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Steroid Profiles, Disease and Gender Verification - Inborn Errors of Metabolism –
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Steroid Profiles, Disease and Gender Verification

- Inborn Errors of Metabolism -

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Urinary steroid profiles of neonatal and infant have been determined for the classification of steroid converting enzyme deficiency. Urine samples of the patient with 21–hydroxylase deficiency (Case–1), 17 α–hydroxylase deficiency (Case–2) and P–450 side chain cleavage enzyme (P–450scc) deficiency (Case–3) were analyzed. The major differences in the urinary steroid profiles of the patients were the excretion in much greater absolute and relative amount of: (1) 17 α–hydroxypregnenolone and 16 α–hydroxy dehydroepiandrosterone, (2) 5 α–tetrahydrocorticosterone (5 α–THB) respectively. 5 α–Tetrahydrocortisone (α –THE) and 20 β–cortolone were the only steroids excreted in the urine of the patient with P–450 scc deficiency before medical treatment(3), but the following application of cortisol (F) to the patient resulted in the urinary excretion of cortisol, cortisone and their tetrahydro metabolites. The results demonstrated that the patient with P–450 scc shows the presence of the other steroid converting enzyme activity, and the analytical results are informative for the research on the metabolism of corticosteroid. This report also re–validated the usefulness of steroid profiling on the positive case of gender verification.

Introduction
The classification of the congenital adrenocortical hyperplasia (CAH) using GC/MS is one of the important requirement in the routine urinalysis. The analytical procedure for the clinical diagnosis of CAH was developed by Shackleton1) and Axelson2). Their methods consisted of deconjugation, extraction, methoxy–TMS derivatization and GC/MS analysis. However, the presented reports in the literature focused on the individual case, and only limited information available that could be applied on the medical control in sports.
The several recent drug–testing incidents involved the doping cases with naturally occurring steroids. Further, some positive cases of gender verification may involve the patient with steroid converting enzyme deficiency and the athlete with transsexual
operation. Thus, the basic research of the steroid metabolism becomes more important also for maintaining the analytical procedure and the criteria for steroid doping.

**Materials and methods**

All the urine samples were collected from the neonatal and were analyzed for combined fraction steroids. All solvents were of reagent grade or analytical grade. Methoxyamine hydrochloride and trimethylsilylimidazole were purchased from Wako pure chemicals (Tokyo). β-Glucuronidase/arylsulfatase form *Ampullaria* (Tokyo zouki) was used. Sample preparation is shown in Figure-1.

**MS conditions**

- **Instrument:** Double focusing GC/MS type JMA-DX303 / DA5000 (JEOL, Tokyo)
- **Ionization:** Electron impact (70 eV, 300  μ A)
- **Ion multiplier:** 1.3 KV
- **Mass range:** 100 – 700 (m/z)
- **Scan rate:** 0.7 sec/scan
- **Ion source temp.:** 200°C

**GC conditions**

- **GC:** MS–GC G06 (JEOL, Tokyo)
- **Column:** Methylsilicone OV–1, 0.243 mm ID x 30 m L  
  0.25 m  μ film thickness(J&W,CA USA).
- **Carrier gas:** He, 1.85 kg/cm³ (ca 1.5ml/min. at 100°C)
- **Temperature:**  
  **Injector:** 300°C  
  **Column:** 200–300°C (8°C/min)  
  hold at 300°C for 10 min.

