Simultaneous Determination of 25-Hydroxyvitamin D2 and 25-Hydroxyvitamin D3 in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry Employing Derivatization with a Cookson-Type Reagent

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A rapid and highly sensitive LC-MS-MS method for simultaneous determination of 25-hydroxyvitamin D2 [25(OH)D2] and 25-hydroxyvitamin D3 [25(OH)D3] in human plasma has been developed using derivatization with a Cookson-type reagent, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQTAD). The derivatization with DMEQTAD significantly improved the ionization efficiencies of 25(OH)D2 and 25(OH)D3, with detection limits of 20 and 12.5 fmol (8 and 5 pg) per injection, respectively. The method employed two steps of solvent extraction but did not require chromatographic purifications for sample pretreatment. The determination was carried out by mass chromatography of the protonated molecular ions formed by atmospheric pressure chemical ionization operating in the positive-ion mode after the derivatization, and 25-hydroxyvitamin D2 was used as an internal standard. The intra-assay coefficients of variation were below 4.02 and 3.24% for 25(OH)D2 and 25(OH)D3, respectively, and the analytical recoveries of both compounds were quantitative. Assay linearity was obtained in the range of 0.05—1 ng per tube and the determination limit was 3 ng/ml for a 20 μl plasma aliquot, for each compound. The developed method was applied to plasma samples obtained from volunteers, two of whom had received vitamin D3 supplementation, and gave satisfactory results.

Key words 25-hydroxyvitamin D; LC-MS-MS; Cookson-type reagent; derivatization; human plasma

Vitamin D actually consists of two different compounds, vitamin D3 (D3) and vitamin D2 (D2), which differ in their side-chain structure at the 17β-position of the secosteroid. Although D3 and D2 are both absorbed from the diet, only D3 is biosynthesized in the skin upon irradiation of 7-dehydrocholesterol with UV light. Vitamin D is hydroxylated in the liver to the circulating form, 25-hydroxyvitamin D [25(OH)D], which is further metabolized in the kidney to the active form, 1,25-dihydroxyvitamin D.1)

Measurement of the concentration of 25(OH)D in serum/plasma is widely used as a means of assessing vitamin D status in man. Although the values of D2 and its metabolites in biological fluids are usually less than one-tenth of those of D3 and its metabolites, their significant rise is observed in the subjects who had taken D2 enriched foods and/or had received D2 for nutritional and/or clinical purposes. Therefore, separate determination of 25(OH)D2 and 25(OH)D3 in serum/plasma is important in the field of nutritional and clinical studies.

The serum/plasma 25(OH)D levels have conventionally been measured by HPLC with UV detection, but the method requires large sample volumes (0.5—2 ml) and time-consuming chromatographic purifications prior to quantification.2—4) RIA for 25(OH)D utilizing a radiiodinated tracer has also been developed, which requires only 50 μl of plasma or serum and eliminates the need for chromatographic purifications before assay.5) However, this method is not able to individually quantify 25(OH)D2 and 25(OH)D3 because the antibody used in the RIA reacts equally with these two metabolites,5,6) and its assay validity in the samples with low concentration has been questioned.7)

LC-MS is considered to be a rapid and specific method for the determination of vitamin D compounds in biological fluids. However, the ionization efficiencies of vitamin D compounds are low in various ionization methods, such as electrospray ionization and atmospheric pressure chemical ionization (APCI). Because of the relatively high level at which it circulates in serum (10—40 ng/ml), the levels of 25(OH)D3 can be determined by the usual LC-MS. On the other hand, those of 25(OH)D2 cannot be determined without large sample volumes, so that several chromatographic purifications are necessary to remove interfering substances derived from the specimens.

We have developed the LC-MS method for 24,25-dihydroxyvitamin D3 [24,25(OH)2D3] in human plasma using the derivatization with a Cookson-type reagent (4-substituted 1,2,4-triazoline-3,5-dione).8) In the study, 4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione (MBOTA-D),9) which is rich in proton-affinitive atoms (oxygen and nitrogen atoms), was very effective in increasing the ionization efficiency of the resulting derivative in APCI operating in the positive-ion mode. These data indicate that the derivatization with a Cookson-type reagent having a different substituent at the 4-position, for example, one having more proton-affinitive atoms is capable of providing higher sensitivity in the positive APCI-MS. In the present paper, we describe an LC-APCI-MS-MS method for the simultaneous determination of 25(OH)D2 and 25(OH)D3 in human plasma employing derivatization with a Cookson-type reagent which does not require chromatographic purifications for sample pretreatment.

