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A novel Cookson-type reagent for enhancing sensitivity and specificity in assessment of infant vitamin D status using liquid chromatography/tandem mass spectrometry

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RATIONALE: 25-Hydroxyvitamin D_3 [25(OH)D_3] is the best-established indicator of vitamin D status. 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD), a representative Cookson-type reagent, has often been employed for enhancing the sensitivity in the trace determination of 25(OH)D_3 in a neonatal dried blood spot (DBS), which contains only 2.65 μL of whole blood, using liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). The objective of this study was the development of a novel Cookson-type reagent surpassing PTAD in terms of sensitivity and specificity in the LC/ESI-MS/MS assay of 25(OH)D_3.

METHODS: A novel Cookson-type reagent, 4-(4′-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione (DAPTAD), was synthesized from 4-dimethylaminobenzoyl chloride. The DAPTAD-derivative of 25(OH)D_3 was prepared and its LC/ESI-MS/MS behavior was examined. The applicability of the DAPTAD-derivatization in the determination of 25(OH)D_3 in neonatal DBSs was also examined.

RESULTS: The derivatization was completed at room temperature within 1 h. The DAPTAD-derivative of 25(OH)D_3 provided a characteristic product ion derived from the cleavage of the vitamin D skeleton during MS/MS. The limit of detection of the DAPTAD-derivative during selected reaction monitoring was 0.25 fmol on the column, which was 30 and 2 times lower than those of the intact 25(OH)D_3 and the PTAD-derivative, respectively. The DAPTAD-derivatization followed by LC/ESI-MS/MS enabled the detection of a trace amount (in the low-ng/mL range) of 25(OH)D_3 in DBSs with a simple pretreatment (only methanol extraction) and short chromatographic run time (10 min). The DAPTAD-derivatization was also useful for the separation of 25(OH)D_3 from a potent interfering metabolite, 3-epi-25-hydroxyvitamin D_3 [3-epi-25(OH)D_3]. On the contrary, the assay using the PTAD-derivatization might lead to overestimation of the true 25(OH)D_3 levels due to the co-elution of 25(OH)D_3 and 3-epi-25(OH)D_3.

CONCLUSIONS: We developed DAPTAD for enhancing the sensitivity and specificity of the LC/ESI-MS/MS assay of 25(OH)D_3. Our new method using DAPTAD can reduce the overestimation of the 25(OH)D_3 levels, and will prove helpful in the diagnosis of vitamin D deficiency in infants. Copyright © 2013 John Wiley & Sons, Ltd.

The measurement of 25-hydroxyvitamin D_3 [25(OH)D_3], which is the major circulating metabolite of vitamin D_3 and the best-established indicator of vitamin D status, in biological fluids is widely used for the diagnostic assessment and the follow-up of several bone metabolic diseases, such as rickets and osteoporosis.[1] In addition to these diseases, vitamin D deficiency in an infant is associated with a wide range of adverse health outcomes, such as type 1 diabetes,[2] multiple sclerosis,[3] and schizophrenia,[4] in later life; screening for vitamin D deficiency in an infant based on the measurement of the blood 25(OH)D_3 is important for the early supplement of vitamin D to the diagnosed infant.

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Recently, liquid chromatography (LC) coupled with electrospray ionization (ESI) tandem mass spectrometry (MS/MS) has been used for the analysis of vitamin D metabolites due to its specificity and versatility.[5,6] The serum/plasma concentration of 25(OH)D_3 can be measured using LC/ESI-MS/MS without much difficulty when a relatively large volume (over 100 μL) of sample is used due to its relatively high serum/plasma level (normal range, 10–40 ng/mL).[7–11] However, the ionization efficiency of 25(OH)D_3 is not high in ESI, and, therefore, in the analysis of 25(OH)D_3 in dried blood spots (DBSs), the insufficient sensitivity often becomes a major problem; the DBS technique is minimally invasive and offers a simpler storage and easier transport, but a disk of DBS contains only approximately 3 μL of whole blood. To enhance the assay sensitivity of vitamin D metabolites including 25(OH)D_3 in various biological samples, derivatization with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), a representative Cookson-type reagent, has often been employed.[5,12–19]

PTAD is a powerful dienophile which rapidly and quantitatively reacts with the *s-cis*-diene of vitamin D metabolites to form the Diels-Alder adducts. The PTAD-derivatives of the vitamin D metabolites are much more easily ionized in positive ESI-MS and efficiently produce characteristic product ions during the MS/MS compared to the underivatized vitamin D metabolites.[5,12] Thus, the PTAD-derivatization has been proven to enhance the sensitivity and be very useful in the LC/ESI-MS/MS of the vitamin D metabolites. However, a Cookson-type reagent having a higher proton-affinitive moiety will enable the more sensitive analysis of vitamin D metabolites using a smaller volume of biological specimens. Furthermore, one of the complicated problems in the 25(OH)D_3 assay is the potential interference from an inactive epimer of 25(OH)D_3, 3-epi-25-hydroxyvitamin D_3 [3-epi-25(OH)D_3], leading to overestimation of the true 25(OH)D_3 concentrations. Because 25(OH)D_3 and 3-epi-25(OH)D_3 have the same molecular masses and their derivatives with a Cookson-type reagent show the same fragmentation pattern during MS/MS, chromatographic separation of these epimers is necessary for the accurate determination of 25(OH)D_3. However, our previous study demonstrated that the PTAD-derivatives of 25(OH)D_3 and 3-epi-25(OH)D_3 co-eluted in the reversed-phase LC without acetylation of the hydroxy group at the 3-position.[19] A Cookson-type reagent that is effective to enhance not only the ESI-MS/MS sensitivity of 25(OH)D_3, but also the resolution of the epimers in reversed-phase LC, will be highly beneficial in the assessment of vitamin D status.

