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EXCRETION PATTERN OF 3β-HYDROXYSTEROIDS IN PATIENTS WITH ADRENAL TUMOR, CUSHING'S DISEASE AND 21-HYDROXYLASE DEFICIENCY, AND IN PREGNANCY, USING THIN-LAYER CHROMATOGRAPHY AND COLOR DEVELOPMENT OF 3β-HYDROXYSTEROIDS WITH 3β-HYDROXYSTEROID OXIDASE

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SUMMARY

The fractional assay is described of 3β-hydroxysteroids in various patients by thin-layer chromatography and color development using an enzyme that reacts specifically with some 3β-hydroxysteroids. Together with dehydroepiandrosterone, androst-5-ene-3β,17β-diol and 16α-hydroxydehydroepiandrosterone can be detected, but their concentrations differ with each disease. An unknown fraction, a more polar 3β-hydroxysteroid than 16α-hydroxydehydroepiandrosterone, is also detected in moderate amounts in patients with adrenal tumor (18.3 mg/day), 21-hydroxylase deficiency (3.2 to 1.2 mg/day), and Cushing's syndrome (0.9—2.3 mg/day, as pregn-5-ene-triol).

INTRODUCTION

Dehydroepiandrosterone (DHEA*) was considered to be a main urinary 3β-hydroxysteroid in normal subjects, but once the excretion of some other 3β-hydroxysteroids was found in adrenal disease and in infant or pregnant urine, the presence of other 3β-hydroxysteroids such as 16α-hydroxy-dehydroepiandrosterone (16-OH-DHEA) and androst-5-ene-3β,17β-diol in normal subjects was demonstrated by many investigators [1—6].

The methods used so far for detecting steroids by color development are the Zimmermann reaction for 17-ketosteroids, the Porter—Silber reaction for 17-hydroxycorticosteroids, the Kober reaction for estrogens, sulfuric acid reaction

*Non-standard abbreviations used: DHEA, 3β-hydroxy-androst-5-en-17-one; 16α-OH-DHEA, 3β,16α-dihydroxy-androst-5-en-17-one; A-diol, androst-5-ene-3β,17β-diol; A-triol, androst-5-ene-3β,16α,17β-triol.

for pregnanediol or triol, and the Pincus reaction using antimony trichloride, after paper or thin-layer chromatographic (TLC) separation, etc.

In this paper, a new method for the fractional determination of 3β-hydroxysteroids using TLC and an enzyme that reacts specifically with some 3β-hydroxysteroids [7] is described, using urine samples from patients with adrenal tumor, Cushing's syndrome, and 21-hydroxylase deficiency, and from pregnant women.

MATERIALS

The TLC plates used in this study were Kieselgel 60 F245 (Merck), is heated at 110° for 30 min before use. Analytical-grade organic solvents were used; β-glucuronidase (bacterial powder from *E. coli*, EC 3.2.1.31) and all steroids were purchased from Sigma, St. Louis, Mo., U.S.A.

The enzyme reagent for color development of 3β-hydroxysteroids on thin-layer plates was prepared by dissolving 2 mg of 4-aminoantipyrine and 15 mg of phenol in 20 ml of 0.1 *M* phosphate buffer (pH 7.0) containing, per 20 ml, 10 U of 3β-hydroxysteroid oxidase from *B. sterolicum* (Kyowa Hakko Co., Machida-shi, Tokyo, Japan), 100 U of peroxidase (EC 1.11.1.7) and 0.02 ml of the surfactant Triton X-100.

METHODS

Preparation of sample

Pipette 10 ml of urine (in cases of nephrosis, wash with petroleum ether twice to remove cholesterol) into a 40 ml tube and adjust to pH 6.5 using bromthymol blue paper as indicator. Add 1 ml of β-glucuronidase (1,000,000 Fishman units/l), 1 ml of 0.5 *M* phosphate buffer (pH 6.5) and a few drops of chloroform to the tube and mix well. Incubate the mixture for 24 h at 37°, then adjust to pH 1 with 6 *M* HCl and saturate with 5 g of sodium chloride. Shake the solution with 20 ml of ethyl acetate for 5 min. After centrifugation, discard the urine layer and keep the ethyl acetate layer for another 24 h at 37° to achieve complete solvolysis of the sample. Wash the ethyl acetate layer successively with 2 ml of NaOH (80 g/l), with concd. Na_2CO_3, 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 a thin-layer plate with marker dye (sudan III and isatine) and standards. The plate is developed in the solvent mixture ethyl acetate—benzene (1:1, v/v) for 40—60 min at 20°, the distance of the front from the origin being 15—16 cm.

