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Note

Separation of the metabolites of [26,27-^{3}H]25-hydroxycholecalciferol in plasma extracts by high-pressure liquid chromatography on a preparative column

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Several high-pressure liquid chromatographic (HPLC) systems have been developed recently to separate metabolites of vitamin D_2 (calciferol) and vitamin D_3 (cholecalciferol) in human and animal plasma[1–11] and to identify vitamins D_2 and D_3 in pharmaceutical preparations[12–14]. The first described method was reversed-phase liquid–liquid chromatography using aqueous methanol for elution from a column packed with octadecylsilane permanently bonded to glass beads[1]. This chromatographic system has been shown to be useful in the analysis of pharmaceutical forms[13], but has been found less useful in the analysis of plasma extracts due to a minimal resolution of 1α,25-$(OH)_2$-D_3 from 25,26-$(OH)_2$-D_3* (see ref. 2).

Small porous silica gel columns have found wide application in the HPLC separation of the metabolites of vitamins D_2 and D_3 in plasma. Two 25 cm × 2.1 mm Zorbax-Sil columns in series, eluted with 1–20% isopropanol in Skellysolve B have been used for the separation of vitamins D_2 and D_3 and their metabolites[2]. A recent communication has described the separation of synthetically prepared vitamin D derivatives, on a Zorbax-Sil column, using dichloromethane with 2% methanol[3]. The separation of 1α,25-$(OH)_2$-D_2 and 25-OH-D_3 has been achieved on a Porasil column (30 × 0.4 cm), eluted with hexane containing 5% absolute ethanol[9] and on two Porasil columns in series eluted with dichloromethane containing 2% isopropanol[10].

Recently we have studied the metabolism of intraveneously injected [^{3}H]25-OH-D_3 and investigated the concentrations of labelled metabolites in human plasma. The separation of labelled metabolites on Sephadex LH-50 columns indicated, as expected, the formation of the polar metabolites [^{3}H]24,25-$(OH)_2$-D_3 and of a mixture of [^{3}H]1,25- and 25,26-$(OH)_2$-D_3[15]. We found that Merckosorb SI 60, dry packed in a 1 m × 5.5 mm preparative high pressure column separated the three important polar metabolites of [^{3}H]25-OH-D_3 in human plasma extracts without preliminary chromatographic separation of lipids; a procedure necessary when analytical and semipreparative silica gel columns have been used[2,4–6,8–10].

* Abbreviations: 25-OH-D_2 = 25-hydroxycalciferol; 25-OH-D_3 = 25-hydroxycholecalciferol; 1α,25-$(OH)_2$-D_3 = 1α,25-dihydroxycholecalciferol; 24,25-$(OH)_2$-D_3 = 24,25-dihydroxycholecalciferol; 25,26-$(OH)_2$-D_3 = 25,26-dihydroxycholecalciferol.

MATERIALS

[26,27-^{3}H]25-OH-D_3, specific activity *ca.* 10,000 mCi/mmol was purchased from the Radiochemical Centre, Amersham, Great Britain. It was found to be about 99% pure by HPLC in a 1 m × 5.5 mm Merckosorb SI 60 column, eluted with dichloromethane containing 3% methanol.

[26,27-^{3}H]24,25-$(OH)_2$-D_3 and [26,27-^{3}H]25,26-$(OH)_2$-D_3 were prepared by chick kidney homogenate hydroxylation of tritiated 25-OH-D_3 by the method of Tanaka *et al.*[17]. Tritiated 25,26-$(OH)_2$-D_3 was further identified by co-chromatography with unlabelled 25,26-$(OH)_2$-D_3 (a gift from Dr. J. Redel, Hôpital Cochin, Paris, France) on a 25 cm × 4.6 mm Zorbax-Sil column. [26,27-^{3}H]1α,25-$(OH)_2$-D_3 was a gift from Dr. M. Uskoković, Hoffmann-La Roche, Nutley, N.J., U.S.A.

A Varian 8500 chromatograph (Varian, Palo Alto, Calif., U.S.A.) was used for HPLC. A 1 m × 5.5 mm I.D. column was dry packed with 15 g of Merckosorb SI 60, mean particle size 20 μm. Methanol (analytical reagent grade) and dichloromethane distilled over a 100-cm column (fraction 40–41°) were used. The scintillator contained 2,5-diphenyloxazole (PPO, 15 g) and 1,4-bis-(5-phenyloxazolyl-2)-benzene (POPOP, 500 mg) in 2.5 l toluene. Radioactivity was measured in a Beckman LS-233 liquid scintillation counter.

