Original Article

A Method for Simultaneous Determination of 25-Hydroxyvitamin D₃ and Its 3-Sulfate in Newborn Plasma by LC/ESI-MS/MS after Derivatization with a Proton-Affinitive Cookson-Type Reagent

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A method for the simultaneous determination of 25-hydroxyvitamin D₃ [25(OH)D₃] and its 3-sulfate [25(OH)D₃S] in newborn plasma, which is expected to be helpful in the assessment of the vitamin D status, using stable isotope-dilution liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) has been developed and validated. The plasma was pretreated based on the deproteinization and solid-phase extraction, then subjected to derivatization with 4-(4-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione (DAPTAD). The derivatization enabled the accurate quantification of 25(OH)D₃ without interference from 3-epi-25(OH)D₃ and also facilitated the simultaneous determination of the two metabolites by LC/positive ESI-MS/MS. Quantification was based on the selected reaction monitoring with the characteristic fragmentation of the DAPTAD-derivatives during MS/MS. This method was reproducible (intra- and inter-assay relative standard deviations of 7.8% or lower for both metabolites) and accurate (analytical recovery, 95.4–105.6%). The limits of quantification were 1.0 ng/mL and 2.5 ng/mL for 25(OH)D₃ and 25(OH)D₃S, respectively, when using a 20-µL sample. The developed method was applied to the simultaneous determination of plasma 25(OH)D₃ and 25(OH)D₃S in newborns; it was recognized that the plasma concentration of 25(OH)D₃S is significantly higher than that of 25(OH)D₃, and preterm newborns have lower plasma 25(OH)D₃S concentrations than full-term newborns.

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INTRODUCTION

The plasma/serum concentration of 25-hydroxyvitamin D₃ [25(OH)D₃], the major circulating metabolite of vitamin D₃, is well recognized as an indicator of the vitamin D status. Vitamin D deficiency/insufficiency in a newborn infant is associated not only with several bone metabolic diseases, such as rickets, but also with a wide range of adverse health outcomes, such as type 1 diabetes, multiple sclerosis, and schizophrenia, in later life. 25-Hydroxyvitamin D₃ 3-sulfate [25(OH)D₃S], the sulfated conjugate of 25(OH)D₃, is another major metabolite of vitamin D₃, and its circulating level was found to be much higher than that of 25(OH)D₃ in infants. 25(OH)D₃S might be the storage form of vitamin D₃; 25(OH)D₃S may be utilized after deconjugation to 25(OH)D₃. Therefore, it is expected that the simultaneous determination of 25(OH)D₃ and 25(OH)D₃S in plasma/serum is also helpful in the assessment of the vitamin D status and diagnosis for vitamin D deficiency/insufficiency of newborns/infants.

Liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) is now the most commonly used method to determine vitamin D metabolites in various biological samples due to its high specificity and accuracy. Many LC/ESI-MS/MS assays for the serum/plasma 25(OH)D₃ have been reported, but not without a problem in these assays. One of the complicated problems
in the 25(OH)D$_3$ quantification is the potential interference from its inactive epimer, 3-epi-25-hydroxyvitamin D$_3$ [3-epi-25(OH)D$_3$], leading to overestimation of the true 25(OH)D$_3$ concentrations$^{11,12}$; it was reported that the epimer contributed 9–61% of the total 25(OH)D$_3$ in infants aged 0. To overcome this problem, we have developed 4-(4-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione (DAPTAD) as a novel Cookson-type reagent$^{13}$ (Fig. 1). The Cookson-type reagent is the 4-substituted-1,2,4-triazoline-3,5-dione (PTAD), a representative Cookson-type reagent.$^{14}$ Furthermore, the detection response of the DAPTAD-derivatized 25(OH)D$_3$ in the positive ESI-MS/MS responded well in the 25(OH)D$_3$ assay when only a limited blood sample volume is obtained from newborns/infants.$^{15}$ Thus, DAPTAD is a promising derivatization reagent for the trace quantification of 25(OH)D$_3$, and 25(OH)D$_3$S in preterm and full-term newborns is also described.

