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Dried Blood Spot Multiplexed Steroid Profiling Using Liquid Chromatography Tandem Mass Spectrometry in Korean Neonates

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Background: Screening for congenital adrenal hyperplasia (CAH) using immunoassays for 17α-hydroxyprogesterone generates many false-positive results. We developed and validated a liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay for simultaneous quantification of nine steroid hormones in dried blood spot (DBS) samples, and established reference intervals for these hormones.

Methods: We examined our method for linearity, precision, accuracy, extraction recovery, and matrix effects and determined the reference intervals of cortisol, 17α-hydroxyprogesterone, 11-deoxycortisol, 21-deoxycortisol, androstenedione, corticosterone, 11-deoxycorticosterone, testosterone, and progesterone in 1,146 DBS samples (from 272 preterm and 874 full-term neonates). Immunoassay and LC-MS/MS methods were compared for 17α-hydroxyprogesterone. Fourteen additional samples were tested to validate the clinical applicability of the LC-MS/MS method.

Results: The linearity range was 2.8–828.0 nmol/L for cortisol and 0.9–40.0 nmol/L for the other steroids ($R^2 > 0.99$). Intra-day and inter-day precision CVs were 2.52–12.26% and 3.53–17.12%, respectively. Accuracy was 80.81–99.94%, and extraction recovery and matrix effects were 88.0–125.4% and 61.7–74.2%, respectively. There was a negative bias, with higher values measured by immunoassay compared with LC-MS/MS ($r = 0.8104$, $P < 0.0001$). The LC-MS/MS method was successfully applied to the analysis of nine steroids in DBS for screening and diagnosis of CAH using the 14 additional samples.

Conclusions: Our method enables highly sensitive and specific assessment of nine steroids from DBS and is a promising tool for clinical analysis of CAH.

Key Words: 17α-hydroxyprogesterone, Steroid hormones, Congenital adrenal hyperplasia, reference intervals, LC-MS/MS, Dried blood spot

INTRODUCTION

Serum steroid assays play an important role in the clinical evaluation of many common endocrine disorders [1]. Congenital adrenal hyperplasia (CAH), the most common adrenal gland disorder in infants and children, is a group of autosomal recessive disorders [2]. The 21-hydroxylase catalyzes hydroxylation of 17α-hydroxyprogesterone to 11-deoxycortisol in the glucocorticoid pathway [1]. In particular, 21-hydroxylase deficiency is the cause of approximately 95% of CAH cases [2]. Most neonatal screening pro-

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grams worldwide include the detection of CAH, and the standard test parameter is 17α-hydroxyprogesterone [3, 4].

Although immunoassays to evaluate 17α-hydroxyprogesterone are easy to handle, rapid, and highly sensitive, they have questionable reliability because of a lack of specificity to CAH and matrix effects [5]. Conventional screening for CAH using immunoassays for 17α-hydroxyprogesterone generates many false-positive results [3, 5], which not only upset parents and medical staff but also necessitate subsequent costly clinical and laboratory analyses [3].

While the inclusion of extraction steps improves immunoassay specificity, all interfering molecules cannot be completely eliminated [5]. Several studies attribute the low specificity to cross-reactivity of antibodies with steroids other than 17α-hydroxyprogesterone, such as steroid sulfates, 17α-hydroxypregnenolone, and 17α-hydroxypregnenolone sulfate [1, 3, 5]. Poor immunoassay specificity is an issue, especially in preterm or stressed infants, who have high concentrations of delta-5 cross-reacting steroids [2]. Preterm neonates often have high concentrations of 17α-hydroxyprogesterone because of stress or delayed maturation of 11-hydroxylase [1], which can reduce diagnostic specificity in screening for CAH by antibody-based methods, potentially resulting in false-positive diagnosis and generating demand for a second-tier confirmation test [2]. Using steroid profiling by liquid chromatography–tandem mass spectrometry (LC-MS/MS) in neonate screening for CAH yields fewer false-positive results because of its high analytical specificity and potential to quantify several compounds in a single run [2].

