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Newborn Screening for Congenital Adrenal Hyperplasia: Additional Steroid Profile using Liquid Chromatography-Tandem Mass Spectrometry

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Background: Neonatal screening programs for congenital adrenal hyperplasia (21-CAH) using an immunoassay for 17α-hydroxyprogesterone (17-OHP) generate a high rate of positive results attributable to physiological reasons and to cross-reactions with steroids other than 17α-OHP, especially in preterm neonates and in critically ill newborns.

Methods: To increase the specificity of the screening process, we applied a liquid chromatography-tandem mass spectrometry method quantifying 17α-OHP, 11-deoxycortisol, 21-deoxycortisol, cortisol, and androstenedione. The steroids were eluted in aqueous solution containing d_8-17α-OHP and d_2-cortisol and quantified in multiple reaction mode.

Results: Detection limit was below 1 nmol/liter, and recovery ranged from 64% (androstenedione) to 83% (cortisol). Linearity was proven within a range of 5–100 nmol/liter (cortisol, 12.5–200 nmol/liter), and total run time was 6 min. Retrospective analysis of 6151 blood samples and 50 blood samples from newborns with clinically confirmed 21-CAH, as well as prospective analysis of 1609 samples of a total of 242,500 testing positive in our routine 17-OHP immunoassay, allowed clear distinction of affected and nonaffected newborns. High levels of 21-deoxycortisol were only found in children with 21-hydroxylase deficiency. Calculating the ratio of 17α-OHP to 21-deoxycortisol divided by cortisol further increased the sensitivity of the method.

Conclusion: Our liquid chromatography-tandem mass spectrometry procedure as a second-tier test can be used to reduce false-positive results of standard 21-CAH screening. The short total run time of 6 min allows for immediate reanalysis of all immunoassay results above the cutoff. **(*J Clin Endocrinol Metab* 92: 2581–2589, 2007)**

CONGENITAL ADRENAL HYPERPLASIA (CAH) (OMIM: 201910) is caused by a group of autosomal recessive disorders of adrenal cortisol biosynthesis. Excess production of hormones proximal to the enzymatic defect results in various clinical phenotypes. The most frequent forms of CAH are 21-hydroxylase (CYP21A2) and 11β-hydroxylase (CYP11B1) deficiency (1). Defective mineralocorticoid synthesis may lead to life-threatening salt-wasting crisis. Clinical symptoms include poor feeding and failure to thrive, vomiting, hyperkalemia, hyponatremia, dehydration, metabolic acidosis, and apathy. In the Caucasian population, 21-hydroxylase deficiency, the classical form of CAH, accounts for more than 90% of all cases, whereas 5% are caused by 11-hydroxylase deficiency. Other enzyme deficiencies and clinical phenotypes are less frequent (1).

Neonatal screening for CAH was first introduced in 1977 when immunoassays became available (2). Since then, most screening laboratories have used these tests, which, however, produce a large number of false-positive results (3). Preterm babies often show high levels of 17α-hydroxyprogesterone (17-OHP) because of stress or delayed maturation of 11-hydroxylase. Furthermore, cross-reactions of the specific antibody with other steroids add to the high number of false-positive results (4). Compared with other neonatal screening tests, the specificity of screening for 21-CAH by immunoassay is low.

There are a number of different techniques for diagnosing 21-CAH, and some of them are still in use. 17α-OHP has been measured in both whole blood and dried blood by radioimmunological assays (2, 5). Most of these have been replaced by immunoassays that determine time-resolved fluorescence of europium-labeled 17α-OHP (6) or work as an ELISA. Torresani *et al.* (7) as well as Allen *et al.* (8) established cutoff values for 17α-OHP depending on gestational age to reduce the number of false-positive results in preterm babies. To minimize false-positive results attributable to cross-reactivity, Wudy *et al.* developed a method to analyze 17-OHP and other steroids in blood and urine by gas chromatography (9) and liquid chromatography (10) mass spectrometry.

To reduce the high number of false-positive results produced by immunoassays, several research groups applied various techniques for steroid analysis, including 17α-OHP (11). Kushnir *et al.* (12) evaluated quantification of 11-deoxycortisol (S) (Reichstein's substance S; 17α,21-dihydroxy-4-pregnene-3,20-dione), 17-OHP, 17-hydroxypregnenolone, and pregnenolone after oxime formation and subsequent solid-phase extraction and chromatographic analysis by liquid chromatography-tandem mass spectrometry (LC-MS/

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Abbreviations: 4-A, Androstenedione (4-androstene-3,17-dione); CAH, congenital adrenal hyperplasia; F, cortisol (Reichstein's substance M; 11β,17α,21-trihydroxypregn-4-ene-3,20-dione); 21-F, 21-deoxycortisol (11β,17α-dihydroxy-4-pregnene-3,20-dione); LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; m/z, mass-to-charge ratio; 17-OHP, 17α-hydroxyprogesterone (17α-hydroxy-4-pregnene-3,20-dione); S, 11-deoxycortisol (Reichstein's substance S; 17α,21-dihydroxy-4-pregnene-3,20-dione); TRFIA, time-resolved fluorescence immunoassay.

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MS). Lacey *et al.* (13) and Minutti *et al.* (14) were able to improve the specificity of conventional 17-OHP screening by adding steroid profiling using MS/MS as a second-tier method. They measured 17α-OHP, cortisol (F) [Reichstein's substance M; 11β,17α,21-trihydroxypregn-4-ene-3,20-dione], and androstenedione (4-A) (4-androstene-3,17-dione). Total run time was 12 min. Kosel *et al.* (15) used real-time PCR to confirm 21-CAH by a second-tier screening.

Our aim was to further improve MS/MS steroid profiling as a second-tier test for neonatal standard 21-CAH screening. We developed a method for steroid analysis in blood spots allowing precise detection of 17α-OHP, F, as well as three other steroids that are relevant for diagnosis of 21-CAH.

