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Note

Enzymatic determination of urinary 17β-hydroxysteroids on thin-layer chromatograms

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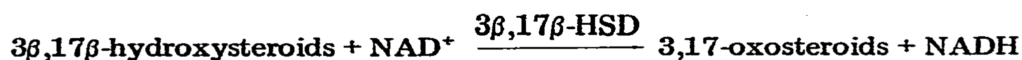
The enzymatic detection of steroids such as neutral 3α-hydroxysteroids [1, 2], 3β-hydroxysteroids [3—5], acidic 3α-hydroxysteroids [6, 7], and 17β-hydroxysteroids [8] has been reported. In this paper, the enzymatic detection of urinary 17β-hydroxysteroids on thin-layer plates is described. The principle of the method is as follows: after separation of urinary 17β-hydroxysteroids by thin-layer chromatography, color development is performed by use of the enzyme 3β,17β-hydroxysteroid dehydrogenase (3β,17β-HSD), which catalyzes the reaction



$$3\beta,17\beta\text{-hydroxysteroids} + NAD^{+} \xrightarrow{3\beta,17\beta\text{-HSD}} 3,17\text{-oxosteroids} + NADH$$

$$NADH + p\text{-iodonitrotetrazolium violet} \xrightarrow{\text{diaphorase}} \text{formazan}$$

Total urinary 17β-hydroxysteroids can be determined by two enzymatic reactions described previously [8], but by using chromatography only 17β-hydroxysteroids could be detected on the thin-layer plate, which may serve as a diagnostic tool in clinical tests.

MATERIALS AND METHODS

Preparation of the enzyme color development reagent used has been described previously [8]. Briefly, dissolve 10 U of 3β,17β-HSD, 8 mg of NAD^{+}, 40 mg of disodium EDTA and 30 U of diaphorase in 20 ml of aqueous K_2HPO_4 solution (0.2 *M*, pH 8.5).

The thin-layer plates used in this study were Kieselgel 60 (E. Merck, Darmstadt, G.F.R.) heated at 110°C for 30 min before use.

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Preparation of urine samples

Pipette 10 ml of urine into a 40-ml tube and adjust to pH 6.5 with bromothymol blue paper as an indicator. Add 1 ml of β-glucuronidase (1000 Fishman units/ml), 1 ml of 0.5 *M* phosphate buffer (pH 6.5) and a few drops of chloroform and mix well. Incubate the mixture for 24 h at 37°C, then adjust to pH 1 with 6 *M* sulfuric acid and saturate with 5 g of sodium chloride. Shake the solution with 15 ml of ethyl acetate for 5 min. After centrifuging, discard the urine layer and keep the ethyl acetate layer for another 24 h at 37°C. Wash the ethyl acetate layer successively with 3 ml of concentrated sodium carbonate, and 2 ml of water. After centrifugal separation, transfer 10 ml of the ethyl acetate extract to a tube and evaporate to dryness.

Thin-layer chromatography

To the dry residue, a few drops of a chloroform—methanol mixture (1:1, v/v) are added, and the sample is applied to a thin-layer plate with marker dye and standards. The plate is developed in the solvent mixture ethyl acetate—benzene (1:1, v/v) for 60 min at 20°C. The distance of the front from the starting point was about 15 cm [5].

Color development of 17β-hydroxysteroids on thin-layer plates

Place the thin-layer plate over a water-bath at 40°C, and spray with the enzyme reagent. Incubate for 30 min so that a pink-colored zone is visible. Densitometric scanning at 500 nm can be also be performed.

RESULTS AND DISCUSSION

The selectivity of 3β,17β-HSD was tested with a series of steroids in solution and on thin-layer plates using 20 μg of steroids. The data are shown in Table I. The R_F values of steroids are presented in Table II.

The excretion of 17β-hydroxysteroids in some patients and in a normal subject are shown in Fig. 1.

TABLE I

THE SELECTIVITY OF 3β,17β-HYDROXYSTEROID DEHYDROGENASE

Each steroid at 20 μg per tube or per spot was determined with enzyme solution at 40°C for 30 min and the intensity of reaction was expressed as a percentage relative to testosterone. The detection limit for testosterone was 1—2 μg per spot on the thin-layer plate.

Compound	Selectivity	
	in solution (%)	on thin-layer plate (%)
Testosterone	100	100
Estradiol	99	75
5α-Androstane-3β,17β-diol	153	80
Dehydroepiandrosterone	98	60
5-Pregnene-3β,17α,20-triol	50	45
3α-hydroxysteroids	0	0
Estriol	0	0
Cholesterol	0	0

TABLE II

R_F VALUES OF STEROIDS

Solvent system: ethyl acetate—benzene (1:1).