LC-MS/MS is the most accurate method currently available for measuring small molecules, and it can measure multiple steroid hormones simultaneously [6]. Steroid profiling with LC-MS/MS allows for evaluation of the status of enzymes involved in adrenal steroid biosynthetic pathways; thus, it is a better diagnostic tool than the evaluation of a single steroid [6]. A specific LC-MS/MS method with simultaneous detection of multiple steroids has been introduced to minimize unnecessary follow-up analyses [3, 6]. Although previous studies examined multiple steroid hormonal analyses using LC-MS/MS in Korea, they included limited numbers of steroids [6, 7] or did not evaluate clinical screening for CAH using patient samples [6]. Testosterone and progesterone, steroid hormones useful for the screening and diagnosis of CAH, have not been studied using dried blood spots (DBS) in Korean neonates [6, 7].

We developed and validated an LC-MS/MS method that accurately detects cortisol, 17α-hydroxyprogesterone, 11-deoxycortisol, 21-deoxycortisol, androstenedione, corticosterone,

11-deoxycorticosterone, testosterone, and progesterone in DBS, so as to be suitable for the screening and diagnosis of CAH in neonates. As the reference interval is not only method-specific [8] but also age-specific [9], we also determined age-specific reference intervals for the nine aforementioned steroids for healthy Korean neonates, including preterm neonates.

METHODS

Reagents, instruments, analytical conditions, and sample preparation

Samples were collected on DBS cards (Honeywell Burdick & Jackson, Morristown, NJ, USA) between May 2013 and November 2016 at Samsung Medical Center, Seoul, Korea. Reagents, instruments, analytical conditions, and sample preparation were as described previously [6, 8] with modifications for two more steroid hormones (testosterone and progesterone). Quantitative analyses were conducted in multiple reaction monitoring mode using an Agilent 6490 Triple Quadrupole Mass Spectrometer equipped with an Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA).

Operating conditions are shown in Table 1. To determine reference intervals for the nine steroids in Korean subjects, the 1,146 DBS samples from neonates (272 from preterm and 874 from full-term neonates) were analyzed using the LC-MS/MS method, and the data were stratified by sex.

Assay performance characteristics

Assay performance characteristics, including assay range, lower limit of quantification (LLOQ), linearity, precision, accuracy, extraction recovery, and matrix effects were evaluated according to the updated guidelines and literature for MS/MS method validation and neonate screening [2, 10-27]. Accuracy was additionally assessed by participating in the second-tier Congenital Adrenal Hyperplasia Proficiency Testing Program (CAHPT), a proficiency test for immunoassay and LC–MS/MS methods published quarterly by the Newborn Screening Quality Assurance Program of the Centers for Disease Control and Prevention [4]. In total, 1,146 anonymized DBS samples submitted for routine clinical testing were used to compare 17α-hydroxyprogesterone concentrations determined by immunoassay (AutoDELFIA; PerkinElmer, Waltham, MA, USA) and LC-MS/MS.

Reference intervals

Reference intervals were determined according to the CLSI guidelines [9] and compared with previously reported intervals

Table 1. HPLC gradient conditions and mass spectrometer operating conditions

HPLC gradient condition		
Time (min)	Mobile A (%)	Mobile B (%)
0	90	10
4	58	42
8	51	49
10.5	34	66
11.5	10	90
13	10	90
13.5	90	10

Mass spectrometer operating conditions						
Time segment	Compound name	RT (min)	Dwell time (msec)	Precursor-Product ion (m/z)		CE (eV)
1	Cortisol	5.903	200	363.1	121	30
	d4-Cortisol	5.879	200	367.4	121.1	22
2	21-Deoxycortisol	6.751	100	347.2	311	16
	d8-21-Deoxycortisol	6.683	100	355.3	100	32
	Corticosterone	7.084	100	347.2	121	22
	d8-Corticosterone	6.987	100	355.2	337.1	14
	11-Deoxycortisol	7.438	100	347.2	109	28
	d2-11-Deoxycortisol	7.405	100	349.3	109	34
3	Androstenedione	8.281	50	287.2	97	16
	d7-Androstendione	8.201	50	294.2	100	22
	Testosterone	9.043	50	289.2	109.1	22
	d5-Testosterone	8.916	50	294.2	99.9	22
	11-Deoxycorticosterone	9.200	500	331.3	97.1	32
	d8-11-Deoxycorticosterone	9.115	50	339.5	100	27
	17α-Hydroxyprogesterone	9.775	50	331.3	97.2	32
	d8-17α-Hydroxyprogesterone	9.697	50	339.5	100.1	27
4	Progesterone	11.196	100	315.2	109.2	30
	d9-Progesterone	11.133	100	324.2	113.1	26