MATERIALS AND METHODS

Materials and Reagents 25(OH)D2 was synthesized in our laboratories according to the known method.10) 25(OH)D3 and 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQTAD)11) were obtained from Wako Pure Chemical Ind. (Osaka, Japan).
Japan). 25(OH)D₃ [internal standard (IS)] was donated by Dr. Yoji Tachibana (Nissin Flour Milling Co., Saitama, Japan). MBOTAD was synthesized in our laboratories as previously reported, and now its precursor (urazole form) is available from Dojindo Laboratories (Kumamoto, Japan). All other reagents and solvents were of analytical grade. Stock solutions of 25(OH)D₂ and 25(OH)D₃ were prepared as 50 µg/ml solutions in EtOH, the concentrations of which were confirmed by UV spectroscopy using the molar absorptivity, ε = 18900 and 18200, respectively, at 265 nm. Subsequent dilutions were carried out with EtOH to prepare 5, 10, 20, 50 and 100 ng/ml solutions. Ethanolic solution of IS with a concentration of 100 ng/ml was also prepared.

**Plasma Samples** Plasma samples were obtained from 7 healthy volunteers (4 males and 3 females, age range: 21—32 years) known not to have received vitamin D supplementation and 2 healthy male volunteers (age: 22 and 32 years) who had received successive oral administrations of a vitamin supplement (Kawai Liver Oil Drop S) (Kawai Pharmaceutical Co., Tokyo, Japan) containing 400 IU/d of D₂ for 2 weeks. In the D₂ supplementation study, informed consent was obtained from the volunteers. All of the plasma samples were stored at −20°C prior to use.

**LC-MS-(MS) ** LC-MS-(MS) was performed using a ThermoQuest LCQ (San Jose, CA, U.S.A.) liquid chromatograph–ion trap–mass spectrometer connected to a JASCO PU-980 (Tokyo, Japan) chromatograph, and APCI was used in the positive-ion mode. A J'Sphere ODS-H-80 (4.6 mm i.d.) (YMC, Kyoto, Japan) column was used at a flow rate of 1 ml/min at 40°C. For MS-MS analysis, helium was used as the collision gas. The source current, the capillary voltage, the tube lens offset and the sheath gas flow rate were 5 μA, 3 V, 15 V and 80 units, respectively. The heated capillary temperature, the vaporizer temperature and the mobile phase for each analyte were as follows: 175°C, 550°C and MeOH–H₂O (9:1, v/v) for 25(OH)D₂; 225°C, 450°C and MeCN–H₂O (5:1, v/v) for the MBOTAD derivative; 225°C, 550°C and MeCN–H₂O (3:2, v/v) for the DMEQTAD derivative.

**Pretreatment of Plasma Sample** The plasma sample (20 µl) was added to MeCN (100 µl) containing IS (1 ng), vortex-mixed for 30 s and subjected to centrifugation at 1500 × g for 10 min. The supernatant was evaporated under a N₂ gas stream, and the residue was dissolved in AcOEt (200 µl). H₂O (100 µl) was added to this solution and vortex-mixed for 30 s. The AcOEt layer was saved, and the H₂O layer was mixed with another AcOEt (100 µl). The AcOEt layers were combined, evaporated under a N₂ gas stream and then subjected to derivatization.

**Derivatization with Cookson-Type Reagents** The samples were extensively dried and then dissolved in AcOEt (25 µl) containing MBOTAD or DMEQTAD (2.5 µg). The mixture was kept at room temperature for 30 min, then an additional reagent (2.5 µg/25 µl of AcOEt) was added and the entire mixture was further kept at room temperature for 1 h. After addition of EtOH (40 µl) to decompose excess reagent, the solvent was evaporated and, unless otherwise indicated, the residue was dissolved in the mobile phase (40 µl), 15 µl of which was subjected to LC-MS.

**Effect of Derivatization for Detection Responses** The effect of the derivatization for the detection responses was evaluated by the limit of detection [LOD; the amount of 25(OH)D₃ or derivatives per injection giving a signal to noise ratio (S/N) of 3]. Two hundred picograms (500 fmol) of 25(OH)D₃ was derivatized with MBOTAD or DMEQTAD as described above. These derivatives were dissolved in the mobile phase (200 µl) to prepare the solutions of 2.5 fmol/µl and subjected to LC-MS. By decreasing the injection volume of the resulting solution stepwise, the amount of derivative giving an S/N of 3 was determined. The LOD of intact 25(OH)D₃ was determined using the solution of 20 ng/ml in the same way.