METHODS

Separation of [^{3}H]25-OH-D_3 and its metabolites by HPLC

About 5000 cpm of [^{3}H]25-OH-D_3, 3500 cpm of [^{3}H]24,25-$(OH)_2$-D_3, 1700 cpm of [^{3}H]25,26-$(OH)_2$-D_3 and 4000 cpm of [^{3}H]1α,25-$(OH)_2$-D_3 in 100 μl dichloro-

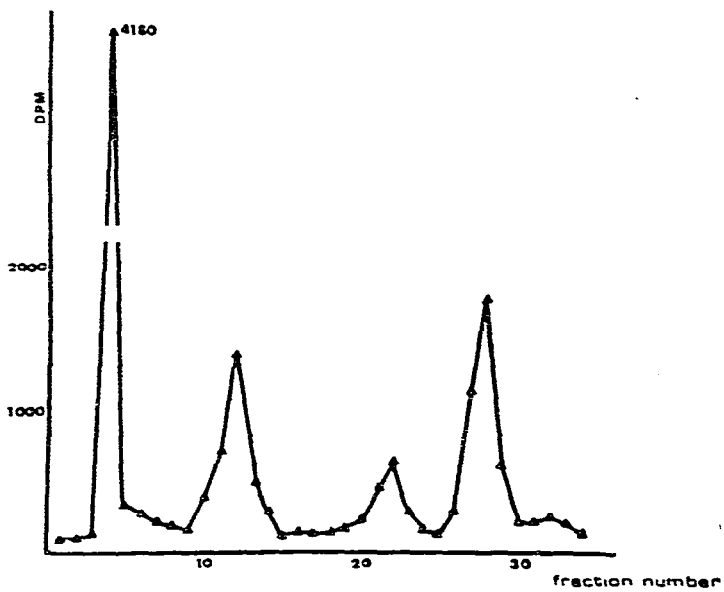

Fig. 1. Separation of tritiated 25-OH-D_3 and its metabolites on a 1 m × 5.5 mm I.D. column packed with Merckosorb SI 60, mean particle size 20 μm. Eluted with dichloromethane containing 3% methanol, 10-ml fractions collected, evaporated and counted. Fractions 4–7: 25-OH-D_3; fractions 11–15: 24,25-$(OH)_2$-D_3; fractions 21–23: 25,26-$(OH)_2$-D_3 and fractions 26–29: 1α,25-$(OH)_2$-D_3.

methane was injected into a 1 m × 5.5 mm column, and eluted with dichloromethane, containing 3% methanol. 10-ml fractions were collected (flow-rate, 200 ml/h) and thereafter the whole fractions were evaporated and counted (Fig. 1).

Extraction of metabolites from human plasma

Patients were injected with 10 μCi of [^{3}H]25-OH-D$_3$ which was dissolved in 0.5 ml ethanol and diluted to 10 ml with a saline solution shortly before injection. Blood samples (10–20 ml) were collected 24 h later and the plasma separated. A trace of ascorbic acid (as antioxidant) was added and lipids and metabolites were extracted with 40 ml ethanol, vortexed for 3 min and centrifuged. The second extract was obtained after suspension of the protein cake in 20 ml ethanol, vortexing and centrifuging. Pooled extracts were evaporated under vacuum at 40°. The residue was dissolved in dichloromethane (10 ml) a small quantity of insoluble material was filtered off, the filtrate concentrated under CO_2, redissolved in 0.2 ml dichloromethane and transferred quantitatatively onto the column. The tube was washed with an additional 0.2 ml dichloromethane, transferred to the column and the radioactive derivatives eluted with dichloromethane, containing 3% methanol. Whole fractions were counted (Fig. 2).

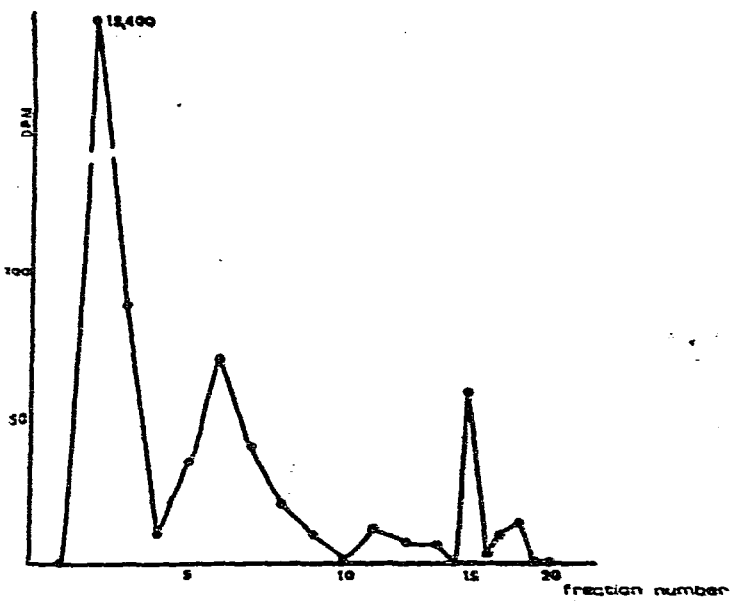

Fig. 2. Separation of metabolites, extracted from 8.7 ml of human plasma. For convenience 20-ml fractions (fraction 1–13) and 10-ml fractions (fractions 14–20) were collected. Fractions 2–3: 25-OH-D$_3$; fractions 5–8; 24,25-(OH)$_2$-D$_3$; fractions 11–12: 25,26-(OH)$_2$-D$_3$ and fraction 15: 1α,25-(OH)$_2$-D$_3$.

