Journal of Chromatography, 230 (1982) 263—270
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1280

ENZYMATIC DETECTION OF URINARY CONJUGATED STEROIDS AFTER GEL CHROMATOGRAPHY

YOSHIHISA YAMAGUCHI*,* and CHOZO HAYASHI

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

(First received December 1st, 1981; revised manuscript received February 23rd, 1982)

SUMMARY

An enzymatic detection method is described for urinary conjugated steroids after chromatographic fractionation with Sephadex G-25. The principle of the method is as follows. Part of a 24-h urine sample, (1—2 ml of urine) is applied directly, to a short column of Sephadex G-25 and eluted with acetate buffer solution. Steroid conjugates in each fraction are hydrolyzed with steroid sulfatase—β-glucuronidase. After enzymatic hydrolysis, an enzymatic color development reagent for steroids, either 3α-hydroxysteroid dehydrogenase or 3β-hydroxysteroid oxidase, are added and the dye formed is measured spectrophotometrically. Excretion patterns of steroid-3β-sulfates, and steroid-3α-glucuronides and steroid-3α-sulfates are shown with some patients' samples. A precision of the assay values for steroid-3α-glucuronide, steroid-3α-sulfate and steroid-3β-sulfates in urine samples and assay values for normal subjects are also studied.

This simple enzymatic method for detecting the excretion patterns of urinary conjugated steroids may have a diagnostic value for clinical tests.

INTRODUCTION

Urinary steroids have been determined by colorimetric methods such as the Porter—Silber reaction and the Zimmerman reaction after hydrolysis of steroid conjugates and extraction with organic solvent [1,2].

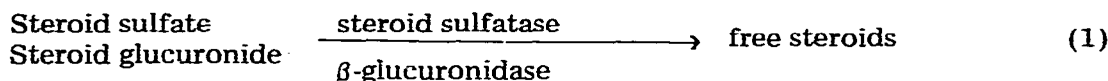
Enzymatic detection of steroids such as neutral 3α-hydroxysteroids [3,4], 3β-hydroxysteroids [5,6], acidic 3α-hydroxysteroids [7], and 17β-hydroxysteroids [8,9] has been reported previously.

Amberlite XAD-2 and Sephadex G-25 resins have been used for extraction of conjugated steroids instead of organic solvent [10,11], and Sephadex LH-20 and DEAE-Sephadex have been used for fractionation of free steroids and conjugated steroids [12,13].

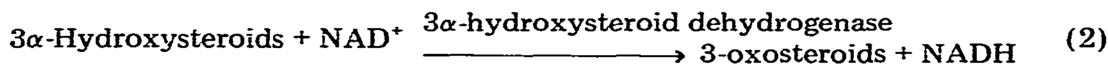
*Present address: College of Bio-Medical Technology, Osaka University, 1-1 Machikaneyamacho, Toyonaka, Osaka 560, Japan.

0378-4347/82/0000—0000/$02.75 © 1982 Elsevier Scientific Publishing Company

In this paper, direct enzymatic detection of urinary conjugated steroids such as steroid-3β-sulfate, steroid-3α-glucuronide and steroid-3α-sulfate is described. The principle of the reactions is as follows:

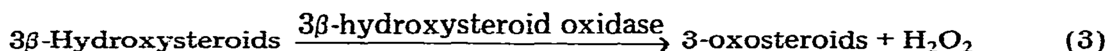


$$\text{Steroid sulfate, Steroid glucuronide} \xrightarrow[\beta\text{-glucuronidase}]{\text{steroid sulfatase}} \text{free steroids} \quad (1)$$



$$3\alpha\text{-Hydroxysteroids} + NAD^+ \xrightarrow{3\alpha\text{-hydroxysteroid dehydrogenase}} \text{3-oxosteroids} + NADH \quad (2)$$

$$NADH + INT \xrightarrow{\text{diaphorase}} \text{formazan}$$



$$3\beta\text{-Hydroxysteroids} \xrightarrow{3\beta\text{-hydroxysteroid oxidase}} \text{3-oxosteroids} + H_2O_2 \quad (3)$$

$$H_2O_2 + \text{4-Aminoantipyrine} + EMAE \xrightarrow{\text{peroxidase}} \text{quinoneimine dye}$$

