Gross and Separative Determination of 1α,25-Dihydroxyvitamin D₂ and D₃ in Plasma Using Calf Thymus Receptor

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Summary Determination of 1α,25-dihydroxyvitamin D [1,25(OH)₂D, gross amounts of 1,25(OH)₂D₂ and 1,25(OH)₂D₃] and separative determination of 1,25(OH)₂D₂ and 1,25(OH)₂D₃ in plasma using calf thymus receptor have been investigated. A lipid extract from 1 ml of plasma is applied to a Bond Elut C₁₈OH column and an eluate corresponding to 1,25(OH)₂D including both 1,25(OH)₂D₂ and 1,25(OH)₂D₃ is applied to calf thymus receptor to assay a gross amount of the two compounds. On the other hand, when separative assay of the two compounds is performed, the 1,25(OH)₂D₂ eluate obtained from the Bond Elut C₁₈OH column is further applied to HPLC using a Zorbax SIL column with 5% isopropanol in methylene chloride as a developing solvent to separate the two compounds from one another. The separated eluates are independently applied to the receptor to assay the two compounds. Since less amounts of unknown components non-specifically bound to interfering concomitants besides 1,25(OH)₂D exist in the calf thymus receptor, complicated purification steps to eliminate the concomitants are unnecessary. The detection limit by this method is 1.25 pg/tube which is sensitive enough for a routine method to assay 1,25(OH)₂D in plasma.

Key Words vitamin D, 1,25-dihydroxyvitamin D, 1,25-dihydroxyvitamin D₂, 1,25-dihydroxyvitamin D₃, radioreceptor assay, calf thymus receptor

Vitamin D (vitamin D is used as a general name for vitamins D₂ and D₃ and the same nomenclature system is also applied to the metabolites) is metabolized to 25-hydroxyvitamin D (25-OH-D) in the liver and subsequently to 1α,25-dihydroxyvitamin D (1,25(OH)₂D) or 24R,25-dihydroxyvitamin D (24,25(OH)₂D) in the kidney (1,2). 1,25(OH)₂D is known as an active form of vitamin D which promotes intestinal calcium absorption and remodeling in bone to keep calcium homostasis. Recent investigations have revealed that 1,25(OH)₂D has the activity

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in suppressing proliferation and inducing cell differentiation besides the calcemic activity (3, 4). The finding has accelerated clinical uses of 1,25(OH)\(_2\)D\(_3\) and 1α-hydroxyvitamin D\(_3\) (1α-OH-D\(_3\)) as a prodrug of 1,25(OH)\(_2\)D\(_3\) to leukemia, psoriasis, skin cancer, and other diseases. According to the development of clinical uses, establishment of a simplified and precise method for determination of 1,25-(OH)\(_2\)D in plasma has been required for clinical studies. However, since plasma levels of 1,25(OH)\(_2\)D are extremely low (normal range: 20–80 pg/ml), it is very difficult to establish a simplified and precise method. Since high-performance liquid chromatography (HPLC) using an ultraviolet (UV) detector and gas-liquid chromatography/mass spectrometry (GC-MS) cannot be applied to the determination of 1,25(OH)\(_2\)D in plasma because of their low sensitivity (5), radioreceptor assay (RRA) is now most popular for the purpose (6–13). Cytosol-chromatin receptors (6–13) have been used for RRA. Recently, Reinhardt et al. (14) isolated a receptor from calf thymus showing high affinity to 1,25(OH)\(_2\)D and established a method for the determination using the receptor (15). Hollis (16) also reported a non-HPLC method for determination of 1,25(OH)\(_2\)D HPLC method for determination of 1,25(OH)\(_2\)D using C\(_{18}\)OH column and the receptor. Since calf thymus receptor is now commercially available, we have studied gross and separative determination of 1,25(OH)\(_2\)D\(_2\) and 1,25(OH)\(_2\)D\(_3\) in plasma using the receptor.