**Results and discussion**

The major fragments of methoxyme–TMS steroids were M⁺, M⁺−15 (loss of methyl radical), M⁺−90 (loss of trimethylsilyl alcohol radical in case of hydroxy steroids) and M⁺−31 (loss of methoxy radical in case of ketosteroids). Some other characteristic fragments were also observed. The cleavage between C13–C17 of steroid skeleton results in the formation of D–ring fragment. D–ring fragment of 20  ζ–hydroxypregnanes and 21–hydroxypregnanes, or 17 hydroxy–20–ketopregnanes and 21–hydroxy–20–ketopregnanes give rise a signal of fragment ion (m/z) 117 [C₂H₄OSi(CH₃)₃]⁺ or 188 [C₄H₈Si(CH₃)₃NOCH₃]⁺ respectively. The major A–ring fragment of 3  ζ–hydroxy pregnane was 129 [C₃H₄OSi(CH₃)₃]⁺. Molecular ion could hardly be detected in case
of non-keto poly hydroxy pregnanes, for example, pregnanediol, pregnanetriol, pregnenetriol and pregnanetetrol. Carbonyl group at C11 position of C-ring could not be derivatized into methyl oxime and was detected as the native form. Some typical EI mass spectrums of methoxyme-TMS steroids were shown in Figure-2-1 to Figure-2-5.

**Authentic steroids:**
Standard mass chromatograms were given in Figure-3 and Figure-4. About 40 important endogenous steroids can be monitored simultaneously over one GC/MS analysis. Syn- and anti-isomers of dehydroepiandrosterone(DHEA), corticosteroids and some other ketosteroids were formed by methoxyme-TMS derivatization, and were detected as coupled peaks. The relative retention time and the EI mass spectra of authentic methoxyme-TMS steroids were in good agreement of those published in the literature (Table-1).

**Steroid profiles in urine of normal infants:**
The major difference in the urinary steroid profiles between newborn and normal adult is the increased excretion of 3-\(\beta\)-hydroxy-5-ene steroids. Some unknown isomers of 16-hydroxydehydroepiandrosterone, namely androst-ene-diol-mono-one, could be detected. Elevated levels of 16-\(\alpha\)-hydroxylase activity is also expected. The concentration of 17-\(\alpha\)-hydroxyprogesterone (17-OHP), a typical neonatal steroid, is highly correlated to the body weight and gestational age of newborn. In mature infants, the concentration of neonatal steroids fell rapidly soon after birth, and the period was around first 33 weeks of the life after gestation.\(^4\)

**Case-1 CAH:**

**21-Hydroxylase deficiency (Figure-5)**

Social sex (external genitalia type): Male
Sex chromatin: XX Female

The urine sample was collected on 6th. day after birth. The steroid profiles represented the large increase of the urinary absolute excretion of 16-\(\alpha\)-hydroxy pregnenolone, DHEA, and 16-\(\alpha\)-hydroxy DHEA. Concentration of pregnanetriol(P3) and pregnantriolone(11K-P3) were relatively higher than those of normal infants. Absence of 21-hydroxylase activity resulted in the suppressed urinary excretion of 5-\(\alpha\)-THE, 20-\(\beta\) -Cortolone. It was expected that external expression of sexuality in this case was regulated by the elevated endogenous production of androgens.
Case-2 CAH: 17α-Hydroxylase deficiency (Figure-6)

Social sex (female type external genitalia): Female castrated
Sex chromatin: XY Male

The urinary steroid profiles of the patient were measured at one year after delivery. Because neonatal steroids still seen in the urine sample of this patient at one year old, this case was firstly expected as 3β-hydroxy steroid dehydrogenase deficiency. Elevated level of total 17-hydroxycorticosterone (17-OHCS) was measured by colorimetry, but the value was not in good agreement with the summation of 17-OHCS fractionation determined by GC-FID. Confirmation analysis by GC/MS was requested because the major unknown peak of GC chromatogram had the same retention time as those of 5β-tetrahydro cortisol (5β-THF) and the result was not agree with the other test results.

It was confirmed by MS analysis with modified GC temperature program that the main peak of the chromatogram was any of the isomer of pregnantriolones, and the peak was eluted slightly faster than 5β-THF. By comparison with authentic pregnantriolones, the unknown peak was finally assigned as 5α-THB. Thus, the elevated level of total 17-OHCS was validated. Major significant difference of the steroid profiles were the excretion in much greater absolute amount of 5α-THB, and relative amount of β-THA and 16α-hydroxypregnenolone. Suppressed excretion of 3β-hydroxysteroids (DHEA and 16α-hydroxy DHEA for example) was not caused by the deficiency of 3-hydroxysteroid dehydrogenase but caused by the lack of their parent compound.