MATERIALS AND METHODS

All the reagents used were of analytical grade, and together with β-glucuronidase from *Escherichia coli* (EC 3.2.1.31) and bovine liver, and sulfatase—β-glucuronidase (EC 3.1.6.1 and EC 3.2.1.31) from *Helix pomatia*, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

All standards of steroids and conjugated steroids, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride (INT) and diaphorase (EC 1.6.99.2, from *Clostridium kluyveri*) were also purchased from Sigma. 3α-Hydroxysteroid dehydrogenase (3α-HSD) from *Pseudomonas testosteroni* (EC 1.1.1. 50) and β-NAD^+ were purchased from Nyegaard (Oslo, Norway). 3β-Hydroxysteroid oxidase from *Brevibacterium sterolicum* (EC 1.1.3.6), peroxidase from horseradish (EC 1.11.1.7), 4-aminoantipyrine (4-AA) and N-ethyl-N-(3-methylphenyl)-N'-acetylethylenediamine (EMAE) were purchased from Kyowa Medics Co., Tokyo, Japan.

Sephadex G-25 fine and the column were purchased from Pharmacia (Uppsala, Sweden).

Preparation of reagents

Sulfatase—β-glucuronidase (200 units of sulfatase and 3000 Fishman units of β-glucuronidase per 1 ml of 0.05 *M* acetate buffer, pH 5.0) from *H. pomatia* (type H-1), β-glucuronidase (500 Fishman units per 1 ml of 0.1 *M* phosphate buffer, pH 6.8) from *E. coli*, and β-glucuronidase (1500 Fishman units per 1 ml of 0.05 *M* acetate buffer, pH 5.0) from bovine liver (type B-3) were prepared. All these enzymes were used without addition of activator for a step in the hydrolysis of steroid conjugates.

For the preparation of enzyme reagent for color development of 3α-hydroxysteroids [3,4] dissolve 60 mg of INT in 100 ml of 0.2 *M* K_2HPO_4 (pH 9.0) containing per 100 ml: 10 U of 3α-HSD, 500 U of diaphorase and 50 μmol of

β-NAD$^+$. The enzyme reagent for color development of 3β-hydroxysteroids was as described previously [5,6]. Determiner FC '555' was purchased and used for color development of 3β-hydroxysteroids.

Preparation of urine sample

A 24-h urine specimen is collected and an aliquot is centrifuged for 3 min at 2500 *g*. A volume of 1—2 ml of the supernatant is directly applied to the Sephadex G-25 column (1 × 24 to 29 cm).

Sephadex gel chromatography

Sephadex G-25 is swollen by heating a suspension of the particles in 0.05 *M* acetate buffer (pH 5.0) for 4 h at 90°C under constant stirring. The fines are removed by several decantations and the slurry is poured directly into the column which is then washed for 3 h with acetate buffer solution.

After application of the urine sample, chromatographic separation is performed with 0.05 *M* acetate buffer (pH 5.0). One fraction of effluent contains 1.3 ml; ten fractions are run within 30 min and 35 fractions are collected.

Procedure for detection of steroid-3α-glucuronide and steroid-3α-sulfate

To 0.4 ml of each chromatographic effluent is added 0.1 ml of sulfatase—β-glucuronidase solution and incubated for 20 h at 37°C. After hydrolysis of steroid conjugates, 1 ml of color development reagent for 3α-hydroxysteroids is added and incubated for 30 min at 37°C. Absorbance at 500 nm is read against the first effluent fraction.

Procedure for determination of steroid-3β-sulfate

To 0.6 ml of each effluent fraction is added 0.1 ml of sulfatase-β-glucuronidase solution and incubated for 20 h at 37°C. After hydrolysis, 1 ml of color development reagent for 3β-hydroxysteroids is added and incubated for 20 min at 37°C. Absorbance at 550 nm is read against the first effluent fraction.