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

Place the thin-layer plate on a heater at 37° (or over a water-bath at 40°), and spray with enzyme reagent. Incubate for 30 min so that a pink-colored zone is visible. Quantitative densitometric scanning at 500 nm can also be per-

formed for high concentrations of 3β-hydroxysteroids in the sample. The instrument used for the assay was a dual-wavelength TLC scanner CS-910 (Shimadzu).

RESULTS

Specificity of 3β-hydroxysteroid oxidase

The specificity of 3β-hydroxysteroid oxidase was tested with a series of steroids in solution and on thin-layer plates, using 25 μg of steroids per tube. The data are shown in Table I. The R_F values of steroids on thin-layer plates are presented in Table II.

TABLE I

SPECIFICITY OF 3β-HYDROXYSTEROID OXIDASE

Compound	Specificity	
	In solution (%)	On TLC (%)
Dehydroepiandrosterone	100	100
Epiandrosterone	100	65
Androst-5-ene-3β,17β-diol	100	57
16α-Hydroxy-dehydroepi-androsterone	44	39
Pregnenolone	75	20
Cholesterol	65	<20
Androsta-5,16-dien-3β-ol	43	<20
5α-Androst-16-en-3β-ol	5	N.D.*
5β-Pregnane-3β,20α-diol	1	N.D.
3α-Hydroxysteroids	0	N.D.
Estradiol	0	N.D.
Estriol	0	N.D.

*N.D., Not detectable.

TABLE II

R_F VALUES OF STEROIDS ON THIN-LAYER PLATES

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

Compound	R_F
Cholesterol	0.49
Androsta-5,16-dien-3β-ol	0.45
Pregnenolone	0.39
Dehydroepiandrosterone	0.36
Epiandrosterone	0.35
5β-Pregnane-3β,20α-diol	0.30
Androst-5-en-3β,17β-diol	0.28
16α-Hydroxy-dehydroepi-androsterone	0.22
Androst-5-en-3β,16α,17β-triol	0.08
Marker dyes:	
Sudan III	0.63
Isatine	0.31

The excretion patterns of 3β-hydroxysteroids in a patient with adrenal tumor (sample 1), 21-hydroxylase deficiency in an adult (sample 2) and in a child (sample 3), in pregnancy (samples 4—6), Cushing's syndrome (samples 7 and 10), in a normal subject (sample 8) and after administration of ACTH (sample 9) are shown in Fig. 1. The densitometric scanning patterns of the thin-layer plates are shown in Fig. 2. The percentage of each steroid fraction is presented in terms of peak area, not corrected with reactivity of 3β-hydroxysteroid oxidase for each steroid, in Table III.

Precision

In five repeated assays on thin-layer plates using a standard solution containing 10 μg of DHEA, 10 μg of androst-5-en-3β,17β-diol and 10 μg of 16-α-

