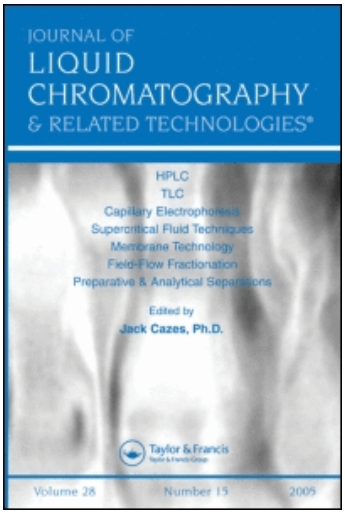

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Enzymatic Detection of Urinary Steroids on Thin-Layer Chromatograms

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ENZYMATIC DETECTION OF URINARY STEROIDS ON THIN-LAYER CHROMATOGRAMS

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ABSTRACT

Enzymatic detection of urinary steroids on thin-layer chromatograms (TLC) is described. Steroids in urine such as neutral 3α-hydroxysteroids, 3β-hydroxy steroids, acidic 3α-hydroxysteroids and 17β-hydroxy-steroids are detected on thin-layer plate using enzyme reactions.

Combination of chromatographic techniques and enzyme reactions give more specific analysis of the compounds in a body fluid. Some clinical application by the use of these enzymatic methods is also described and these enzymatic detection methods are shown to be useful for the diagnosis of some disease of abnormal steroid metabolism.

INTRODUCTION

The enzymatic determination of steroids such as neutral 3α-hydroxysteroids (1,2), 3β-hydroxysteroids (3,4,5), acidic 3α-hydroxysteroids(6,7), and 17β-hydroxysteroids (8,9) has been reported. Advantages of TLC and enzyme reaction must be considered to give more specific detection and determination, in this paper

some clinical application using enzymatic detection of urinary steroids on TLC is described.

The principle of the method is as follows:

$$\text{Substrate} \xrightarrow{\text{enzyme}} \text{Product}$$

$$\text{Product} \xrightarrow{\text{catalyzer}} \text{formation of dye}$$

Hydroxysteroid dehydrogenase reactions (HSD)

$$\text{I.}\quad 3\alpha\text{-hydroxysteroids} + NAD^{+} \xrightarrow{3\alpha\text{-HSD}} \text{3-oxosterois} + NADH$$

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

$$\text{III.}\quad NADH + INT \xrightarrow{\text{diaphorase}} \text{formazan}$$

3β-Hydroxysteroid oxidase reaction

$$\text{IV.}\quad 3\beta\text{-hydroxysteroids} \xrightarrow{3\beta\text{-hydroxysteroid oxidase}} \text{3-oxosteroids} + H_2O_2$$

$$\text{V.}\quad H_2O_2 + \text{phenol} + \text{4-aminoantipyrine} \xrightarrow{\text{peroxidase}} \text{quinoneimine dye}$$

EXPERIMENTAL

All reagents used were of analytical grade, and preparation of the enzyme color development reagent used has been described peviously (1-9).

Preparation of samples

Urine is pipetted into a tube and hydrolysis with β-glucuronidase and solvolysis are performed. The ethyl acetate layer is then washed successively with concentrated sodium carbonate and water. After centrifugal separation, the ethyl acetate extract is transferred to a tube and the ethyl acetate evaporated (neutral fraction). This fraction is used for the assay of neutral 3α, 3β, and 17β-hydroxysteroids.

The alkali and water washes of the ethyl acetate extract are combined and the combined solution is acidified to pH 1 and sodium chloride added. Shake the solution with ethyl acetate for 5 min. After centrifuging, transfer 15 ml of the extract to a tube. Evaporate the ethyl acetate (acidic fraction). This fraction is used for the assay of acidic steroids.

RESULTS AND DISCUSSION

Effects of the enzyme specificity for an assay on color intensity.

Problems for quantitative determination on TLC arise form its enzyme specificity. For example, a relation of color intensity and reaction time using 3β-hydroxysteroid oxidase reaction in solution is shown in FIGURE 1. On thin-layer plate, color intensity of each steroids depend on solubility of steroid and the function of reaction time.

Other enzyme reactivity for each steroids is also different as being described previously (1-9).

Application of enzymatic detection method for clinical diagnosis.

