Journal of Chromatography, 273 (1983) 87—94
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1535

SIMPLE ENZYMATIC DETECTION METHOD FOR URINARY SULFATED 7α-HYDROXY BILE ACIDS IN NORMAL SUBJECTS AND IN PATIENTS WITH ACUTE HEPATITIS

YOSHIHISA YAMAGUCHI*, TOKUICHIRO SEKI and SHINICHIRO WATANABE

College of Bio-Medical Technology, Osaka University, 1-1 Machikaneyamacho, Toyonaka, Osaka (Japan)

and

CHOZO HAYASHI and KIYOSHI MIYAI

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

SUMMARY

Urinary sulfated primary bile acids, 7α-hydroxy bile acids, are detected by an enzymatic method using 7α-hydroxysteroid dehydrogenase (EC 1.1.1.-, 7α-HSD) after chromatographic fractionation on Sephadex G-25.

Urinary sulfated or glucuronated bile acids are hydrolyzed by β-glucuronidase/sulfatase (EC 3.2.1.31/EC 3.1.6.1) from *Helix pomatia* and then released 7α-hydroxy bile acids are detected with 7α-HSD in the presence of β-NAD^+, diaphorase (EC 1.6.99.2, from *Clostridium kluyveri*) and 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride. The absorbance of formazan formed during the enzymic reaction is measured at 500 nm.

Excretion values of 7α-hydroxy bile acids in normal subjects and in patients with acute hepatitis were compared. This enzymatic detection method for the excretion pattern of urinary 7α-hydroxy bile acids may be useful for clinical diagnosis.

INTRODUCTION

The enzymatic assay of 3α-hydroxy bile acids in serum by the use of NAD-linked 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50, 3α-HSD, from *Pseudomonas testosteroni*) is widely studied as a simple, specific and sensitive method [1—3].

The enzyme, 7α-HSD, isolated from *Escherichia coli* which uses NAD as cofactor and from *P. testosteroni* which uses NADP as cofactor has also been introduced [4, 5].

It is well known that the major bile acids in urine are chenodeoxycholic

0378-4347/83/0000—0000/$03.00

acid, cholic acid and their taurine or glycine conjugates. These bile acids are excreted as sulfates, being sulfated at the 3-hydroxy group [6—10].

In this paper, an enzymatic detection method for conjugated 7α-hydroxy bile acid is described. The principle of the method is as follows:

$$\text{Conjugated 7}\alpha\text{-hydroxy bile acid} \xrightarrow[\beta\text{-glucuronidase}]{\text{sulfatase}} \text{free 7}\alpha\text{-hydroxy bile acid}$$

$$\text{7}\alpha\text{-Hydroxy bile acid} + \text{NAD}^+ \xrightarrow{\text{7}\alpha\text{-HSD}} \text{7-keto bile acid} + \text{NADH}$$

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

EXPERIMENTAL

All the reagents used were of analytical grade, and β-glucuronidase from *E. coli* (EC 3.2.1.31) and sulfatase/β-glucuronidase (EC 3.1.6.1 and EC 3.2.1.31) from *Helix pomatia* were purchased from Sigma, St. Louis, MO, U.S.A.

All standards of steroids and bile acids, INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride), diaphorase (EC 1.6.99.2) from *Clostridium kluyveri* and 7α-HSD from *E. coli* (EC 1.1.1.-) were purchased from Sigma. 3α-Hydroxysteroid dehydrogenase (3α-HSD) from *P. testosteroni* (EC 1.1.1.50) and β-NAD^+ were purchased from Nyegaard & Co. (Oslo, Norway). Sephadex G-25 Fine and the column used were purchased from Pharmacia (Uppsala, Sweden).

Preparation of reagent

Enzyme solution for hydrolysis of conjugates: sulfatase/β-glucuronidase from *H. pomatia* (Type H-1), 200 units as sulfatase/3000 Fishman units as β-glucuronidase per 1 ml of 0.05 *M* acetate buffer (pH 5.0), and β-glucuronidase from *E. coli*, 500 Fishman units per 1 ml of 0.1 *M* phosphate buffer (pH 6.8), were prepared. All of these enzymes were used without the addition of an activator during the hydrolysis of steroid conjugates.

Preparation of enzyme reagent for color development of 7α-hydroxy bile acids: 20 mg of INT, 10 U of 7α-HSD, 100 U of diaphorase and 40 mg of β-NAD were dissolved in 100 ml of 0.2 *M* K_2HPO_4 (pH 9.0).

