Determination of circulating 25,26-dihydroxycholecalciferol in man by radioimmunoassay

L. J. FRAHER, T. L. CLEMENS, S. E. PAPAPOULOS, J. REDEL* AND J. L. H. O’RIORDAN

Department of Medicine, The Middlesex Hospital, London, and *Institute de Rhumatologie, Hôpital Cochin, Paris, France

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Summary

1. A radioimmunoassay for 25,26-dihydroxycholecalciferol has been developed with an antiserum raised in a sheep, tritiated 1,25-dihydroxycholecalciferol as tracer and synthetic 25,26-dihydroxycholecalciferol as standard. The metabolite was purified from serum extracts by Sephadex LH 20 and high-pressure liquid chromatography; recovery was monitored with biologically generated, tritiated 25,26-dihydroxycholecalciferol.

2. The mean ± SEM concentration of 25,26-dihydroxycholecalciferol in serum from 18 healthy subjects was 587 ± 65 pmol/l. Seven Asian patients with osteomalacia due to vitamin D deficiency had very low or undetectable (<96–231 pmol/l) circulating 25,26-dihydroxycholecalciferol concentrations.

3. The metabolite was detectable in the sera from seven anephric patients (mean 262 ± 43 pmol/l), indicating that extrarenal sites for the 26-hydroxylation of 25-hydroxycholecalciferol exist in man.

4. A strong positive correlation between the concentrations of 25-hydroxycholecalciferol and those of 25,26-dihydroxycholecalciferol in serum was obtained. Thus it appears that in man the production of this dihydroxy metabolite of vitamin D depends on the concentration of its precursor, 25-hydroxycholecalciferol.

Key words: 25,26-dihydroxycholecalciferol, 25-hydroxycholecalciferol, nephrectomy, radioimmunoassay, vitamin D deficiency.

Correspondence: Dr J. L. H. O’Riordan, Department of Medicine, The Middlesex Hospital, London W1N 8AA.

Abbreviations: 1,25-(OH)\textsubscript{2}D\textsubscript{3}, 1,25-dihydroxycholecalciferol; 24,25-(OH)\textsubscript{2}D\textsubscript{3}, 24,25-dihydroxycholecalciferol; 25,26-(OH)\textsubscript{2}D\textsubscript{3}, 25,26-dihydroxycholecalciferol; 25,26-(OH)\textsubscript{2}D\textsubscript{3}, 25,26-dihydroxyergocalciferol; 25-OHD\textsubscript{3}, 25-hydroxycholecalciferol; 25-OHD\textsubscript{3}, 25-hydroxyergocalciferol; 25R, 26-(OH)\textsubscript{2}D\textsubscript{3}, R isomer of 25,26-dihydroxycholecalciferol; 25S,26-(OH)\textsubscript{2}D\textsubscript{3}, S isomer of 25,26-dihydroxycholecalciferol; [\textsuperscript{3}H]1,25-(OH)\textsubscript{2}D\textsubscript{3}, tritiated 1,25-dihydroxycholecalciferol; [\textsuperscript{3}H]25,26-(OH)\textsubscript{2}D\textsubscript{3}, tritiated 25,26-dihydroxycholecalciferol; [\textsuperscript{3}H]25-OHD\textsubscript{3}, tritiated 25-hydroxycholecalciferol.

Introduction

25,26-Dihydroxycholecalciferol [25,26-(OH)\textsubscript{2}D\textsubscript{3}] is a metabolite of cholecalciferol produced by further hydroxylation of 25-hydroxycholecalciferol (25-OHD\textsubscript{3}). This compound was first isolated from hog plasma (Suda, DeLuca, Schnoes, Tanaka & Holick, 1970) and was later reported to increase intestinal calcium transport in animals (Lam, Schnoes & DeLuca, 1974; Redel, Bell, Bazely, Calando, Delbarre & Kodicek, 1974). More recent reports have also indicated that it can inhibit the secretion of parathyroid hormone in the goat (Care, Pickard, Papapoulos, O’Riordan & Redel, 1978). However, very little is known regarding its physiological role or its tissue of origin in man.

We have previously described the development and clinical application of a radioimmunoassay for 1,25-dihydroxycholecalciferol [1,25-(OH)\textsubscript{2}D\textsubscript{3}], the hormonal form of the vitamin (Clemens, Hendy, Papapoulos, Fraher, Care & O’Riordan, 1979). The antiserum used in that assay also recognizes 25,26-(OH)\textsubscript{2}D\textsubscript{3} and it has
therefore been possible to adapt the method and develop a radioimmunoassay for the measurement of this metabolite in human serum. 25,26-(OH)D3 was found to be present in the serum from normal and anephric subjects and its circulating concentrations were dependent on the concentrations of its precursor.

