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## Application of 3 $\alpha$ -Hydroxysteroid Dehydrogenase Column to the Determination of Bile Acids Fractionated by High-Performance Liquid Chromatography: Advantage of Pretreating Human Bile Acids with Seppak C18 and Piperidinohydroxypropyl Sephadex LH-20

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## Abstract

The analysis of bile acids in human bile was tried by high-performance liquid chromatography (HPLC). Bile acids in human bile were first prefractionated into free, glycine- and taurine-conjugated bile acids using a Seppak C18 cartridge and a piperidinoxypropyl Sephadex LH-20 (PHP-LH-20) column. Each fraction was then processed through a HPLC system consisting of a Zorbax ODS column and a 3  $\alpha$ -hydroxysteroid dehydrogenase (3  $\alpha$ -HSD) column. By these procedures the major 15 bile acids were clearly separated, and each bile acid of 10-125 ng was accurately analyzed. More than 400 times of analysis could be repeated on one 3  $\alpha$ -HSD column without loss of sensitivity. Thus the pretreatment through Seppak C18 and PHP-LH-20 made the HPLC analysis of human bile acids accurate and applicable to clinically obtained materials.

**KEYWORDS:** bile acid, high-performance liquid chromatography, 3 $\alpha$ -hydroxysteroid dehydrogenase, immobilized column, piperidinoxypropyl Sephadex LH-20

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The analysis of bile acids in human bile was tried by high-performance liquid chromatography (HPLC). Bile acids in human bile were first prefractionated into free, glycine- and taurine-conjugated bile acids using a Seppak C18 cartridge and a piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) column. Each fraction was then processed through a HPLC system consisting of a Zorbax ODS column and a 3 $\alpha$ -hydroxysteroid dehydrogenase (3 $\alpha$ -HSD) column. By these procedures the major 15 bile acids were clearly separated, and each bile acid of 10-125 ng was accurately analyzed. More than 400 times of analysis could be repeated on one 3 $\alpha$ -HSD column without loss of sensitivity. Thus the pretreatment through Seppak C18 and PHP-LH-20 made the HPLC analysis of human bile acids accurate and applicable to clinically obtained materials.

*Key words:* bile acid, high-performance liquid chromatography, 3 $\alpha$ -hydroxysteroid dehydrogenase, immobilized column, piperidinohydroxypropyl Sephadex LH-20

Bile acid metabolism has received considerable clinical attention in the field of hepatology and gastroenterology (1, 2). The analysis of bile acids has been performed by thin-layer chromatography (TLC) (3, 4), gas-liquid chromatography (GLC) (5-7) and high-performance liquid chromatography (HPLC)(8-12). By these methods, however, the separation of structurally similar bile acids is often difficult. These methods are also not sensitive enough to detect the minor components of bile acids in clinical materials such as patients' sera.

Recently, HPLC system using immobilized 3 $\alpha$ -hydroxysteroid dehydrogenase (3 $\alpha$ -HSD) has been used to increase the sensitivity of bile acid analysis (13-19). In our prelimi-

nary experiments, however, each bile acid was not satisfactorily separated when crude bile was analyzed by this system. In the present study, the pretreatment of human crude bile with Seppak C18 and piperidinohydroxypropyl Sephadex (PHP-LH-20) gave rather good results in the analysis of bile acids by HPLC with immobilized 3 $\alpha$ -HSD. These results are reported in this paper.

### Materials and Methods

*Chemicals.* Ursodeoxycholic acid (UDCA), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), and their taurine- and glycine-conjugates were kindly donated by Tokyo Tanabe Pharmaceutical

Co. (Tokyo, Japan). They were dissolved in methanol and stored at 4°C.

3 $\alpha$ -HSD, derived from *Pseudomonas testosteroni* (grade II), and  $\beta$ -nicotinamide adenine dinucleotide (NAD, grade II) were purchased from Sigma Chem. Co. (St. Louis, MO, USA). All other reagents and organic solvents used in this work were of HPLC grade.

