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Simultaneous determination of 17α-hydroxypregnenolone and 17α-hydroxyprogesterone in dried blood spots from low birth weight infants using LC–MS/MS

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ABSTRACT

17α-Hydroxypregnenolone (17OHPreg) has heretofore been considered to be the major cause of the false elevated 17α-hydroxyprogesterone (17OHP) value in the immunoassay-based newborn screening for congenital adrenal hyperplasia (CAH). To verify this point, we developed a liquid chromatography–tandem mass spectrometric (LC–MS/MS) method that enables the simultaneous quantification of 17OHPreg and 17OHP in the dried blood filter papers and measured their blood levels in infants, especially in infants with low birth weights. Steroids were extracted from the filter papers with methanol, purified using a Strata-X cartridge, derivatized with 2-hydrazinopyridine and subjected to LC–MS/MS. Validation tests proved that this method was specific and reproducible; endogenous steroids did not interfere with the quantifications, and the intra- and inter-assay coefficients of variation were below 5.2%. The limits of quantitation were 1.0 and 0.5 ng/mL for 17OHPreg and 17OHP, respectively, when 3 disks (3 mm in diameter) of the filter papers (corresponding to 8 μL of whole blood) were used. The blood 17OHPreg level was elevated in the very low birth weight (1000–1500 g) infants and extremely low birth weight (<1000 g) infants, compared to those in the normal birth weight (>2500 g) infants ($P < 0.05$). However, the 17OHPreg concentration was not high enough to cause the false positive results in the enzyme immunoassay-based screening, and it was considered that the false positive results come from other endogenous components rather than 17OHPreg.

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1. Introduction

Congenital adrenal hyperplasia (CAH) caused by a deficiency of 21-hydroxylase is the most common inborn error in the adrenal steroid pathways. The resulting hormone imbalances with decreased glucocorticoids and mineralcorticoids and increased 17α-hydroxyprogesterone (17OHP) can lead to serious salt-wasting crises in the newborn period.

Enzyme immunoassay (EIA) has been conventionally used for measuring 17OHP in the dried blood filter papers, enabling the newborn screening for CAH, and will doubtless continue to be the method of choice for routine use in the screening. The currently used EIA kits, such as Enzaplate Neo-17α-OHP kit (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA), are highly specific for 17OHP; the antibodies were prepared using the haptenic derivatives conjugated with carrier proteins at the C-7 position of 17OHP and show very low cross-reactivity with other endogenous steroids. The Enzaplate Neo-17α-OHP kit provided a very low false positive rate (<0.2%, $n = 44621$) for the normal birth weight infants (NBWI, birth weight, >2500 g), when the cut-off value was set at 4 ng/mL. On the contrary, high false positive rates were still observed for the very low birth weight infants (VLBWI, birth weight, 1000–1500 g, false positive rate, ca. 20%, $n = 268$) and the extremely low birth weight infants (ELBWI, birth weight, <1000 g, false positive rate, ca. 50%, $n = 143$) in the first screening using the kit (unpublished data), and therefore, such infants were subjected to unnecessary follow-up investigations. It has heretofore been considered that one of the probable causes of the false elevated 17OHP level in EIA is that 17α-hydroxypregnenolone (17OHPreg) is abnormally elevated and is accumulated in the blood due to the incomplete adrenal function, especially in the preterm infants [1–3]. Although the antibody used in the Enzaplate Neo-17α-OHP kit shows only

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0.5% cross-reactivity with 17OHPreg, this steroid may still interfere with the EIA-based 17OHP measurement when the blood 17OHPreg concentration is extremely high beyond our expectations in the infants. The simultaneous quantification of 17OHPreg and 17OHP in the dried blood filter papers from infants, especially those with low birth weights, is expected to prove helpful for reducing the false positive results in the first screening. Although a few reports reveal the blood level of 17OHPreg in infants using radioimmunoassay [1] and gas chromatography–mass spectrometry [4], that in the infants with low birth weights has not been well elucidated. Furthermore, these methods require more than 100 μL of plasma and therefore, they cannot be utilized in the filter paper-based assay.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been actively examined as an alternative method for the determination of 17OHP in the dried blood filter papers due to its high specificity [5–7]. Another advantage of LC–MS/MS is simultaneous multi-analyte quantification capability. However, there is no LC–MS/MS method that can measure 17OHPreg in the dried blood filter papers from infants. The main cause of this is a lack of sensitivity for 17OHPreg in LC–MS/MS. Although 17OHP (3-oxo-4-ene-steroid) is satisfactorily ionized during electrospray ionization (ESI), 17OHPreg (3β-hydroxy-5-ene-steroid) is poorly ionized during either ESI or atmospheric pressure chemical ionization. As a procedure to overcome this problem, we have reported a derivatization using 2-hydrazinopyridine (HP, Fig. 1), which can enhance the detection responses of oxosteroids in ESI-MS [8].

