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The Diagnosis of Congenital Adrenal Hyperplasia in the Newborn by Gas Chromatography/Mass Spectrometry Analysis of Random Urine Specimens

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Definitive neonatal diagnosis of congenital adrenal hyperplasia (CAH) is frequently complicated by normal 17-hydroxyprogesterone levels in 21-hydroxylase-deficient patients, residual maternal steroids, and other interfering substances in neonatal blood. In an effort to improve the diagnosis, we developed a gas chromatography/mass spectrometry method for simultaneous measurement of 15 urinary steroid metabolites as early as the first day of life. Furthermore, we developed 11 precursor/product ratios that diagnose and clearly differentiate the four enzymatic deficiencies that cause CAH. Random urine samples from 31 neonatal 21-hydroxylase-deficient patients and 59 age-matched normal newborns were used in the development. Additionally, samples from two 11β-hydroxylase-deficient patients and one patient each for 17 α-hydroxylase and 3β-hydroxysteroid dehydrogenase deficiencies were used. The throughput for one bench-top gas chromatography/mass spectrometry instrument is 20 samples per day. Thus, this method affords an accurate, rapid, noninvasive means for the differential diagnosis of CAH in the newborn period without the need for invasive testing and ACTH stimulation. (*J Clin Endocrinol Metab* 87: 3682–3690, 2002)

CONGENITAL ADRENAL HYPERPLASIA (CAH) is an autosomal recessive disorder involving deficiency of one of the four enzymes, or a cholesterol-trafficking protein, necessary for adrenal cortisol biosynthesis (1–6). Diagnosis and treatment of affected infants in the neonatal period is necessary to minimize the morbidity and mortality associated with adrenal insufficiency. The diagnosis is usually accomplished by measuring blood levels of adrenal hormones and precursor steroids (1). However, definitive neonatal diagnosis is complicated by nonelevated 17-hydroxyprogesterone (17OHP) levels in some infants with 21-hydroxylase deficiency and by the presence of residual maternal-placental and fetal steroid products, leading to misdiagnoses (7–10). Even when very specific RIAs are used, interfering substances in neonatal plasma increase the apparent 17OHP in the samples and reduce the discrimination between normal individuals and those with CAH. Reliable neonatal diagnosis requires extraction and chromatography before serum steroid immunoassay to assure accurate differential diagnosis. In an earlier study, one of the authors (C.H.L.S.) identified 17 α-hydroxypregnenolone and its sulfate as major contributing factors to the 17-OHP overestimation (11).

An alternative diagnostic method uses gas chromatography/mass spectrometry (GC/MS), which has been used for the diagnosis of 21-hydroxylase deficiency for 25 yr (12, 13). However, it is only recently that instrumentation has become reliable and inexpensive enough for routine use. There are multiple reports of GC/MS being used for diagnosis of all forms of CAH in the newborn period (12–17), and straightforward and comprehensive methods that allow diagnosis of each form have been reported (18, 19). There are several advantages of urine analysis for CAH diagnosis. The sample collection is noninvasive, a random sample is suitable, and a full spectrum of steroid hormone metabolites and precursor/product ratio assessments can be measured simultaneously, increasing accuracy and confidence in the diagnosis. Universal methodology can be developed for distinguishing the separate enzymatic forms of the disorder.

Materials and Methods

Normal subjects

Using premature infant diapers, random urine samples were collected from 59 infants in a newborn nursery and outpatient clinics at Children's Hospital, San Diego, and the University of California, San Diego. The top liner of the diaper was removed and discarded. After removing the plunger of a 20-ml syringe, the wetted diaper padding was placed into the syringe barrel. The plunger was replaced, and 3–5 ml urine was extracted by compressing the diaper padding. Studies were approved by the University of California San Diego Human Subjects Institutional Review Board.

Abbreviations: 6α-Hydroxy-THA, 6α-Hydroxy-tetrahydrocompound A; 6α-hydroxy-THS, 6α-hydroxytetrahydro substance S; 3β-HSD, 3β-hydroxysteroid dehydrogenase; CAH, congenital adrenal hyperplasia; CM, cortisol metabolite; GC/MS, gas chromatography/mass spectrometry; 17OHP, 17-hydroxyprogesterone; SIM, selected ion monitoring; TIC, total ion current.

Patients with CAH

Urine samples were obtained from 36 neonates with CAH before starting glucocorticoid therapy.

21-Hydroxylase deficiency. Samples from 31 patients with 21-hydroxylase deficiency were stored at −20 C. Of these, 28 were from patients aged 1–11 d, and three were from older patients (14, 18, and 23 d, respectively). Creatinine measurements were performed on 21 of these samples.

11 β-Hydroxylase deficiency. Samples from two patients aged 14 d and 7 wk were obtained.

17-Hydroxylase deficiency. One urine sample from a 7-d-old patient was obtained.

3β-Hydroxysteroid dehydrogenase (3β-HSD) deficiency. Samples were obtained at 7 and 15 d of age from one individual in whom 3β-HSD deficiency was later confirmed by mutation analysis.