**Absolute Recoveries of 25(OH)D₂ and IS from Plasma Specimen** Because a plasma sample not containing 25(OH)D₃ could not be prepared, the absolute recoveries from a plasma specimen were examined only for 25(OH)D₂ and IS. To the blank plasma, in which 25(OH)D₃ was not detected, 25(OH)D₂ was added to a concentration of 5 or 50 ng/ml. Twenty microliters of blank plasma and 25(OH)D₂-spiked plasma were pretreated as above, and then 100 pg of 25(OH)D₂ was added to only the blank sample. Both samples were added with IS (1 ng) and derivatized. The absolute recovery of 25(OH)D₂ during pretreatment was calculated from the peak area ratios [25(OH)D₂/IS] of the spiked sample and the blank sample. Next, 20 µl of blank plasma and IS-spiked plasma (50 ng/ml) were pretreated, and 1 ng of IS was added to only the blank sample. Both samples were added with 25(OH)D₂ (200 pg) and derivatized. The absolute recovery of IS was calculated from the peak area ratios [IS/25(OH)D₂] of the spiked sample and the blank sample.

**Influence of Endogenous Substances for Derivatization** The influence of endogenous substances for derivatization was examined as follows. IS (1 ng) was placed in tubes with plasma (20 µl) extract prepared as described above. After derivatization, the peak area was compared with that obtained from the sample without the plasma extract. Incidentally, an excess of DMEQTAD produces the adduct quantitatively on condition that the substrate is pure.

**RESULTS AND DISCUSSION**

**Selection of Cookson-type Reagent** Although several Cookson-type reagents have been developed, there are a few reagents which are stable and easily available, as well as rich in proton-affinitive atoms. MBOTAD and DMEQTAD meet these requirements and are considered to be useful in LC-MS analysis of vitamin D compounds. Both of them have previously been developed as fluorescence labeling reagents having a highly reactive dienophile and a sensitive fluorophore. When these reagents were utilized in the assay of the vitamin D metabolites in plasma using HPLC with fluorescence detection, a complicated pretreatment to remove the excess reagent was necessary. However, a compound having a different molecular weight does not interfere in an LC-MS experiment, which indicates that the step to remove the excess reagent can be omitted in this experiment. Furthermore, in LC-MS, the derivatization with these reagents have another advantage in that the molecular weight of the analyte is shifted to a higher mass range, where background noise is relatively low.

Our initial efforts were directed toward selecting a Cookson-type reagent giving higher sensitivity. The sensitivity
was estimated by examining the LOD (S/N=3) using 25(OH)D$_3$ as the model compound. The adduct of a vitamin D compound with the Cookson-type reagent consisted of 6S and 6R isomers (Fig. 1), because the reagent attacked at the s-cis-diene of the compound from the $\alpha$- and $\beta$-sides. In the case of 25(OH)D, the 6S-isomer was the main product (6S:6R ca. 7:2); therefore, we used the isomer in the following studies.

The LC-MS conditions for analysis of intact 25(OH)D$_3$, the MBOTAD derivative and the DMEQTAD derivative were optimized as described in the experimental section, in which the mobile phases were selected to give a relatively short chromatographic run-time to obtain a higher run capacity. Intact 25(OH)D$_3$ was ionized more efficiently in the mobile phase using MeOH than in that using MeCN; on the contrary, the ionization efficiencies of the derivatives were higher in the mobile phase using MeCN. 25(OH)D$_3$ gave a protonated molecular ion together with its intense dehydrated ions (Table 1). On the other hand, the DMEQTAD derivatives gave a very intense protonated molecular ion and its dehydrated ion was slight (relative intensity: <5%). The ions listed in Table 1 were used as monitoring ions to evaluate the effect of the derivatization for sensitivity. The DMEQTAD derivative showed the highest sensitivity with an LOD of 20 fmol, equivalent to ca. 8 pg of 25(OH)D$_3$, which was 15 times of that obtained without derivatization. The effect of derivatization with MBOTAD (LOD: 30 fmol) was two-thirds of that of DMEQTAD in its sensitivity, which is due to the fact that the latter is richer in proton-affinitive atoms than the former. Considering these results, we used DMEQTAD as the derivatization reagent in the following studies.

