Journal of Chromatography, 272 (1983) 261–268
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1503

IMMOBILIZED 3α-HYDROXYSTEROID DEHYDROGENASE AND DANSYL HYDRAZINE AS A PRE-LABELING REAGENT FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION OF BILE ACIDS

TAKAO KAWASAKI, MASAKO MAEDA and AKIO TSUJI*

School of Pharmaceutical Sciences, Showa University, Hatanodai, Shinagawa-ku, Tokyo 142 (Japan)

(First received June 4th, 1982; revised manuscript received September 22nd, 1982)

SUMMARY

A high-performance liquid chromatographic method with fluorescence detection is described for the determination of bile acids and their conjugates. After enzymatic conversion of bile acids to 3-oxo-bile acids using the immobilized 3α-hydroxysteroid dehydrogenase reactor column, 3-oxo-bile acids were extracted with a Sep-Pak C_{18} cartridge, labelled with dansyl hydrazine and then separated by high-performance liquid chromatography on a reversed-phase column. The eluate is monitored by a fluorophotometer at 365 nm (excitation) and 520 nm (emission). Reactions proceed quickly under mild conditions to give fluorescent derivatives. Linearity of the fluorescence intensity (peak height) with the amounts of various bile acids and their conjugates was obtained above 0.5—1.0 pmol. The method is sensitive, reliable and useful for the simultaneous determination of bile acids in biological samples.

INTRODUCTION

The identification and determination of various bile acids may be of value in the diagnosis of liver disease. Many methods have been reported for the simultaneous determination of individual unconjugated and conjugated bile acids, including thin-layer chromatography [1, 2], gas—liquid chromatography [3] and gas chromatography—mass spectrometry [4, 5]. Recently, high-performance liquid chromatographic (HPLC) methods [6—9] have been developed, combining the advantages of mild separation conditions. However, the sensitivity of HPLC is low due to the use of a refractive index or UV detector, because most common bile acids have no strong UV-absorbing groups in their molecules. Therefore, bile acids have been derivatized before column separation

0378-4347/83/0000—0000/$03.00

by UV-absorbing reagents [10—13] and fluorescent derivatizing reagents [14, 15] in order to improve their detectability. However, taurine-conjugated bile acids could not be determined using these reagents because they react only with carboxyl groups. Baba et al. [16] reported a highly sensitive and selective fluorescence HPLC method for bile acids using 3α-hydroxysteroid dehydrogenase (3α-HSD) and cofactor (NAD^+). Recently, modified methods have been developed using an immobilized enzyme reactor column instead of enzyme solution by Okuyama et al. [17] and Arisue et al. [18] with a fluorescence detector and by Kamada et al. [19] with an electrochemical detector.

Dansyl hydrazine is a favorable reagent for the formation of hydrazones because of its high reactivity under mild conditions. We used it as a fluorescent labeling reagent in the HPLC determination of various oxosteroids [20—22]. In this paper, we have attempted to develop a new fluorescence HPLC method for the determination of bile acids and their conjugates using immobilized 3α-HSD reactor and dansyl hydrazine as a pre-labeling reagent.

EXPERIMENTAL

Reagents and materials

Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA) and their glycine (G) and taurine (T) conjugates were obtained from Sigma or PL Biochemical Co. β-Nicotinamide-adenine dinucleotide (NAD^+, Grade I) was from Boehringer Mannheim-Yamanouchi Co. 3α-Hydroxysteroid dehydrogenase (Grade II) and dansyl hydrazine (Grade II) were from Sigma. Amino glass beads used as the solid phase of immobilized enzyme were Amino Propyl-CPG 180 Å of Electro-Nucleonics and the Sep-Pak C_{18} cartridge was from Waters. All other chemicals were obtained commercially.

Bile acid stock solution: each bile acid was dissolved in methanol and made up to 10 μmol/ml.

NAD^+ solution: 0.5 m*M* NAD^+ solution was prepared by dissolving NAD^+ in 0.1 *M* pyrophosphate buffer (pH 9.0).

Immobilized 3α-HSD reactor column: 3α-HSD was coupled to amino glass beads (120—200 mesh) by the glutaraldehyde method [23] and packed in a glass syringe (35 × 6 mm I.D.).

Dansyl hydrazine solution: a 0.2% (w/v) solution was prepared by dissolving 20 mg of dansyl hydrazine in 10 ml of benzene; it was stored in a refrigerator until use.

Trichloroacetic acid (TCA) was in benzene solution (0.1%, w/v).