Subjects and Methods

Reagents and calibrators

For routine screening, we used the Delfia Neonatal 17α-OHP competitive immunoassay from PerkinElmer (Turku, Finland) labeled with europium. Blood was collected on filter cards type S&S 903 from Whatman Schleicher & Schuell (Dassel, Germany).

For our LC-MS/MS procedure, we purchased 17-OHP, F, 4-A, 21-deoxycortisol (21-F) (11β,17α-dihydroxy-4-pregnene-3,20-dione), and S from Sigma (Taufkirchen, Germany), and deuterated standards d_8-17-OHP and d_2-F were from Cambridge Isotope Laboratories (LCG Eurisotop, Saarbruecken, Germany). Acetone, acetonitrile, formic acid, methanol, and HPLC-grade water were from Merck (Darmstadt, Germany) and had highest purity grades.

Dried blood calibrators, controls, and samples for testing precision, stability, and recovery were prepared from venous blood using a modified method reported by Lacey *et al.* (13): red blood cells were washed three times with saline and then diluted with steroid-free serum (MP Biomedicals, Eschwege, Germany) to obtain a hematocrit of 55%. To ensure complete hemolysis, samples were frozen twice at −18 C and thawed again. Cortisol and the remaining steroids were dissolved in methanol/water at 50:50 (vol/vol) with a final concentration of 10 and 4 μmol/liter, respectively. These stock solutions were added to the "whole blood pool" to give calibrators for 17-OHP, 4-A, 21-F, and S at final concentrations of 0 (blank), 5, 10, 25, 50, and 100 nmol/liter and for F at concentrations of 0, 12.5, 25.0, 62.5, 125, and 250 nmol/liter. Consequently, calibrators were spotted on filter cards (S&S 903) with each spot containing 25 μl. The cards were dried for more than 12 h at room temperature and stored at −20 C.

For precision and recovery analysis, whole blood was spotted on S&S 903 filter paper, dried, and stored (blank sample). An additional aliquot was spiked with steroids to achieve final concentrations of 5, 15, and 50 nmol/liter and 12.5, 63, and 125 nmol/liter, respectively, and spotted on filter paper. Steroid concentrations of the spiked samples were calculated by subtracting the concentrations obtained from the blank samples.

To evaluate the recovery, aqueous calibrator solutions containing steroids at concentrations of 5, 10, 25, 50, and 100 nmol/liter (F: 12.5, 25, 62.5, and 125 nmol/liter) were measured three times. Then, the recovery was calculated taking into account the calibrators as well as the blank sample.

Stock solutions of internal standards d_8-17-OHP (300 μmol/liter) and d_2-F (100 μmol/liter) were diluted with methanol to give final concentrations of 15 nmol/liter for d_8-17-OHP and 100 nmol/liter for d_2-F.

Samples

In a retrospective study, 6151 dried blood samples from our neonatal screening program that had been analyzed in the routine program by time-resolved fluorescence immunoassay (TRFIA) and 50 dried blood samples from neonates with confirmed 21-hydroxylase deficiency were analyzed by LC-MS/MS. In the prospective part of the study, 1609 samples that had exceeded the respective cutoff levels in the TRFIA test were immediately analyzed by LC-MS/MS. This group included an additional 16 cases of 21-hydroxylase deficiency. All cases (n = 66) were confirmed in accordance with the Consensus Statement of 21-Hydroxylase Deficiency (16); in 40 cases, additional molecular genetic analysis was performed.

External control material

For quality control measurements, we used proficiency testing material from several institutions: 17-OHP in dried blood samples from Centers for Disease Control (Atlanta, GA), serum samples of F and 17-OHP from the Society of Clinical Chemistry and Laboratory Medicine (Karlsruhe, Germany), and cortisol from Instand (Düsseldorf, Germany). In addition, dried blood control samples were taken from the Delfia immunoassay that contained 17α-OHP only.

Assay procedure

Routine screening. The TRFIA was performed as described in the assay procedure manual delivered by the manufacturer. Blood spot diameter was 3 mm. The cutoffs were adjusted to the gestational age given on the request form. Cutoffs were calculated in relation to percentiles and rounded to tens. Percentiles used were 99.5 for term babies, 99.0 for 35–36 wk gestational age, 98.0 for 30–34 wk, and 90.0 for 28–29 wk. When the gestational age was younger than 28 wk, a cutoff of 180 nmol/liter was used.

LC-MS/MS. Six-millimeter-diameter blood spots equaling 12 μl whole blood each were punched into polypropylene microtiter plates. Twenty-microliter acetone/acetonitrile at 50:50 (vol/vol) and 20 μl of both of the internal standards d_8-17-OHP (15 nmol/liter) and d_2-F (100 nmol/liter) were added per well. The plate was sealed and shaken for 50 min at ambient temperature. The supernatant was transferred to a polypropylene microtiter plate and carefully dried at 60–70 C. After reconstitution in 80 μl methanol/water at 50:50 (vol/vol) containing 50 mmol/liter formic acid, the plate was vigorously shaken for 30 min. Then the solutions were transferred to a 384-well microtiter plate and centrifuged for 10 min at 2000 U/min to remove residues of the filter paper.

HPLC and tandem mass spectrometer

For HPLC analysis, the Atlantis dC18 3 μm, 2.1 × 20 mm IS Column (Waters, Eschborn, Germany) was used. The precolumn was a C18 ODS 4.0 × 2.0 mm (Phenomenex, Aschaffenburg, Germany).

The mobile phase consisted of two solvents: HPLC-grade water (solvent A) containing 32 mmol/liter formic acid, and methanol (solvent B). The initial solvent mixture was 45% solvent A and 55% solvent B. The gradient increased up to 78% solvent B within 1.9 min, then up to 100% solvent B within 0.1 min, held for 0.5 min, returning via a step gradient to the initial state after 2.42 min. The total run time was 6 min. The flow rate was 250 μl/min. Chromatography was done at ambient temperature.