Reagent for color development for 3α-hydroxysteroid and 3β-hydroxysteroid was described previously [11—14].

Preparation of Sephadex G-25 column

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

Preparation of urine sample

A 24-h urine specimen was collected and an aliquot of the 24-h urine was centrifuged for 3 min at 2500 *g*; 1—2 ml of supernatant of urine were directly applied to the Sephadex G-25 column (27 × 1 cm).

Sephadex chromatography

The supernatant from a 1—2-ml urine sample was applied to the column. After application of the sample, chromatographic separation was performed with acetate buffer (0.05 *M*, pH 5.0). One fraction of the effluent contains 1.3 ml; ten fractions were collected within 30 min followed by another 35 fractions.

Procedure for detection of sulfated 7α-hydroxy bile acids

To each fraction collected during chromatography was added 0.1 ml of sulfatase/β-glucuronidase solution and incubated for 20 h at 37°C. After hydrolysis of the conjugates, 1 ml of the color-developing reagent for 7α-hydroxy bile acids was added, and after incubation for 20 min at 37°C the absorbance at 500 nm was read against the first fraction of the effluent.

RESULTS

Specificity of the 7α-HSD from *E. coli* is shown in Table I. As shown in the table, this enzyme reacted only with the 7α-hydroxy group of steroids.

TABLE I

SPECIFICITY OF 7α-HYDROXYSTEROID DEHYDROGENASE FROM *E. COLI*

A 2-ml volume of 7α-HSD solution was added to a tube containing 20 μl of 2.5 m*M* of standard solutions.

Steroids	Absorbance at 500 nm (20 μl of 2.5 m*M*)
Cholic acid	0.304
Taurochenodeoxycholic acid	0.352
Glycochenodeoxycholic acid	0.295
Taurocholic acid	0.346
Glycocholic acid	0.335
Taurolithocholic acid	0.000
Androsterone	0.000
Testosterone	0.000
Dehydroepiandrosterone	0.000
Tetrahydrocortisol	0.000
Estradiol	0.000

The precision of the method using the sample of a patient with acute hepatitis was C.V. = 7.5% (five repeated assays, mean 32 ± 2.5 mg/day) and with a normal subject C.V. = 18% (mean 2.8 ± 0.5 mg/day). When glycochenodeoxycholic acid added to urine as a standard for 7α-hydroxy bile acid was analyzed in triplicate, the C.V. obtained was 5.7% at a level of 20 μg/ml with a mean recovery of 93.5%.

Excretion pattern of 7α-hydroxysteroids with normal subjects and a patient with acute hepatitis

A typical chromatogram for normal subjects showing the excretion pat-

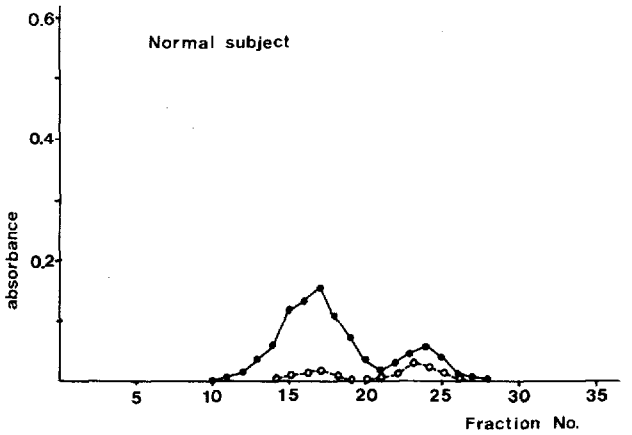

Fig. 1. Excretion pattern of 7α-hydroxy bile acids, obtained from a normal subject. (o— — — o), 7α-Hydroxy bile acids (color-developed with 7α-HSD as described in Experimental); (•——•), 3α-hydroxysteroids.

tern of 7α-hydroxysteroids and 3α-hydroxysteroids is given in Fig. 1. A typical excretion pattern for 7α-hydroxy bile acids in acute hepatitis is shown in Fig. 2.