**EXPERIMENTAL**

**Apparatus.** Vac Elut SPS-24 (Analyticalchem International Co., USA) was used as a sample processing station for use with Bond Elut C\(_{18}\)OH cartridge column (C\(_{18}\)OH column). HPLC was performed with a high-performance liquid chromatograph (LC6A, Shimadzu Co., Kyoto, Japan) equipped with an ultraviolet (UV) detector (SPD-6A, Shimadzu Co.). Radioactivities were measured by a liquid scintillation counter (LSC-700, Aloka Co., Tokyo, Japan).

**Compounds and reagents.** Commercially available crystalline 25-OH-D\(_3\), 24,25(OH)\(_2\)D\(_3\), and 1,25(OH)\(_2\)D\(_3\) (Solvay Duphar Co., The Netherlands) were used. 1,25(OH)\(_2\)D\(_2\) was kindly donated by Prof. DeLuca, University of Wisconsin-Madison (USA). [23,24 (\(n\)-\(^3\)H)-25-OH-D\(_3\) (specific activity, 3.3 TBq/mmol), [23,24 (\(n\)-\(^3\)H)-24,25 (OH)\(_2\)D\(_3\) (specific activity, 2.3 TBq/mmol), and [26,27-methyl-\(^3\)H]-1,25(OH)\(_2\)D\(_3\) (specific activity, 6.7 TBq/mmol) were purchased from Amersham Co. (UK). A commercially available assay kit including calf thymus and a Bond Elut C\(_{18}\)OH column of Nichols Co. (Switzerland) was kindly donated by Nihon Medi-Physics Co. (Agent of Nichols Co. in Japan, Tokyo). Commercially available chick embryonal intestine receptor of Yamasa Shoyu Co. (Tokyo, Japan) was used. Commercially available liquid scintillation cocktail, Opti-Fluor (Packard Co., USA) was used. Organic solvents of analytical grades were distilled before use. Other guaranteed reagents were used.

**Procedure for determination of 1,25(OH)\(_2\)D (gross amounts of 1,25(OH)\(_2\)D\(_2\) and 1,25 (OH)\(_2\)D\(_3\)) in plasma.** Determination of 1,25(OH)\(_2\)D (gross amounts of
1,25(OH)₂D₂ and 1,25(OH)₂D₃ in plasma is performed by using a commercially available assay kit including a C₁₈OH column and calf thymus receptor (Nichols Co., Switzerland) without separating 1,25(OH)₂D₂ and 1,25(OH)₂D₃. Exactly 1.0 ml of a plasma sample is placed in a glass tube and 1,25(OH)₂D is assayed according to the procedure reported by Hollis (16).

Procedure for separative determination of 1,25(OH)₂D₂ and 1,25(OH)₂D₃ in plasma. The 1,25(OH)₂D fraction containing both 1,25(OH)₂D₂ and 1,25(OH)₂D₃ obtained by the above C₁₈OH column chromatography is evaporated to dryness under reduced pressure. The resulting residue is dissolved in 200μl of 5% (v/v) isopropanol in methylene chloride and applied to the following HPLC to separate 1,25(OH)₂D₂ and 1,25(OH)₂D₃.

Column: Zorbax SIL (4.6×250mm); mobile phase: 5% (v/v) isopropanol in methylene chloride; flow rate: 1.1ml/min (40 kg/cm²); collection of 1,25(OH)₂D₂ and 1,25(OH)₂D₃ fractions: 1,060–1,220 drops (retention time, 10.6–12.2 min) for 1,25(OH)₂D₂ and 1,250–1,410 drops (retention time, 12.4–14.2 min) for 1,25(OH)₂D₃.

The respective 1,25(OH)₂D₂ and 1,25(OH)₂D₃ fractions obtained above are applied to the procedure using calf thymus receptor described in the kit of Nichols Co. The concentrations are calculated by the following formula.

\[
\text{Concentrations of 1,25(OH)₂D₂ or 1,25(OH)₂D₃} = \frac{A}{V} \times \frac{0.2}{0.05} \times \frac{1}{R} \times 100
\]

A: a value of 1,25(OH)₂D₂ or 1,25(OH)₂D₃ (pg/tube) obtained from a calibration curve described by a standard compound of 1,25(OH)₂D₃. R: recovery (%). V = volume of a plasma sample. A recovery value obtained by [³H]-1,25(OH)₂D₃ is also applied to determination of 1,25(OH)₂D₂.