Based on this background information, we developed and validated an LC–ESI-MS/MS method combined with derivatization for the simultaneous determination of 17OHPreg and 17OHP in the dried blood filter papers. Using the developed method, we measured the blood 17OHPreg and 17OHP levels in the infants with low birth weights and normal birth weights and verified the influence of the blood 17OHPreg level on the EIA results.

2. Materials and methods

2.1. Chemicals and reagents

17OHPreg and 17OHP were purchased from Sigma–Aldrich, Japan (Tokyo, Japan). Stock solutions of the steroids were prepared at 100 μg/mL in ethanol, and subsequent dilutions were carried out with ethanol to prepare 1, 2, 5, 10, 15 and 20 ng/mL solutions. [21,21,21-$^2H_3$]-17OHPreg (IS_1) and [2,2,6,6,21,21,21-$^2H_7$]-17OHP (IS_2) used as the internal standards (ISs) were synthesized in our laboratories according to the known methods [9,10] and then dissolved in and diluted with ethanol. Other steroids were purchased from Steraloids (Newport, RI, USA) or Tokyo Kasei Kogyo (Tokyo). HP was purchased from Tokyo Kasei Kogyo. Strata-X cartridges (60 mg adsorbent; Phenomenex, Torrance, CA, USA) were successively preconditioned with ethyl acetate (2 mL), methanol (2 mL) and water (2 mL) prior to use. All other reagents and solvents were of analytical grade.

2.2. LC–MS/MS

LC–MS/MS was performed using an Applied Biosystems API 2000 triple stage quadrupole-mass spectrometer (Foster City, CA, USA) connected to a Shimadzu LC-20AD chromatograph (Kyoto, Japan). A YMC-Pack Pro C18 RS column (5 μm, 150 mm × 2 mm i.d.; YMC, Kyoto) was used at a flow rate of 0.2 mL/min and 40 °C. Acetonitrile–methanol–10 mM ammonium formate (5:3:1, v/v/v) was used as the mobile phase. The HP derivatives of the steroids

