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Original

A Case of a Preterm Infant with 21-Hydroxylase Deficiency: Implications of the Biochemical Diagnosis with Urinary Pregnanetriolone by Gas Chromatography/Mass Spectrometry in Selected Ion Monitoring (GCMS-SIM)

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Abstract. The biochemical diagnosis of 21-hydroxylase deficiency (21-OHD) is difficult in preterm infants. To date, no marker for the biochemical diagnosis of 21-OHD has been found. Seventeen α-hydroxyprogesterone (17-OHP), is not useful because of interference by delta 5 steroids from the fetal adrenal cortex. A 5-d-old female infant, born at 31 wk of gestation, was suspected of having 21-OHD based on physical findings (mild clitoromegaly, pigmentation of the tongue and gingiva) as well as laboratory data (17-OHP >93.5 ng/ml by ELISA 7 prime extractive method in filter paper-dried blood spot and 718.3 ng/ml by RIA after high performance liquid chromatography extraction in serum; plasma ACTH 690 pg/ml; and serum testosterone 3,169 ng/dl). We examined her urinary steroid profiles by gas chromatography/mass spectrometry in selected ion monitoring (GCMS-SIM) at 8 d of age. The pregnanetriolone (Ptl) level was noticeably high (0.80 mg/g creatinine), which was strongly suggestive of 21-OHD. Gene analysis of *CYP21A2* showed compound heterozygosity, one allele having a cluster mutation in exon 6 and the other having a large deletion including *CYP21A2*, confirming the diagnosis of 21-OHD. This case suggested that, in preterm infants, urinary Ptl by GCMS-SIM can be useful for the biochemical diagnosis of 21-OHD.

Key words: urinary steroid profiles, pregnanetriolone (Ptl), 21-hydroxylase deficiency (21-OHD), preterm infants

Introduction

The early diagnosis of 21-hydroxylase deficiency (21-OHD) is mandatory for both correct sexual assignment and early treatment to prevent adrenal crisis. In term infants, 17α-hydroxyprogesterone (17-OHP) in a filter paper-

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dried blood spot has proven to be useful in newborn mass-screening for 21-OHD. Serum levels of 17-OHP are very sensitive markers for biochemical diagnosis of 21-OHD; however, in premature infants no marker for biochemical diagnosis of 21-OHD has been found. In preterm infants, 17-OHP is not useful because of interference by delta 5 steroids from the fetal adrenal cortex. The serum levels of 17-OHP can be high in unaffected preterm infants, precluding the biochemical diagnosis of 21-OHD (1–4).

We present here a case of a female infant with 21-OHD, born at 31 wk of gestation. Analysis of urinary steroid profiles by gas chromatography/mass spectrometry in selected ion monitoring (GCMS-SIM) showed a high pregnanetriolone (Ptl) level, which was strongly suggestive of 21-OHD. The diagnosis of 21-OHD was confirmed by gene analysis of *CYP21A2*. This is the first report which presents the usefulness of urinary Ptl by GCMS-SIM for the biochemical diagnosis of 21-OHD in preterm infants.

Case Report

A female Japanese infant was born to unrelated parents, as the first child of trichorionic, triamniotic triplets. None of her family had 21-OHD. She was delivered at 31 wk of gestation by an emergency cesarean section because of preterm labor; the birth weight was 1,464 g and the Apgar score was 6 and 8 at 1 and 5 min, respectively. Mechanical ventilation and medical treatment were required for respiratory distress syndrome, patent ductus arteriosus, and heart failure. Phototherapy was given from 3 through 6 and from 8 through 11 d of age for hyperbilirubinemia. At 5 d of age, we found mild clitoromegaly as well as pigmentation of the tongue and gingiva. Laboratory data and imaging studies revealed hyponatremia (127 mEq/l), hyperkalemia (7.7 mEq/l), disseminated intravascular coagulopathy (DIC), pulmonary hemorrhage, and subependymal hemorrhage. Medical treatment successfully controlled the respiratory failure, heart failure, and DIC. A provisional diagnosis of 21-OHD was made, prompting us to do endocrinological studies: 17-OHP >93.5 (ELISA 7 prime extractive method in filter paper-dried blood spot) and 718.3 ng/ml (RIA after high performance liquid chromatography extraction in serum); plasma ACTH 690 pg/ml; plasma renin activity 77.4 ng/ml/h; serum 21-deoxycortisol (21-DOF) 368 ng/ml; serum androstenedione 292 ng/ml; and serum testosterone 3,169 ng/dl. Ultrasonography found bilaterally enlarged adrenal glands. Urine steroid profile analysis was performed according to the method previously reported (5). In brief, the method consisted of enzymatic hydrolysis of 0.5–2 ml of urine sample, extraction, derivative formation, purification, GCMS-SIM analysis and quantification. The urinary steroid profiles at 8 d of age showed a high Ptl level (0.80 mg/g creatinine; Table 1). This high level of Ptl strongly suggested 21-OHD when correlated with the cut-off level in term infants. We analyzed *CYP21A2* gene on the patient and her parents with genomic DNA extracted from their peripheral blood leukocytes by PCR direct sequencing (6). Informed consent for gene analaysis was obtained from her parents. The patient had compound heterozygosity, one allele having a cluster mutation in exon 6 (I236K, V237E, M239K) and the other having a large deletion including *CYP21A2*. Each parent had heterozygous abnormality. The diagnosis of 21-OHD was confirmed by these results.

