Rapid Communication

Effects of Age and Diseases on Human Serum 25-Hydroxycholecalciferol Determined by Competitive Protein-Binding Assay*

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Synopsis

Competitive protein-binding assay of 25-OH-D was developed by the use of specific vitamin D-binding proteins from vitamin D-deficient rat serum. Ether extract of serum sample which was dried and dissolved in ethanol, or standard solution of 25-OH-D₃, was incubated with ³H-25-OH-D₃ and vitamin D-binding protein for 2 hours at 4°C. Free and bound ³H-25-OH-D₃ were separated through dextran-coated charcoal. The sensitivity of the assay system was 0.22 ng/tube. Percent cross reaction in the assay was 2.18% in vitamin D₃, 0.70% in 1, 25-(OH)₂-D₃, less than 0.28% in 1 α -OH-D₃, and less than 0.06% in dihydrotachysterol, cholesterol and cortisol.

Human serum 25-OH-D is 28.9 ± 2.9 ng/ml in 19 normal subjects. Serum 25-OH-D in the old-age group (50-70 years of age) was significantly decreased, compared with that in the young-age group (20-40 years of age). Serum 25-OH-D was significantly decreased in gastrectomized and osteoporotic patients as well as in the patients with liver cirrhosis, in comparison with their age-controls.

In the recent years, it has been established that 25-hydroxylation of vitamin D₃ is performed in liver to produce 25-hydroxycholecalciferol (25-OH-D₃) and 1-hydroxylation (Fraser and Kodicek, 1970) or 24-hydroxylation of 25-OH-D₃ is performed in kidneys to produce 1, 25-dihydroxycholecalciferol (1, 25-(OH)₂-D₃) or 24, 25-dihydroxycholecalciferol (24, 25-(OH)₂-D₃), and that there exists feedback mechanism between these two hydroxylation reactions in the kidney (Tanaka and DeLuca, 1974). On the other hand, the mechanism of the actions of vitamin D or its metabolites upon calcium absorption from the intestinal tract, bone mobilization and the homeostasis of blood calcium level are being further clarified.

The methods for measuring 25-OH-D by competitive protein-binding assay were first reported by Belsey *et al.* (1971, 1974) and Haddad *et al.* (1971). We also have established competitive protein-binding assay of 25-OH-D in order to elucidate the effects of age and various diseases on the metabolism of vitamin D or its metabolites.

Materials and Methods

In order to produce vitamin D-binding proteins, male Holtzman weanling rats were fed with vitamin D deficient food (Suda *et al.*, 1970) for 3-4 weeks, and blood was collected by cutting carotid vein under ether anesthesia. The obtained serum was 500 times, 1,000 times, 2,000 times, or 4,000 times diluted with 0.04 M barbital-acetate-buffer (pH 8.6) and 1 ml of each diluted serum was incubated with 50 μ l of ethanol containing 10,000 cpm of ³H-25-OH-D₃.

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(Amersham Searle) for 2 hours at 4°C for the determination of optimal concentration of binding protein. Ether was used for the extraction of 25-OH-D₃ from the serum (recovery rate was more than 90%). A half ml of the ether extract of human blood sample was evaporated to dryness and the residue was dissolved in ethanol, a portion of which was employed for assay, while another portion was used for the measurement of the recovery of sample.

To the 50 μ l ethanol-dissolved standard or sample, were added the same volume of 10,000 cpm [³H]-25-OH-D₃ and 1 ml of 1,000 times-diluted vitamin D-deficient rat serum, and the mixture was incubated for 2 hours at 4°C. After adding 0.25 ml of dextran-coated charcoal to each tube for separation of bound and free tracer, we left it standing for about 10 min at 4°C followed by centrifugation at 3,000 rpm at 4°C for 15 min. The radioactivity of 500 μ l of the supernatant was determined by a liquid scintillation counter (Fig. 1).

Human blood samples collected mainly in Tokyo University Hospital during the period between April and August, 1975 were used for the assay.

Fig. 1. Outline of the assay method of 25-OH-D₃ by competitive protein-binding assay.

Results

I. Dilution curves of vitamin D-binding protein

Fig. 2 represents dilution curves of vitamin D-binding protein. The maximum binding percent of ³H-25-OH-D₃ was 53% in 500 times diluted serum, 49% in 1,000 times diluted serum, 14% in 2,000 times diluted serum and 5% in 4,000 times diluted serum. The most sensitive curve was obtained when 1,000 times diluted vitamin D deficient rat serum was used.

Fig. 2. Dilution curves of vitamin D binding protein. Vitamin D-deficient rat serum was 500 times, 1,000 times, 2,000 times or 4,000 times diluted with 0.04 M barbital acetate buffer, respectively. Ordinate represents bound % of ³H-25-OH-D₃ (B/T × 100) to binding protein. Abscissa represents an increasing amount of standard 25-OH-D₃ added to the test tube.

II. 25-OH-D₃ Standard curve

Fig. 3 represents a standard curve of 25-OH-D₃ when 1,000 times-diluted solution of vitamin D deficient rat serum was used. Ten percent fall from 0 standard was 0.22 ng/tube and the measurable range was between 0.22–10 ng/tube.

III. Cross reaction of various substances

Percent cross reaction (i.e. $X/Y \cdot 100$, where X is the weight of 25-OH-D₃ and Y the weight of the interfering steroid required to produce 50% inhibition of binding of ³H-25-OH-D₃) in the assay was 2.18% in vitamin D₃, 0.70% in 1, 25-(OH)₂-D₃, less than 0.28% in 1 α -OH-D₃, and less than 0.06% in dihydrotachysterol, cholesterol and cortisol (Fig. 3).