Based on this background information, the objective of this study was the development of a novel Cookson-type reagent for enhancing sensitivity and specificity in the LC/ESI-MS/MS assay of trace 25(OH)D_3. We designed and synthesized 4-(4′-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione (DAPTAD), a PTAD analogue having a dimethylamino group as a highly proton-affinitive moiety. The DAPTAD-derivative of 25(OH)D_3 was prepared and its behavior during LC and ESI-MS/MS was examined. The application of the DAPTAD-derivatization in the analysis of 25(OH)D_3 in neonatal DBSs is also described.

EXPERIMENTAL

Chemicals and reagents

25(OH)D_3 and 3-epi-25-hydroxyvitamin D_3 [3-epi-25(OH)D_3] were obtained from Wako Pure Chemical Industries (Osaka, Japan) and the Cayman Chemical Company (Ann Arbor, MI, USA), respectively. $^2H_3$-25(OH)D_3 purchased from IsoSciences (King of Prussia, PA, USA) was used as an internal standard. PTAD was synthesized from 4-phenylurazole (Nacalai Tesque, Kyoto, Japan) according to the known method,[20] but it is now available from several reagent-manufacturing companies, such as Tokyo Chemical Industry (Tokyo, Japan). 4-[4-(6-Methoxy-2-benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione (MBOTAD) and 4-(4-nitrophenyl)-1,2,4-triazoline-3,5-dione (NPTAD) were synthesized as previously reported.[21,22] The stock solutions of 25(OH)D_3 and its epimer were prepared as 20.0 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 with ethanol to prepare 0.100, 1.00, 10.0, 100 and 1000 ng/mL solutions. An ethanolic solution of $^2H_3$-25(OH)D_3 with a concentration of 5.00 ng/mL was also prepared. Silica gel and octadecylsilyl-silica gel (ODS) column chromatography were carried out with Merck silica-gel 60 (60–200 μm; Darmstadt, Germany) and Wakogel® 100C18 (63–212 μm; Wako Pure Chemical Industries), respectively. All other reagents and solvents were of analytical or LC/MS grade.

Synthesis of DAPTAD

The synthetic route is shown in Fig. 1.

4-Dimethylaminobenzoyl azide (II)

A solution of sodium azide (100 mg, 1.5 mmol) in water (0.5 mL) was added to a solution of the carbonyl chloride (I, 200 mg, 1.1 mmol) in acetone (15 mL) when cooled in ice. The mixture was stirred for 1 h on ice. The reaction mixture was diluted with ethyl acetate (25 mL) and then washed with saturated saline (25 mL, 3×). The organic layer was dried over $MgSO_4$ and evaporated *in vacuo*. The residue was chromatographed on a silica gel column [150 × 12 mm i.d., hexane/ethyl acetate (4:1, v/v)] to give the carbonyl azide (II, 157 mg) as a colorless powder.

1-Ethoxycarbonyl-4-(4′-dimethylaminophenyl)semicarbazide (IV)

A solution of II (157 mg, 0.8 mmol) in toluene (5 mL) was refluxed for 20 min for conversion into the isocyanate (III). To this solution, a solution of ethyl carbazate (100 mg, 1.0 mmol) in benzene (5 mL) was then added. The reaction mixture was stirred at room temperature for 1 h and then refluxed for 1 h.

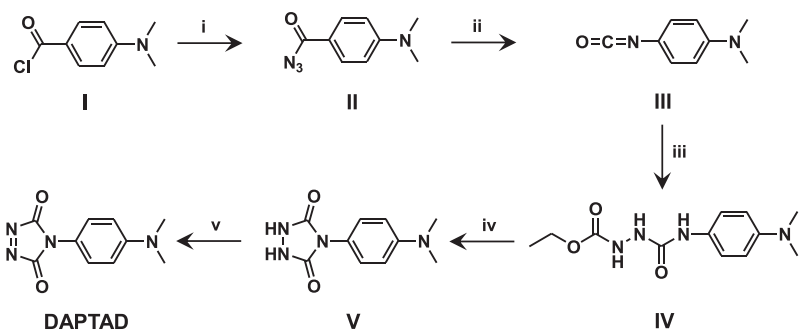

Figure 1. Synthesis of DAPTAD. (i) NaN_3 aq., acetone, 0°C, 1 h; (ii) toluene, reflux, 20 min; (iii) ethyl carbazate, benzene, r.t., 1 h and then reflux, 1 h; (iv) K_2CO_3 aq., 90°C, 3 h; (v) iodobenzene diacetate, ethyl acetate, r.t., 3 h.

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After cooling, the precipitate, the semicarbazide (IV, colorless powder, 213 mg), was separated. IV was subjected to the next reaction without purification.

4-(4′-Dimethylaminophenyl)-1,2,4-triazolidine-3,5-dione (V)

A mixture of IV (100 mg, 0.4 mmol) and K_2CO_3 (100 mg, 0.7 mmol) in water (10 mL) was stirred at 90°C for 3 h. The pH of the reaction mixture was adjusted to approximately 6 by the addition of acetic acid and the solvent was evaporated *in vacuo*. The residue was chromatographed on a Wakogel® 100C18 column [300 × 10 mm i.d., methanol/water (1:1, v/v)]. After removal of the solvent, the crude product was recrystallized from water to give the triazolidine (V, 52 mg) as brown amorphous solid. The ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra of V in $^{2}H_4$-methanol were recorded using a JEOL JNM-LD400 spectrometer with tetramethylsilane as the internal standard; ^{1}H-NMR (400 MHz) δ (ppm): 2.97 [6H, s, -N(CH_3)$_2$], 6.82 (2H, d, J = 9.2 Hz, 3′- and 5′-H), 7.20 (2H, d. J = 9.0 Hz, 2′- and 6′-H). ^{13}C-NMR (100 MHz) δ (ppm): 40.7 [−N(CH_3)$_2$], 113.6 (C-3′ and -5′), 121.1 (C-1′), 128.5 (C-2′ and -6′), 152.1 (C-4′), 156.5 (C-3 and -5).

