Steroid Alcohols in Urine

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At present the most common method of determining urinary steroids is the specific estimation of 17-ketosteroids according to *Zimmermann*[1] or a modification of this method[2,3]. However, as the majority of urinary steroids are hydroxysteroids they can also be determined with steroid dehydrogenases[4].

Principle

3α-Hydroxysteroid dehydrogenase catalyses the following reaction:

(1) 3α-Hydroxysteroid + DPN^+ ⇌ 3-ketosteroid + DPNH + H^+

3β,17β-Hydroxysteroid dehydrogenase catalyses several steroid oxidations:

(2) 3β-Hydroxysteroid + DPN^+ ⇌ 3-ketosteroid + DPNH + H^+

(3) 17β-Hydroxysteroid + DPN^+ ⇌ 17-ketosteroid + DPNH + H^+

(4) 16β-Hydroxysteroid + DPN^+ ⇌ 16-ketosteroid + DPNH + H^+

The 17β- and 16β-hydroxyl groups only react when the adjacent carbon atoms have no hydroxyl or keto groups; for example, oestriol (3,16α,17β-OH) is not oxidized. The 16β-hydroxyl group reacts at an appreciably slower rate than the 3β- and 17β-hydroxyl groups.

The ketosteroids already present in the urine and those formed by the action of the enzymes are trapped as the hydrazones. In this way the equilibrium of the oxidative process is displaced in favour of the quantitative formation of the ketosteroids. The increase of absorption at 340 mμ due to the formation of DPNH is a measure of the reaction[5]. If the 3α-hydroxysteroid dehydrogenase is added to the reaction mixture first and then the 3β,17β-hydroxysteroid dehydrogenase, this allows the successive determination of the corresponding hydroxysteroids in the same reaction mixture. The order of addition of the enzymes must be adhered to because the 3β,17β-hydroxysteroid dehydrogenase preparations still contain 1 to 2% of the 3α-dehydrogenase. On the other hand, the 3α-dehydrogenase is not contaminated with the 3β,17β-dehydrogenase.

The hydroxysteroids are only present in very low concentration in urine and therefore they must first be concentrated by extraction with organic solvents. Other constituents of urine do not affect the enzymatic reaction. The recovery of hydroxysteroids added to urine is about 93%.

Reagents

1. Diphosphopyridine nucleotide, DPN
   free acid; commercial preparation, see p. 1010.
2. Hydrazine sulphate, A. R.
3. Sulphuric acid, A. R., 2 N
4. Sodium hydroxide, A. R., 1 N
5. Methanol, A. R.
6. Glycine, A. R.

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[1] *W. Zimmermann*, Hoppe-Seylers Z. physiol. Chem. *245*, 47 [1936].
[2] *H. L. Mason* and *W. W. Engstrom*, Physiol. Rev. *30*, 321 [1950].
[3] *P. L. Munson* and *A. D. Kenny*, Recent Prog. Hormone Res. *9*, 135 [1954].
[4] *B. Hurlock* and *P. Taladay*, Endocrinology *62*, 201 [1958].
[5] *O. Warburg* and *W. Christian*, Biochem. Z. *287*, 291 [1936].

7. β-Glucuronidase
   5000 *Fishman* units[6]/ml.; commercial preparation, see p. 975.
8. 3α-Hydroxysteroid dehydrogenase
   ca. 10 units*/mg. Isolation and purity of the preparation, see Appendix p. 489.
9. 3β,17β-Hydroxysteroid dehydrogenase
   ca. 10 units*/mg. Isolation and purity of the preparation, see p. 489.
10. Methylene chloride
11. Sodium hydrogen carbonate, $NaHCO_3$, A. R.
12. n-Hexane
13. Amberlite MB 1 **)
14. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 2\ H_2O$, A. R.
15. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
16. Acetic acid, A. R.
17. Ethylene-diamine-tetra-acetic acid, EDTA
    disodium salt, EDTA-$Na_2H_2 \cdot 2\ H_2O$.

Preparation of Solutions

I. Diphosphopyridine nucleotide (*ca.* $6 \cdot 10^{-3}$ M β-DPN):
   Dissolve 41 mg. DPN in 10 ml. doubly distilled water. Store the solution at 0°C and freeze again after use.

