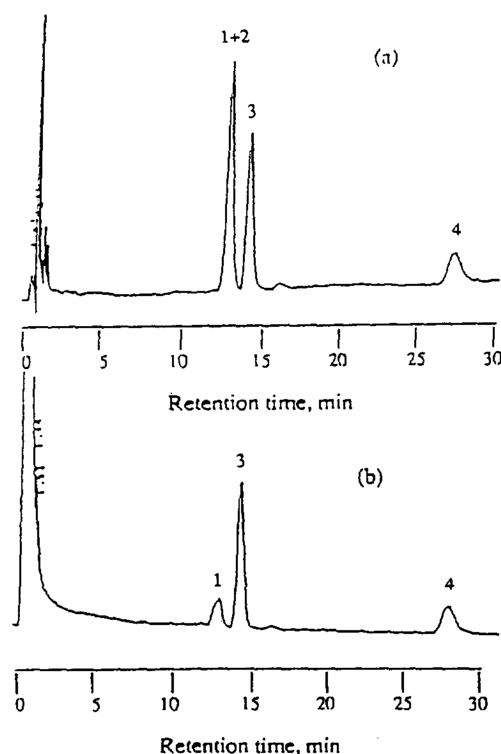

Fig. 4. Chromatogram of provitamin D_3 irradiated for 40 s and heated for 12 h at 60°C (study A). (a) Before adding maleic anhydride; (b) 15 min after addition. Peaks: 1 = previtamin D_3; 2 = tachysterol; 3 = vitamin D_3; 4 = residual provitamin D_3.

previously by analysing a heated vitamin D_3 solution.

During irradiation, between 0.5 and 20 min the appearance of previtamin D_3 and tachysterol could be followed, in addition to the disappearance of provitamin D_3. After 3 min, the level of previtamin D_3 remained constant (about 10% of the provitamin taking account of the UV response factors); the level of tachysterol reached about 48% of the provitamin D_3 after 10 min of irradiation and 65% after 20 min.

Heating the provitamin D_3 solution for 5 h at 60–70°C, after a 10-min irradiation at 254 nm, produced a mixture of residual provitamin (4), vitamin D_3 (3), tachysterol (2) and previtamin D_3 (1) (Fig. 9). As before, addition of maleic anhydride decreased the tachysterol peak.

Under these operating conditions with a FAp

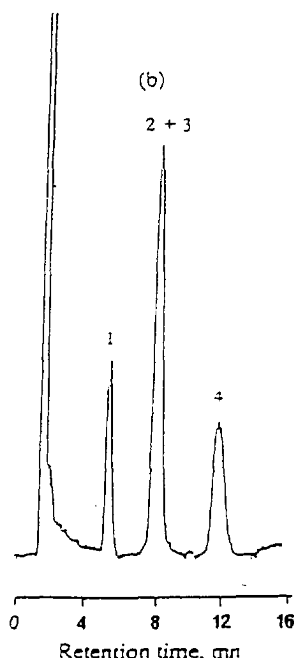

Fig. 5. Chromatogram of provitamin D_3 irradiated for 1 min (study B) with addition of vitamin D_3. Peaks: 1 = previtamin D_3; 2 = tachysterol; 3 = vitamin D_3; 4 = residual provitamin D_3.

phase, vitamin D_3 and its related products are conveniently separated. The determination of vitamin D_3 (60 mg/l) with various amounts of provitamin D_3 (3, 6 and 12 mg/l) gave an accuracy of 95%.

4. Discussion

The work demonstrated the interesting properties of two new apatite materials in HPLC, HAp and FAp, as an original means of separation by their qualitative application to the separation of provitamin D_3, previtamin D_3, vitamin D_3 and tachysterol. These compounds are not completely resolved under optimized standard conditions using normal- or reversed-phase materials. They are resolved, however, with the new HAp and FAp stationary phases, with retention times compatible with frequent repeated injections. The elution order is similar using CN-silica or an apatite stationary phase.

