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3-*Epi*-25-hydroxyvitamin D_3 is a poor substrate for SULT2A1: Analysis of its 3-sulfate in cord plasma and recombinant human SULT2A1 incubate

Yusuke Yoshimura[a], Moeka Togashi[a], Shoujiro Ogawa[a,b], Tatsuya Higashi[a,*]

[a] Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba 278-8510, Japan
[b] Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, 1 Sanzo, Gakuen-cho, Fukuyama-shi, Hiroshima 729-0292, Japan

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ABSTRACT

A variety of metabolites derived from 25-hydroxyvitamin D_3 [25(OH)D_3], including its 3-epimer [*Epi*-25(OH)D_3] and 3-*O*-sulfate [25(OH)D_3-3S], is found in human plasma/serum. We hypothesized that the 3-*O*-sulfate of *Epi*-25(OH)D_3 [*Epi*-25(OH)D_3-3S] might be present in plasma/serum. Clarifying this point could improve our understanding of the metabolism of vitamin D_3. In this study, we first carefully analyzed the cord plasma samples by derivatization-assisted liquid chromatography/electrospray ionization-tandem mass spectrometry and demonstrated the occurrence of *Epi*-25(OH)D_3-3S in the plasma. However, the concentration ratio of *Epi*-25(OH)D_3-3S to 25(OH)D_3-3S (sulfated form) was infinitely lower than the ratio of *Epi*-25(OH)D_3 to 25(OH)D_3 (unconjugated form). To determine what caused this result, we next performed an *in vitro* experiment of the 3-*O*-sulfation for 25(OH)D_3 and *Epi*-25(OH)D_3 using the recombinant human sulfotransferase (SULT) 2A1. This *in vitro* experiment revealed that *Epi*-25(OH)D_3 is a poor substrate for the 3-*O*-sulfation catalyzed by SULT2A1 as compared to 25(OH)D_3. This substrate specificity of SULT2A1 would be the main cause for the result obtained from the analysis of the cord plasma samples.

1. Introduction

Vitamin D_3 is metabolically activated to 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] by the sequential 25- and 1α-hydroxylation, which are catalyzed by cytochrome P450 (CYP) 2R1 in the liver and CYP27B1 in the kidney, respectively. 1,25$(OH)_2D_3$ plays several biological roles in humans, such as the maintenance of calcium homeostasis and bone formation. 25-Hydroxyvitamin D_3 [25(OH)D_3, Fig. 1a] occurs as the major circulating form of vitamin D_3 as well as the intermediate in this metabolic activation of vitamin D_3. The bio-conversion to 1α,25$(OH)_2D_3$ from 25(OH)D_3 is strictly regulated by the serum calcium level; 1α,25$(OH)_2D_3$ is reported to be in the plasma/serum at a concentration of up to 150 pM, which is almost 1,000 times lower than the normal concentration of 25(OH)D_3 (40–100 nM) [1,2]. The excess 25(OH)D_3 is partly converted to inactive and/or conjugated metabolites. The major inactivation pathway of 25(OH)D is the CYP-dependent side-chain oxidation, including the conversion into 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$]. The plasma/serum concentration of 24,25$(OH)_2D_3$ has been reported to be about one-tenth that of 25(OH)D_3 [1,2]. We recently identified 24,25$(OH)_2D_3$ 24-glucuronide as the major urinary vitamin D_3 metabolites in humans [3]; this finding indicates that the 24-hydroxylation is the first step in the conversion of 25(OH)D_3 into a metabolite that is excreted into the urine.

25(OH)D_3 is also converted into its 3-epimer, 3-*epi*-25-hydroxyvitamin D_3 [*Epi*-25(OH)D_3, Fig. 1a], whose serum/plasma concentration has been reported to be an average of several percent that of 25(OH)D_3 [4,5]. A recent study demonstrated that the C3-epimerization of 25(OH)D_3 is primarily catalyzed by 25(OH)D_3 3-epimerase present in liver microsomes [6]. It has also been reported that the concentration ratio of *Epi*-25(OH)D_3 to 25(OH)D_3 in the plasma/serum is higher in infants than in adults [7,8]. The 3-epimerization is thought to be one of the metabolic pathways to reduce the biological activity of vitamin D metabolites [9].

25(OH)D_3 3-sulfate [25(OH)D_3-3S, Fig. 1a] is another major circulating metabolite of vitamin D_3, and its plasma level (mean, ca. 60 nM) was found to be much higher than that of 25(OH)D_3 (mean, ca 20 nM) in newborns [10,11]. 25(OH)D_3-3S has a high binding affinity for the vitamin D binding protein [12], which would be the main cause for its relatively high abundance in the circulation. Recently, human sulfotransferase (SULT) 2A1 in the liver was identified as the major enzyme responsible for the 3-*O*-sulfation of 25(OH)D_3 [12,13]. 25(OH)D_3-3S might be the storage form of vitamin D_3 because 25(OH)D_3 can be regenerated from this sulfate by deconjugation.

Thus, a variety of metabolites derived from 25(OH)D_3 is found in

* Corresponding author.
E-mail address: higashi@rs.tus.ac.jp (T. Higashi).

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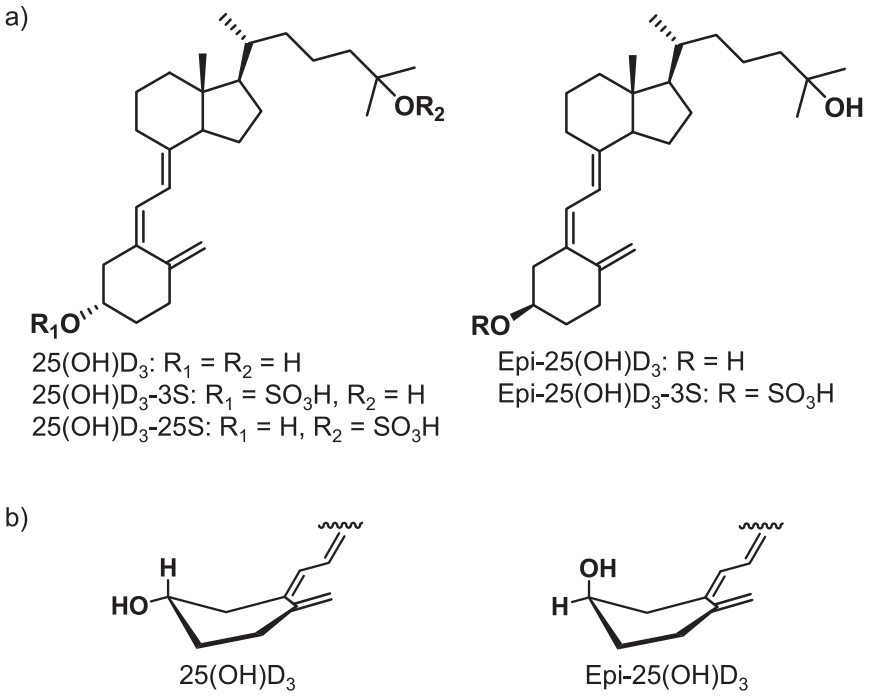

