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Fundamental Studies on Measurement of Plasma 1α,25-Dihydroxycholecalciferol Concentration by Radioreceptor Assay

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ABSTRACT. It is well known that 1α,25-dihydroxyvitamin D(1α,25 $(OH)_2D$) plays an important role in the pathophysiology of bone and calcium metabolism. In present paper, we established a relatively convenient assay system, using radioreceptor assay, for 1α,25$(OH)_2D$. Fundamental studies showed that this assay system had good sensitivity enough to detect 2 pg of 1α,25$(OH)_2D$ per tube, and had good reproducibility. Therefore, it was shown that this assay system could be applied for clinical use.

The plasma concentrations of 1α,25$(OH)_2D$, measured by this assay system, in healthy young males and females, and in aged females were 55.7±16.1, 44.8±22.1 and 23.5±13.0 pg/ml (mean±s.d.), respectively. Thus, the aged females showed significantly lower ($p<0.005$) 1α,25$(OH)_2D$ levels than the young group. This fact suggests that vitamin D deficient state might exist in aged females.

After 1α,25$(OH)_2D_3$ was identified as the final active form of vitamin D_3, evidences were being accumulated of its wide actions on bone and calcium metabolism, cell differentiation[1] and immuno-regulating systems,[2] as well. Now that the synthetic 1α,25$(OH)_2D_3$ and 1αOHD_3 are available for the treatment of metabolic bone diseases, precise measurement of blood levels of 1α,25$(OH)_2D_3$ is mandatory in managing these patients as well as in investigating the pathophysiology of the diseases. For the measurement of blood levels of 1α, 25$(OH)_2D$, several techniques such as radioreceptor assay (RRA), radioimmunoassay (RIA), and bioassay have been reported. However, because of 10s pg/ml of very low concentration and the complicated procedure of extraction and purification, those measurements have not got the popularity.

We have developed a sensitive, specific and relatively convenient RRA for plasma 1α,25$(OH)_2D$ in clinical use. We have performed the fundamental studies of this RRA, and measured plasma 1α,25$(OH)_2D$ concentration in normal adults by this method.

MATERIALS AND METHODS

Reagents

1α,25$(OH)_2D_3$, 24,25-dihydroxycholecalciferol (24,25$(OH)_2D_3$), 25-hydroxycholecalciferol (25OHD_3) and cholecalciferol (D_3) were gifted kindly from

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Chugai Pharm. Co. (Tokyo). 1α,25-dihydroxy [26,27-methyl-^{3}H] cholecalciferol (^{3}H-1α,25(OH)$_2$D$_3$) (130-180 Ci/mmol) was purchased from Amersham Int. plc (England). Chick embryo intestinal 1α,25(OH)$_2$D$_3$ receptor was supplied from Yamasa-shoyu Co. (Tokyo). All solvents used in chromatography were in purity of HPLC grade and all other reagents were in purity of reagent grade.

Preparation of vitamin D-deficient chick serum extract (DDCSE)

White leghorn cockerels at 1 week of age were fed with a vitamin D-deficient diet (Oriental Yeast Co.) for 3 weeks and then sacrificed. Their blood was collected, and the serum was separated by the centrifugation at 1,000 g for 15 min. Five-volume of absolute ethanol was added to the serum, and the tube was vigorously shaken and centrifuged at 3,000 g for 15 min. The supernatant was stored at -20°C overnight and thereafter centrifuged again. This supernatant was kept at -20°C as DDCSE.

Preparation of equivalent particle sized dextran-coated charcoal (eqDCC)

Fifteen grams of charcoal (Norit A, Sigma Chemical Co.) was suspended with 300 ml of 0.14 M phosphate buffer containing 0.15 M NaCl, 0.015 M NaN_3 and 0.1% gelatin (pH 7.0) (G-P buffer), and stirred overnight. The suspension was stood without stirring for 30 s. The supernatant was separated from the heavy particles at the bottom by decantation. After centrifuging at 60 g for 10 min, the light particles suspending in the supernatant were discarded and the pellet was re-suspended in 300 ml of G-P buffer. This procedure was repeated 5 times to make the particle size uniform. Then, to the suspension of the uniformly arranged charcoal particles was added 3 g of Dextran T-70 (Pharmacia Co.). The Dextran-charcoal suspension was stirred overnight and was centrifuged at 60 g for 10 min. The pellet was lyophilized and stored at -20°C. Prior to use, this eqDCC was suspended in G-P buffer at 3% (w/v) and centrifuged at 60 g for 10 min. The supernatant was removed, and the pellet was re-suspended with G-P buffer to adjust to 3% (w/v).