Abbreviations: RT, retention time; CE, collision energy.

in the other populations [6, 8, 23-25, 27]. To validate the reference intervals, we used additional anonymized samples from eight neonates without CAH (normal neonates), three full-term neonates with CAH (21-hydroxylase deficiency) whose 17α-hydroxyprogesterone was >18.2 nmol/L by immunoassay, two girls (4 and 14 years old) with CAH (21-hydroxylase deficiency), and a 3-year-old girl with congenital adrenal lipoid hyperplasia due to *StAR* mutation.

Statistical analyses

All data for steroid hormones with or without stratification by age and sex were not normally distributed. We used the Mann–Whitney U test to compare them. Reference inervals were estimated

as the central 95% (the 2.5th and 97.5th percentiles) of the distribution of test results according to the CLSI guidelines [9]. $P<0.05$ was considered significant. Bland–Altman plots were created, and the nonparametric Passing–Bablock regression was conducted to compare the immunoassay and LC-MS/MS for 17α-hydroxyprogesterone, using MedCalc software for Windows, version 17.9.7 (MedCalc Software, Ostend, Belgium) [17].

Ethics

This study was conducted according to the Declaration of Helsinki, and all procedures involving human subjects were approved by the Institutional Review Board of Samsung Medical Center (SMC 2012-08-058), Seoul, Korea.

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RESULTS

Performance of LC-MS/MS for nine steroid hormones

Analyses of steroid-free samples showed no interfering peaks at either steroid or internal standard retention times. The total run time for each sample was 16 minutes. There were linear correlations between steroid concentration and signal intensity for cortisol (2.8–828.0 nmol/L) as well as for the other steroids (0.9–40.0 nmol/L; $R^2 > 0.99$). In addition, steroid concentrations that were 10 times higher than the highest calibrator still complied with linearity [14, 24]. Intra-day precision and inter-day precision CVs were 2.52–11.54% and 3.53–17.12%, respectively. Accuracy was 80.81–99.94% (difference: 0.06–19.19%). High accuracy was assured by participating in CAHPT and having acceptable results for four quarters [4, 14]. Extraction recovery (the average percent of the target concentration recovered from DBS calibrators run as patient samples) was 64.3–74.2%, and the matrix effect was 88.0–125.4%.

In the comparison between the LC-MS/MS method and immunoassay for 17α-hydroxyprogesterone, the LLOQ of the immunoassay for 17α-hydroxyprogesterone determination was 0.3 nmol/L. The correlation coefficient (r) was 0.8104 (95% CI: 0.7896–0.8294, $P < 0.0001$), and there was a negative bias, with higher values measured by immunoassay than by LC-MS/MS over the entire data range (Fig. 1).

Reference intervals and clinical application to 21-hydroxylase deficiency

Reference intervals of all steroid hormones except testosterone and progesterone significantly differed between preterm and full-term neonates (Table 2, $P < 0.0001$). Reference intervals and median values of the nine steroids determined in this study in comparison with those reported by other studies are presented in Tables 3 and 4. Of the nine steroids, only testosterone concentrations differed significantly by sex. The LC-MS/MS method allowed reliable differentiation of samples from 21-hydroxylase-deficient and unaffected neonates (Fig. 2). Of the two children with 21-hydroxylase deficiency, the 14-year-old girl was identified as screen positive for 21-hydroxylase deficiency with increased 17α-hydroxyprogesterone, 21-deoxycortisol, and androstenedione, and the 4-year-old girl was screen positive with only increased 17α-hydroxyprogesterone (her cortisol concentration was higher than the upper limit of the reference interval for full-term neonates). The ratio of androstenedione+17α-hydroxyprogesterone to cortisol of the children was not higher than the upper limit of the reference interval for either full-term neonates or preterm neonates. For the 3-year-old girl with congenital adrenal lipoid hyperplasia caused by *StAR* mutation, the cortisol concentration was higher than the upper limit of the reference interval for full-term neonates, whereas the other steroids were within the reference interval for full-term neonates.

