Synthesis of Deuterium-Labeled Vitamin D Metabolites as Internal Standards for LC-MS Analysis

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Abstract: Blood levels of the vitamin D3 (D3) metabolites 25-hydroxyvitamin D3 (25(OH)D3), 24R,25-dihydroxyvitamin D3, and 1α,25-dihydroxyvitamin D3 (1,25(OH)2D3) are recognized indicators for the diagnosis of bone metabolism-related diseases, D3 deficiency-related diseases, and hypercalcemia, and are generally measured by liquid-chromatography tandem mass spectrometry (LC-MS/MS) using an isotope dilution method. However, other D3 metabolites, such as 20-hydroxyvitamin D3 and lactone D3, also show interesting biological activities and stable isotope-labeled derivatives are required for LC-MS/MS analysis of their concentrations in serum. Here, we describe a versatile synthesis of deuterium-labeled D3 metabolites using A-ring synthons containing three deuterium atoms. Deuterium-labeled 25(OH)D3 (2), 25(OH)D3-23,26-lactone (6), and 1,25(OH)2D3-23,26-lactone (7) were synthesized, and successfully applied as internal standards for the measurement of these compounds in pooled human serum. This is the first quantification of 1,25(OH)2D3-23,26-lactone (7) in human serum.

Keywords: vitamin D; deuterium labeling; liquid-chromatography tandem mass spectrometry; measurement of vitamin D metabolites in blood

1. Introduction

Vitamin D3 (D3) (1) is metabolized by members of the cytochrome P450 (CYP) family to generate more than 50 compounds in vivo. Among them, 25-hydroxyvitamin D3 (25(OH)D3) (2) is generated from D3 (1) by CYP2R1 and/or CYP27A1-mediated hydroxylation at C25 in the liver, and the resulting 25(OH)D3 (2) is further metabolized to the active form of D3, 1α,25-dihydroxyvitamin D3 (1,25(OH)2D3) (3), by CYP27B1-mediated oxidation at Clα in the kidneys (Figure 1). 1,25(OH)2D3 (3) plays a key role in the regulation of bone metabolism in vivo [1]. In normal conditions, production of 3 from 2 is strictly controlled by the concentrations of calcium and parathyroid hormone (PTH) in the blood, and thus the concentration of 3 in the blood is a useful indicator of functional status [2] and is helpful in the diagnosis of diseases such as hypercalcemia, hyperphosphatemia, rickets, and bone metabolism-related diseases [3,4]. The concentration of 3 in the blood is also useful as an indicator for the diagnosis of various vitamin D deficiency-related diseases [3,4]. Recently, various D3 metabolites, mostly oxidized at the D-ring side chain, have also been found to show biological activities. For example, 20S,25(OH)2D3, which is generated by CYP11A1, inhibits the growth of keratinocytes, leukemia cells, and melanoma cells [5–9], while 24R,25(OH)2D3 (4), produced by CYP24A1, shows inhibitory activity against various cancer cell lines [10,11]. Further, 25(OH)D3-23,26 lactone (6) and 1,25(OH)2D3-23,26 lactone (7), which are thought to be final metabolites of D3, show antagonistic activity.
towards 1,25(OH)$_2$D$_3$ [12–14], thereby, inhibiting bone formation and resorption. Recently, compound 7 was reported to inhibit fatty acid oxidation [15]. Thus, there is a need to measure the blood levels of these metabolites.

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**Figure 1.** Structures of vitamin D$_3$ (1) and its metabolites (2)–(7).

The concentrations of metabolites 2 and 3 in blood have been measured for clinical purposes by radioimmunoassay (RIA) or chemiluminescent enzyme immunoassay (CLEIA) [16]. They, however, have disadvantages such as the need to handle radioactive materials, and insufficient discrimination of vitamin D metabolites by antibodies [17]. More recently, a liquid-chromatography tandem mass spectrometry (LC-MS/MS) method has been developed to determine the concentration of multiple vitamin D metabolites simultaneously in blood [18]. However, LC-MS/MS-based measurement also has some problems, such as the low ionization efficiency of vitamin D derivatives and interference by contaminants including multiple D$_3$ metabolites in the blood. To address these issues, several approaches have been investigated. Cookson-type reagents have been developed to improve the ionization efficiency of D$_3$ metabolites, affording high sensitivity even at low abundance [19,20]. The isotope dilution method has also been applied to avoid interference from contaminants in the blood. This method requires a stable isotope-labeled compound as an internal standard, and so far, deuterium-labeled 25(OH)$_2$D$_3$ (2), 1,25(OH)$_2$D$_3$ (3), and 24R,25(OH)$_2$D$_3$ (4), in which deuterium is introduced at C26, C27, C6, and C19, have been synthesized (Figure 2) [21–25].