The LC-MS/MS system consisted of a Waters 1525μ HPLC pump

TABLE 1. MRM functions and settings for detecting steroids

Function	Time (min)	Name	Parent $(M+H)^+$	Daughter $(M+H)^+$	Cone voltage (V)	Collision energy (eV)
1	0–2.55	F	363.3	121.1	50	24
		d_2-F	365.3	122.1	50	22
2	2.10–2.95	S	347.3	109.1	25	30
		21-F	347.3	311.2	50	16
3	2.50–3.35	4-A	287.3	97.0	60	22
4	2.55–6.00	17α-OHP	331.3	109.0	64	28
		d_8-17α-OHP	339.4	113.2	60	32

with internal solvent degasser unit connected to a Waters Ultima ESI triple quadrupole mass spectrometer and an autosampler/injection system CTC Pal (Axel Semrau, Sprockhoefel, Germany). All parts were controlled by the MassLynx Software 4.0 (Waters); for integration and quantification, the QuanLynx software (Waters) was used.

The settings were optimized for maximum ion yield. For each steroid, the best settings and fragments were chosen after injection of aqueous single samples as described in Table 1. Capillary voltage was 3.5 kV, source temperature was 120 C, desolvation temperature was 300 C, and argon gas cell pressure was 2.2×10^{-3} mbar. During HPLC-MS/MS performance, the ambient temperature was kept at 23 C. All steroids were measured in the positive ion mode using electrospray ionization. The interchannel delay time, interscan time, and dwell time were 0.05 sec each. Functions 1–4 (Table 1) refer to the multiple reaction monitoring (MRM) transitions of the respective steroids.

Quantification

Quantitative results were obtained by dividing the peak area of each steroid by the peak area of the internal standard (d_2-F for F, d_8-17-OHP for the remaining steroids). Concentrations were calculated using the QuanLynx Software via linear regression with reciprocal fit weighting to ensure maximum accuracy at the lower concentration range.

Assay performance characteristics

Method interassay was determined analyzing three replicates per run of samples containing steroids at final concentrations of 5, 15, and 50 nmol/liter (F: 12.5, 37.5, and 125 nmol/liter) in one run per day over a period of 10 d. For intraassay analysis, a sequence of 10 replicates of samples was measured.

The method linearity was evaluated using dried blood samples containing steroid concentrations between 5 and 100 nmol/liter and cortisol concentrations from 12.5 to 250 nmol/liter.

To determine the limit of detection, a liquid control sample containing known concentrations of steroids was diluted with steroid-free serum (MP Biomedicals). The dilution was stopped when a signal-to-noise ratio of 10:1 was reached.

The ion suppression was determined by injecting a sample in a constant flow (10 μl/min) of 17-OHP (25 nmol/liter) and consequently measuring the signal reduction of the mass transition [mass-to-charge ratio (m/z), 331.3 → 109.0] after injection.

Statistical analysis of the data was done using MedCalc version 9 (MedCalc, Mariakerke, Belgium) as well as SigmaPlot (Systat, Erkrath, Germany).

Results

Figure 1 shows the total ion chromatogram reflecting all MRM functions of one sample. The separate MRM functions

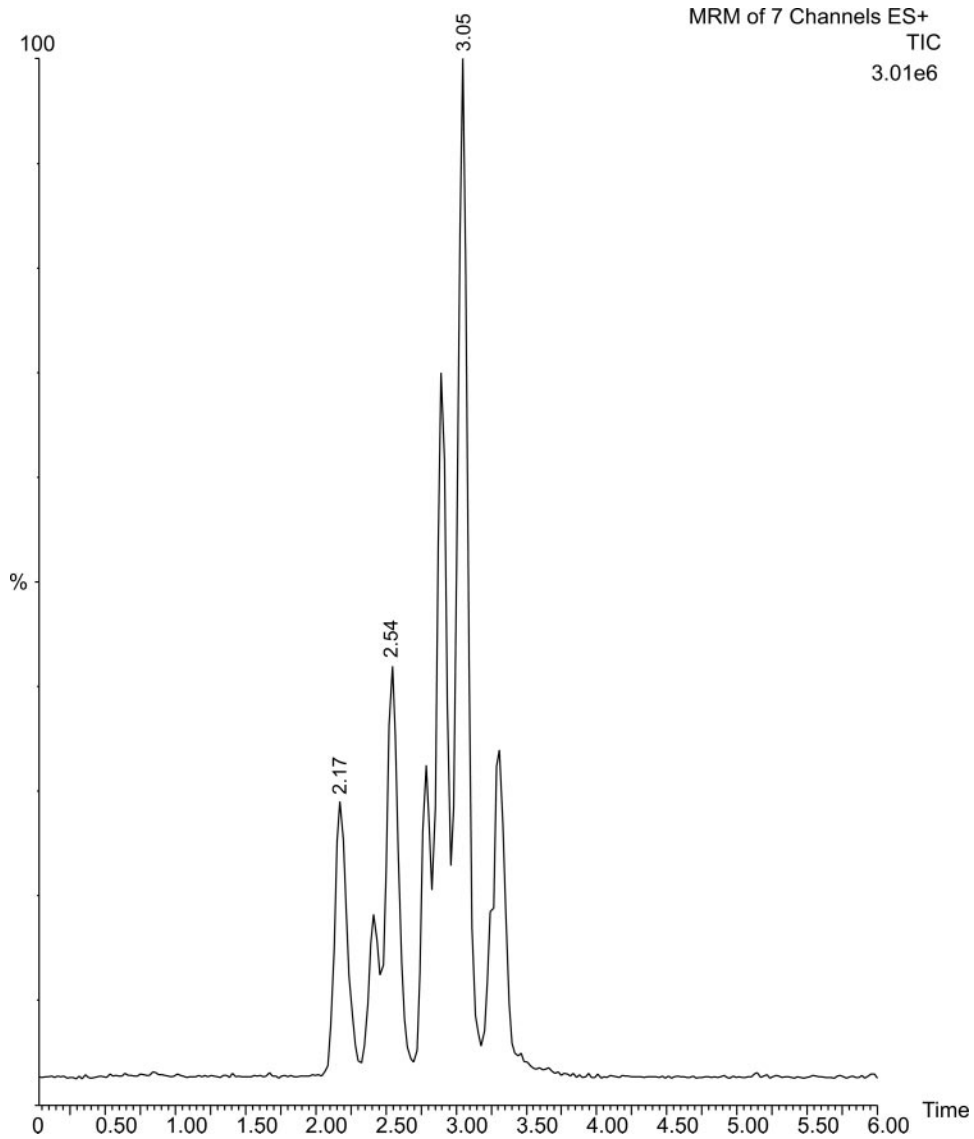

FIG. 1. Total ion chromatogram (TIC) of steroids including all MRM functions. Functions 1–4 shown in Table 1 are superimposed in this diagram.