Instrumentation

We used a Hitachi Model 635 high-performance liquid chromatograph equipped with a Kyowa Seimitsu KHP-UI-130 injection valve and a Jasco Model FP-110 fluorescence spectrophotometer. A Radial-Pak A column (5 μm, Waters), a μBondapak Phenyl column (10 μm, 300 × 6 mm, Waters) and a Zorbax ODS column (5—6 μm, 250 × 4.6 mm, DuPont) were used. The detector wavelengths were 365 nm for excitation and 520 nm for emission.

Analytical procedure

The bile acids were dissolved by adding 100 μl of methanol, transferred onto the 3α-HSD reactor column and eluted from the column with 2 ml of NAD^+ solution at a flow-rate of 0.5 ml/min. To the eluent were added 2 ml of 0.5 *M* phosphate buffer (pH 6.0), the solution was then poured into the syringe attached to the Sep-Pak C_{18} cartridge. The cartridge was washed with 4 ml of water, followed by elution with 2 ml of methanol and the methanol eluent was evaporated to dryness under reduced pressure. The residue was dissolved by adding 0.2 ml of TCA—benzene solution, left to stand for 10 min at 30°C and evaporated to dryness under a stream of nitrogen gas. The labelled residue was dissolved in 500 μl of methanol and an aliquot of the solution was injected into the chromatograph.

Extraction of bile acids from serum

A 0.1-ml serum sample was mixed with 0.5 ml of methanol and ultrasonicated for 15 min. Then 0.3 ml of the supernatant was evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in 1.0 ml of 0.05 *M* phosphate buffer (pH 7.0) and applied onto a Sep-Pak C_{18} cartridge, and washed with 2 ml of 2% methanol. Bile acids were eluted with 4 ml of 80% methanol and the eluent was then evaporated under reduced pressure at 40°C.

Fractionation of bile acids

The fractionation of three different groups (unconjugated, glycine-conjugated and taurine-conjugated) was carried out using a piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) column (13 × 7.5 mm I.D.) as reported by Goto et al. [24]. The resultant residue of each fraction was assayed by the analytical procedure.

Preparation of 3-oxo-bile acids and dansyl hydrazones of bile acids

3-Oxo-bile acids were prepared from bile acids by selective oxidation of the hydroxyl group at carbon-3 using silver carbonate adsorbed on Celite as a mild oxidizing reagent according to the descriptions of Fetizon and Golfier [25] and Dayal et al. [26]. For example, cholic acid was oxidized to 3-oxo-7α,12α-dihydroxy-5β-cholan-24-oic acid. (Anal. calc. for $C_{24}H_{38}O_5$: C, 70.90; H, 9.42. Found: C, 70.58, H, 9.48.)

Dansyl hydrazones of bile acids were synthesized from 3-oxo-bile acids and dansyl hydrazine in TCA—benzene solution under the same conditions as described in the analytical procedure.

Calculation

The enzymatic conversion ratio from bile acids to 3-oxo-bile acids with immobilized 3α-HSD column, the recoveries of 3-oxo-bile acids from Sep-Pak C_{18} cartridge extraction and the yields of dansyl hydrazones of 3-oxo-bile acids were calculated as follows: percentage enzymatic conversion = $H_1/H_2 \times 100$, percentage Sep-Pak extraction = $H_2/H_3 \times 100$, percentage dansylation yield = $H_3/H_4 \times 100$, where H_1 = peak height of bile acid in chromatogram obtained according to the analytical procedure, H_2 = peak height of 3-oxo-bile

acid obtained by the analytical procedure without the enzymatic reaction step, H_3 = peak height of 3-oxo-bile acid in chromatogram obtained by dansylation step, H_4 = peak height of synthesized dansyl hydrazone of 3-oxo-bile acid.

Recovery test

A synthetic mixture of 1.0 nmol of each bile acid was added to 0.1 ml of normal human serum and then assayed by the present method. Recoveries were calculated against a pure standard bile acid mixture carried through the procedure.

RESULTS AND DISCUSSION

The assay procedure is illustrated schematically in Fig. 1. The following parameters were examined in order to obtain the optimum conditions for the assay procedure such as enzymatic reaction, extraction of 3-oxo-bile acids and labelling reaction.

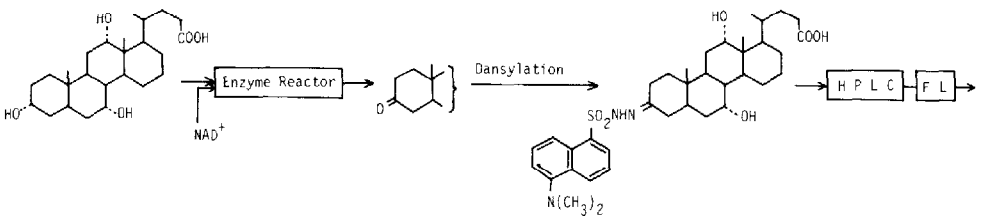

Fig. 1. Flow diagram of the fluorescence HPLC method. Conditions: enzyme reactor, immobilized 3α-HSD (35 × 6 mm I.D.); 0.5 m*M* NAD^+ in 0.1 *M* pyrophosphate buffer (pH 9.0); fluorophotometer (excitation 365 nm, emission 520 nm).

