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Note

Enzymatic color development of 3α-hydroxysteroids on thin-layer chromatograms for determination of excretion pattern of 3α-hydroxysteroids in patients with some adrenogenital syndrome

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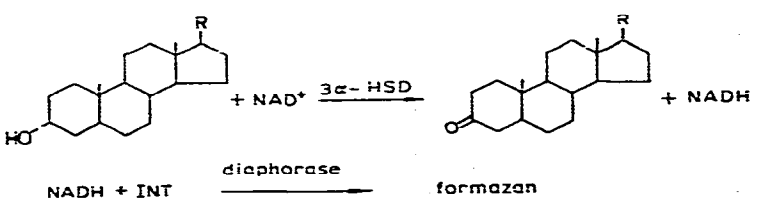
The differential diagnosis of adrenogenital syndrome is diffcult without the use of chromatographic techniques, but gas—liquid or column liquid chromatography are time-consuming and need high techniques of analysis.

The simpler methods, however, often do not provide adequate separation and identification of the individual steroid compounds and lack the accuracy of the original procedures, but many simple methods have been reported for the detection of steroids after chromatographic separation such as the Zimmermann reaction for 17-ketosteroids [1], some other chemical reactions and enzymatic color development of 3β-hydroxysteroids [2].

Although the paper chromatographic—enzyme spray technique for the detection of sugars is well known [3], in this paper a thin-layer chromatographic (TLC)—enzyme solution spray technique is described for the determination of the excretion pattern of 3α-hydroxysteroids in patients with some adrenogenital syndrome. The principle of the reaction is as follows:



The dye formed has a maximum absorption at 500 nm.

MATERIALS AND METHODS

3α-Hydroxysteroid dehydrogenase (3α-HSD) (from *Pseudomonas testosteroni*, EC 1.1.1.51) and β-NAD^+ were purchased from Nyegaard & Co. (Oslo, Norway). All steroids, β-glucuronidase (bacterial powder from *Escherichia coli*, EC 3.2.1.31), diaphorase (from *Clostridium kluyveri*, EC 1.6.99.2) and other reagents for color development were purchased from Sigma (St. Louis, MO, U.S.A.). To prepare the enzyme reagent for color development of 3α-hydroxysteroids on thin-layer plates: dissolve 6 mg of 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride in 10 ml of 0.2 *M* K_2HPO_4 (pH 8.5) containing, per 10 ml, 1 U of 3α-HSD, 50 U of diaphorase and 5 μmol of β-NAD.

Preparation of sample

Pipette 5 ml of urine (in case of high excretion of urinary steroids, use 2 ml of sample urine) into a 40-ml tube and adjust to pH 6.5 with bromthymol blue paper as a indicator. Add 1 ml of β-glucuronidase (1,000,000 Fishman units/l), 1 ml of 0.5 *M* phosphate (pH 6.5) and a few drops of chloroform to the tube and mix well. Incubate the mixture for 24 h at 37°C, then adjust to pH 1 with 6 *M* sulphuric acid and saturate with 5 g of sodium chloride. Shake the solution with 20 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 to achieve complete solvolysis of the sample. Wash the ethyl acetate layer successively with 2 ml of sodium hydroxide (80 g/l), with concentrated sodium carbonate, and water. After centrifugal separation, transfer 15 ml of the ethyl acetate extract to a tube. Evaporate the ethyl acetate aliquots.

Thin-layer chromatography

To the dry residue, a few drops of chloroform are added, and the sample is applied to an activated thin-layer plate with marker dye (sudan III and isatin) and standards. The development of the thin-layer plate was performed with a solution of chloroform–methanol (9:1, v/v) for 60 min at 25°C. The distance of the front from the starting point was about 18 cm.

Color development of 3α-hydroxysteroids on thin-layer plates

Place the thin-layer plate on a heater at 37°C (or above a water bath at 40°C), and spray the enzyme reagent. Incubate for 30 min so that the zone of pink color can be seen. Then quantitative denistometric scanning at 500 nm can also be performed for the determination of 3α-hydroxysteroids in the sample. The instrument used for the assay is a dual-wavelength TLC scanner CS-910 (Shimadzu, Tokyo, Japan).

RESULTS

The absorption curve of the dye formed has a maximum at 500 nm.

Selectivity of 3α-HSD with some steroids

Selectivity of 3α-HSD was tested with some steroids (Table I). Phenolic hydroxy, 3β-hydroxy and 3-keto groups did not react with this enzyme.

Excretion patterns of 3α-hydroxysteroids

The excretion pattern of 3α-hydroxysteroids in patients with some adrenogenital syndrome are shown in Fig. 1 and each R_F value of the standards is listed in Table II.

Precision

The percentages of each fraction in five repeated assays on thin-layer plates were calculated and the average of each C.V. was 7.8% using 10 μg of each steroids, and a convenient range for quantitative analysis was 5—50 μg of each steroid in the residue of the extracts.

TABLE I

SELECTIVITY OF 3α-HYDROXYSTEROID DEHYDROGENASE

Each steroid of 25 μg per tube was determined with 2 ml of enzyme solution at 37° C for 20 min.

Steroid	Intensity of reaction (O.D. at 500 nm; 25 μg)
Etiocholanolone	0.560
Androsterone	0.525
Cortol	0.504
Tetrahydrocortisol, tetrahydrocortisone	0.490
Tetrahydro-11-deoxycortisol	0.470
Pregnanediol	0.190
Pregnanetriol	0.090
Cortisol	0.000
Dehydroepiandrosterone	0.000
Testosterone	0.000
Estriol	0.000

TABLE II

R_F VALUES OF SOME 3α-HYDROXYSTEROIDS

The development was performed by chloroform—methanol (9:1, v/v).