Methodology

The methodology described is an adaptation of that published previously (18, 20). Conjugated and unconjugated steroids in a random urine sample were absorbed onto a Varian Bond Elut C_{18}/OH solid phase extraction column previously primed with methanol and water. The volume of urine extracted was dependent on the urine creatinine concentration. A volume of urine containing 75 μg creatinine was determined to be acceptable for analysis by GC/MS. Thus, the formula for determining the volume of urine (in microliters) to be extracted was 75,000/creatinine value in mg/liter. Following water washing, the steroids were eluted with methanol (4 ml) and dried under nitrogen. The steroid extract was subjected to enzyme hydrolysis using 36 μl per sample β-glucuronidase/arylsulfatase (Roche Diagnostics, Indianapolis, IN) and 10 mg per sample sulfatase (type H-1, from Helix pomatia, Sigma, St. Louis, MO) in 3 ml acetate buffer (pH 4.7) to release the steroids from conjugation. The samples were then incubated for 3 h at 55 C. The unconjugated steroids were again extracted on a C_{18}/OH column. After the addition of internal standards (cholesteryl butyrate and stigmasterol) to the dried extract, steroids were initially derivatized using 2% methoxamine HCl in pyridine (1 h at 55 C), which protects carbonyl groups (except those at C-11). The samples were dried and subjected to a secondary derivatization with trimethylsilylimidazole (4 h at 120 C), which resulted in a virtually complete silylation of all hydroxyl groups. The derivatizing reagents were removed by chromatography on hydroxyalkoxypropyl dextran type IX (formerly Lipidex, Sigma) columns using cyclohexane as the mobile phase (18). The cyclohexane eluates were dried under nitrogen and reconstituted in 200 μl cyclohexane in preparation for GC/MS analysis.

GC/MS analysis was carried out on an HP 5973 instrument (Hewlett-Packard, Wilmington, DE) housing a 15 m DB1 column (J and W Scientific, Folsom, CA). The samples were injected using an HP 7683 series autosampler and were eluted using cold trapping, with the column temperature ramping from 50 C to 285 C over 41 min. The flow of helium through the column was 1 ml/min. The eluting steroids were detected by selected ion monitoring (SIM). The specific ions used for each steroid are listed in Table 1.

The instrument was calibrated by running standard mixtures containing known amounts of reference steroids and internal standards. For almost all the steroid measurements, authentic steroids were available from Sigma or Steraloids Inc. (Wilton, NH). Other reference steroids were from our own collection. For those steroids having available reference material, five levels of calibrator were prepared for each analyte in five separate mixes. Each mix was analyzed on the GC/MS in all assays, the area of the resulting peaks was measured in SIM mode, and a five-point calibration curve was established for each analyte. Reference materials were not available for 6α-hydroxy-tetrahydrocompound A (6α-hydroxy-THA) or 6α-hydroxytetrahydro substance S (6α-hydroxy-THS), which are important neonatal markers for 17α-hydroxylase and 11β-hydroxylase deficiency syndromes, respectively (15, 17). Calibration for these was achieved using urine extract from affected patients as follows: The steroid extracts with internal standards were analyzed by

TABLE 1. Pediatric steroid reference ranges

Abbreviation	Trivial name	Ion monitored (SIM mode)	1 d old Mean value range (μg/g Creat)	2–4 d old Mean value range (μg/g Creat)	16–30 d old Mean value range (μg/g Creat)
17-HP	17α-Hydroxy-pregnanolone	476	187 30–420	109 15–200	58 40–90
5β-PT′ONE,15β	15β, 17α-Dihydroxypregnanolone		257 85–490	116 25–270	57 45–80
16α-OH-DHEA	16α-Hydroxy-dehydroepiandrosterone	266	53492 500–220000	34040 2000–180000	6977 200–16650
PT	Pregnanetriol	255	337 45–620	171 20–280	37 5–70
THS	Tetrahydro-11-deoxycortisol	564	236 70–670	176 30–470	30 15–60
5-PT′ONE,15β	15β, 17α-Dihydroxypregnanolone	562	15285 4900–43900	9603 1800–59000	1526 630–3300
PT′ONE	Pregnanetriolone	449	114 20–280	106 ≤260	8 ≤10
16-HP	16α-Hydroxy-pregnenolone	490	36692 4400–98900	28088 3460–150840	4169 75–10910
5-PT	5-Pregnenetriol	433	206 ≤360	207 ≤390	61 20–180
THE	Tetrahydrocortisone	578	2431 620–13160	2487 530–7430	3426 1600–5570
6α-OH-THS	6α-Hydroxy-tetrahydrosubstance S	562	121 ≤120	100 ≤170	ND <40
5α-THA	5α-Tetrahydro-11-dehydrocorticosterone	490	ND	ND	736 320–1490
α-Cortolone	α-Cortolone	449	208 25–520	198 20–380	274 40–600
β-Cortolone	β-Cortolone	449	323 120–1360	590 150–1175	538 170–1350
6α-OH-THA	6α-Hydroxy-tetrahydro-11-dehydro-corticosterone	519	210 30–540	100 20–250	47 10–100

ND, Not detected; value below detectable limit of assay.

