Competitive Protein Binding Assay for 1,25-Dihydroxyvitamin D in Human Plasma

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Synopsis

A sensitive and simplified radioreceptor assay for 1, 25-dihydroxyvitamin D ($1, 25-(OH)_2D$) in human plasma was described and applied to preliminary clinical studies. Tritium-labeled $1, 25-(OH)_2D_3$ was produced by incubating chick kidney homogenate with tritium labeled 25-hydroxyvitamin D_3 ($25-OHD_3$). A cytosol receptor was obtained from rachitic chick intestine ($K_d = 5.3 \times 10^{-11}$ M). Lipids in 5 ml of heparinized human plasma were extracted with dichloromethane, and $1, 25-(OH)_2D$ was isolated by a Sephadex LH-20 column followed by high pressure liquid column chromatography. Recovery of $1, 25-(OH)_2D_3$ after the plasma extraction and chromatography ranged from 58 to 100%. The assay was sensitive to 5 pg/tube. Diluted plasma from a patient on a high dose of $1\alpha-OHD_3$ showed a dilution curve parallel to the standard curve. The cytosol receptor showed a cross reactivity to various vitamin D_3 metabolites physiologically present in the circulation and it was thought to be essential to eliminate other vitamin D_3 metabolites than $1, 25-(OH)_2D$ from plasma samples by high pressure liquid chromatography. Plasma concentrations of $1, 25-(OH)_2D$ were, in the case of most normal subjects, distributed from 7 to 33 pg/ml and the range of distribution became greater in relation to age, indicating that plasma values should be matched to age. Whereas markedly high values of $1, 25-(OH)_2D$ in plasma were found in some cases of primary hyperparathyroidism with prominent bone resorption, relatively low values were seen in some patients with chronic renal failure, senile osteoporosis, osteomalacia and hypercalcemia due to bone metastasis.

It is widely considered that 1,25-dihydroxyvitamin D_3 ($1,25-(OH)_2D_3$) is the most active hormonal form of vitamin D_3 which stimulates intestinal calcium transport and bone mineral mobilization. Therefore, it is important to know the circulating $1,25-(OH)_2D$ concentration in order to investigate calcium and phosphate metabolism. The present report describes a sensitive and simplified radioassay for $1,25-(OH)_2D$ in human plasma and its application to preliminary clinical studies.

Materials and Methods

25-Hydroxyvitamin D_3 ($25-OHD_3$) and vitamin D_3 (D_3) were obtained from Philips-Duphar (Amsterdam, Netherlands). $1, 25-(OH)_2D_3$ and $24, 25-(OH)_2D_3$ were generously supplied by Chugai Pharmaceutical Co. (Japan). [$^{23}, 24\text{-}^3H$] $25-OHD_3$ (80 Ci per mmol) was obtained from Radiochemical Centre (Amersham, England).

Material

Plasma concentration of $1, 25-(OH)_2D$ was measured by a competitive protein binding assay in 30 normal subjects; 3 young subjects ranging from 10 to 16 years old, 8 adults from 20 to 35 years old and 19 aged subjects from 60 to 85 years old, and

27 cases with various diseases: 6 patients with primary hyperparathyroidism, 2 with post-operative hypoparathyroidism, 6 with thyrotoxicosis, 6 with chronic renal failure on hemodialysis, 5 with osteoporosis, 1 with osteomalacia and 1 with hypercalcemia due to bone metastasis.

Preparation of [23, 24-³H] 1, 25-(OH)₂D₃

Labeled 1, 25-(OH)₂D₃ was produced according to the method of Boyle *et al.* (1972). One-week-old White Leghorn chicks were raised for 2 weeks in a dark room on a low calcium and vitamin D-deficient diet (Ca: less than 10 mg per 100 g, P: 100 mg per 100 g, Oriental Yeast Co.) Then the chicks were sacrificed and 1 g of the kidney tissue was homogenized in 4 volumes of 0.5 M Tris-acetate. Three ml of the homogenate and 25 ng of [23, 24-³H] 25-OHD₃ were incubated with 1.5 ml of the buffer containing 75 mM sodium succinate, under 100% of O₂ for 30 min in duplicate. Lipids were extracted twice with 13.5 ml of a methanol-chloroform (2:1) mixture. The 1, 25-(OH)₂D₃ fraction was separated by Sephadex LH-20 column chromatography with a solvent of hexane and chloroform (35:65 v/v), and the purity was examined by high pressure liquid chromatography with a pressure of 70 kg/cm² in a solvent of 10% isopropanol in hexane, using a Zorbax SIL column (Shimadzu-Dupond).

