Steroid Profile for Urine: Reference Values


We describe a project, participated in by 24 institutions in The Netherlands and Belgium, to determine normal reference values for steroids in urine by capillary gas chromatography. Urine samples from 288 healthy volunteers were analyzed in triplicate. Reference values, expressed in μmol/24 h, were determined for androsterone, etiocholanolone, dehydroepiandrosterone, 11-keto-androsterone, 11-keto-etiocholanolone, 11-hydroxyandrosterone, 11-hydroxyetiocholanolone, pregnanediol, pregnanetriol, 11-desoxycorticosterone, tetrahydrocortisol, tetrahydrocortisone, tetrahydrocortisol, alloxotetrahydrocortisol, and 17-keto- and 17-hydroxysteroids. We also determined reference ratios for etiocholanolone/androsterone, tetrahydrocortisone/tetrahydrocortisol, and tetrahydrocortisol/allo-tetrahydrocortisol; an upper limit of a discriminant function to establish polycystic ovarian disease; and reference values for 24-h urine volume and creatinine excretion. Reference values were determined separately for men and women, each in six age categories: 0–3 months, 4 months–12 years, 13–16 years, 17–50 years, 51–70 years, and older than 70 years. We conclude that these reference values are reliable and form a basis for quantitative interpretation of steroid profiles.

Additional Keyphrases: sex- and age-related effects, interlaboratory comparison, chromatography, capillary gas, polycystic ovarian disease

There are two primary methods for determining steroid hormones: immunochemistry, to assay the specific hormones, mainly in serum (I); and chromatography, e.g., gas chromatography and HPLC of androgens, progestagens, and corticosteroids (2).

Laboratorians at 24 institutions in The Netherlands and Belgium decided to initiate a project for improving the quality of their gas-chromatographic assays for urinary steroid profiles. An external quality-control program (3) has been introduced, and calibrators and lyophilized control materials with assigned values have been made available. Regular meetings have been held to discuss the results. In addition, our experiences have been published in a series of articles. The first, published some years ago (4), dealt with a comparison of extraction methods. The present work was intended to determine a series of reference values for both men and women in six age categories, because recent changes in methodology have made the literature on reference values either incomplete or outdated.

Because the reference values used in the various institutions were too diverse for results to be pooled, we decided to attack the problem empirically. We plan to compile an atlas of pathological steroid profiles, as an aid to interpretation of the results.

Materials and Methods

Table 1 summarizes analytes and variables for which reference values have been determined: individual steroids (4, 5), classical groups of 17-OH- and 17-ketosteroids (6, 7), steroid ratios for diagnosis of Cushing's syndrome (8), Declercq and van de Calseyde's discriminant function for diagnosis of polycystic ovarian disease (POD) (9), and the volume and creatinine content of 24-h urine.

Samples

At each of the 24 institutions, urine specimens were collected from 12 volunteers, one from each sex in each of the following age categories: 0–3 months, 4 months–12 years, 13–16 years, 17–50 years, 51–70 years, and older than 70 years. All were in good health, free of medication, and the women were neither pregnant nor using oral contraceptives. The volunteers were carefully instructed to collect two consecutive 24-h urine specimens: the first specimen from 0800 h on day one until 0800 h on day two, the second from 0800 h on day two until 0800 h on day three. Each specimen was collected in a separate 2-L container and stored at 4°C during collection. On day three, immediately after completion of the collections, the two specimens were brought to the laboratory and collection was judged correct when the difference between the creatinine values for the two specimens did not exceed 2 mmol/24 h (10). From correctly collected urines, three 15-mL portions of the second specimen were lyophilized, to prevent putrefaction, and sent to three different laboratories according to a schedule that assured an equal workload for all institutions. Thus each of the 24 institutions collected 12 specimens and analyzed 3 lyophilized samples. In our experience, lyophilization does not influence the measured steroid concentrations (3).

