ISOLATION AND PURIFICATION OF IN VIVO-GENERATED 25-HYDROXYVITAMIN D2 BY PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY1

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Summary In order to obtain a standard compound of 25-hydroxyvitamin D2 (25-OH-D2), a method for isolating in vivo-generated 25-OH-D2 from the blood of rats or rabbits was established by using several steps of preparative high-performance liquid chromatography (HPLC). When the unsaponifiable matter of the plasma obtained from rats or rabbits receiving a large dose of vitamin D2 was applied to the preparative HPLC using a Zorbax SIL column, a peak denoted as peak X was observed on the chromatogram. Since the peak X was thought to be due to 25-OH-D2 from the experiments of time course and dose-response, it was purified by subjecting it to successive preparative HPLC using several kinds of columns. From the results of ultraviolet (UV) absorption spectrum, gas chromatography-mass spectrometry (GC-MS) and mass chromatography, the purified peak X compound was confirmed to be 25-OH-D2. The proposed method for isolating in vivo-generated 25-OH-D2 is very convenient, because the time to perform each HPLC is very short though several steps of HPLC are used.

Keywords ergocalciferol, gas chromatography-mass spectrometry, 25-

1 Following abbreviations are used: D2/D3, vitamin D2/D3 (ergocalciferol/cholecalciferol); 25-OH-D2/D3, 25-hydroxyvitamin D2/D3 (25-hydroxy-ergocalciferol/-cholecalciferol); 25-OH-pyro-D2, 25-hydroxypyrovitamin D2 (25-hydroxy-pyroergocalciferol); 25-OH-isopyro-D2, 25-hydroxisopyrovitamin D2 (25-hydroxy-isopyroergocalciferol); 1,25-(OH)2-D2/D3, 1,25-dihydroxyvitamin D2/D3 (1α,25-dihydroxy-ergocalciferol/-cholecalciferol); HPLC, high-performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; UV, ultraviolet; AUFS, a unit of full scale.

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hydroxyvitamin D$_2$, 25-hydroxy-ergocalciferol, high-performance liquid chromatography, preparative HPLC, mass chromatography, rat plasma, vitamin D$_2$

It has been documented that vitamin D should be metabolized to 25-OH-D in the liver and subsequently to 1,25-(OH)$_2$-D in the kidney to show its physiological effects. It has also been established that the major circulating metabolite in the blood is 25-OH-D$_1$.

There are two forms of vitamin D, vitamin D$_2$ and D$_3$, which show practically the same biological activity for mammals including humans. Since the cost of vitamin D$_2$ is much cheaper than that of vitamin D$_3$, the former has been used predominantly in commercial drugs (e.g. vitamin D preparations and multivitamin preparations) and enriched foods (e.g. enriched dry milk). Therefore, when humans exposed to sunlight are received such drugs or enriched foods, a mixture of exogenous 25-OH-D$_2$ and endogenous 25-OH-D$_3$ circulates in their blood. For evaluating vitamin D status from the viewpoints of the nutritional and clinical sciences, it is very important to establish a simplified method for separately measuring 25-OH-D$_2$ and 25-OH-D$_3$ in plasma or serum. In order to develop the investigation, authentic 25-OH-D$_2$ is essential. However, it is difficult to obtain a standard compound of 25-OH-D$_2$. Although 25-OH-D$_3$ has been synthesized by many routes, few reports have been appeared on the synthesis of 25-OH-D$_2$ except that of SALMOND and SOBALA (2). The synthetic route reported by them is an attractive one, but it is rather complicated and time-consuming to obtain 25-OH-D$_2$ as a standard compound. Prior to investigation of separately measuring 25-OH-D$_2$ and 25-OH-D$_3$ in plasma or serum, we tried to find a convenient method for isolating in vivo-generated 25-OH-D$_2$ as a standard compound from the blood of rats or rabbits given large doses of vitamin D$_2$. EISMAN et al. (3) and JONES (4) isolated tritiated 25-OH-D$_2$ generated in vivo or in vitro for separate assay of 25-OH-D$_2$ and 25-OH-D$_3$ by HPLC. We think the proposed method using several steps of preparative HPLC is useful for isolating a highly purified standard compound of 25-OH-D$_2$ from the blood. The detailed procedure and identification of the isolated compound are described in the paper.

