Gas-Liquid Chromatographic Analysis of Urinary Steroids

J. L. BARRON, J. MAXWELL, R. P. MILLAR

SUMMARY
A method for gas-liquid chromatographic separation and quantitation of urinary steroid metabolites is described. A normal reference range has been established and classic examples of urinary steroid profiles of the major steroid endocrine disorders are given.


The advent of gas-liquid chromatography (GLC) of individual steroid metabolites has considerably facilitated the differential diagnosis of steroid hormone disease states, which were previously diagnosed by less specific measurements. Colorimetric methods for 17-oxosteroids and 17-oxogenic steroids are still the most commonly used techniques for the determination of groups of urinary steroids. Urinary steroid profiling by GLC allows individual quantitation of the majority of the urinary steroid metabolites which are important for endocrine investigations. The biosynthetic pathway of the adrenal steroids and the origin of the urinary steroid metabolites is set out in Fig. 1. Previously steroid profiling was confined to 17-oxosteroids, pregnanediol and pregnanetriol, because analysis of corticosteroid metabolites was hindered by thermal instability and destruction during acid hydrolysis. Recently it has been shown that corticosteroid metabolites may be determined by GLC.

CHOLESTEROL
PREGNENOLONE
PREGNENOLONE
PREGNENOLONE
PREGNENOLONE
17-OH
17-DOC
17-DOC
11-DOC
11-DOC
ALDOSTERONE
CORTISOL
17-DOC
17-DOC
11-DOC
11-DOC

Fig. 1. The biosynthetic pathway of adrenal cortico-steroids and androgens with their metabolites in the blocks.

We present the methodology for the quantitation of urinary steroid metabolites by GLC, a normal reference range and examples of steroid profiles characteristic of the major steroid endocrine disorders.

MATERIALS AND METHODS
Reagents: β-glucuronidase (2350 Fishman U/mg) containing significant amounts of sulphatase (Miles Laboratories, Cape Town); tetracosan (Fluka, Buchs, Switzerland); dichloromethane (Fluka, Buchs, Switzerland), redistilled; methoxyamine HCl, N,O-bis (trimethylsilyl) acetamide (BSA); trimethylsilylimidazole (TSIM), trimethylchlorosilane (TMCS) (Pierce Chemical Co., Rockford, Ill., USA); pyridine redistilled over NaOH; OV 101 on Supelcoport 80/100 mesh (Supelco Inc., Bellefonte, Pa, USA); androsterone (A), etiocholanolone (E), dehydroepiandrosterone (D), 11-keto-androsterone (KA), 11-keto-etiocholanolone (KE), 11-hydroxyandrostenedione (HA), 11-hydroxyetiocholanolone (HE), pregnanediol (P3), pregnanetriol (P4), 11-deoxycortisol (THF), tetrahydrocortisol (TSH), tetrahydrocortisone (THE), tetrahydrocortisol (TFS), cortisol (Cort), β-cortol or cholesterol (Chol) (Ikapharm, Tel Aviv, and Sigma Chemical Co., St Louis, Mo., USA).

Specimen collection: 24-hour urine samples were kept at 4°C. To each 100-ml urine aliquot were added 1 ml 3N sodium acetate (pH 4,6) containing 800 μmol boric acid and 1 - 2 ml chloroform. The sample was stored at 4°C or -20°C.

Hydrolysis of urinary steroid conjugates: 0,6 ml 1M sodium acetate (pH 4,6) and 1 ml β-glucuronidase-sulphatase suspension (containing 8 mg enzyme) were added to 6 ml urine. The mixture was incubated at 55°C for 20 - 24 hours.