Assay procedure

Sample extraction and purification

The procedure of separation and purification of 1α,25(OH)$_2$D from plasma is shown schematically in Fig. 1. In order to quantitate the recovery rate of 1α,25(OH)$_2$D through the purification procedure, to each plasma sample (1 ml), 1,500 cpm (5 pg) of ^{3}H-1α,25(OH)$_2$D$_3$ in 50 μl of 75% ethanol was added. For the removal of protein and other impurities, and the extraction of lipid fraction, Chem Elut (Analytichem Int. Co.) was used. Immediately after the addition of 0.2 ml 0.1 M phosphate buffer (pH 8.6) to the column, 1 ml of plasma sample was applied. Then 2 ml of solvent (dichloromethane-aceton 9 : 1) was added, and column was kept standing for 1 h. The column was eluted with 13 ml of the same solvent, and then with another 10 ml. Both eluents were collected and evaporated together under N_2 gas stream. Next, the sample was subjected to HPLC on a CLC-SIL column (Shimazu) at a flow rate of 1.5 ml/min with an elution solvent of *n*-hexane-*iso*-propanol (9 : 1). Fractions between 18-20 min at the position of 1α,25(OH)$_2$D, as determined previously by standard 1α,25(OH)$_2$D$_3$ or ^{3}H-1α,25(OH)$_2$D$_3$, were collected, dried under N_2,

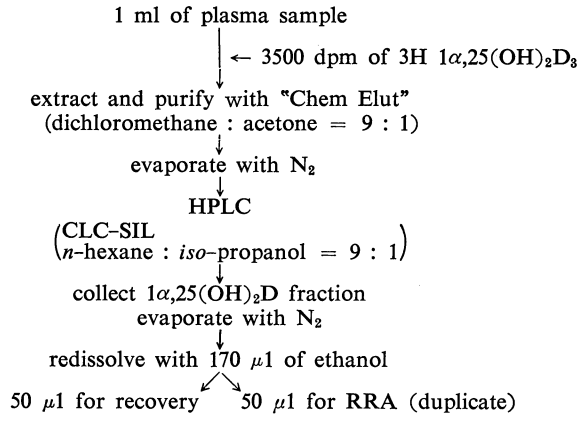

Fig. 1. Extraction and isolation method of 1α,25(OH)$_2$D from plasma.

and redissolved in 170 μl of absolute ethanol. Out of the 170 μl, 50 μl was used to check for the recovery rate of 1α,25(OH)$_2$D from plasma, and two 50 μls were used for RRA in duplicate.

Radioreceptor assay

The outline of RRA procedure is shown in Fig. 2. To a glass assay tube, about 15 pg of ^{3}H-1α,25(OH)$_2$D (c.a. 4,000 cpm) in 50 μl of ethanol, and standard 1α,25(OH)$_2$D$_3$ or sample in 50 μl of ethanol were added, dried under N$_2$ gas, and dissolved with 20 μl of DDCSE. The receptor was dissolved, immediately prior to use, in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose, 0.025 M KCl, 0.005 M MgCl$_2$, 0.001 M EDTA, 0.012 M thioglycerol and 0.01 M dithiothreitol. The solution of 200 μl containing 83 μg receptor was added to each tube, and the tube was mixed by Vortex and incubated for 6-8 h at 4°C. Thereafter, 200 μl of 3% eqDCC suspension was added, and

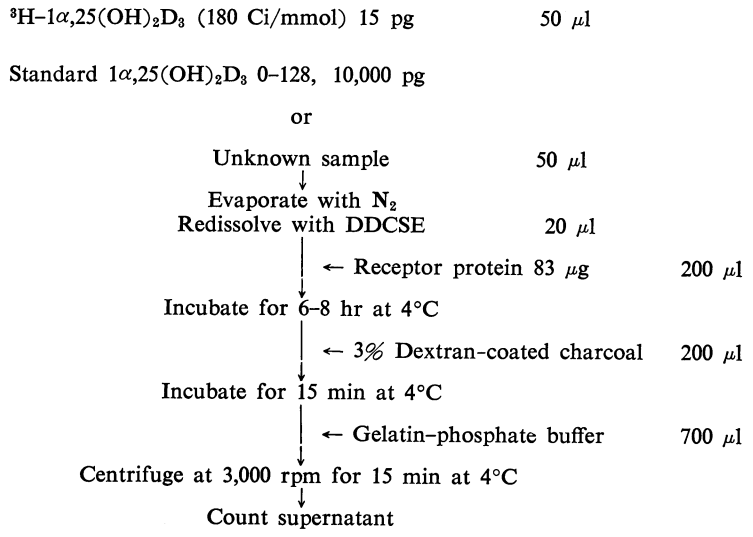

Fig. 2. Radioreceptor assay procedure for 1α,25(OH)$_2$D.