Seppak C18 cartridges were obtained from Waters Assoc. (Milford, MA, USA), and PHP-LH-20 from Shimadzu Co. (Kyoto, Japan). Amino propyl-CPG glass beads used as the solid support for immobilizing 3 $\alpha$ -HSD were purchased from Electro-Nucleonics Inc. (MS, USA).

The mobile phase for HPLC was ethanol and 0.1 M potassium phosphate buffer, pH 6.8, (9 : 10, v/v) filtered through a 4.5  $\mu$ m filter (Millipore Co., USA) and degassed by ultrasonic agitation. NAD (1.5 mM) was prepared in 0.1 M Tris-citric acid (pH 8.0) containing 2.7 mM ethylenediaminetetraacetic acid (EDTA).

**Apparatus.** Fig. 1 shows a schematic diagram of the HPLC system. The system consists of Model 6000 A HPLC pump (Waters Assoc.) equipped with Universal injector Model U6K (Waters Assoc.) and pre-filter (Waters Assoc.), RF 530 fluorescence spectromonitor (Shimadzu Co.), CTO-2A incubator (Shimadzu Co.), 2209 Multitemp. (LKB, Bromma, Sweden) and Chromatopac C-R2AX recorder (Shimadzu Co.). As a separating column, Zorbax ODS (0.5  $\mu$ m) (Shimadzu Co.) was packed in a stainless steel column (4.6 mm  $\times$  150 mm). Model 6000 A HPLC pump was used to carry the NAD solution to the enzyme column.

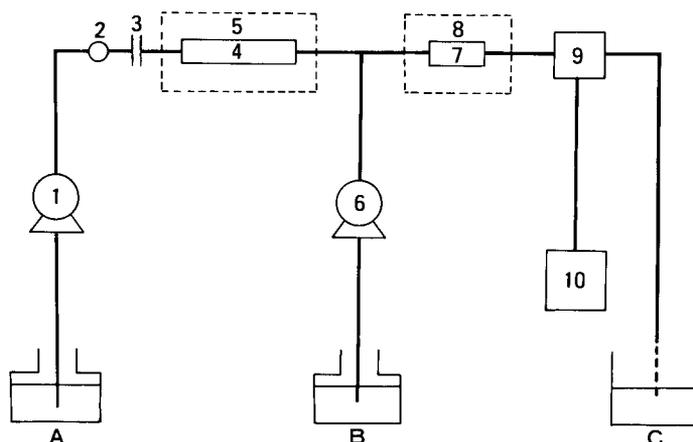
*Preparation of immobilized 3 $\alpha$ -HSD column.*

Two hundred mg of amino propyl-CPG (120-200 mesh) glass beads were added to 2 ml of 2.5% glutaraldehyde. The suspension was degassed for 30 min under reduced pressure and incubated at room temperature for 1 h. After washing with 50 mM sodium phosphate buffer (pH 7.5) three times, 5 mg of 3 $\alpha$ -HSD in 1 ml of the same buffer were added. The suspension was further degassed for 30 min in an ice bath and incubated overnight at 4°C. The beads, on which 3 $\alpha$ -HSD was immobilized, were washed three times with the enzyme column buffer (0.1 M potassium phosphate buffer, pH 7.0, containing 3 mM sodium azide, 6.4 mM mercaptoethanol, 0.1 mM EDTA and 40 mM saccharose). After being suspended in the same buffer, the beads were packed in a stainless steel column (4.6 mm  $\times$  120 mm).

**Pre-treatment of bile.** Gallbladder bile was collected with a duodenal tube after fasting and stored at -20°C until analysis. Ten  $\mu$ l of bile was mixed well with 1 ml of 0.5 M sodium phosphate buffer (pH 7.0). The mixture was passed through a Seppak C18 cartridge, which had been pretreated successively with 5 ml ethanol, 5 ml acetonitrile and 10 ml water. After washing the cartridge successively with 2 ml each of the sodium phosphate buffer, water and 1.5% ethanol, bile acids were eluted with 5 ml of 90% ethanol. The eluate was evaporated to dryness under reduced pressure at 40°C.