Extraction: After cooling and addition of 43 mM sodium chloride, 30 nmol tetracosan was added as an internal standard and the urine was extracted twice with 10 ml dichloromethane. The pooled dichloromethane extracts were washed with 1 ml of a solution containing 0,5M NaOH and 1M NaCl and subsequently with 0,5 ml 1M sodium acetate (pH 4,6). The dichloromethane extract was dried with ± 2,5 g anhydrous sodium sulphate and evaporated to dryness at 45°C. Initially this extract was also filtered through phase separation paper (Whatman PS1) to remove any remaining water, but this was found to yield a peak between P3 and P4, and consequently use of phase separation paper was discontinued.

Derivative formation: The residue was dissolved in 100 μl 60 mM methoxyamine hydrochloride in pyridine, incubated at 90°C for 60 minutes, and then evaporated to dryness at 45°C under nitrogen. The silylation mixture (60 μl) (BSA : TSIM : TMCS : pyridine, 9 : 9 : 6 : 1) was added and left at 60°C overnight. An aliquot, 1,0 - 1,8 μl, of this solution was injected into the gas chromatograph. Periodically, problems arose in achieving complete silylation of the steroids. It had been suggested that adequate silylation may be achieved using TSIM alone. This simplified procedure was adopted in our laboratory recently and appears to be reliable.

Gas chromatography. A Pye 104 or a Packard 421 gas chromatograph fitted with FID and glass columns measuring 2 m × 2,5 mm (internal diameter) and containing 3% OV 101 on Supelcoport 80/100 mesh was used. The initial temperature was 200°C, increasing by 2°C/min...
to 265° - 270°C. The carrier gas was nitrogen at a flow rate of 35 ml/min. The column life was 24 - 3 months and the detectors were cleaned every 2 weeks. The elution of the steroid derivatives was 30 - 35 minutes.

**Standards:** A, E, D, KA, KE, HA, HE, P₁, P₃, THS, THE, THF, cortolone, β-cortol and the internal standard, tetracosan, were dissolved in dichloromethane and ethyl acetate (9: 1) by standing at room temperature for 48 hours. Aliquots containing 10 µg of each steroid were dried down, and derivatives were formed along with each batch of urine samples assayed. From the chromatogram of the standards, a factor was calculated for each of the steroids:

$$f = \frac{\text{surface area of internal standard}}{\text{surface area of steroid}}$$

A run was regarded as satisfactory only if the factor for each of the steroid standards was in the range 0,8 - 1,2. A reagent blank was passed through all stages. The steroid concentrations in the test urine samples were calculated as follows:

$$\frac{\text{dU steroid (µmol)}}{\text{surface area of steroid}} = \frac{\text{conc. of int. std. (µmol/m² × M.W. steroid)}}{\text{MW steroid × 24-h urine volume (ml) × urine aliquot used (µl)}}$$

The urinary steroid (µmol) per gram creatinine was calculated as follows:

$$\frac{\text{dU urinary steroid (µmol)}}{\text{dU creatinine (g)}}$$

**Determination of reference range:** 24-hour urine samples were collected from 22 male and 23 female members of the laboratory staff, none of whom were overweight. All females had regular menstrual cycles. Urinary 17-oxosteroids and 17-oxogenic steroids were determined by the Zimmermann reaction and creatinine levels by the Jaffe reaction.

**RESULTS**

**Quality Control**

A control urine sample was assayed with each run and the mean and standard deviation for steroid metabolites in 65 consecutive assays over 3 years is shown in Table I. As an additional check on the methodology, for each routine urine specimen the 17-oxosteroid and 17-oxogenic steroid values, as determined by the Zimmermann reaction, were compared with the values obtained by summing the appropriate GLC urinary steroid fractions. In general, GLC results correlated well with the Zimmermann estimations, but the latter yielded slightly higher results.

**Reference Range**

The urinary steroid levels in normal persons are shown in Table II. Non-parametric methods were used to calculate the reference range, since the number of samples was low. This range included 90% of the population studied.