Preparation of binding protein

A binding cytosol of 1, 25-(OH)₂D was prepared by the method of Eisman *et al.* (1976 a and b). Seven-day-old White Leghorn chicks were raised for 5 weeks in a dark room on a vitamin D-deficient diet with minimum calcium (less than 0.01%) and low phosphate (0.1%). The removed duodenal mucosa was minced in 5 volumes of phosphate buffer (0.05 M potassium phosphate, 0.05 M potassium chloride) and washed twice by centrifugation at 700 × g. Then the precipitate was homogenized and the 220,000 × g supernatant was lyophilized and stored under N₂ gas at -20°C. The binding ability of the binding protein decreased by about 10% over 3 months under these conditions. When it was used, the lyophilizate was resolved with the phosphate buffer to the concentration of 0.6 mg/ml.

Extraction of 1, 25-(OH)₂D from each plasma sample and its purification

Thirty ml of dichloromethane was added to 5 ml of a plasma sample containing 10 pg (about 1,500 dpm) of the labeled tracer. Lipids were extracted and 1, 25-(OH)₂D was isolated from the lipid extract by a chromatography on a Sephadex LH-20 column (0.7 × 9 cm) with a mixed solvent of hexane, chloroform and methanol (9:1:1) and by high pressure liquid chromatography on a silicic acid column (Zorbax SIL 0.21 × 25 cm) with a solution of 10%

isopropanol in hexane. The recovery of 1, 25-(OH)₂D₃ after plasma extraction and chromatography was determined as the recovery of the labeled 1, 25-(OH)₂D₃ initially added to each plasma sample and the assay results were corrected for loss of tritium.

Radioreceptor assay

Mixtures of 40 pg of labeled 1, 25-(OH)₂D₃ in 50 μl of absolute ethanol, various amounts of standard 1, 25-(OH)₂D₃ or sample in 50 μl of absolute ethanol, and 0.6 mg of cytosol protein in 1 ml of the buffer were incubated in polypropylene tubes at 25°C for 1 hr. One ml of ice cold 40% polyethylene glycol was added to each tube and centrifuged at 10,000 × g at 4°C for 1 hr. The bottom of the tubes were then transferred to vials with 10 ml of Bray's solution (counting efficiency 35%) and counted in a liquid scintillation counter (Packard, model 3385) with automatic external standardization.

Dilution of plasma

For the measurement of plasma 1, 25-(OH)₂D levels, a dilution curve of the plasma must be parallel to the standard curve. A plasma sample obtained from a patient on a high dose of 1α-hydroxyvitamin D₃ (1α-OHD₃) was diluted to 1/2 and 1/4 with normal pooled plasma to study the effect of dilution.

Cross reactivity of cytosol to other vitamin D₃ derivatives

Various amounts of 25-OHD₃, 24, 25-(OH)₂D₃ and D₃ were incubated with labeled 1, 25-(OH)₂D₃ tracer and a cytosol receptor to study the cross reactivity of the cytosol in relation to D₃ derivatives other than 1, 25-(OH)₂D₃.

Results

Labeled 1,25-(OH)₂D₃

Fig. 1 shows a chromatographic profile of the chloroform-methanol extract after kidney homogenates have been incubated with labeled 25-OHD₃. More than 70% of radioactivity was found in the region of 1,25-(OH)₂D and trace amounts of radioactivity were detected in the region of 24,25-(OH)₂D₃. The major radioactive fraction from Sephadex LH-20 column migrated exactly in the same position as synthetic 1,25-(OH)₂D₃ on co-chromatography on high pressure liquid chromatography (Fig. 2).

