Age and Seasonal Variations in the Serum Levels of 25-Hydroxyvitamin D and 24,25-Dihydroxyvitamin D in Normal Humans

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Abstract

Serum concentrations of 25-hydroxyvitamin D (25-OH-D) and 24, 25-dihydroxyvitamin D [24,25-(OH)2-D] were measured in lipid extracts of 1 ml of human sera by a competitive protein binding assay. 25-OH-D and 24, 25-(OH)2-D were isolated from the lipid extracts by Sephadex LH-20 column chromatography. The 24, 25-(OH)2-D fraction on the Sephadex LH-20 columns were further purified by high pressure liquid chromatography prior to assay. Serum levels of 25-OH-D and 24, 25-(OH)2-D in normal males were similar to those in normal females throughout each age group. Serum levels of 25-OH-D and 24, 25-(OH)2-D in newborns were 8.28±1.68 ng/ml (mean±SD) and 0.55±0.16 ng/ml, respectively, which were significantly (p<0.001) lower than those in adults (21.3±4.8 ng/ml of 25-OH-D and 1.55±0.31 ng/ml of 24, 25-(OH)2-D). A seasonal variation was demonstrated in serum levels of 24, 25-(OH)2-D as well as those of 25-OH-D. Serum levels of 24, 25-(OH)2-D were highly correlated (r=0.884, p<0.001) with those of 25-OH-D in normal human subjects, and the percentage ratio of 24, 25-(OH)2-D/25-OH-D was 7.4±1.4% (mean±SD), irrespective of age or seasonal variations.

It is now well established that vitamin D3 is first metabolized to 25-hydroxyvitamin D$_3$ (25-OH-D$_3$) in the liver, and subsequently in the kidney to 1α, 25-dihydroxyvitamin D$_3$ [1α, 25-(OH)$_2$-D$_3$], a biologically active metabolite, or to 24,25-dihydroxyvitamin D$_3$ [24,25-(OH)$_2$-D$_3$] (DeLuca, 1976). Recently, Henry and Norman (1978) reported that 24,25-(OH)$_2$-D$_3$ is essential, along with 1α, 25-(OH)$_2$-D$_3$ for chicken egg hatchability. The biologic significance of 24,25-(OH)$_2$-D$_3$ in man, however, is still unknown.

The percentage ratio of 24, 25-(OH)$_2$-D/25-OH-D in sera of healthy adults has been reported by several investigators (Taylor et al., 1976; Haddad et al., 1977; Graham et al., 1977; Kremer and Guillemant, 1978), but the reported ratio values are variable, ranging from 5 to 57%. The difference appears mainly due to differences in the degree of purification of the 24,25-(OH)$_2$-D fraction.

Recently, Horst et al., (1979a) and our group (Kano et al., 1979a) independently revealed that the determination of 24,25-(OH)$_2$-D has to be further purified by high pressure liquid chromatography (HPLC) after Sephadex LH-20 column chromatography prior to competitive protein binding assay (CPBA). We now report the results obtained with this newly developed method on the serum levels of 25-OH-D and 24, 25-(OH)$_2$-D in normal human subjects.
The study was carried out with particular attention to sex, age and seasonal variations.

Materials and Methods

Chemicals

Crystalline 25-OH-D₃ was purchased from Philips-Duphar Co., the Netherlands. Crystalline 24R, 25-(OH)₂-D₃ was kindly donated by Dr. M. R. Uskokovic, Hoffman-LaRoche Inc., Nutley, New Jersey. [26, 27-³H]-25-OH-D₃ (9.0Ci/mmol) and [23, 24-³H]-25-OH-D₃ (110Ci/mmol) were purchased from Radiochemical Centre, Amersham, England. [26, 27-³H]-24, 25-(OH)₂-D₃ was synthesized biologically by incubating kidney homogenates from chicks with radioactive 25-OH-D₃, as previously reported (Takasaki et al., 1978).

Subjects

Serum concentrations of 25-OH-D and 24, 25-(OH)₂-D were determined in sera collected in June, 1978, from 88 normal Japanese: 12 term newborns ranging from 3 to 7 days old, 12 infants from 3 to 12 months old, 16 preschool children, 16 early school children, 16 adolescents, and 16 adults. Each age group consisted of the same number of males and females. The seasonal variation was examined serially in 6 healthy early school children every 3 months from February to December, 1978 (Group I: February—March, Group II: May—June, Group III: August—September, Group IV: November—December).