**EXPERIMENTAL**

1. *Materials and reagents*

   Crystalline vitamin D$_2$. Commercial grades of Philips-Duphar Co. (The Netherlands) were recrystallized from acetone–water (4:1), mp 115–116°C.

   Organic solvents were purified according to the usual methods and distilled before use. Other materials and guaranteed reagents were used according to previous papers (5-7).
2. Instrumentation

1) Ultraviolet (UV) absorption spectra. A Hitachi 323 automatic spectrometer (Japan) was used to estimate UV spectra.

2) High-performance liquid chromatography (HPLC). HPLC was performed on either a Shimadzu-DuPont 841 high-performance liquid chromatograph equipped with a UVD-1 detector (fixed at 254 nm, AUFS 0.01, Japan) or a Shimadzu LC-2F high-performance liquid chromatograph equipped with a UVD-2 detector (fixed at 254 nm, AUFS 0.001, Japan). The former was used mainly for preparative HPLC to isolate a fraction which was considered to contain 25-OH-D$_2$, whereas the latter was used mainly for analytical HPLC to identify 25-OH-D$_2$. Three types of stainless steel tubes (250 × 6.2 mm i.d., 250 × 4.6 mm i.d. and 250 × 4.0 mm i.d.) packed with microparticulated Zorbax SIL (DuPont Co., USA), Zorbax ODS (DuPont Co.) or µ-Porasil (Waters Assoc., USA) were used for the separation columns. Column size and conditions of separation are referred to in the following sections and Fig. 5.

3) Gas chromatography-mass spectrometry (GC-MS) and mass chromatography. The GC-MS was performed on a Shimadzu LKB 9000 gas chromatograph-mass spectrometer equipped with a unit of electron impact (EI) as an energy source. A glass column (100 × 0.3 cm i.d.) packed with 3% OV-17 on Gas Chrom Q (80–100 mesh) was run at 290°C with a helium flow of 30 ml/min. Separator's temperature, ionizing current and ionizing voltage were controlled at 320°C, 60 μA and 25 eV, respectively. When the peaks were observed on a gas chromatogram, mass spectra were estimated. Mass chromatography was simultaneously estimated by means of a computer. The analysis of GC-MS and mass chromatography were kindly performed by the Analytical Center of Shimadzu Co.

3. Animals

Male rats of the Wistar strain fed a normal diet (MF diet containing 200 I.U. of vitamin D$_3$/100 g, Oriental Yeast Co., Japan) weighing about 200 g (10–12 weeks old) were used. Female rabbits fed a normal diet (RC diet containing 200 I.U. of vitamin D$_3$/100 g, Oriental Yeast Co.) weighing 4–5 kg were also used.

4. Procedure for the experiments described in Sections 1–4 of RESULTS

The rats were each given 10,000–100,000 I.U. of vitamin D$_2$ intraperitoneally in 0.1 ml of 95% ethanol. After being left for various periods (see the Sections), blood was drawn by cardiac puncture using a heparinized syringe, and about 3 ml of plasma per rat was obtained. The rats which were not given vitamin D$_2$ were used as controls.

1) Isolation of the unsaponifiable matter from the plasma. The plasma (0.5–2.0 ml) was saponified for 30 min at 80°C by adding 4 ml of ethanol, 2 ml of 20% pyrogallol–ethanolic solution and 4 ml of 90% (w/v) KOH–ethanolic solution. After immediate cooling, the unsaponifiable matter was isolated with 30 ml of benzene according to the directions in a previous paper (5). The resulting benzene
solution, including the unsaponifiable matter, was filtered through a Whatman 1PS filter paper and then evaporated to dryness under reduced pressure.

2) Separation of a 25-OH-D₂ fraction by preparative HPLC using a Zorbax SIL column. The resulting residue was dissolved in 0.1 ml of 5.5% isopropanol in n-hexane and then applied to preparative HPLC using a Zorbax SIL column (250 × 6.2 mm i.d.). Elution with the same solvent mixture as the mobile phase was carried out at a constant flow of 1.7 ml/min (column pressure: 30 kg/cm²) and every 50-drop aliquot (0.72 ml) of the eluate was automatically collected in a small test tube by a mini-fraction collector. If necessary, the chromatogram was simultaneously recorded by estimating UV absorption. Fraction nos. 24–28, 29–33 and 34–39 were respectively combined and denoted F1, F2 and F3. After evaporating the solvent of each fraction under reduced pressure, each resulting residue was dissolved in 0.1 ml of a mixture of methanol–isopropanol–water (70:12:18).