the mixture was incubated for 15 min at 4°C. Immediately after addition of 700 μl of G-P buffer to each tube, bound and free hormone was separated by a centrifugation at 3,000 g for 15 min at 4°C. The supernatant was decanted into a counting vial and counted by a liquid scintillation counter to obtain the bound radioactivity (B). After counting total radioactivity (T), B/T (%) of each sample was calculated. A standard curve for $1\alpha,25(OH)_2D$ was made, and the $1\alpha,25(OH)_2D$ value in the sample was read from the curve. This value was corrected by recovery rate and plasma $1\alpha,25(OH)_2D$ concentration was obtained.

Fundamental studies

Extraction rate of $1\alpha,25(OH)_2D$ from plasma

Extraction rate of $1\alpha,25(OH)_2D$ from sample plasma was examined on Chem Elut. Recovered counts of ^3H-$1\alpha,25(OH)_2D_3$ within the first 13 ml of eluent and those within both the first 13 ml and the second 10 ml of eluent were compared.

Recovery rate of $1\alpha,25(OH)_2D$ through extraction and purification

The recovery rate of $1\alpha,25(OH)_2D$ from plasma through whole extraction and purification procedure, employing with Chem Elut and HPLC, was estimated with ^3H-$1\alpha,25(OH)_2D_3$ added to the plasma sample.

Determination of the minimal detection limit of RRA

The minimal detection limit of this assay system for $1\alpha,25(OH)_2D$ was settled to be the value which showed significantly different (mean-2s.d.) B/T (%) from that of 0 concentration on the standard curve.

Specificity

The specificity of RRA for $1\alpha,25(OH)_2D$ was estimated by the crossreactivity of vitamin D derivatives to the receptor used in this assay system. Various amounts of $25OHD_3$ (50 pg-12.8 ng), $24,25(OH)_2D_3$ (200 pg-51.2 ng) and D_3 (3.9-500 ng) were added to assay tube, instead of standard $1\alpha,25(OH)_2D_3$, and the RRA was carried out. A standard curve for each derivative was made, and the concentration which showed 50% inhibition of specific binding of ^3H-$1\alpha,25(OH)_2D_3$ was read and compared to that of $1\alpha,25(OH)_2D_3$.

Reproducibility

Intra- and inter-assay variation estimated as CV (%) was calculated from multiple measurements of pooled serum ; 7 times for same assay and 4 times for different assay, respectively.

Analytical recovery

To the 1 ml of plasma with known concentration of $1\alpha,25(OH)_2D$, 32, 64 or 128 pg of $1\alpha,25(OH)_2D_3$ was added and $1\alpha,25(OH)_2D$ concentration in each sample was measured by this RRA. Recovery rate (%) was obtained from comparison of measured and theoretical value.

Dilution test

The sample, which $1\alpha,25(OH)_2D$ concentration was previously determined to be 69.1±4.0 pg/ml (mean±s.d.), was diluted to two volumes with ethanol and the effect of dilution was estimated.

Measurement of plasma concentration of $1\alpha,25(OH)_2D$ in normal human subjects

Using this assay system, the plasma $1\alpha,25(OH)_2D$ concentration was measured in 10 normal young males (23.2±3.0 yrs.), 9 normal young females (22.4±2.2 yrs.) and 24 aged females (80.3±4.4 yrs.). Plasma $1\alpha,25(OH)_2D$ concentration in each group was compared and evaluated.

RESULTS

Fundamental studies of RRA

Recovery of $1\alpha,25(OH)_2D$ from plasma through extraction and purification

$1\alpha,25(OH)_2D$ recovery rate, indicated by counts of ^3H-$1\alpha,25(OH)_2D_3$, was 71.9±10.2% for single extraction with 13 ml of the solvent and 84.2±2.0% for the double extraction with total 23 ml of the solvent (N=4), respectively. Therefore, we employed double extraction procedure as a routine extraction method. The recovery of $1\alpha,25(OH)_2D$ through whole extraction and purification including HPLC was 74.3±6.3% (N=20).