Fig. 1. (a) Chemical structures of 25(OH)D_3, *Epi*-25(OH)D_3 and their sulfates and (b) energetically favored A-ring conformation of 25(OH)D_3 and *Epi*-25(OH)D_3.

human plasma/serum, and it is thought that the metabolism of 25(OH)D_3 plays a primary role in regulating the biological activities of vitamin D_3 in humans. As just described, 25(OH)D_3-3S is the most abundant circulating metabolite of vitamin D_3 in newborns [10,11]. The plasma/serum concentration ratio of *Epi*-25(OH)D_3 to 25(OH)D_3 is higher in newborns/infants than in adults [7,8]. Taken these two findings together, the question about the presence or absence of *Epi*-25(OH)D_3 3-sulfate [*Epi*-25(OH)D_3-3S] in the plasma/serum of newborns/infants arose. Clarifying this question could improve our understanding of the metabolism of vitamin D_3.

Based on this background information, we conducted two experiments in this study. First, the presence or absence of *Epi*-25(OH)D_3-3S in umbilical cord plasma was examined by liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS). To enhance the assay specificity, the derivatization using 4-(4-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione (DAPTAD) (Fig. 2) [14] was incorporated into the LC/ESI-MS/MS. Second, the *in vitro* sulfation efficiencies toward *Epi*-25(OH)D_3 and 25(OH)D_3 by the recombinant human SULT2A1 were compared. The substrate specificity of the human SULT2A1 was evaluated based on the amounts of the sulfate formed *in vitro*.

2. Experimental

2.1. Materials and chemicals

25(OH)D_3, *Epi*-25(OH)D_3, 25(OH)D_3-3S and 25-hydroxy-7-dehydrocholesterol 3-sulfate [25(OH)-7DHC-3S] were the same as those used in previous studies [10,11]. 25-Hydroxyvitamin D_3 25-sulfate [25(OH)D_3-25S] was obtained as a byproduct during the synthesis of 25(OH)D_3-3S [10]. *Epi*-25(OH)D_3-3S was synthesized from *Epi*-25(OH)D_3 in our laboratories according to a known method [15]. Briefly, *Epi*-25(OH)D_3 (230 μg, 0.5 μmol) was reacted with the sulfur trioxide-trimethylamine complex (1.0 mg, 7.2 μmol) in pyridine (100 μL) at room temperature for 90 min, then the reaction mixture was purified by silica-gel column chromatography [packing material, Wakogel® 60 N (63–200 μm; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and eluent, chroloform-methanol (4:1, v/v)] and reversed-phase HPLC [column, J'sphere ODS-H80 (YMC, Kyoto, Japan) and mobile phase, methanol-10 mM ammonium formate (5:2, v/v)]. The chemical structure of *Epi*-25(OH)D_3-3S was confirmed by negative ESI-MS (*m/z* 479.1 $[M-H]^-$) and UV spectroscopy (λ_{max} 265 nm and λ_{min} 228 nm), and its purity determined by HPLC was over 99.5%. [26,26,26,27,27,27-$^2H_6$]-25(OH)D_3 [d_6-25(OH)D_3] and [26,26,26,27,27,27-$^2H_6$]-25(OH)D_3-3S [d_6-25(OH)D_3-3S] were purchased from IsoSciences (King of Prussia, PA, USA) and used as the internal standards (ISs). Standard solutions of the vitamin D_3 metabolites and the ISs were prepared by dissolving their precisely-weighed quantities in ethanol and serial dilutions with ethanol. Recombinant human SULT2A1 and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) were purchased from R & D systems (Minneapolis, MN, USA). Dithiothreitol (DTT) was from Nacalai Tesque, Inc. (Kyoto). A HEPES buffer (50 mM, pH 7.5) was used for the *in vitro* sulfation. DAPTAD was synthesized in our laboratories [14]. An Oasis® HLB cartridge (30 mg adsorbent; Waters, Milford, MA, USA) was used after successive washing with ethyl acetate (1 mL), methanol (1 mL) and water (1 mL). The organic solvents and additives used for the mobile phases were of LC/MS grade. All other reagents and solvents were of analytical grade.

2.2. LC/ESI-MS/MS

A Waters Quattro Premier XE triple quadrupole-mass spectrometer connected to an LC-e2695 chromatograph was used for the LC/ESI-MS/MS analysis. The ESI-MS/MS parameters are described in the Supplementary Data.