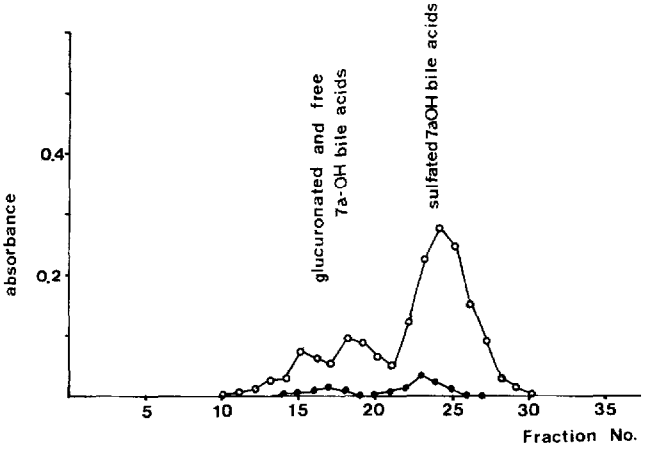

Fig. 2. Excretion pattern of 7α-hydroxy bile acids obtained from a patient with acute hepatitis. (o — — — o), 7α-Hydroxy bile acids obtained from a patient with acute hepatitis; (•——•), 7α-hydroxy bile acids in a normal subject.

In order to obtain information about the conjugated form of 7α-hydroxy bile acids, the sample was treated with β-glucuronidase from *E. coli* to hydrolyse the glucuronate, and with sulfatase/β-glucuronidase from *H. pomatia* to hydrolyse the sulfate; the results are shown in Fig. 3.

Comparison of the excretion patterns of 3α-hydroxysteroids in a normal subject and a patient with acute hepatitis is shown in Fig. 4; elevated steroid-3α-sulfate was observed in patients with acute hepatitis. In order to study the conjugated form of 3α-hydroxysteroids, the sample was treated step-

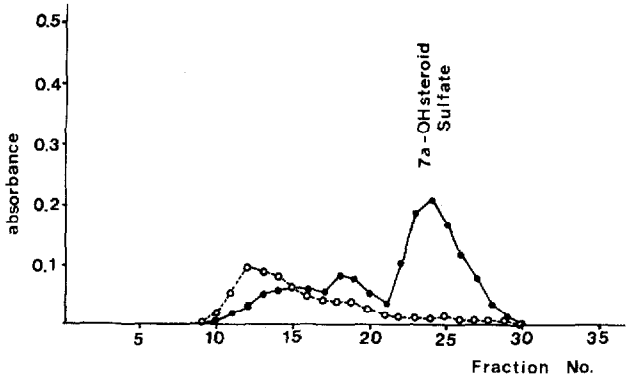

Fig. 3. Effect of β-glucuronidase on hydrolysis of conjugated 7α-hydroxy bile acids. Sample used is from a patient with acute hepatitis. (•———•), Sulfatase/β-glucuronidase; (○ — — —○), β-glucuronidase.

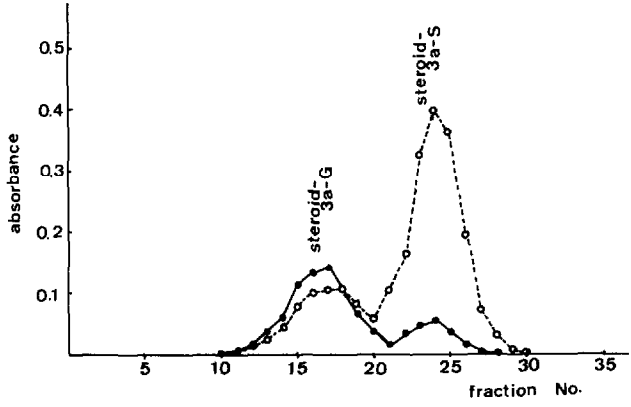

Fig. 4. Comparison of excretion patterns of 3α-hydroxysteroids between a normal subject and a patient with acute hepatitis. (○ — — — ○), 3α-Hydroxysteroids in a patient with acute hepatitis; (•———•), 3α-hydroxysteroids in a normal subject.