II. Glycine buffer (1 M; pH 9.4):
   Dissolve 7.5 g. glycine, 5.2 g. hydrazine sulphate and 0.2 g. EDTA-$Na_2H_2 \cdot 2\ H_2O$ in 85 ml. 1 N NaOH, adjust to pH 9.4 with 1 N NaOH and dilute to 100 ml. with doubly distilled water.

III. Phosphate buffer (0.03 M; pH 7.2):
   a) Dissolve 5.34 g. $Na_2HPO_4 \cdot 2\ H_2O$ in doubly distilled water and make up to 1000 ml.
   b) Dissolve 4.08 g. KH_2PO_4 in doubly distilled water and make up to 1000 ml.
   Mix solutions a) and b) in the ratio of 72.6 to 27.4 parts by volume.

IV. Acetate buffer (1 N; pH 4.5):
   Mix 43 ml. 1 N NaOH with 100 ml. 1 N acetic acid.

V. 3α-Hydroxysteroid dehydrogenase (*ca.* 100 units*/ml.):
   Dissolve the enzyme preparation in phosphate buffer (solution III).

VI. 3β,17β-Hydroxysteroid dehydrogenase (*ca.* 100 units*/ml.):
   Dissolve the enzyme preparation in phosphate buffer (solution III).

VII. β-Glucuronidase (5000 units[6]/ml.):
   Use the commercial preparation undiluted.

Stability of the solutions

The buffer solutions are stable practically indefinitely. If turbidity or a sediment (micro-organisms) is observed in the buffer solutions, then they should be prepared afresh. Prepare the DPN solution

*) 1 unit is the amount of enzyme which converts 1 μmole of substrate in 1 min.

**) Mixed-bed ion exchange resin (anion-cation) manufactured by Rohm & Haas Company, Philadelphia, Pa., USA.

[6] *P. Talalay, W. H. Fishman* and *Ch. Huggins*, J. biol. Chemistry *166*, 757 [1946]; see also p. 463, 873.

freshly each week or dispense in small portions and store frozen. During the analysis keep the dilute enzyme solutions at 0° C and then store frozen. In this state they are stable for several weeks.

Procedure

Extraction of the urinary steroids

Pipette into 60 ml. centrifuge tubes with tapered polyethylene stoppers:

25 ml. urine.

Adjust to pH 4.5 with 2 N H_2SO_4. Add

1.3 ml. acetate buffer (solution IV)

5.0 ml. β-glucuronidase solution (VII)

and incubate at 30° C (incubator or water bath) for 24 hours. Extract three times (shake 30 times for each extraction) with

15 ml. methylene chloride

each time; if necessary, centrifuge for 10 min. at 4000 g to separate the phases. Suck off the methylene chloride (lower phase) with a 20 ml. Record syringe and a long needle (as for lumbar puncture). Adjust the aqueous residue to pH 1.0 with 2 N H_2SO_4 and allow to stand 24 hours at room temperature. Again extract three times with

15 ml. methylene chloride

as described above. Evaporate the combined extracts to dryness in a rotatory evaporator under reduced pressure or with a stream of nitrogen at about 50° C. Dissolve the residue in

5 ml. methanol

and within *ca.* 10 min. pass once or twice through an ion exchange column (0.7 cm. diameter, 10 cm. high, Amberlite MB 1) to absorb the oestrogens. Elute the column with

25 ml. methanol.

Evaporate the eluates (*ca.* 30 ml.) to dryness as previously described and dissolve the residue in

15 ml. aqueous methanol (70% v/v).

Extract the solution three times with

5 ml. n-hexane

each time*) (separating funnel or centrifuge tube), evaporate the methanol-water phase to dryness (see above) and dissolve in

0.5 ml. methanol.

Use portions of this solution for the assay.

Spectrophotometric measurements

Wavelength: 340 mμ; light path: 1 cm.; final volume: 3 ml.; temperature: 25° C. Measure against a control cuvette containing methanol instead of urine extract.

Pipette successively into the cuvettes:

2.00 ml. glycine buffer (solution II)

0.10 ml. DPN solution (I)

0.01 – 0.10 ml. urine extract (methanol in the control cuvette)

0.09 – 0 ml. methanol

distilled water to 2.96 ml.

*) Lipids are extracted; otherwise they would cause turbidity in the aqueous assay mixture and so interfere with the determination.

Mix and read the optical density E_1. Mix into the experimental and control cuvette

0.02 ml. 3α-hydroxysteroid dehydrogenase solution (V).