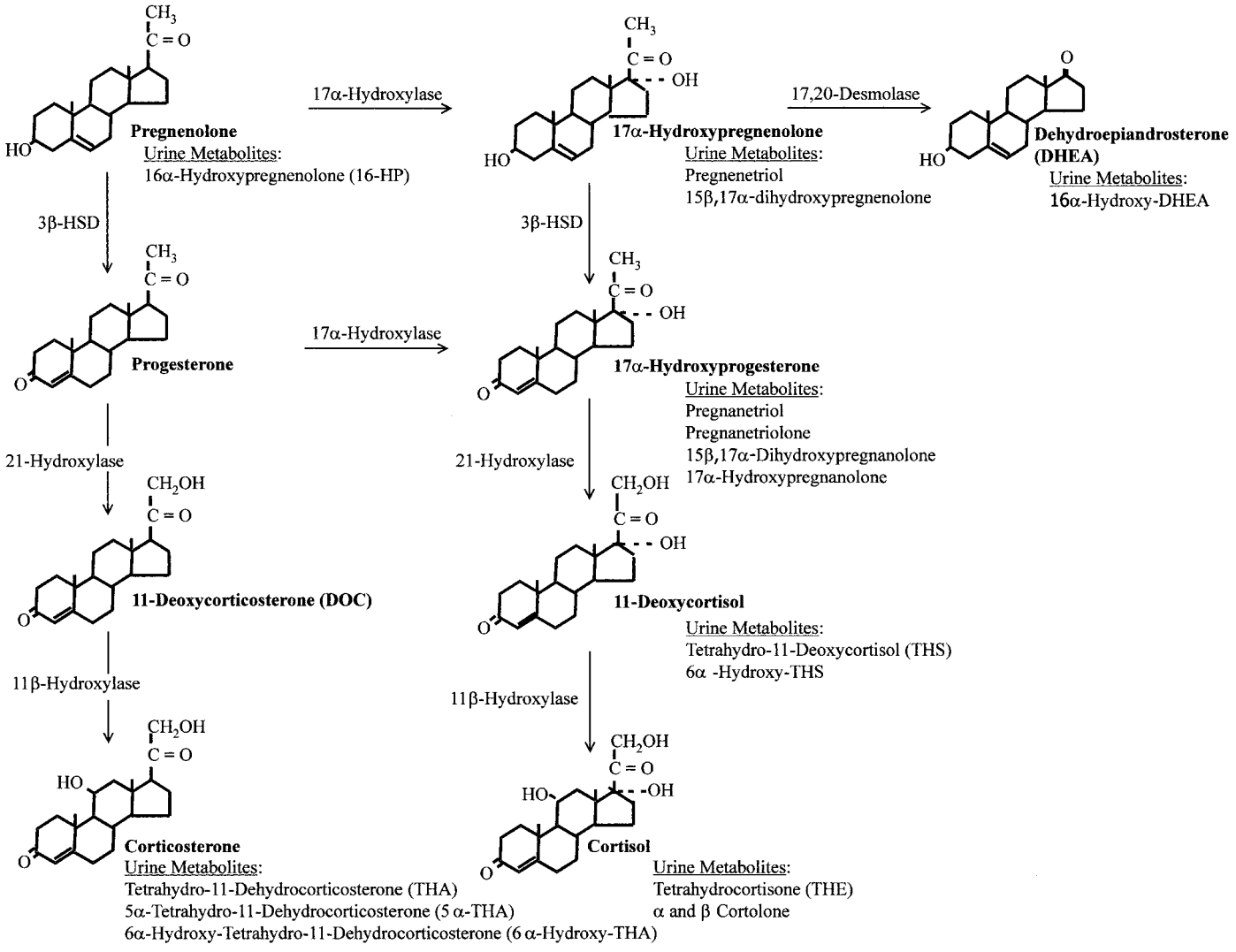


FIG. 1. Neonatal steroid synthesis and metabolism. Diagram illustrates the steroid precursors for all the metabolites quantitated in the assay as well as the locations of the enzyme blocks in the pathway for the four types of CAH studied. Many important metabolites of hormonal steroids and precursors are not listed or measured.

GC/MS in scanning mode, and the total ion current (TIC) peak areas of components identified as 6α-hydroxy-THA and 6α-hydroxy-THS were integrated. These steroids elute between the first and second internal standards, and we related the peak areas to the mean TIC peak areas of the two internal standards. We assumed for the purpose of this study that peak area responses in TIC of different compounds were identical for components of similar retention time (an approximate but practical assumption). Based on this assumption, we determined the approximate concentration of 6α-hydroxy-THA and 6α-hydroxy-THS in the patient samples so they could be used for instrument calibration. The samples were analyzed again in SIM mode to obtain calibration response curves to be used in quantitating these compounds in the patient samples.

After response curves had been obtained for each steroid analyte to be measured, quantitation of the steroids was performed using the HP ChemStation software. Peak areas for the steroids were obtained and compared with calibration response curves for quantitation, using the internal standard stigmasterol to correct for procedural losses. The raw results obtained were multiplied by the ChemStation software by 13,333 to obtain the desired microgram per gram creatinine units.

The quantitated steroid data were exported to an Excel spreadsheet (Microsoft, Seattle, WA) in which the diagnostic ratios were calculated (described in *Results* and *Discussion*).

Statistics

The Mann-Whitney *U* test was used to statistically compare the steroid ratio data for 21-hydroxylase deficiency with data from the normal and diseased groups at the different ages. These statistics should be interpreted with caution because of the relatively small study population. Additional studies are required to extend this to the general population. Statistical analysis was not performed on the steroid ratio data for the 3β-HSD, 11β-hydroxylase, and 17-hydroxylase deficiencies because of the low number of disease state samples available.

Results

Quantitative analysis

Normals. Thirty-four adrenal steroid hormone metabolites were measured. Reference ranges were developed for the 3β-hydroxy-5-ene steroids as well as for the DHEA and pregnenolone metabolites produced in the adrenal fetal zone. Table 1 lists the mean and observed range for the 15 metabolites. Figure 1 shows the adrenal steroid biosynthetic path-

way and the steroid precursor for each of the 15 referenced metabolites.

CAH patients. Table 2 lists the quantitative excretions for 28 patients with CAH. Although the mean excretion of all 3β-hydroxy-5-ene steroids is higher than normal in affected infants, there is considerable overlap in the ranges. Specific analytes for 21-hydroxylase, 17-hydroxylase, and 3βHSD defects are also notably elevated. Individual steroid values for 11β-hydroxylase deficiency were not listed because creatinine values were unavailable. The excretion of cortisol metabolites is not markedly different between normals and CAH patients, probably reflecting remaining maternal contribution.