DAPTAD

Iodobenzene diacetate (6 mg, 18.5 μmol) was added to a suspension of V (4 mg, 18.2 μmol) in ethyl acetate (4 mL) and stirred at room temperature for 3 h; the color of the mixture changed to red. After centrifugation (1000 *g*, 10 min), the supernatant was stored as the DAPTAD solution at −18°C. After a five-fold dilution with ethyl acetate, the solution was used for the derivatization.

LC/ESI-MS/MS

LC/ESI-MS/MS was performed using a Waters Quattro Premier XE triple quadrupole-mass spectrometer connected to a LC-2795 chromatograph (Milford, MA, USA). A YMC-Pack Pro C18 RS column (5 μm, 150 × 2.0 mm i.d.; YMC, Kyoto, Japan) was used at a flow rate of 0.2 mL/min at 40°C. The intact and derivatized vitamin D compounds were analyzed in the positive-ion mode, and the conditions were as follows: capillary voltage, 3.3 kV; cone voltage, 20 V (intact and NPTAD-derivative), 40 V (DAPTAD-derivative) or 30 V (PTAD- and MBOTAD-derivatives); collision energy, 8 eV (intact), 25 eV (DAPTAD-derivative), 15 eV (PTAD- and NPTAD-derivatives) or 30 eV (MBOTAD-derivative); source temperature, 120°C; desolvation temperature, 350°C; desolvation gas (N_2) flow rate, 600 L/h; cone gas (N_2) flow rate, 50 L/h; and collision gas (Ar) flow rate, 0.19 mL/min. The mobile phases and selected reaction monitoring (SRM) transitions for the respective compounds are described in Table 1. Masslynx software (version 4.1, Waters) was used for the system control and data processing.

Derivatization

The standard and pretreated DBS samples were dried and then dissolved in ethyl acetate (50 μL) containing a Cookson-type reagent (DAPTAD, PTAD, MBOTAD or NPTAD, 10 μg). The mixture was kept at room temperature for 1 h and then ethanol (20 μL) was added to the mixture to terminate the reaction. After the solvent had evaporated, the residue was dissolved in the mobile phase, an aliquot of which was subjected to LC/ESI-MS/MS.

Effect of derivatization for detection response

The effect of the derivatization for the detection response was evaluated by the limit of detection [LOD; the amount of intact or derivatized 25(OH)D_3 per injection giving a signal-to-noise ratio (S/N) of 4]. The S/N value was manually calculated by division of the peak height of the intact or derivatized 25(OH)D_3 by the noise level around the peak. The transitions listed in Table 1 were monitored in the SRM mode. 25(OH)D_3 (2.00 pg) was derivatized with a Cookson-type reagent as already described. The derivatives were dissolved in the mobile phases (100 μL) described in Table 1 and then subjected to LC/ESI-MS/MS. By stepwise decreasing the injection volume of the resulting solution, the LOD was determined. The LOD of the intact 25(OH)D_3 was determined using a solution of 10.0 ng/mL in the same way.

Analysis of 25(OH)D_3 in neonatal DBSs

Neonatal DBSs on filter paper (PKU test paper, Advantec Toyo, Tokyo, Japan) from Japanese infants of both sexes were used in this study. Written informed consent was obtained from their parents.

Table 1. LODs for intact and derivatized 25(OH)D_3

	Mobile phase[a]	t_R (min)	SRM transition (precursor ion, m/z → product ion[b], m/z)	LOD (fmol)	Increasing sensitivity[c]
25(OH)D_3 (intact)	10:1	5.5	401.5 $[M+H]^+$ → 383.2 $[M+H–H_2O]^+$	7.5	1
25(OH)D_3-DAPTAD	5:1	5.8[d]	619.6 $[M+H]^+$ → 341.1 $[A]^+$	0.25	30
25(OH)D_3-PTAD	5:1	5.7[d]	558.6 $[M+H–H_2O]^+$ → 298.0 $[A]^+$	0.50	15
25(OH)D_3-MBOTAD	9:1	5.8[d]	723.1 $[M+H]^+$ → 445.0 $[A]^+$	0.50	15
25(OH)D_3-NPTAD	6:1	5.9[d]	638.0 $[M+NH_4]^+$ → 342.8 $[A]^+$	0.75	10

[a]Mixture of methanol and 10 mM ammonium formate (v/v).
[b]$[A]^+$ is the product ion derived from the cleavage of the C-6–7 bond of the vitamin D skeleton (see Fig. 3).
[c]The detection response of intact 25(OH)D_3 is taken as 1.
[d]Retention time (t_R) of the major isomer.

A disk of 3 mm diameter (equivalent to 2.65 μL of whole blood) was excised from a 10 mm diameter DBS and used to analyze 25(OH)D_3. Methanol (100 μL) and an ethanolic solution (10 μL) of $^2H_3$-25(OH)D_3 (50 pg) was added to the disk placed into a test tube and the disk was subjected to an ultrasonic extraction (oscillation frequency, 46 kHz) using a CS-20 water bath (Shibata Scientific Technology, Tokyo, Japan) at ambient temperature (*ca*. 20°C) for 30 min. The extract was transferred to another test tube and the solvent was evaporated. The residue was subjected to the derivatization. After evaporation of the solvent, the residue was dissolved in the mobile phase [methanol/10 mM ammonium formate (4:1, v/v), 60 μL], 15 μL of which was subjected to LC/ESI-MS/MS. The SRM transitions for the intact and derivatized 25(OH)D_3 are described in Table 1.