Recovery

Recovery of 1,25-(OH)₂D after plasma extraction and chromatography was found to be $74.9 \pm 16.4\%$ (mean $\pm$ SD, $n=61$).

Sensitivity studies

Fig. 3 shows a typical standard reference curve for 1,25-(OH)₂D₃. On the semilogarithmic scale, the ratio of bound tritiated 1,25-(OH)₂D₃ to the total decreased as a

straight line in the range of 6 to 100 pg of non radioactive 1,25-(OH)₂D₃ per tube. In this assay system, the maximum binding of the tracer to the binding cytosol when incubated without standard 1,25-(OH)₂D₃, was found to be $29.4 \pm 1.4\%$ (mean $\pm$ SD, $n=6$). The assay was sensitive to about 5 pg added to the test tube, of which the value was significantly different from the zero point of the standard curve.

Dilution curve

Diluted plasma from a patient treated with 1α -OHD₃ gave a parallel inhibition curve with standard curve as shown in Fig. 3. The results showed that with this cytosol receptor there was no detectable difference in sterol-receptor binding behavior between the endogenous 1,25-(OH)₂D and the synthetic 1,25-(OH)₂D₃.

Cross reactivity

Fig. 4 shows displacement curves for the natural metabolites of D₃. Cross reactivity of 25-OHD₃ with the binding protein was 0.16% at 50% displacement of the labeled 1,25-(OH)₂D₃, and that of 24,25-(OH)₂D₃ was 0.04%. No displace-

Fig. 1. A chromatographic profile on a Sephadex LH-20 column of lipid extracts of the kidney homogenate incubated with labeled 25-OHD₃ for 30 min.

Fig. 2. High pressure liquid chromatography of the major radioactive peak from the Sephadex LH-20 column. Before application to the column, 10 μ g of synthetic 1, 25-(OH)₂D₃ was added. The solid line shows radioactivity and the dotted line shows optical density at 254 nm.

Fig. 3. A representative standard curve (●) and a dilution curve of plasma from a patient with a high dose of 1α -OHD₃ (○). A plasma sample was diluted to 1/2 and 1/4. The ordinate represents B/T ratio in % of the tracer. The abscissa represents an increasing amount of standard 1, 25-(OH)₂D₃ added to the test tube.

ment of the labeled $1,25-(\text{OH})_2\text{D}_3$ occurred with D_3 even at 1,000 ng/tube.

Scatchard plot

Scatchard analysis of $1,25-(\text{OH})_2\text{D}_3$ binding to the receptor showed that the receptor possessed two or more different classes of binding sites as shown in Fig. 5. The dissociation constant for the higher affinity site was 5.3×10^{-11} M with a capacity of 0.1 pmol/mg at 25°C .

Plasma $1,25-(\text{OH})_2\text{D}$ levels

Plasma concentrations of $1,25-(\text{OH})_2\text{D}$ as measured by this method are shown in Fig. 6. In normal subjects, no significant difference of mean concentration was seen in different age groups. However, the range of distribution became greater in the aged group than in the younger, giving mean values of 29.0 ± 1.37 pg/ml (mean $\pm$ SD) for subjects ranging from 10 to 16 years old, 19.6 ± 8.28 pg/ml (mean $\pm$ SD) for the subjects 20 to 35 years old, and 17.3 ± 8.82 pg/ml (mean $\pm$ SD) for the subjects more than 60 years old. In primary hyperparathyroidism there were 4 cases with prominent bone resorption, in which plasma $1,25-(\text{OH})_2\text{D}$ concentrations were markedly high. In 2 other cases of renal stones without prominent bone resorption, the plasma levels were distributed within normal range. In four groups of the patients, one with hypoparathyroidism, one with chronic renal failure, one with osteomalacia and one with hypercalcemia due to bone metastasis, the concentrations of $1,25-(\text{OH})_2\text{D}$ appeared to be slightly lower than those among normal subjects. In most patients with hyperthyroidism or senile osteoporosis, plasma $1,25-(\text{OH})_2\text{D}$ concentrations were distributed within normal range.