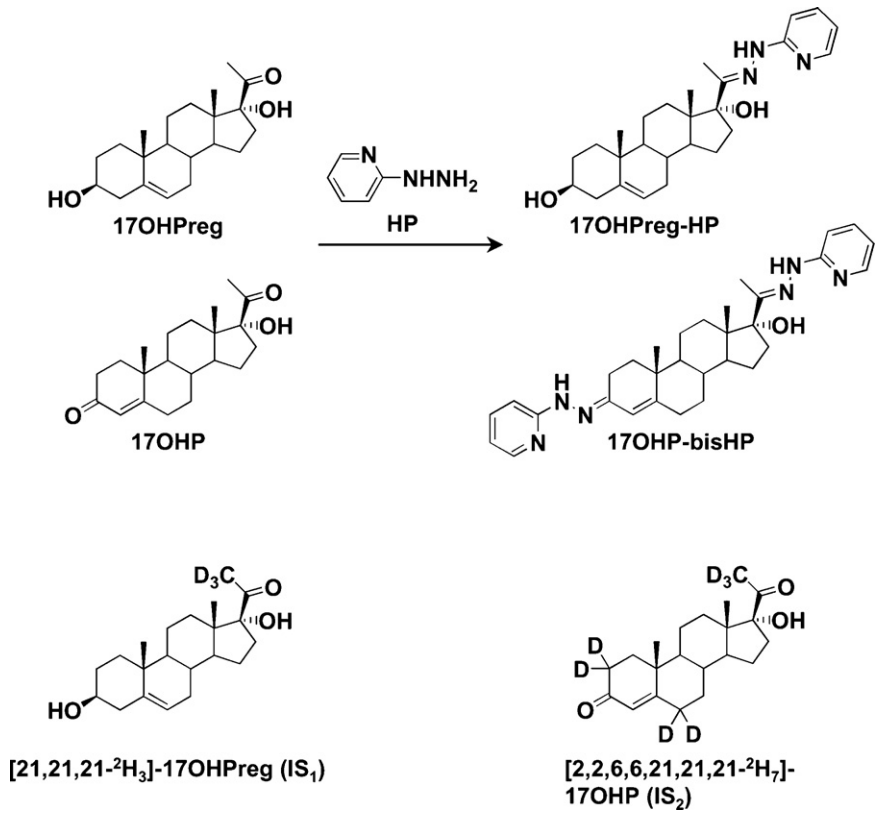

Fig. 1. Derivatization of 17OHPreg and 17OHP with HP and chemical structures of ISs (D = ^{2}H).

were analyzed by ESI-MS in the positive-ion mode and the conditions were as follows: declustering potential, 30 V (condition 1) or 80 V (condition 2); focusing potential, 200 V; entrance potential, 10 V; ion spray voltage, 5 kV; curtain gas, 45 psi; ion source gas 1, 80 psi; ion source gas 2, 80 psi; turbo gas temperature, 500 °C and interface heater, on. Nitrogen gas was used as the collision gas in the selected reaction monitoring (SRM) mode with a collision energy of 40 eV (condition 1) or 50 eV (condition 2) and a collision cell exit potential of 10 V. The transitions in SRM were as follows: 17OHPreg-HP, m/z 424.3 → 253.0; 17OHP-bisHP, m/z 513.4 → 364.1; IS_1-HP, m/z 427.3 → 253.0; IS_2-bisHP, m/z 520.4 → 368.1. The derivatized 17OHPreg and IS_1 were analyzed under condition 1 (0–3.5 min after injection), and the derivatized 17OHP and IS_2 were analyzed under condition 2 (3.5–7 min). The ESI-MS and -MS/MS spectra of the HP derivatives of progestogens, corticoids and androgens were recorded under the above described LC–MS(/MS) conditions. The data were collected and quantified using the Applied Biosystems Analyst software (Version 1.3.1).

2.3. Pretreatment procedure

A disk of 3-mm in diameter (equivalent to 2.65 μL of whole blood) was excised from a 10-mm diameter dried blood spot. Three disks of the dried blood filter paper were used unless otherwise stated. Methanol (200 μL) containing IS_1 and IS_2 (100 pg each) was added to the filter papers placed into a test tube and the papers were subjected to an ultrasonic extraction (oscillation frequency, 46 kHz) using a CS-20 water bath (Shibata Scientific Technology, Tokyo) at ambient temperature (ca. 20 °C) for 30 min. The methanolic extract was diluted with water (1 mL) and purified using a Strata-X cartridge. After successive washing with water (2 mL), methanol–water (1:1, v/v, 2 mL) and hexane (1 mL), the steroids were eluted with ethyl acetate (1 mL). After evaporation, the residue was subjected to derivatization with HP as described in Section 2.4. When the steroid concentrations were over 20 ng/mL (the highest concentrations on the calibration curves when 3 disks were used), 1/2, 1 or 2 disks of the samples were used. The numbers of the disk used did not influence the measured values (see Section 3.1).

2.4. Derivatization

A freshly prepared solution of HP (10 μg) in ethanol (50 μL) containing 25 μg of trifluoroacetic acid was added to the pretreated samples dissolved in ethanol (50 μL), and the mixture was subjected to an ultrasonic treatment (oscillation frequency, 46 kHz) in a CS-20 water bath at ambient temperature (ca. 20 °C) for 15 min. After the removal of the solvent, the derivatives were dissolved in methanol–10 mM ammonium formate (1:1, v/v, 30 μL), 10 μL of which was subjected to LC–MS/MS.