The first step of this method is the enzymatic oxidation of bile acids to 3-oxo-bile acids with immobilized 3α-HSD reactor column. Fig. 2 shows the effects of type and pH of buffer solution in enzymatic reaction on the fluorescence intensity (peak height in chromatogram). The pyrophosphate buffer (pH 9.0) gave the highest peaks and the peak height increased with increasing concentration of pyrophosphate up to 0.1 *M*, it reached a constant

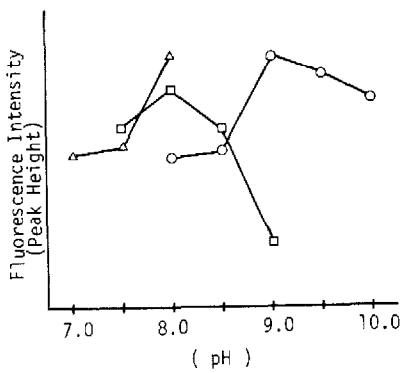

Fig. 2. Effects of type and pH of buffer in enzymatic reaction of fluorescence intensity. (○) Pyrophosphate buffer, (□) Tris–HCl buffer, (△) phosphate buffer.

at 0.2 *M* but sodium pyrophosphate was deposited occasionally at this concentration. Therefore, 2 ml of 0.1 *M* pyrophosphate buffer (pH 9.0) containing 0.5 m*M* NAD was used as the enzyme reaction medium because the peak height reached a plateau at 0.2 m*M* NAD. Under these conditions, the percentage conversion of bile acids (cholic acid, deoxycholic acid, lithocholic acid) to 3-oxo-bile acids ranged from 75% to 95%.

3-Oxo-bile acids, formed by enzymatic oxidation, were extracted successfully by the Sep-Pak C_{18} cartridge. Recoveries of unconjugated, glycine- and taurine-conjugated bile acids in the extraction step using the Sep-Pak C_{18} cartridge were approximately 96% and the coefficient of variation was 2--4%.

The derivatization conditions of 3-oxo-bile acids with dansyl hydrazine were examined. Fig. 3 shows the effects of the concentration of TCA solution on the fluorescence intensity (peak height in the chromatogram). The peak height reached a maximum with 0.1% TCA—benzene solution, and decreased slightly with increasing TCA concentration in benzene. Though the peak height reached a constant value within 5 min with this TCA solution, the reaction time was held for 10 min. The reaction temperature was set at 30°C, because the reaction rate was independent of temperature between 20 and 50°C. Under these conditions, the yield of dansyl hydrazone of each bile acid was about 98%.

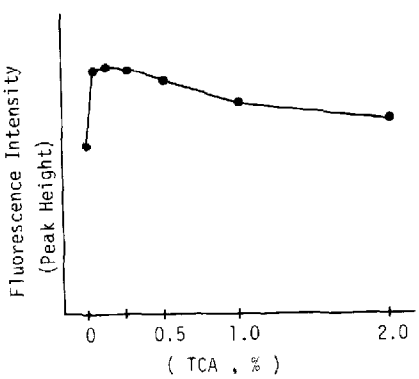

Fig. 3. Effect of TCA concentration in dansylation on fluorescence intensity.

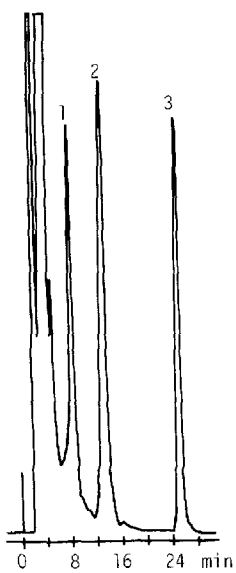

Fig. 4. Chromatogram of standard mixture of unconjugated bile acids. Peaks: 1 = cholic acid, 2 = deoxycholic acid, 3 = lithocholic acid. Column: Zorbax ODS (250 × 4.6 mm I.D.). Mobile phase: methanol—acetonitrile–0.03 *M* phosphate buffer (pH 3.2) (68:15:17), 1 ml/min.

