

## Original Article

# Measurement of Serum 17 $\alpha$ -hydroxyprogesterone in Newborn Infants by Stable Isotope Dilution—Gas Chromatography/Mass Spectrometry

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**Abstract.** Immunochemical measurement of serum 17 $\alpha$ -hydroxyprogesterone (17OHP), the most important parameter for diagnosis of classical 21-hydroxylase deficiency (21OHD) in newborn infants, is known to be inaccurate due to the cross-reactivity of antibodies with a large quantity of fetal adrenal steroids. The aims of this study were 1) to establish reference values for the serum 17OHP level in Japanese newborn infants using non-immunochemical stable isotope dilution—gas chromatography/mass spectrometry (SID-GC/MS) and 2) to compare the serum 17OHP levels determined by SID-GC/MS with those determined by radioimmunoassay (RIA). The first study subjects were used for determination of reference values and included 57 healthy full-term newborn infants (4–5 d of age). The second study subjects were used for comparison of SID-GC/MS with RIA and included 27 healthy full-term newborn infants (3–6 d of age) and two subjects with neonatal transient hyper 17OHPnemia; these two subjects were 16 and 27 d of age, respectively. In the first study subjects, the intra-assay coefficient of variation for SID-GC/MS was 3% (n=5), the recovery rate was 98%, the sensitivity was 0.2 ng/ml, and the range of linearity was 0.5–200 ng/ml. The reference values for the serum 17OHP level determined by SID-GC/MS ranged from 0.3–1.5 (0.6) (ng/ml) (median). In the second study subjects, the serum 17OHP levels determined by SID-GC/MS were lower in one of the 27 subjects and both of the two subjects with neonatal transient hyper 17OHPnemia compared with the levels determined by RIA. Measurement of the serum 17OHP level using SID-GC/MS may be clinically useful for definitive diagnosis of classical 21OHD in newborn infants.

**Key words:** 17 $\alpha$ -hydroxyprogesterone, newborn infants, 21-hydroxylase deficiency, SID-GC/MS, RIA

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Received: January 14, 2009

Accepted: April 6, 2009

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## Introduction

Newborn mass screening for classical 21-hydroxylase deficiency (21OHD) by measurements of the 17OHP level in filter paper blood using an enzyme-linked immunosorbent

assay has been used in Japan with high reliability (1). Definitive diagnosis of classical 21OHD is, however, not easy, partly because immunochemical measurement of the serum 17OHP level is problematic due to the cross-reactivity of antibodies with a large number of fetal adrenal steroids (2).

Theoretically, measurement of the serum 17OHP level by non-immunochemical stable isotope dilution - gas chromatography/mass spectrometry (SID-GC/MS) is more accurate than measurement by immunochemical methods (3–5). However, few reference values for the serum 17OHP level in newborn infants using SID-GC/MS have been reported (6). Furthermore, no studies comparing measurement of the 17OHP level by SID-GC/MS with other immunochemical methods have been reported.

The aims of this study were 1) to establish the reference values for the serum 17OHP level in Japanese newborn infants using SID-GC/MS and 2) to compare the levels of serum 17OHP determined using SID-GC/MS with those determined by radioimmunoassay (RIA) in newborn infants.

### Subjects and Methods

The first subjects were used to establish reference values by SID-GC/MS and included 57 healthy full-term Japanese newborn infants (3–5 d of age, 32 boys and 25 girls, 37–42 wk gestation, birth weight 2,500–3,824 g). The second subjects were used for comparison of SID-GC/MS with RIA and included 1) 27 healthy full-term Japanese newborn infants (3–6 d of age, 16 boys and 11 girls, 37–41 wk gestation, birth weight 2,603–4,162 g) and 2) two subjects with neonatal transient hyper 17OHPnemia; these two subjects were 16 and 27 d of age, respectively. Diagnosis of neonatal transient hyper 17OHPnemia was based on the combination of the following; 1. no clinical signs and symptoms of classical 21OHD, 2. normal serum Na and K level, 3. normal serum glucose level, 4. no acidosis, and 5. normal serum

17OHP level later in life. The parent(s) of the subjects gave informed consent.

The 17 $\alpha$ -hydroxyprogesterone (17OHP) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), and [11,11,12,12-d<sub>4</sub>]-17OHP was synthesized by us (N.Y.) as described previously (7). Heptafluoro-n-butyric anhydride (HFBA) was obtained from GL Sciences (Tokyo, Japan). Dichloromethane, isooctane, cyclohexane, acetone and ethanol were of analytical reagent grade and were used without further purification. A DPC 17 $\alpha$ -OH progesterone kit was purchased from Mitsubishi Kagaku Iatron (Tokyo, Japan).

The serum 17OHP level (ng/ml) was measured by SID-GC/MS using 100  $\mu$ l of serum. In brief, the method consisted of extraction, derivatization, and GC/MS analysis. The procedures were as follows; 1. Five ng of [11,11,12,12-d<sub>4</sub>]-17OHP, as an internal standard, in 5  $\mu$ l of ethanol and 1 ml of distilled water was added to 100  $\mu$ l of neonate serum or 17OHP standard solution (0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, or 20 ng/ml in ethanol). 2. Steroids were extracted with 3 ml of dichloromethane / isooctane (1:2 v/v). 3. The organic extracts were evaporated under a stream of nitrogen. 4. One hundred microliters of acetone and 25  $\mu$ l of HFBA were added to the mixture. 5. The mixture was left for 1 h at room temperature. 6. The excess reagent was removed under a stream of nitrogen. 7. The residue was dissolved in 30  $\mu$ l of cyclohexane. 8. Four microliters of the solution was analyzed using GC/MS. GC/MS analysis was performed on an HP 5890II GC with an HP fused silica column (25 m) coupled to an HP 5971 MS (Agilent Technologies, Palo Alto, CA, U.S.A.). The temperature programming was 100 C (2 min), 100–230 C (30 C/min), 230–290 C (3 C/min) and 290 C (10 min). Selected ion monitoring (SIM) analysis was performed with two characteristic mass ions selected for each steroid (465.3 and 369.2 m/z for 17OHP; 469.3 and 373.2 m/z d<sub>4</sub>-17OHP). The turnaround time of the assay was 12 h.