As soon as the optical density is constant, read E_2. Then mix into both cuvettes

0.02 ml. 3β,17β-hydroxysteroid dehydrogenase solution (VI)

and read E_3 when the optical density is constant.

Calculations

The optical density changes $E_2-E_1 = \Delta E_\alpha$ and $E_3-E_2 = \Delta E_\beta$ are used for the calculations. ΔE_α corresponds to the concentration of 3α-hydroxysteroid in the cuvette, and ΔE_β to the sum of the 3β-, 17β- and 3β,17β-hydroxysteroids (abbreviated to 3β,17β-hydroxysteroids in the following).

Therefore

$$\frac{\Delta E \times 3}{6.22} = \mu\text{moles 3}\alpha\text{-hydroxysteroid/assay mixture or } \mu\text{moles}^{*)} \text{ 3}\beta,17\beta\text{-hydroxysteroid/assay mixture}$$

where

3 = volume of the assay mixture [ml.]

6.22 = extinction coefficient of DPNH at 340 mμ [cm.²/μmole]

Example

25 ml. of urine were extracted, the extract was dissolved in 0.5 ml. of methanol and 0.1 ml. of this was added to the cuvette. After the addition of 3α-hydroxysteroid dehydrogenase the optical density change was $\Delta E_\alpha = 0.300$. The patient excreted 1 500 ml. of urine in 24 hours, that is 60 times the amount extracted.

$$\frac{0.300 \times 3}{6.22} \times 5 \times 60 = 43 \text{ μmoles 3}\alpha\text{-hydroxysteroid per 24 hours}$$

5 = conversion of portion taken (0.1 ml.) to the total volume of extract (0.5 ml.)

60 = conversion of the volume of urine extracted (25 ml.) to the total volume of the 24 hour urine (1 500 ml.)

Normal Values

With this method *Talalay*[4] found the following normal values:

Subjects examined	Number of determinations	3α-Hydroxysteroids [μmoles/24 hrs.]	[μmoles/l.]	3β,17β-Hydroxysteroids [μmoles/24 hrs.]	[μmoles/l.]
Men (20 to 47 years)	13	43.7 ± 13.0 (29.1 to 70.7)	41.8 ± 14.1 (18.7 to 66.7)	7.52 ± 2.71 (3.16 to 13.7)	6.90 ± 2.75 (3.29 to 13.2)
Women (17 to 37 years)	11	42.4 ± 18.5 (16.4 to 74.2)	44.1 ± 16.1 (26 to 78)	6.07 ± 2.65 (2.61 to 11.6)	6.44 ± 2.87 (2.90 to 14.6)

The average values and the standard deviation are given. In the brackets are the range of the analytical values. In the adrenogenital syndrome the 3β,17β-hydroxysteroids increase considerably, while in adrenal hyperplasia the increase is less marked[4].

*) Strictly speaking this is μmoles 3β-hydroxysteroid + μmoles 17β-hydroxysteroid + 0.5 μmoles 3β,17β-hydroxysteroids.

Other Methods of Determination

The enzymatic determination of urinary steroids can be improved by first separating the steroids chromatographically. In this way it is possible to determine 3β- and 17β-hydroxysteroids separately. A method for the paper chromatographic separation of hydroxysteroids and their subsequent determination enzymatically has been described by *Talalay*[4]. The solvent system used for the paper chromatography depends on the type of steroids to be separated, while the spectrophotometric assay is similar to the one described above.

Appendix

Preparation of 3α-hydroxysteroid dehydrogenase

Method: The enzyme is purified from *Pseudomonas testosteroni* by ammonium sulphate precipitation, protamine precipitation and acetone precipitation to yield a preparation with a turnover number of about 2500 to 5000 moles androsterone/min./10^5 g. protein (25° C)[7-9]. In particular, it is separated from the 3β,17β-dehydrogenase by ammonium sulphate fractionation: the 3β,17β-dehydrogenase precipitates between 30 and 40% saturation, while the 3α-dehydrogenase is only precipitated between 40 and 55% saturation.

Purity: The 3α-dehydrogenase contains practically no 3β,17β-dehydrogenase or alcohol dehydrogenase. However, it is contaminated with a Δ^5-3-ketosteroid isomerase.

Substrates of the enzyme are 3α-hydroxysteroids containing 19, 21 and 24 carbon atoms (A/B-*cis* and A/B-*trans*). C_{27} steroids do not react.