In the synthesis of the deuterium-labeled metabolites 2–4-$d_6$, deuterium was introduced into the side chain at C26 and C27 by reacting esters 8 with deuterated Grignard reagent, CD$_3$MgBr (Figure 2B) [21,22]. On the other hand, 2–3-$d_3$ were synthesized by reacting SO$_2$ adducts of cyclic compounds 9 derived from D$_3$ with deuterium oxide (D$_2$O) [23–25]. In both strategies, the range of metabolites that can be synthesized is limited due to the restrictions imposed by the use of steroid precursors. Therefore, a more versatile approach is required. Convergent strategies, with coupling between CD-ring and A-ring moieties, have been widely applied for the synthesis of D$_3$ derivatives [26,27]. Since the CD-ring structures of the metabolites are diverse, whereas the A-ring structures are relatively constant, we considered that deuterium-labeled A-ring synthons would be suitable for the preparation of a variety of deuterium-labeled D$_3$ metabolites (Figure 2D). In addition, labeling in the A-ring has an advantage in metabolism studies because the side chains of the D3 are well known to be enzymatically metabolized easily. In this study, we have developed a synthesis of deuterium-labeled A-ring precursors 13-$d_3$ and 16-$d_3$ incorporating three deuterium atoms. These precursors were coupled with CD-ring moieties 17 and 18 to afford deuterium-labeled 25(OH)D$_2$D$_3$-d$_3$ (2-$d_3$) and vitamin D lactones 25(OH)D$_2$-23,26-lactone-$d_3$ (6-$d_3$) and 1,25(OH)$_2$D$_2$-23,26-lactone-$d_3$ (7-$d_3$). We also confirmed that the concentrations of 2, 6, and 7 in human serum could be measured by LC-MS/MS using the corresponding deuterium-labeled compounds as the internal standards (IS) (see Supplementary Materials).
11 The hydroxyl group in alkyne 11 was protected with TBS ether, followed by deprotection of the TMS and pivaloyl groups with NaOMe in MeOH to give the alcohol 12 followed by epoxidation with NaH and reaction with TMS-acetylene (22% yield from α-deuteration). In this reaction, the stereochemistry of the D3 were well known to be enzymatically metabolized easily. In this study, we have in a 96% yield with over 93% deuteride content [28]. In this reaction, the stereochemistry of alcohol 13 was isomerized (4:1 ratio of α-deuteration and β-deuteration) with deuterium labels at C3 and C4 (enantiomeric mixture) was converted into alkyne 11 by tosylation of the primary alcohol followed by epoxidation with NaH and reaction with TMS-acetylene (22% yield from α-deuteration). The hydroxyl group in alkyne 11 was protected with TBS ether, followed by deprotection of the TMS and pivaloyl groups with NaOMe in MeOH to give the alcohol 12 in an 86% yield from 11. Enyne 13 was obtained in a 51% yield from 12 via 4 steps, (i) tosylation of the primary alcohol; (ii) cyation with NaCN; (iii) reduction of the nitrile group with DIBAL-H to aldehyde; and (iv) a Wittig reaction with Ph3PCH3I and NaHMDS. It was confirmed by HM-NMR that the deuteration rate did not decrease in these reaction steps [30].

Figure 2. Synthetic strategy of deuterium-labeled D3 metabolites. (A) Structures of reported deuterium-labeled D3 metabolites. (B) Previous work on the synthesis of 2-4-d3. (C) Previous work on the synthesis of 2-4-d3 (D) This work: general synthesis of deuterium-labeled D3 metabolites.