Diagnostic ratios

Table 3 reports the range of precursor and product metabolite ratios for the four types of CAH. The rationale for choice of the numerator in the ratio is addressed in the discussion. The product (cortisol) metabolites used as denominator are the summation of tetrahydrocortisone, α-cortolone, and β-cortolone, referred to as cortisol metabolites (CM). Two ratios are not based on the CM denominator: pregnanetriol/pregnanetriolone (5-pregnene-3β, 17α, 20α-triol/5β-pregnane-3α, 17α, 20α-triol-11-one) for discriminating the 21-hydroxylase defect from 3βHSD deficiency and 16α-hydroxypregnenolone/16α-hydroxy DHEA for diagnosis of 17-hydroxylase deficiency.

It is evident that for the chosen ratios, there is no overlap between affected patients and normals (Figs. 2 through 5).

Discussion

We describe the development of a robust method independent of 24-h excretion or values relative to creatinine and based on the measurement of steroid precursor/product ratios. These ratios represent the ratio of the urinary metabolite of an adrenal precursor (*e.g.* 17-OHP) to the urinary metabolites of the product (cortisol). When testing both children and adults, we used the summation of three important CM for the denominator, namely: tetrahydrocortisone, tetrahydrocortisol, and 5α-tetrahydrocortisol. The latter two steroids are virtually immeasurable in neonates because of the dominance of the 11-keto-containing steroids in the perinatal period. In neonates, we would have preferred to use the three dominant neonatal metabolites (tetrahydrocortisone, 6α-hydroxytetrahydrocortisone, and 6α-hydroxycortolone) (21, 22); however, authentic steroids are not available for the two latter compounds, rendering instrument calibration difficult. Therefore, we decided to use the sum of tetrahydrocortisone, α-cortolone, and β-cortolone (abbreviated as CM) as the denominator. Typically, this group represents 20–50% of excreted cortisol metabolites (21, 22). Any use of a CM denominator is potentially compromised in the first few days of life because some cortisol precursor presumably originated in the mother. However, the maternal contribution to neonatal cortisol metabolites is probably negligible after 4 d of life. This, of course, also applies to the numerator analyte because maternal derived pregnanetriol, for example, will contribute to neonatal excretion.

21-Hydroxylase deficiency

Three diagnostic ratios use well-known analytes, *i.e.* 17α-hydroxypregnenolone × 100/CM (ratio 1), pregnanetriol/CM (ratio 2), and pregnanetriolone × 100/CM (ratio 3) (18). We also measured the 15β,17α-dihydroxypregnanolone × 100/CM ratio (ratio 4) based on the numerator being a specific neonatal diagnostic analyte for CAH (16). It is clear from Fig. 2 that in all cases the patients with 21-hydroxylase deficiency exhibited no overlap with the normal neonates. We had a sufficiently large sample size to break down the result into three age groups (*i.e.* first day of life, d 2–4, and 5 d and above. It is evident that, although discrimination between normal and affected individuals is readily achievable on the first day of life, the separation between them increases with age of the infants owing to both the ratio increasing for affecteds and decreasing for normals. The wid-

TABLE 2. Steroid levels in 24 CAH patients

Steroid	21-OH deficiency			17-OH deficiency	3β-HSD deficiency	
	1 d old	2–4 d old	5–11 d old	7 d old infant	7 d old infant	15 d old infant
	Mean (μg/g Creat) (n = 6)	Mean (μg/g Creat) (n = 8)	Mean (μg/g Creat) (n = 14)	(μg/g Creat)	(μg/g Creat)	(μg/g Creat)
17-HP	10 241	8541	22 402	65.8	5315	30 724
5β-PT′ONE,15β	9536	6513	23 558	36.1	4770	20 660
16α-OH-DHEA	96 846	70 002	85 741	956	1 155 224	1 133 638
PT	13 512	3753	15 365	33.8	1607	14 217
THS	896	614	1575	117	1107	3705
5-PT′ONE,15β	18 196	6586	12 605	594	467 245	380 230
PT′ONE	10 786	3032	8774	105	592	629
16-HP	232 560	239 128	323 660	149 513	1 008 220	463 264
5-PT	951	332	1474	ND	25 356	20 430
THE	2693	951	1218	253	3853	9369
6α-OH-THS	ND	ND	ND	ND	ND	ND
5α-THA	ND	ND	ND	146	ND	ND
α-Cortolone	263	117	267	42.3	ND	455
β-Cortolone	1621	851	1199	523	3255	3888
6α-OH-THA	ND	ND	ND	245 415	ND	ND

ND, Not detected, level below detectable limit of assay; Creat, creatinine.