Fig. 4 shows a typical chromatogram of unconjugated bile acids obtained using a Zorbax ODS column with a mixed solvent system of methanol–phosphate buffer. Under these chromatographic conditions, deoxycholic acid and

chenodeoxycholic acid could not be separated. Linearity of the relationship between fluorescence intensity (peak height) and amounts of bile acids was obtained between 3 and 40 pmol, and the detection limit for bile acids was about 0.5 pmol (signal-to-noise ratio = 3.5). The sensitivity of this method was superior to that of other HPLC methods using fluorescence derivatization. As shown in Table I, the precision of this method was satisfactory. In order to obtain good separation, many chromatographic conditions were examined. Shimada et al. [6] used 0.3% ammonium carbonate (pH 7.8)—acetonitrile, but in this method the peaks of the bile acids were interfered with by excess dansyl hydrazine under the alkaline conditions. As shown in Table II, complete separation could not be obtained with methanol—phosphate buffer. Ion-pair chromatography with tetrabutylammonium phosphate was suitable for the separation of all unconjugated and taurine-conjugated bile acids (Table II, Fig. 5).

TABLE I

REPRODUCIBILITY OF PEAK HEIGHTS OF BILE ACIDS*

nmol	C.V. (%) (n = 5)					
	Unconjugated			Taurine-conjugated		
	CA	DCA	LCA	CA	DCA	LCA
10.0	1.5	2.5	5.1	1.9	2.0	3.4
5.0	3.9	2.5	2.3	2.1	1.8	2.9
2.5	2.2	2.3	2.4	2.5	2.3	2.2
1.25	2.9	3.1	5.0	3.2	3.0	3.6

*For abbreviations see Reagents and materials.

TABLE II

CAPACITY FACTORS OF UNCONJUGATED BILE ACIDS AS ANALYSED ON THE ZORBAX ODS, RADIAL-PAK A AND μBONDAPAK PHENYL COLUMNS WITH VARIOUS SOLVENT SYSTEMS*

	Capacity factor							
	Zorbax ODS			Radial-Pak A		μBondapak Phenyl		
	I	II	III	IV	III	IV	V	III
Ursodeoxycholic acid	6.4	4.8	3.7	9.0	3.2	4.7	3.5	2.0
Cholic acid	7.8	5.4	4.1	9.6	3.7	5.2	3.6	2.3
Chenodeoxycholic acid	12.0	8.7	7.3	19.2	6.1	8.5	5.5	3.1
Deoxycholic acid	12.4	8.8	8.3	19.4	6.9	8.5	6.5	3.4
Lithocholic acid		22.8	14.8	45.2	12.2	16.0	10.4	4.8

*I = methanol—acetonitrole—water—acetic acid (68:15:17:1); II = methanol—0.03 M phosphate buffer (pH 2.5) (75:25); III = 0.05 M tetrabutylammonium phosphate in methanol—water (75:25); IV = methanol—0.03 M phosphate buffer (pH 2.0) (75:25); V = methanol—0.03 M phosphate buffer (pH 4.5) (75:25).

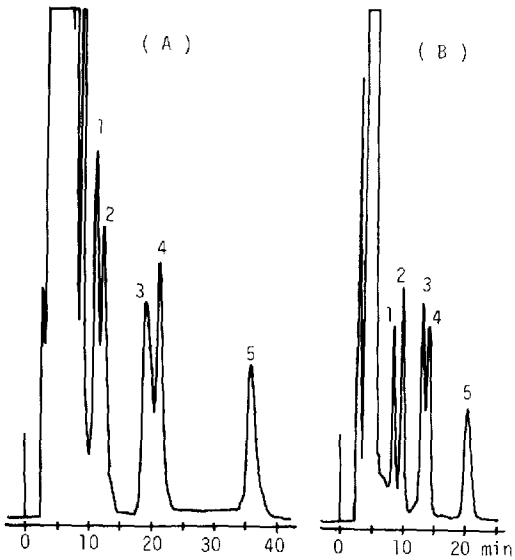

Fig. 5. Chromatograms of standard mixture of bile acids. (A) Unconjugated and taurine-conjugated bile acids. Peaks: 1 = ursodeoxycholic acid, 2 = cholic acid, 3 = chenodeoxycholic acid, 4 = deoxycholic acid, 5 = lithocholic acid. Column: Radial-Pak A. Mobile phase: 0.05 *M* tetrabutylammonium phosphate in methanol—water (75:25), 0.8 ml/min. (B) Glycine-conjugated bile acids. Peaks: 1 = urosdeoxycholic acid, 2 = cholic acid, 3 = chenodeoxycholic acid, 4 = deoxycholic acid, 5 = lithocholic acid. Column: μBondapak Phenyl (300 × 6 mm I.D.). Mobile phase: 0.03 *M* KH_2PO_4 (pH 2.0)—methanol (25:75), 1 ml/min.