The calibration curve was constructed using the calibration DBS samples which had been prepared in the same way as our previous report[19]; the calibration DBS samples contained 1.5, 3.0, 6.0, 15.0, or 30.0 ng/mL of 25(OH)D_3 and same amount of 3-epi-25(OH)D_3, because 3-epi-25(OH)D_3 might affect the calibration of 25(OH)D_3. The calibration curve was constructed by plotting the peak area ratio [25(OH)D_3/$^2H_3$-25(OH)D_3, y] versus the concentration of 25(OH)D_3 (x). The major peaks of the DAPTAD-derivatized 25(OH)D_3 [retention time (t_R) 8.5 min] and $^2H_3$-25(OH)D_3 (t_R 8.4 min) were used for the quantification.

RESULTS AND DISCUSSION

Synthesis of DAPTAD

DAPTAD was synthesized in five steps from 4-dimethylaminobenzoyl chloride (I) according to the method of Shimizu *et al*.[23] with some modifications (Fig. 1). The carbonyl chloride (I) was converted into the semicarbazide (IV) by treatment with sodium azide, Curtius rearrangement and then condensation with ethyl carbazate. The semicarbazide (IV) was cyclized to give the triazolidine (V), which was then oxidized with iodobenzene diacetate in ethyl acetate to form DAPTAD as a red solution. This red solution was used for the derivatization without purification since DAPTAD was difficult to isolate from the reaction mixture. The DAPTAD solution could be used for at least 2 months when stored at −18°C. Ethyl acetate was preferable as the reaction solvent for the oxidation of triazolidine and for the derivatization of 25(OH)D_3.

Derivatization

25(OH)D_3 was derivatized with DAPTAD at room temperature for 1 h by reference to the conditions of the PTAD-derivatization.[12,13,16,17,19] When 25(OH)D_3 (1.0 ng) was derivatized with DAPTAD under these conditions and one-quarter of the derivative [equivalent to 250 pg of 25(OH)D_3] was then subjected to LC/ESI-MS/MS, the underivatized 25(OH)D_3 was not detected. Our LC/ESI-MS/MS method can detect 3.0 pg of intact 25(OH)D_3 (see a later section) and this amount was 1.2% of the injected amount (250 pg) if the entire 25(OH)D_3 had remained underivatized. In this study, the absolute derivatization yield was not determined, but the yield is inferred to be satisfactory (almost quantitative), because the amount of 25(OH)D_3 that remained underivatized was negligible (less than 1.2%) as presented above.

LC and ESI-MS/MS behavior of intact and derivatized 25(OH)D_3

The DAPTAD-derivative of 25(OH)D_3 [25(OH)D_3-DAPTAD] consists of the 6*R*- and 6*S*-isomers, because the reagent attacks at the *s-cis*-diene of the metabolite from the α- and β-sides of the vitamin D skeleton (Fig. 2), as does the PTAD-derivative.[24] 25(OH)D_3-DAPTAD gave twin peaks at 5.2 and 5.8 min under the LC conditions described in the Experimental section and Table 1. Concerning the formation ratio of the 6*R*/*S*-isomers during the derivatization with a Cookson-type reagent, it has been reported that the 6*S*-isomer

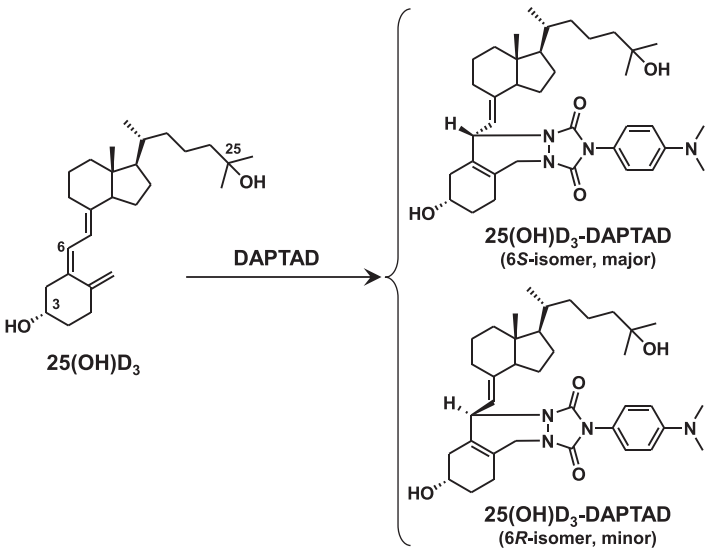

Figure 2. Derivatization reaction scheme of 25(OH)D_3 with DAPTAD.

forms in preference to the 6*R*-isomer for 25(OH)D_3.[24] For the DAPTAD-derivatization, the ratio of 6*S*-isomer to 6*R*-isomer was *ca*. 5:1.

25(OH)D_3-DAPTAD provided $[M+H]^+$ at *m/z* 619.6 as the base peak together with $[M+H-H_2O]^+$ (*m/z* 601.6, relative intensity 17%), $[M+Na]^+$ (*m/z* 641.5, relative intensity 4%) and $[M+K]^+$ (*m/z* 657.5, relative intensity 14%) in the positive ESI-MS experiment. The collision-induced dissociation (CID) of $[M+H]^+$ gave the characteristic A-ring fragment ion at *m/z* 341.1, which was derived from the cleavage of the C-6–7 bond of the vitamin D skeleton with a sufficient intensity (Fig. 3). Based on these results, the SRM mode with the transition of *m/z* 619.6 → 341.1 was used for the detection of 25(OH)D_3 as its DAPTAD-derivative.