Case-3 Prader's syndrome: P-450 scc deficiency (Figure-7)

Social sex (female type external genitalia): Female castrated
Sex chromatin: XY Male
Age: one year old

No significant amount of urinary steroids, except cholesterol exogenously taken from foods could be seen in the urine sample of the patient with P-450 scc deficiency. An additional urine specimen was also collected after single dose oral application of cortisol (F). Parent compound (F) and the metabolites e.g. cortisone(E), THF and THE were detected in the urine sample that collected after administration of F. This results indicated the presence of the other steroid converting enzymes. Since no biological background of E and F present in this case, the metabolites of exogenous F could be identified easily. The proposed metabolic pathway of the neonatal steroids was summarized in Figure-8. Expression of external sexuality in case-1 was regulated by the elevated endogenous production of androgens. On the other hand, suppressed maturity of sexual organ in case-2 and case-3 was due to the lack of androgens.
Conclusion
This report re-validated the usefulness of the traditional steroid profiling not only for doping control but also for the following up procedure of gender verification.

Acknowledgment
All the urine samples were collected by Dr. Keiko Honma at the Keio University Hospital School of Medicine, Endocrinology Department of Clinical Laboratories\(^5\), and were analyzed in Tokyo laboratory.

References
<table>
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<th>Abbreviations</th>
<th>Summary Structure</th>
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Figure-1 Sample preparation procedure for the steroid profiling

Urine sample 5.0ml
  Add 2 ml of saturated NH₄OH (removal of phosphate)
  Centrifuge

Supernatant
  Adjust pH to 5.0±0.5 using acetic acid
  Add β-glucuronidase from Ampullaria (320 Fishman units)
  Incubation. 60°C 2hrs
  10% saturation of Na₂SO₄
  Extraction by 10ml of CH₂Cl₂

Organic Phase
  Wash with 5ml of 8% of NaOH under 10% saturation of Na₂SO₄
  Wash with 5ml of aqueous solution under 10% saturation of Na₂SO₄

Organic Phase (5ml)
  Internal Standards (5 μg each of Stigmasterol and Cholesterol butylate)
  Dry up under the stream of nitrogen

Dry Residue
  100 μl of 2%-Methoxyamine HCl in pyridine
  Stand for 1hr at 60°C
  100 μl of Trimethylsilyl imidazole
  Stand for over night at 75°C
  Extraction by 1.5ml of CH₂Cl₂
  Centrifuge

Organic Phase
  Wash with 1ml of water
  Add Na₂SO₄ anhydrous
  Dry up under the stream of nitrogen
  Re-dissolve in 20 μl of n-hexane

GC/MS analysis
Figure 2-1 El mass spectra of Methoxyme-TMS steroids.
Figure 2-2: EI mass spectra of Methoxyme-TMS steroids.
Figure 2–3 El mass spectra of Methoxyene-TMS steroids.
Figure 2-4: EI mass spectra of Methoxy me-TMS steroids.
Figure 2-5 El mass spectra of Methoxyene-TMS steroids.
Figure 3 Mixture Group 1

Figure 4 Mixture Group 2

Mass chromatogram of the authentic standard mixture of methoxyme-TMS steroids.
The urinary steroid profiles of the patient with Congenital Adrenocortical Hyperplasia (CAH).

Figure 5: 21-Hydroxylase deficiency

Figure 6: 17α-Hydroxylase deficiency
A. Before medical treatment

B. After oral application of Cortisol

Figure-7 The urinary steroid profiles of the infants with P-450 scc deficiency.
Figure-8 Metabolic pathway of steroids