2.3. Cord plasma samples

In this study, newborn plasma was not available; as an alternative sample, umbilical cord plasma was used because there are good correlations in the concentrations between the newborn plasma and cord plasma for both 25(OH)D_3 and 25(OH)D_3-3S [11]. The cord blood samples were obtained during delivery at the Shizuoka Saiseikai General Hospital (Shizuoka, Japan) and initially used for the primary clinical tests, then the remaining amounts of the samples were used for this study. The plasma samples were prepared by centrifugation after

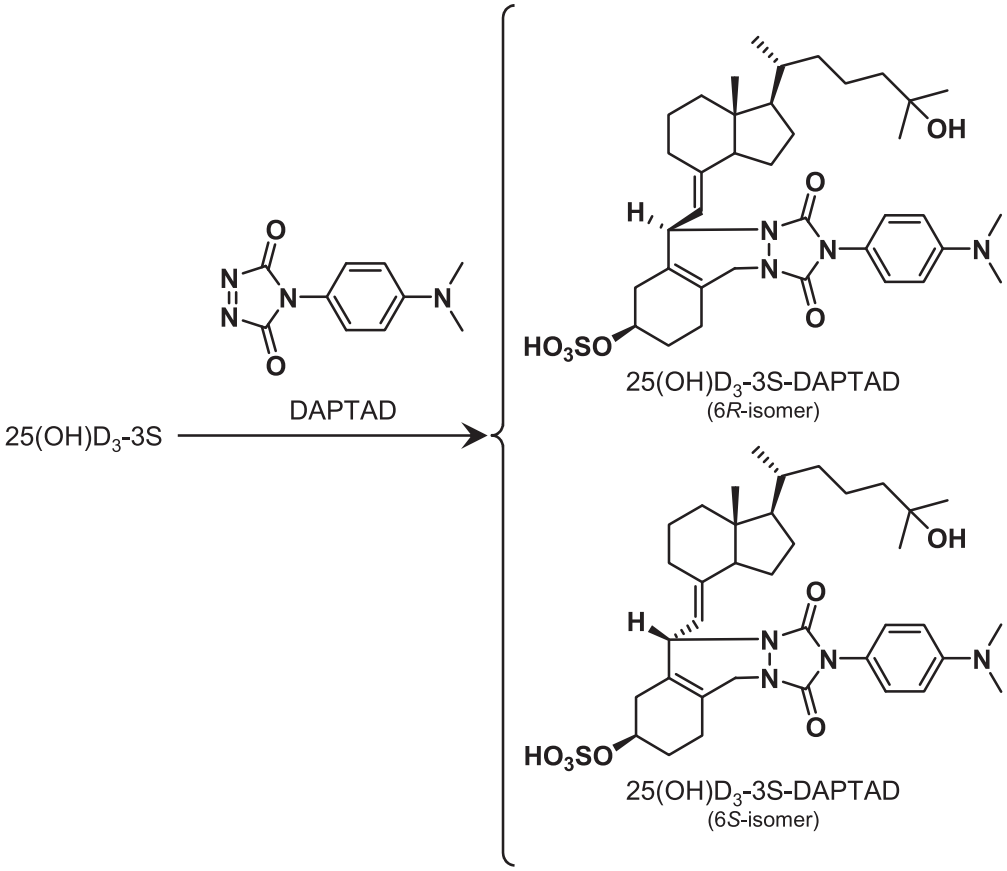

Fig. 2. Derivatization reaction scheme of *Epi*-25(OH)D_3-3S with DAPTAD.

blood coagulation, then stored at –30 °C until used. The experimental procedure was approved by the Ethics Committee of Tokyo University of Science (No. 14001). Written informed consent forms were obtained from all the subjects.

2.4. Analysis of cord plasma sample

A cord plasma sample (50 μL) was added to acetonitrile (100 μL) containing d_6-25(OH)D_3 (1.0 ng) and d_6-25(OH)D_3-3S (1.0 ng), vortex-mixed for 30 s, then centrifuged at 1,000 × *g* for 10 min. The supernatant diluted with water (600 μL) was applied to the Oasis® HLB cartridge. After washing with water (1 mL) and methanol–water (1:1, v/v, 1 mL), the unconjugated vitamin D_3 metabolites including 25(OH)D_3 and *Epi*-25(OH)D_3 were eluted with ethyl acetate (1.5 mL). The sulfated vitamin D_3 metabolites including 25(OH)D_3-3S and *Epi*-25(OH)D_3-3S were then eluted with methanol (1 mL).

For the ethyl acetate fraction, the solvent was evaporated, then the residue was dissolved in ethyl acetate (50 μL) containing DAPTAD (20 μg). After the derivatization was performed as previously reported [11], the solvent was evaporated, then the resulting sample was dissolved in the mobile phase A (60 μL). Fifteen microliters of this sample was injected into the LC/ESI-MS/MS operating in the positive-ion mode. A YMC-Pack Pro C18 RS column (3 μm, 150 × 2.0 mm i.d., YMC) was used at the flow rate of 0.2 mL/min and 40 °C. The mobile phase A [methanol-10 mM ammonium formate (7:2, 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] were used with the following gradient elution program; B = 0% maintained (0–6 min), 100% linearly increased (6–11 min) and maintained (11–13 min), and 0% maintained (13–29 min). The selected reaction monitoring (SRM) transitions were *m/z* 619.5 → 341.3 for the derivatized 25(OH)D_3 and *Epi*-25(OH)D_3, and *m/z* 625.5 → 341.3 for the derivatized d_6-25(OH)D_3.

For the methanol fraction, the solvent was evaporated, then the residue was derivatized with DAPTAD in the same way as the ethyl acetate fraction. The derivatized sample was dissolved in the mobile phase C (60 μL), 15 μL of which was subjected to the LC/ESI-MS/MS operating in the positive-ion mode. An Ascentis Express C18 column (2.7 μm, 100 × 2.1 mm i.d., Merck Ltd., Tokyo, Japan) was used at the flow rate of 0.2 mL/min and 40 °C. Methanol-10 mM ammonium formate (3:2, v/v) containing 0.05% (v/v) formic acid was used as the mobile phase C (isocratic elution). The SRM transitions were *m/z* 699.6 → 421.2 for the derivatized 25(OH)D_3-3S and *Epi*-25(OH)D_3-3S, and *m/z* 705.6 → 421.2 for the derivatized d_6-25(OH)D_3-3S.

2.5. Incubation procedure for in vitro sulfation

The *in vitro* sulfation procedure was based on the study by Kurogi et al. [13]. The mixture of the substrate [25(OH)D_3 or *Epi*-25(OH)D_3, 200 ng in ethanol-HEPES buffer (2:23, v/v, 5 μL)], recombinant human SULT2A1 [1.15 μg in HEPES buffer (5 μL)], PAPS [280 pmol in HEPES buffer (5 μL)] and DTT [20 nmol in HEPES buffer (5 μL)], with a final volume of 20 μL, was incubated in air at 37 °C for 10 min. Negative control samples without the substrate or enzyme were assayed in parallel. The reaction was initiated by adding PAPS and terminated by adding ice-chilled acetonitrile (50 μL) containing d_6-25(OH)D_3-3S (50 ng).