Intact 25(OH)D_3 gave its protonated molecule ($[M+H]^+$, *m/z* 401) as the base peak and the dehydrated ion ($[M+H-H_2O]^+$, *m/z* 383) was formed from $[M+H]^+$ with a satisfactory intensity by CID. The PTAD-derivative gave the base peak at *m/z* 558, which was formed by the elimination of one water molecule from its protonated molecule ($[M+H-H_2O]^+$). The CID of $[M+H-H_2O]^+$ gave the characteristic A-ring fragment ion derived from the cleavage of the C-6–7 bond of 25(OH)D_3 with a sufficient intensity. $[M+H]^+$ and $[M+NH_4]^+$ were observed as the base peaks for MBOTAD- and NPTAD-derivatives, respectively. By the CID of these precursor ions, the A-ring fragment ions derived from the cleavage of the vitamin D skeleton were also formed. Based on the results, the transitions listed in Table 1 were used for the detection of 25(OH)D_3 and its derivatives.

Effect of derivatization for detection response

The effect of the DAPTAD-derivatization for the detection response was evaluated by the LOD in which the mobile phases were adjusted so that the t_R values of the respective compounds were between 5.5 and 6 min (when a derivative gave twin peaks, its major peak was used for this study) (Table 1). The LOD of the 25(OH)D_3-DAPTAD was 0.25 fmol [equivalent to 0.1 pg of intact 25(OH)D_3] on the column; the detection response was increased by 30-fold over the intact 25(OH)D_3 by the DAPTAD-derivatization.

We also compared the detection response of DAPTAD-derivative to those of the derivatives with other Cookson-type reagents. The magnitude of the increase in the detection response by the DAPTAD-derivatization was twice that by the PTAD-derivatization. The principal reason for this is due to the high proton affinity of the dimethylamino group of DAPTAD. Furthermore, the LOD of the DAPTAD-derivative was 2 and 3 times lower than those of the MBOTAD- and NPTAD-derivatives, respectively. Thus, DAPTAD was superior to PTAD, MBOTAD and NPTAD in enhancing assay sensitivity. Although MBOTAD had the same ability as PTAD to enhance the detection response of 25(OH)D_3, MBOTAD was less stable than PTAD (the PTAD solution could be used for at least 2 months when stored at −18°C, whereas the MBOTAD solution could be used only for a few days). Based on these results, the applicability of MBOTAD and NPTAD in the DBS analysis was not examined.

Analysis of 25(OH)D_3 in neonatal DBS as the DAPTAD-derivative

To demonstrate the utility of the DAPTAD-derivatization, the pilot quantitative study of 25(OH)D_3 in neonatal DBSs was performed. The measurement of 25(OH)D_3 in a DBS is expected to be useful for screening of the vitamin D deficiency in infants.[14,19,25] However, a major disadvantage in the use of DBS is the low 25(OH)D_3 abundance, because a disk (3 mm in diameter) from a DBS contains only approximately 2.65 μL of whole blood. Therefore, the PTAD-derivatization has often been employed to enhance the assay sensitivity.[14,19,25] In this study, the advantages of the DAPTAD-derivatization over the PTAD-derivatization in the DBS assay were examined.

After the addition of $^2H_3$-25(OH)D_3 as the internal standard, 25(OH)D_3 was extracted from a DBS disk with methanol and derivatized with DAPTAD. Representative chromatograms from a neonatal DBS are shown in Fig. 4(b), in which the peaks corresponding to the derivatized 25(OH)D_3 (7.4 and 8.5 min) and $^2H_3$-25(OH)D_3 (7.3 and 8.4 min, *m/z* 622.6 → 344.1) were clearly observed. On the contrary, when the same sample was analyzed without any derivatization, the peak corresponding to 25(OH)D_3 was not detected (chromatogram not shown).

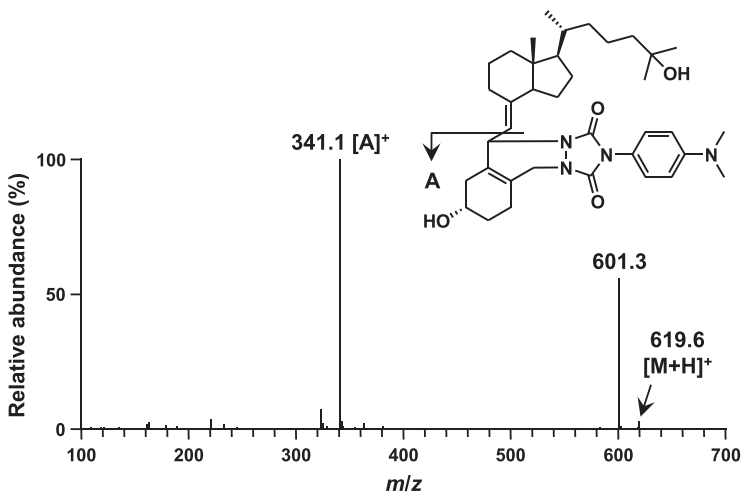

Figure 3. ESI-MS/MS spectrum of 25(OH)D_3-DAPTAD. The spectrum was recorded by the collisional activation of $[M+H]^+$ of the derivative under the conditions described in the Experimental section and Table 1.