To fractionate the free, taurine- and glycine-conjugated bile acids, the dried eluate was dissolved in 1 ml of 90% ethanol and loaded on a PHP-LH-20 column (6 mm  $\times$  18 mm) as described by Goto *et al.* (20) and Nambara *et al.* (21). After

Fig. 1 Schematic diagram of the high-performance liquid chromatography (HPLC) system for bile acid analysis. 1 and 6, HPLC pump; 2, sample injector; 3, pre-filter; 4, Zorbax ODS column; 5 and 8, incubator; 7, 3 $\alpha$ -hydroxysteroid dehydrogenase column; 9, fluorescence spectromonitor; 10, recorder and data processor. A, mobile phase: ethanol / 0.1 M potassium phosphate buffer (9/10, v/v), pH 6.8; B, 1.5 mM  $\beta$ -nicotinamide adenine dinucleotide (NAD) solution in 0.1 M Tris-citric acid (pH 8.0) and 2.7 mM ethylenediaminetetraacetic acid; C, waste.



washing the column with 5 ml of 90% ethanol, the free, glycine- and taurine-conjugated bile acids were eluted stepwisely with 5 ml of 0.1 M acetic acid, 0.2 M formic acid and 0.3 M potassium acetic acid buffer (pH 6.5) in a 90% ethanol solution. The flow rate of the solution was adjusted to 150  $\mu$ l/min. The eluates were evaporated to dryness under reduced pressure at 40°C and finally dissolved in 1 ml of the mobile phase for HPLC.

**Analysis of bile acids.** One hundred  $\mu$ l of the free bile acid fraction, 20  $\mu$ l of the taurine-conjugated bile acid fraction and 5  $\mu$ l of the glycine-conjugated bile acid fraction of the bile prepared as described above, were applied to Zorbax ODS column of the HPLC system through the injector (Fig. 1). The flow rate was 0.7 ml/min, and the column temperature was kept at 45°C in CTO-2A incubator. The eluate from the Zorbax ODS column was continuously carried to a 3 $\alpha$ -HSD column in a 22°C water bath. To the enzyme column, the NAD solution was injected at 0.5 ml/min. The amount of reduced NAD (NADH) was measured in RF 530 fluorescence spectromonitor at an excitation wavelength of 350 nm and an emission wavelength of 460 nm. All data were recorded and analyzed by C-R2AX Chromatopac at the attenuation of 1. The chart speed was 2 mm/min.

Immediately after the analysis, the temperature of the 3 $\alpha$ -HSD column was cooled to 4°C, and the column was washed thoroughly with the enzyme-column buffer to preserve the enzyme activity.

**Construction of calibration curves.** Ten to 125 ng of free, taurine-conjugated and glycine-conjugated standard bile acids were mixed with methanol and analyzed as described above. The

calibration curve was constructed by plotting the peak areas for each bile acid.

**Recovery rate of bile acids from Seppak C18 and PHP-LH-20.** A mixture of 50 ng of each standard bile acid was passed through a Seppak C18 cartridge or a PHP-LH-20 column. The eluate was analyzed by HPLC, and the recovery rate was calculated.

**Determination of total bile acids.** The total amount of bile acids obtained by the present system was calculated by the summation of the peak areas. The amount obtained was compared with that determined using an Enzabile kit (Nihon Shoji, Tokyo, Japan). The bile was appropriately diluted with saline for determination by the Enzabile kit.

**Stability study of 3 $\alpha$ -HSD column.** To examine the deterioration of 3 $\alpha$ -HSD immobilized in column, a mixture of 50 ng each standard bile acid was analyzed at every analysis of human bile. Peak areas were compared with that obtained with a new column. Effects of time and numbers of analysis were examined.