2. Results

We employed a convergent strategy using the palladium-catalyzed coupling reaction of enyne-type deuterium-labeled A-ring precursors 13-d3 and 16-d3 with bromoolefins 17 and 18 as the CD-ring moieties. The deuterium atoms in 13-d3 and 16-d3 were introduced by the H/D exchange at the a-position of the alcohol, as reported by Sajiki et al. [28]. Our synthesis of deuterium-labeled enyne 13 commenced with the H/D exchange reaction of alcohol 10, which was obtained from L(-)-malic acid in 4 steps (Scheme 1) [29]. The alcohol 10 was subjected to the H/D exchange reaction with a catalytic amount of Ru/C in D2O at 80 °C under an H2 atmosphere to afford 10-d3 deuterium-labeled at C3 and C4 in a 96% yield with over 93% deuteride content [28]. In this reaction, the stereochemistry at C3 was isomerized (4:1 ratio of α-10a and β-10b). The deuterium-labeled alcohol 10 (enantiomeric mixture) was converted into alkyne 11 by tosylation of the primary alcohol followed by epoxidation with NaH and reaction with TMS-acetylene (22% yield from 10-d3). The hydroxyl group in alkyne 11 was protected with TBS ether, followed by deprotection of the TMS and pivaloyl groups with NaOMe in MeOH to give the alcohol 12 in an 86% yield from 11. Enyne 13 was obtained in a 51% yield from 12 via 4 steps, (i) tosylation of the primary alcohol; (ii) cyation with NaCN; (iii) reduction of the nitrile group with DIBAL-H to aldehyde; and (iv) a Wittig reaction with Ph3PCH3I and NaHMDS. It was confirmed by HM-NMR that the deuteration rate did not decrease in these reaction steps [30].
Next, the deuterium-labeled enyne 16 bearing a hydroxyl group at C1α was synthesized (Scheme 2). The alcohol moiety in 12 was oxidized with an IBX and the resulting aldehyde was reacted with a HWE Wittig reagent to give an unsaturated ester, whose ester group was reduced with a DIBAL-H to give allyl alcohol 14 in a 70% yield from 12 [31,32]. The allyl alcohol 14 was subjected to a Sharpless asymmetric epoxidation with a TBHP in the presence of Ti(OiPr)₄ and L-(+)-DET [33], and the resulting epoxy alcohol was subjected to iodination with iodine and triphenylphosphine followed by treatment with zinc to give a secondary alcohol 15 in a 79% yield (3 steps) [31,32]. The diastereomer ratio at C1 in 15 was 10:1, and the undesired C1β diastereomer was removed by kinetic resolution, using acylation with isopropyl acid anhydride in the presence of (2S,3R)-HyperBTM [34], to give (−)-15 in an 82% yield as a single diastereomer. The undesired diastereomer at C3 was also removed via silica gel column purification. The deuterium-labeled enyne 16, in which the secondary alcohol was protected as the TBS ether, was obtained in an 82% yield.

The palladium-catalyzed coupling reaction of 13-d₃ and bromooliefin 17 followed by deprotection of the silyl groups provided 25(OH)D₃-d₃ (2-d₃) in a 36% yield [35]. Next, 25(OH)D₃-23,26-lactone-d₃ (6-d₃) and 1,25(OH)₂D₃-23,26-lactone-d₃ (7-d₃) were similarly synthesized by reacting bromooliefin 18 and enynes 13-d₃ and 16-d₃, respectively [36]. In the synthesis of 2-d₃ and 6-d₃, the undesired diastereomers at C3α were separated by an HPLC (Scheme 3).
Scheme 3. Synthesis of vitamin D₃ metabolites-d₃ (2-d₃, 6-d₃, 7-d₃).

2.1. Derivatization of 2, 6, 7 for LC-MS/MS, and Preparation of Calibration Curves

With the deuterium-labeled D₃ metabolites of 2-d₃, 6-d₃, and 7-d₃ in hand, we next examined the quantitative analysis of the three D₃ metabolites in pooled human serum by LC-MS/MS. First, we confirmed that our deuterium-labeled D₃ metabolites were suitable as the internal standards for the isotope dilution method in an LC-MS/MS analysis. As described above, D₃ and its metabolites have low ionization efficiency in an LC-MS/MS, and derivatization is necessary to improve the ionization efficiency. Thus, the D₃ metabolites 2, 6, and 7, as well as 2-d₃, 6-d₃, and 7-d₃, were derivatized with a recently developed reagent DAP-PA (4-(4′-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione-phenyl anthracene) [20], and the ion peaks of the DAP adducts were detected by selective reaction monitoring (SRM) under the LC-MS/MS conditions shown in Table 1 (Figure 3).