2.5. Calibration curves for 17OHP and 17OHPreg

Whole blood (10 mL) of a healthy volunteer was collected into a heparinized tube and centrifuged at 1500 × g (room temperature, 15 min) to be separated into plasma and blood red cells. The obtained plasma (4 mL) was stirred overnight with activated charcoal (40 mg, Norit EXW, Nacalai Tesque, Kyoto) and centrifuged at 1500 × g (room temperature, 20 min). The red blood cells were washed with saline (30 mL) and centrifuged at 1500 × g (room temperature, 15 min), and the supernatant was discarded. This procedure was repeated four times. The washed cells were combined with the charcoal-treated plasma in the ratio of 55:45 (v/v). This artificial steroid-free blood was used to construct calibration curves. The artificial blood (1 mL) was spiked with 17OHPreg and 17OHP (0.5, 1, 2, 5, 10 or 20 ng each, corresponding to 0.5, 1, 2, 5, 10 or 20 ng/mL), and 50 μL of this solution was spotted on a No. 545 filter paper (Advantec Toyo, Tokyo). After drying at room temperature, the filter paper was stored at −20 °C until use. Each filter paper (3 disks) was pretreated, derivatized and subjected to LC–MS/MS. The calibration curves were constructed by plotting the peak area ratios (17OHPreg/IS_1 or 17OHP/IS_2) versus the concentrations of the steroids.

2.6. Recoveries of 17OHPreg and 17OHP during pretreatment

The filter-paper form prepared with a healthy male volunteer's blood was pretreated, and 17OHPreg, 17OHP (16 pg each, corresponding to 2.0 ng/mL) and ISs were then added. The sample was derivatized and subjected to LC–MS/MS; the 17OHPreg or 17OHP concentration obtained here was F_0. To the same whole blood (1 mL) obtained from the volunteer, 17OHPreg and 17OHP (2.0 ng each) were added. The filter-paper form prepared from this spiked blood sample was pretreated, and ISs were then added. The sample was derivatized and subjected to LC–MS/MS; the 17OHPreg or 17OHP concentration obtained from the spiked sample was F. The recovery rates were calculated by $(F - F_0 + 2)/2$.

2.7. Assay precision and accuracy

To the whole blood (1 mL) obtained from a healthy male volunteer, 17OHPreg and 17OHP (2.0 or 15 ng each) were added, and then 50 μL of this solution was spotted on the filter paper [sample A (2.0 ng/mL each spiked) and sample B (15 ng/mL each spiked)]; because the steroid concentrations of the healthy male volunteer were less than our limits of quantification (LOQs), we used the steroid-spiked blood for the study of assay precision. The intra-assay precision was assessed by determining the steroids in these filter-paper forms on one day. The inter-assay precision was assessed by analyzing these samples over five days. The precision was determined as the coefficient of variation (CV).

Sample B was also analyzed using the different numbers (1/2, 1, 2 and 3) of the disk. The assay accuracy was evaluated as the parallelism between the measured values and numbers of disk.

2.8. Dried blood filter-paper forms from infants

Dried blood filter-paper forms from 108 infants of both sexes were collected and divided into four groups according to their birth weights; NBWI (birth weight, >2500 g, n = 65), low birth weight infants [LBWI, birth weight, 1500–2500 g (mean ± standard deviation (S.D.), 2231 ± 249 g), n = 17], VLBWI [birth weight, 1000–1500 g (1196 ± 100 g), n = 9] and ELBWI [birth weight, <1000 g (746 ± 128 g), n = 17]. The NBWI was further divided into two groups; CAH-negative (birth weight, 3065 ± 305 g, n = 48) and CAH-false positive (birth weight, 2842 ± 244 g, n = 17) according to the results in the EIA-based first screening. All the LBWI, VLBWI and ELBWI examined in this study were diagnosed to be false positive in the EIA-based first screening. The blood was collected from the heel at 4–8 days (NBWI), 5–18 days (LBWI), 5–13 days (VLBWI) or 7–28 days (ELBWI) after birth. Some infants showed high blood 17OHP values, but they proved negative for the 21-hydroxylase deficient-CAH by the follow-up examinations. Informed consent was obtained from the parents of the subjects.

2.9. EIA

The EIA of 17OHP was carried out with the Enzaplate Neo-17α-OHP kit according to its operation manual.

2.10. Statistical analysis

The statistical analyses were performed using the Mann–Whitney *U*-test for the two groups comparison and Kruskal–Wallis test followed by Steel–Dwass test for the multiple comparison. A *P*-value of <0.05 was considered statistically significant. For statistical purposes, steroid concentrations that were less than our LOQs were treated as 0.5 ng/mL and 0.25 ng/mL for 17OHPreg and 17OHP, respectively.