3) Analytical HPLC using a Zorbax ODS column. Each solution obtained above was individually applied to analytical HPLC equipped with a Zorbax ODS column (250 × 4.6 mm i.d.). Elution with the same solvent mixture was carried out at a constant flow of 0.7 ml/min (column pressure: 50 kg/cm²). HPLC profiles were automatically recorded by estimating UV absorption of the eluate.

5. Procedure for the experiment described in Section 5 of RESULTS

The F2 fraction obtained from preparative HPLC mentioned above was successively applied to preparative HPLC using various kinds of columns. The columns and conditions for the HPLC are given in Fig. 5. HPLC profiles were automatically recorded by estimating UV absorption of the eluate and every 50-drop aliquot (0.72 ml) of the eluate was simultaneously collected in a small test tube by a mini-fraction collector.

6. Procedure for the experiment described in Section 6 of RESULTS

A rabbit, of the above-mentioned type, was intravenously given 500,000 I.U. of vitamin D₂ in 0.5 ml of 95% ethanol. After being left for various periods, small quantities of blood were taken from the aural vein and plasma was obtained. According to Section 4 of EXPERIMENTAL, the unsaponifiable matter isolated from the plasma was applied to preparative HPLC and then the peak height corresponding to vitamin D₂ on the chromatogram was estimated. The level of vitamin D₂ in the plasma was determined by comparison with that of authentic vitamin D₂. On the other hand, the F2 fraction collected from the preparative HPLC was subsequently applied to analytical HPLC as described in the same Section and the peak height corresponding to 25-OH-D₂ on the chromatogram was estimated. The level of 25-OH-D₂ in the plasma was determined by comparison with that of authentic 25-OH-D₂ (obtained in the following Section).

7. Procedure for the experiment described in Section 7 of RESULTS

Three rabbits were each given 500,000 I.U. of vitamin D₂ intravenously in
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0.5 ml of 95% ethanol. After being left for 30 hr, blood was drawn by carotid cannulization using a heparinized cannule yielding about 20 ml of plasma per rabbit. Sixty ml of the plasma obtained from the three rabbits was divided into 30 parts each of 2 ml. According to Section 4 of EXPERIMENTAL, the unsaponifiable matter isolated from a part of plasma was applied to the preparative HPLC and the F2 fraction was separated. The same procedure was repeatedly applied to the other parts of plasma and the resulting F2 fractions were combined. The combined fraction was subsequently applied to preparative HPLC using various kinds of columns according to Section 5 of EXPERIMENTAL.

8. Procedure for the experiment described in Section 8 of RESULTS

The 25-OH-D$_2$ fraction purified by the above procedure was finally applied to the GC-MS described in Section 2 of EXPERIMENTAL with or without trimethylsilylation. Trimethylsilylation was performed according to a previous paper (5).

RESULTS

1. Profiles of the preparative HPLC using a Zorbax SIL column on the unsaponifiable matter of rat plasma (according to Section 4 of EXPERIMENTAL)

A rat that had received 10,000 I.U. of vitamin D$_2$ was left for 24 hr and then the plasma was obtained. The plasma of a rat not administered vitamin D$_2$ was also obtained as a control. The unsaponifiable matter obtained from the plasma samples was individually applied to preparative HPLC using a Zorbax SIL column. The HPLC profiles are shown in Fig. 1. When the profile of the vitamin D$_2$-dosed rat was compared with that of the control, peaks Y and X had newly appeared in the former (Fig. 1b), while they were undetectable in the latter (Fig. 1a). Peak Y was identified as vitamin D$_2$ from the co-chromatography performed by mixing the eluate with authentic vitamin D$_2$. On the other hand, since peak X included in the F2 fraction was considered to contain a metabolite derived from vitamin D$_2$, the following experiments were carried out.