**Patient Material**

During the period 1972 - 1978 we processed 900 urinary steroid profiles of patient material from the routine endocrine service. The incidence of confirmed, classically abnormal steroid profiles is shown in Table III. On some patients several urinary steroid analyses were carried out to confirm the diagnosis and to assess the response to therapy.

**Gas Chromatography**

The urinary steroid profile of a normal adult female is shown in Fig. 3. Figs 4 - 6 show the profiles in patients with Cushings disease, adrenal adenoma and adrenal carcinoma. Examples of profiles from persons with congenital adrenal hyperplasia due to the 21- and 11-hydroxylase enzyme deficiencies are shown in Figs 7 and 8. The urinary steroid profile in a patient with a typical polycystic ovary syndrome (Stein-Leventhal syndrome) is shown in Fig. 9.
TABLE II. ADULT URINARY STEROID REFERENCE RANGE (90% CONFIDENCE LIMITS)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Trivial name</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Androsterone</td>
<td>1.7 - 10.0 μmol/24 h</td>
<td>6.5 - 20.0 μmol/24 h</td>
</tr>
<tr>
<td>E</td>
<td>Etiolcholanololone</td>
<td>3.1 - 10.7 μmol/24 h</td>
<td>7.6 - 24.1 μmol/24 h</td>
</tr>
<tr>
<td>D</td>
<td>Dehydroepiandrosterone</td>
<td>0.1 - 1.4 μmol/24 h</td>
<td>0.0 - 6.6 μmol/24 h</td>
</tr>
<tr>
<td>KA</td>
<td>11-keto-androsterone</td>
<td>0.3 - 1.3 μmol/24 h</td>
<td>0.3 - 2.3 μmol/24 h</td>
</tr>
<tr>
<td>KE</td>
<td>11-keto-etiocholanolone</td>
<td>0.3 - 1.3 μmol/24 h</td>
<td>0.3 - 2.3 μmol/24 h</td>
</tr>
<tr>
<td>HA</td>
<td>11-hydroxyandrosterone</td>
<td>1.0 - 2.6 μmol/24 h</td>
<td>1.6 - 5.3 μmol/24 h</td>
</tr>
<tr>
<td>HE</td>
<td>11-hydroxy-etiocholanolone</td>
<td>1.0 - 2.6 μmol/24 h</td>
<td>1.6 - 5.3 μmol/24 h</td>
</tr>
<tr>
<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Pregnanediol</td>
<td>0.6 - 4.7 μmol/24 h</td>
<td>0.4 - 2.6 μmol/24 h</td>
</tr>
<tr>
<td>P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Pregnanetriol</td>
<td>0.6 - 4.2 μmol/24 h</td>
<td>0.5 - 2.6 μmol/24 h</td>
</tr>
<tr>
<td>THS</td>
<td>11-deoxytetrahydrocortisol</td>
<td>0 - 1.4 μmol/24 h</td>
<td>0 - 2.0 μmol/24 h</td>
</tr>
<tr>
<td>THE</td>
<td>Tetrahydrocortisone</td>
<td>1.6 - 6.9 μmol/24 h</td>
<td>4.1 - 11.8 μmol/24 h</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrocortisol</td>
<td>1.6 - 7.8 μmol/24 h</td>
<td>4.6 - 14.7 μmol/24 h</td>
</tr>
<tr>
<td>Cort</td>
<td>Cortolone</td>
<td>0.6 - 4.4 μmol/24 h</td>
<td>1.1 - 5.2 μmol/24 h</td>
</tr>
<tr>
<td>Chol</td>
<td>β-cortol or cholesterol</td>
<td>0.3 - 3.3 μmol/24 h</td>
<td>1.1 - 5.7 μmol/24 h</td>
</tr>
</tbody>
</table>

These values are based on the urine analysis of 23 adult females and 22 adult males. The 17-oxosteroids and 17-oxogenic steroid levels were determined by the Zimmermann reaction and the creatinine levels by the Jaffe reaction.