DISCUSSION

We developed and validated an LC-MS/MS method for the simultaneous quantification of nine steroids in DBS. In addition, we determined age-specific reference intervals of the nine steroids for Korean neonates (preterm and full-term). To the best of our knowledge, this is the first attempt to determine reference intervals for testosterone and progesterone concentrations in
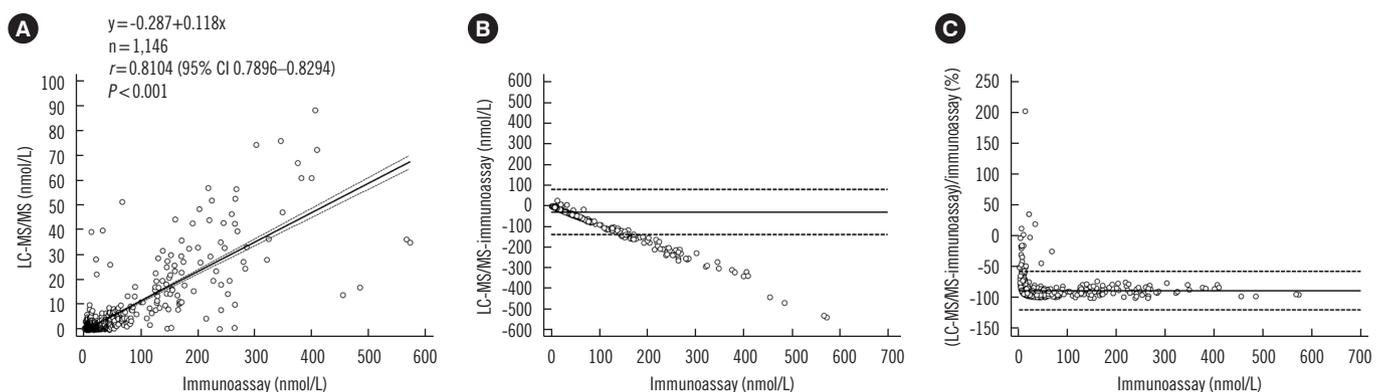
Fig. 1. Comparison of LC–MS/MS and immunoassay. Correlation (A), absolute difference (B), and percent bias (C) between LC-MS/MS and immunoassay results for 17α-hydroxyprogesterone in 1,146 dried blood spots from Korean neonates. The solid line in (A) is the best-fit regression line and dashed lines in (A) represent 95% confidence interval. Solid lines in (B) and (C) represent average differences, and dashed lines in (B) and (C) represent limit of agreement.
Abbreviation: LC-MS/MS, liquid chromatography–tandem mass spectrometry.

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Table 2. Reference intervals (nmol/L) for nine steroid hormones in dried blood spots from Korean preterm and full-term neonates

	Preterm neonates			Full-term neonates			P
	N	Median	Central 95th percentile	N	Median	Central 95th percentile	
Cortisol	272	29.31	<2.76–1,026.66	874	18.77	<2.76–188.88	0.0001
21-Deoxycortisol	272	1.36	<0.90–16.24	874	<0.90	<0.90–5.36	<0.0001
Corticosterone	250	1.79	<0.90–28.55	830	0.98	<0.90–24.45	<0.0001
11-Deoxycortisol	272	2.18	<0.90–19.59	874	1.13	3.30–3.53	<0.0001
Androstenedione	272	2.02	<0.90–29.87	874	<0.90	<0.90–6.27	<0.0001
11-Deoxycorticosterone	272	<0.90	<0.90–1.55	874	<0.90	<0.90	<0.0001
Testosterone	M 149	<0.90	<0.90–3.96	M 428	<0.90	<0.90–2.63	0.1651
	F 123	<0.90	<0.90–1.54	F 446	<0.90	<0.90–1.80	0.0572
17α-Hydroxyprogesterone	272	2.70	<0.90–59.72	874	<0.90	1.32–6.11	<0.0001
Progesterone	272	4.29	<0.90–29.19	874	4.87	<0.90–30.41	0.0703
(Androstenedione+17α-hydroxyprogesterone)/ cortisol ratio	272	0.19	0.01–3.84	874	0.11	0.01–0.78	<0.0001