**EXPERIMENTAL**

**Chemicals and reagents**

25(OH)D$_3$ and 3-epi-25(OH)D$_3$ were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Cayman Chemical Company (Ann Arbor, MI, USA), respectively. 25(OH)D$_3$S and 7-dehydro-25-hydroxycholesterol 3-sulfate were synthesized from 25(OH)D$_3$ and 7-dehydro-25-hydroxycholesterol, respectively, according to the method of Iida et al.$^{16}$ in our laboratories. $[6,19,19-^2$H$_3]$, 25(OH)D$_3$ [H$_3$-25(OH)D$_3$] and [26,26,26,27,27,27-^2$H$_6$]-25(OH)D$_3$ [H$_6$-25(OH)D$_3$S] [internal standards (ISs)] were purchased from IsoSciences (King of Prussia, PA, USA). The stock solutions of 25(OH)D$_3$ and 25(OH)D$_3$S were prepared as 2.5 µg/mL solutions in ethanol, and their concentrations were confirmed by UV spectroscopy using the molar absorptivity (ε) of 18200 at 265 nm. Subsequent dilutions were carried out using ethanol to prepare 2.0, 5.0, 10, 20, 50 and 100 ng/mL solutions. The ethanolic solutions of the ISs (25 ng/mL) were also prepared. DAPTAD was the same as used in a previous study.$^{17}$ The DAPTAD precursor (urazole form), which can be easily converted into DAPTAD by the oxidation, is now commercially available from several manufacturers. An Oasis® HLB cartridge (30 mg adsorbent; Waters, Milford, MA, USA) was successively washed with methanol (1 mL) and water (1 mL) prior to use. All other reagents and solvents were of analytical grade or LC/MS grade.

**LC/ESI-MS/MS**

LC/ESI-MS/MS was performed using a Waters Quattro Premier XE triple quadrupole-mass spectrometer connected to an LC-e2695 chromatograph. A YMC-Pack Pro C-18 RS (3 µm, 150×2.0 mm i.d., Kyoto Japan) was used at the flow rate of 0.2 mL/min at 40°C. A gradient elution program with mobile phase A [methanol–10 mM ammonium formate (4:1, v/v) containing 0.05% (v/v) formic acid] and mobile phase B [acetonitrile–10 mM ammonium formate (9:1, v/v) containing 0.05% (v/v) formic acid] was performed for quantification of the vitamin D$_3$ metabolites in the plasma; B=0% maintained (0–3 min), 100% linearly increased (3–8 min) and maintained (8–10 min), and 0% maintained (10–26 min). The DAPTAD-derivatized vitamin D$_3$ metabolites and ISs were analyzed in the positive-ion mode unless otherwise noted, and the conditions were as follows: capillary voltage, 2.8 kV; cone voltage, 40 V [for
25(OH)D₃, determination] or 35V (for 25(OH)D₃S determination); collision energy, 25 eV; column temperature, 120°C; desolvation temperature, 350°C; desolvation gas (N₂) flow rate, 600 L/h; cone gas (N₂) flow rate, 50 L/h; and collision gas (Ar) flow rate, 0.19 mL/min. The selected reaction monitoring (SRM) transitions were as follows: 25(OH)D₃-DAPTAD: m/z 619.6→341.3, 2H₃-25(OH)D₃-DAPTAD: m/z 622.7→344.2, 25(OH)DS-DAPTAD: m/z 699.6→421.2 and 2H₆-25(OH)DS-DAPTAD: m/z 705.6→421.2. When the DAPTAD-derivatized 25(OH)DS were analyzed in the negative-ion mode, the cone voltage and collision energy were set at 40 V and 60 eV, respectively. The other parameters were the same as used in the positive-ion mode. The SRM transition was as follows: 25(OH)DS-DAPTAD: m/z 697.6→96.9. Masslynx software (version 4.1, Waters) was used for the system control and data processing.

Pretreatment of plasma

The plasma (20 µL) was added to acetonitrile (50 µL) containing the ISs (0.050 ng each), vortex-mixed for 30 s and centrifuged at 10000 × g (10 min). The supernatant was diluted with water (300 µL), and the sample was passed through an Oasis HLB cartridge. After washing with water (1 mL) and methanol–water (1:1, v/v) (1 mL), the vitamin D₃ metabolites and ISs were eluted with methanol (1 mL). After removal of the solvent, the residue was subjected to derivatization with DAPTAD. When the 25(OH)D₃S concentration was over 50 ng/mL, 10 µL of the plasma was used for the quantification.