RESULTS

1. Determination of 1,25(OH)₂D (gross amounts of 1,25(OH)₂D₂ and 1,25(OH)₂D₃) in plasma

1) Comparison between the calf thymus and chick embryonal intestinal receptors. Figure 1 shows an elution profile of standard compounds of [³H]-vitamin D₃ metabolites from the C₁₈OH column. [³H]-1,25(OH)₂D₃ was eluted in the eluate of 5% (v/v) isopropanol in n-hexane with good separation from the other metabolites. Figure 2 shows calibration curves of 1,25(OH)₂D₂ and 1,25(OH)₂D₃ for calf thymus receptor. Since there was no significant difference between the two, a calibration curve described by a standard compound of 1,25(OH)₂D₃ can be also applied to the determination of 1,25(OH)₂D₂. Detection limit and 50% displacement of bound tracer (Bₒₐ) obtained by the calibration curve were 1.25 and 8 pg/tube, respectively. An averaged value of overall recovery by the procedure was 81.7±4.6% (M±SEM) and intra- and inter-assay coefficient of variation (CV)
Fig. 1. Elution profiles of 25-OH-D₃, 24,25(OH)₂D₃, and 1,25(OH)₂D₃ from a C₁₈OH column. Each 5,000 dpm of [³H]-25-OH-D₃, [³H]-24,25(OH)₂D₃, and [³H]-1,25(OH)₂D₃ in 50 µl of ethanol was mixed with each 1 ml of water, acetonitril and 0.4 M K₂HPO₄ buffer solution (pH 10.5). Whole volume of the mixed solution was applied onto a C₁₈OH column and successively eluted by each 5 ml of water, 70 (v/v)% methanol in water, 10 (v/v)% methylene chloride in n-hexane, 1 (v/v)% isopropanol in n-hexane and 5 (v/v)% isopropanol in n-hexane. Each 1 ml of the eluate was fractionated and the radioactivities were measured.

Fig. 2. Calibration curve of 1,25(OH)₂D₂ and 1,25(OH)₂D₃ for calf thymus receptor.
Fig. 3. Profiles of C180H column chromatography and HPLC of a plasma sample containing [3H]-1,25(OH)2D3. A plasma sample containing 2,000 dpm of [3H]-1,25(OH)2D3 was applied to the proposed procedure described in the text and a profile of C18OH column chromatography was described by radioactivity and receptor binding affinity with calf thymus receptor. The 1,25(OH)2D fraction eluted from the column was further applied to HPLC using a Zorbax SIL column and a profile was described by UV absorption at 265 nm, radioactivity and binding affinity with calf thymus receptor.

Values 11.7 and 8.1%, respectively.

A liquid extract from a plasma sample was applied to a C18OH column and the 1,25(OH)2D fraction was further applied to HPLC using a Zorbax SIL column with 5% isopropanol in methylene chloride. Figure 3 shows the chromatographic profiles described by radioactivity, receptor binding, and UV absorption. Although the two peaks due to receptor binding were observed in the profiles of C18OH column chromatography, no receptor binding other than 1,25(OH)2D was observed in the profile of HPLC. The assayed values (29.8 pg/ml) of 1,25(OH)2D in the 1,25(OH)2D fraction of C18OH column chromatography was close to that after application to the HPLC (33.3 pg/ml). These results suggest that existing interfering substances in plasma can be effectively eliminated by the C18OH column chromatography alone and no further purification by HPLC is necessary for assaying 1,25(OH)2D.