RESULTS

Gel chromatography of conjugated steroids

One millilitre of dehydroepiandrosterone-sulfate (DHEA-S), androsterone-glucuronide (A-G), estrone-sulfate (E_1-S) and estriol-16α-glucuronide (E_3-G) solution (100—500 μg/ml) was applied to the column. DHEA-S and A-G were detected by the present methods, and E_1-S and E_3-G were detected spectrophotometrically at 260 nm. Their chromatogram is shown in Fig. 1.

Sulfuric ion in samples on chromatogram

Sulfuric ion in urine, which is an inhibitor of sulfatase, was detected with barium chloride solution; the elution position of sulfuric ion is shown in Fig. 2. The sample used is from a patient with adrenal virilizing tumor.

Effect of incubation time with sulfatase on hydrolysis rate

After the addition of sulfatase solution, the hydrolysis rates at 2 h and 20 h were determined and found to be 59% and 86%, respectively.

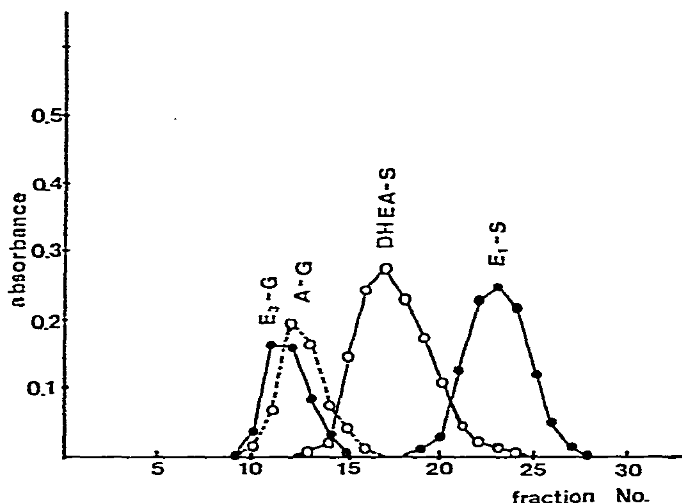


Fig. 1. Gel chromatography of steroid conjugates of standard. DHEA-S = dehydroepiandrosterone-sulfate; A-G = androsterone-3-glucuronide; E_1-S = estrone-3-sulfate; E_3-G = estriol-16-glucuronide. Color development of DHEA-S and A-G was performed as described in Methods; E_1-S and E_3-G were detected spectrophotometrically at 260 nm.

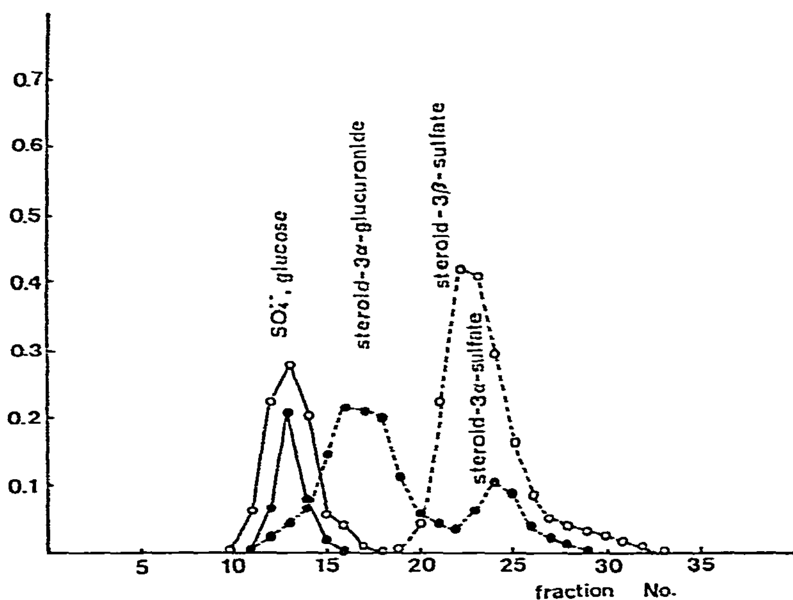


Fig. 2. Elution position of sulfuric ion in urine sample. (○) sulfuric ion; (●) glucose (detected by the glucose oxidase method). Column length was 29 cm. The sample used to detect the elution position on the chromatogram is from a patient with adrenal virilizing tumor.

