Journal of Chromatography, 224 (1981) 111—115
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 850

Note

Enzymatic detection of urinary acidic 3α-hydroxysteroids on thin-layer chromatograms

YOSHIHISA YAMAGUCHI*, CHOZO HAYASHI and KIYOSHI MIYAI

The Central Laboratory for Clinical Investigation, Osaka University Hospital, Fukushima-ku, Osaka (Japan)

(First received December 31st, 1980; revised manuscript received January 27th, 1981)

It is well known that an abnormal metabolism of bile acids occurs in some liver diseases and a number of papers have been published so far [1—3]. Recently, carboxylic acid metabolites of steroids have also been reported by Bradlow and co-workers [4—6], and some aspects of these metabolites have been described. Four carboxylic acid metabolites of cortisol in man have been isolated and identified using [4-^{14}C]cortisol and an Amberlite XAD-2 column.

In this paper the enzymatic detection of these steroids carrying 3α-hydroxy and carboxylic acid groups is described. The principle of the method used [7] is as follows:

$$\text{Acidic 3}\alpha\text{-hydroxysteroid} + \text{NAD}^+ \xrightarrow{3\alpha\text{-HSD}} \text{acidic 3-ketosteroid} + \text{NADH}$$

$$\text{NADH} + \text{2-}p\text{-iodophenyl-3-}p\text{-nitrophenyl-5-phenyltetrazolium chloride} \xrightarrow{\text{diaphorase}} \text{formazan (pink color dye)}$$

MATERIALS AND METHODS

All reagents used were of analytical grade, and 3α-hydroxysteroid dehydrogenase (3α-HSD) and β-NAD were purchased from Nyegaard (Oslo, Norway).

Preparation of the enzyme color development reagent used has been described previously [7]. Briefly, dissolve 6 mg of 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride in 10 ml of 0.2 *M* K_2PO_4 (pH 8.5) containing (per 10 ml) 1 U of 3α-HSD, 50 U of diaphorase and 5 μmol of β-NAD.

0378-4346/81/0000—0000/$02.50

Preparation of acidic and neutral fractions

Urine (15 ml) is pipetted into a 40-ml tube and hydrolysis with β-glucuronidase and solvolysis are performed [7]. The ethyl acetate layer is then washed successively with 3 ml of concentrated sodium carbonate twice and water. After centrifugal separation, the ethyl acetate extract is transferred to a tube and the ethyl acetate evaporated (neutral fraction).

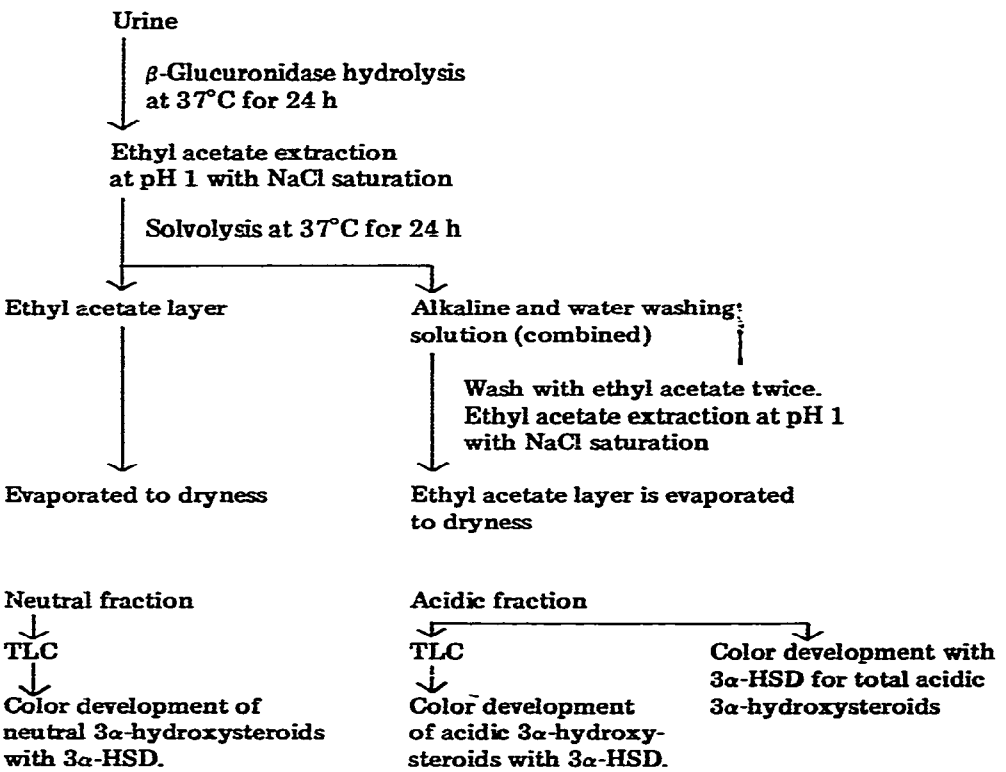
The alkali and water washes of the ethyl acetate extract are combined and the combined solution is acidified to pH 1 and sodium chloride added. Shake the solution with 20 ml of ethyl acetate for 5 min. After centrifuging, transfer 15 ml of the extract to a tube. Evaporate the ethyl acetate (acidic fraction).