Separation of [^{3}H]25,26-(OH)$_2$-D$_3$ and [^{3}H]1α,25-(OH)$_2$-D$_3$ isolated first on a Sephadex LH-20 column

A mixture of the two metabolites was eluted from a Sephadex LH-20 column as a 50-ml fraction (between 250–300 ml) using a chloroform–hexane (2:1) mixture

according to ref. 15. On chromatography on a 1 m × 5.5 mm column packed with Merckosorb SI 60 it was resolved into two peaks, corresponding to the two metabolites (Fig. 3).

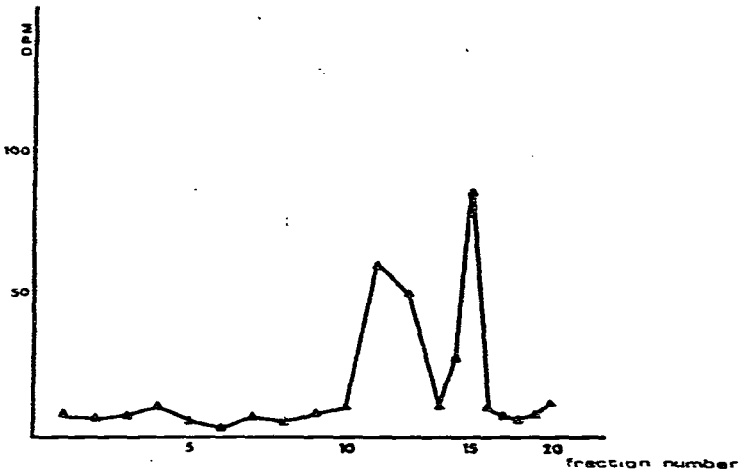

Fig. 3. Separation of a synthetic mixture of [^{3}H]25,26- and [^{3}H]1α,25-(OH)$_2$-D$_3$, isolated first on a Sephadex LH-20 column. For convenience 20-ml fractions (fractions 1–13) and 10-ml fractions (fractions 14–20) were collected. Fractions 11–12: 25,26-(OH)$_2$-D$_3$ and fraction 15: 1α,25-(OH)$_2$-D$_3$.

RESULTS AND DISCUSSION

Since the introduction of Sephadex LH-20 in the chromatography of metabolites of vitamin D (ref. 15) only one communication to our knowledge has described the metabolism of labelled cholecalciferol in man[16]. The results of this study were limited by a very low conversion of cholecalciferol to the final active metabolite 1α,25-(OH)$_2$-D$_3$. Using labelled 25-OH-D$_3$ we tried to improve the precision of calculation of the conversion of [^{3}H]25-OH-D$_3$ to the 1α,25-dihydroxy derivative. Porous silica gel columns in HPLC are capable of resolving the polar metabolites of cholecalciferol[2,4–6,8–10]. A prepurification step has been found necessary to remove a large quantity of lipids from the plasma. We achieved a good separation of metabolites of 25-OH-D$_3$ on a 1-m preparative column without preliminary separation of lipids. A synthetic mixture of 25,26-(OH)$_2$-D$_3$ and 1α,25-(OH)$_2$-D$_3$, isolated first on a Sephadex LH-20 column, was resolved into two peaks (Fig. 3).

Two similar chromatographic systems have recently been published[3,10]. The former used a mixture of dichloromethane with 2% methanol, the latter dichloromethane with 2% isopropanol, in both cases on analytical columns. We believe that our chromatographic system represents a reasonable compromise between Sephadex LH-20 and microporous analytical columns, packed with 5–10 μm size particles. Our method is especially useful when larger quantities of human or animal plasma extract have to be used.

In our study of the metabolism of labelled 25-OH-D$_3$ in man we were limited by two factors: the permitted dose of tritium and the size of blood samples. Blood samples of 20 ml were collected, giving usually 8–10 ml plasma. In one of the patients (K.P., body weight 40 kg, demonstrated in Fig. 2) an attempt was made to evaluate

the conversion of tritiated 25-OH-D_3 to other metabolites. The extract from 8.7 ml plasma gave, after chromatography, 15,500 dpm of [^{3}H]25-OH-D_3. This corresponds to $3.56 \cdot 10^6$ dpm (1.6 μCi) in the whole plasma. Calculations were based on the assumption that total plasma content is equal to 5% of the body weight, *i.e.* 16% of the injected dose was circulating in plasma at that time. The whole plasma content of tritiated 24,25-$(OH)_2$-D_3 and 1α,25-$(OH)_2$-D_3 was calculated to be 38,000 dpm and 13,600 dpm respectively. This would correspond to 0.17% conversion of tritiated 25-OH-D_3 to 24,25-dihydroxy derivative and 0.06% conversion to 1α,25-dihydroxy derivative.

Recently published mean levels of vitamin D ($D_2 + D_3$) and its important metabolites in human plasma[5] showed that the quantity of 24,25-$(OH)_2$-D is about 8.3% of that of 25-OH-D and that for 1α,25-$(OH)_2$-D the corresponding quantity is about 0.14%. This would mean that in our patient there is a feedback control limiting production of both labelled polar metabolites of 25-OH-D_3 (refs. 18 and 19).

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