Serum Levels of 16-Dehydropregnenolone Sulfate during the Early Neonatal Period

Noriko Tagawa, Yuichi Nakata, Satoshi Kusuda, Yoshiharu Kobayashi, and Fukuko Watanabe

Clinical Chemistry Laboratory, Kobe Pharmaceutical University, 4-19-1, Motoyamakita-machi, Higashinada-ku, Kobe, 658, Japan, and Department of Neonatology, Prenatal Center of Osaka City General Hospital, 2-13-22, Miyakoimahondoni, Miyakojima, Osaka, 534, Japan. Received August 5, 1996; accepted October 28, 1996

We have established a method for quantifying serum 16-dehydropregnenolone (3β,16α-pregnadiene-20-one) sulfate (16-DHP S) by GC-MS. The levels of 16-DHP S at birth were compared in infants grouped as extremely immature (gestational age: 22—27 weeks), pre-term (gestational age: 28—36 weeks) and full-term (gestational age: 37—41 weeks). The average of the serum concentration of 16-DHP S in full-term infants was 0.172 ± 0.104 μmol/l (n = 10, mean ± S.D.) which was significantly higher than the levels of the extremely immature (0.106 ± 0.054 μmol/l, n = 14, p < 0.05) and pre-term infants (0.088 ± 0.066 μmol/l, n = 33, p < 0.01). However, 16-DHP S was not detected in sera from normal adults (age 22—73 years, n = 40).

We investigated chronological changes in serum levels of 16-DHP S during the early neonatal period. In extremely immature and pre-term infants, these levels were significantly higher at 2—7 days than those of 16-DHP S at day 0 (p < 0.001). The levels at 9—18 d were still significantly higher than those at day 0 (p < 0.05), but in full-term infants, these levels did not change at days 0 and 2—7.

These results indicate that 16-DHP S is a steroid specific to fetuses and neonates and the involution of the fetal adrenal gland does not affect its serum levels in the early neonatal period.

Key words 16-dehydropregnenolone sulfate; neonate; serum level; GC-MS; pre-term infant; full-term infant

At term, fetal adrenal glands are twenty times as large as adult glands when compared as a ratio to body weight. The fetal adrenal glands consist primarily of a fetal zone (80%) which rapidly involutes after birth. This may account for the remodeling of the fetal zone into the adult zona fasciculata. As the histological changes of the fetal zone proceed after birth, the hormonal milieu of the fetus dramatically changes to that of the adult.

The fetal liver also produces the precursor of estriol (1,3,5(10)-estratriene-3β,16α,17β-triol), 16-hydroxydehydroepiandrosterone (3β,16α-dihydroxy-5-androsten-17-one) sulfate from dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one) sulfate.

We identified 16-dehydropregnenolone (3β-hydroxy-5,16-pregnadiene-20-one) (16-DHP) in the serum of immature infants and suggested it to be a form of a sulfated conjugate. This steroid was initially identified in mono-sulfate conjugate steroid fraction of neonatal feces by Gustafsson et al. Subsequently, 16-DHP was also identified in human placenta, urine, gallbladder, meconium and amniotic fluid. Although there are many reports describing 16-DHP sulfate (16-DHP S), its physiological significance and metabolic fate remains unclear. Ascertaining the hormonal milieu of 16-DHP S in the early neonatal period is important for elucidation of the metabolic fate of this steroid in fetuses and neonates. We therefore established a method for the determination of serum 16-DHP S in early neonates using GC-MS, and applied it to measurement of its serum levels during the early neonatal period.

MATERIALS AND METHODS

The sources of materials were as follows: 16-DHP from Steraloids (Wilton, N.H.), stigmasterol (3β-hydroxy-24-ethyl-5,22-cholestadiene) from GL Sciences (Tokyo, Japan), N,O-bis(trimethylsilyl) acetamide (BSTFA) from Wako Pure Chemical Industries (Osaka, Japan), arylsulfatase (EC 3.1.6.1. from Helix pomatia) from Boehringer Mannheim (Mannheim, Germany), ASPEC pak® (disposable octadeyl silane cartridge, 0.9 mm i.d. x 20 mm) column from M&S Instruments Trading Inc. (Osaka, Japan), Acell Plus QMA® (disparsable quaternary methly ammonium conjugated silica cartridge, 300 mg) column from Waters Corp. (Milford, M.A.). We synthesized 16-DHP S from 16-DHP according to the method described by Maeda. Steroid free serum was prepared according to Heyns et al. Water was purified with a Milli-Q system (Nippon Millipore Ltd., Tokyo, Japan).

GC-MS Conditions GC-MS was performed on a Hewlett-Packard model 5890 gas chromatograph equipped with a Hewlett-Packard model 5971 mass detector (electron impact, 70 eV). The J&W DB-1 column, 0.25 mm i.d. x 15 m, 0.1 μm film thickness, was run under the following conditions: 1 ml/min of helium gas flow rate; injection port temperature, 300 °C; oven temperature, 50 °C for 0.5 min followed by 50—300 °C at 30 °C/min and finally held at 300 °C for 2 min.