3. Result and discussion

3.1. Development and validation of LC–ESI-MS/MS method for the simultaneous quantification of 17OHPreg and 17OHP

As mentioned in Section 1, the ionization efficiency of 17OHPreg was very poor during ESI, and therefore, we employed the derivatization with HP (Fig. 1), which rapidly and quantitatively reacts with the oxo-group of the steroids, to enhance the detection response [8]. For the ESI-MS operating in the positive-ion mode, the HP derivatives of 17OHPreg and 17OHP provided intense protonated molecules, $[M+H]^+$, as the base peak ions. Characteristic product ions at *m/z* 253.0 and 364.1 were also produced from the protonated molecules of derivatized 17OHPreg and 17OHP, respectively, during the MS/MS. Based on these results, the SRM mode using the transitions described in Section 2.2 was employed in this study.

The extract from the dried blood filter-paper forms was purified using a Strata-X cartridge. The recovery rates of 17OHPreg and 17OHP during this pretreatment were 78.8 ± 1.1 and 81.3 ± 2.0% [mean ± S.D., $n = 5$], respectively.

Due to the formation of the *E*- and *Z*-isomers during the derivatization of the oxosteroids with HP [8], the derivatives sometimes produce twin peaks on their chromatograms. In principle, the higher sensitivity could be realized with the convergence of twin peaks into a single peak. When a YMC-Pack Pro C18 RS column with the mobile phase of acetonitrile–methanol–10 mM ammonium acetate (5:3:1, v/v/v) was used, the derivatized 17OHPreg and 17OHP gave single peaks at 2.7 and 5.1 min, respectively, though the peak shape of 17OHP-HP was not symmetrical. The chromatograms obtained from an LBWI are shown in Fig. 2, in which the peaks corresponding to the derivatized 17OHPreg [retention time (t_R), 2.7 min] and 17OHP (t_R, 5.1 min) were clearly detected. The chromatographic run time was 7 min per sample.

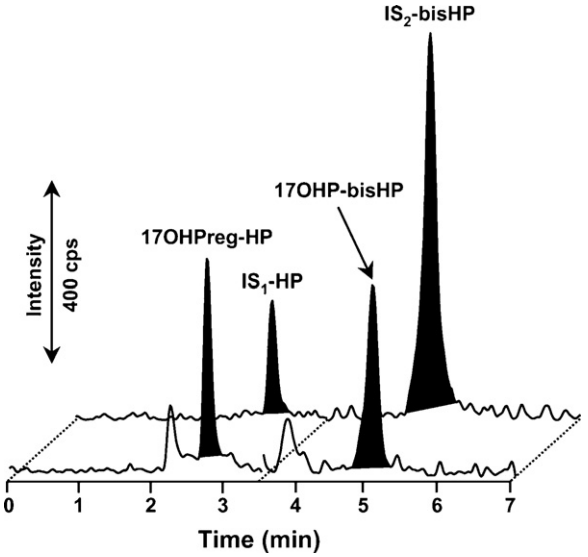

Fig. 2. Chromatograms obtained from the dried blood filter paper from an LBWI. The measured concentrations of 17OHPreg and 17OHP were 26.9 and 4.1 ng/mL, respectively.