*P values were calculated using the Mann–Whitney U test for nonparametric data.
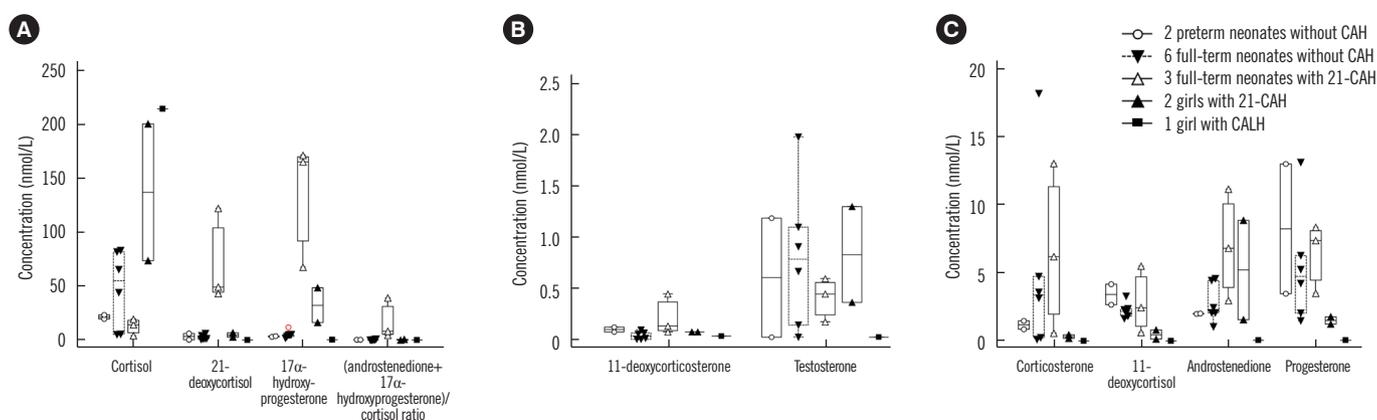
Fig. 2. Steroid profiles for 14 additional samples from neonates and children used to evaluate the clinical applicability of the LC-MS/MS method. Full-term neonates with 21-CAH (Δ) had distinctively low concentrations of cortisol, high concentrations of 21-deoxycortisol, 17α-hydroxyprogesterone, 11-deoxycorticosterone, and high values of (androstenedione+17α-hydroxyprogesterone)/cortisol ratio (A & B). Other steroid hormone concentrations in full-term neonates with 21-CAH were variable (C). Two children with 21-CAH had high concentrations of cortisol and 17α-hydroxyprogesterone, progesterone, and androstenedione. Concentrations of other steroids were not distinctive. One child with CALH had high concentration of cortisol and low concentrations of other steroids.
Abbreviations: CAH, congenital adrenal hyperplasia; 21-CAH, congenital adrenal hyperplasia caused by 21-hydroxylase deficiency; CALH, congenital adrenal lipoid hyperplasia due to the StAR mutation; LC-MS/MS, liquid chromatography–tandem mass spectrometry.

DBS samples from Korean neonates.

Intra-day precision and inter-day precision CVs were comparable with results in the literature and guidelines [2, 12, 13, 16, 22]. Accuracy was within the acceptable range for neonatal screening using MS/MS [2, 4, 12, 13, 16, 17, 22]. Extraction recovery and the matrix effect were comparable to the previous findings and existing guidelines for neonatal screening by MS/MS [2, 11-13, 16, 17, 20-23]. In our study, the bias between LC-MS/MS and immunoassay findings changed over the measurement interval in a linear fashion, and there was a negative bias, with higher values measured by immunoassay than LC-MS/MS over the entire range.