Sensitivity

Fig. 3 shows a representative standard curve of this RRA. The minimal

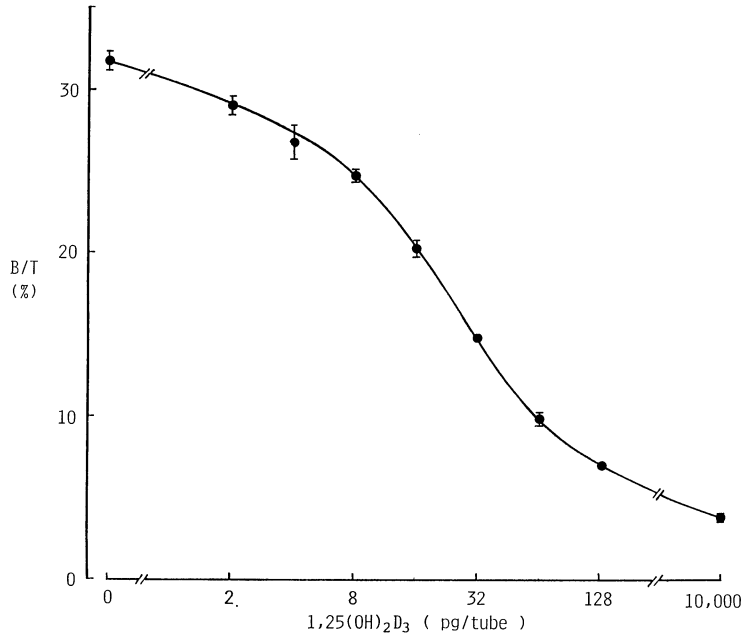

Fig. 3. Representative standard curve of radioreceptor assay for $1\alpha,25(OH)_2D$.

detection value was settled to be 2 pg/tube of which B/T (%) (28.8±1.2%) was significantly differerent from that of 0 pg/tube (31.7±1.3%). Therefore, 2–128 pg/tube of $1\alpha,25(OH)_2D$ could be measurable by this RRA.

Specificity of RRA

The concentrations of vitamin D derivatives which showed 50% inhibition for the specific binding of ^3H-$1\alpha,25(OH)_2D_3$ to the receptor were presented in Table 1. $25OHD_3$, showing the highest crossreactivity to $1\alpha,25(OH)_2D_3$ receptor, needed 430 pg/tube for 50% inhibition, while $1\alpha,25(OH)_2D_3$ did 22 pg/tube. The crossreactivities of other vitamin D derivatives were relatively lower than that of $25OHD_3$.

TABLE 1. The concentration of vitamin D derivatives showing 50% binding inhibition of ^3H-$1\alpha,25(OH)_2D_3$ to the receptor.

Vitamin D derivative	50% Binding inhibition
$1\alpha,25(OH)_2D_3$	22 pg/tube
$25OHD_3$	430
$24,25(OH)_2D_3$	2,000
D_3	56,000

Reproducibility

The reproducibility of this RRA was shown in Table 2. The intra- and inter-assay variation was 9.3% (N=7) and 6.6% (N=4), respectively.

TABLE 2. Reproducibility study in radioreceptor assay for $1\alpha,25(OH)_2D$.

	Mean ± S.D.	CV
Intraassay	41.7±3.9 pg/ml (N=7)	9.3 %
Interassay	44.6±3.0 pg/ml (N=4)	6.6 %

Analytical recovery

As shown in Table 3, added $1\alpha,25(OH)_2D_3$ was recovered within the range between 80% and 110%.

TABLE 3. Analytical recovery of $1\alpha,25(OH)_2D$ from plasma.

	Added $1\alpha,25(OH)_2D_3$			
	0	32	64	128 pg/ml
Measured value	49.0	64.6	117.3	196.8
Theoretical value		81.0	113.0	177.0
Recovery		79.8	104	111 %

Dilution test

After double dilution of the plasma sample of 69.1±4.0 pg/ml of $1\alpha,25(OH)_2D$ concentration, the concentration was determined as 32.2±4.9 pg/ml.

Blood concentration of 1α,25(OH)$_2$D in normal subjects

The plasma concentration of 1α,25(OH)$_2$D measured by this assay system was shown in Fig. 4. The mean plasma concentration was 55.7±16.1 pg/ml (N=10) for the normal young male, 44.8±22.1 pg/ml (N=9) for the normal young female and 23.5±13.0 pg/ml (N=24) for the aged female, respectively. There was no significant difference between the young male and female. However, the values in aged female were significantly lower than those in young female ($p<0.005$).