Materials and methods

Materials

Chemically synthesized 25S,26-dihydroxycholecalciferol [25S,26-(OH)2D3] the natural human metabolite (Redel, Bazely, Mawer, Hann & Jones, 1979; 1980), and 25R,26-dihydroxycholecalciferol [25R,26-(OH)2D3] were prepared as described previously (Redel, Miravet, Bazely, Calando, Carre & Delbarre, 1977). 25,26-(OH)D3 as a synthetic mixture of the R and S isomers was kindly provided by Dr Phillip Bell. 25-Hydroxycholecalciferol (25-OHD3) was obtained from the Upjohn Company, Kalamazoo, MI, U.S.A.

High-pressure liquid chromatography was performed by using a model LC 204 fitted with a model 6000A pumping system, and a model 440 ultraviolet, fixed wavelength (254 nm) detector [all from Waters Associates (Inst) Ltd, Northwich, Cheshire, U.K.]. Samples were injected via a Rheodyne model 7280 valve (Rheodyne, Berkeley, CA, U.S.A.). Stainless-steel columns (25 cm x 6.2 mm internal diameter) packed with Zorbax-SIL silica were purchased from Dupont Instruments Ltd, Hitchin, Herts., U.K. High-pressure liquid chromatography grade solvents were purchased from Rathburn Chemicals, Walkerburn, Scotland. All other solvents were AnalaR grade and those used for chromatography were redistilled before use. Glassware used in extraction and purification procedures was routinely prerinsed in acetone and rinsed in the working solvent.

Tritiated 25,26-(OH)2D3 and 1,25-(OH)2D3 were synthesized biologically by incubation of [23,24-3H]25-OHD3 (sp. radioactivity, 76 Ci/mmoll, The Radiochemical Centre, Amersham, Bucks., U.K.) with chick kidney homogenates. The specific radioactivity of these biosynthesized metabolites was assumed to be the same as that of tritiated 25-OHD3 as the amounts generated were not sufficient to permit direct determination (Clemens, Fraher, O'Riordan, Little & Dale, 1980). The identity of each tritiated metabolite was confirmed by co-chromatography with authentic crystalline preparations on high-pressure liquid chromatography. Fig. 1 illustrates co-chromatography of purified [3H]25,26-(OH)2D3 with crystalline 25,26-(OH)2D3 on a Zorbax-SIL column eluted with hexane/propan-2-ol (85:15, v/v) at a flow rate of 1.5 ml/min.

Extraction and purification of serum

Six hundred c.p.m. (7 fmol) of [3H]25,26-(OH)2D3 in 50 μl of ethanol (for estimation of recovery) were added to 3–5 ml of serum (or plasma) in a 15 ml round-bottomed tube. After a 30 min equilibration, the sample was extracted with 1 vol. of cyclohexane/ethyl acetate (1:1, v/v) as suggested by Dr R. Bouillon (personal communication) by using a mechanical shaker. The upper layer was removed, the aqueous phase re-extracted twice with 1 vol. of cyclohexane/ethyl acetate and the combined extracts were dried under nitrogen. This extract was chromatographed on a short Sephadex LH 20 column (0-8 cm x 9 cm) developed with hexane/methanol/chloroform (9:1:2:1, by vol.). Elution was performed with the same solvent and an elution volume of 10–22 ml was collected and dried under nitrogen. This extract was chromatographed on a short Sephadex LH 20 column (0-8 cm x 9 cm) developed with hexane/methanol/chloroform (9:1:2:1, by vol.). Elution was performed with the same solvent and an elution volume of 10–22 ml was collected and dried under nitrogen. This system largely separates 25,26-(OH)2D3 from 25-OHD3 but does not resolve it from 1,25-(OH)2D3 and only partially separates 24,25-(OH)2D3. Final purification of 25,26-(OH)2D3 was achieved by high-pressure liquid chromatography on a Zorbax-SIL column (25 cm x 6.2 mm internal diameter) eluted with hexane/propan-2-ol (85:15, v/v) at a flow rate of 1.5 ml/min. This system separates 25,26-
(OH)2D3 from other vitamin D3 metabolites. Under these conditions 25,26-(OH)2D3 eluted between 15 and 17 ml (that is 11 and 12.33 min) after injection; the exact elution position of crystalline 25,26-(OH)2D3 was established before each run of samples.