Assays for 25-OH-D and 24, 25-(OH)₂-D

One ml of sera was extracted with methanol-chloroform (2:1, v/v) according to the method of Bligh and Dyer (1959). The extracts were applied to Sephadex LH-20 microcolumns (0.75×14 cm) which were eluted with a mixed solvent of chloroform and n-hexane (55:45, v/v). The 25-OH-D fraction was collected from 5 to 11.5 ml by Sephadex LH-20 column chromatography. The 24, 25-(OH)₂-D fraction (11.5-27 ml) of the Sephadex column was further applied to HPLC (Waters, Model 204) equipped with a Zorbax-Sil column (4.6 mm×15 cm) which was eluted with 2.5% methanol in dichloromethane at a flow rate of 0.9 ml/min (Kano et al., 1979a). The 24, 25-(OH)₂-D fraction on HPLC was collected from 7 to 10.5 min. The assays for 25-OH-D and 24, 25-(OH)₂-D were performed by CPBA using plasma from rats fed a low Ca, vitamin D-deficient diet (Suda et al., 1970) for 3 weeks. The details of the CPBA method have been described elsewhere (Kano et al., 1979a). The sensitivity of the assay for 24, 25-(OH)₂-D was increased to 12.5 pg/tube by using [23, 24-³H]-25-OH-D₃ with a specific activity of 110 Ci/mmol (Kano et al., 1979a). To compensate for recovery during extraction and chromatography, 2000 dpm of [³H]-25-OH-D₃ and [³H]-24, 25-(OH)₂-D₃ were added to each sample prior to extraction. The mean recovery of [³H]-25-OH-D₃ was 92.5±7.3 (SD)% and that of [³H]-24, 25-(OH)₂-D₃ was 65.4±5.5 (SD)%. Results were evaluated by Student's t test and a p value less than 0.05 was taken as significant.

Results

Purification of 25-OH-D and 24,25-(OH)₂-D:

Purification by HPLC of the 25-OH-D fraction on the Sephadex column did not appear necessary. When the 25-OH-D fraction of the Sephadex column was applied to HPLC, some unknown materials other than 25-OH-D₃ were detected by CPBA. The peak just prior to [³H]-25-OH-D₃ detected by CPBA was considered to be 25-OH-D₂ (Kano et al., 1979b) (Fig. 1A). Similar unknown materials were detected by CPBA in the extracts of 1 ml distilled water (Fig. 1B). The levels of the unknown materials, however, were much lower than those of 25-OH-D (Fig. 1). The 25-OH-D levels determined by CPBA after the Sephadex column were highly correlated with those after HPLC (r=0.998, p<0.001. The former mean value was 106.3±4.2 (SD)% of the latter one. In addition, the intra-assay variance and the inter-assay variance were lower in the former (4.4% and 7.3%) than in the latter (10.9% and 12.5%).