In order to examine the assay specificity, the ESI-MS and -MS/MS spectra of the HP derivatives of endogenous progestogens, corticoids and androgens were recorded under the LC–MS(/MS) conditions described in Section 2.2. The base peaks and most abundant product ions of the respective steroids were as follows: 16α-hydroxypregnenolone (*m/z* 424.3 $[M+H]^+$ and *m/z* 95.0), 21-hydroxypregnenolone (*m/z* 424.3 $[M+H]^+$ and *m/z* 95.0), 16α-hydroxyprogesterone (*m/z* 513.3 $[M+H]^+$ and *m/z* 362.0), 11-deoxycorticosterone (*m/z* 513.3 $[M+H]^+$ and *m/z* 389.0), pregnenolone (*m/z* 408.3 $[M+H]^+$ and *m/z* 95.0), progesterone (*m/z* 497.4 $[M+H]^+$ and *m/z* 348.0), 11-deoxycortisol (*m/z* 529.3 $[M+H]^+$ and *m/z* 364.0), 21-deoxycortisol (*m/z* 529.4 $[M+H]^+$ and *m/z* 380.0), cortisol (*m/z* 545.3 $[M+H]^+$ and *m/z* 380.0), cortisone (*m/z* 543.3 $[M+H]^+$ and *m/z* 378.0), androstenedione (*m/z* 469.3 $[M+H]^+$ and *m/z* 322.1), testosterone (*m/z* 380.2 $[M+H]^+$ and *m/z* 95.0) and dehydroepiandrosterone (*m/z* 380.2 $[M+H]^+$ and *m/z* 94.9). Among these steroids, 21-hydroxypregnenolone, 11-deoxycorticosterone, pregnenolone, progesterone, 11-deoxycortisol, 21-deoxycortisol, cortisol, cortisone, androstenedione, testosterone and dehydroepiandrosterone were not at all detected by the selected transitions for 17OHPreg (*m/z* 424.3 → 253.0) and 17OHP (*m/z* 513.0 → 364.0). The HP derivatives of 16α-hydroxypregnenolone (t_R 2.0 min, single peak) and 16α-hydroxyprogesterone (t_R 3.4 and 3.9 min, twin peaks), the positional isomers of 17OHPreg and 17OHP, gave product ions at *m/z* 253 and 364 with low intensities as well as the above mentioned abundant product ions, and therefore, they could be detected by the above transitions (*m/z* 424.3 → 253.0 and *m/z* 513.0 → 364.0). However, these derivatized steroids were chromatographically well separated from 17OHPreg-HP and 17OHP-HP. These results show that the endogenous steroids do not interfere with the quantification of 17OHPreg and 17OHP. In addition, significant ion suppression or ion enhancement for the derivatized steroids due to the blood matrix was not observed.

The calibration curves were constructed using the dried blood filter-paper forms prepared from five different volunteers. The regression lines obtained from the combination of five calibration curves were $y = 0.121x - 0.021$ with a correlation coefficient (r) of 0.999 within the range of 1.0–20 ng/mL for 17OHPreg and $y = 0.135x - 0.011$ with an r of 0.999 within the range of 0.5–20 ng/mL for 17OHP. The CVs of the slopes ($n = 5$) in both the curves were below 2.1%; this result demonstrates that the individual difference in the blood matrix does not affect the calibration curves. The CVs and relative errors of the back-calculated concentration at 1.0 ng/mL for 17OHPreg were 6.0 and 3.8%, respectively. Those at 0.5 ng/mL for 17OHP were 5.3 and 4.0%, respectively. The peaks corresponding to the respective steroids at these concentrations were clearly observed with a signal-to-noise ratio of more than 5. Based on these results, the LOQs of 17OHPreg and 17OHP were determined to be 1.0 and 0.5 ng/mL, respectively, when three disks of the filter-paper forms (corresponding to 8 μL of whole blood) were used. These LOQs were 160 and 2.5 times, respectively, greater than those of the intact steroids.

The assay precision was examined using two dried blood filter-paper forms with different concentrations of 17OHPreg and 17OHP (Table 1). The intra-assay CVs for both the steroids were less than 3.5%, and good inter-assay CVs (less than 5.3%) were also obtained. The assay accuracy was evaluated as the parallelism between the measured values and numbers of disk. As shown in Table 2, the 17OHPreg and 17OHP concentrations obtained from the analysis using 3 disks of the sample and those using 1/2, 1 or 2 disk(s) of the sample were quite similar. This result demonstrates that the accuracy of the method is satisfactory.

Table 1
Intra- and inter-assay precision for determination of 17OHPreg and 17OHP in dried blood filter papers

	17OHPreg		17OHP	
	Sample A	Sample B	Sample A	Sample B
Intra-assay ($n=5$)				
Mean ± S.D. (ng/mL)	2.85 ± 0.10	15.86 ± 0.35	2.42 ± 0.06	15.45 ± 0.28
CV (%)	3.5	2.2	2.5	1.8
Inter-assay ($n=5$)				
Mean ± S.D. (ng/mL)	2.81 ± 0.11	15.85 ± 0.34	2.46 ± 0.13	15.39 ± 0.30
CV (%)	3.9	2.1	5.3	1.9

Thus, the validation study data prove that the developed method is specific, reproducible and accurate.