2.6. Analysis of incubation sample

An incubation sample terminated with ice-chilled acetonitrile was subjected to centrifugation at 1,000 × *g* for 10 min. The supernatant was diluted with water (300 μL) and the sample was applied to the Oasis® HLB cartridge. After washing with water (2 mL) and

methanol–water (1:1, v/v, 1 mL), the remaining substrate [25(OH)D_3 or *Epi*-25(OH)D_3] was washed away with ethyl acetate (1.5 mL). The formed sulfated vitamin D_3 metabolite was then eluted with methanol (1 mL). After the solvent was evaporated, the residue was derivatized with DAPTAD and analyzed by LC/ESI-MS/MS. The LC conditions and SRM transitions were the same as those for the analysis of the sulfated vitamin D_3 metabolites in the cord plasma.

2.7. Quantification of vitamin D_3 metabolites

The quantification of the vitamin D_3 metabolites was carried out by the previously-developed methods [11] with some modifications. These methods were based on the internal standard method, in which d_6-25(OH)D_3 and d_6-25(OH)D_3-3S were used as the ISs for the unconjugated and sulfated metabolites, respectively. The calibration ranges for the cord plasma analysis were 1.0–50 ng/mL for 25(OH)D_3, 0.1–2.0 ng/mL for *Epi*-25(OH)D_3, 5.0–100 ng/mL for 25(OH)D_3-3S and 0.4–8.0 ng/mL for *Epi*-25(OH)D_3-3S. Those for the analysis of the incubation sample were 1.0–20 ng/incubation sample for 25(OH)D_3-3S and 0.4–8.0 ng/incubation sample for *Epi*-25(OH)D_3-3S.

3. Results and discussion

3.1. LC/ESI-MS/MS behavior of DAPTAD-derivatized Epi-25(OH)D_3-3S

It has been demonstrated that the DAPTAD derivatization enhances the detectability and specificity in the positive-ESI-MS/MS not only for the unconjugated vitamin D_3 metabolites [14,16,17] but also for the sulfated [11,12,18] and glucuronidated metabolites [3,18]. The derivatization also works well for LC resolution of the stereoisomers, such as 25(OH)D_3 and *Epi*-25(OH)D_3 [14,16]. These advantages prompted us to use this derivatization to identify *Epi*-25(OH)D_3-3S in the cord plasma (Fig. 2).

Our first effort was directed toward an understanding of the mass spectrometric and chromatographic behavior of the DAPTAD-derivatized *Epi*-25(OH)D_3-3S. *Epi*-25(OH)D_3-3S-DAPTAD produced a protonated molecule ($[M + H]^+$) at *m/z* 699.6 in the positive ESI-MS. When this ion was collisionally activated, a characteristic product ion at *m/z* 421.2 was efficiently produced similar to that for the derivatized 25(OH)D_3-3S [11] (Fig. 3). Based on this result, the SRM transition of *m/z* 699.6 → 421.2 was used for the detection of *Epi*-25(OH)D_3-3S as well as 25(OH)D_3-3S in the assay samples as their DAPTAD derivatives.

As previously reported, the 6*R*- and 6*S*-isomers are formed during the DAPTAD derivatization for the vitamin D_3 metabolites (Fig. 2) [14]. Therefore, two peaks appear on the chromatogram for some vitamin D_3 metabolites after the derivatization. The 6*R*- and 6*S*-isomers of 25(OH)D_3-3S-DAPTAD co-eluted as a single peak [retention time (t_R) 28.1 min]. On the other hand, the 6*R*/*S*-isomers of *Epi*-25(OH)D_3-3S-DAPTAD separately eluted under the stated LC conditions and their t_Rs were 25.7 and 30.5 min. The early-eluted peak (t_R 25.7 min) was used to identify *Epi*-25(OH)D_3-3S in the cord plasma because the late-eluted peak (t_R 30.5 min) was hard to detect when the relatively large peak of 25(OH)D_3-3S-DAPTAD appeared. For identification of *Epi*-25(OH)D_3-3S formed by *in vitro* sulfation, both of the peaks were used as will be described later.

3.2. Analysis of cord plasma sample

The unconjugated and sulfated vitamin D_3 metabolites were fractionated using an Oasis® HLB cartridge. In the model experiment, the mixture of 25(OH)D_3, *Epi*-25(OH)D_3, 25(OH)D_3-3S and *Epi*-25(OH)D_3-3S (1.0 ng each) were loaded on the cartridge, then a two-step elution was performed. The ethyl acetate fraction of the first elution step contained 93.0 ± 2.4% [mean ± standard deviation (SD), n = 3] of 25(OH)D_3, 94.1 ± 5.2% of *Epi*-25(OH)D_3, and no sulfated metabolites. On the other hand, 84.7 ± 2.9% of 25(OH)D_3-3S, 86.0 ± 4.2% of *Epi*-25(OH)D_3-3S and negligibly small quantities (≤0.2%) of 25(OH)D_3 and *Epi*-25(OH)D_3 eluted into the methanol fraction of the second elution step. Thus, our procedure could almost completely fractionate the unconjugated and sulfated metabolites.

The cord plasma samples were subjected to deproteinization followed by the above fractionation using an Oasis® HLB cartridge. The ethyl acetate fraction was first analyzed by LC/ESI-MS/MS after the derivatization with DAPTAD. As shown in Fig. 4b, three peaks were observed in the SRM chromatogram (*m/z* 619.5 → 341.3) of the cord plasma sample; these peaks were identified as the DAPTAD-derivatized 25(OH)D_3 (6*R*-isomer, t_R 9.7 min), *Epi*-25(OH)D_3 (t_R 10.5 min) and 25(OH)D_3 (6*S*-isomer, t_R 11.3 min) by comparison to the authentic standards (Fig. 4a) [14]. As is obvious from this chromatogram, *Epi*-25(OH)D_3 was definitely present together with 25(OH)D_3 in the cord plasma. The plasma concentrations of these metabolites were determined by the internal standard method using d_6-25(OH)D_3 as the IS