Gel chromatography of urine samples for detection of steroid-3α-glucuronide and steroid-3α-sulfate

β-Glucuronidase from *E. coli*, bovine liver and *H. pomatia* were used for comparing hydrolysis of urinary steroid conjugates; the results are shown in Fig. 3.

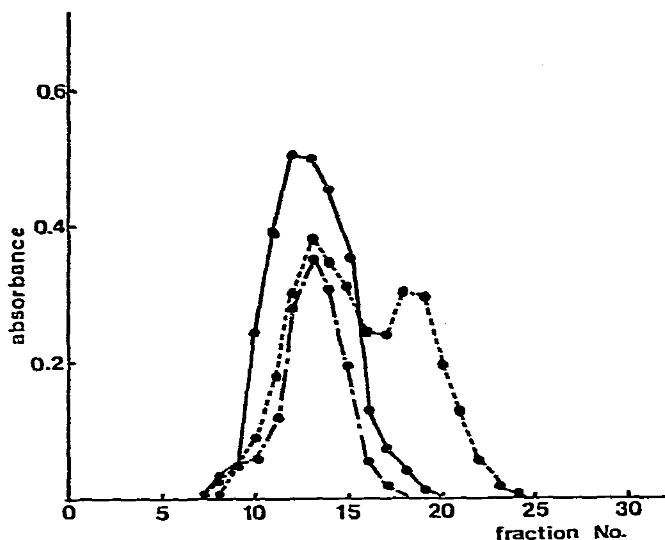


Fig. 3. Color development of 3α-hydroxysteroids after hydrolysis with β-glucuronidase from *E. coli* (● —●), bovine liver (●— · —●), and *H. pomatia* (● — — — ●). Sample used for analysis is from a patient with adrenogenital syndrome. After hydrolysis with various β-glucuronidases, steroids were color-developed with 3α-hydroxysteroid dehydrogenase as described in Methods.

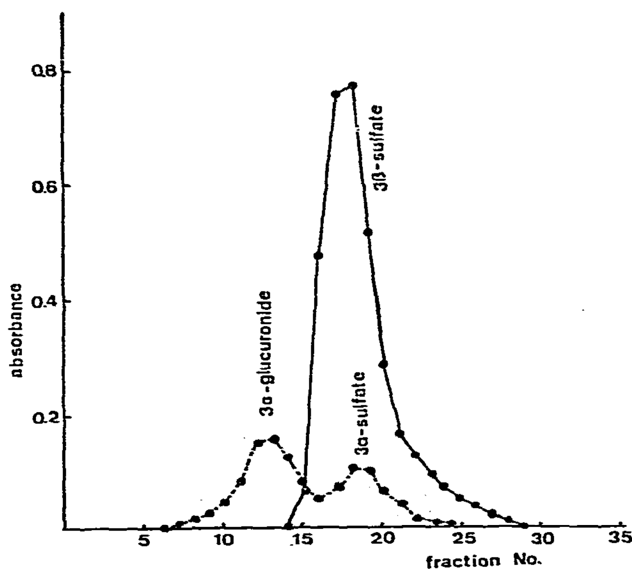


Fig. 4. Excretion patterns of steroid-3α-glucuronide, steroid-3α-sulfate and steroid-3β-sulfate in a urine sample from a patient with adrenal tumor. (● — — — ●) 3α-hydroxysteroids; (●———●) 3β-hydroxysteroids. Hydrolysis of steroid conjugates was performed with sulfatase—β-glucuronidase from *H. pomatia*; enzymic color development was performed by the method described in Methods.