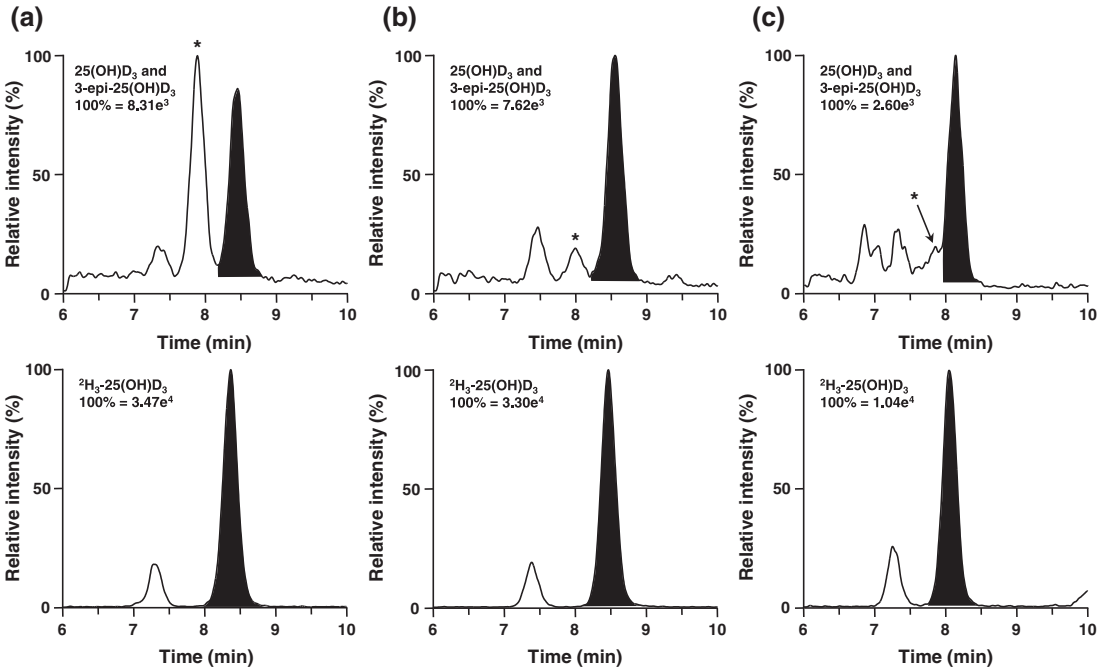

Figure 4. Chromatograms of derivatized 25(OH)D_3, 3-epi-25(OH)D_3 and $^2H_3$-25(OH)D_3 (internal standard) in DBS sample: (a) a calibration DBS sample containing 25(OH)D_3 and 3-epi-25(OH)D_3 (3.0 ng/mL each) was analyzed with the DAPTAD-derivatization, (b) a neonatal DBS sample was analyzed with the DAPTAD-derivatization (the measured value of 25(OH)D_3 was 3.86 ng/mL), and (c) the same DBS sample was analyzed with the PTAD-derivatization. The peaks of the 6*S*-isomers (filled in black) were used for the quantification and the peaks derived from 3-epi-25(OH)D_3 are marked by an asterisk. The LC/MS/MS conditions are described in the Experimental section and Table 1 except for the mobile phase [methanol/10 mM ammonium formate (4:1, v/v)].

As mentioned in the introductory section, one of the complicated problems in the 25(OH)D_3 assay of neonatal DBSs is the potential interference from 3-epi-25(OH)D_3, leading to overestimation of true 25(OH)D_3 concentrations. As described above, 25(OH)D_3-DAPTAD gave twin peaks at 7.4 and 8.5 min under the stated LC conditions. On the contrary, when 3-epi-25(OH)D_3-DAPTAD was analyzed under the same LC conditions, a single peak was eluted at 7.9 min; the 6*R*/*S*-isomers of 3-epi-25(OH)D_3-DAPTAD co-eluted under the stated LC conditions. As shown in Fig. 4(a), which are the chromatograms of the calibration DBS sample containing 3 ng/mL of 25(OH)D_3 and 3-epi-25(OH)D_3, the epimers could be satisfactorily separated using a conventional ODS column by the DAPTAD-derivatization; the resolution for the major peak (t_R 8.5 min) of 25(OH)D_3-DAPTAD and 3-epi-25(OH)D_3-DAPTAD (t_R 7.9 min) was 1.51. The LC/ESI-MS/MS assays using a cyanopropyl-silica gel (CN)[8,11] or pentafluorophenylpropyl-slica gel (PFP) column,[26,27] that enable the resolution of 25(OH)D_3 and 3-epi-25(OH)D_3 were reported, but these methods are hardly applicable to the DBS-based testing because a large volume (over 100 µL) of serum/plasma is required and the LODs of these methods were more than 25 fmol on the column, which was more than 100 times larger than that of our method. Furthermore, these assays required a long chromatographic run time (20–40 min),[8,11] ultra-high-performance liquid chromatography (UHPLC)[26] or two-dimensional HPLC.[27] On the contrary, our method requires 10 min for one chromatographic run and a conventional HPLC. Thus, the DAPTAD-derivatization was useful not only for enhancing the assay sensitivity, but also for separation of 25(OH)D_3 from the potent inferring epimer.

The regression line for 25(OH)D_3 showed a good linearity in the range of 1.5–30.0 ng/mL; $y = 0.0526x + 0.0246$, with a correlation coefficient of 0.999. The reproducibility of the determination of 25(OH)D_3 in a neonatal DBS was good; the relative standard deviation of the measured value [2.61 ± 0.07 ng/mL, mean ± standard deviation (SD)] for four repeated analyses was 2.7%. Although a method validation was not fully performed in this study, the 25(OH)D_3 concentration in the DBS presented in Fig. 4(b) was determined to be 3.86 ng/mL using the above regression line and the concentration ranged from 1.57 to 7.16 ng/mL (4.11 ± 1.72 ng/mL, mean ± SD) for 14 Japanese newborns. Thus, the present method has sufficient practicality for the analysis of 25(OH)D_3 in neonatal DBSs.