Derivation

The standard vitamin D₃ metabolites and pretreated plasma samples were dissolved in ethyl acetate (50 µL) containing DAPTAD (20 µg). The mixture was stored at room temperature for 1 h, then ethanol (40 µL) was added to the mixture to terminate the reaction. The solvent was evaporated, then the residue was dissolved in mobile phase A (60 µL), 15 µL of which was injected into the LC/ESI-MS/MS.

Comparison of positive- and negative-ion modes for detection of 25(OH)D₃S-DAPTAD

The positive- and negative-ion modes were compared for the detection of the DAPTAD-derivatized 25(OH)D₃S. The limits of detection [LODs; the amount of the derivatized 25(OH)D₃S per injection giving a signal to noise ratio (S/N) of 3] were determined in both modes. The S/N value was manually calculated by division of the peak height of the derivatized 25(OH)D₃S by the noise level around the peak. 25(OH)DS (100 pg) was derivatized with DAPTAD and the resulting derivative was dissolved in the mobile phases (100 µL) described below, then subjected to LC/ESI-MS/MS. By stepwise decreasing the injection volume of the resulting solution, the LOD was determined. The mobile phase (isocratic elution) used in the positive-ion mode was methanol–10 mM ammonium formate (4:1, v/v) containing 0.05% (v/v) formic acid, and in the negative-ion mode was methanol–10 mM ammonium formate (4:1, v/v).

Calibration curves

The plasma (20 µL) was added to acetonitrile (50 µL) containing the ISs (0.050 ng each), graduated amounts of standard 25(OH)D₃ (0.020, 0.050, 0.10, 0.20, 0.50 or 1.0 ng; corresponding to 1.0, 2.5, 5.0, 10, 25 or 50 ng/mL) and 25(OH)D₃S (0.050, 0.10, 0.20, 0.50 or 1.0 ng; corresponding to 2.5, 5.0, 10, 25 or 50 ng/mL), and pretreated, then derivatized in the same way as described in the above sections. The peak area ratio [25(OH)D₃/2H₃-25(OH)D₃ or 25(OH)D₃S/2H₆-25(OH)D₃S] (y) was plotted versus the added amount of 25(OH)D₃ or 25(OH)D₃S (ng per tube) (x) with a weighting of 1/x to construct the regression lines.

The ISs (0.50 ng each), 25(OH)D₃ (0.020, 0.050, 0.10, 0.20, 0.50 or 1.0 ng) and 25(OH)D₃S (0.050, 0.10, 0.20, 0.50 or 1.0 ng) were pipetted into tubes, then derivatized. The resulting derivatives were dissolved in mobile phase A (60 µL), 15 µL of which was subjected to LC/ESI-MS/MS. The peak area ratio [25(OH)D₃/2H₃-25(OH)D₃ or 25(OH)D₃S/2H₆-25(OH)D₃S] (y) was plotted versus the amount of 25(OH)D₃ or 25(OH)D₃S (ng per tube) (x) with a weighting of 1/x, and the obtained regression lines were used as the calibration curves.

Precision and accuracy (analytical recovery)

The intra- and inter-assay precisions were assessed by the repeated measurements (n=5) of two plasma samples A and B on one day and over five days, respectively. The precision was determined as the relative standard deviation (RSD, %).

The assay accuracy was determined using plasma samples A and B. The plasma (20 µL) was added to acetonitrile (50 µL) containing the ISs (0.50 ng each), 25(OH)D₃ and 25(OH)D₃S (0.10 or 0.20 ng each; corresponding to 5.0 or 10 ng/mL) (spiked sample), and pretreated, then derivatized in the same way as described in the above sections. The assay accuracy (analytical recovery) was defined as \( F(F₀/A) \times 100 \) (%), where \( F \) is the concentration of 25(OH)D₃ or 25(OH)D₃S in the spiked sample, \( F₀ \) is the concentration of 25(OH)D₃ or 25(OH)D₃S determined in the inter-assay precision test and \( A \) is the spiked concentration.