Compounds	R_F value
Marker dye	
Sudan III	0.82
Isatin	0.58
Steroids	
Androsterone	0.68
Etiocholanolone	0.67
11-Ketoandrosterone	0.63
Pregnanediol	0.56
11β-Hydroxyandrosterone	0.56
Tetrahydro-11-deoxycortisol	0.41
Pregnanetriol	0.37
Tetrahydrocortisone	0.33
Tetrahydrocortisol	0.22
Cortol	0.10

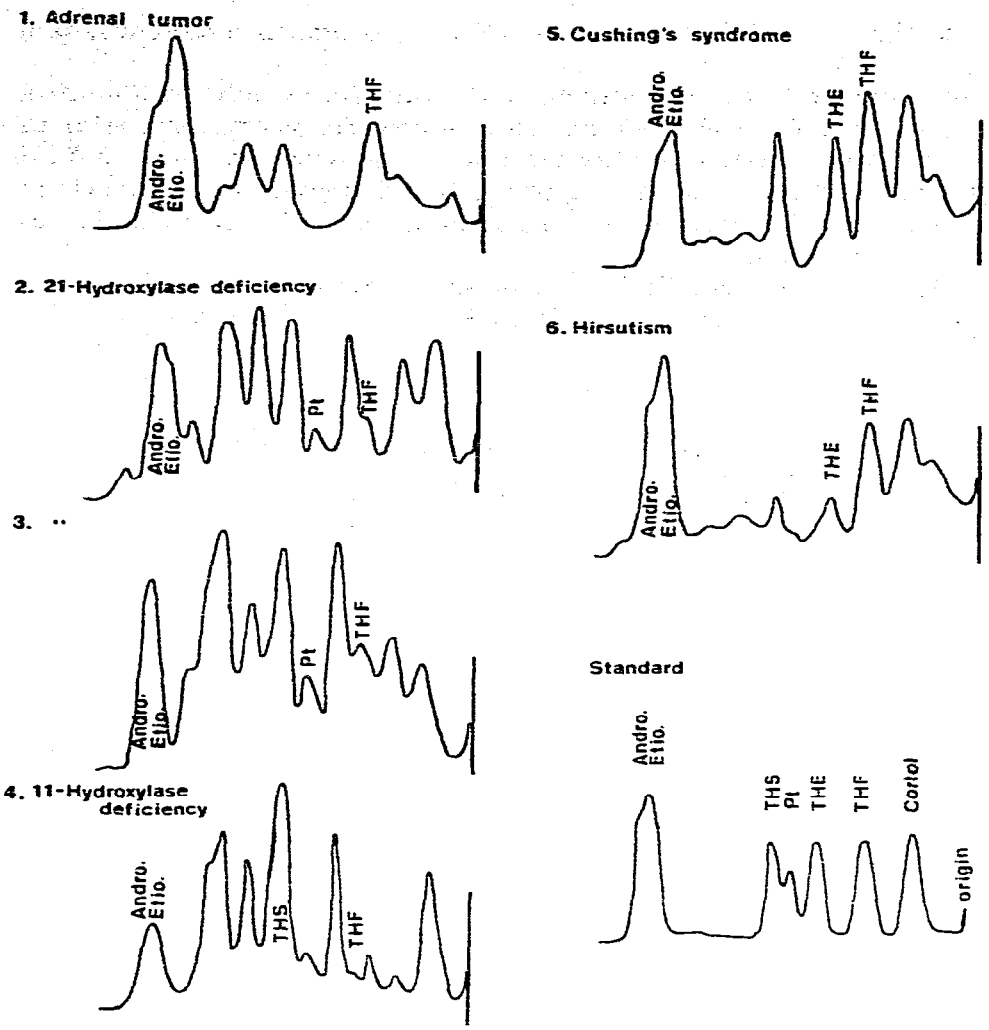


Fig. 1. Excretion pattern of 3α-hydroxysteroids by densitometric scanning. Samples are from 1, adrenal tumor; 2, 21-hydroxylase deficiency; 3, 21-hydroxylase deficiency; 4, 11-hydroxylase deficiency; 5, Cushing's syndrome; 6, hirsutism and some standard compounds. THF, tetrahydrocortisol; THE, tetrahydrocortisone; Andro., androsterone; Etio., etiocholanolone; Pt, 5β-pregnane-3α,17α,20α-triol.

DISCUSSION

The available techniques for quantitation of individual steroids are complicated and time consuming, but this method provides an estimate of groups of 3α-hydroxysteroids and is adequate for certain purposes being a simple and rapid procedure.

It is easily recognized that peaks of tetrahydro-11-deoxycortisol and some C_{21}-steroids can be seen between peaks of 17-ketosteroids and 17-hydroxycorticosteroids in patients with adrenogenital syndrome caused by enzyme

deficiency, so that this method can be used for the diagnosis of adrenogenital syndrome.

Color development with diaphorase, NAD and 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride can also be used for the determination of certain steroids using certain hydroxysteroid dehydrogenases, e.g., NADH formed by the reaction of certain hydroxysteroid dehydrogenases such as 17β-hydroxysteroid dehydrogenase can be used for specific determination of the corresponding steroids.

The new detection method, TLC—enzyme spray technique, for 3α-hydroxysteroids is of value for the diagnosis of some adrenogenital syndrome.

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