To establish the efficiency of the purification procedures, [3H]25-OH-D3, [3H]24,25-(OH)2D3 and [3H]1,25-(OH)2D3 (10000 c.p.m. each) were added to separate serum extracts which were then purified on Sephadex LH 20 and high-pressure liquid chromatography in the usual manner. The carry-over of tritium into the 25,26-(OH)2D3 eluting fraction on high-pressure liquid chromatography was 0.01% for 25-OH-D3 and 0.10% for 24,25-(OH)2D3. No radioactivity attributable to 1,25-(OH)2D3 was detected in the 25,26-(OH)2D3 region. Therefore contamination of the 25,26-(OH)2D3 fraction by these metabolites would not be significant. The 25,26-(OH)2D3 fraction purified from serum extracts was stored at −20°C in the dark until assay, at which time it was dried under nitrogen and redissolved in 0.5 ml of ethanol. A portion (0.166 ml) of this solution was dried under nitrogen and dissolved in liquid scintillant (0.5% PPO and 0.025% POPOP-Packard) in toluene containing 30% (v/v) Triton X 100). Samples were counted for radioactivity for 10 min each at an efficiency of 35–40%.

A displacement curve was obtained by plotting the radioactivity counts bound in the supernatant against the amount of 25,26-(OH)2D3 added to each tube. In this way, the concentration of 25,26-(OH)2D3 in the sample could be calculated after correction for losses during the purification procedure.

**Measurement of 25-OHD3**

Serum 25-OH-D3 was measured with a competitive protein-binding assay (Preece, O'Riordan, Lawson & Kodicek, 1974). The normal range for this assay is 7.2–79 nmol/l and the limit of detection is 1.9 nmol/l. This assay measures both 25-hydroxyergocalciferol (25-OHD2) as well as 25-OH-D3 but it has been previously shown that the concentration of 25-OHD2 in serum from normal British adults is low or unmeasurable (Preece, Tomlinson, Ribot, Pietrek, Korn, Davies, Ford, Dunningan & O’Riordan, 1975; Poskitt, Cole & Lawson, 1979).

**Results**

**Sensitivity of assays**

Fig. 2 shows calibration curves obtained for the racemic mixture of 25,26-(OH)2D3 and the separated R and S isomers. All three forms were equipotent in the displacement of [3H]1,25-(OH)2D3 from the antibody and therefore for practical reasons the racemic mixture was used as reference preparation for further studies. Addition of 0.046 pmol (20 pg) of 25,26-(OH)2D3 resulted in significant displacement of [3H]1,25-(OH)2D3 compared with tubes with no added sterol. Taking this value as the sensitivity of the assay, the limit of detection of the method when a 5 ml sample is extracted would be 96 pmol/l (40 pg/ml).

**Circulating 25,26-(OH)2D3 in man**

In order to evaluate assay variation, a pool of serum was obtained from a healthy subject and
FIG. 2. Displacement curves for synthetic 25,26-(OH)₂D₃ (racemic mixture; ○) and the separated 25R,26-(OH)₂D₃ (△) and 25S,26-(OH)₂D₃ (▲) isomers. Antiserum 02282 was used at a final dilution of 1:75,000 and [³H]1,25-(OH)₂D₃ was used as tracer.

frozen in portions. Repetitive purification and radioimmunoassay of serum samples from this pool showed an interassay variation of 17%. When serum samples from this pool were purified in parallel and assayed in one assay, the intra-assay variation was 10%.

The accuracy of the method was further assessed by addition of 25,26-(OH)₂D₃ to two samples, increasing their concentrations by 300 pmol/l. These samples were then purified and assayed together with two sera from the same pool which contained only endogenous 25,26-(OH)₂D₃. The samples with no added 25,26-(OH)₂D₃ had a mean concentration of 353 pmol/l and those spiked with exogenous sterol had a mean value of 714 pmol/l. In another experiment, addition of large amounts of 25-OHD₃ (400 nmol/l) and 24,25-(OH)₂D₃ (42 nmol/l) to this pool of serum did not significantly affect the measured concentrations of 25,26-(OH)₂D₃.

These results support the findings of the tracer studies indicating that the carry-over of other vitamin D metabolites into the assay fraction is negligible.