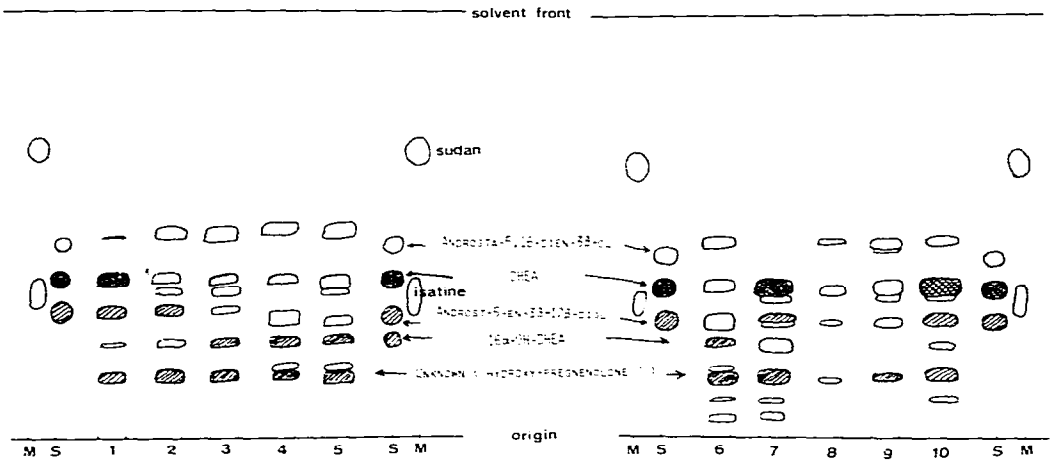

Fig. 1. TLC of 3β-hydroxysteroids. The numbered samples are from: 1, adrenal tumor; 2, 21-hydroxylase deficiency (22-year-old female); 3, 21-hydroxylase deficiency (6-year-old female); 4, pregnancy; 5, pregnancy; 6, pregnancy; 7, Cushing's syndrome; 8, a normal subject; 9, ACTH administration; 10, Cushing's syndrome. M is a marker dye of sudan III (R_F = 0.63) and isatine (R_F = 0.31); S is a mixture of standards of androsta-5,16-dien-3β-ol, dehydroepiandrosterone, and androst-5-en-3β,17β-diol.

TABLE III

PERCENTAGE OF EACH 3β-HYDROXYSTEROID FRACTION IN VARIOUS DISEASES

	% fraction				
	DHEA	A-diol	16-OH-DHEA	Unknown	A-triol
Adrenal tumor (Sample 1)	64.6	16.9	2.2	12.1	4.2
21-Hydroxylase deficiency					
Sample 2	12.3	14.0	11.4	49.7	13.0
Sample 3	15.5	3.2	24.7	48.2	8.3
Pregnancy (Sample 6)	16.2	12.8	10.7	30.3	29.9
Cushing's disease					
Sample 7	44.3	12.5	10.5	17.7	15.0
Sample 10	44.2	17.7	13.2	19.4	5.5