IV. Serum 25-OH-D level in normal subjects and gastrectomized patients

Table 1 shows the serum 25-OH-D values of 19 normal subjects and 15 gastrectomized patients. In the old-age group (50–70 years of age) in the normal subjects, their serum 25-OH-D level was significantly decreased compared to that of the younger-

age group (20–49 years of age). The average value for the total of 19 normal subjects was 28.9 ± 2.9 ng/ml.

In gastrectomized patients, the average value for the 20–49 years of age group was significantly decreased compared to that of age matched normal subjects, while in the group of 50–70 years of age there was no significant difference from the same age group in the normal subjects. The average value for the total of 15 gastrectomized patients was 18.5 ± 2.5 ng/ml, which was significantly decreased compared to that of the normal subjects.

Table 1. Effects of Age and Gastrectomy on Human Serum 25-OH-D

Group	Age	Number	Serum 25-OH-D (ng/ml)
Normal subject	20–49	9	$36.9 \pm 4.0^*$
	50–70	10	$23.1 \pm 3.1^{**}$
	Total	19	$28.9 \pm 2.9^{***}$
Gastrectomized patient	20–49	3	$18.4 \pm 1.7^\dagger$
	50–70	12	18.6 ± 2.9
	Total	15	$18.5 \pm 2.5^{\dagger\dagger}$

Mean $\pm$ S.E.

* vs. ** $P < 0.02$

* vs. $\dagger$ $P < 0.01$

*** vs. $\dagger\dagger$ $P < 0.02$

Fig. 3. Standard curves for 25-OH-D₃, vitamin D₃, 1, 25-(OH)₂-D₃ and 1 α -OH-D₃ in the competitive protein-binding assay system.

V. Serum 25-OH-D level in various diseases

As shown in Fig. 4, the average value of serum 25-OH-D was 22.9 ± 5.0 ng/ml for 5 uremic patients, 11.5 ± 2.0 ng/ml for 5 liver-cirrhotic patients, and 14.7 ± 1.6 ng/ml for 9 osteoporotic patients (all of the osteoporotic patients were between 50–70 years of age), respectively. The average of 25-OH-D value for 19 healthy persons was 28.9 ± 2.9 ng/ml (the average of 10 subjects of 50–70 years of age was 23.1 ± 3.1 ng/ml). There was a significant decrease of serum 25-OH-D level in cirrhotic patients and in osteoporotic patients compared to their age-normal controls ($P < 0.01$ and $P < 0.05$, respectively).

Discussion

In the recent years, 25-hydroxylation of vitamin D₃ in liver to produce 25-OH-D₃ and its 1-hydroxylation (Fraser and Kodicek, 1970) or 24-hydroxylation in kidneys to produce 1,25-(OH)₂-D₃ or 24, 25-(OH)₂-D₃ respectively have been established. Preliminary observations are consistent with the view that the conversion of 25-OH-D₃ to either of these products is regulated by a second control mechanism and is modulated by circulating parathyroid hormone, calcitonin and inorganic phosphate levels: increased parathyroid hormone or hypophosphatemia stimulates the production of 1,25-(OH)₂-D₃ and increased calcitonin or hyperphosphatemia stimulates the production of 24,25-(OH)₂-D₃ (Avioli and Haddad, 1973; Fraser and Kodicek, 1973). The determination of serum 25-OH-D is still important means for the understanding of vitamin D metabolism, because the determination of 1,25-(OH)₂-D₃ is still not possible except for only a few places (Brumbaugh *et al.*, 1974). We modified the competitive protein binding assay method of 25-OH-D reported by

Fig. 4. Human serum 25-OH-D in normal subjects and in various diseases.

Belsey *et al.* (1974) and applied the method to the clinical study to elucidate the effects of age and various diseases on human serum 25-OH-D levels.

It is known that after gastrectomy, the frequency of the occurrence of osteomalacia and osteoporosis is increased. In addition to the report that the absorption of calcium from the intestinal tract and the clavicular cortical thickness were decreased in gastrectomized patients (Fujita *et al.*, 1971), our result that serum 25-OH-D value was decreased in gastrectomized patients, supports a possibility that the disorder of vitamin D metabolism as well as the decrease of calcium absorption might be involved in gastrectomy, causing various disorders of bone metabolism. One of the causes for the decrease of serum 25-OH-D level in gastrectomized patients, among others, would be the decreased absorption of vitamin D from the intestine due unappropriate timing of gastric hormone secretion as well as bile secretion.

Likewise, for the causes of the decrease in serum 25-OH-D in the higher-age group, the deterioration of vitamin D absorption

from the intestinal tract as well as insufficient intake of vitamin D might be considered the main factors.

The decrease in serum 25-OH-D level in osteoporotic patients in this study, is compatible with the recent conception that deficiency in vitamin D intake is one of the factors to cause osteoporosis (Rasmussen, 1974). On the other hand, the coexistence of osteoporosis and osteomalacia in the aged was reported (Mizuno *et al.*, 1974), and the role of vitamin D in the pathogenesis of osteoporosis still remained to be further elucidated.

Bayard *et al.* (1973) reported the decrease of serum 25-OH-D in uremic patients, while Shen *et al.* (1975) reported the increase of serum 25-OH-D in the patients having uremic osteodystrophy. Although we have confirmed in our experiment no significant changes of serum 25-OH-D₃ in uremic patients, it is well considerable that nutritive conditions and intake rates of vitamin D in uremic patients have greatly influenced serum 25-OH-D level. However, this problem still remains to be further elucidated.

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