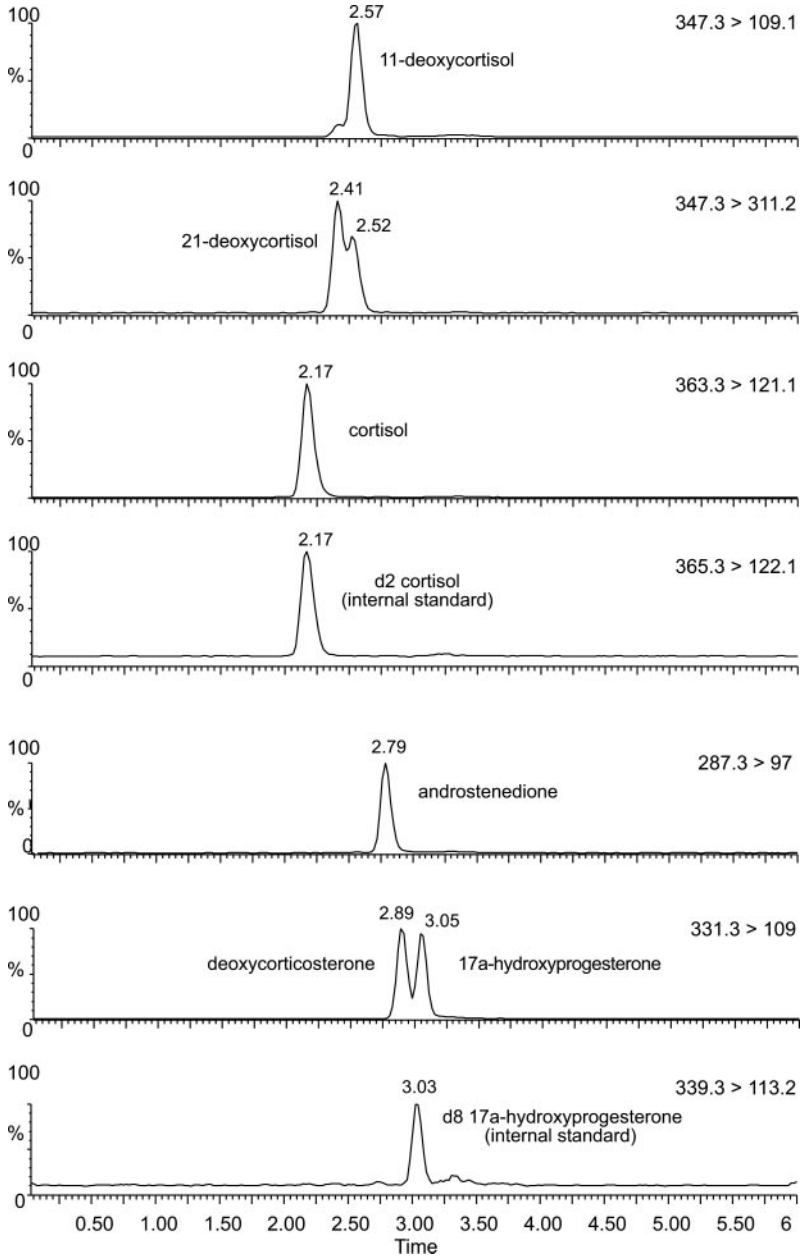

FIG. 2. MRM transitions of the respective steroids.

are shown in Fig. 2. There was a double peak at transition m/z of 347.3. This consisted of the fragments of both 21-F and S. 21-F showed two transitions: m/z of 347.3 → 311.2 as well as an additional fragment at m/z of 109.1. However, chromatographic separation still allowed quantification of 21-F. The mass transition m/z of 331.9 → 109.0 also showed a double peak consisting of the fragments of 17-OHP and deoxycorticosterone. Chromatographic separation allowed quantification of 17-OHP.

With a flow rate of 250 μl/min, ion suppression began at

TABLE 2. Coefficient of variation (CV) (intraassay and interassay) at different steroid concentrations measured in spiked dried blood samples

Compound	CV (%) intraassay (n = 10)			CV (%) interassay (n = 10)		
	5 nM	15 nM	50 nM	5 nM	15 nM	50 nM
17-OHP	9.9	4.5	4.2	10.9	7.9	8.3
S	8.4	8.2	8.7	11.2	10.6	9.8
21-F	9.4	9.3	7.4	12.2	11.0	10.8
4-A	6.6	6.8	3.3	12.3	9.4	9.0
	7.5 nM	125 nM	200 nM	7.5 nM	125 nM	200 nM
F	6.1	7.6	4.7	13.7	11.2	8.3

TABLE 3. Recovery data at different steroid concentrations measured in spiked dried blood samples

Compound	Blood spots with added concentrations		
	5 nmol/liter	15 nmol/liter	50 nmol/liter
17-OHP	73.4%	78.8%	79.3%
S	89.4%	80.7%	78.7%
21-F	80.8%	81.8%	80.8%
4-A	76.0%	66.5%	60.8%
	12.5 nmol/liter	25.5 nmol/liter	125 nmol/liter
F	72.5%	81.0%	84.5%

a retention time of 0.7 min with a decline of the baseline and recovery by 1.2 min. Of all steroids, F eluted first at 2.17 min. There was no ion suppression at any of the retention times of the steroids.