Compound	R_F
Marker dyes	
Sudan III	0.63
Isatine	0.31
Estradiol	0.39
Dehydroepiandrosterone	0.36
5-Androstene-3β,17β-diol	0.28
5α-Androstane-3β,17β-diol	0.27
Testosterone	0.26
5-Pregnene-3β,17α,20-triol	0.15
5-Androstene-3β,16α,17β-triol	0.08

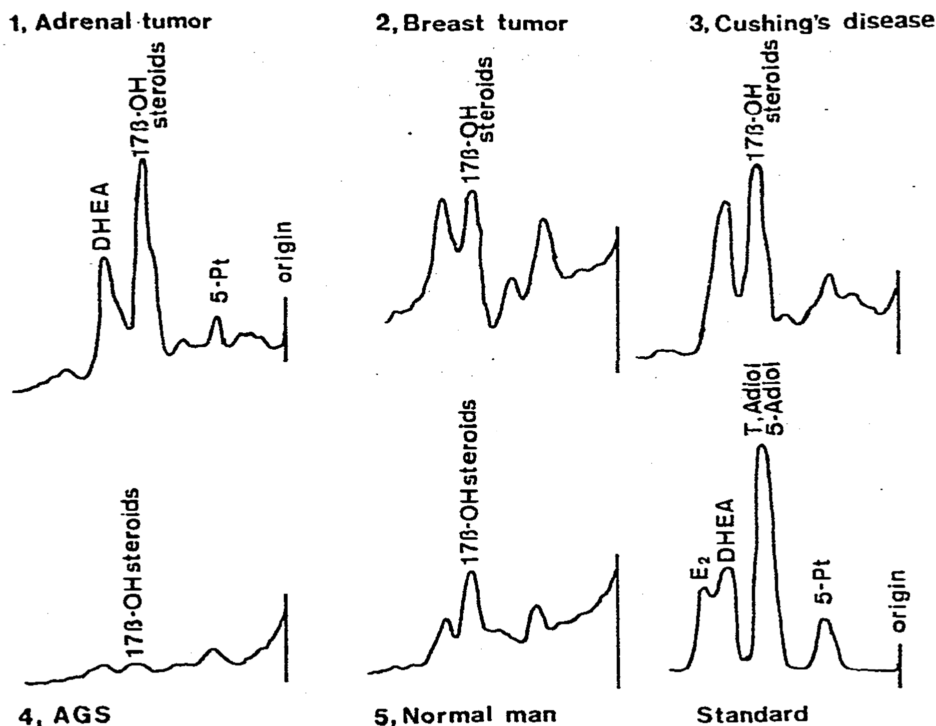

Fig. 1. Chromatograms of 17β-hydroxysteroids detected by 3β,17β-hydroxysteroid dehydrogenase. DHEA = dehydroepiandrosterone; 17β-hydroxysteroids = testosterone, 5-androstene-3β,17β-diol and 5α-androstane-3β,17β-diol; E_2 = estradiol; 5-Pt = 5-pregnene-3β,17α,20-triol; T = testosterone; Adiol = 5-androstene-3β,17β-diol; 5-Adiol = 5α-androstane-3β,17β-diol; AGS = adrenogenital syndrome.

The excretion values of total 17β-hydroxysteroids, determined by a previously described method [8], were 18.3 mg/day for adrenal tumor, 7.8 mg/day for breast tumor, 7.5 mg/day for Cushing's disease, 0.5 and 0.2 mg/day for 21-hydroxylase deficiency, and 1.5—4.5 mg/day for normal subjects.

Although urinary total 17β-hydroxysteroids were determined by subtracting the value for 3β-hydroxysteroids from the value for 3β,17β-hydroxysteroids [8], it is difficult to avoid interference from 3β-hydroxysteroids on thin-layer plates. Most of the urinary 3β-hydroxysteroids can be separated using the chromatographic conditions described in the Methods section [5], so it can be said that it is the urinary 17β-hydroxysteroids that are detected on the thin-layer plates. Detection of 17β-hydroxysteroids on thin-layer plates is just for screening for abnormal androgen metabolism. For a more accurate determination of urinary 17β-hydroxysteroids, use of high-performance liquid chromatography or column chromatography with enzymatic detection is preferable. In this paper a simple detection method for 17β-hydroxysteroids has been described as a useful clinical test.

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