TABLE 3. Diagnostic ratios in normal neonates and patients with CAH

Steroid ratio	Age (days)	Normals (n = 59)	21-OH deficiency (n = 31)	3β-HSD deficiency (n = 2)[a]	11β-OH deficiency (n = 2)[b]	17-OH deficiency (n = 1)[c]
17α-Hydroxypregnanolone × 100	0–1	2.0–22.0	29.9–423			
CM	2–4	1.0–11.0	103–974			
	5–30	1.0–4.0	248–1470	74.8, 224	36.4, 22.0	8.0
Pregnanetriol × 100	0–1	2.0–30.0	36.6–648			
CM	2–4	1.0–13.0	112–286			
	5–30	≤3.0	76.6–1344	22.6, 103	16.4, 5.8	4.1
Pregnanetriolone × 100	0–1	0.7–15.9	40.6–461			
CM	2–4	≤14.5	45.1–331			
	5–30	≤0.2	83.3–961	8.3, 4.6	6.4, 3.4	12.9
15β, 17α-Dihydroxypregnanolone × 100	0–1	3.8–17.4	47.4–400			
CM	2–4	1.0–14.4	116–498			
	5–30	1.0–2.6	170–1627	67.1, 150	33.6, 21.4	4.4
Pregnenetriol × 100	0–1	≤24.4	≤27.4			
CM	2–4	≤17.1	≤36.6			
	5–30	0.5–2.4	≤287.9	356, 149	36.4, 1.2	ND
Pregnenetriol	0–1	0.1–10.8	≤0.4			
Pregnanetriolone	2–4	0.2–95.2	≤0.4			
	5–30	5.6–28.6	≤1.5	42.8, 32.5	2.6, 1.7	ND
THS × 100	0–1	2.9–25.0	13.6–34.0			
CM	2–4	1.1–26.0	10.4–49.1			
	5–30	0.5–1.4	1.5–136	15.6, 27.0	366, 97.2	14.2
6α-Hydroxy-THS × 100	0–1	≤13.0	ND			
CM	2–4	≤11.0	ND			
	5–30	ND_d	ND	ND, ND	190 482, 117 723	ND
5α-THA × 100	0–1	≤8.0	≤55.4			
CM	2–4	≤11.0	≤7.8			
	5–30	≤34.3	≤75.4	ND, 57.0	ND, ND	50.4
6α-Hydroxy-THA × 100	0–1	1.0–28.0	ND			
CM	2–4	1.0–11.0	ND			
	5–30	1.0–2.0	ND	ND, ND	NMC, NMC	29 983
16α-Hydroxypregnenolone	0–1	0.3–1.3	1.2–4.4			
16α-Hydroxy-DHEA	2–4	0.1–2.2	1.9–11.4			
	5–30	0.2–0.7	1.0–11.7	0.9, 0.4	9.5, 8.1	156

ND, not detected, numerator level below detectable limit of assay; NMC, not measured due to contamination of numerator component.
[a] One patient; samples collected when 7 and 15 d of age.
[b] Two patients, 14 and 49 d of age.
[c] One 7-d-old patient.

ening gap between normal and affected ranges can be attributed to reduction in the maternal contribution to steroid excretion and increasing neonatal adrenal stimulation in response to falling cortisol availability.

3βHSD deficiency

The two samples from a single patient with diagnosed 3βHSD II deficiency showed a profile distinctive for the disorder. The excretion of 3β-hydroxy-5-ene steroids (relative to creatinine) were extremely elevated (Table 2). Except for their high excretion, the quantitatively major neonatal 3β-hydroxy-5-ene steroids do not provide diagnostic specificity for the condition. The classical analyte of the condition, pregnenetriol (5-pregnene-3β,17α,20α-triol), first identified by Bongiovanni (23, 24) in urine from older patients with the disorder, is a relatively minor component of neonatal 3βHSD urine but nonetheless remains the discriminating analyte. Pregnenetriol is the major urinary metabolite of 17α-hydroxypregnenolone. The pregnenetriol × 100/CM ratio (ratio 5) was 356 on d 7 of life and 149 on d 14 of life, and the highest recorded ratio for 2- to 4-d-old normal infants was 17 (Fig. 3).

Distinguishing 3βHSD deficiency from 21-hydroxylase has always been challenging. Bongiovanni (23) noted early that the 21-hydroxylase deficiency analytes pregnanetriol and 17-hydroxypregnanolone were also elevated in 3βHSD deficiency, and this has been verified by other authors (14). In addition, serum analysis of 17α-hydroxyprogesterone results in elevated values that may lead to false diagnosis (25). In our 3βHSD-deficient patient, ratios 1, 2, and 4 (Fig. 2) all were elevated, possibly indicating the presence of 21-hydroxylase deficiency. In addition, two 21-hydroxylase-deficient patients had ratio 5 values within the range of the 3βHSD patient (Fig. 3). One feature of 3βHSD noted by Bongiovanni (23, 24) is the virtual absence of pregnanetriolone, and this proves to be a necessary discriminant. Ratio 3, alone among the 21-hydroxylase deficiency ratios, is normal. Thus, the two primary diagnostic ratios, pregnanetriol × 100/CM (ratio 5), which should be elevated, and pregnanetriolone × 100/CM (ratio 3), which should be normal, could be combined to give the secondary ratio pregnenetriol/pregnanetriolone (ratio 6). Plots of this ratio against normals and 21-hydroxylase patients are shown in Fig. 3. This ratio clearly distinguishes between the 21-hydroxylase and 3β-HSD deficiencies.

In summary, for 3βHSD the following criteria must be met:

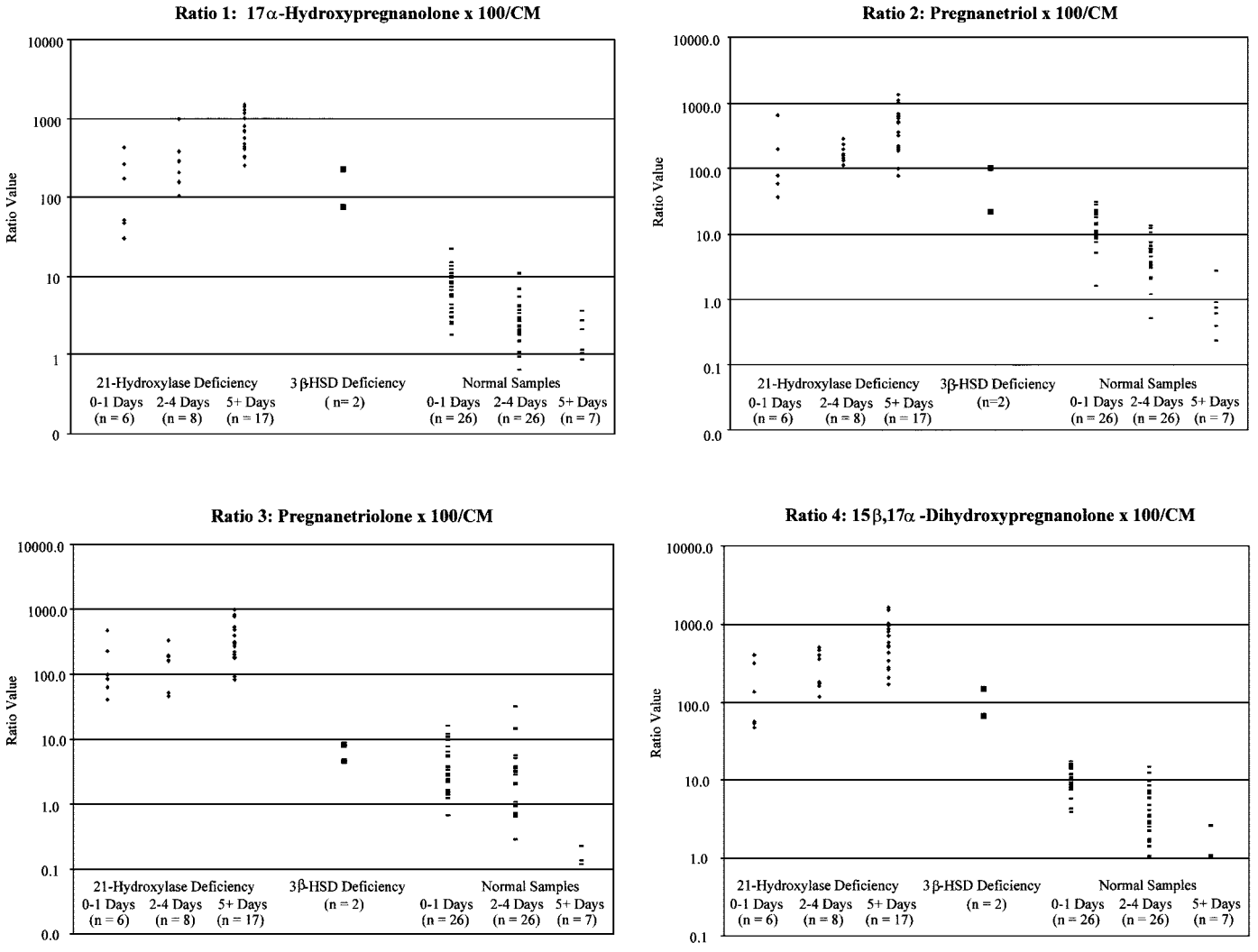


FIG. 2. Diagnostic ratios for 21-hydroxylase deficiency. Ratios 1–4 illustrate the excretions of four urinary steroid markers for 21-hydroxylase deficiency relative to major CMs on a log scale. The patient and normal data have been grouped according to age at urine collection (d 1, d 2–4, and more than 5 d). The 3βHSD patient (analysis at 7 and 11 d) has also been included because ratios 1, 2, and 4 give values in the range of 21-hydroxylase deficiency. For 3βHSD deficiency, ratio 3 is normal. Statistical analysis of ratios 1–4 from normal patients *vs.* the 21-hydroxylase-deficient patients at the different age groups gave the following *P* values: <0.001, <0.001, and 0.001 for 0–1 d, 2–4 d, and 5 d or more, respectively, for all four ratios.

elevated pregnenetriol × 100/CM, normal pregnanetriolone/CM, and elevated pregnenetriol/pregnanetriolone.

11β-Hydroxylase deficiency

Reichstein's substance S (11-deoxycortisol) is the primary steroid overproduced in 11β-hydroxylase deficiency, and its tetrahydro derivative has for many years been recognized as the urinary marker of the disorder when excreted in excess. In children and adults, the absence of 11-oxygenated androgens and highly elevated androsterone and etiocholanolone levels are also notable features that are less evident in neonates. In newborns, the active 6α-hydroxylase results in 6α-hydroxy-THS being the second most important urinary analyte (15). Both analytes are normally excreted in extremely low amounts and could not be quantified in all normal neonates. Plots of the ratios THS/CM (ratio 7) and 6α-hydroxy-THS/CM (ratio 8) are shown in Fig. 4. The two patients with 11β-hydroxylase defect had extremely high values for these diagnostic ratios.

17-Hydroxylase deficiency

The major hormonal steroid overproduced in 17-hydroxylase deficiency is corticosterone, and in previous studies we have shown that Kendall's compound A (11-dehydrocorticosterone) is dominant in the neonate. Both 5α-tetrahydro compound A (5αTHA) and tetrahydro compound A are quantitatively important, but the major corticosterone metabolite is 6α-hydroxy-THA (17, 26). This is in contrast to children and adults in whom metabolites of corticosterone (Kendall's compound B) dominate, *e.g.* tetrahydrocorticosterone or 5α-tetrahydrocorticosterone. Thus, the primary analytes we measure for diagnosis of 17-hydroxylase deficiency are tetrahydro compound A, 5αTHA, and 6α-hydroxy-THA. Deficiency of 17-hydroxylase prevents the formation of C_{19}