2. Profiles of analytical HPLC on the F1, F2 and F3 fractions (according to Section 4 of EXPERIMENTAL)

The F1, F2 and F3 fractions obtained from preparative HPLC as mentioned above were individually applied to analytical HPLC. The profiles are shown in Fig. 2. A peak showing a retention time of 18.4 min was observed in both chromatograms of the F3 fraction irrespective of the administration of vitamin D$_2$, which was confirmed to be due to 25-OH-D$_3$ from co-chromatography with the authentic compound. On the other hand, the peak showing a retention time of 17.6 min observed in both chromatograms of the F1 fraction irrespective of the administration of vitamin D$_2$ could not be confirmed as there was no vitamin D-related compound showing the same retention time as the peak. Since peak X
Fig. 1. Profiles of the preparative HPLC on the unsaponifiable matter of rat plasma. The unsaponifiable matter obtained from the plasma of the rats who had or had not received 10,000 I. U. of vitamin D$_2$ was individually applied to preparative HPLC equipped with a Zorbax SIL column (250 × 6.2 mm i.d.). (a) (-D$_2$) without receipt of vitamin D$_2$; (b) (+D$_2$) having received vitamin D$_2$.

(retention time: 20.0 min) that was observed only in the chromatogram of the F2 fraction with the administration of vitamin D$_2$ was thought to be due to 25-OH-D$_2$, further experiments as described in the following Sections were carried out.

3. **Time course of the appearance of peak X (according to Section 4 of EXPERIMENTAL)**

Rats that had received 10,000 I.U. of vitamin D$_2$ were left for 16, 24 or 41 hr and then plasma samples were obtained. The unsaponifiable matter of the samples was individually applied to preparative HPLC and then the F2 fractions were collected. The fractions were individually applied to analytical HPLC and the profiles are shown in Fig. 3. The height of peak X showed a maximum value in the plasma sample obtained from the rat left for 24 hr.

4. **Dose-response of the appearance of peak X (according to Section 4 of EXPERIMENTAL)**

Rats that had received either 10,000, 50,000 or 100,000 I.U. of vitamin D$_2$
Fig. 2. Profiles of analytical HPLC on the F1, F2 and F3 fractions. The F1, F2 and F3 fractions obtained from the preparative HPLC of Fig. 1 were individually applied to analytical HPLC equipped with a Zorbax ODS column (250 × 4.6 mm i.d.). (−D₂) without receipt of vitamin D₂; (+D₂) having received vitamin D₂.

Fig. 3. Time course of the appearance of peak X. The rats that had received 10,000 I.U. of vitamin D₂ were left for 16, 24 and 41 hr. After individually applying the unsaponifiable matter of their plasma to preparative HPLC equipped with the Zorbax SIL column, each separated F2 fraction including the peak X compound was subjected to analytical HPLC equipped with the Zorbax ODS column.
were left for 24 hr and then plasma samples were obtained. The unsaponifiable matter of the samples was individually applied to analytical HPLC and the profiles are shown in Fig. 4. The heights of peak X increased in proportion to the increase of the dose. The dose-response strongly suggested that the peak X was due to 25-OH-D₂.

Fig. 4. Dose response of the appearance of peak X. The rats that had received 10,000, 50,000 or 100,000 I. U. of vitamin D₂ were left for 24 hr. After individually applying the unsaponifiable matter of their plasma to preparative HPLC, each separated F2 fraction including the peak X compound was subjected to analytical HPLC equipped with the Zorbax ODS column.