Figure 4 shows comparison of chick embryonal intestinal (9) and calf thymus receptors. A lipid extract from a plasma sample was purified by the three steps of HPLC described in our previous report (13) and applied to both chick intestinal and calf thymus receptors. The assayed values of 1,25(OH)2D by the two methods were 49.6 and 59.8 pg/ml, respectively, which were close to one another. On the
Fig. 4. Comparison of values in a plasma sample assayed by RRA using either chick intestinal or calf thymus receptors. A plasma sample containing 2,000 dpm of [3H]-1,25(OH)2D3 was applied to the procedure described in a previous paper (13) and the 1,25(OH)2D fraction purified by application to the 3 steps of HPLC was applied to RRA using either chick intestinal or calf thymus receptor to determine concentrations of 1,25(OH)2D (upper panel). In contrast, the same plasma sample was applied to the procedure described in the text and the 1,25(OH)2D fraction eluted from a C18OH column was successively applied to the 3 steps of HPLC described in a previous paper (13). Each 1,25(OH)2D fraction obtained by each step was applied to RRA using either chick intestinal or calf thymus receptor (lower panel).

Other hand, when the same lipid extract was purified by the C18OH column alone and the eluate corresponding to 1,25(OH)2D was directly applied to either chick intestinal or calf thymus receptor, the assayed values were 114.2 and 52.5 pg/ml, respectively—quite different from one another. When the eluates were successively applied to the three steps of HPLC and each 1,25(OH)2D fraction was applied to RRA using either chick intestinal or calf thymus receptor, the high value by chick intestinal receptor decreased after applying to the 2nd steps of HPLC while the values by the calf thymus receptor were not changed after applying to the 3 steps of HPLC as shown in Fig. 4. The results suggest that the amounts of unknown components non-specifically bound to interfering concomitants besides 1,25(OH)2D in the calf thymus receptor were smaller than those in the chick intestinal receptor. Therefore, a complete clean-up procedure using at least two steps of HPLC (or a Sep-Pak silica cartridge and one step of HPLC) is necessary to eliminate interfering substances in the assay using chick intestinal receptor.
Fig. 5. Profiles of 3 steps of HPLC of a plasma sample containing [3H]-1,25(OH)2D3.
A plasma sample containing 2,000dpm of [3H]-1,25(OH)2D3 was applied to the proposed procedure described in the text and the 1,25 (OH)2D fraction eluted from a C18OH column was successively applied to the 3 steps of HPLC described in a previous paper (13). Each profile at each step was described by UV absorption at 265 nm, radioactivity and binding affinity with chick intestinal receptor.

whereas a simple clean-up procedure using a C18OH column alone is enough to perform the assay of 1,25(OH)2D by calf thymus receptor because of its specificity to 1,25(OH)2D. In order to clarify the properties of interfering concomitants non-specifically bound to the chick intestinal receptor, a lipid extract from a plasma sample was applied to the C18OH column and the 1,25(OH)2D fraction eluted from a C18OH column was successively applied to the 3 steps of HPLC. Figure 5 shows the chromatographic profiles described by radioactivity and receptor binding. Many peaks due to receptor binding were observed in the profiles of the 1st step of HPLC and these included both less and more polar than 1,25(OH)2D. The profile of the 2nd step of HPLC still showed the existence of both less and more polar interfering substances than 1,25(OH)2D. The profile of the 3rd steps of HPLC showed a single peak corresponding to 1,25(OH)2D and those of the interfering substances were eliminated from the 1,25(OH)2D fraction. The results suggest that the 1,25(OH)2D fraction obtained from the 1st step of HPLC still contained interfering substance to chick intestinal receptor which could be separated from 1,25(OH)2D by the 2nd step of HPLC, because the profile of the 3rd step of HPLC gave a receptor binding peak corresponding to 1,25(OH)2D alone.