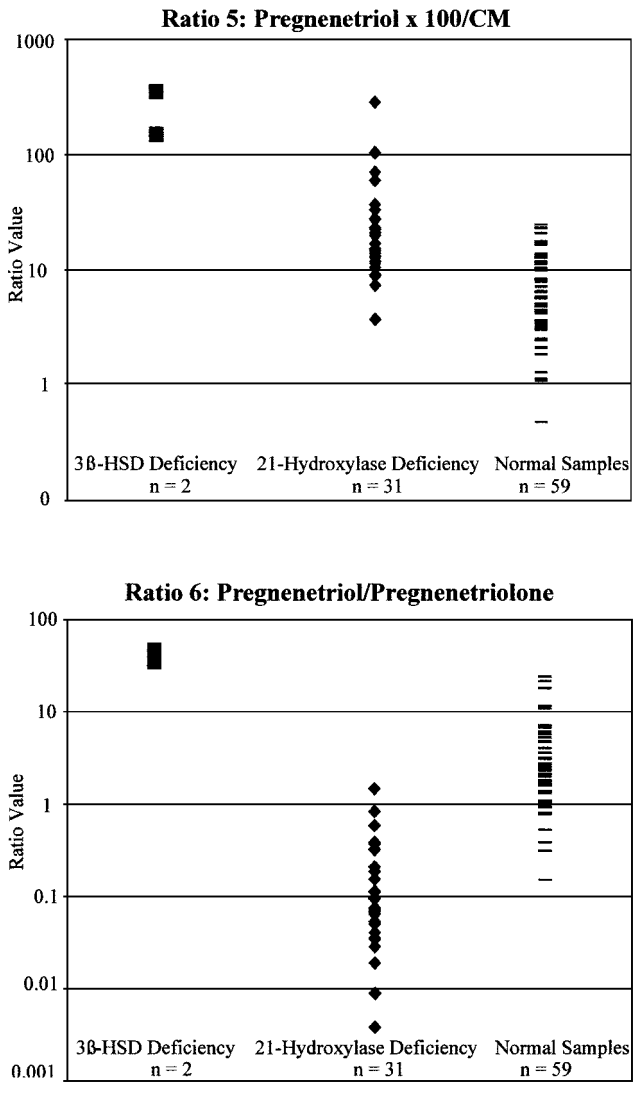


FIG. 3. Diagnostic ratios for 3βHSD deficiency. Two samples from a patient with 3βHSD deficiency have been compared with both normals and patients with 21-hydroxylase deficiency because they can share overproduction of some of the diagnostic analytes. Although ratio 5 is higher for 3βHSD than all normals, two patients with 21-hydroxylase have a similar value. A secondary ratio 6 clearly discriminates the two conditions because of low pregnanetriolone excretion in 3βHSD deficiency. Statistical analysis ratios 5 and 6 of normals *vs.* the 21-hydroxylase-deficient patients gave P values of <0.001 and <0.001, respectively.

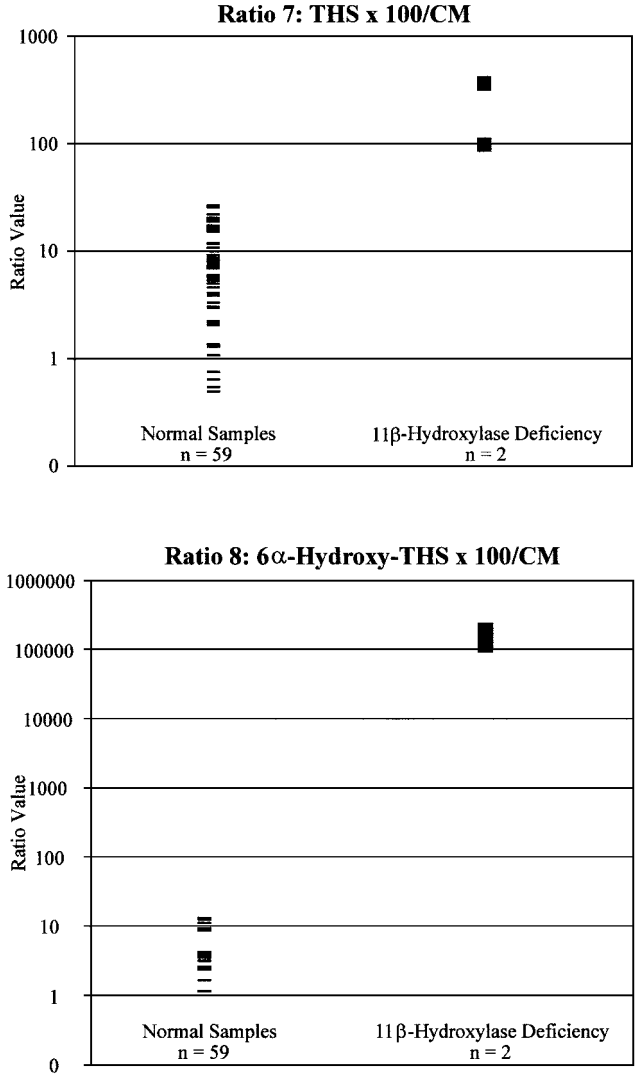


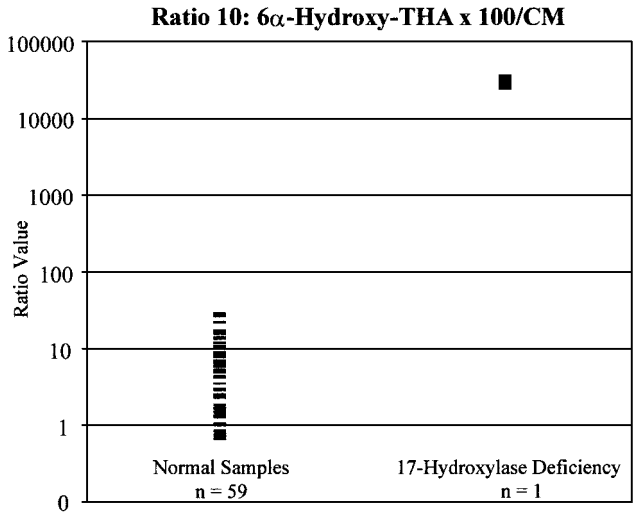
FIG. 4. Diagnostic ratios for 11β-hydroxylase deficiency. Ratios including tetrahydro S (THS) (ratio 7) and 6α-hydroxy-THS (ratio 8) clearly distinguish 11β-hydroxylase deficiency from normal.