Apparatus

Each of the participating laboratories used a gas chromatograph (different suppliers) equipped with a flame ionization detector and fitted with a CP-Sil 5 CB fused-silica capillary column (25 m × 0.32 mm i.d.) with a film thickness of 0.12 μm (Chrompack, Middelburg, The Netherlands). For extraction, the Baker-10SPE System (Baker Chemicals BV, Deventer, The Netherlands) was used, fitted with 6-mL octadecyl (C18) columns and 3-mL amino (NH₂) columns (different suppliers).

Reagents and Standards

Chemicals. Helix pomatia juice contained 10⁸ Fishman units of β-glucuronidase (EC 3.2.1.31) and 10⁶ Roy units of
Trivial IUPAC

THF/aTHF
THEITHF
THE
A
ACS
Poitet-Girard,
arylsulfatase
Creat

androstane-3α,17α-diol
and
4.6,
liter.

Chemical
formed
mixture
the
column
and
which
in
function
Steroid
17-OH
Individual
7-Keto
Standards.

7-Keto steroids: the sum of A, E, D, 11-KA, 11-KE, 11-HA, and 11-HE

17-OH
17-Hydroxy steroids: the sum of P3, THS, THE, THF, and aTHF

Steroid ratios
E/A
Ratio of etiocholanolone and androsterone
THE/THF
Ratio of tetrahydrocortisone and tetrahydrocortisol
THF/aTHF
Ratio of tetrahydrocortisol and allo-tetrahydrocortisol

Van de Calsyde's discriminant
function
Patient is classified as having polycystic ovarian disease if 0.09 (A + E + 11 – HA + A/E) > 3.0

Other
Creat 24 h
Creatinine, mmol/24 h
Volume 24 h
Urine vol, L/24 h

arylsulfatase (EC 3.1.6.1) per milliliter (Société Chimique Poitet-Girard, Villeneuve-la-Garonne, France). Methoxyamine hydrochloride and "Trasil-TBT" were from Pierce Chemical Co., Rockford, IL 61101. All other chemicals were ACS reagent grade (different suppliers).

Reagents. Methoxyamine HCl, 40 g/L, in pyridine. Acetate buffer, pH 4.6, 3 mol/L, containing 50 g of boric acid per liter. Acetate buffer, pH 4.6, 1 mol/L. Acetate buffer, pH 4.6, 0.2 mol/L. NaOH–carbonate buffer, NaOH 0.5 mol/L and Na₂CO₃ 1 mol/L.

Standards. All steroid standards were from Sigma Chemical Co., St. Louis, MO 63178. A 200 mg/L solution in methanol was prepared. The internal standard was 5α-androstane-3α,17α-diol (National Institute of Biological Standards and Control, London, U.K.). A 170 mg/L solution in methanol was used.

Procedures

Hydrolysis. Reconstitute the lyophilized sample in 15 mL of distilled water. Allow to dissolve for 30 min, adjust the pH to 4.6 with boric acid–acetate buffer, and centrifuge. Aspirate through the column 10 mL of centrifuged urine to which 100 µL of internal standard solution has been added and wash with two 5-mL portions of water. Elute the column with two 1-mL portions of methanol and evaporate the eluate at 50 °C in a stream of air. Dissolve the residue in 100 µL of methanol, add 5 mL of the 0.2 mol/L acetate buffer and 50 µL of Helix pomatia juice, and incubate the mixture overnight at 50 °C.

Extraction. Extraction of the hydrolyzed urines was performed by method A or method B.

Method A: Condition a C18 column as described above, then aspirate the incubation mixture through the column and wash with two 5±mL portions of water. Place about 1 g of anhydrous sodium sulfate on the top of an amino column and connect the C18 column on top of this. Elute the column with two 1.5-mL portions of ethyl acetate. Evaporate the eluate at 50 °C under air.

Method B: After cooling, add 2.5 g of sodium chloride and extract the urine twice with 10 mL of chloroform. Pool the chloroform and wash in 1 mL of a NaOH–carbonate solution and subsequently in 0.5 mL of 1 mol/L acetate buffer. Dry the chloroform with 2.5 g of anhydrous sodium sulfate and evaporate at 50 °C in a stream of air.