TABLE III

RECOVERY AND REPRODUCIBILITY OF BILE ACIDS ADDED TO HUMAN SERUM

Normal human serum (0.1 ml) was used to which 1 nmol of each of nine bile acids was added $n = 5$.

	Unconjugated		Taurine-conjugated		Glycine-conjugated	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
Cholic acid	98.1	3.6	94.8	3.8	92.6	7.0
Deoxycholic acid	86.4	4.2	89.2	4.4	90.0	5.1
Lithocholic acid	92.0	5.1	88.6	4.8	94.1	4.1

Reproducibility was tested by measuring simultaneously the recoveries of bile acids (1 nmol) in a standard mixture in normal human serum (0.1 ml). The bile acids in serum were extracted with the Sep-Pak C_{18} cartridge and then fractionated to three different groups (unconjugated, glycine-conjugated and taurine-conjugated) using the PHP-LH-20 column according to the procedure reported by Goto et al. [24]. Bile acids in each fraction were assayed by the present fluorescence HPLC method. The recovery of each bile acid was calculated from the peak height ratio of each bile acid against the peak

height of pure standard bile acid mixture carried through the analytical procedure. As illustrated in Table III, the recoveries ranged from 86.4% to 98.1% with the coefficients of variation (C.V.) ranging from 3.6% to 7.0%. Application of this method to the assay of serum bile acids or other physiologically important substances such as 3α-hydroxysteroids is being conducted in our laboratory; the details will be reported elsewhere in the near future.

ACKNOWLEDGEMENT

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1 A.K. Batta, G. Salen and S. Shefer, J. Chromatogr., 168 (1979) 557.
2 R.W. Shepherd, Clin. Biochem., 11 (1978) 106.
3 P.P. Nair and C. Garcia, Anal. Biochem., 29 (1969) 164.
4 G. Karlaganis and G. Paumgartner, Clin. Chim. Acta, 92 (1979) 19.
5 J. Yanagisawa, M. Itoh, M. Ishibashi, H. Miyazaki and F. Nakayama, Anal. Biochem., 104 (1980) 75.
6 K. Shimada, M. Hasegawa, J. Goto and T. Nambara, J. Chromatogr., 152 (1978) 431.
7 J. Goto, M. Hasegawa, H. Kato and T. Nambara, Clin. Chim. Acta, 87 (1978) 141.
8 N. Parris, Anal. Biochem., 100 (1979) 260.
9 M.S. Sian and A.J. Harding Ranis, Clin. Chim. Acta, 98 (1979) 243.
10 F. Stellaard, D.L. Hachey and P.D. Klein, Anal. Biochem., 87 (1978) 359.
11 B. Shaikh, N.J. Pontzen, J.E. Molina and M.I. Kelsey, Anal. Biochem., 85 (1978) 47.
12 S. Okuyama, D. Uemura and Y. Hirata, Bull. Chem. Soc. Jap., 52 (1979) 124.
13 D.P. Mattees, Anal. Chim. Acta, 109 (1979) 161.
14 S. Okuyama, D. Uemura and Y. Hirata, Chem. Lett., (1979) 461.
15 J.B.F. Lloyd, J. Chromatogr., 189 (1980) 359.
16 S. Baba, R. Uenoyama, K. Suminoe, F. Takeda, S. Hasegawa and Y. Kameno, Kobe J. Med. Sci., 26 (1980) 89.
17 S. Okuyama, N. Kokubun, S. Higashidate, D. Uemura and Y. Hirata, Chem. Lett., (1979) 1443.
18 K. Arisue, Z. Ogawa, K. Kohda, C. Hayashi and Y. Ishida, Jap. J. Clin. Chem., 9 (1980) 104.
19 S. Kamada, M. Maeda, A. Tsuji, Y. Umezawa and T. Kurahashi, J. Chromatogr., 239 (1982) 773.
20 T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 163 (1979) 143.
21 T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 226 (1981) 1.
22 T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 232 (1982) 1.
23 H.H. Weetall, Methods Enzymol., 44 (1976) 104.
24 J. Goto, H. Kato, Y. Saruta and T. Nambara, J. Liquid Chromatogr., 3 (1980) 991.
25 M. Fetizon and M. Golfier, C.R. Acad. Sci. Ser. C, 267 (1968) 900.
26 B. Dayal, E. Bagan, G.S. Tint, S. Shefer and G. Salen, Steroids, 34 (1979) 259.