Precision of the method

The coefficient of variation (C.V.) of day-to-day assay using a sample of a patient with Cushing's disease was 6.9% (mean 25.5 ± 1.8 mg/day, duplicate assay for three days) for steroid-3α-glucuronides, 7.2% (mean 23.7 ± 1.5 mg/day) for steroid-3α-sulfates, and 20.1% (mean 4.2 ± 0.8 mg/day) for steroid-3β-sulfates. Values of the individual steroid groups are calculated from standard curves of androsterone-glucuronide for steroid-3α-glucuronides, androsterone-sulfate for steroid-3α-sulfate, and dehydroepiandrosterone-sulfate for steroid-3β-sulfates. All values were obtained by submitting these standard compounds to the whole procedure and the sum of the measured absorbance of each fraction was used for calculation of the sample values.

Normal values of urinary steroid conjugates

Values determined for five normal subjects were 13.8 ± 6.2 mg/day for steroid-3α-glucuronides, and 5.7 ± 2.1 mg/day for steroid-3α-sulfates; steroid-3β-sulfates were not detectable.

Excretion pattern of steroid conjugates in some patients

The excretion patterns of urinary steroid conjugates in patients with adrenal tumor and Cushing's disease are shown in Figs. 4 and 5, respectively.

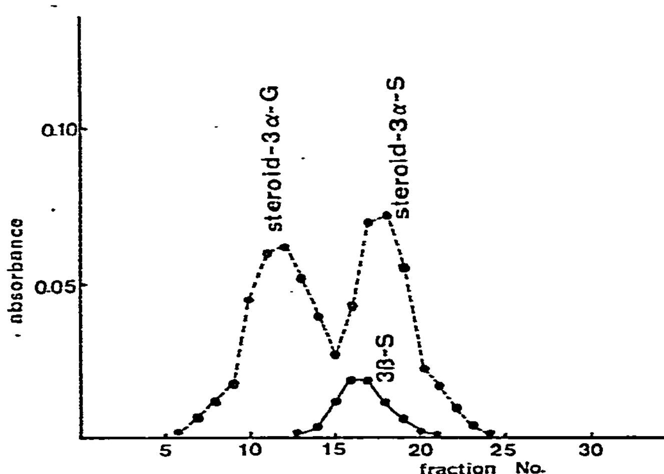


Fig. 5. Excretion patterns of steroid-3α-glucuronide, steroid-3α-sulfate and steroid-3β-sulfate in a urine sample from a patient with Cushing's disease. (• – – – •) 3α-hydroxysteroids; (•——•) 3β-hydroxysteroids. Hydrolysis of steroid conjugates was performed with sulfatase—β-glucuronidase from *H. pomatia*; enzymic color development was performed by the method described in Methods.

DISCUSSION

The hydrolysis enzyme β-glucuronidase from various sources has a well-known specifity for substrate and some inhibitors or activators [14,15], but in the present method chromatography on Sephadex G-25 was used to eliminate the influence of inhibitor. A selection of suitable enzymes for hydrolysis is still an important factor (Fig. 3).

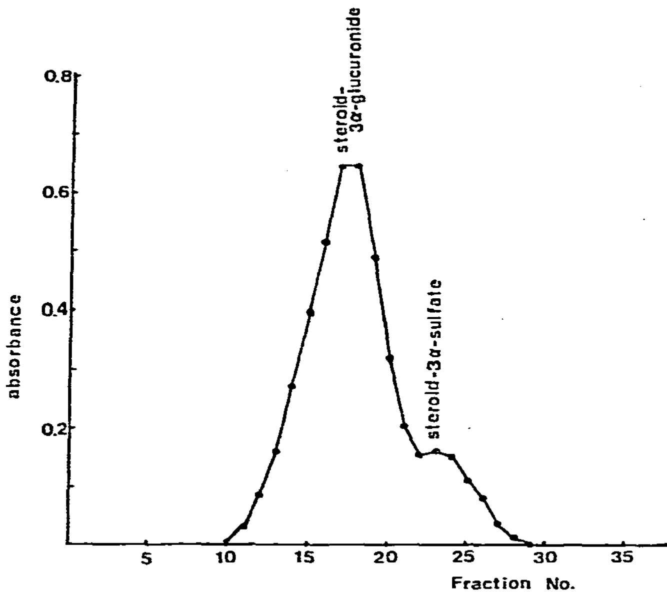


Fig. 6. Excretion pattern of steroid-3α-glucuronide and steroid-3α-sulfate in a urine sample from a patient with adrenogenital syndrome caused by enzyme deficiency.