Table 1. Parameters for the LC/MS/MS analysis.

| Compound | SRM Transition (m/z) | Cone Voltage (kv) | CE (eV) |
|----------|----------------------|------------------|--------|
| 25(OH)D₃-DAP (2-DAP) | 619.4 > 341.2 [M + H]⁺ [A]⁺ | 48 | 28 |
| 25(OH)D₃-23,26-lactone-DAP (6-DAP) | 647.4 > 341.2 | 48 | 28 |
| 1,25(OH)₂D₃-23,26-lactone-DAP (7-DAP) | 663.4 > 357.2 | 48 | 28 |
| 25(OH)D₃-d₃-DAP (2-d₃-DAP) | 622.4 > 344.2 | 48 | 28 |
| 25(OH)D₃-23,26-lactone-d₃-DAP (6-d₃-DAP) | 650.4 > 344.2 | 48 | 28 |
| 1,25(OH)₂D₃-23,26-lactone-d₃-DAP (7-d₃-DAP) | 666.4 > 360.2 | 48 | 28 |

Figure 3. SRM chromatograms for the DAP adducts of D₃ metabolites 2, 6, and 7, as well as 2-d₃, 6-d₃, and 7-d₃.

In the case of the DAP-adducts of 2 and 2-d₃ (Figure 3A,B), we observed identical ion peaks at the retention time of 5.60 min (abbreviated as tᵣ: 5.60 min). Similarly, 6 and 6-d₃ showed the same tᵣ of 3.50 min, and 7 and 7-d₃ showed the same tᵣ of 2.15 min, indicating that the deuterium-labeled compounds are suitable as internal standards for the...
isotope dilution method. We also observed small peaks at the retention times of 5.20 min (Figure 3A,B), 2.65 min (Figure 3C,D), and 2.37 min (Figure 3E,F) for 2/2-d3, 6/6-d3, and 7/7-d3, respectively. These peaks are due to the epimers at C6 of the DAP adducts, because DAP-PA reacts from both the α- and β-faces.

Next, the calibration curves were prepared as follows (Figure 4). A total of 100 μL of each one of the calibrator solutions was mixed with 200 μL of the internal standard solution and evaporated to dryness. After derivatization with DAP-PA, an LC-MS/MS analysis of the unlabeled and labeled DAP-adducts was performed, and the calibration curves were prepared by plotting the concentration of unlabeled DAP-adduct against the ion peak area ratio of unlabeled versus labeled DAP-adduct. All of the calibration curves showed good linearity.

![Figure 4](image_url)

**Figure 4.** Calibration curve of D3 metabolites; (A) 25(OH)D3 (2); (B) 25(OH)D3-23,26-lactone (6); and (C) 1,25(OH)2D3-23,26-lactone (7).

### 2.2. Quantification of the D3 Derivatives in Human Serum

The levels of 2, 6, and 7 in pooled human serum were quantified by the LC-MS/MS using the isotope dilution method with the constructed calibration curves. The serum was pretreated as follows. An aliquot of serum (100 μL) was mixed with the internal standards solution (200 μL). Each sample was loaded onto a supported liquid extraction column (ISOLUTE SLE+ 300 μL sample Volume, Biotage, Uppsala, Sweden) and eluted three times with 600 mL hexane/ethyl acetate (1/1, v/v) using a PRESSURE+48 positive pressure manifold (Biotage, Uppsala, Sweden). The combined eluates were evaporated to dryness in a centrifugal evaporator. The ion peaks of the metabolites matched well with those of the corresponding internal standards in the pretreated samples (Figure 5).

![Figure 5](image_url)

**Figure 5.** SRM chromatograms for D3 derivatives 2, 6, and 7 in pooled human serum.

The concentrations of 2, 6, and 7 in human serum were calculated to be 5.1 ng/mL, 38.3 pg/mL, and 8.9 pg/mL, respectively, based on the area ratios of the detected peaks. The concentrations of 2 and 6 were in agreement with previously reported values [37], while this is the first quantification of 1α-lactone 7 in human serum.
We synthesized deuterium-labeled A-ring-d₃ synths 13 and 16 and utilized them for the convergent synthesis of deuterium-labeled D₃ derivatives 25(OH)D₃ (2), 25(OH)D₃-23, 26-lactone (6), and 1,25(OH)₂D₃-23, 26-lactone (7). These deuterium-labeled D₃ metabolites were successfully applied as internal standards for the quantification of the metabolites in pooled human serum by LC-MS/MS using the isotope dilution method. This is the first quantification of 1,25(OH)₂D₃-23, 26-lactone (7) in human serum.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27082427/s1, Experimental procedures for synthesis and characterization of compounds, Experimental procedure for LC-MS/MS analysis using the isotope dilution method, ¹H and ¹³C NMR spectra.

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