**Table 1. Limit of Detection in 25(OH)D$_3$ and Derivatives$^{(a)}$**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Monitoring ions (m/z)</th>
<th>$t_R$ (min)</th>
<th>LOD (per injection)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D$_3$</td>
<td>Sum of 401 [M+H]$^+$ (24), 383 [401−H$_2$O]$^+$ (100) and 365 [401−2H$_2$O]$^+$ (21)</td>
<td>5.0</td>
<td>300 fmol (120 pg)</td>
</tr>
<tr>
<td>25(OH)D$_3$-MBOTAD</td>
<td>Sum of 723 [M+H]$^+$ (100) and 705 [723−H$_2$O]$^+$ (35)</td>
<td>4.4</td>
<td>30 fmol</td>
</tr>
<tr>
<td>25(OH)D$_3$-DMEQTAD</td>
<td>746 [M+H]$^+$ (100)</td>
<td>4.6</td>
<td>20 fmol</td>
</tr>
<tr>
<td>25(OH)D$_3$-DMEQTAD (MS-MS)$^c$</td>
<td>746 [M+H]$^+$ (100)</td>
<td>4.6</td>
<td>12.5 fmol</td>
</tr>
</tbody>
</table>

$^a$ The $t_R$ and LOD values of the derivatives are those of the 6S-isomer.  
$^b$ The values in parentheses are relative intensities.  
$^c$ Precursor ion: m/z 746, relative collision energy: 15%.

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that the MS-MS mode of monitoring these product ions was not advantageous in sensitivity. On the other hand, when the MS-MS mode with 15% of the relative collision energy using the protonated molecular ions as the precursor ions and the same residual ions as the monitoring ions was used, the noise ions were reduced without decreasing the intensities of the monitoring ions, and as a result, a smaller LOD value was obtained [LOD of 25(OH)D3-DMEQTAD: 12.5 fmol (equivalent to ca. 5 pg of 25(OH)D3)] (Table 1). Based on these results, the MS-MS mode with 15% of the relative collision energy was utilized in the following studies.

**Calibration Curve** In the present study, 25(OH)D3 was used as an IS, because this compound is not endogenous in man and its DMEQTAD derivative showed similar behavior in APCI-MS and -MS-MS as well as that of 25(OH)D2 or 25(OH)D4. That is, the derivative of IS gave the protonated molecular ion (m/z 760) as the base ion (Fig. 2 d), which was not fragmented by 15% of the relative collision energy.

When MeCN–H2O (3:2, v/v) was used as mobile phase, the retention times (tR, min) of each DMEQTAD derivative were as follows; 25(OH)D2: 4.4 (6R) and 5.8 (6S), 25(OH)D3: 3.7 (6R) and 4.6 (6S), and IS: 5.1 (6R) and 6.0 (6S). As mentioned above, only the 6S-isomers were used in the present study, so that the LC eluent entered the mass spectrometer from 4 to 6.5 min after injection by a diversion valve.

Considering all the data, a single LC-MS-MS analysis was divided into two segments and the following mass chromatographic method was used for the determination of 25(OH)D2 and 25(OH)D3 in plasma; the first segment (4—5.2 min), precursor ion: m/z 746, scan range: m/z 740—750, monitoring ion: m/z 746, the second segment (5.2—6.5 min), precursor ions: m/z 758—760, scan range: m/z 755—765, monitoring ions: m/z 758 for 25(OH)D2 and m/z 760 for IS, respectively. Under these conditions, the LOD of the 25(OH)D3 derivative was 12.5 fmol as already mentioned and that of the 25(OH)D2 derivative was 20 fmol [equivalent to ca. 8 pg of 25(OH)D2] (S/N=3).

The calibration curves were constructed by plotting peak area ratios [25(OH)D2/IS or 25(OH)D3/IS] against the amounts of 25(OH)D2 or 25(OH)D3 per tube. The regression line obtained from the combination of five standard curves were y (peak area ratio)=0.8526x (amount, ng)+0.0098 [correlation coefficient (r²)=0.999] for 25(OH)D2 and y= 0.9134x+0.0031 (r²=0.997) for 25(OH)D3. The coefficients of variation (CV) of the y values at 0.05 ng per tube of 25(OH)D2 and 25(OH)D3 (lower limit of quantitation) were 4.9 and 6.3%, respectively. The data demonstrated good linearity in the range of 0.05—1 ng per tube.