Radioimmunoassay of 25,26-(OH)₂D₃ in serum from a patient treated with large doses of cholecalciferol (40,000 i.u./day) revealed a concentration of 9.3 pmol/l (3.9 ng/ml). His serum 25-OHD₃ concentration was 368 nmol/l (147 ng/ml). Measurement of 25,26-(OH)₂D₃ in serial dilutions of the purified serum extract (Fig. 3) showed that all values obtained for these sample dilutions fell on an assay curve for this metabolite; this finding further validates the assay method. 25,26-Dihydroxyergocalciferol [25,26-(OH)₂D₃] is not available and therefore its reactivity with the antiserum and its behaviour in the chromatographic systems could not be determined. To investigate the possible contribution of 25,26-(OH)₂D₃ to the observed concentration of 25,26-(OH)₂D₃, sera were obtained from two patients treated with large doses of ergocalciferol (50,000 and 100,000 i.u./day respectively). Their serum 25-hydroxyvitamin D concentrations were very high, being 873 and 1246 nmol/l. The activity measured as 25,26-(OH)₂D₃ in the sera of these two patients was 336.5 and 411 pmol/l respectively; these values are much lower than that observed in the patient who had been treated with high doses of cholecalciferol.

Fig. 4 shows circulating 25,26-(OH)₂D₃ concentrations in man measured by radioimmunoassay. The mean ± SEM concentration determined in 18 healthy adult subjects was 587 ± 65 pmol/l (range 135–1017 pmol/l). Seven patients with dietary vitamin D deficiency had concentrations ranging from undetectable (<96 pmol/l) to 231 pmol/l. These patients had histologically proven osteomalacia and their serum 25-OHD₃ concentrations were below 7 nmol/l (3 ng/ml). Seven anephric subjects had detectable 25,26-(OH)₂D₃ concentrations with values ranging from 135 to
FIG. 4. Concentrations of 25,26-(OH)₂D₃ in normal subjects, in vitamin D-deficient patients and in anephric patients. The horizontal broken line denotes the limit of detection of the assay method (96 pmol/l or 40 pg/ml).

457 pmol/l (mean 262 ± 43 pmol/l). This value was significantly different ($t = 2.78$, $P < 0.02$) from the mean obtained for healthy adults (587 pmol/l).

**Relationship between serum 25-OHD₃ and 25,26-(OH)₂D₃ concentrations**

When the 25,26-(OH)₂D₃ concentrations for normal and anephric subjects were related to their serum 25-OHD₃ concentrations, a strong positive correlation was observed (Fig. 5). This was true for both groups considered together ($r = 0.81$, $P < 0.01$) as well as individually ($r = 0.81$, $P < 0.001$ for normal; $r = 0.89$, $P < 0.05$ for anephric patients). The slopes of the two regression lines were not significantly different. However, comparison of 25,26-(OH)₂D₃ concentrations at a given concentration of 25-OHD₃ showed a significant difference between the groups. At a 25-OHD₃ concentration of 32.9 nmol/l (mean value for the anephric patients) the predicted mean f SEM concentrations for the anephric and normal group were 291 f 56 and 476 f 156 pmol/l respectively; $t = 4.09$, $P < 0.001$ (Student’s $t$-test). These data indicate that for the same 25-OHD₃ concentration the anephric individuals have lower 25,26-(OH)₂D₃ concentrations than normal subjects.

**Discussion**

The scarcity of information about the physiological significance of 25,26-(OH)₂D₃ in man is largely attributable to the lack of assays for its measurement in serum. We have, therefore, developed a sensitive radioimmunoassay and applied it to the measurement of this metabolite in man. The assay uses an antiserum raised against 1,25-(OH)₂D₃-25-hemisuccinate-bovine serum albumin conjugate. This same antiserum has also been used successfully in a radioimmunoassay for 1,25-(OH)₂D₃ (Clemens et al., 1979) and since 25,26-(OH)₂D₃ was found to displace effectively [³H]1,25-(OH)₂D₃ from the antibody, it has been possible to use the antiserum to develop a radioimmunoassay for 25,26-(OH)₂D₃. The use of [³H]25,26-(OH)₂D₃ as tracer instead of [³H]1,25-(OH)₂D₃ conferred no advantage and therefore the latter was used because of its greater availability.