Assay performance

The limit of detection was less than 1 nmol/liter for all steroids. There was a linear correlation between steroid concentration and signal intensities for 17-OHP, S, 21-F, and 4-A from 3–125 nmol/liter. This was also true for F and the signal intensities from 12.5–250 nmol/liter ($r > 0.999$). The coefficient of regression was more than 0.998 in all cases. Concentrations that were well above the highest calibration standard were extrapolated. In addition, by dilution of those samples, we were able to show that steroid concentrations that were 10-fold higher than the highest calibrator still complied with linearity. In Table 2, the precision data are given.

As shown in Table 3, recovery ranged from 60.8 to 89.4% for 17-OHP, S, 21-F, and 4-A and F. 4-A showed the lowest recovery with 60.8%, whereas the degree of extraction of S from the dried blood spots was highest with 89.4% recovery. There was no significant correlation between the rate of recovery and the concentration of the respective steroid.

Analyzing control samples of TRFIA spiked with 17-OHP as the only steroid yielded comparable results with LC-MS/MS. The regression analysis performed according to the Deming method between the TRFIA (y) and the LC-MS/MS (x) results yielded a slope of 1.436 ± 0.197 nmol/liter (mean ± SE) and a y-intercept of 30.5 ± 2.55 nmol/liter (mean ± SE) with a coefficient of variation (r) of 0.866. The Bland-Altman difference plot (17–19) revealed greater disagreement between 17-OHP measured by TRFIA and LC-MS/MS (Fig. 3). The TRFIA method has positive bias, especially for lower concentrations; the difference decreased with higher concentrations.

Analytical results obtained with the control samples of the TRFIA kit are depicted in Fig. 4. The values given by the manufacturer were compared with those obtained by both the TRFIA test kit and LC-MS/MS. The results obtained by LC-MS/MS were well within the limits given by the kit producer, showing less variation compared with those produced by the TRFIA. Measured results of TRFIA exceeded

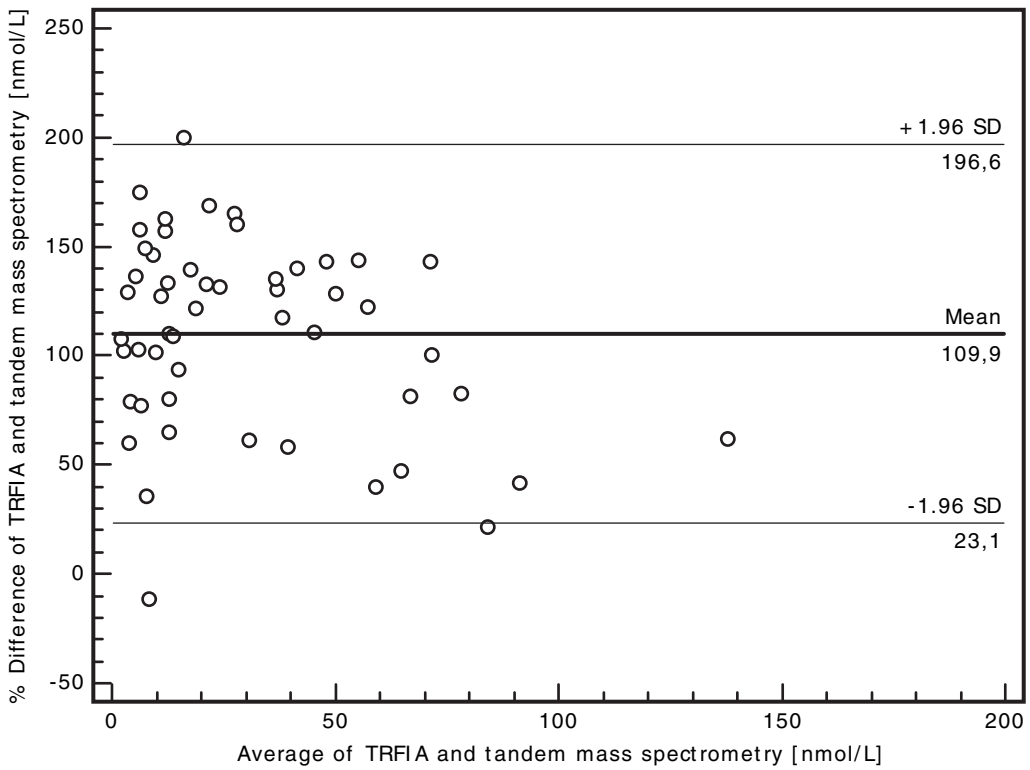

FIG. 3. Bland-Altman plot comparing 17α-OHP concentration in dried blood spots obtained with TRFIA and LC-MS/MS assays.