5. Purification of peak X compound by several steps of preparative HPLC (according to Section 5 of EXPERIMENTAL)

A rat that had received 10,000 I. U. of vitamin D₂ was left for 24 hr and then the plasma was obtained. The unsaponifiable matter of the plasma was applied to preparative HPLC using a Zorbax SIL column and the separated F2 fraction containing the peak X compound was successively applied to several steps of preparative HPLC to purify the compound. The used column, mobile phases and flow rates of the elution are shown in Fig. 5. The chromatograms were simultaneously recorded with fractionation, which are shown in Fig. 5. The large irrelevant peaks prior to peak X, which might be due to coexisting impurities, were observed in the first chromatogram as shown in Fig. 5a, but these peaks gradually diminished during successive HPLC (Fig. 5b–5d) and finally no detectable peaks
Fig. 5. Purification of the peak X compound by several steps of preparative HPLC. The rats that had received 10,000 I.U. of vitamin D$_2$ was left for 24 hr. After applying the unsaponifiable matter of the plasma to preparative HPLC equipped with the Zorbax SIL column, the separated F$_2$ fraction including the peak X compound was successively subjected to several steps of preparative HPLC described in the figure. * peak X.
other than peak X were observed in the last chromatogram as shown in Fig. 5e. When the UV spectrum of the fraction corresponding to peak X obtained from the fourth HPLC (Fig. 5d) was estimated, a comparatively large UV absorption was observed in the region below 230 nm and it suggested that impurities still remained in the fraction. Therefore, further purification by application of the fraction to the fifth HPLC (Fig. 5e) was performed and then absorption in the region of short wavelength decreased as shown in Fig. 6. The UV spectrum giving a maximum at 264 nm and a minimum at 228 nm showed the characteristic cis-triene structure in the vitamin D molecule. Furthermore, the absorption ratio of the minimum to maximum \( \frac{A_{228}}{A_{264}} \) giving 0.55 showed that the purification was complete.

6. **Time course of the plasma levels of vitamin D\(_2\) and peak X compound (25-OH-D\(_2\)) in a rabbit (according to Section 6 of Experimental)**

In order to obtain large amounts of the peak X compound, the same procedures as those in rats were applied to rabbits. Prior to isolation of the compound, experimental work on the time course was carried out. A rabbit that had received a single dose of 500,000 I.U. of vitamin D\(_2\) was left and then the blood was taken at appropriate intervals from the aural vein to obtain plasma samples. After treating the samples according to the procedure described in Experimental, the levels of vitamin D\(_2\) and the peak X compound (25-OH-D\(_2\)) in the plasma samples were determined. As shown in Fig. 7, the level of vitamin D\(_2\) after 2 hr (1.8 \( \mu \)g/ml) gradually decreased to reach a nearly constant value (about
0.6 μg/ml) after 30 hr. On the other hand, the levels of the peak X compound (25-OH-D₂) gradually increased until a maximum level was observed after 30 min. From these results, we decided that the plasma for isolating the peak X compound (25-OH-D₂) should be collected from the rabbits left for 30 hr after administration of vitamin D₂.

7. *Isolation of the peak X compound from the plasma of vitamin D₂-dosed rabbits (according to Section 7 of EXPERIMENTAL)*

Three rabbits that had received a single dose of 500,000 I.U. of vitamin D₂ were left for 30 hr and then the plasma was obtained. By treating the plasma according to the procedure described in EXPERIMENTAL, about 30 μg of the purified peak X compound was obtained from 60 ml of the plasma.

8. *Identification of the peak X compound as 25-OH-D₂ by GC-MS (according to Section 8 of EXPERIMENTAL)*

The purified peak X compound thus obtained was subjected to GC-MS with or without trimethylsilylation. The two peaks derived from 25-OH-pyro- and -isopyro-D₂ or their trimethylsilyl ethers were observed in the gas chromatograms. The respective eluates corresponding to the peaks were subjected to MS analysis.
The mass spectra corresponding to the two peaks without trimethylsilylation were nearly same, which gave the molecular ion \( \text{M}^+ \) at \( m/e \) 412 (the molecular weight of vitamin D\(_2\) + mass number of 16) and the fragment ions at \( m/e \) 379 (\( \text{M}^+ - \text{H}_2\text{O} - \text{CH}_3 \)), 354 (\( \text{M}^+ - \text{C}_3\text{H}_6\text{O} \) (residue of cleavage at C24–25 bond)) and 253 (\( \text{M}^+ - \text{side chain} - \text{H}_2\text{O} \)) which were characteristic for the pyro- and isopyro-compounds of 25-OH-D\(_2\). On the other hand, the mass spectra corresponding to the two peaks with trimethylsilylation were also nearly the same. The mass spectrum of 25-OH-pyro-D\(_3\) trimethylsilyl ether is shown in Fig. 8 as being representative. The molecular ion (\( \text{M}^+ \)) at \( m/e \) 556 and the fragment ions at \( m/e \) 466 [\( \text{M}^+ - \text{HOSi(CH}_3)_3 \)], 451 [\( \text{M}^+ - \text{HOSi(CH}_3)_3 - \text{CH}_3 \)], 361 [\( \text{M}^+ - 2\times \)]
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HOSi(CH₃)₃-CH₃, 343 (M⁺ - side chain), 253 [M⁺ - side chain - HOSi(CH₃)₃] and 131 [(CH₃)₂C-O-Si(CH₃)₃, cleavaged ion at C24-25 bond, base peak] strongly supported the structure of trimethylsilyl ether of 25-OH-pyro-D₂. Furthermore, when the mass chromatograms of the trimethylsilyl ether of peak X compound were described by using the molecular ion at m/e 556 and the fragment ion at m/e 131, they gave two peaks due to the trimethylsilyl ethers of 25-OH-pyro- and -isopyro-D₂ with the retention times of 8.0 and 9.3 min, respectively, as shown in Fig. 9. From the results of GC-MS, mass chromatography and UV absorption spectrum, we concluded that the peak X compound was 25-OH-D₂.