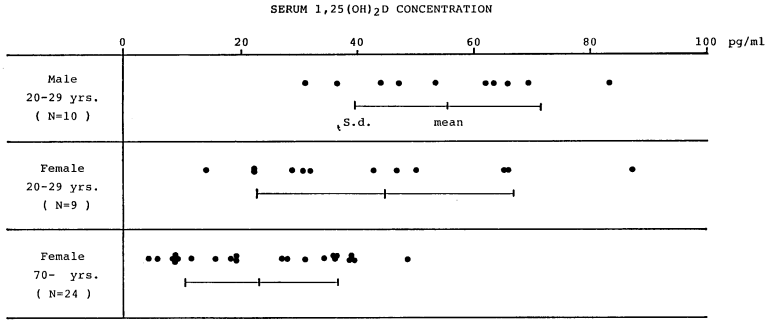

Fig. 4. Plasma concentration of 1α,25(OH)$_2$D in normal young male and female, and aged female.

DISCUSSION

For elucidating the mechanisms of diseases with derangement of bone and calcium metabolism, measurement of plasma 1α,25(OH)$_2$D concentration is essential. But plasma concentration of 1α,25(OH)$_2$D is very low, while other vitamin D derivatives circulate in relatively high concentrations. Furthermore, 1α,25(OH)$_2$D$_3$ receptors, obtained from chick embryo, vitamin D deficient chick or calf thymus,[3] and antibodies against 1α,25(OH)$_2$D$_3$[4] are all known to have some crossreactivity to other vitamin D derivatives. Therefore, the isolation and purification of 1α,25(OH)$_2$D from plasma is indispensable in assay system using these binding proteins, and the isolation and purification make the measurement troublesome. At first, the isolation and purification procedure including extraction with organic solvent, silicic acid, Sephadex LH-20 and Celite column chromatography was reported.[5] Then, more simplified method consisting of extraction with organic solvent, Sephadex LH-20 and HPLC was presented.[6] To be more convenient, the extraction and purification were done with a first column alone and a disposable mini-column was used.[7] Recently, a method without HPLC was reported.[8] With the non-HPLC method, however, it is known that non-specific high value might be measured in some particular cases, and the validity for that method has not been established. Cytoreceptor assay, based on the selective internalization and binding of 1α,25(OH)$_2$D to its receptor inside the cultured target cells, was also reported.[9] However, at present, RRA is most widely being carried out among various assay methods for 1α,25(OH)$_2$D, and the purification with HPLC seems to be most reliable. In this paper, we developed relatively convenient RRA for 1α,25(OH)$_2$D. Our RRA has such characteristics as follows ; using HPLC, to be

simplified purification procedure, using disposable mini-column for extraction, and being able to treat more numbers of plasma samples per one assay. Furthermore, fundamental studies showed that this assay system had good sensitivity, reproducibility, recovery and dilution test, and this assay was suggested that it could be applied to clinical use.

Many reports have been made on $1\alpha,25(OH)_2D$ concentration in normal plasma.[7,9,10] According to them, normal concentration is supposed to range from 20 pg/ml to 70 pg/ml. By using our RRA, we found that $1\alpha,25(OH)_2D$ concentration in plasma was 55.7 ± 16.1 pg/ml for normal young male and 44.8 ± 22.1 pg/ml for normal young female. On the other hand, aged females showed lower $1\alpha,25(OH)_2D$ levels than youngs. It is well known that relation between osteoporosis and $1\alpha,25(OH)_2D$ level has been noticed.[11–13] As well-known fact, hydroxylation of 25OHD at 1α-position occurs in kidney. It is indicated that the decreased activity of 1α-hydroxylase accompanied with aging might induce low $1\alpha,25(OH)_2D$ plasma concentration. Furthermore, the deficiency of sex hormone, especially estrogen, is also known to reduce the production of 1α-hydroxylase.[13] The decreased $1\alpha,25(OH)_2D$ plasma concentration in aged female is supposed to reflect the changes of these states.[11] Anyway, the decreased plasma concentration of $1\alpha,25(OH)_2D$ in aged female is interesting, and it suggests that vitamin D deficient state might exist in aged female.

Our RRA system for plasma $1\alpha,25(OH)_2D$ is reliable and sensitive, and expected to be a useful tool for elucidating the mechanisms of various diseases with abnormal bone and calcium metabolism.

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