Freeze/thaw stability

The freeze/thaw stabilities of 25(OH)D₃ and 25(OH)D₃S in the plasma were determined by analyzing plasma samples A and B without/with 3 additional freeze/thaw cycles.

Absolute recovery

The plasma specimen not containing the vitamin D₃ metabolites could not be prepared by the charcoal-treatment because they are tightly bound to the plasma protein. Therefore, the absolute recovery from the plasma specimen was evaluated using the ISs (2H₃-25(OH)D₃ and 2H₆-25(OH)D₃S, 0.50 ng/20 µL plasma). The recovery rates were calculated from the peak area ratio [25(OH)D₃/2H₃-25(OH)D₃ or 25(OH)D₃S/2H₆-25(OH)D₃S] in samples I and II as described below; recovery=peak area ratio in sample II/peak area ratio in sample I.

Sample I (n=6): The plasma (20 µL) was added to acetonitrile (50 µL) containing the ISs (0.050 ng each), pretreated and then derivatized as already described.

Sample II (n=6): The plasma (20 µL) was added to acetonitrile (50 µL) and pretreated in the same way as already described. After the addition of the ISs (0.050 ng each) to this pretreated plasma, the resulting sample was subjected to the derivatization.

Newborn plasma sample

The anonymized plasma samples from 59 Japanese
newborns (gestational age, 30.1–41.0 weeks) including 39 preterm newborns of both sexes were examined. Blood was collected from their dorsal hand vein within 28 days after birth at the Shizuoka Saiseikai General Hospital (Shizuoka, Japan). Written informed consent was obtained from their parents. Thirteen cord blood samples were also obtained at delivery, and then the plasma was separated. The experimental procedures were approved by the Ethics Committees of the Tokyo University of Science and Shizuoka Saiseikai General Hospital.

Although the plasma was used in this study for no particular reason, it was reported that there is no significant difference between the 25(OH)D₃ concentration in plasma and that in serum.15) 25(OH)D₃ in plasma/serum has also been demonstrated to be extremely stable; when it is stored at room temperature, marked degradation is not observed at least for 24 h.15)

RESULTS AND DISCUSSION

Derivatization of 25(OH)D₃ and 25(OH)D₃S with DAPTAD

The DAPTAD-derivatization for the vitamin D₃ metabolites was carried out at room temperature for 1 h by reference to previous studies13,16 (Fig. 1). Our previous study demonstrated that the derivatization rate was almost quantitative for 25(OH)D₃ under this reaction condition.13) When 25(OH)D₃S (1.0 ng) was derivatized with DAPTAD under the same condition and one-quarter of the reaction mixture [equivalent to 250 pg of the intact 25(OH)D₃S] was then subjected to LC/ESI-MS/MS, the underivatized 25(OH)D₃S was not detected. Our LC/ESI-MS/MS (negative-ion mode) can detect 3.0 pg of intact 25(OH)D₃S as previously reported5); this amount was 1.2% of the injected amount (250 pg) if the entire 25(OH)D₃S had remained underivatized. This result demonstrated that the derivatization rate was also almost quantitative for 25(OH)D₃S.

ESI-MS/(MS) behavior of DAPTAD-derivatized 25(OH)D₃ and 25(OH)D₃S

The positive ESI-MS and -MS/MS behaviors of the DAPTAD-derivatized 25(OH)D₃ [25(OH)D₃-DAPTAD] have been previously reported,13) and a similar behavior was observed for 25(OH)D₃S-DAPTAD; 25(OH)D₃S-DAPTAD provided a protonated molecule ([M+H]+) at m/z 699.6 as the base peak in the ESI-MS, and the CID of [M+H]+ gave the characteristic fragment ion at m/z 421.2, which was derived from the cleavage of the C6–7 bond of the vitamin D skeleton, with a sufficient intensity (Fig. 2a). Thus, 25(OH)D₃S could be detected in the positive ESI-MS/MS

Fig. 2. Product ion spectra of DAPTAD-derivatized 25(OH)D₃S in (a) positive-ion mode and (b) negative-ion mode.