2) Comparison between the respective assayed values of 1,25(OH)2D in plasma
Table 1. Comparison between the respective assayed values of 1,25(OH)₂D in plasma obtained by the proposed method without and with applying one step of HPLC.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Assayed value of 1,25(OH)₂D (pg/ml)</th>
<th>Ratio (a/b) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without HPLC (a)</td>
<td>With HPLC (b)</td>
</tr>
<tr>
<td>1</td>
<td>114.8</td>
<td>104.2</td>
</tr>
<tr>
<td>2</td>
<td>161.6</td>
<td>163.0</td>
</tr>
<tr>
<td>3</td>
<td>182.0</td>
<td>169.6</td>
</tr>
<tr>
<td>4</td>
<td>180.0</td>
<td>177.0</td>
</tr>
</tbody>
</table>

¹ Plasma samples were obtained from patients with leukemia orally administered 3–12 μg/day of 1α-OH-D₃. ² Condition of HPLC: column, Zorbax SIL (4.6 × 250 mm); mobile phase, 5% isopropanol/methylene dichloride; flow rate, 1.1 ml/min.

using calf thymus with and without applying HPLC. We chose four plasma samples obtained from patients with leukemia receiving daily oral administration of 3–12 μg of 1α-OH-D₃. Lipid extracts of the plasma samples were applied to the C₁₈OH column and the 1,25(OH)₂D fractions obtained from the column were assayed by applying to RRA using calf thymus receptor. In order to check the reliability of the assayed values obtained by the proposed method, we compared the respective assayed values of 1,25(OH)₂D in the plasma of patients with and without applying HPLC using Zorbax SIL as a column and 5% isopropanol in n-hexane as a mobile phase. As shown in Table 1, the respective assayed values were close to each other which were satisfactory.

3) Assayed values of 1,25(OH)₂D in plasma. Plasma samples obtained from humans (normal subjects and patients with diabetes mellitus, hypoparathyroidism, primary hyperparathyroidism, and osteoporosis) and rats (normal and vitamin D-deficient rats) were applied to the proposed method to determine concentrations of 1,25(OH)₂D. As shown in Fig. 6, satisfactory results were obtained.

4) Correlation between the assayed values obtained by the proposed method and a previously reported method. Figure 7 shows the correlation between the assayed values obtained by the presently proposed method using calf thymus receptor and the previously reported method using chick intestinal receptor with three steps of HPLC (13). A significant correlation was observed between the two (p < 0.01).

2. Separative determination of 1,25(OH)₂D₂ and 1,25(OH)₂D₃ in plasma

1) Chromatographic profiles of 1,25(OH)₂D₂ and 1,25(OH)₂D₃. Figure 8a shows an elution profile of standard compounds of 1,25(OH)₂D₂ and 1,25(OH)₂D₃ by the C₁₈OH column. Since the two compounds were eluted in the same fraction, the eluate was successively applied to HPLC to separate the both compounds. Figure 8b shows an HPLC profile of standard compounds of 1,25(OH)₂D₂ and 1,25(OH)₂D₃. Since the two compounds were clearly separated from one another,
Fig. 6. Concentrations of 1,25(OH)₂D in human and rat plasma assayed by the presently proposed method. Plasma samples obtained from humans (normal subjects and patients with diabetes mellitus, hypoparathyroidism, primary hyperparathyroidism, and osteoporosis) and rats (normal and vitamin D-deficient rats) were applied to the presently proposed method to determine concentrations of 1,25(OH)₂D.

Fig. 7. Correlation between the values assayed by the presently proposed and previously reported methods.

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Fig. 8. Profiles of C18OH column chromatography and HPLC of 1,25(OH)2D2 and 1,25(OH)2D3. Standard compounds of 1,25(OH)2D2 and 1,25(OH)2D3 were applied to the C18OH chromatography (a) and HPLC (b) according to the procedures described in the text.