To avoid unnecessary tests, a sensitive and specific test, such as the LC-MS/MS method, is advantageous [1, 7]. Our method allows for simultaneous and rapid quantitation of nine steroids related to CAH with high specificity and without compromising

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Table 3. Reference intervals (nmol/L) of nine steroid hormones in dried blood spots from Korean preterm neonates and comparisons with previous studies

| | This study | | | Kim et al. [6] | | | Janzen et al. [24] | | | Boelen et al. [25] | | | | | |
| | | | | | | | | | | GA≤33W | | 33<GA≤35W | | 35<GA≤36W | |
	N	Median	Central 95th percentile	N	Median	Central 95th percentile	N	Median	Central 90th percentile	N	Mean±2SD	N	Mean±2SD	N	Mean±2SD
Cortisol	272	29.31	<2.76–1,027	76	28.76	<2.76–208.93	864	807	81.6–5,126	107	7.8–346	107	4.9–337	106	3.0–238
21-Deoxycortisol	272	1.36	<0.90–16.24	76	<1.45	<1.45–3.73	864	5.60	2.23–14.9	91	<1	93	<1	89	<1
Corticosterone	250	1.79	<0.90–28.55	76	3.06	<1.45–21.27				105	<36	105	<28	105	<20
11-Deoxycortisol	272	2.18	<0.90–19.59	76	1.85	<1.45–18.41	864	35.1	21.5–707	106	1.3–14	106	<6.0	106	<4.0
Androstenedione	272	2.02	<0.90–29.87	76	<1.75	<1.75–5.30	864	15.9	4.64–79.4	105	<7.0	105	<6.9	106	<3.9
11-Deoxycorticosterone	272	<0.90	<0.90–1.55	76	<1.52	<1.52				94	<1	83	<1	69	<1
Testosterone	M 149	<0.90	<0.90–3.96							51	<2.6	51	<3.2	53	<2.0
	F 123	<0.90	<0.90–1.54							52	<1	54	<1	53	<1
17α-Hydroxyprogesterone	272	2.70	<0.90–59.72	76	3.79	<1.52–25.00	864	75.6	37.2–171	106	1.1–29	106	<14	107	<5.0
Progesterone	272	4.29	<0.90–29.19												
(Androstenedione + 17α-hydroxyprogesterone)/cortisol ratio	272	0.19	0.01–3.84												

*To convert ng/mL to nmol/L for data comparison, multiplication factors of 2.76 for cortisol, 2.89 for 21-deoxycortisol, corticosterone, and 11-deoxycortisol, 3.49 for androstenedione, 3.03 for 11-deoxycorticosterone and 17α-hydroxyprogesterone, 3.18 for progesterone, and 3.67 for testosterone were applied [26].
Abbreviations: GA, gestational age; W, week.

Table 4. Reference intervals (nmol/L) of nine steroid hormones in dried blood spots from Korean full-term neonates and comparisons with previous studies

| | This study | | | Kim et al. [6] | | | Magnisali et al. [8] | | Janzen et al. [23] | | Janzen et al. [24] | | | Boelen et al. [25] | | Monostori et al. [27] | | |
| | | | | | | | | | (Neonates and children) | | | | | | | | | |
	N	Median	Central 95th percentile	N	Median	Central 95th percentile	N	Ref. interval	N	Range	N	Median	Central 90th percentile	N	Mean±2SD	N	Median	1-99th percentile
Cortisol	874	18.77	<2.76–188.88	78	23.07	<2.76–194.8	M 23	5.0–188.23	5	143.9–339.2	729	176	14.7–642	128	3.9–152	81	34.8	4.6–423.7
							F 27	16.01–211.14										
21-deoxycortisol	874	<0.90	<0.90–5.36	78	<1.45	<1.45–4.42			5	0.00–2.1	729	2.90	<1–7.85	101	<1	81	<5.0	<5.0–5.9
Corticosterone	830	0.98	<0.90–24.45	78	1.73	<1.45–15.49			5	2.1–10.3				128	<12			
11-Deoxycortisol	874	1.13	3.30–3.53	78	<1.45	<1.45–3.70	M 23	<1.45–3.32	5	5.3–7.7	729	6.30	1.48–23.3	129	<1.8	81	<2.5	<2.5
							F 27	<1.45–3.15										
Androstenedione	874	<0.90	<0.90–6.27	78	<1.75	<1.75–2.51	M 23	<1.75–2.72	5	0.00–3.2	729	5.80	1.10–17.8	128	<2.4	81	<1.0	<1.0–3.0
							F 27	<1.75–2.30										
11-Deoxycorticosterone	874	<0.90	<0.90	78	<1.52	<1.52			5	0.8–4.2				96	<1			
Testosterone	M 428	<0.90	<0.90–2.63						5	0.00–10.9				63	<1			
	F 446	<0.90	<0.90–1.80											66	<1			
17α-Hydroxyprogesterone	874	<0.90	1.32–6.11	78	<1.52	<1.52–4.51	M 23	<1.52–3.03	5	4.7–8.1	729	10.2	3.38–24.9	128	<2.5	81	2.5	<2.5–8.3
							F 27	<1.52–4.12										
Progesterone	874	4.87	<0.90–30.41				50	<0.01–0.15	5	0.00–11.0								
(Androstenedione + 17α-hydroxyprogesterone)/cortisol ratio	874	0.11	0.01–0.78													81	0.04	0.01–0.43