Our new method based on the DAPTAD-derivatization was compared to the method based on the PTAD-derivatization.[14,19,25] Figure 4(c) represents the chromatograms obtained by analyzing the DBS sample with the PTAD-derivatization. The peak corresponding to the derivatized 25(OH)D_3 was observed, but its intensity and S/N were not as high as those of the DAPTAD-derivative (Fig. 4(b)); thus, a more sensitive detection was achieved by the DAPTAD-derivatization. Furthermore, even with the use of several ODS columns [YMC-Pack Pro C18 RS (5 µm, 150 × 2.0 mm i.d.; YMC), J'sphere ODS-H80 (4 µm, 150 × 2.0 mm i.d.; YMC) and Cadenza CD-C18 (3 µm, 75 × 2.0 mm i.d.; Intakt, Kyoto)] and

a CN column [YMC-Pack CN (5 μm, 150 × 2.0 mm i.d.; YMC)], combined with different mobile phases, the satisfactory separation of 25(OH)D_3 and 3-epi-25(OH)D_3 could not be achieved as the PTAD-derivatives; the t_Rs were 7.3 and 8.1 min (twin peaks) for the 25(OH)D_3-PTAD and 7.6 and 7.8 min (twin peaks) for 3-epi-25(OH)D_3-PTAD, when a YMC-Pack Pro C18 RS column was used with the mobile phase of methanol/10 mM ammonium formate (4:1, v/v). The peak derived from 3-epi-25(OH)D_3 partly overlapped with the major peak of 25(OH)D_3-PTAD (Fig. 4(c)). Based on these results, the PTAD-derivatization-based assay may lead to overestimation of the 25(OH)D_3 levels, i.e., false diagnosis of vitamin D deficiency in infants. On the contrary, our new method with the DAPTAD-derivatization can avoid the overestimation of the 25(OH)D_3 levels, which is the greatest advantage of our method over the method employing the PTAD-derivatization. To separate 25(OH)D_3-PTAD from 3-epi-25(OH)D_3-PTAD, additional acetylation of the hydroxy group at the 3-position was needed.[19]

CONCLUSIONS

We developed a novel Cookson-type reagent, DAPTAD, for enhancing sensitivity and specificity in the LC/ESI-MS/MS assay of 25(OH)D_3. The detection response of the DAPTAD-derivative was 30-fold and twice as high as that of the intact 25(OH)D_3 and the PTAD-derivative, respectively, and the LOD was 0.25 fmol. The derivatization procedure with DAPTAD has a sufficient applicability for the assay of neonatal DBSs; the DAPTAD-derivatization followed by LC/ESI-MS/MS enabled the detection of trace amounts (in the low-ng/mL range) of 25(OH)D_3 with a simple pretreatment (only methanol extraction), small sample volume (a disk with a 3-mm diameter, equivalent to 2.65 μL of whole blood) and short chromatographic run time (10 min). Based on the DAPTAD-derivatization, 25(OH)D_3 was well separated from a potent interfering metabolite, 3-epi-25(OH)D_3. Thus, our new method using DAPTAD can reduce the overestimation of the 25(OH)D_3 levels, and will prove helpful in the diagnosis of vitamin D deficiency in infants.

We are now studying the application of the DAPTAD-derivatization for the quantitative analysis of 1α,25-dihydroxyvitamin D_3, an active form of vitamin D_3, in plasma (in the pg/mL range). Details of these results will be reported in the future.