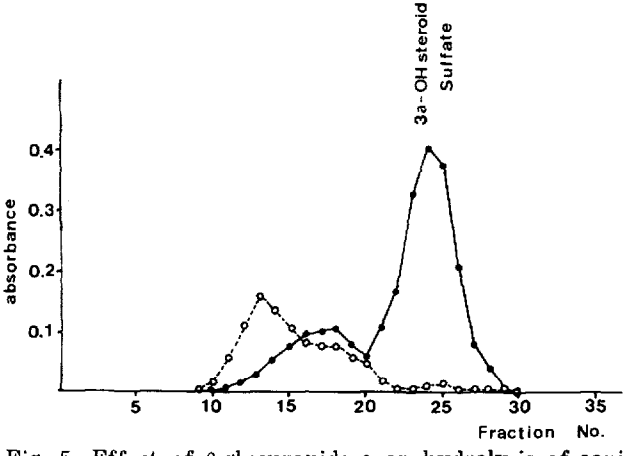

Fig. 5. Effect of β-glucuronidase on hydrolysis of conjugated 3α-hydroxysteroids. Sample used is from a patient with acute hepatitis. (•———•), Sulfatase/β-glucuronidase; (○ — — — ○), β-glucuronidase.

wise with β-glucuronidase and with sulfatase/β-glucuronidase; the result is shown in Fig. 5.

The excretion rate of total 7α-hydroxy bile acids in normal subjects was 3.5 ± 2.8 mg/day (n = 15). Excretion rates for patients with acute hepatitis varied from 7.3 to 57 mg/day (n = 6).

DISCUSSION

In this paper, typical excretion patterns of urinary 7α-hydroxy bile acids obtained for a patient with acute hepatitis and a normal subject are shown. In the chromatogram obtained with the healthy subject the amount of 7α-hydroxy bile acids was too low to promote the detector response. On the other hand, in diseases such as liver cirrhosis, which show elevated excretion of bile acids, the excretion pattern of 7α-hydroxy bile acids was distinctly increased (Fig. 6).

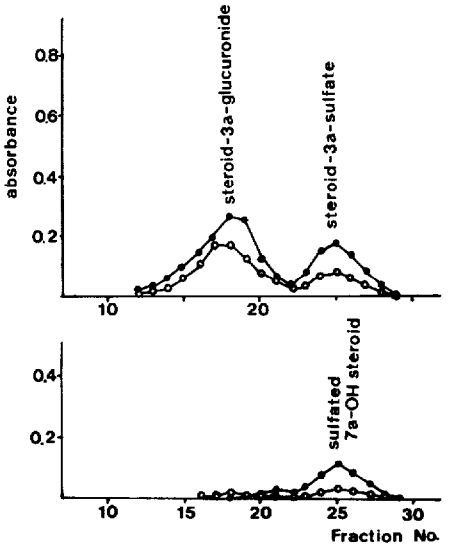

Fig. 6. Excretion patterns of 7α-hydroxy bile acids obtained from a patient with liver cirrhosis. (o- - - - - - o), Normal subject, (•——•), liver cirrhosis (compensative stage). 3α-Hydroxysteroids were color-developed with 3α-hydroxysteroid dehydrogenase as described in a previous paper [12].

The excretion patterns of other conjugated steroids obtained from patients with acute hepatitis was also investigated by the method previously reported [11, 12]. Our own data are presented in Fig. 7 for comparison. Urinary 3β-hydroxysteroids eluted at the same position as sulfated 7α-hydroxy bile acids. The 3β-hydroxysteroids are suggested to be bile acids because the neutral 3β-hydroxysteroids were not detected in any significant amount (using 3β-hydroxysteroid oxidase from *Brevibacterium sterolicum*). It is known that this enzyme reacts mainly with neutral 3β-hydroxysteroids [11, 15].

The elution position of standards of non-sulfated bile acids was also determined (Fig. 8) and compared with the chromatogram of Fig. 5. At the posi-

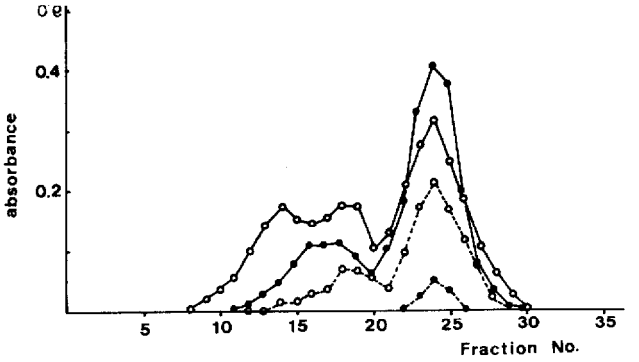

Fig. 7. Excretion patterns of some other urinary steroids obtained from a patient with acute hepatitis. (•———•), 3α-Hydroxysteroids; (○———○), 3β,17β-hydroxysteroids; (○ — — — ○), 7α-hydroxy bile acids, (•— — — •), neutral 3β-hydroxysteroids.