Derivatization. Add 100 µL of methoxyamine solution to the residue and warm at 80 °C for 90 min. Evaporate the pyridine in a stream of air. Add 100 µL of Trasil-TBT and warm overnight at 80 °C.

Conditions for gas chromatography. The conditions for gas chromatography were not identical for all institutions because they used different types of gas chromatographs. The injection temperature was 270 ± 10 °C and the detector temperature 300 ± 10 °C. Oven temperature programmes ranged from 200 ± 5 °C to 270 ± 20 °C at 2–3 °C/min. Hydrogen and helium were used as carrier gas at 30 ± 10 mL/min. Split and splitless injection were used. Some laboratories used "make-up" gas.

Data processing. The computer program executed a validity control on all data with the Q-test (11) at the 95% confidence level to detect analytical errors (triplicate results were compared) and specimen errors: within an age group, 24-h volume and creatinine concentration were compared to detect and exclude specimens from abnormal
volunteers. Data exceeding the Q-test’s values were rejected. Then the final calculations were carried out to establish the reference values, defined as the range covering 95% of all data in a given age/sex group.

Results

All the urine samples requested from the 288 volunteers in the 24 institutions were provided, but some institutions performed a part of their analyses too late: 3% of the planned analytical results were not available in time for data processing.

Validity-Control Data

Of the analytical data, 1.7% did not pass the Q-test and were rejected, as were seven specimens: four that showed a pathological profile, two that showed interfering peaks, and one that was rejected owing to an excessive 24-h volume. The data on 20 babies were excluded because, in babies younger than five weeks, 3β-hydroxy-5-ene steroids (residuals of fetoplacental steroid metabolism) predominate, rendering measurement of adult-type steroids difficult: androsterone, etiocholanolone, and other 17-keto steroids are not seen, and some cortisol metabolites differ from those in adults (12).

Reports. Figure 1 shows excretion/age plots for androsterone and tetrahydrocortisone for women. We constructed 42 plots of this type for all parameters mentioned in Table 1, for both men and women. The excretion/age plots were converted into reference values: Table 2 contains a survey of all variables for both sexes in six age categories, and Figure 2 shows a form applicable in daily routine.

Discussion

Extensive comparison of our results with previously reported reference values is impossible, because publica-

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<th>Table 2. Reference Values (95th Percentile) for Men and Women in Six Age Categories</th>
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<td><strong>Age of subjects</strong></td>
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<td><strong>μmol/24 h</strong></td>
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<td>Creat, mmol/24 h</td>
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tions on this theme are scarce and often out of date.

In the case of the individual steroids a comparison can be made with the reference values published by van de Calseyde et al. (5). Our values for androsterone and etiocholanolone are approximately 30% lower, whereas our values for tetrahydrocortisone are 15% higher for men and 15% lower for women. The small number of specimens (15 adult men and 15 adult women in their paper) and the fact that we used capillary columns whereas van de Calseyde et al. used packed columns might explain the differences. A comparison of our results with former reference values of the participating institutes shows that, for any of the individual steroids, a laboratory can be found that has higher and lower reference values.

Quantification of adult-type steroids proved to be difficult in young babies; owing to the interfering 3β-hydroxy-5-ene steroids, the reliability of our results for this age group is limited. Work is in progress to provide guidelines for qualitative interpretation. Philioppou’s (8) ratios for etiocholanolone/androsterone (0.5–2.1), tetrahydrocortisone/tetrahydrocortisol (1.1–2.6), and tetrahydrocortisol/allo-tetrahydrocortisol (0.5–3.5) in women are similar to ours in the 17–50 years category. The upper limit of 3.0 for the modified discriminant function of Declercq and van de Calseyde (9, modification not published, personal communication) agrees with the value 3.2 that we established. Reference values for creatinine and 24-h volume are similar to previously published values (13).

We conclude that, owing to (a) the good compatibility between our results and those previously available, (b) the size of the project, and (c) the collection of samples and assays being performed by 24 laboratories, we have achieved the purpose of the project: the determination of reliable reference values for quantitative interpretation of urinary steroid profiles.

We thank all colleagues who participated in the project, especially Mr. C. Dekkers, who prepared the software for the data processing.

References