Discussion

The biochemical diagnosis of 21-OHD was made by the finding of a high urinary Ptl level that had been determined via GCMS-SIM in this preterm infant. The level of Ptl was compatible with 21-OHD previously reported in term infants (7). The diagnosis was ultimately confirmed by molecular analysis.

In premature infants, the diagnosis of 21-OHD is difficult because: [1] Preterm infants with 21-

Table 1 Urinary steroid profile by GCMS-SIM at 8 d of age

Urinary steroids	Patient	21OHD*	Reference value**
Ptl	0.80	2.25–124	<0.01–0.07
PT	2.94	1.46–58.2	0.02–0.52
An	0.99	0.18–8.66	0.01–0.22
11HA	4.24	0.52–21.0	0.01–0.21
16HP5	138	4.71–596	0.83–101
16αHD	32.1	1.27–122	0.76–76.5
5βTHE	0.92	0.33–10.3	1.19–17.8

(mg/g creatinine)

*ranges in spot urine of 28 term newborn infants with classical 21OHD from 7 to 14 d of age. **ranges in spot urine of 29 control term newborn infants from 6 to 14 d of age.

Ptl: pregnanetriolone, PT: pregnanetriol, An: androsterone, 11HA: 11β-hydroxyandrosterone, 16HP5: 16α-hydroxypregnenolone, 16αHD: 16α-hydroxyDHEA, 5βTHE: 5β-tetrahydrocortisone.

OHD may have subtle ambiguous genitalia due to prematurity of the labia majora pudenda; [2] Preterm infants without 21-OHD often show hyponatremia and hyperkalemia, which are common laboratory findings in 21-OHD; [3] Although gene analysis of *CYP21A2* is a good test for the diagnosis of 21-OHD, it is time consuming and expensive (8); [4] Most importantly, a marker for the biochemical diagnosis of 21-OHD has not been found. In preterm infants, 17-OHP is not useful because of the interference of delta 5 steroids from the fetal adrenal cortex. An unaffected preterm infant may have high serum levels of 17-OHP (1–4). Gruneiro-Papendieck *et al.* reported 17-OHP levels had a statistically significant negative correlation with gestational age and birth weight (9). In newborn mass-screening for 21-OHD, a modification of the cut-off limits of 17-OHP in a filter paper-dried blood spot has been reported; this modification has been found to have slightly increased specificity in preterm infants. Allen *et al.* reported weight-adjusted criteria for 17-OHP to reduce the false-positive rate of 21-OHD in preterm infants (10). Their weight-adjusted criteria, however, do not factor in small for gestational age infants. Ohkubo *et al.* reported cut-off limits allowing for the equivalent age of gestation at blood sampling (11). This method may prove to be useful for screening, but cannot provide a definitive diagnosis.

Urinary steroid profiles, especially Ptl, by GCMS-SIM can be useful for the biochemical diagnosis of 21-OHD in preterm infants because: [1] they can detect a full spectrum of steroid hormone metabolites (Fig. 1); [2] the sample collection is noninvasive and a random sample is appropriate; [3] reference values of urinary steroids in term newborn infants by GCMS-SIM have been established (5); [4] precursor/product ratio assessments can be measured, which may increase both the sensitivity and specificity of the diagnosis (7). The literature contains reports that describe urinary steroid profiles by GCMS-SIM for the biochemical diagnosis of 21-OHD in newborn infants. Via urinary steroid profiles with GCMS-SIM, Malunowicz *et al.* studied 161 newborns and infants, with clinical suspicion of congenital adrenal hyperplasia (CAH). In eight cases (two full-term and six preterm infants), a false-positive diagnosis of CAH was made based on serum steroid evaluation (12). Caulfield *et al.* reported three diagnostic ratios, measured by GCMS-SIM: 17α-