Excretion patterns of 3β-hydroxysteroids with normal subjects, patients of some adremogenital syndrome (AGS) and pregnant woman were compared. (FIGURE 2)

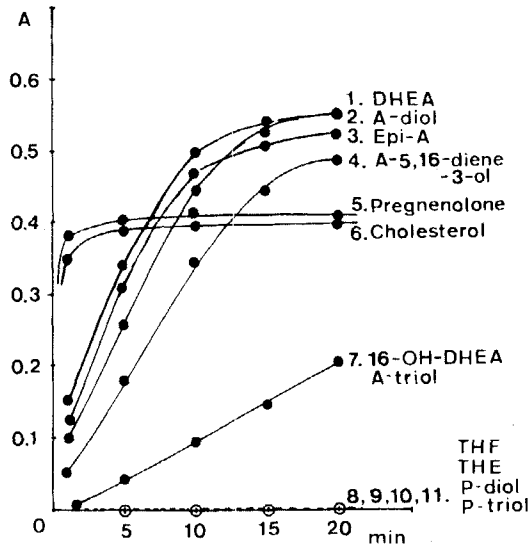

FIGURE 1. Specificity of 3β-hydroxysteroid oxidase.

Each steroids of 50 μg per tube was color-developped and its color intensity was measured.

Abbreviations used: DHEA, dehydroepiandrosterone; A-diol, androstene-3β,17β-diol ; epi-A, epiandrosterone; A-5,16-diene-3-ol, androsta-5,16-diene-3β-ol ; 16-OH-DHEA, 16α-hydroxy dehydroepiandrosterone ; A-triol , androstene-3β,16α,17β-triol; THF, tetrahydrocortisol; THE, tetrahydrocortisone; P-diol , pregnanediol; P-triol , pregnanetriol.

Effect of Su-4885,which is an inhibitor of 11-hydroxy lase of steroids,on excretion of 3β-hydroxysteroids was shown in FIGURE 3. Excretion of 5-pregnenetriol was remarkably increased when compared with before of the test.

Enzymatic detection of acidic 3α-hydroxysteroids with patient of liver function disorder was shown in FIGURE 4 and as a comparison of another steroid excretion, neutral 3α-hydroxysteroids excretion pattern of the

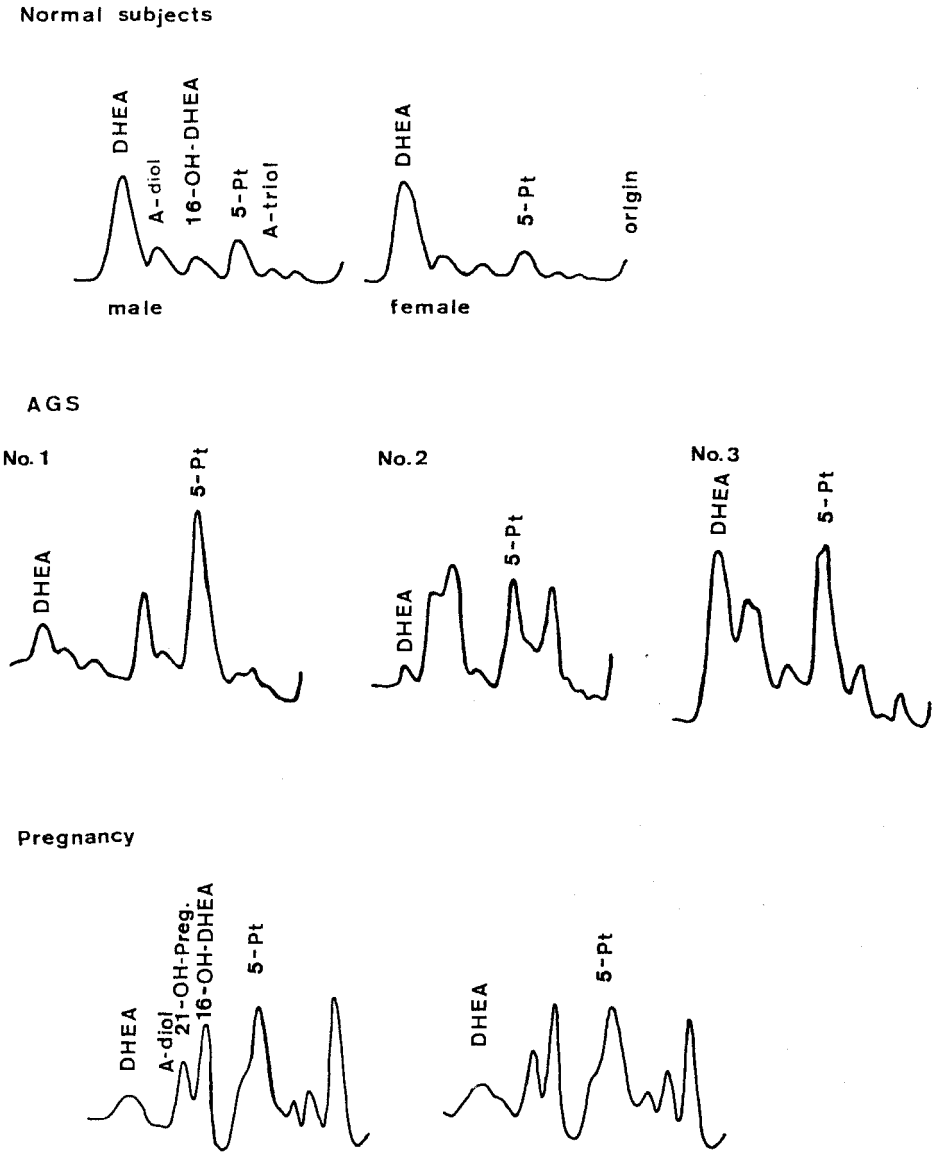

FIGURE 2. Excretion patterns of urinary 3β-hydroxysteroids with normal subjects, patients of adrenogenital syndrome (AGS), and pregnant women . TLC scanner used was Shimazu-CS-910.