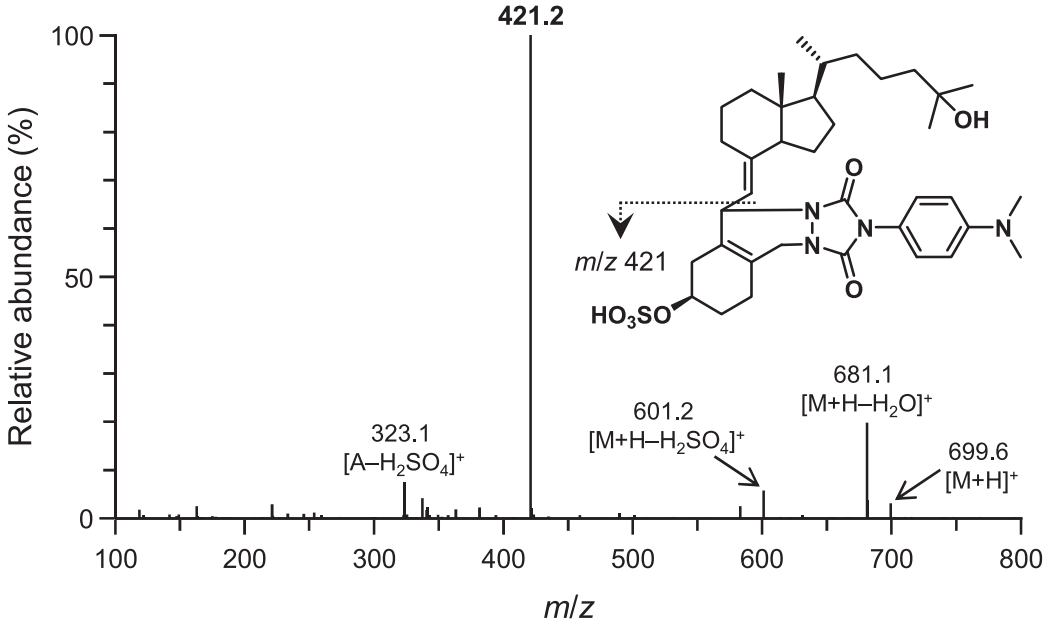

Fig. 3. Product ion spectrum of DAPTAD-derivatized *Epi*-25(OH)D_3-3S.

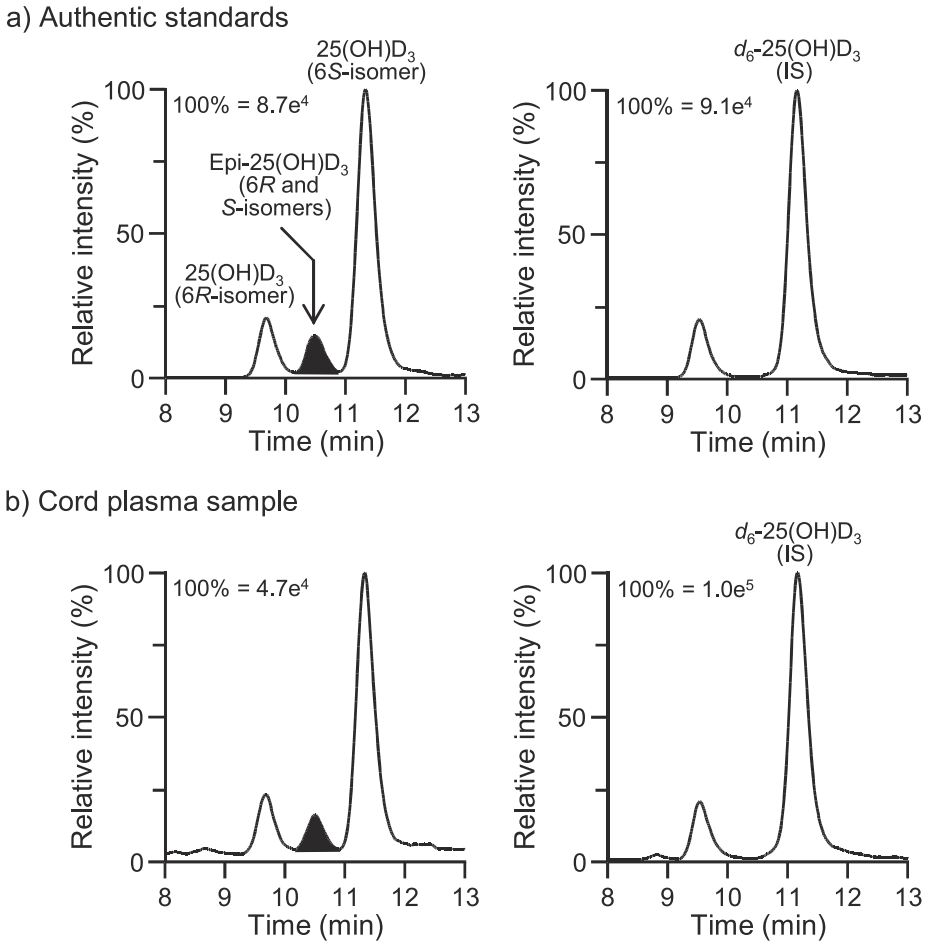

Fig. 4. SRM chromatograms of DAPTAD-derivatized 25(OH)D_3 and *Epi*-25(OH)D_3 with IS [d_6-25(OH)D_3]. (a) Authentic standards; the mixture of 25(OH)D_3 (1.0 ng) and *Epi*-25(OH)D_3 (100 pg) was derivatized, then one-quarter of the sample was injected into the LC/ESI-MS/MS. (b) Cord plasma sample. The measured concentrations of 25(OH)D_3 and *Epi*-25(OH)D_3 were 8.0 and 0.9 ng/mL, respectively.

(Table 1). The concentration ratio of *Epi*-25(OH)D_3 to 25(OH)D_3 was 9.3 ± 1.3% (mean ± SD, n = 5).