Fig. 4. Displacement curves for the natural metabolites of D_3 . $[23, 24\text{-}^3\text{H}]$ $1, 25-(\text{OH})_2\text{D}_3$ (70 pg; 80 Ci/mmol) was incubated with an increasing amount of various nonradioactive sterol in the reconstituted cytosol-receptor system. The percentage of the bound tritiated tracer was plotted as a function of the amount of nonradioactive sterols in the incubation mixture. Each point represents the average of duplicate assays.

Fig. 5. Scatchard analysis. Small amounts of the cytosol (0.6 mg of protein per tube) were incubated with 70 pg of $[23, 24\text{-}^3\text{H}]$ $1, 25-(\text{OH})_2\text{D}_3$ in the presence of various amounts of the nonlabeled hormone for 1 hour at 25°C . The ordinate represents B/F ratio and the abscissa represents the amount of $1, 25-(\text{OH})_2\text{D}_3$ bound to the cytosol. A site with high affinity ($K_d = 5.3 \times 10^{-11}$ M) to $1, 25-(\text{OH})_2\text{D}_3$ was thought to be specific and a site with low affinity to be nonspecific.

Discussion

A radioreceptor assay for 1,25-(OH)₂D in human plasma was first developed by Brumbaugh *et al.* using a chromatin receptor from the rachitic chick small intestine (Brumbaugh *et al.*, 1974 a and b). Two years later, a simplified radioreceptor assay for 1,25-(OH)₂D in human plasma was developed by Eisman *et al.* (1976) using intestinal cytosol from rachitic chicks. The sensitivity of these assays was reported as 80 pg/tube and 10 pg/tube, respectively. The present study showed that the lower limit of the assay was 5 pg per tube, which was sensitive enough to measure normal plasma concentrations of 1,25-(OH)₂D. This assay system is more sensitive than that of previous studies. The high sensitivity was obtained by using the binding protein which had a higher affinity ($K_d = 5.3 \times 10^{-11}$ M) than that used by

Brumbaugh *et al.* ($K_d = 2.2 \times 10^{-9}$ M), and by using the labeled tracer with higher specific activity (80 Ci/mmol) than that used by Eisman *et al.* (9.3 Ci/mmol). Two different binding sites were observed in the cytosol receptor, and the high affinity site was thought to be specific (Brumbaugh and Haussler, 1974). Though the cytosol receptor was specific for 1,25-(OH)₂D₃, the labeled tracer was displaced by much higher concentrations of D₃ (more than 100,000 fold excess), 25-OHD₃ (95-fold excess), and 24,25-(OH)₂D₃ (1,750-fold excess). To eliminate possible interference by circulating metabolites of vitamin D, purification with high pressure liquid chromatography should be required prior to the assay. However, there still remains a possibility that 1,25-(OH)₂D₂ might be involved in this assay, since 1,25-(OH)₂D₃ cannot be separated from 1,25-(OH)₂D₂ by this method.

This study demonstrated that no difference was detected in the assay between

Fig. 6. Plasma concentration of 1,25-(OH)₂D in normal subjects and various diseases. PHP: Primary hyperparathyroidism, Hypo P: Post-operative hypoparathyroidism, HT: Hyperthyroidism, CRF: Chronic renal failure, SOP: Senile osteoporosis, OM: Osteomalacia, HC: Hypercalcemia due to bone metastasis.

the endogenous sterol and synthetic sterol upon dilution of plasma from a patient treated with a high dose of 1α -OHD₃.

The present results suggest that in most of the normal subjects plasma $1,25$ -(OH)₂D concentration was distributed from 7 to 33 pg/ml, which corresponded to previous reports by Brumbaugh *et al.* (39 ± 8 pg/ml, mean $\pm$ SEM, $n=6$), and Eisman *et al.* (29 ± 2 pg/ml, mean $\pm$ SEM, $n=20$). The sensitivity of this assay should make the method useful for both physiological and clinical studies. The present study showed markedly high $1,25$ -(OH)₂D levels in some cases of primary hyperparathyroidism with prominent bone resorption. Relatively low values were seen in some cases with chronic renal failure, senile osteoporosis, osteomalacia and hypercalcemia due to bone metastasis. At the present time, the exact implications of these findings are not known, but they can provide an important clue in clarifying the derangement of calcium metabolism.

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