*To convert ng/mL to nmol/L for data comparison, multiplication factors of 2.76 for cortisol, 2.89 for 21-deoxycortisol, corticosterone, and 11-deoxycortisol, 3.49 for androstenedione, 3.03 for 11-deoxycorticosterone and 17α-hydroxyprogesterone, 3.18 for progesterone, and 3.67 for testosterone were applied [26].
Abbreviations: F, female; GA, gestational age; LLOQ, lower limit of quantification; M, male; W, week.

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chromatographic resolution.

Differential diagnosis of CAH subtypes is possible by steroid profiling using the LC–MS/MS method [1]. In this method, diagnosis of CAH depends on not only the absolute quantification of 17α-hydroxyprogesterone but also the ratio of multiple steroids to determine a positive result: 17α-hydroxyprogesterone (a direct substrate for 21-hydroxylase), cortisol (the final product of the adrenal enzyme's action), and androstenedione (which secondarily accumulates in 21-hydroxylase deficiency) [2]. Although the prevalence of CAH other than 21-hydroxylase deficiency is very low, and samples from patients with those diseases were not tested in this study, steroid profiling on the basis of ratios of multiple steroids, including precursor hormone and product hormones, used in this method has diagnostic strengths.

According to the CLSI guidelines [9], at least 120 samples for analysis are required to establish reference intervals. Some previous studies on the development and application of mass spectrometry for steroid profiling included less than 120 samples for establishing reference intervals [6, 8, 23, 25, 27]. Although the various reference intervals presented in these studies corresponded to different analytical conditions, they were quite similar to the present ones, as shown in Tables 3 and 4 [6, 8, 23-25, 27]. We included more than 120 samples for all nine steroids, especially testosterone and progesterone, which previous studies did not do [8, 25, 27].

Furthermore, we successfully demonstrated the clinical applicability of steroid profiling to differentiate between neonates with 21-hydroxylase deficiency and normal neonates. However, the reference interval was not established using samples from children to appropriately differentiate children with CAH from the normal ones. Considering that there was a 3-year-old girl with congenital adrenal lipoid hyperplasia due to *StAR* mutation whose cortisol concentration was higher than the upper limit of the reference interval for full-term neonates, whereas the other steroids were within the reference interval for full-term neonates, the reference interval of hormone analyses should be established separately for different age groups [6, 9].

The limitation of this study was the limited numbers of samples for validation, because the prevalence of CAH is low [25]. Previous studies also included limited numbers of CAH patients for clinical application due to the low prevalence of CAH, but they reported successful clinical application of the methods in a manner similar to this study [2, 5, 7].

In conclusion, our method produced lower values for 17α-hydroxyprogesterone than an immunoassay, consistent with the relatively low analytical specificity of immunoassays compared with LC-MS/MS. We also established reference intervals for nine steroid hormones in Korean neonates, which were comparable with those in other populations. Steroid profiling by LC–MS/MS highlights the utility of secondary diagnostic markers as a means of reducing false-positive results due to stress- or illness-induced transient elevation of 17α-hydroxyprogesterone. Thus, our sensitive, specific, and accurate steroid profiling method is suitable for routine neonatal screening for CAH.

Authors' Disclosures of Potential Conflicts of Interest

The authors declare that they have no conflicts of interest.

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