The spectra were recorded by the collisional activation of [M+H]+ and [M−H]−, respectively, under the conditions described in Experimental section.
after conversion to the DAPTAD-derivative due to the enhanced proton-affinity by the derivatization. On the other hand, 25(OH)D₃-DAPTAD could also be detected in the negative-ion mode because it has a sulfate group; it provided a deprotonated molecule ([M–H]⁻) at m/z 697.6 as the base peak in the negative ESI-MS, and the CID of [M–H]⁻ gave only the fragment ion derived from the sulfate group at m/z 96.9 (Fig. 2b).

The LODs in the positive-ion mode were 0.37 and 6.3 fmol for the derivatized 25(OH)D₃ and 25(OH)D₃S, respectively. The detection response of 25(OH)D₃-DAPTAD was approximately 30-fold higher than that of the intact 25(OH)D₃ as previously reported.¹³ The use of ammonium acetate and acetic acid as the mobile phase additives instead of ammonium formate and formic acid caused little change in the chromatographic behaviors of the derivatized vitamin D₃ metabolites, but decreased the assay sensitivity to one-half for 25(OH)D₃. The LOD of 25(OH)D₃S-DAPTAD in the positive ESI-MS/MS [6.3 fmol, equivalent to 3.0 pg of intact 25(OH)D₃S] was quite similar to that of the intact 25(OH)D₃S analyzed in the negative ESI-MS/MS (m/z 479.1 [M–H]⁻→96.6 [SO₃H]) ¹⁰. Because 25(OH)D₃S has a strong acidic functional group, a sulfate group, its DAPTAD-derivative might be less efficiently protonated during the positive ESI than the neutral compounds, such as 25(OH)D₃-DAPTAD. Recently, Gomes et al. reported that the PTAD-derivatization followed by positive ESI-MS/MS did not increase the sensitivity for the analysis of the sulfated vitamin D₃ compounds;¹⁷ the detection response of 25(OH)D₃S-PTAD in the positive-ion mode was approximately 90% less than that of the intact 25(OH)D₃S analyzed in the negative-ion mode. Despite the inefficiency of PTAD for increasing the sensitivity, the serum 25(OH)D₃S was quantified together with other non-sulfated vitamin D₃ metabolites as the PTAD-derivatives by LC/positive-ESI-MS/MS. As described above, the DAPTAD-derivatization enabled the positive ESI-MS/MS detection of 25(OH)D₃S without reducing the assay sensitivity, contrary to the PTAD-derivatization. The calculated proton affinity of the N,N-dimethylamino moiety in DAPTAD is reported to be 941 kJ/mol, which is much higher than that of benzene (750 kJ/mol) in PTAD.¹⁰ This is a major reason why the DAPTAD-derivatization was superior to the PTAD-derivatization for the analysis of 25(OH)D₃S by the positive ESI-MS/MS.

As already described, two SRM modes with transitions of m/z 699.6→421.2 (positive-ion mode) and m/z 697.6→96.9 (negative-ion mode) were applicable for the detection of 25(OH)D₃S-DAPTAD, but the LOD in the negative-ion mode (6.3 fmol) was higher than that in the positive-ion mode (0.37 fmol). Based on this result, the positive-ion mode was employed for the detection of the 25(OH)D₃S as the DAPTAD-derivative in the following studies.

**LC behavior of DAPTAD-derivatized 25(OH)D₃ and 25(OH)D₃S**

The DAPTAD-derivatives of the vitamin D₃ metabolites consist of the 6R- (minor) and 6S-isomers (major),¹³ therefore, the derivatives sometimes give characteristic two peaks on their chromatograms. Under the LC conditions used in this study, the retention times (tᵣ) of 25(OH)D₃-DAPTAD were 7.1 (6R) and 8.2 min (6S). A satisfactory separation of 25(OH)D₃ and 3-epi-25(OH)D₃, which is a potential interfering metabolite in the 25(OH)D₃ assay,¹¹,¹² was achieved by the DAPTAD-derivatization; the resolution (Rs) for the major peak (tᵣ 8.2 min) of 25(OH)D₃-DAPTAD and 3-epi-25(OH)D₃-DAPTAD (tᵣ 7.7 min; the 6R/S-isomers of 3-epi-25(OH)D₃-DAPTAD co-eluted as a single peak) was 1.40.