It has been shown previously that an assay of comparable sensitivity could be achieved with the serum vitamin D-binding protein (Graham, Preece & O’Riordan, 1977). Nevertheless, the use of either the antibody or the serum binding protein to measure 25,26-(OH)₂D₃ in serum requires that the sterol be efficiently separated from other cross-reacting metabolites before assay. In the method described here, 25,26-(OH)₂D₃ was purified by chromatography on
Sephadex LH 20 followed by high-pressure liquid chromatography. In this way, a very sensitive method was achieved capable of detecting as little as 96 pmol/l of serum.

The mean concentration of 25,26-(OH)₂D₃ in serum from healthy adults by radioimmunoassay was 587 pmol/l. This is consistent with our previous estimations using a competitive protein-binding assay that measured the combined concentrations of 24,25-(OH)₂D₃ and 25,26-(OH)₂D₃ (Papapoulos, Lewin, Clemens, Hendy & O’Riordan, 1979). In normal subjects a strong positive correlation was found between circulating concentrations of 24,25-(OH)₂D₃ and 25,26-(OH)₂D₃. Patients with clinical osteomalacia due to dietary vitamin D deficiency had low or undetectable concentrations of 25,26-(OH)₂D₃. Conversely, the concentration of this dihydroxy metabolite was high in a patient whose serum 25-OHD₃ was elevated during treatment with large doses of cholecalciferol. It therefore appears that in man the production of 25,26-(OH)₂D₃ is dependent on the concentration of its 25-hydroxyvitamin D precursor. Such a relationship has previously been suggested from indirect measurements in studies investigating the metabolism of labelled cholecalciferol in man (Stanbury & Mawer, 1978). A similar relationship has also been shown to exist for the concentrations of 24,25-(OH)₂D₃ (Taylor, Mawer, Wallace, St John, Cochran, Russell & Kanis, 1978).

The contribution of 25,26-(OH)₂D₂ to the assayed concentration of 25,26-(OH)₂D₃ could not be fully investigated due to a non-availability of synthetic 25,26-(OH)₂D₂. However, it was shown that patients treated with high doses of ergocalciferol had inappropriately low concentrations of 25,26-(OH)₂D₃ for their total circulating 25-hydroxyvitamin D. This indicates that the assay method discriminates against 25,26-(OH)₂D₂, but this would be of little consequence in normal British subjects since it is known that their serum contains predominantly 25-OHD₃ (Preece et al., 1975; Poskitt et al., 1979).

Horst, Shepard, Jorgensen & DeLuca (1979) have used a competitive protein-binding assay to measure 25,26-dihydroxyvitamin D, which was isolated from serum extracts by Sephadex LH 20 chromatography and high-pressure liquid chromatography. With this assay they found the concentration in four healthy American adults to average 1923 ± 961 pmol/l (mean ± SD). These subjects also had a higher mean serum concentration of 25-hydroxyvitamin D (67.2 nmol/l) than the mean concentration obtained for the subjects reported here (44 nmol/l). Although this difference in the 25-hydroxyvitamin D concentrations may in part explain the higher 25,26-dihydroxyvitamin D concentrations reported by Horst et al. (1979), the number of subjects they studied was too small to draw any definite conclusions.

The sites of synthesis of 25,26-(OH)₂D₃ in man are not yet known though the chick kidney can carry out 26-hydroxylation of 25-OHD₃ in vitro (Tanaka, Shepard, DeLuca & Schnoes, 1978) and indeed has been used to generate the tritiated metabolite for the studies reported here. In order to investigate the importance of the kidney in the production of 25,26-(OH)₂D₃ in man, we have measured its concentration in the sera of anephric subjects. Despite the absence of the kidneys, these patients had detectable concentrations of 25,26-(OH)₂D₃ in serum which were positively correlated to the circulating concentrations of 25-OHD₃. However, when compared with normal subjects these anephric individuals had lower concentrations of 25,26-(OH)₂D₂ for a given concentration of 25-OHD₃, suggesting that in man the kidney may contribute in part to the total circulating concentration of this metabolite. Another less likely explanation for this difference may be that 25,26-(OH)₂D₃ is more susceptible to loss than 25-OHD₃ during haemodialysis. Alternatively, if these patients had taken at some stage any multivitamin preparations containing small amounts of ergocalciferol, it might be that 25,26-(OH)₂D₂ if formed was not measured. However, it is entirely clear from our studies that extrarenal sites for 26-hydroxylation of 25-OHD₃ exist in man.

It is hoped that further application of this assay in conjunction with the assays for other vitamin D metabolites will shed light on the physiological significance of 25,26-(OH)₂D₃ in man.

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References


25,26-Dihydroxycholecalciferol in man