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REFERENCES

[1] B. W. Hollis, R. L. Horst. The assessment of circulating 25(OH)D and 1,25$(OH)_2$D: where we are and where we are going. *J. Steroid Biochem. Mol. Biol.* **2007**, *103*, 473.
[2] E. Hyppönen, E. Läärä, A. Reunanen, M. R. Järvelin, S. M. Virtanen. Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. *Lancet* **2001**, *358*,1500.
[3] C. J. Willer, D. A. Dyment, A. D. Sadovnick, P. M. Rothwell, T. J. Murray, G. C. Ebers. Canadian Collaborative Study Group. Timing of birth and risk of multiple sclerosis: population based study. *BMJ* **2005**, *330*, 120.
[4] J. McGrath, D. Eyles, B. Mowry, R. Yolken, S. Buka. Low maternal vitamin D as a risk factor for schizophrenia: a pilot study using banked sera. *Schizophr. Res.* **2003**, *63*, 73.
[5] T. Higashi, K. Shimada, T. Toyo'oka. Advances in determination of vitamin D related compounds in biological samples using liquid chromatography–mass spectrometry: a review. *J. Chromatogr. B* **2010**, *878*, 1654.
[6] J. M. El-Khoury, E. Z. Reineks, S. Wang. Progress of liquid chromatography-mass spectrometry in measurement of vitamin D metabolites and analogues. *Clin. Biochem.* **2011**, *44*, 66.
[7] M. Vogeser. Quantification of circulating 25-hydroxyvitamin D by liquid chromatography–tandem mass spectrometry. *J. Steroid Biochem. Mol. Biol.* **2010**, *121*, 565.
[8] S. S.-C. Tai, M. Bedner, K. W. Phinney. Development of a candidate reference measurement procedure for the determination of 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 in human serum using isotope-dilution liquid chromatography–tandem mass spectrometry. *Anal. Chem.* **2010**, *82*, 1942.
[9] J. M. W. van den Ouweland, A. M. Beijers, P. N. M. Demacker, H. van Daal. Measurement of 25-OH-vitamin D in human serum using liquid chromatography tandem-mass spectrometry with comparison to radioimmunoassay and automated immunoassay. *J. Chromatogr. B* **2010**, *878*, 1163.
[10] C. S. Højskov, L. Heickendorff, H. J. Møller. High-throughput liquid-liquid extraction and LCMSMS assay for determination of circulating 25(OH) vitamin D3 and D2 in the routine clinical laboratory. *Clin. Chim. Acta* **2010**, *411*,114.
[11] H. C. M. Stepman, A. Vanderroost, K. Van Uytfanghe, L. M. Thienpont. Candidate reference measurement procedures for serum 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 by using isotope-dilution liquid chromatography–tandem mass spectrometry. *Clin. Chem.* **2011**, *57*, 441.
[12] T. Higashi, Y. Shibayama, M. Fuji, K. Shimada. Liquid chromatography–tandem mass spectrometric method for the determination of salivary 25-hydroxyvitamin D_3: a noninvasive tool for the assessment of vitamin D status. *Anal. Bioanal. Chem.* **2008**, *391*, 229.
[13] P. A. Aronov, L. M. Hall, K. Dettmer, C. B. Stephensen, B. D. Hammock. Metabolic profiling of major vitamin D metabolites using Diels-Alder derivatization and ultra-performance liquid chromatography–tandem mass spectrometry. *Anal. Bioanal. Chem.* **2008**, *391*, 1917.
[14] D. Eyles, C. Anderson, P. Ko, A. Jones, A. Thomas, T. Burne, P. B. Mortensen, B. Nørgaard-Pedersen, D. M. Hougaard, J. McGrath. A sensitive LC/MS/MS assay of 25OH vitamin D_3 and 25OH vitamin D_2 in dried blood spots. *Clin. Chim. Acta* **2009**, *403*, 145.
[15] X. Duan, B. Weinstock-Guttman, H. Wang, E. Bang, J. Li, M. Ramanathan, J. Qu. Ultrasensitive quantification of serum vitamin D metabolites using selective solid-phase extraction coupled to microflow liquid chromatography and isotope-dilution mass spectrometry. *Anal. Chem.* **2010**, *82*, 2488.
[16] S. Ding, I. Schoenmakers, K. Jones, A. Koulman, A. Prentice, D. A. Volmer. Quantitative determination of vitamin D metabolites in plasma using UHPLC-MS/MS. *Anal. Bioanal. Chem.* **2010**, *398*, 779.
[17] Z. Wang, T. Senn, T. Kalhorn, X. E. Zheng, S. Zheng, C. L. Davis, M. F. Hebert, Y. S. Lin, K. E. Thummel.

Simultaneous measurement of plasma vitamin D_3 metabolites, including 4β,25-dihydroxyvitamin D_3, using liquid chromatography–tandem mass spectrometry. *Anal. Biochem.* **2011**, *418*, 126.
[18] F. G. Strathmann, T. J. Laha, A. N. Hoofnagle. Quantification of 1α,25-dihydroxy vitamin D by immunoextraction and liquid chromatography–tandem mass spectrometry. *Clin. Chem.* **2011**, *57*, 1279.
[19] T. Higashi, M. Suzuki, J. Hanai, S. Inagaki, J. Z. Min, K. Shimada, T. Toyo'oka. A specific LC/ESI-MS/MS method for determination of 25-hydroxyvitamin D_3 in neonatal dried blood spots containing a potential interfering metabolite, 3-epi-25-hydroxyvitamin D_3. *J. Sep. Sci.* **2011**, *34*, 725.
[20] R. C. Cookson, S. S. Gupte, I. D. R. Stevens, C. T. Watts. 4-Phenyl-1,2,4-triazoline-3,5-dione. *Org. Synth.* **1971**, *51*, 121.
[21] K. Shimada, T. Mizuguchi. Sensitive and stable Cookson-type reagent for derivatization of conjugated diens for high-performance liquid chromatography with fluorescence detection. *J. Chromatogr.* **1992**, *606*, 133
[22] T. Higashi, A. Yamauchi, K. Shimada. Application of 4-(4-nitrophenyl)-1,2,4-triazoline-3,5-dione to analysis of 25-hydroxyvitamin D_3 in human plasma by liquid chromatography/electron capture atmospheric pressure chemical ionization-mass spectrometry. *Anal. Sci.* **2003**, *19*, 941.
[23] M. Shimizu, S. Kamachi, Y. Nishii, S. Yamada. Synthesis of a reagent for fluorescence-labeling of vitamin D and its use in assaying vitamin D metabolites. *Anal. Biochem.* **1991**, *194*, 77.
[24] M. Shimizu, S. Yamada. New fluorescence-labeling reagent targeting conjugated dienes: Application to the fluorometric analysis of vitamin D and A metabolites. *Vitamins* (Japan) **1994**, *68*, 15.
[25] D. W. Eyles, R. Morley, C. Anderson, P. Ko, T. Burne, M. Permezel, P. B. Mortensen, B. Nørgaard-Pedersen, D. M. Hougaard, J. J. McGrath. The utility of neonatal dried blood spots for the assessment of neonatal vitamin D status. *Paediatr. Perinat. Epidemiol.* **2010**, *24*, 303.
[26] R. L. Schleicher, S. E. Encisco, M. Chaudhary-Webb, E. Paliakov, L. F. McCoy, C. M. Pfeiffer. Isotope dilution ultra performance liquid chromatography-tandem mass spectrometry method for simultaneous measurement of 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and 3-epi-25-hydroxyvitamin D3 in human serum. *Clin. Chim. Acta* **2011**, *412*, 1594.
[27] S. Baecher, A. Leinenbach, J. A. Wright, S. Pongratz, U. Kobold, R. Thiele. Simultaneous quantification of four vitamin D metabolites in human serum using high performance liquid chromatography tandem mass spectrometry for vitamin D profiling. *Clin. Biochem.* **2012**, *45*, 1491.