25(OH)D₃S-DAPTAD was eluted at 6.1 min as a single peak. 7-Dehydro-25-hydroxycholesterol 3-sulfate, a possible endogenous steroid, has the same molecular weight as 25(OH)D₃S and also reacts with DAPTAD. The DAPTAD-derivatized 7-dehydro-25-hydroxycholesterol 3-sulfate (tᵣ 1.8 min) was chromatographically well separated from the derivatized 25(OH)D₃S under the LC conditions and did not provide a fragment ion at m/z 421.2 during the MS/MS measurement. Thus, this steroid did not interfere with the 25(OH)D₃S measurement.

**Pretreatment of plasma**

The plasma (20 μL) was deproteinized in acetonitrile and the supernatant was then purified using an Oasis® HLB cartridge. The absolute recovery rates [ mean±standard deviation (S.D.), n=5] of 1H₂-25(OH)D₃ and 2H₆-25(OH)D₃S from the plasma specimen were 83.1±2.1 and 84.8±4.0%, respectively. Because the present method employed a stable isotope dilution technique, the absolute recovery rates of 25(OH)D₃ and 25(OH)D₃S from a plasma specimen were considered to be similar to those of the ISs.

The chromatograms obtained from a newborn with ISs are shown in Fig. 3, in which the peaks corresponding to 25(OH)D₃ (tᵣ 7.1 and 8.2 min) and 25(OH)D₃S (tᵣ 6.1 min) were clearly observed with satisfactory shapes.

**Linearity and calibration curves**

In order to assess the linearity, the ISs (0.50 ng each), graduated amounts of 25(OH)D₃ (0.020–1.0 ng/20 μL plasma) and 25(OH)D₃S (0.050–1.0 ng/20 μL plasma) were added to five different plasma samples and analyzed by LC/ESI-MS/MS after the pretreatment and derivatization. The regression lines showed a satisfactory linearity with determination coefficients (r²) of greater than 0.997 and reproducible slopes [2.082±0.0489 (mean±S.D.) and 2.4% (RSD)] were obtained for 25(OH)D₃. The r² values and slopes of the regression lines for 25(OH)D₃S were >0.997 and 1.8265±0.0464 (RSD 2.5%), respectively. When compared to the lines obtained using standard solutions of 25(OH)D₃ [range; 0.020–1.0 ng per tube, r² >0.998 and slope; 2.142±0.0283 (RSD 1.3%)] and 25(OH)D₃S [range; 0.050–1.0 ng per tube, r² >0.997 and slope; 1.8279±0.0637 (RSD 3.5%)], there were no significant differences between the slopes of the lines obtained from the standard-added plasma samples and the standard solutions. These results demonstrated that the plasma matrix had no influence on the determination of 25(OH)D₃ and 25(OH)D₃S as the DAPTAD-derivatives. Furthermore, it has been reported that calibration curves constructed with the standard samples (non-matrix sample) can be used for the quantification as long as the ISs co-elute with the analytes of interest; the matrix matching is not always necessary for the stable-isotope dilution LC/MS/MS. Based on these data, the calibration curves constructed using the standard solutions were used for the quantification in the following studies.
Simultaneous determination of newborn plasma 25(OH)D\textsubscript{3} and 25(OH)D\textsubscript{3S}

The 25(OH)D\textsubscript{3} and 25(OH)D\textsubscript{3S} concentrations in the newborn plasma were simultaneously determined based on the developed method (Fig. 4). The plasma concentration of 25(OH)D\textsubscript{3S} was 30.1±12.2 ng/mL (mean±S.D., n=59) with the range of 3.9–65.1 ng/mL and significantly higher than that of 25(OH)D\textsubscript{3} (7.1±3.0 ng/mL, 2.7–17.0 ng/mL); these agreed with previously reported results.\textsuperscript{5–7} As mentioned in the introduction, 25(OH)D\textsubscript{3S} might be the storage form of vitamin D\textsubscript{3} and utilized after deconjugation to 25(OH)D\textsubscript{3}.\textsuperscript{5–7}