## Results

**Recovery rate of standard bile acids after pretreatment through Seppak C18 cartridge or PHP-LH-20 column.** Table 1 shows the mean recovery rate of standard bile acids after each pretreatment step. The recovery rate ranged from 88.3 to 98.7% after passage through Seppak C18 cartridge, and from 91.7 to 101.1% after passage through

**Table 1** Recovery rate of bile acid after passage through a Seppak C18 cartridge or a piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) column<sup>a</sup>

	Seppak C18			PHP-LH-20		
	Free	Taurine-conjugates	Glycine-conjugates	Free	Taurine-conjugates	Glycine-conjugates
Ursodeoxycholic acid	96.8 $\pm$ 1.6 <sup>b</sup>	90.8 $\pm$ 4.4	96.6 $\pm$ 1.4	97.5 $\pm$ 2.0	98.9 $\pm$ 4.0	101.1 $\pm$ 1.8
Cholic acid	96.5 $\pm$ 3.2	88.3 $\pm$ 4.4	93.9 $\pm$ 2.3	92.7 $\pm$ 3.0	91.7 $\pm$ 5.2	95.7 $\pm$ 3.7
Chenodeoxycholic acid	98.7 $\pm$ 1.9	93.7 $\pm$ 5.9	98.6 $\pm$ 1.9	100.2 $\pm$ 1.5	96.9 $\pm$ 5.4	98.6 $\pm$ 4.5
Deoxycholic acid	97.7 $\pm$ 1.9	93.6 $\pm$ 5.0	95.0 $\pm$ 1.8	99.6 $\pm$ 2.1	95.4 $\pm$ 4.2	97.1 $\pm$ 2.0
Lithocholic acid	97.6 $\pm$ 2.1	94.4 $\pm$ 5.6	96.1 $\pm$ 2.2	98.2 $\pm$ 0.9	99.0 $\pm$ 3.5	98.5 $\pm$ 1.0

<sup>a</sup>: Fifty nanograms of a mixture of each standard bile acid were applied to a Seppak C18 cartridge or a PHP-LH-20 column as described in "Materials and Methods".

<sup>b</sup>: Values are mean  $\pm$  SD (%) of 4 determinations. No statistically significant difference of the recovery rate was observed among the bile acids.

PHP-LH-20 column. As for the recovery rate, no statistically significant difference was observed among the standard bile acids examined.

**Separation of standard bile acids.** Fig. 2 shows the chromatograms of the standard bile acids analyzed by HPLC with immobilized  $3\alpha$ -HSD. UDCA, CA, CDCA, DCA and LCA of the free (Fig. 2A), glycine-conjugated (Fig. 2B) and taurine-conjugated (Fig. 2C) forms were clearly separated and eluted within 20 min.

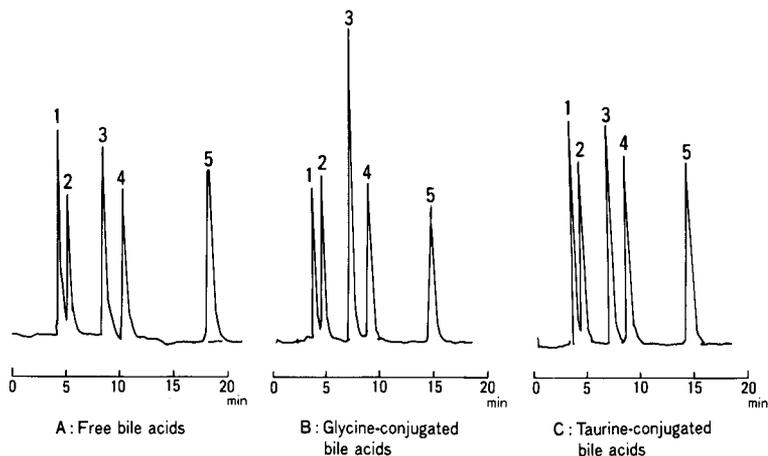
**Quantitative accuracy with standard bile acids.** The calibration curves for the standard bile acids analyzed by HPLC with im-

mobilized  $3\alpha$ -HSD are shown in Fig. 3. Peak areas for the 5 bile acids of free (Fig. 3A), glycine-conjugated (Fig. 3B) and taurine-conjugated (Fig. 3C) forms increased linearly with the increase of the amount of bile acid up to 125 ng.