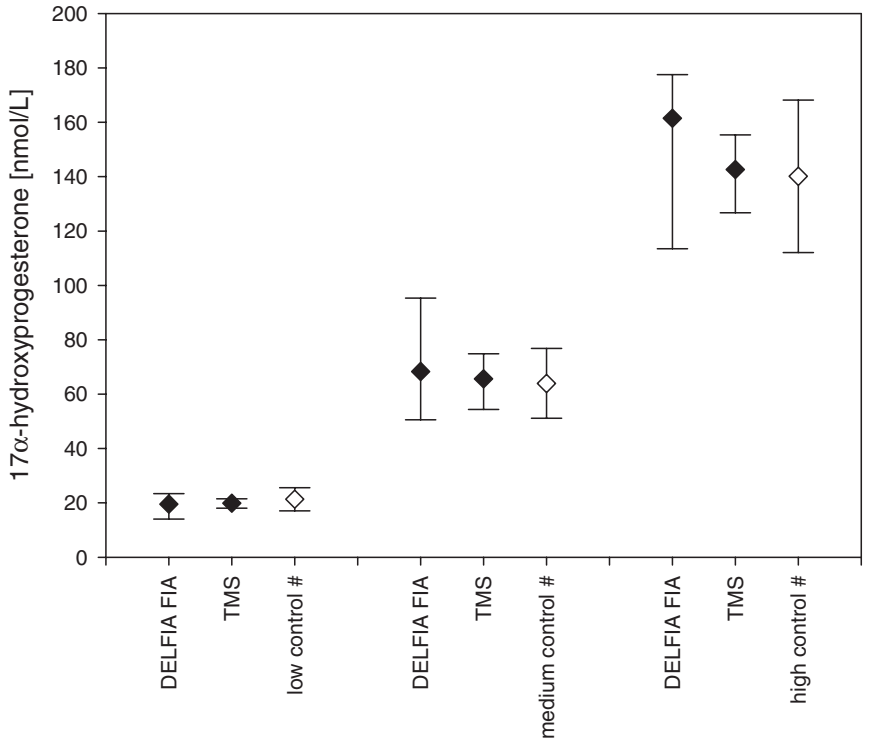

FIG. 4. 17α-OHP concentrations in control samples (low, medium, and high control) supplied with the TRFIA test kits (mean, 2-s range); number range given by the manufacturer (mean, 2-s range). TMS, Tandem mass spectrometry.

the upper limits defined in the kit for the medium as well as the high control. Moreover, values obtained by TRFIA were below the given limits of the low control.

Samples

Data of children of the retrospective group without 21-CAH are listed in relation to the gestational age in Table 4. For all gestational age groups, 17-OHP was significantly higher in the TRFIA compared with LC-MS/MS ($P < 0.001$, paired *t* test). Values of 17-OHP, S, and 4-A decreased with increasing gestational age. F and 21-F showed no correlation, but F variation was higher in the group with lower gestational ages.

Table 5 shows the inverse correlation of 17-OHP levels and gestational age for female and male newborns. There was no significant difference in the 17-OHP values of male compared with female newborns (ANOVA, $P = 0.190$). The 17-OHP concentration correlated inversely with the gestational age groups: $r^2 = 0.681$ in males and $r^2 = 0.782$ in females.

Table 6 and Fig. 5 show the results of 1609 prospective samples that exceeded the gestational age-specific cutoff during neonatal screening by TRFIA in a total of 242,500 newborns. According to clinical data, patients were divided into three groups: healthy term infants, preterms without 21-CAH (gestational age < 37 wk), and infants with proven 21-CAH. 17-OHP levels were significantly higher in samples of preterms and newborns with 21-CAH compared with those of healthy term infants (ANOVA, $P < 0.001$).

21-F levels were significantly higher in newborns with 21-CAH compared with those of the other two groups (ANOVA, $P < 0.001$). There was no significant difference between 21-F values of preterms compared with term infants (ANOVA, $P = 0.42$). The 95th percentile of the preterm group, however, overlapped marginally with the 5th percentile of newborns with 21-CAH.

The F levels varied widely in all three groups. In the preterm group, F levels were significantly higher compared with the other two groups (ANOVA, $P < 0.001$). F was found in low concentrations in samples of infants with 21-CAH. However, there was no significant difference compared with

TABLE 4. Steroid concentrations of 6151 newborns except confirmed cases with 21-CAH (retrospective study) depending on gestational age groups (median, 5th, and 95th percentiles)

Steroid	Gestational age (wk)					
	<28 (n = 433)	28–29 (n = 303)	30–31 (n = 401)	32–34 (n = 764)	35–36 (n = 702)	>36 (n = 3.548)
17-OHP (TRFIA)	92.4 (33.6–>250)	71.6 (28.2–222)	63.3 (31.1–146)	54.2 (23.4–115)	45.0 (16.4–86.4)	17.6 (<7.50–56.7)
17-OHP (TMS)	25.1 (4.70–104)	21.4 (3.84–70.2)	16.4 (3.91–52.8)	11.5 (2.30–25.4)	6.90 (1.60–23.8)	2.10 (<1.10–9.70)
S	11.9 (1.48–46.6)	9.70 (2.18–35.4)	8.10 (1.36–26.8)	5.70 (<1–17.4)	4.20 (<1–12.7)	2.20 (<1–8.60)
21-F	2.25 (<1–8.20)	2.30 (<1–7.40)	2.40 (<1–7.85)	2.30 (<1–7.60)	2.30 (<1–7.22)	1.70 (<1–6.00)
F	137 (116.6–1.702)	102 (14.9–971)	110 (12.7–508)	92.9 (10.1–356)	105 (9.48–415)	92.4 (10.4–366)
4-A	5.25 (<1–27.4)	5.30 (<1–26.4)	5.50 (<1–19.7)	4.80 (<1–17.8)	3.70 (<1–15.2)	1.60 (<1–6.96)

TMS, Tandem mass spectrometry.

TABLE 5. 17α-OHP in males and females (median, 5th, and 95th percentiles, retrospective study)

	Male (nmol/liter)		Female (nmol/liter)	
Gestational age	n	17-OHP	n	17-OHP
<28 wk	211	23.5 (4.20–90.9))	222	24.0 (3.20–126)
28–29 wk	158	24.0 (4.44–74.4)	145	18.9 (3.60–67.9)
30–31 wk	196	16.4 (3.80–49.8)	205	16.0 (3.98–57.6)
32–34 wk	393	12.0 (2.43–38.4)	371	10.5 (1.92–30.75)
35–36 wk	368	7.50 (1.19–25.4)	334	6.15 (1.41–24.7)
>36 wk	1.783	2.40 (1.90–12.6)	1.765	2.90 (1.10–10.7)

TABLE 6. Prospective study: steroid profiles of healthy infants, preterms, and infants with 21-CAH (median, 5th, and 95th percentile)