HPLC Conditions HPLC was performed on a Waters model 6000A pump, a Waters model 710B auto sampler and a WakoSil-II 5C18-HG 4.6 mm i.d. x 150 mm column (Wako Pure Chemical Industries, Osaka, Japan) run at 40 °C. Data reduction was carried out using a Uvidec-100-IV detector (240 nm) and a Waters 805 data station. Samples were dissolved in 200 μl of mobile phase of acetonitrile:water = 1:1 (v/v) and 150 μl was applied to the HPLC column. The flow rate was 1 ml/min.

Preparation of Serum Samples for Measurement of 16-DHP S Serum samples (50 μl) were diluted with 1 ml of 0.9% NaCl and applied to an ASPEC pak® column © 1997 Pharmaceutical Society of Japan
(0.9 mm i.d. x 20 mm) which was washed with 20 ml of water. A QMA® column was then connected to the ASPEC pak® outlet. From the top of the ASPEC pak®, 6 ml of 72% ethanol was passed through these columns. Negatively charged steroid sulfates and glucuronides bound to the QMA® column whereas neutral steroids passed through. The ASPEC pak® and QMA® columns were disconnected.

To remove steroid glucuronides, 6 ml of 0.3 M acetic acid in 72% ethanol was passed through the QMA® column. Mono-conjugated sulfates were then eluted from it with 3 ml of 0.3 M acetate-sodium acetate buffer (pH 6.3) in 72% ethanol. The eluate was collected and evaporated in vacuo. The residue was dissolved in 1.5 ml of 250 mM acetate-sodium acetate buffer (pH 6.0) to which 20 µl of arylsulfatase was added, and the mixture was incubated at 40°C overnight. Steroids liberated by this procedure were extracted twice with 2 ml of dichloromethane and then evaporated in vacuo. The residue was dissolved in 200 µl of HPLC mobile phase solution (acetoni-trile: water = 1:1) and 150 µl of the sample was applied to HPLC. The elute between 16.8 and 20.3 min was collected and referred to as the 16-DHP fraction. After evaporation, 10 ng of stigmasterol in methanol (100 µl) was added as the internal standard. The methanol was evaporated in vacuo and 175 µl of BSTFA: acetonitrile (1:1) was added to the residue. After incubation at 40°C for 30 min, the solvent was evaporated in vacuo. Dichloromethane (100 µl) was added to the residue and 2 µl of the sample was analyzed by GC-MS as described above. Ion 386 (M⁺) and 484 (M⁺) for trimethylsilyl derivatives of 16-DHP and stigmasterol were monitored, respectively (selected ion monitoring mode).

**GC-MS Calibration Curve for 16-DHP**

Serially diluted authentic 16-DHP dissolved in methanol was added to tubes containing 10 ng of stigmasterol in methanol (100 µl) as the internal standard. The solvent was evaporated in vacuo. The trimethylsilylation and GC-MS conditions were as described above.

**Statistical Analysis**

Values are expressed as the means ± S.D. Differences between two groups were statistically analyzed by the Mann-Whitney U test. A p value below 0.05 was considered significant.

**RESULTS**

**Overall Recovery Test of 16-DHP S from Serum**

To investigate the overall recovery of 16-DHP S from serum, 100 and 200 ng of 16-DHP S in methanol (100 µl) were placed in tubes, then the solvent was evaporated and 1 ml of steroid-free serum was added. Sera containing 16-DHP S were stored at 4°C for overnight. To measure the concentration of 16-DHP S, 50 µl of serum (5 and 10 ng/50 µl) were processed as described in Methods. The recovery of 16-DHP S from each serum sample (5 and 10 ng/50 µl) was 95.9 ± 1.2% (n = 6) and 97.6 ± 1.5% (n = 6), respectively. These recoveries were used to correct the serum concentration of 16-DHP S.

**Sample Dilution Study**

Two serum samples from neonates were serially diluted with steroid free serum and 16-DHP S levels were determined. The relationship between the dilution and the 16-DHP S concentration for each sample was linear and the line passed through the zero point.

**Intra- and Inter-Assay Precision**

Intra- and inter-assay precisions were determined from assays of serum samples. The intra-assay coefficient of variation (C.V.) for the 5 samples was 2.1% (0.077 ± 0.002 µmol/l). The value for the inter-assay C.V. (n = 4) was 18.4% (0.091 ± 0.017 µmol/l).

**Serum Levels of 16-DHP S in Infants and Adults**

Serum levels of 16-DHP S at birth and in adults were measured using our new method. These levels were compared among infants grouped as extremely immature (gestational age: 22–27 weeks), pre-term (gestational age: 28–36 weeks) and full-term (gestational age: 37–41 weeks). The average of the serum concentration of 16-DHP S in full-term infants was 0.172 ± 0.104 µmol/l (n = 10, mean ± S.D.). As shown in Fig. 1, these levels in full-term infants were significantly higher than those in the extremely immature (0.106 ± 0.054 µmol/l, n = 14, p < 0.05) and pre-term infants (0.088 ± 0.066 µmol/l, n = 33, p < 0.01). However, 16-DHP S was undetectable in serum from normal adults (age 22–73 years, n = 40; data not shown).