DISCUSSION

The modified method for urinary steroid profiling by GLC described here has proved to be a reliable and useful technique. Inter-assay precision for most of the major steroid metabolites (Table I) compares reasonably well with that in other reports. A comprehensive assessment of the standard deviations for all the steroids, including minor metabolites, is not usually mentioned in other reports. It is perhaps salient to draw attention to the fact that the standard deviation is higher for minor metabolites (such as D) and hence caution should be exercised in the interpretation of profiles when these minor metabolites are marginally raised. The normal range established by us is similar to but not as wide as that given in other reports.

Table II shows considerable difference between the upper limits of the urinary androgen metabolites for males and females, but expressed in terms of urinary creatinine, the values are of the same order.

The major steroid hormone endocrine disorders are easily distinguished by GLC. Adrenal carcinomas are frequently characterized by a rise in androgen metabolites and THS levels and the appearance of abnormal steroid metabolites. The elevation in THS levels could be due to...

Fig. 3. Urinary steroid profile of a normal adult female in the luteal phase of the menstrual cycle.

Fig. 4. Urinary steroid profile of a female with Cushing's disease (pituitary adenoma). THE and THF are raised while androgen metabolites are normal.
Fig. 5. Urinary steroid profile of a patient with adrenal adenoma. THE and THF are raised and the androgen metabolites are low.

Fig. 6. Urinary steroid profile from a female (aged 27) with adrenal carcinoma showing elevated A, E and THS. Note the unknown metabolite between P3 and THS.

Fig. 7. Urinary steroid profile of a child with 21-hydroxylase deficiency showing elevated A, E and P3.

Fig. 8. Urinary steroid profile of a male (aged 17) with 11-hydroxylase deficiency on treatment with cortisol shows elevated THS, A, E and P3. The peak between THS and THE is an unknown metabolite.

A relative inactivity of the 11-hydroxylation enzyme, since this enzyme is ACTH-dependent and ACTH secretion is clearly suppressed in patients with autonomously secreting adrenocortical tumours. The profile from the patient with adrenocortical carcinoma (Fig. 6) shows this classic picture, with a raised A, E, and THS, and an unusual peak. On the other hand, the profile from the patient with adrenocortical adenoma (Fig. 5) displayed no abnormal peaks; the diminished androgen production is consistent with the theory that the adenoma is composed of a single cell line and that the raised cortisol levels suppress ACTH, resulting in atrophy of normal adrenal cortex.

Virilization occurs in both 21- and 11-hydroxylase deficiency states, and the two entities are sometimes difficult to separate clinically. In both, diminished cortisol production results in excessive ACTH release, stimulation of the adrenal and shunting of the steroids into androgen production. The two conditions are clearly distinguished from each other by GLC. In 21-hydroxylase deficiency (Fig. 7), 17α-hydroxyprogesterone accumulates, and large
Fig. 9. Urinary steroid profile of a female (aged 26) with polycystic ovary syndrome shows elevated A and E with normal cortisol metabolites.

TABLE III. PATIENTS WITH ABNORMAL STEROID PROFILES

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cushing's syndrome</td>
<td>6</td>
</tr>
<tr>
<td>Pituitary adenoma</td>
<td>1</td>
</tr>
<tr>
<td>Ectopic ACTH</td>
<td>5</td>
</tr>
<tr>
<td>Adrenal adenoma</td>
<td>2</td>
</tr>
<tr>
<td>Adrenal carcinoma</td>
<td></td>
</tr>
<tr>
<td>Congenital adrenal hyperplasia</td>
<td>23 (6 salt-losing)</td>
</tr>
<tr>
<td>21-hydroxylation deficiency</td>
<td></td>
</tr>
<tr>
<td>11-hydroxylation deficiency</td>
<td>1</td>
</tr>
<tr>
<td>Polycystic ovary syndrome</td>
<td>27</td>
</tr>
</tbody>
</table>

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