Steroid	Healthy term infants (nmol/liter), n = 729	Preterms (nmol/liter), n = 864	Infants with 21-CAH (nmol/liter), n = 16
17-OHP	10.2 (3.38–24.9)[a]	75.6 (37.2–171)	74.1 (15.8–1105)
S	6.30 (1.48–23.3)	35.1 (21.5–707)[a]	5.70 (<1–20.5)
21-F	2.90 (<1–7.85)	5.60 (2.23–14.9)	48.2 (<13.6–320)[a]
F	176 (14.7–642)	807 (81.6–5126)[a]	82.5 (14.1–250)
4-A	5.80 (1.10–17.8)[a]	15.9 (4.64–79.4)	19.6 (2.24–427)

[a] Significance calculated per row.

the levels in samples of healthy term infants (ANOVA, $P = 0.773$). 4-A concentrations were significantly lower in healthy term infants compared with both other groups (ANOVA, $P < 0.001$). Comparison of 4-A levels of preterms and infants with 21-CAH showed no significant difference (ANOVA, $P > 0.263$). S levels (Table 6) were significantly higher in preterms

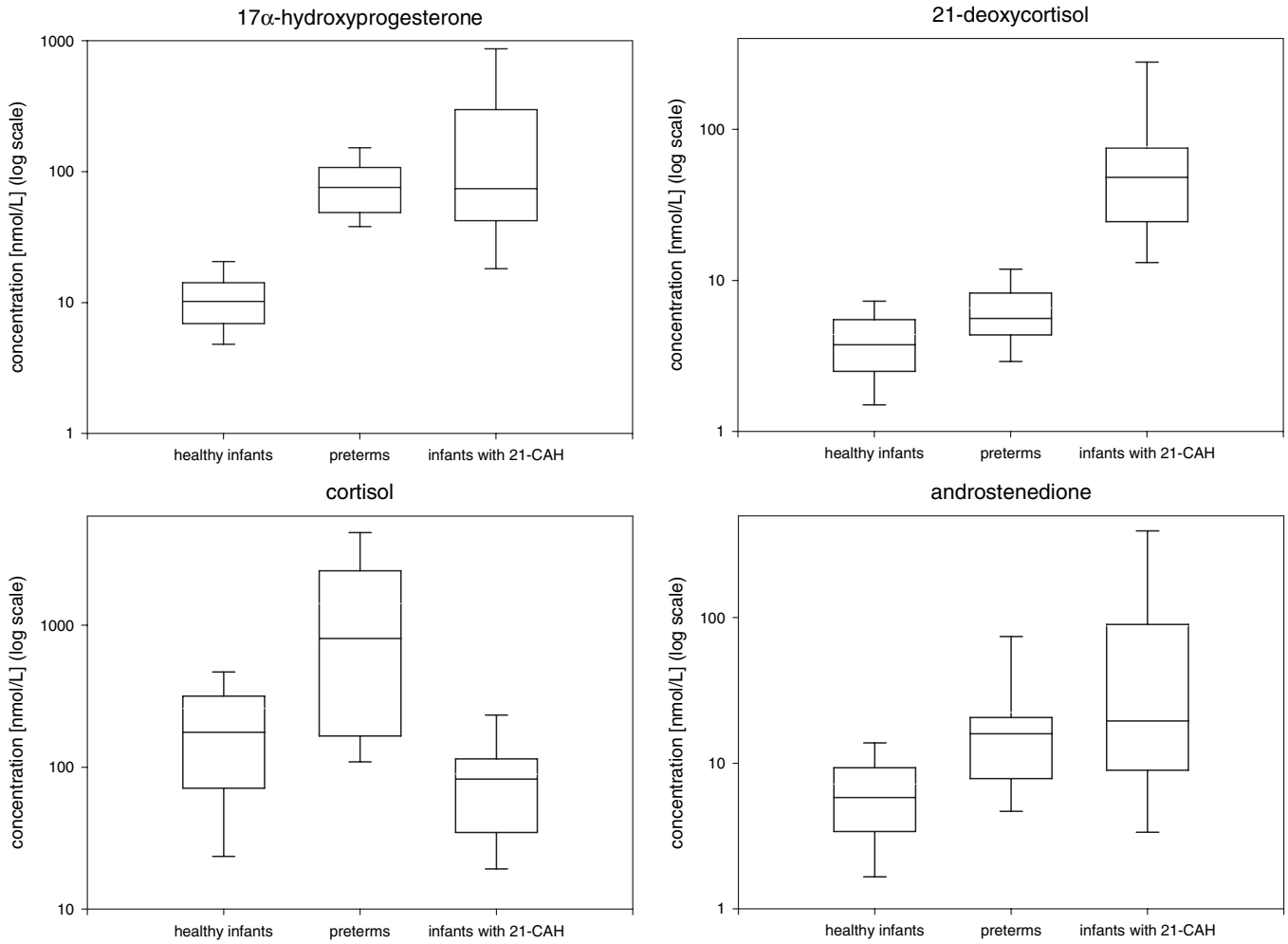

FIG. 5. Box plots (mean, 5th, 25th, 75th, and 95th percentile). Values measured by LC-MS/MS of all samples testing positive on TRFIA (prospective study, 1609 samples).

compared with both healthy term infants and newborns with 21-CAH (ANOVA, $P < 0.001$).

The TRFIA showed a sensitivity of 1.0, the specificity was 0.994, and the positive predictive value was 0.01. The LC-MS/MS method, based on absolute concentrations of 17-OHP and the ratio of (21-F +17-OHP)/F, showed the same sensitivity, higher specificity with 1.0, and a positive predictive value of 1.0.

For all samples of the retrospective study, the following ratios were calculated and compared: 21-F/F; 17-OHP/F; and (21-F + 17-OHP)/F. All three ratios were clearly elevated in the 50 newborns with 21-CAH. Elevation of the ratio (21-F + 17-OHP)/F correlated most significantly with proven 21-CAH. In healthy newborns (including preterms), this ratio was less than 0.07, whereas it was more than 0.53 in newborns with 21-CAH (Fig. 6). Similarly, in the prospective study, the quotient (21-F + 17-OHP)/F was 0.516–12.7 in cases of 21-CAH, and it was less than 0.07 for all remaining samples.