We investigated chronological changes in serum levels of 16-DHP S in the early neonatal period (Fig. 2). In extremely immature and pre-term infants, serum levels of 16-DHP S were significantly elevated at 2–7 d compared with those at 0 d. The levels at 8–18 d were still significantly higher than those at day 0. The 16-DHP S serum levels did not change at days 0 and 2–7 in full-term infants.

**Fig. 1. Serum Levels of 16-DHP S at Birth in Extremely Immature (Gestational Age: 22–27 Weeks), Pre-term (Gestational Age: 28–36 Weeks) and Full-term (Gestational Age: 37–41 Weeks) Infants**

The number of infants is shown in parentheses. *p < 0.05, n.s.: not significant.

**Fig. 2. Chronological Changes of Serum Levels of 16-DHP S during the Early Neonatal Period**

(a) Extremely immature and pre-term infants (gestational age: 22–36 weeks); (b) full-term infants (gestational age: 37–41 weeks). The number of infants is shown in parentheses. *p < 0.05, ***p < 0.001.
DISCUSSION

The structure of 16-DHP S is unique in that it bears a double bond at C-16 of the steroid skeleton. Over four decades ago, Fukushima \textit{et al.} found the 16-ene steroid, 3α-hydroxy-16-pregnene-20-one in the urine of a patient with Cushings’s syndrome. Since then, many investigators have found 16-ene steroids in human sources, namely 16-DHP in urine, meconium, amniotic fluid and gallbladder bile.\textsuperscript{10} 16-dehydroproganelone (3β-hydroxy-16-pregnene-20-one) in urine, andandrosta-4,16-diene-3-one in plasma.\textsuperscript{11} We also identified 16-DHP, maybe 16-DHP S, in serum from immature infants.\textsuperscript{3} Reynolds \textit{et al.}\textsuperscript{18} reported that 16-ene double bond might be introduced in the placenta from their finding that 16-hydroxyprogesterone (16α-hydroxy-4-pregnene-3,20-dione) was converted to 16-dehydroprogesterone (4,16-pregnadiene-3,20-dione) in incubations with human placenta. Smith and Axelrod\textsuperscript{5} also identified 16-DHP in the incubation medium containing 17-hydroxyprogrenenolone (3β,17α-dihydroxy-5-pregnene-20-one) as a precursor and human placenta. On the other hand, Huhtaniemi\textsuperscript{19} presented evidence that the 16-ene steroid, 16-DHP, is formed from pregnenolone (3β-hydroxy-5-pregnene-20-one) by the fetal liver in \textit{vitro} and suggested that 16-hydroxyprogrenenolone (3β,16α-dihydroxy-5-pregnene-20-one) produced in liver from pregnenolone is the precursor of 16-DHP. If the introduction of the double bond at C-16 occurs in the placenta, neonatal serum levels of 16-DHP S should be reduced immediately after parturition. Our present data showed that the serum levels of 16-DHP S at 2—18 d were not lower than the levels at day 0 in extremely immature, pre-term or full-term infants (Fig. 2). These results suggest that 16-DHP S is produced in the fetal and/or neonatal liver rather than in the placenta \textit{in vivo}.

Although the precursor of 16-DHP S remains unknown, the involvement of pregnenolone S produced by the fetal adrenal gland should be considered, because pregnenolone S is the key steroid for the biosynthesis of various steroid hormones. As the fetal adrenals involute, the serum levels of pregnenolone S at 2—10 d decreased to 60% of the levels at birth.\textsuperscript{20} However, in our present study, the serum levels of 16-DHP S did not decrease in full-term infants during the 7 d after birth. Moreover, the 16-DHP S levels significantly increased at 2—7 d compared with the levels at day 0 in extremely immature and pre-term infants (Fig. 2). These results did not mirror the changes of pregnenolone S levels in the early neonatal period. There are some possible explanations for these conflicting results. It is likely that the precursor of 16-DHP S is 16-hydroxyprogenolone S. Several investigators\textsuperscript{19,21,22} have reported that 16-hydroxyprogenolone S is derived from pregnenolone S in the fetal adrenal or liver. Meanwhile, the 16-hydroxylation of dehydroepiandrosterone and pregnenolone is inhibited by estriol and other estrogens.\textsuperscript{23} We therefore speculate that the discrepancy between the serum levels of pregnenolone S and 16-DHP S is due to the regulation of 16-hydroxylation by estriol, which is produced by the placenta, uncontrolled ex-utero, at least during the early neonatal period.

Haruyama\textsuperscript{24} reported that serum levels of 16-hydroxy-pregnolone S in low birthweight neonates increased immediately after birth, whereas there were no changes of the level in normal birthweight neonates. If the precursor of 16-DHP S is 16-hydroxyprogenolone S, their findings are consistent with our present data (Fig. 2).

Further studies on the metabolic fate and physiological significance of 16-DHP S in fetuses and neonates are now under way in our laboratory.

REFERENCES