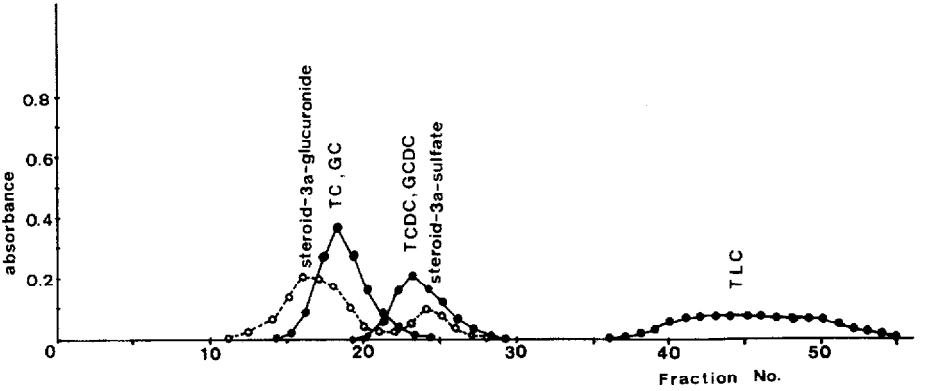

Fig. 8. Chromatogram of non-sulfated bile acids. TC = Taurocholic acid, GC = glycocholic acid, TCDC = taurochenodeoxycholic acid, GCDC = glycochenodeoxycholic acid, TLC = taurolithocholic acid. For each of TC, GC, TCDC, GCDC and TLC 100 μg per 1 ml were applied to the column of Sephadex G-25 and, after fractionation, 3α-HSD was added.

tions of taurochenodeoxycholic acid and glycochenodeoxycholic acid no peaks were seen (see Fig. 5, β-glucuronidase hydrolysis). Taurocholic acid and glycocholic acid overlapped with steroid-3α-glucuronide; therefore it was necessary to make use of the enzymatic detection via 3α-HSD without hydrolysis to distinguish between steroid-3α-glucuronide and non-conjugated (3-hydroxy) bile acids.

Urinary metabolites of bile acids are mainly 7α-hydroxy, 7-keto and 7-deoxy bile acids, the 7α-hydroxy bile acids being the parent compounds. It appears that this rather simple detection method for sulfated 7α-hydroxy bile acids can be used for the diagnosis of some liver diseases.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Ministry of Education, Japan, which is gratefully acknowledged.

REFERENCES

1 F. Mashige, K. Imai and T. Osuga, Clin. Chim. Acta, 70 (1976) 79.
2 F. Mashige, T. Osuga, N. Tanaka, K. Imai and M. Yamanaka, Clin. Chem., 24 (1978) 1154.
3 S. Miyazaki, H. Tanaka, R. Horikawa, H. Tsuchiya and K. Imai, J. Chromatogr., 181 (1980) 177.
4 G.A.D. Haslewood, G.M. Murphy and J.R. Richardson, Clin. Sci., 44 (1973) 95.
5 B.A. Skålhegg and O. Fausa, Scand. J. Gastroenterol., 12 (1977) 433.
6 I. Makino, K. Shinozaki, S. Nakagawa and K. Mashimo, J. Lipid Res., 15 (1974) 132.
7 A. Cantafora, M. Angelico, A.F. Attili, L. Ercoli and L. Capocaccia, Clin. Chim. Acta, 95 (1979) 501.
8 G.P. van Berge Henegouwen and A.F. Hofmann, Clin. Chim. Acta, 73 (1976) 469.
9 I. Makino, K. Shinozaki and S. Nakagawa, Lipids, 8 (1973) 47.
10 J. Goto, H. Kato, Y. Saruta and T. Nambara, J. Chromatogr., 226 (1981) 13.
11 Y. Yamaguchi, J. Liquid Chromatogr., 5 (1982) 743.
12 Y. Yamaguchi and C. Hayashi, J. Chromatogr., 230 (1982) 263.
13 Y. Yamaguchi, J. Chromatogr., 163 (1979) 253.
14 Y. Yamaguchi, C. Hayashi and K. Miyai, J. Chromatogr., 182 (1980) 430.
15 R.S. Strumfel and J.B. Sidbury, J. Clin. Endocrinol. Metab., 24 (1964) 367.