Discussion

The method presented here allows rapid quantitative analysis of the steroids 21-F, 17-OHP, and F as downstream product and is therefore a highly useful tool to detect 21-CAH in newborn screening.

The specificity of the presented method is greater than any of the other current methods used for 21-CAH screening. During the reference period, blood samples of 242,500 newborns were analyzed by TRFIA. A total of 1609 samples showed a positive TRFIA test result. In all of these cases, the samples were subsequently analyzed by LC-MS/MS. Taking both the elevated 17-OHP and the ratio (21-F + 17-OHP)/F into account, 16 newborns were suspected with 21-CAH. These cases were subsequently clinically (16) and molecular genetically confirmed. The prevalence of 21-CAH was 1:15,500 in our study group.

We improved the method of Lacey *et al.* (13) by including 21-F and S into the steroid profile. 21-F is elevated in cases of 21-CAH, as demonstrated by Fiet *et al.* (20) and later by Christoni *et al.* (21). S is elevated in 11β-hydroxylase deficiency. S and 21-F, which are of the same molecular mass, must be separated by chromatography because some of their fragments are identical. No case of 11β-hydroxylase deficiency was detected in our study.

Our method allows reliable measurement of 17-OHP without cross-reactions and also of 21-F, the most important marker for detection of 21-CAH. In those cases in which 21-F was not well above the upper normal limit, the quotient (21-F + 17-OHP)/F was significantly elevated so that diagnosis of 21-CAH could be made reliably.

Quantitative analysis of the relevant steroids by LC-MS/MS has been shown to be a useful tool to improve 21-CAH screening by significantly reducing the rate of false-positive results. In our prospective study group, the rate of false-positive results was about 1:100. This could be reduced to nearly zero if steroid profiling was implemented as a standard second-tier method.

At this stage, the method is not fast enough to replace the TRFIA completely. However, it allows rapid confirmation of positive test results. The method might be also used for

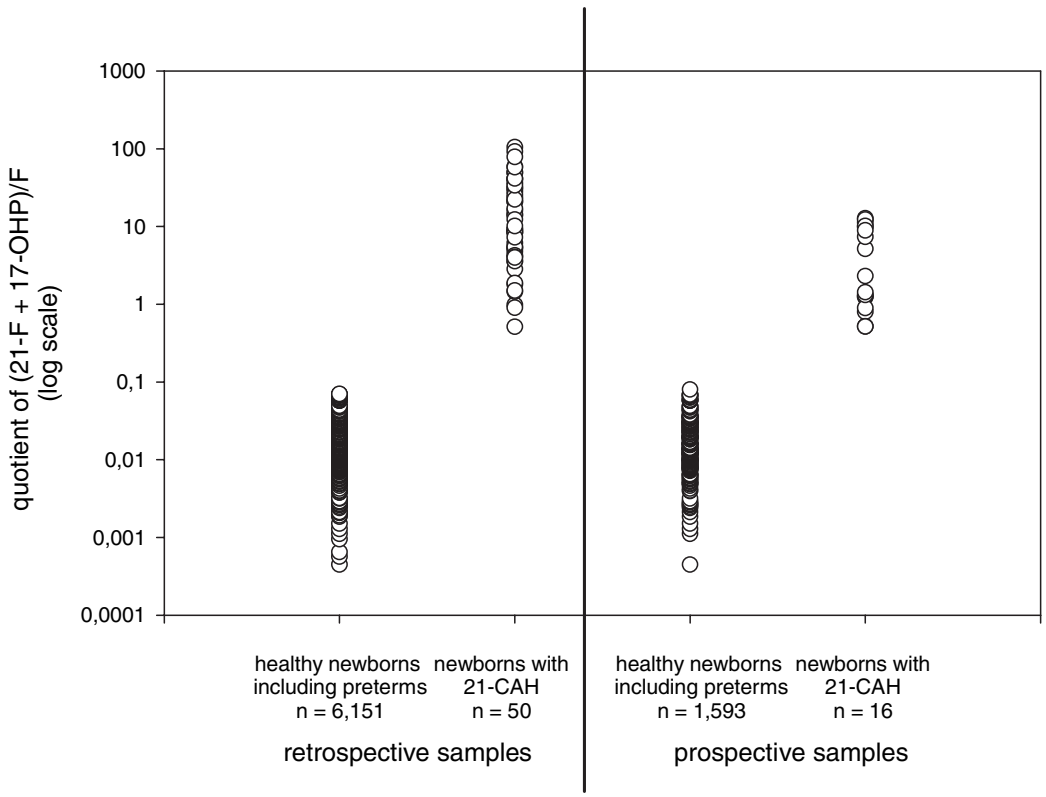

FIG. 6. Logarithmic plot of the ratio (21-F + 17-OHP)/F comparing both study groups.

primary analysis of 17-OHP in preterms, because the probability to obtain a false-positive TRFIA result is very high even when gestational age-specific cutoff values are applied. The method is robust and reproducible. Compared with other second-tier methods such as PCR (15), the analysis does not depend on the chromosomal localization of the CYP21 gene defect. The LC-MS/MS method allows immediate confirmation of 21-hydroxylase deficiency, because only a small amount of material is needed, which can be taken from the original filter card. Most importantly, the steroid profile includes 21-F, the most relevant marker for 21-CAH, and a sensitive ratio can be used to distinguish between true 21-CAH and false-positive results. We also suggest performing the LC-MS/MS method in all cases, including preterms in which 17-OHP levels exceed the lowest cutoff in the TRFIA test. In that case, gestational age-specific cutoffs might not be necessary anymore.

In conclusion, the main advantage of LC-MS/MS confirmation of suspected 21-CAH over PCR is seen by the fact that the results are available in the same laboratory that did the initial screening within a few hours after the results of the TRFIA are obtained. MS/MS instrumentation is now available in most screening laboratories, which is not the case with molecular genetic methods. We cannot give details on the cost of tandem mass spectrometry as a second-tier test yet, but it should be much less than current costs of molecular genetic testing.

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