**Pretreatment of Plasma Sample and Derivatization**

Fig. 2. APCI Mass Spectra of (a) 25(OH)D2-DMEQTAD and (b) 25(OH)D3-DMEQTAD. (c) Product Ion Mass Spectrum of 25(OH)D3-DMEQTAD (Precursor Ion: m/z 746, Relative Collision Energy: 25%). (d) APCI Mass Spectrum of IS-DMEQTAD
As mentioned in the introductory section, the hitherto developed HPLC methods for 25(OH)D required large sample volumes and chromatographic purifications prior to quantification.\(^1\) On the contrary, RIA is able to determine 25(OH)D levels with 50μl of sample and employs only organic solvent extraction for sample purification before assay.\(^5\) The proposed derivatization–LC-MS-MS method is so highly sensitive and selective that the plasma 25(OH)D\(_2\) and 25(OH)D\(_3\) levels were determined with very small sample volumes and a simple pretreatment equal to that in the RIA. The plasma specimen (20μl) was extracted with MeCN, and then the extract was dissolved in AcOEt and washed with H\(_2\)O. The obtained fraction was treated with a large excess of DMEQTAD at room temperature. The derivatization procedure was very simple, simply mixing the analytes and the reagent, and no post cleanup step was necessary. The derivatization efficiency of the plasma sample was almost equal to that of the standard sample [101.2±2.2%, mean±standard deviation (S.D.), n=5], which demonstrated that endogenous substances preventing the reagent reacting were essentially removed by the two steps of solvent extraction. The absolute recovery rates of 25(OH)D\(_2\) at 5 and 50 ng/ml from the plasma specimen were 83.9±1.2% (n=3) and 84.5±1.9% (n=3), respectively; and that of IS at 50 ng/ml was 83.2±2.3% (n=3); there was no significant difference between the recoveries of 25(OH)D\(_2\) and IS. Although the absolute recovery rate of 25(OH)D\(_3\) was not determined, it was considered to be about the same as that of IS, judging from the results of analytical recovery studies described later. Based on these data, the practical minimal measurable amount of this method was ca. 3 ng/ml for both 25(OH)D\(_2\) and 25(OH)D\(_3\) when 20 μl of the plasma specimen was used, which surpassed that of the RIA (LOD: 2.8 ng/ml for 50 μl of serum/plasma aliquot).\(^5\)

Typical chromatograms are shown in Fig. 3. Although both chromatograms shown in Figs. 3a and b were obtained from the same plasma of the subject who had received successive oral administrations of 400 IU/d of D\(_2\) for 2 weeks, in the case of the former, the derivatization was obstructed by the addition of 50% EtOH. These chromatograms revealed that there was no interfering peak derived from endogenous plasma components and the reagent at elution positions of the DMEQTAD derivatives of 25(OH)D\(_2\) (m/z 758), 25(OH)D\(_3\) (m/z 746) and IS (m/z 760). On the other hand, the peaks with satisfactory shapes corresponding to 25(OH)D\(_2\) and 25(OH)D\(_3\) were observed in Fig. 3b, which gave the results of 3.2 and 14.6 ng/ml, respectively.

**Validation and Application of the Present Method**

The plasma specimens to which known amounts of 25(OH)D\(_2\) and 25(OH)D\(_3\) had been added were pretreated and analyzed by the present method. Satisfactory analytical recovery rates ranging from 92.9 to 109.8% were obtained (Table 2). Excellent intra-assay CV values (less than 4.02%, n=5) were also obtained. These data indicate that the present method is accurate and highly reproducible.

The developed method was applied to the plasma samples obtained from 7 healthy subjects known not to have received vitamin D supplementation. The plasma concentration of 25(OH)D\(_2\) was 14.95±2.63 ng/ml (range: 10.75—18.80 ng/ml), which was compatible with results previously reported,\(^1,5\) and that of 25(OH)D\(_2\) was less than the determination limit in all subjects. On the contrary, the plasma 25(OH)D\(_2\) concentrations of the 2 subjects who had received D\(_2\) supplementation (400 IU/d) for 2 weeks were 3.23 and 4.81 ng/ml, respectively. Incidentally the plasma 25(OH)D\(_3\) levels of these subjects before and after administration of D\(_2\) were 16.51 and 14.56 ng/ml, respectively, and 10.75 and 8.82 ng/ml, respectively, for each subject.

**CONCLUSION**

We have demonstrated the LC-MS-MS method for determination of 25(OH)D\(_2\) and 25(OH)D\(_3\) in human plasma. Derivatization with DMEQTAD was employed in the present study, which was very useful in improvement of the ionization efficiency of the analytes in APCI-MS and made possible simultaneous determination of the two vitamin D metabolites with only 20 μl of plasma. The method has a high run capacity; no chromatographic purifications are necessary for sample pretreatment, and the analysis time from...
one injection to the next is also short (within 7 min). It is expected that this derivatization method can be applied in the LC-MS(-MS) assay of other small amounts of vitamin D compounds, such as a prodrug of the active form of D3, 1α-hydroxyvitamin D3, in biological fluids. Such studies are now in progress in our laboratories.

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REFERENCES