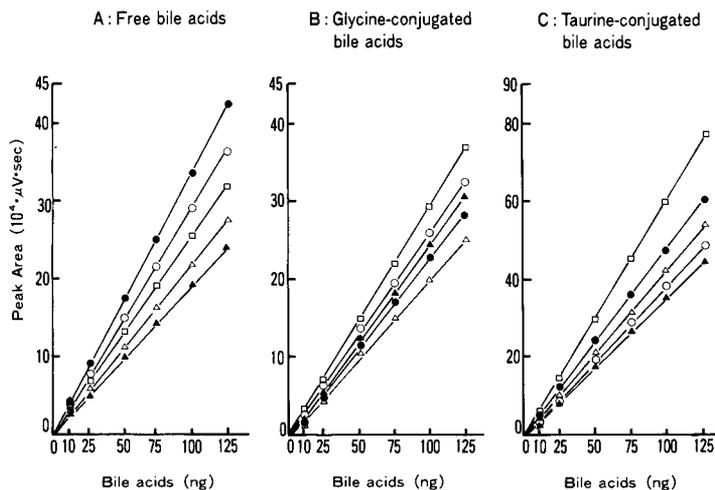
**Experiments with human bile.** Human bile was pretreated through a Seppak C18 cartridge and fractionated by a PHP-LH-20 column into three bile acid fractions; free, taurine-conjugated and glycine-conjugated. These three fractions were analyzed by HPLC with immobilized  $3\alpha$ -HSD.

Fig. 4 shows the chromatograms of the bile acids in human bile analyzed by this

**Fig. 2** Chromatograms of standard bile acids. A mixture of 50 ng of each standard bile acid was analyzed as described in "Materials and Methods". 1, ursodeoxycholic acid (UDCA); 2, cholic acid (CA); 3, chenodeoxycholic acid (CDCA); 4, deoxycholic acid (DCA); 5, lithocholic acid (LCA).



**Fig. 3** Calibration curves for bile acids. Each standard bile acid (10 to 125 ng) was analyzed as described in "Materials and Methods".  $\circ$ , ursodeoxycholic acid (UDCA);  $\bullet$ , cholic acid (CA);  $\Delta$ , chenodeoxycholic acid (CDCA);  $\blacktriangle$ , deoxycholic acid (DCA);  $\square$ , lithocholic acid (LCA).



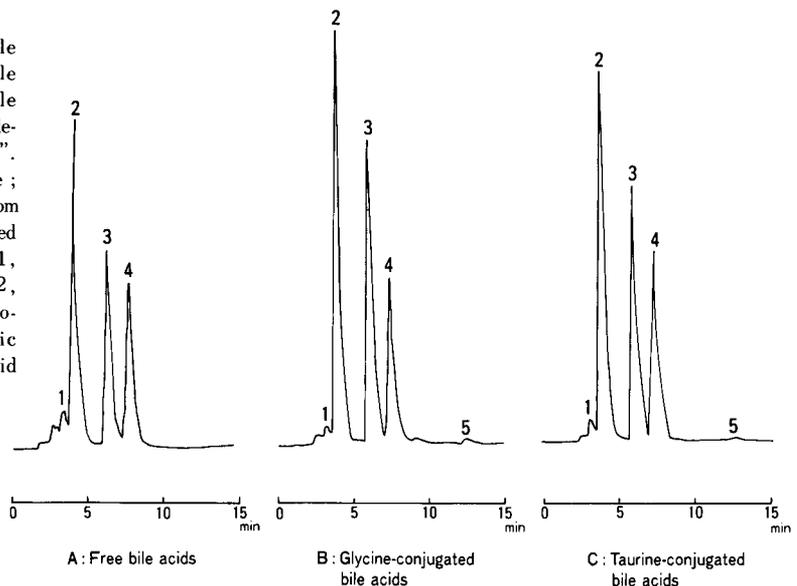
system. Free (Fig. 4A), glycine-conjugated (Fig. 4B) and taurine-conjugated (Fig. 4C) forms from 1  $\mu$ l, 0.05  $\mu$ l and 0.2  $\mu$ l bile materials, respectively, were analyzed. Although not as sharply as with the standard bile acids, the separation among 5 peaks of UDCA, CA, CDCA, DCA and LCA of all free and conjugated forms appeared to be clear enough for the practical analysis of these bile acids.