The methanol fraction containing the sulfated metabolites was then analyzed as the DAPTAD derivatives. 25(OH)D_3-3S was detected at a significantly higher concentration (24.1 ± 9.4 ng/mL, mean ± SD, n = 5) than its unconjugated form [25(OH)D_3, 6.4 ± 1.8 ng/mL] from all the samples, which was consistent with previous studies [11]. The occurrence of *Epi*-25(OH)D_3-3S in the cord plasma was carefully examined according to the LC/ESI-MS/MS behavior described in Section 3.1. As shown in the chromatograms of Fig. 5b, a small peak (t_R 25.7 min) was observed before the peak of the derivatized 25(OH)D_3-3S. This t_R was completely identical to that of the authentic standard of *Epi*-25(OH)D_3-3S-DAPTAD (Fig. 5a).

To more reliably identify this peak, we examined the LC/ESI-MS/MS behavior of the DAPTAD derivatives of 25(OH)D_3-25S and 25(OH)-7DHC-3S, whose molecular weights are the same as that of *Epi*-25(OH)D_3-3S. Although 25(OH)D_3-25S and 25(OH)-7DHC-3S have not been yet identified in human body fluids, we could not initially exclude the possibility that the peak at 25.7 min might be derived from these compounds. In the ESI-MS of the derivatized 25(OH)D_3-25S, only the sulfuric acid-eliminated ion ($[M + H–H_2SO_4]^+$, m/z 601.5) was detected. When this ion was collisionally activated, an ion at m/z 341.1 was produced by the cleavage of the C6-7 bond of the vitamin D skeleton (Fig. S1a, Supplementary Data). Thus, the derivatized 25(OH)D_3-25S provided quite different ions from the derivatized *Epi*-25(OH)D_3-3S in the ESI-MS and -MS/MS. Although the derivatized 25(OH)-7DHC-3S provided its protonated molecule at m/z 699.6, the product ion formed in MS/MS was observed at m/z 163.1 but not at m/z 421.2 (Fig. S1b, Supplementary Data). These results verified that the derivatied 25(OH)D_3-25S and 25(OH)-7DHC-3S were not detected by the SRM transition used for the detection of *Epi*-25(OH)D_3-3S (m/z 699.6 → 421.2). In other words, this SRM transition was highly specific for the detection of

Table 1
Concentrations of unconjugated and sulfated forms of 25(OH)D_3 and *Epi*-25(OH)D_3 in cord plasma.

Sample	25(OH)D_3 (ng/mL, *A*)	*Epi*-25(OH)D_3 (ng/mL, *B*)	Ratio (*B*/*A* × 100, %)	25(OH)D_3-3S (ng/mL, *C*)	*Epi*-25(OH)D_3-3S (ng/mL, *D*)	Ratio (*D*/*C* × 100, %)
Plasma 1	6.4	0.5	7.8	36.2	Trace[a]	–
Plasma 2	8.0	0.9	11.3	27.2	0.6	2.2
Plasma 3	7.4	0.7	9.5	28.1	0.4	1.4
Plasma 4	6.7	0.6	9.0	14.3	Trace[a]	–
Plasma 5	3.3	0.3	9.1	14.8	Trace[a]	–
Mean ± SD	6.4 ± 1.8	0.6 ± 0.2	9.3 ± 1.3	24.1 ± 9.4	≤ 0.6	≤ 2.2

[a] Below limit of quantification (0.4 ng/mL).