Based on this concept, the simultaneous determination of the plasma 25(OH)D\textsubscript{3} and 25(OH)D\textsubscript{3S} will be more helpful than the determination of 25(OH)D\textsubscript{3} alone in the assessment of the vitamin D status and diagnosis for vitamin D deficiency/insufficiency of newborns. The preterm newborns have lower plasma 25(OH)D\textsubscript{3S} concentrations (Pearson’s correlation coefficient test, \(p<0.01\)), whereas the plasma

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**Table 1. Assay precision and accuracy.**

|                          | Plasma A (\textsubscript{n}=5) | Plasma B (\textsubscript{n}=5) | Plasma A (\textsubscript{n}=5) | Plasma B (\textsubscript{n}=5) |
|--------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| **Intact sample (intra-assay, \(n=5\))** |                                |                                 |                                |                                 |
| Measured\textsuperscript{a} (ng/mL) | 7.92±0.36                      | 6.29±0.13                       | 20.62±0.43                      | 15.58±0.43                      |
| Precision (RSD, %)       | 4.5                             | 2.1                             | 2.1                             | 2.7                             |
| **Intact sample (inter-assay, \(n=5\))** |                                |                                 |                                |                                 |
| Measured\textsuperscript{a} (ng/mL) | 8.02±0.23                      | 5.76±0.45                       | 22.65±1.35                      | 15.16±0.32                      |
| Precision (RSD, %)       | 2.9                             | 7.8                             | 5.9                             | 2.1                             |
| **Spiked sample (+5.0 ng/mL, \(n=2\))** |                                |                                 |                                |                                 |
| Measured\textsuperscript{a} (ng/mL) | 12.83                           | 10.70                           | 29.19                           | 20.93                           |
| Accuracy (%)             | 98.5                            | 99.4                            | 105.6                           | 103.8                           |
| **Spiked sample (+25 ng/mL, \(n=2\))** |                                |                                 |                                |                                 |
| Measured\textsuperscript{a} (ng/mL) | 31.51                           | 29.95                           | 49.29                           | 41.50                           |
| Accuracy (%)             | 95.4                            | 97.4                            | 103.4                           | 103.3                           |

\textsuperscript{a} Mean or mean±S.D.
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25(OH)D₃ concentration was not related to the gestational age (p=0.36). The low level of 25(OH)D₃S may be a possible cause of rickets that is more common in the preterm newborns. The anonymized samples were examined in this study; no subject information other than the gestational age was provided. Therefore, the gender differences in the plasma concentrations of the vitamin D₃ metabolites were not examined in this study.

Using the developed method, the concentrations of 25(OH)D₃ and 25(OH)D₃S in the cord plasma were also determined and compared with those in the plasma of newborns of 0 or 1 day old (n=13). For both metabolites, there were good correlations in the concentrations between the newborn plasma and cord plasma as shown in Fig. 5 (Pearson’s correlation coefficient test, p<0.01). This result indicates that the cord plasma can also be used as the specimen for the assessment of the vitamin D status for newborns.

CONCLUSION

We have developed the stable isotope-dilution LC/ESI-MS/MS method for the simultaneous determination of 25(OH)D₃ and 25(OH)D₃S in newborn plasma. The method employed the DAPTAD-derivatization, which enabled the accurate quantification of 25(OH)D₃ without interference from 3-epi-25(OH)D₃ and also facilitated the simultaneous determination of the two metabolites by positive ESI-MS/MS. The method was able to quantify 1.0–50 ng/mL of 25(OH)D₃ and 2.5–50 ng/mL of 25(OH)D₃S with satisfactory accuracy and reproducibility using a 20-µL plasma sample. The developed method was successfully applied to the analysis of newborn plasma; it was recognized that the plasma concentration of 25(OH)D₃S is significantly higher than that of 25(OH)D₃ in newborns, and preterm newborns have lower plasma 25(OH)D₃S concentrations than full-term newborns. Thus, this well-characterized method will prove helpful in the assessment of the vitamin D status for newborns.

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