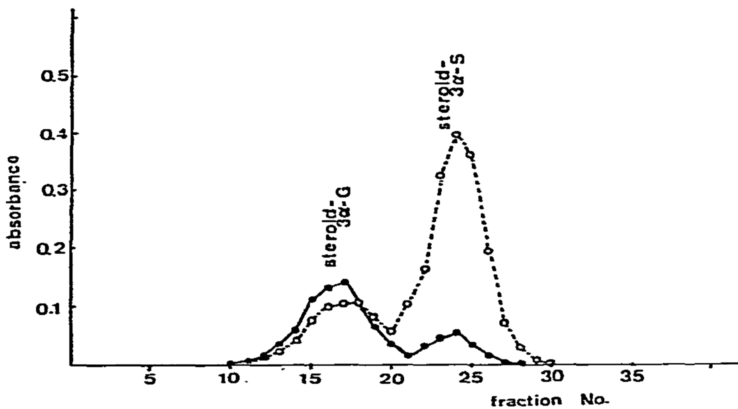


Fig. 7. Excretion patterns of steroid-3α-glucuronide and steroid-3α-sulfate in a urine sample from a patient with acute hepatitis (o) and from a normal subject (•).

The sample volume which can be applied to the column is small, so a high excretion of steroid conjugates is required as in the case of adrenogenital syndrome, Cushing's disease and some other forms of abnormal steroid metabolism.

In application to clinical diagnosis, significant differences between normal subjects and pathological urine were observed; for example, elevated steroid-3β-sulfate in patients with adrenal virilizing tumor (Fig. 4), elevated excretion of steroid-3α-glucuronide in patients with adrenogenital syndrome caused by enzyme deficiency (Figs. 3 and 6), and elevated steroid-3α-sulfate in patients with acute hepatitis (Fig. 7). From these observations, we conclude that this simple enzymatic method for detecting the excretion patterns of steroid conjugates may have a significance for diagnosis of steroid abnormal metabolism.

REFERENCES

1 C.C. Porter and R.H. Silber, J. Biol. Chem., 185 (1950) 201.
2 W. Zimmermann, Z. Physiol. Chem., 233 (1935) 257.
3 Y. Yamaguchi, Clin. Chem., 26 (1980) 491.
4 Y. Yamaguchi, C. Hayashi and K. Miyai, J. Chromatogr., 182 (1980) 430.
5 Y. Yamaguchi, K. Tateishi and C. Hayashi, Clin. Chim. Acta, 66 (1976) 449.
6 Y. Yamaguchi, J. Chromatogr., 163 (1979) 253.
7 Y. Yamaguchi, C. Hayashi and K. Miyai, Clin. Chem., 27 (1981) 777.
8 Y. Yamaguchi and C. Hayashi, Anal. Lett., 14 (1981) 433.
9 Y. Yamaguchi, J. Chromatogr., 228 (1982) 317.
10 D. Gupta and J. Goodwin, Steroids, 8 (1966) 195.
11 C.G. Beling, Acta Endocrinol. Suppl., 79 (1963) 58.
12 P.A. Drewes and A.J. Kowalski, Clin. Chem., 20 (1974) 1451.
13 R. Hobkirk, P.I. Musey and M. Nilsen, Steroids, 14 (1969) 191.
14 V. Graef and M. Fuchs, Z. Clin. Chem., 13 (1975) 163.
15 V. Graef, E. Furuya and O. Nishikaze, Clin. Chem., 23 (1977) 532.