3.2. Blood 17OHPreg and 17OHP levels in infants

Although the Enzaplate Neo-17α-OHP kit used in this study is highly specific for 17OHP as described in Section 1, the false positive rate is not nil even for the NBWI and is fairly high for the VLBWI and ELBWI. To investigate the cause of this, we first compared two NBWI groups whose EIA-based screening had proved false positive and negative, respectively, in the blood 17OHPreg and 17OHP levels (Fig. 3). The difference between the two groups was more significant in the 17OHPreg concentration than in the 17OHP concentration. However, the 17OHPreg concentration was not more than 10 times the 17OHP concentration even in the false positive group. When this result and the fact that the EIA kit shows only 0.5% cross-reactivity with 17OHPreg are summarized, for the NBWI, 17OHPreg is considered to be one of the causes but a minor cause of the false positive results in the EIA.

Although the 17OHP concentration determined by LC–MS/MS was also statistically higher in the false positive group than in the negative group ($P<0.05$), the measured values in 16 out of 17 false positive infants were below 4 ng/mL (cut-off value in EIA) (Fig. 3b). This result indicates that the false positive rate can be reduced in the CAH screening for the NBWI, when the LC–MS/MS is used in place of the EIA.

Next, to examine the relation between the birth weight and the steroid levels, LC–MS/MS-based 17OHPreg and 17OHP values were compared between the NBWI, LBWI, VLBWI and ELBWI, all of whom had given false positive results in the EIA-based screening (Fig. 3). Although no difference was observed in the steroid concentrations between the NBWI and LBWI, both the 17OHPreg and 17OHP concentrations were significantly higher in the VLBWI and ELBWI than in the NBWI. Generally, the steroid concentrations varied inversely with the birth weight, and the subjects with high 17OHPreg concentration showed a high 17OHP concentration.

Fig. 3b demonstrates that the blood 17OHP concentration is not always a good index in the CAH diagnosis for the VLBWI and ELBWI, because the concentrations were actually higher than the cut-off value (4 ng/mL) in most of the subjects. In other words, for these subjects, the measurement of the blood 17OHP using EIA or even the more specific method, LC–MS/MS, cannot anticipate so much of the positive effects for the CAH screening. As an alternative means for the 21-hydroxylase deficient-CAH diagnosis for the subjects with a high 17OHP concentration, such as the VLBWI and ELBWI, confirming the low cortisol concentration may be useful [7].

As mentioned in Section 1, there is higher incidence of the overestimation of the 17OHP value by EIA in infants with low birth weights than in the NBWI. Therefore, we compared the EIA-based 17OHP value with the LC–MS/MS-based 17OHP concentration in the preterm infants diagnosed to be false positive (LBWI, VLBWI and ELBWI were lumped together) in the EIA (Fig. 4a). As it is obvious from Fig. 4a, the EIA significantly overestimated the 17OHP concentration. This substantial difference between the EIA- and LC–MS/MS-based values cannot be explained by only the cross-reaction with 17OHPreg; although the blood 17OHPreg was surely elevated in the false positive group (Fig. 3a), its concentration is

Table 2
Parallelism between measured values and numbers of disk

Number of disk	Measured value (ng/mL)[a]	
	17OHPreg	17OHPreg
1/2	15.95 (100.6)	15.50 (100.7)
1	16.39 (103.4)	16.34 (106.2)
2	15.74 (99.3)	15.94 (103.6)
3	15.85 (100.0)	15.39 (100.0)

[a] The values in parentheses are percentage of the measured values obtained from the analysis using three disks of the sample. Data represent the mean of duplicate assay.