It was necessary, on the other hand, to further purify the 24,25-(OH)₂-D fraction of the Sephadex column by HPLC before the CPBA determination could be made. At least four compounds (Peaks I, II, III and V) other than 24,25-(OH)₂-D₃ exhibiting some binding activity could be eliminated by HPLC (Fig. 2A). A peak similar to peak I has been reported by Horst et al., (1979a and b). However, peak I was also found when 1 ml of distilled
water was extracted and purified on the Sephadex column and HPLC in the same manner as described in the MATERIALS AND METHODS (Fig. 2B (a)). Peak I was also detected by CPBA when the same volume of the distilled solvent which was run through the Sephadex column was applied to HPLC and the peak height was almost the same as that shown in Fig. 2B (a). The level of peak I, on the other hand, was considerably reduced when the same volume of the solvent used for the elution on the Sephadex column was directly applied to HPLC (Fig. 2B (b)). Peak I was not found when the sample prepared from 1 ml of distilled water instead of serum was directly applied to HPLC (data not shown). Peak I was therefore considered to be mainly derived from contaminants of Sephadex LH-20. Peak II seemed to be a mixture of 25-OH-D$_3$ and an metabolite of 24,25-(OH)$_2$-D$_3$ (peak C) recently reported by Takasaki et al. (1978) because these 2 radioactive metabolites comigrated to exactly the same position on HPLC when the column was eluted with 2.5% methanol in
Table 1. Serum levels of 25-OH-D and 24, 25-(OH)₂-D in normal human subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>N</th>
<th>25-OH-D (ng/ml)</th>
<th>24, 25-(OH)₂-D (ng/ml)</th>
<th>24, 25-(OH)₂-D/25-OH-D (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Term newborns¹</td>
<td>M</td>
<td>6</td>
<td>8.5±1.2*</td>
<td>0.56±0.20*</td>
<td>6.60±1.61</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6</td>
<td>8.1±2.2*</td>
<td>0.53±0.12*</td>
<td>6.70±0.97</td>
</tr>
<tr>
<td>Infants²</td>
<td>M</td>
<td>6</td>
<td>21.1±4.7</td>
<td>1.30±0.22</td>
<td>6.33±1.40</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6</td>
<td>19.4±3.3</td>
<td>1.33±0.36</td>
<td>6.87±1.47</td>
</tr>
<tr>
<td>Preschool children³</td>
<td>M</td>
<td>8</td>
<td>23.9±4.6</td>
<td>1.86±0.30</td>
<td>7.94±1.38</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>8</td>
<td>22.2±2.4</td>
<td>1.72±0.32</td>
<td>7.78±1.30</td>
</tr>
<tr>
<td>Early school children⁴</td>
<td>M</td>
<td>8</td>
<td>20.9±4.4</td>
<td>1.58±0.30</td>
<td>7.73±1.44</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>8</td>
<td>21.0±5.3</td>
<td>1.56±0.48</td>
<td>7.33±1.21</td>
</tr>
<tr>
<td>Adolescents⁵</td>
<td>M</td>
<td>8</td>
<td>21.8±5.3</td>
<td>1.55±0.50</td>
<td>7.10±1.31</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>8</td>
<td>18.2±3.1</td>
<td>1.39±0.27</td>
<td>7.94±1.38</td>
</tr>
<tr>
<td>Young adults⁶</td>
<td>M</td>
<td>8</td>
<td>21.6±5.4</td>
<td>1.63±0.31</td>
<td>7.71±1.30</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>8</td>
<td>21.0±4.6</td>
<td>1.47±0.29</td>
<td>7.20±1.71</td>
</tr>
</tbody>
</table>

Sera were collected from normal Japanese in June, 1978. Data represent mean±SD. *significant (p<0.001) to the “young adults”

¹: 3-7 days old; ²: 3-12 months old; ³: 1-5 years old; ⁴: boys 6-11 years old and girls 6-9 years old; ⁵: boys 12-15 years old and girls 10-15 years old; ⁶: 20-29 years old.

dichloromethane. These 2 metabolites were clearly separated when HPLC was eluted with 7.5% isopropanol in n-hexane (data not shown). Peak III was considered to be 24,25-(OH)₂-D₂, since the levels for peak III rose markedly in rats given large amounts of vitamin D₂ (Kano et al., 1979b). Peak IV comigrated to exactly the same position as authentic 24R,25-(OH)₂-D₃. Peak V is an unknown metabolite.

Serum levels of 25-OH-D and 24, 25-(OH)₂-D

There was no significant difference between males and females of the respective age groups in the serum levels of 25-OH-D and 24,25-(OH)₂-D (Table 1). The mean serum levels of 25-OH-D (8.28±1.68 ng/ml) and 24,25-(OH)₂-D (0.55±0.16 ng/ml) in newborns were significantly (p<0.001) lower than those in adults (21.3±4.8 ng/ml and 1.55±0.31 ng/ml). The serum levels of 25-OH-D and 24,25-(OH)₂-D in infants, preschool children, early school children, and adolescents were almost identical to those in adults (Table 1).

Figure 3 illustrates seasonal changes in the serum levels of 25-OH-D and 24,25-(OH)₂-D serially determined in 6 healthy early school children. The mean serum levels of 25-OH-D were 19.3±5.4 ng/ml in group I, 22.7±5.9 ng/ml in group II, 30.7±6.2 ng/ml in group III, and 23.0±5.3 ng/ml in group IV. The mean serum levels of 24,25-(OH)₂-D were 1.41±0.33 ng/ml in group I, 1.65±0.46 ng/ml in group II, 2.38±0.68 ng/ml in group III, and 1.62±0.32 ng/ml in group IV. These results indicate a rise of serum 25-OH-D and 24,25-(OH)₂-D levels during the summer time and a fall during the winter time. The serum levels of 25-OH-D and 24,25-(OH)₂-D were significantly (p<0.05) higher in the subjects after sea bathing (40.4±3.4 ng/ml in 25-OH-D and 3.26±0.40 ng/ml in 24,25-(OH)₂-D) than those in group III, respectively. The serum levels of 24,25-(OH)₂-D were highly correlated with those of 25-OH-D in normal subjects (r=0.884, p<0.001) (Fig. 4). The percentage ratios of 24,25-(OH)₂-D/25-OH-D were almost identical (7.4±1.4%) in all of the age and seasonal variations.
Discussion

The assay procedure described here offers a reasonably fast, sensitive, accurate and reproducible method for 25-OH-D and 24,25-(OH)₂-D.