Retention tends to be longer with HAp than with FAp. Presumably this reflects the greater interaction of OH groups than F groups with the secondary alcoholic groups of vitamin D_3 and related compounds. On the other hand, no significant difference was noted when these two adsorbents were used to separate proteins (unpublished data). In this case, Ca and PO_4 sites interact with the charged groups of proteins, as described in the literature [19,21].

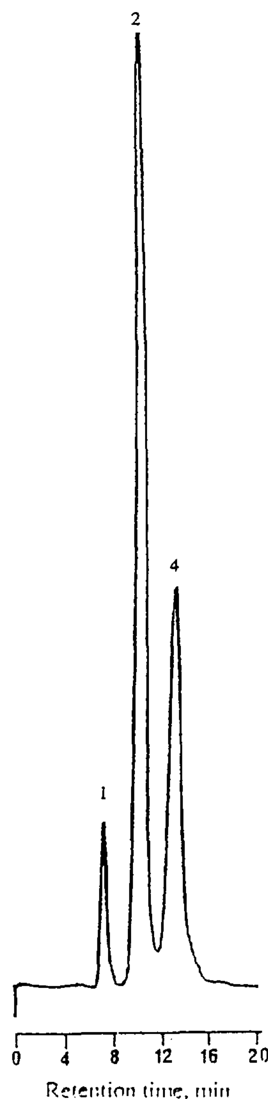

Fig. 6. Chromatogram of provitamin D_3 irradiated for 10 min (study C). Peaks: 1 = previtamin D_3; 2 = tachysterol; 4 = residual provitamin D_3.

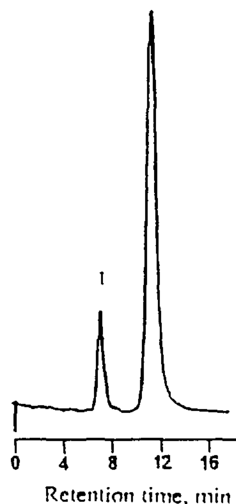

Fig. 7. Chromatogram of vitamin D_3 after heating at 80°C for 5 h (study C). Peaks: 1 = previtamin D_3; 3 = residual vitamin D_3.

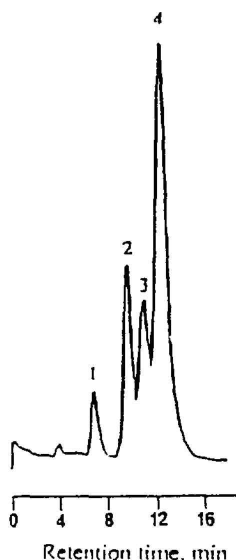

Fig. 8. Chromatogram of provitamin D_3 irradiated for 10 min and heated for 5 h at 70°C, 40 min after adding maleic anhydride (study C). Peaks: 1 = previtamin D_3; 2 = tachysterol; 3 = vitamin D_3; 4 = residual provitamin D_3.

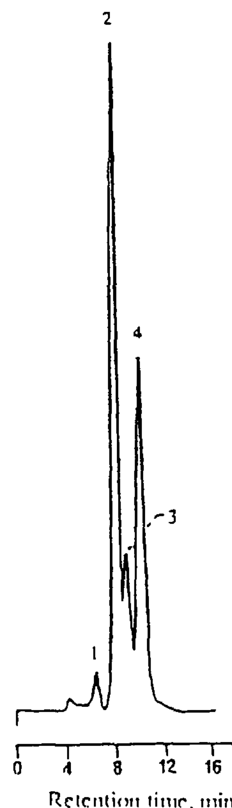

Fig. 9. Chromatogram of provitamin D_3 irradiated for 10 min and heated for 5 h at 70°C (study D). Peaks: 1 = previtamin D_3; 2 = tachysterol; 3 = vitamin D_3; 4 = residual provitamin D_3.

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