steroids; therefore, the excretion of all such steroids is minimal. The ratio of 16α-hydroxypregnenolone/16α-hydroxy-DHEA is useful as a discriminant. In a previous study of a different patient (17), this ratio was reported to be more than 100 rather than approximately unity for normals. The elevated ratio has now been verified in the patient studied here (Fig. 5).

We have adopted the following ratios for 17-hydroxylase deficiency: 5αTHA $\times$ 100/CM (ratio 9), 6α-hydroxy-THA $\times$ 100/CM (ratio 10), and 16α-hydroxypregnenolone/16α-hydroxy-DHEA (ratio 11). Values for ratios 10 and 11 are plotted in Fig. 5 and clearly demonstrate the deficiency of 17α-hydroxylase. Ratio 9 is equally discriminating but is not illustrated.

In summary, methodology that can be used for routine analysis of neonatal urine for four causes of CAH has been described. This methodology is noninvasive; requires no ACTH stimulation; and provides discrimination of 21-hydroxylase, 17α-hydroxylase, 3βHSD, and 11β-hydroxylase deficiencies in the first days of life. For 21-hydroxylase deficiency, the predominant enzyme deficiency for CAH, we have shown that reliable diagnosis can be made on samples collected within 24 h of birth. The method can be used independently or in conjunction with serum steroid assays. It allows for diagnosis of CAH within 36 h upon receipt of the urine in the laboratory. Specificity of analysis is achieved by requiring at least two, and sometimes more, specific analytes for each condition to be measured.

There is one additional form of CAH that is not addressed in this manuscript, namely congenital lipoid adrenal hyperplasia. This is a rare variant of the disorder caused by defi-



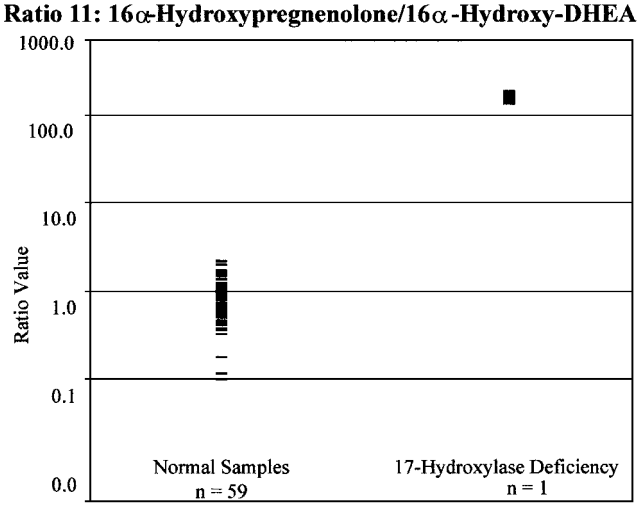


FIG. 5. Diagnostic ratios for 17-hydroxylase deficiency. The 6α-hydroxy-THA × 100/CM ratio (ratio 10) is strikingly elevated in a patient with the disorder. Similar results are found with the 5αTHA/CM ratio (not shown). The decreased production of C_{19} steroids is illustrated by ratio 11.

ciency of steroid acute regulatory protein (StAR). This is a protein associated with cholesterol transport from outer to inner mitochondrial membrane (6, 27), a process necessary for introducing the precursor to the steroidogenic cascade. This is a disorder causing severe adrenal insufficiency and male pseudohermaphroditism. Neonates with lipoid adrenal hyperplasia excrete almost no steroids; those that are excreted in the first days of life are of maternal origin. A combination of adrenal hyperplasia and negligible steroid excretion is diagnostic of the condition.

Current trends in diagnosis are moving toward molecular rather than biochemical analysis. Direct mutation analysis for diagnosis of CAH is possible, particularly for 21-hydroxylase deficiency. It is used for prenatal diagnosis of the condition and as a complement to neonatal screening because the serum 17-hydroxyprogesterone immunoassay, in the absence of preextraction and chromatography, can produce false-positive results (28–32). However, routine molecular diagnosis for the 21-hydroxylase defect remains difficult because of the presence of the CYP21 pseudogene and the wide range of mutations reported in affected patients (1, 2, 28). These include gene deletions, large gene conversions, point mutations, and small deletions. Screening for common mutations has been proposed, but the best way to avoid missing uncommon mutations is to sequence the entire gene (2, 32, 33). Although automated sequencing methods are not yet widely available, DNA chip technology is the most attractive option for routine high throughput diagnosis but remains a research technique (2). Mutation analysis for the 11β-hydroxylase, 17-hydroxylase, and 3β-HSD defects also remain research methods.

Thus, it is our view that mass spectrometry provides the best bridging technology between multiple serum immunoassay methods and routine DNA analysis for CAH diagnosis. The random urine steroid metabolite profile provides a rapid, simplified, comprehensive, differential diagnosis for CAH.

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