Thin-layer chromatography

To the dry residue, a few drops of a chloroform and methanol mixture (1:1, v/v) are added, and the sample is applied to an activated thin-layer plate with marker dye and standards. The thin-layer plate (Kieselgel 60) is developed with a solution of chloroform—methanol (85:15, v/v) for 60 min at 20°C, to a distance of the front from the starting point of about 16 cm.

Color development of neutral 3α-hydroxysteroids and acidic 3α-hydroxysteroids was performed by the method described previously [7].

An outline of the method is shown in Scheme 1.



Scheme 1.

RESULTS

The selectivity of 3α-HSD for steroid molecules is shown in Table I, for which the intensity of the reaction with some steroids has been described in a previous paper [7].

TABLE I

SELECTIVITY OF 3α-HSD FOR SOME STEROIDS AND CHOLIC ACID

Each compound, at 0.1 μmol per tube, was incubated with 2 ml of enzyme solution at 37°C for 20 min.

Compound	Intensity of reaction (absorbance at 500 nm)
Cholic acid	0.555
Etiocholanolone	0.650
Androsterone	0.610
Tetrahydrocortisol Tetrahydrocorticosterone	0.717

Absorption curves of the dye formed by the enzymatic reaction with acidic fractions obtained from various urine samples are shown in Fig. 1.

The excretion pattern of acidic 3α-hydroxysteroids in a sample from a patient with adrenal disease (Cushing's disease) is shown in Fig. 2 together with the excretion pattern of neutral 3α-hydroxysteroids.

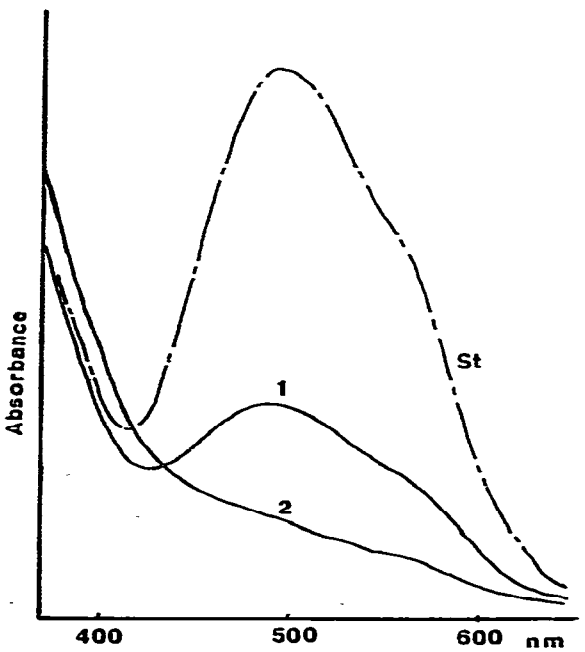


Fig. 1. Absorption curves of dye formed by reaction with the acidic fraction from urine. St = standard; 1 = Cushing's disease; 2 = normal subject.