Table 2. Comparison between the values of 1,25(OH)2D2 and 1,25(OH)2D3 assayed by the presently proposed (calf thymus receptor) and previously reported (chick intestinal receptor) methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Assayed value (pg/ml)</th>
<th>1,25(OH)2D2</th>
<th>1,25(OH)2D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proposed method</td>
<td>18.5±4.6 (n = 12)</td>
<td>43.8±3.6 (n = 12)</td>
<td></td>
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<tr>
<td>Previous method</td>
<td>21.9±5.7 (n = 12)</td>
<td>36.3±3.9 (n = 12)</td>
<td></td>
</tr>
</tbody>
</table>

$r$  
$P$  
$Y$  

Date are shown as $M±SE$. Numbers in parentheses mean those of plasma samples.

separative assay of the both compounds can be achieved by applying each separated eluate to RRA using calf thymus receptor. When plasma samples were applied to the proposed method with addition of 50 pg of standard compounds of either 1,25(OH)2D2 or 1,25 (OH)2 D3, averaged analytical recoveries were 95.8 and 113.4% for 1,25(OH)2D2 and 1,25(OH)2D3, respectively, which were satisfactory.

2) Comparison between the values of 1,25(OH)2D2 and 1,25(OH)2D3 assayed by the presented proposed and previously reported methods. Table 2 shows comparison between the values of 1,25(OH)2D2 and 1,25(OH)2D3 assayed by the presently proposed method using calf thymus receptor and a previously reported method using chick intestinal receptor (13). Significant correlations were observed between the respective values.
DISCUSSION

In the last decade, a number of assay methods for 1,25(OH)$_2$D in plasma has been reported by using biological procedures, i.e., bioassay (17), RRA (6–16), and radioimmunoassay (RIA) (18, 19). These methods have shown great advantages in sensitivity and specificity. Although physicochemical methods using GC-MS (5) are very attractive, these have not yet been developed as routine methods in biological fluids. RIA has been developed by using antisera raised against 3- or 25-hemisuccinate of 1,25(OH)$_2$D$_3$ (18, 19), but a good antibody with high sensitivity has not been obtained. RRA has been developed by many research groups (6–16) and it is most popular as a routine method for determination of 1,25(OH)$_2$D in plasma because of its high sensitivity. In a previous paper (13), we have established a method for determination of 1,25(OH)$_2$D using chick embryonal intestinal receptor. However, since the receptor contains significant amounts of unknown components non-specifically bound to interfering concomitants besides 1,25(OH)$_2$D, two or three clean-up steps using HPLC are required to avoid an unexpected abnormal value due to interfering concomitants. Reinhardt et al. (15) isolated 1,25(OH)$_2$D receptor from calf thymus and established an assay method for 1,25(OH)$_2$D using the receptor with Silica Sep-Pak and C$_{18}$ Silica cartridges for clean-up without use of HPLC. Hollis (16) revised the method to a simplified one by using the C$_{18}$OH column which can be used as reversed solid-phase extraction and subsequent normal phase purification. Since the RRA method has been simplified by use of C$_{18}$OH column in the purification step, a kit with combined use of the column and calf thymus receptor has been commercially available and we have checked the kit to establish simplified methods for gross and separative determination of 1,25(OH)$_2$D$_2$ and 1,25(OH)$_2$D$_3$ in plasma.

As shown in Fig. 4, when a lipid extract obtained by a plasma sample was applied to chick embryonal intestinal and calf thymus receptors after purification by the C$_{18}$OH column to compare the receptors with one another, an abnormally high value was obtained by the chick intestinal receptor but not by the calf thymus receptor. The high value by the former diminished by stepwisely applying the eluate to HPLC. The results suggest that the amounts of unknown components non-specifically bound to interfering concomitants besides 1,25(OH)$_2$D in the calf thymus receptor are smaller than those in the chick intestinal receptor. Kubo et al. (20) also observed that chick embryonal intestinal receptor contained large amounts of such interfering components which could be eliminated by a purification step using precipitation with ammonium sulfate solution. The results suggested that low specificity observed in the chick embryonal intestinal receptor might be due to existence of unknown protein impurities. They also checked specificity of calf thymus receptor by reaction with 1,25(OH)$_2$D fraction of a serum sample obtained through a C$_{18}$OH column and found that existence of impurities was much lower than in chick embryonal intestinal receptor. However, non-specific
binding components were still observed in the fractions other than 1,25(OH)₂D though these were not so large, while our used commercial kit in this study showed no other binding reaction than 1,25(OH)₂D fraction as shown in Fig. 3. The results suggest that our used receptor is more pure than that used by Kubo et al. (20).