Equilibrium constant:

$$K_H = \frac{[\text{androstane-3,17-dione}] \times [\text{DPNH}] \times [H^+]}{[\text{androsterone}] \times [\text{DPN}^+]} = 5.8 \times 10^{-9} \text{ mole/l. (25° C; pH 6–9.0)}^{8)}$$

At pH 9.1 and with a ten-fold excess of DPN androsterone is practically quantitatively oxidized to androstanedione. Therefore it is possible in this way to estimate 3α-hydroxysteroids without the addition of hydrazine sulphate.

The *Michaelis constant* for androsterone is 1.6×10^{-6} M (pH 9.1 and 25° C); K_M for DPN is 1.04×10^{-4} M (pH 9.1; 10^{-5} M androsterone as substrate).

Assay of activity: see under the 3β,17β-dehydrogenase. Androsterone is used as the substrate instead of testosterone.

Stability: Solutions of the enzyme containing 50 mg. protein/ml. are stable for years at −20° C.

Preparation of 3β, 17β-dehydrogenase

Method: The formation of 3β,17β-dehydrogenase is induced in *Pseudomonas testosteroni* by the addition of testosterone[10]. The cells are disintegrated by exposure to ultrasonic radiation and the enzyme is purified by ammonium sulphate precipitation, protamine precipitation and acetone precipitation to yield a preparation with a turnover number of about 2500 to 5000 moles testosterone/min./10^5 g. protein[7-9].

Purity: The preparation is free from alcohol dehydrogenase, but still contains a steroid isomerase which catalyses the conversion of Δ^5-androstene-3,17-dione to Δ^4-androstene-3,17-dione. It is also contaminated with about 1 to 2% of the 3α-dehydrogenase.

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7) *P. I. Marcus* and *P. Talalay*, J. biol. Chemistry *218*, 661 [1956].
8) *P. Talalay* and *P. I. Marcus*, J. biol. Chemistry *218*, 675 [1956].
9) *B. Hurlock* and *P. Talalay*, J. biol. Chemistry *227*, 37 [1957].
10) *P. Talalay*, *M. M. Dobson* and *D. F. Tapley*, Nature [London] *170*, 620 [1952].

Equilibrium constant:

$$K_H = \frac{[\Delta^4\text{-androstene-3,17-dione}] \times [DPNH] \times [H^+]}{[\text{testosterone}] \times [DPN^+]} = 3.78\,(\pm 1.1) \times 10^{-8}\,\text{moles/l.}$$

(25° C; pH 6 to 10)[8,12].

If the enzymatic assay is carried out at pH 9.0 with a ten-fold excess of DPN compared to the amount of testosterone, then at equilibrium there is about 378 times more androstenedione than testosterone present. This method is therefore suitable for the quantitative determination of testosterone.

Affinity constant[11]*:* Relatively low concentrations of testosterone (6×10^{-6}M) inhibit the 3β,17β-hydroxysteroid dehydrogenase. Apart from the Michaelis complex between the substrate and enzyme (ES) a further complex is formed with increasing testosterone concentration, which is composed of two molecules of substrate and one molecule of enzyme (ES_2). The affinity constant K_1 for ES (25° C and testosterone as substrate) is 0.93×10^{-6} M; the constant K_2 for ES_2 is 39.0×10^{-6} M[11].

Inhibitors: The enzymatic reaction is strongly inhibited by natural and synthetic oestrogens (*e.g.* diethylstilboestrol).

Assay of activity: Pipette into a 1 cm. cuvette, 1 ml. pyrophosphate buffer (0.1 M; pH 8.9), 0.5 μmoles DPN and 15 μg. testosterone. Dilute with distilled water to 3 ml. The reaction mixture is pH 9.1. Start the reaction (at 25° C) by the addition of 0.02 to 0.1 ml. enzyme solution. A unit is the amount of enzyme which causes an optical density change of 0.001/min. at 340 mμ.

Stability: Solutions of the enzyme containing 50 mg. protein per ml. are stable for years at −20° C.

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[11] *P. J. Marcus* and *P. Talalay*, Proc. Roy. Soc. [London] Ser. B, *144*, 116 [1955].
[12] *P. Talalay*, Record Chem. Progr. Kresge-Hooker Sci. Lib. *18*, 31 [1957].