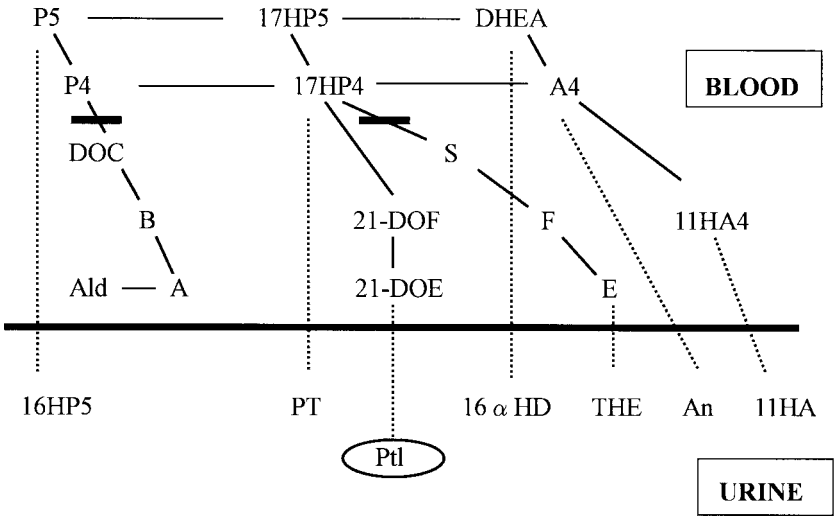

Fig. 1 Chief steroid metabolites in 21-OHD. Urinary steroid profiles obtained by GCMS-SIM can detect all urinary metabolites shown here.

P5: pregnenolone
P4: progesterone
B: corticosterone
A: 11-dehydrocorticosterone
S: deoxycortisol
F: cortisol
E: cortisone
Ald: aldosterone
11HP5: 17α-hydroxypregnenolone
17HP4: 17α-hydroxyprogesterone
DOC: deoxycorticosterone
21-DOF: 21-deoxycortisol
21-DOE: 21-deoxycortisone
DHEA: dehydroepiandrosterone
A4: androstenedione
11HA4: 11 hydroxyandrostenedione
16HP5: 16α-hydroxypregnenolone
PT: pregnanetriol
Ptl: pregnanetriolone
16αHD: 16α-hydroxyDHEA
THE: tetrahydrocortisone
An: androsterone
11HA: 11β-hydroxyandrosterone

hydroxypregnanolone × 100/cortisol metabolites (CM); pregnanetriol (PT) × 100/CM; and Ptl × 100/CM having no overlap between 21-OHD and normal infants (7).

Among several metabolites of 17-OHP, urinary Ptl may be the best biochemical marker for the diagnosis of 21-OHD in term infants. Ptl exhibited no overlap between the 21-OHD cases and the control when we compared several urinary steroid metabolites by means of GCMS-SIM (Fig. 2). Other metabolites, such as PT and androsterone, exhibited overlap between the control and 21-OHD (data not shown). The mechanisms which explain why Ptl is superior to PT, in the early biochemical diagnosis of 21-OHD, despite the fact that both are specific metabolites of 21-OHD (Fig. 1), are not completely understood. One possible reason for the disparity may be because of a relatively greater inactivity of the enzymes metabolizing 17-OHP to PT than 21-DOF to Ptl in newborns.

In preterm infants, urinary Ptl with GCMS-SIM may have good sensitivity and specificity for the biochemical diagnosis of 21-OHD. Our study suggests that urinary Ptl with GCMS-SIM may have the ability to make a biochemical diagnosis of 21-OHD (true positive). It is of note that Furukawa *et al.* reported urinary Ptl levels obtained by GCMS-SIM were helpful in diagnosing two very low birth

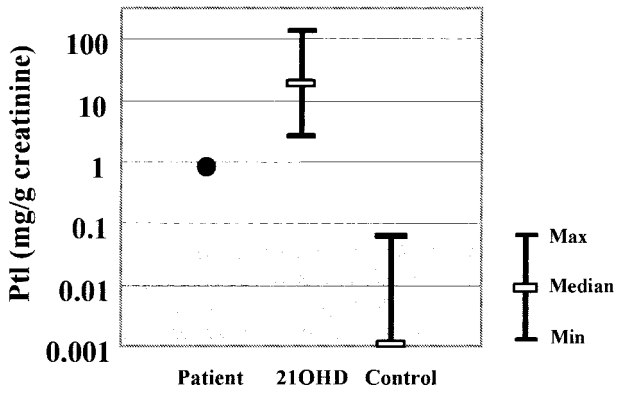

Fig. 2 Urine pregnanetriolone (Ptl) in the patient, 21-OHD, and control. Ptl levels measured by GCMS-SIM in term control infants (number=29, 6–14 d of age), term infants with 21-OHD (number=28, 7–14 d of age), and our patient (8 d of age). There was no overlap between the control and 21-OHD. The level of Ptl in our patient was beyond that of control infants.

weight infants as having transient high 17-OHP (43.6 and 27.7 ng/ml at 35 and 10 d of age, respectively) and transient high 21-DOF (6.0 and 4.2 ng/ml at 35 and 16 d of age, respectively) (false positive) (13).

Finally, we propose that the measurement of urinary Ptl by GCMS-SIM is a useful biochemical marker for the diagnosis of 21-OHD even in preterm infants. We recommend all neonatologists and pediatricians to measure urinary Ptl by GCMS-SIM in any premature infants who have high 17-OHP with filter paper-dried blood spot in newborn screening. Additional premature infant case studies are indicated to establish and confirm the usefulness of urinary Ptl by GCMS-SIM.

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