*Quantitative accuracy with bile acids in human bile.* The total amounts of bile acids in human bile analyzed by this system were

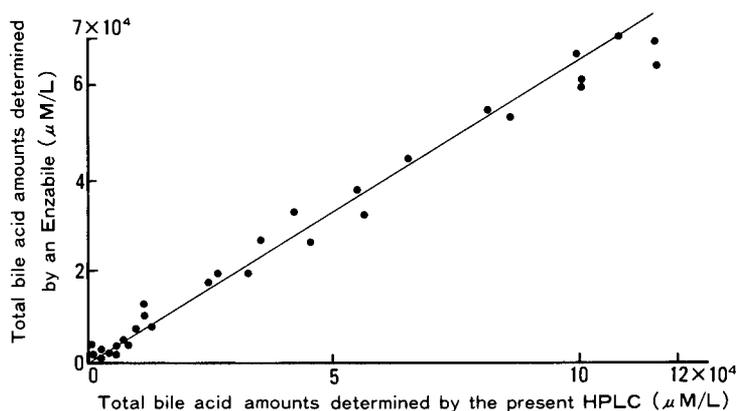
calculated from the peak area of each bile acid and compared with those determined by the Enzabile kit. As shown in Fig. 5, total amounts determined by these two methods correlated well with each other.

*Stability of 3 $\alpha$ -HSD.* Fig. 6 shows the stability curves of 3 $\alpha$ -HSD immobilized in column. In 24 days, the analysis was performed 408 times with 136 human bile materials without loss of the enzyme activity. After 52 days (522 times of analysis with 174 pretreated human biles), the enzyme activity lowered below half the original.

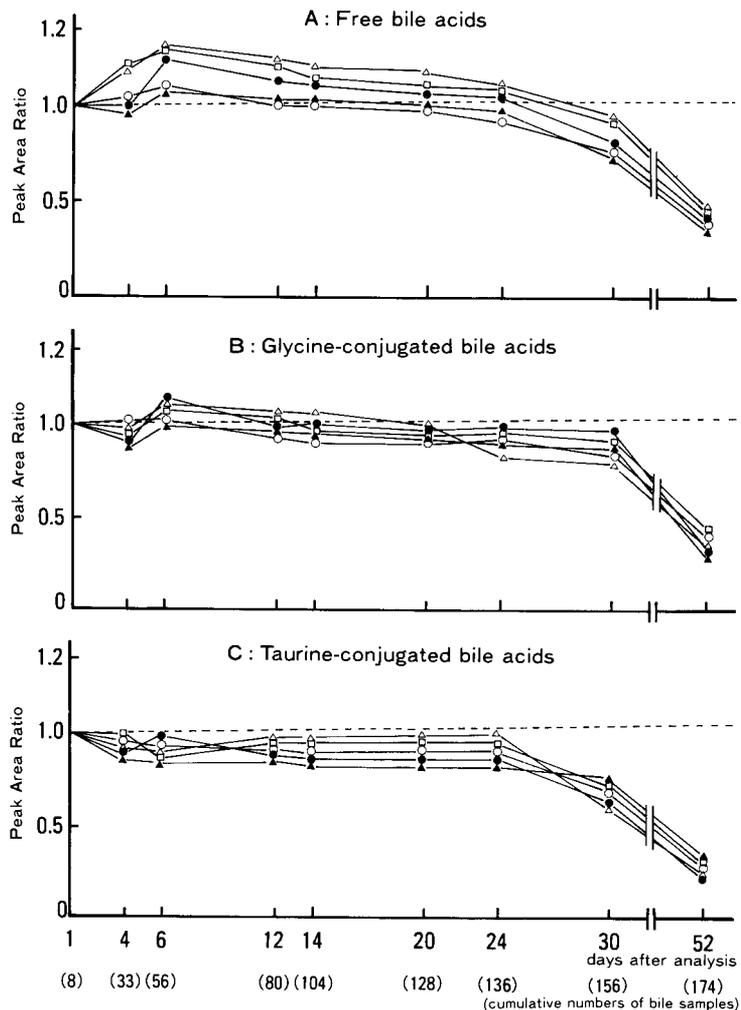
**Fig. 4** Chromatograms of the bile acids in human gallbladder bile. Bile acids from human gallbladder bile were pretreated and analyzed as described in "Materials and Methods". A, free bile acids, from 1  $\mu$ l bile; B, glycine-conjugated bile acids, from 0.05  $\mu$ l bile; C, taurine-conjugated bile acids, from 0.2  $\mu$ l bile. 1, ursodeoxycholic acid (UDCA); 2, cholic acid (CA); 3, chenodeoxycholic acid (CDCA); 4, deoxycholic acid (DCA); 5, lithocholic acid (LCA).