Fig. 9. Mass chromatogram of 25-OH-D₂ trimethylsilyl ether.

DISCUSSION

It is well known that the two forms of vitamin D, vitamin D₂ and D₃, are practically useful. Industrial production of vitamin D₂ and D₃ can be performed by UV irradiation of ergosterol and 7-dehydrocholesterol, respectively, but vitamin D₂ is more common than D₃ because ergosterol, easily obtained by fermentation of yeast is cheaper than 7-dehydrocholesterol. Therefore, vitamin D₂ is predominantly used for commercial drugs and enriched foods. When a human being exposed to sunlight receives such a vitamin D₂ supplement, a mixture of exogenous 25-OH-D₂ and endogenous 25-OH-D₃ circulates in his blood. Therefore, it is very important to establish a simplified method for the separate assay of the two metabolites in the plasma or serum. The biggest obstacle for developing the investigation is a poor supply of authentic 25-OH-D₂ as a standard compound. The side chain of 25-OH-D₂ has an extra double bond at the 22–23 position and an extra methyl group at the 24 position, which differ from that of 25-OH-D₃. Since the synthetic insertion
of a 25-hydroxyl group into the vitamin D$_2$ molecule is very difficult, successful synthesis of 25-OH-D$_2$ has been prevented. From these considerations, we decided to obtain the standard compound of 25-OH-D$_2$ from biological materials.

Isolation of in vivo-generated 25-OH-D$_2$ was first reported by SUDA et al. (8). They isolated the metabolite from the blood of four pigs administered large amounts of vitamin D$_2$ by silicic acid and Celite-partition column chromatography and identified it by mass spectrometry and proton nuclear magnetic resonance. STRYD and GILVERTSON (9) and SHEPARD et al. (10) reported that sufficient separation between 25-OH-D$_2$ and 25-OH-D$_3$ could not be achieved by column chromatography using silica gel or Sephadex LH-20, whereas the separation was possible by HPLC. Since the plasma containing exogenous 25-OH-D$_2$ usually involves endogenous 25-OH-D$_3$, as shown in Fig. 2, we decided to use preparative HPLC for isolating the standard compound of 25-OH-D$_2$.

As shown in Fig. 1, the HPLC profile of the plasma of a rat that had received 10,000 I. U. of vitamin D$_2$ gave new peaks, denoted as peak Y and X, which could not be found in the profile of a rat without administration of vitamin D$_2$. Peak Y was considered to be vitamin D$_2$ from the result of co-chromatography. On the other hand, the time course and dose response of the appearance of peak X, as shown in Figs. 3 and 4, strongly suggested that the peak might be due to 25-OH-D$_2$. Then, the peak X compound was further purified by several steps of preparative HPLC (Fig. 5). The purified compound gave an UV absorption spectrum showing a typical cis-triene structure in the vitamin D molecule (Fig. 6). The same procedure for purification was applied to the three rabbits that had received a single dose of 500,000 I. U. of vitamin D$_2$ and then about 30 μg of the purified compound was isolated. The compound was finally confirmed to be 25-OH-D$_2$ from the results of GC-MS and mass chromatography (Figs. 7 and 8). Establishment of a simplified method for separate assay of 25-OH-D$_2$ and 25-OH-D$_3$ in plasma will be followed by using the purified standard compound of 25-OH-D$_2$ and the results will be reported in a forthcoming paper.

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