The serum 17OHP level was also measured by  $^{125}\text{I}$ -RIA (DPC 17 $\alpha$ -OH progesterone kit) using 25  $\mu\text{l}$  of serum after ether extraction, according to manufacturer's instructions.

## Results

### Basic properties of SID-GC/MS

The recovery rate for serum was 98%. The sensitivity was 0.02 ng/tube (0.2 ng/ml in serum). The intra-assay C.V. (coefficient of variation) was 3% (n=5, 12.8 ng/ml). Linearity was observed from 0.05 ng to 20 ng (0.5–200 ng/ml in serum).

### Reference values

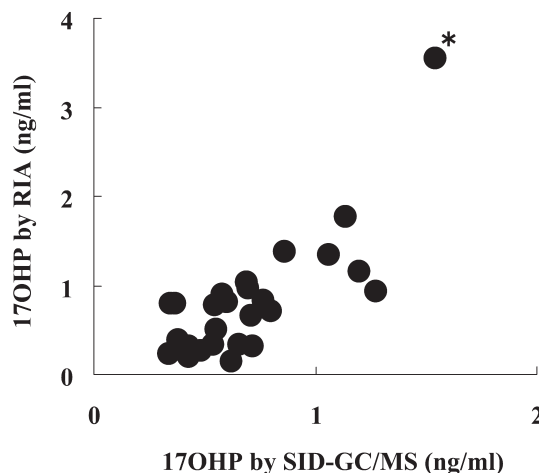
The reference values of serum 17OHP in newborn infants determined by SID-GC/MS ranged from 0.3 to 1.5 ng/ml with a median of 0.6 ng/ml.

### Comparison between SID-GC/MS and RIA

Out of 27 healthy full-term newborn infants, the serum 17OHP level determined by SID-GC/MS was lower in one case (1.5 ng/ml) compared with the level determined by RIA after extraction (3.6 ng/ml). In the other 26 subjects, the serum 17OHP levels determined by SID-GC/MS were almost the same as those by determined by RIA after extraction (Fig. 1). Two subjects with neonatal transient hyper 17OHPnemia had lower serum 17OHP levels determined by SID-GC/MS (3.1 and 1.1 ng/ml) compared with the levels determined by RIA after extraction (9.8 and 4.2 ng/ml).

## Discussion

We have successfully established reference values for the serum 17OHP level in newborn infants by SID-GC/MS, 0.3 to 1.5 ng/ml with a median of 0.6 of ng/ml; this was similar to the values reported by Wudy *et al.* using the same methods, 0.5 to 4.3 ng/ml with a median of 1.8 ng/ml (6). It is noteworthy that the basic properties of our method were good judging from



**Fig. 1** Correlation between the levels of 17OHP determined by SID-GC/MS and RIA after extraction. The asterisk (\*) indicates the case in which the 17OHP level determined by SID-GC/MS was lower than the level determined by RIA after extraction.

the recovery rate, sensitivity, intra-assay C.V., and linearity.

Clinically, some points are noteworthy. First, measurement of the serum 17OHP level by SID-GC/MS was reliable, judging from the method's good basic properties, such as the recovery rate, sensitivity, intra-assay C.V., and linearity. Second, we used 100  $\mu\text{l}$  of serum for 17OHP measurement by SID-GC/MS, while we used 25  $\mu\text{l}$  for determination of the 17OHP level by RIA. The larger volume required for measurement might be a weak point of SID-GC/MS, however the turnaround time of the assay is 12 h. Third and most importantly, the serum 17OHP levels determined by SID-GC/MS were lower than those by RIA in one control subject and two subjects with neonatal transient hyper 17OHPnemia. Theoretically, determination of the serum 17OHP level by SID-GC/MS is more accurate. Namely, the serum 17OHP level determined by RIA was higher than the true value, probably because fetal adrenal steroids

could cross-react with the antibody used in the RIA. In preterm newborn infants, who have more residual fetal adrenal gland than full-term newborn infants, SID-GC/MS would be a good candidate for accurate measurement of the serum 17OHP level. Fourth, measurement of the serum 17OHP level by SID-GC/MS may be more useful than RIA for definitive diagnosis of classical 21-hydroxylase deficiency (21OHD) in newborn infants. We believe that these reference values of serum 17OHP are relevant to definitive diagnosis of 21OHD as well as other adrenal disorders in newborn infants, such as P450 oxidoreductase deficiency (8, 9).

In summary, we have established reference values for the serum 17OHP level in newborn infants by SID-GC/MS. We are currently addressing the clinical utility of our method in differential diagnosis of 21OHD in newborn infants, who show elevated 17OHP level in filter paper blood as determined by ELISA in neonatal screening.

### Acknowledgement

This work was partly supported by the Yamaguchi Endocrine Research Association.

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