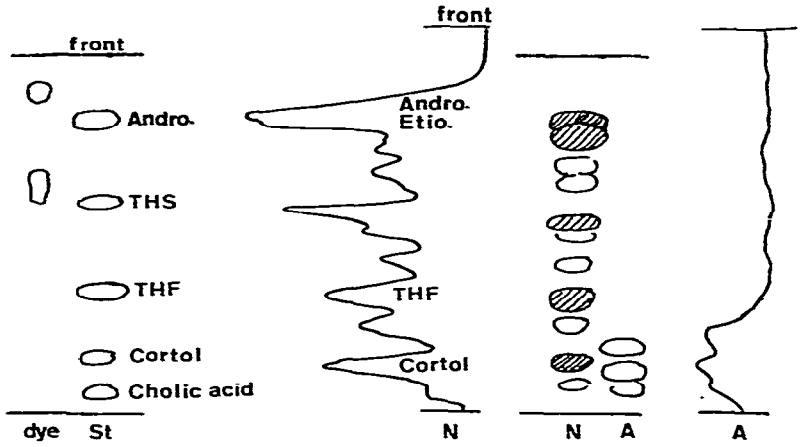


Fig. 2. Detection of acidic 3α-hydroxysteroids and neutral 3α-hydroxysteroids on thin-layer plates using a sample from a patient with adrenal disease. N = neutral fraction; A = acidic fraction.

For determination of total acidic 3α-hydroxysteroids, urine samples from cases of some adrenal and liver diseases and normal subjects (15 cases) were tested. The values obtained were 12.8 mg/day for Cushing's disease, 26.3 and 7.5 mg/day for hepatoma, 12.5 mg/day for some liver function disorder, and not detectable to 4.5 mg/day (N.D., below 0.5 mg/day) in 15 normal subjects. Assay of total acidic 3α-hydroxysteroids was performed by adding 2 ml of enzyme solution for color development to tubes containing the dry residues of the acidic fractions and incubating at 37°C for 30 min. Absorbance was read at 450, 500, and 550 nm and amounts calculated using Allen's formula.

The standard curve for acidic 3α-hydroxysteroids, obtained by submitting cholic acid to the whole procedure was linear for concentrations of cholic acid up to 200 μg.

DISCUSSION

Simple detection and determination of acidic 3α-hydroxysteroids in urine have been demonstrated using a sample from a patient with Cushing's disease. Acidic 3α-hydroxysteroids showed no elevated values in normal subjects by this method. This result seems to be reasonable because increased excretion of acidic steroids has not been reported [6].

This method can be used for detecting urinary bile acids, which are found in some liver diseases, as shown in Fig. 3. Many unidentified steroids and bile acids can easily be seen in an increased amount on chromatograms of neutral and acidic fractions. Use of a different solvent system for development of the thin-layer plates may give more details for diagnosing some metabolic abnormalities.

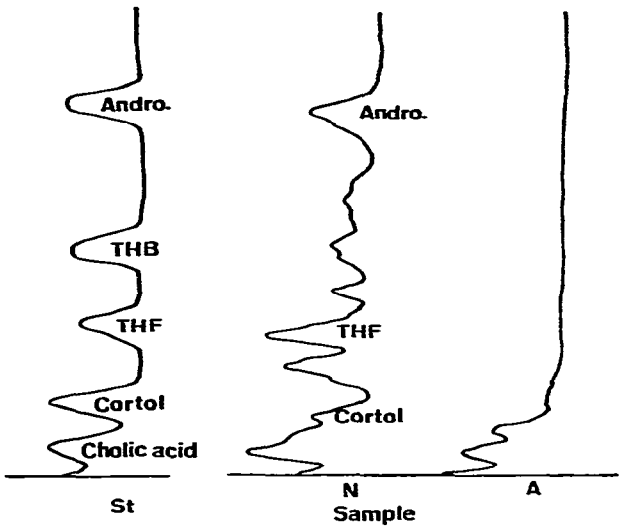


Fig. 3. Chromatograms of neutral and acidic fractions from a patient with a liver function disorder. N = neutral fraction; A = acidic fraction (may contain bile acids); St = standard.

REFERENCES

1 I. Makino, J. Sjövall, A. Norman and B. Strandvik, FEBS Lett., 15 (1971) 161.
2 I. Makino, K. Shinozuki and S. Nakagawa, Lipids, 8 (1973) 47.
3 B. Almé, A. Brennelgaard, J. Sjövall and P. Thomassen, J. Lipid Res., 18 (1977) 339.
4 H.L. Bradlow, B. Zumoff, C. Monder, H.J. Lee and L. Hellman, J. Clin. Endocrinol. Metab., 37 (1973) 811.
5 C. Monder, H.L. Bradlow and B. Zumoff, J. Clin. Endocrinol. Metab., 51 (1980) 312.
6 C.H.L. Shackleton, E. Roitman, C. Monder and H.L. Bradlow, Steroids, 36 (1980) 289.
7 Y. Yamaguchi, C. Hayashi and K. Miyai, J. Chromatogr., 182 (1980) 430.