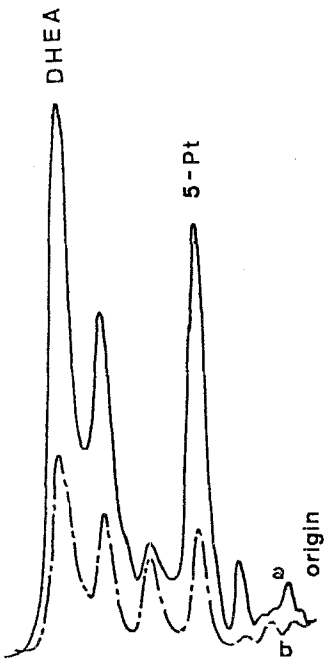

FIGURE 3. Effects of Su-4885 on excretion of 3β-hydroxy steroids with patient of Cushing disease.
(a) after administration of Su-4885 (2nd day)
(b) before administration
Densitometric scanning was performed at 500 nm.

patient was also shown in the figure. Excretion values of total acidic steroids determined by 3α,3β-hydroxy-steroid dehydrogenase was 6.5 mg/day for normal subjects (mean of ten subjects) , and 28.2 mg/day for some liver function disorder patients (mean of 7 patients).

Excretion patterns of neutral 3α-hydroxysteroids, 3β-hydroxysteroids, acidic 3α-hydroxysteroids and 17β-hydroxysteroids using enzymatic detection has been shown by authers (1-9), but these are the model of enzymatic detection method in biological fluids and

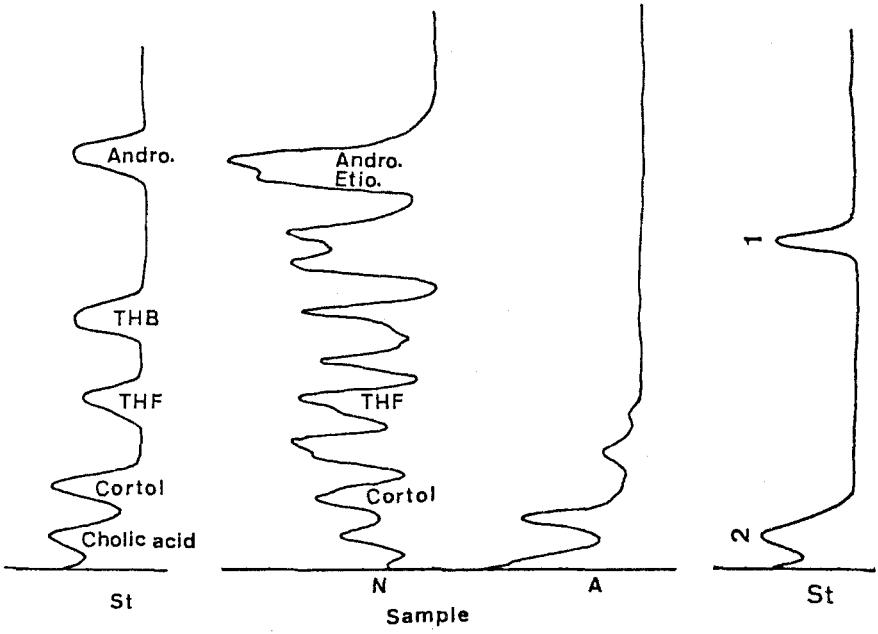

FIGURE 4. Enzymatic detection of acidic 3α-hydroxy-steroids on TLC.

Chromatograms show standards (Androsterone, THB; tetra hydrocorticosterone, THF, cortol, cholic acid), neutral 3α-hydroxysteroids of the sample, acidic 3α-hydroxy-steroids of the sample and then acidic steroid standard, 1; 5β-androstan-3α-ol-17β-carboxylic acid, 2; cholic acid.

many other compounds could be detected by other enzymic methods.

An application of enzymic method to the column chromatography was also performed (10) for the detection of steroid sulfatase activity in enzyme preparation.

Thus the use of the specific enzyme for detection of its substrate gives simple and specific method.

AKNOWLEGMENT

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