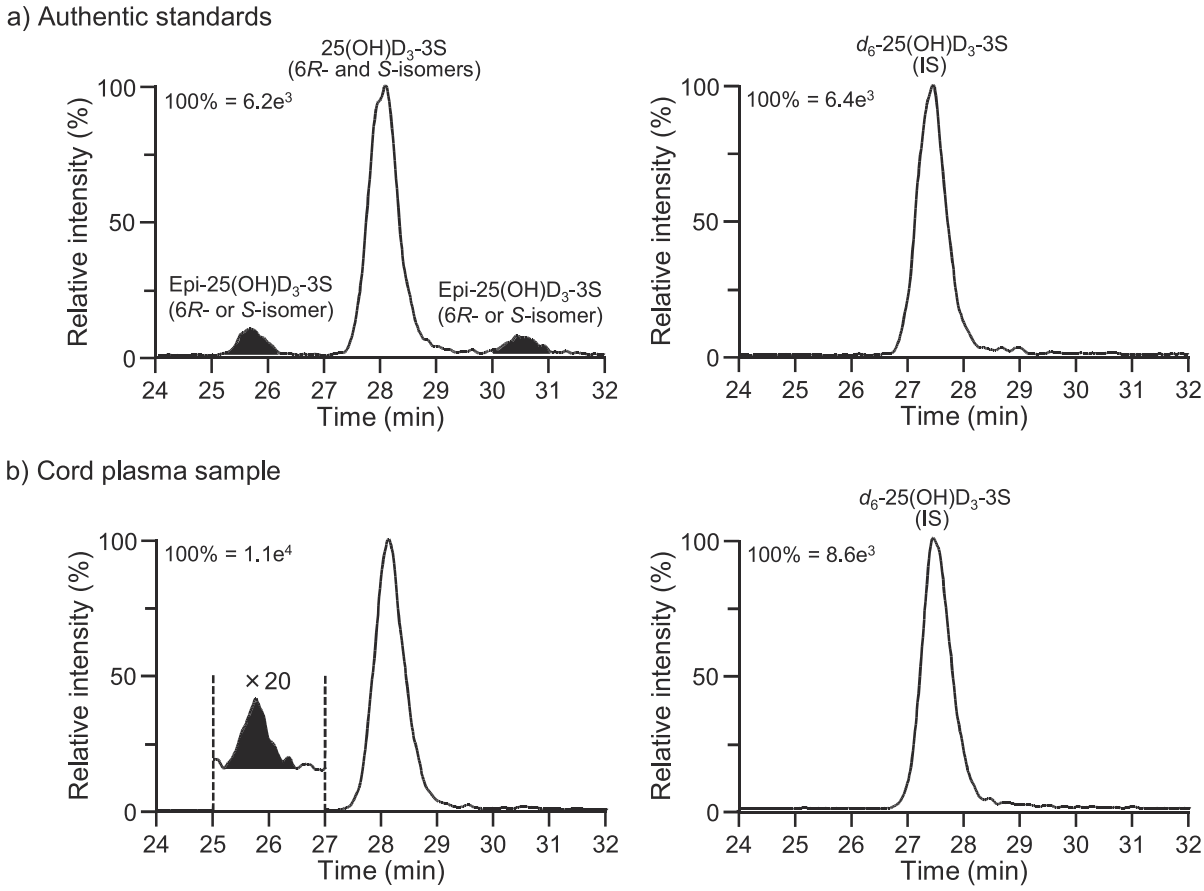

Fig. 5. SRM chromatograms of DAPTAD-derivatized 25(OH)D_3-3S and *Epi*-25(OH)D_3-3S with IS [d_6-25(OH)D_3-3S]. (a) Authentic standards; the mixture of 25(OH)D_3-3S (1.0 ng) and *Epi*-25(OH)D_3-3S (200 pg) was derivatized, then one-quarter of the sample was injected into the LC/ESI-MS/MS. (b) Cord plasma sample; the intensity of the peak derived from *Epi*-25(OH)D_3-3S (25.0–27.0 min) was enlarged 20 times. The measured concentrations of 25(OH)D_3-3S and *Epi*-25(OH)D_3-3S were 27.2 and 0.6 ng/mL, respectively.

Epi-25(OH)D_3-3S as well as 25(OH)D_3-3S. Furthermore, the derivatied 25(OH)D_3-25S (t_R 26.9 min) and 25(OH)-7DHC-3S (t_R 21.0 min) were chromatographically separated from the derivatized *Epi*-25(OH)D_3-3S. Based on all the results considered, the peak at 25.7 min observed in the cord plasma sample was almost certainly derived from *Epi*-25(OH)D_3-3S.

Over the last decade, it has become clear that 20-hydroxyvitamin D_3 [20(OH)D_3], which is formed from vitamin D_3 by action of CYP11A1 [2,19,20], is an active metabolite different from 1,25(OH)$_2D_3$; 20(OH)D_3 exerts anti-proliferative, pro-differentiation and anti-inflammatory effects on epidermal cells [19,20]. 20(OH)D_3 is found in human serum [2,19] and the isomer of 25(OH)D_3 and *Epi*-25(OH)D_3. Assuming that the 3-sulfate of 20(OH)D_3 is present, it is the isomer of 25(OH)D_3-3S and *Epi*-25(OH)D_3-3S. Although separation of the isomers is an important component for positive identification of the metabolites, 20(OH)D_3 and its 3-sulfate could not be examined in this study due to unavailability of their standards. However, our derivatization-based LC/ESI-MS/MS method had a high capability for discrimination of stereoisomers and positional isomers, such as 25(OH)D_3-3S, *Epi*-25(OH)D_3-3S and 25(OH)D_3-25S. Considering all the factors together, the metabolites detected in the cord plasma samples (Fig. 4b and 5b) were arguably identified as 25(OH)D_3, *Epi*-25(OH)D_3, 25(OH)D_3-3S and *Epi*-25(OH)D_3-3S.

The concentration of *Epi*-25(OH)D_3-3S in the cord plasma was much lower than that of 25(OH)D_3-3S (Table 1); the plasma *Epi*-25(OH)D_3-3S concentrations were below the limit of quantification (0.4 ng/mL) in 3 of the 5 samples. Consequently, their ratio [*Epi*-25(OH)D_3-3S/25(OH)D_3-3S, ≤ 2.2%] was significantly lower than the ratio of the unconjugated form [*Epi*-25(OH)D_3/25(OH)D_3, 9.3 ± 1.3%]. Based on these results, we hypothesized that *Epi*-25(OH)D_3 would be the poor substrate in the 3-*O*-sulfation catalyzed by SULT2A1 compared to 25(OH)D_3.

3.3. *In vitro* sulfation using recombinant human SULT2A1

To test our hypothesis, we performed an *in vitro* experiment using the recombinant human SULT2A1. The incubation sample was pretreated, derivatized, then subjected to LC/ESI-MS/MS. The recovery rates of 25(OH)D_3-3S and *Epi*-25(OH)D_3-3S from the incubation sample were almost equal; 86.1 ± 3.4% (mean ± SD, n = 3) and 88.3 ± 3.9%, respectively. When 25(OH)D_3 was incubated as the substrate, a clear peak was observed at the elution position of the derivatized 25(OH)D_3-3S (t_R 28.1 min, Fig. 6a). On the other hand, two very small peaks appeared for the incubation sample with *Epi*-25(OH)D_3 as the substrate and their t_Rs (25.7 and 30.5 min) were identical to those of the authentic derivatized *Epi*-25(OH)D_3-3S (Fig. 6b). No peaks corresponding to 25(OH)D_3-3S and *Epi*-25(OH)D_3-3S were detected in the negative control samples. The amounts (per incubation sample) of the formed sulfates were determined and summarized in Table 2. It should be noted that compared to 25(OH)D_3, *Epi*-25(OH)D_3 was converted into its sulfate at a significantly lower level; the conversion rate of *Epi*-25(OH)D_3-3S from *Epi*-25(OH)D_3 was 0.3 ± 0.1% (mean ± SD, n = 5), which was about one tenth that of 25(OH)D_3-3S from 25(OH)D_3 (2.8 ± 0.9%). Thus, *Epi*-25(OH)D_3 appeared to be a poor substrate for the 3-*O*-sulfation by SULT2A1 than 25(OH)D_3, which indicated that SULT2A1 exhibited a significant substrate specificity between 25(OH)