HPLC allows the complete resolution of all vitamin D metabolites derived from vitamin D₂ and D₃. In addition, the ultra-violet absorption analysis on HPLC offers the distinct advantage of the elimination of CPBA and its associated problems. The ultra-violet analysis on HPLC, however, is less sensitive (lower detection limit; 5 ng) (Horst et al. 1979b). This is a big disadvantage, especially in the pediatric field. Rat serum protein binding analysis, on the other hand, is much more sensitive. The sensitivity of the assay was increased to 12.5 pg per tube by using [23, 24-³H]-25-OH-D₃ with the specific activity of 110 Ci/mmol (Kano et al., 1979a).

Rat serum protein, however, bound to some unknown materials other than 25-OH-D and 24,25-(OH)₂-D. It was therefore, necessary to further purify the 24,25-(OH)₂-D fraction on the Sephadex column by HPLC before CPBA could be made. There were at least four compounds other than 24,25-(OH)₂-D exhibiting some binding activity in the 24,25-(OH)₂-D fraction of the Sephadex column. The levels of 24, 25-(OH)₂-D after HPLC were approximately one third of the values detected by CPBA immediately after purification on the Sephadex column (Fig. 2). Much higher values in serum 24,25-(OH)₂-D have been reported by several investigators (Taylor et al., 1976; Weisman et al., 1977; Kremer and Guillemant, 1978), who did not use HPLC for purification of the 24,25-(OH)₂-D fraction.

Purification by HPLC of the 25-OH-D fraction on the Sephadex column, on the other hand, did not appear to be necessary.
The 25-OH-D fraction on the Sephadex column consisted mainly of 25-OH-D₃ and the metabolite is suspected to be 25-OH-D₂. Measurements of serum 25-OH-D levels were therefore performed by CPBA immediately after purification on the Sephadex column. The elimination of HPLC offers an advantage in determining total levels of 25-OH-D rapidly.

Our results on the age variation of the 24,25-(OH)₂-D levels are consistent with those reported by Weisman et al. (1977). They reported that serum levels of 24, 25-(OH)₂-D in 20 children ranging from 3-18 years of age were significantly greater than those in newborns. Weisman et al. (1977), however, measured serum levels of 24,25-(OH)₂-D by CPBA immediately after purification on Sephadex LH-20 column chromatography. In addition, they collected serum samples randomly throughout the year.

Data on the seasonal change in the serum 24,25-(OH)₂-D levels have not been reported. The present study clearly indicates that there is a significant seasonal variation in the serum 24,25-(OH)₂-D levels as well as in the 25-OH-D levels. The latter has been reported by Haddad et al. (1974), McLauglin et al. (1974) and Stryd et al. (1979). These findings are important in evaluating serum levels of 25-OH-D and 24,25-(OH)₂-D in patients with abnormal vitamin D metabolism.

Our previous animal study (Kano et al. 1979b) revealed that plasma levels of 24, 25-(OH)₂-D₃ were highly correlated with those of 25-OH-D₃ in rats given graded doses of vitamin D₃. The present human study indicates that there is a similar linear relationship between serum levels of 25-OH-D and those of 24,25-(OH)₂-D in normal human subjects. The percentage ratio of 24,25-(OH)₂-D/25-OH-D appeared to be regulated in human subjects, irrespective of age or seasonal variations. The percentage ratio was approximately 30% in rats but only 7.4% in normal human subjects. The cause(s) of the difference in the ratio between species is not known. However, it may be useful to determine the percentage ratio of 24,25-(OH)₂-D/25-OH-D in evaluating pathogenesis of disturbances in calcium metabolism in patients with hepatic, renal or some endocrine diseases. We have actually observed some changes in this ratio in these diseases and the results will be published in a succeeding paper.

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**References**