A simple clean-up procedure using only a C₁₈OH column is sufficient to perform the assay of 1,25(OH)₂D by calf thymus receptor. This is a big merit for determination of 1,25(OH)₂D, because omitting HPLC is not only time-saving but also gives the possibility for simultaneous assay of many samples due to use of cheap open columns. As shown in Fig. 7, there is a significant correlation between the values obtained by the presently proposed method using calf thymus receptor and the previous method using chick intestinal receptor with three steps of HPLC (13). These results suggest that the proposed method using the calf thymus receptor can be widely available as a routine one for determination of 1,25(OH)₂D in plasma.

There are two forms of vitamin D, namely D₂ and D₃, which are structurally different in the side chains, but they are similarly metabolized into 1,25(OH)₂D₂ and 1,25(OH)₂D₃ via 25-OH-D₂ and 25-OH-D₃ to show physiologically similar activity in mammals including humans (1, 2). Since vitamin D₂ is not biosynthesized in the body (21), all of the D₂ compounds detected in the body are derived from exogenous sources, for instance, multivitamin preparations, enriched foods and Shiitake mushroom. Hartwell et al. (22) orally administered 4,000 IU/day of vitamin D₂ to healthy premenopausal women for 8 weeks and observed the changes in plasma levels of 1,25(OH)₂D₂ and 1,25(OH)₂D₃. The plasma levels of 1,25(OH)₂D₂ increased (from an undetected level to 31 pg/ml) with decrease of 1,25(OH)₂D₃ levels (from 42 to 19 pg/ml) to keep a constant level of 1,25(OH)₂D (from 42 to 50 pg/ml). We also observed that oral administration of 400 IU/day of vitamin D₂ to healthy male subjects for 44 weeks increased the plasma levels of 1,25(OH)₂D₂ (from an undetected level to 29.8 pg/ml) with keeping gross levels of 1,25(OH)₂D within the normal range (23). These results suggest that strict feedback regulation of 1α-hydroxylation occurs from 25-OH-D to 1,25(OH)₂D in the kidney without distinguishing the D₂ and D₃ compounds. These results are very interesting in the field of vitamin D₂ and D₃ studies. Therefore, establishment of a simplified method for separative determination of 1,25(OH)₂D₂ and 1,25(OH)₂D₃ plasma is very important to perform the studies in the fields of vitamin D₂ and its analogs, for instance, 25-OH-D₂, 1,25 (OH)₂ D₂, and 1α-OH-D₂. When a 1,25(OH)₂D fraction obtained from the C₁₈OH column chromatography in the proposed method described above was applied to the HPLC, 1,25(OH)₂D₂ and 1,25(OH)₂D₃ were clearly separated from one another as shown in Fig. 8a. The both compounds were satisfactorily determined by applying each separated fraction to RRA using calf thymus receptor. As shown in Fig. 2, 1,25(OH)₂D₂ gave a calibration curve nearly the same as that of 1,25(OH)₂D₃ and the result suggests that a calibration curve described by a standard compound of 1,25(OH)₂D₂ is also available for determination of 1,25(OH)₂D₂. This is very convenient, because a standard compound of 1,25(OH)₂D₃ is easy to get in commercial markets while a
standard compound of 1,25(OH)_2D_3 should be obtained by chemical synthesis or in vitro formation using chick or rat kidney homogenates.

In conclusion, we have established sensitive and simplified methods for gross and separative determination of 1,25(OH)_2D_2 and 1,25(OH)_2D_3 in plasma using calf thymus receptor. These are useful as routine methods for determination of gross amounts of 1,25(OH)_2D_2 and 1,25(OH)_2D_3 and separative determination of the two compounds. These will contribute to development of clinical studies in future.

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