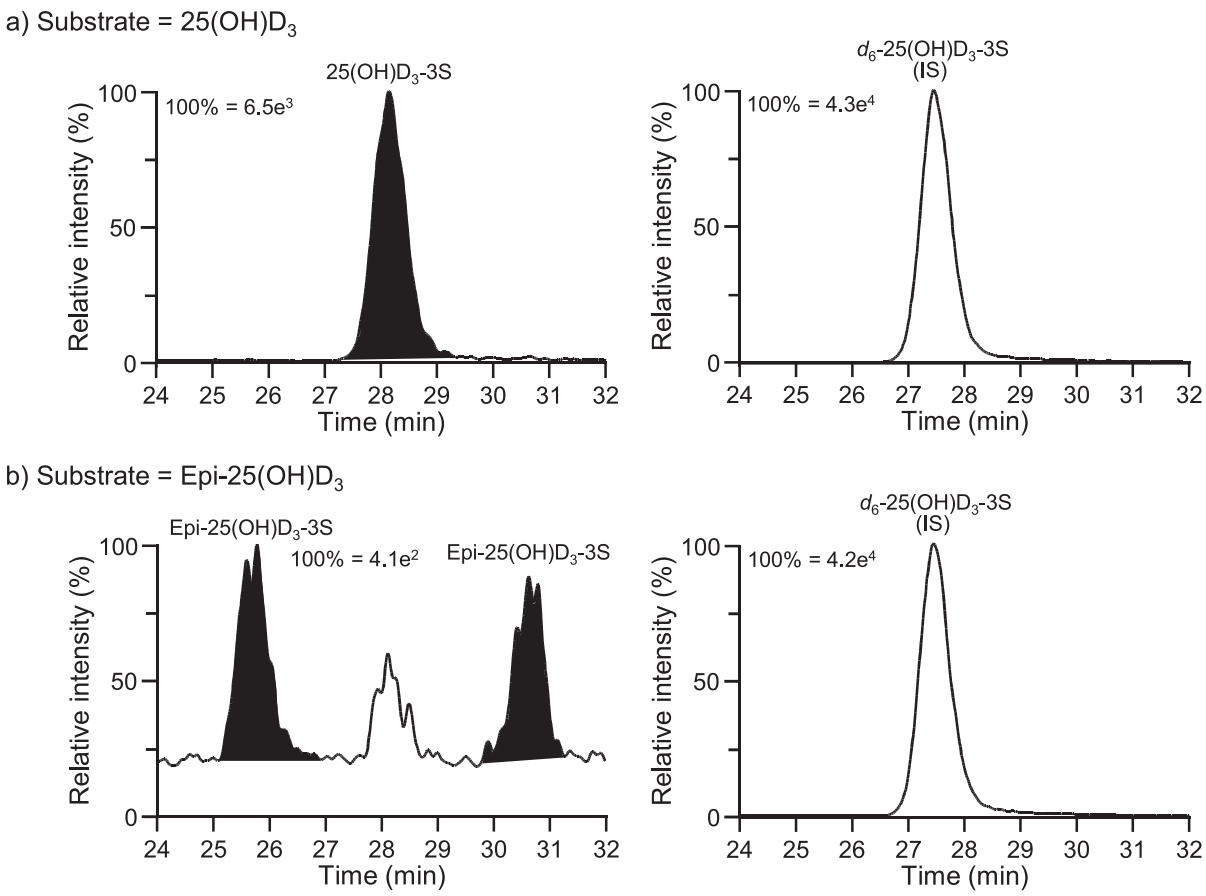

Fig. 6. SRM chromatograms of DAPTAD-derivatized 25(OH)D_3-3S and *Epi*-25(OH)D_3-3S formed *in vitro*. (a) Incubation sample with 25(OH)D_3 as the substrate. The formed amount of 25(OH)D_3-3S was 7.6 ng/sample. (b) Incubation sample with *Epi*-25(OH)D_3 as the substrate. The formed amount of *Epi*-25(OH)D_3-3S was 0.8 ng/sample.

Table 2
In vitro conversion rates of 25(OH)D_3 and *Epi*-25(OH)D_3 to their sulfates by SULT2A1.

Substrate	Amount of formed sulfate[a] (ng/incubation sample, *A*)	Conversion rate[b] (%)
25(OH)D_3	6.7 ± 2.3	2.8 ± 0.9
Epi-25(OH)D_3	0.8 ± 0.2	0.3 ± 0.1

[a] Mean ± SD (n = 5).
[b] Conversion rate (%) = *A* (mean)/200 × 400/480 × 100. 200 is the amount (ng) of the substrate. 400 and 480 are the molecular weights of the substrate and its sulfate, respectively.

D_3 and *Epi*-25(OH)D_3.

Two pathways to produce 25(OH)D_3-3S have been proposed by Kurogi et al. [13]; (1) the direct 3-*O*-sulfation of 25(OH)D_3, and (2) the C25-hydroxylation of vitamin D_3 3-sulfate (D_3-3S). Because of the significant low circulating concentration of D_3-3S [21,22], we inferred that the former would be the main pathway to produce 25(OH)D_3-3S. SULT2A1 has been identified as the most responsible enzyme for the 25(OH)D_3-3S formation among the several SULT isoforms [12,13]. This study found that the sulfating activity of SULT2A1 was much lower toward *Epi*-25(OH)D_3 than 25(OH)D_3. The energetically favored conformers calculated by Chem3D MM2 (PerkinElmer, Waltham, MA) are shown in Fig. 1b; the C3-hydroxy group is at the equatorial and axial positions for 25(OH)D_3 and *Epi*-25(OH)D_3, respectively. The C3-hydroxy group of *Epi*-25(OH)D_3 consequently might be prevented from being located near the active site of SULT2A1. Our *in vitro* experiment revealed that *Epi*-25(OH)D_3 was much less sulfated by SULT2A1 compared to 25(OH)D_3, which would be the main cause that the plasma concentration ratio of *Epi*-25(OH)D_3-3S to 25(OH)D_3-3S was extremely lower than expectation based on the concentration ratio of *Epi*-25(OH)D_3 to 25(OH)D_3.

4. Conclusion

In this study, we found that *Epi*-25(OH)D_3-3S is present in the cord plasma at the trace level by using the specific LC/ESI-MS/MS method combined with the DAPTAD derivatization. Interestingly, the plasma concentration ratio of *Epi*-25(OH)D_3-3S to 25(OH)D_3-3S was much lower than the ratio of *Epi*-25(OH)D_3 to 25(OH)D_3. To determine what led to this result, the *in vitro* experiment using the recombinant human SULT2A1 was performed. This experiment revealed that *Epi*-25(OH)D_3 is a poor substrate for the 3-*O*-sulfation catalyzed by SULT2A1 as compared to 25(OH)D_3. These findings obtained in this study would be of significant help for a deeper understanding of the vitamin D_3 metabolism.

CRediT authorship contribution statement

Yusuke Yoshimura: Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Visualization. **Moeka Togashi:** Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Visualization. **Shoujiro Ogawa:** Methodology, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Tatsuya Higashi:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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