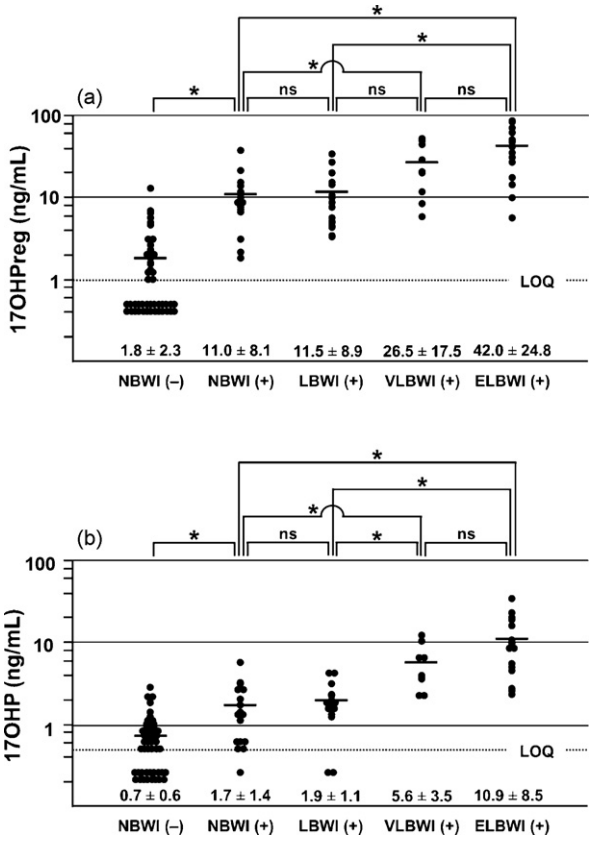

Fig. 3. Blood concentrations of 17OHPreg (a) and 17OHP (b) in infants determined using the developed LC–MS/MS. Values are mean concentrations with S.D. (ng/mL). * and ns represent statistically significant ($P<0.05$) and nonsignificant, respectively. (+) and (–) represent false positive and negative, respectively, in the EIA-based screening.

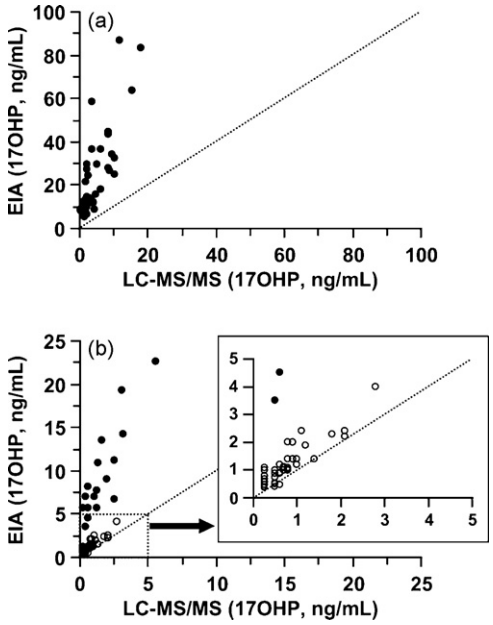

Fig. 4. Scatter diagrams comparing the EIA-based 17OHP value with the 17OHP concentration determined by the developed LC–MS/MS. (a) LBWI, VLBWI or ELBWI: all the subjects were diagnosed to be false positive in the EIA-based screening. Samples whose 17OHP values determined by EIA were beyond the measurable range (>100 ng/mL) were excluded from this examination. (b) NBWI: closed and open circles represent false positive and negative subjects, respectively, in the EIA-based screening. The dotted lines represent the lines of equality.

not high enough to significantly increase the 17OHP value in the EIA. The comparison of EIA-based 17OHP value and the LC–MS/MS-based 17OHP concentration in the NBWI is also shown in Fig. 4b. As expected, the EIA significantly overestimated the 17OHP concentration in the false-positive NBWI (closed circle), whereas the EIA values were slightly higher than the LC–MS/MS values in the negative NBWI (open circle). The false positive NBWI had a higher 17OHPreg concentration than the negative NBWI (Fig. 3a), but it is not high enough to drastically increase the 17OHP value in the EIA, either. These results indicate that other endogenous components rather than 17OHPreg are the main cause of the false positive results in the EIA. Because various organs do not attain their full maturities in the preterm infants, some nonsteroidal compounds may also be elevated and accumulated in the blood and may non-specifically interfere with the 17OHP EIA.

4. Conclusion

We developed a sensitive, accurate and precise LC–ESI-MS/MS method that enables the simultaneous determination of 17OHPreg and 17OHP in the dried blood filter-paper forms. Using this method, we revealed the blood 17OHPreg and 17OHP levels in infants with normal and low birth weights. Contrary to our expectations, the 17OHPreg concentration was not high enough to cause the false positive results in the EIA-based screening.

Because 17OHPreg represents a marker steroid of 3β-hydroxysteroid dehydrogenase (3β-HSD) deficiency and this enzyme is also essential for corticoid biosynthesis, the developed method is expected to be also useful in the diagnosis of the 3β-HSD deficient-CAH.

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