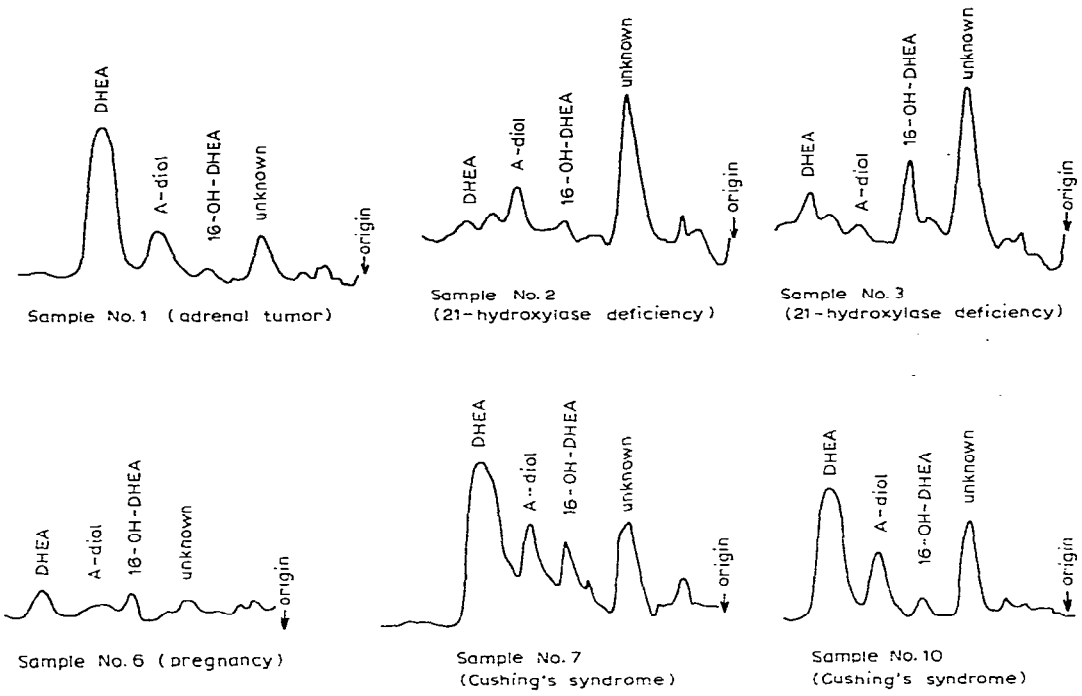

Fig. 2. Densitometric scanning pattern of patients 1, 2, 3, 6, 7 and 10 using a dual-wavelength TLC scanner CS-910 at 500 nm. Samples 2 and 3 are amplified × 2.

OH-DHEA, the percentages of each fraction (mean ± S.D.) obtained from peak areas were 50.75% ± 1.48 for DHEA, 29.00% ± 0.71 for androst-5-en-3β,17β-diol, and 20.25% ± 1.48 for 16α-OH-DHEA. The values for each fraction remained fairly constant up to 20 μg of each steroid per one TLC application.

In five repeated assays using a sample from a patient with Cushing's syndrome, the CV was 4.2% for the DHEA fraction and 10.3% for 16α-OH-DHEA.

DISCUSSION

16-Hydroxylation, which is said to inactivate of biologically active steroids, can be seen for DHEA in all samples, especially in a high percentage in pregnancy and Cushing's syndrome. Although 16α-OH-DHEA is a 17-ketosteroid, the Zimmermann chromogen was not obtained with *m*-dinitrobenzene because there is no active methylene group at position 16.

A moderate amount of an unknown 3β-hydroxysteroid with an R_F of 0.14 was observed in all samples. This unknown steroid seems to be a hydroxypregnenolone derivative (such as pregn-5-ene-3β,17α,20α-triol identified by Hirschmann and Hirschmann [8]) on the basis of the mass number of 334 obtained by mass spectrometry.

The excretion of 3β-hydroxysteroids in the last stage of pregnancy was 2—6 mg/day; most of them were more polar 3β-hydroxysteroids than DHEA itself, as shown in Fig. 3 and Table III.

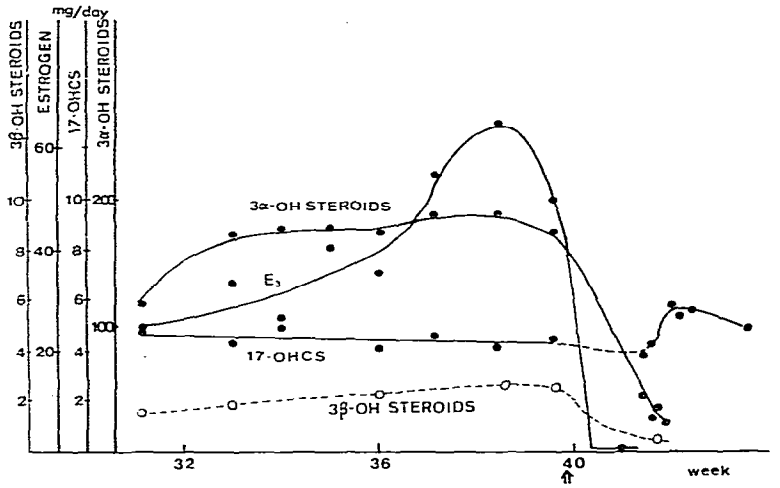

Fig. 3. Excretion of 3β-hydroxysteroids in pregnancy. 3β-Hydroxysteroids were determined by the method described in ref. 7. 17-OHCS was determined by the Porter—Silber reaction after hydrolysis using β-glucuronidase. Estrogen was determined by the Kober—Ittrich method. 3α-Hydroxysteroids were determined after hydrolysis with β-glucuronidase using "Sterognost-3α" (purchases from Nyegaard and Co.).

In this paper, the fractional determination of 3β-hydroxysteroids is shown to be useful for the diagnosis of some adrenal diseases.

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