**Fig. 5** Correlation between the total bile acid amounts measured by the present high-performance liquid chromatography system and the Enzabile kit. Thirty human bile materials were analyzed as described in "Materials and Methods". The regression equation was  $y = 0.662x - 0.09$ . The correlation coefficient was 0.93,  $p < 0.05$ .



**Fig. 6** Stability of  $3\alpha$ -hydroxysteroid dehydrogenase ( $3\alpha$ -HSD) column. Using one  $3\alpha$ -HSD column, the analysis was performed 522 times with 174 human bile materials in 52 days. On day 1, 4, 6, 12, 14, 20, 24, 30 and 52, standard bile acids were analyzed to determine the deterioration of the  $3\alpha$ -HSD column. The peak area on day 1 was taken as 1.0. ○, ursodeoxycholic acid (UDCA); ●, cholic acid (CA); △, chenodeoxycholic acid (CDCA); ▲, deoxycholic acid (DCA); □, lithocholic acid.



The stability curves for the three forms of the bile acids were essentially the same.

## Discussion

Mashige and coworkers recently used  $3\alpha$ -HSD to determine total serum bile acid content (22). This enzymatic method was then employed to determine serum bile acids fractionated by HPLC (13). Although useful in terms of specificity and sensitivity, a considerable amount of purified  $3\alpha$ -HSD was consumed in this method. To overcome this disadvantage and to analyze bile acids in

human bile, Okuyama *et al.* immobilized  $3\alpha$ -HSD in column and connected to a HPLC (14). However, HPLC with a  $3\alpha$ -HSD column as modified by Okuyama (16) still had the following disadvantages: 1) as the  $3\alpha$ -HSD immobilized in column deteriorated quickly, stable and reproducible assays on many materials were difficult, and 2) the major 15 bile acid components, especially tauro-UDCA and glyco-DCA, were not separated satisfactorily. In the present studies, pretreatment of human bile through a Seppak C18 cartridge and a PHP-LH-20 column resulted in the elimination of contaminants

that might harm the 3 $\alpha$ -HSD column. Furthermore, prefractionation of bile acids by a PHP-LH-20 column made possible the clear separation of the major 15 bile acids and their accurate analysis by HPLC. Although 3 times of HPLC analysis were needed to determine the major 15 components by our method, the time needed for analyzing one material was about 60 min comparable to that reported by Okuyama *et al.* (16).

Another modification in the present study was the change of the mobile phase for HPLC. Originally, ammonium carbonate and acetonitrile were used (14) and then substituted by potassium phosphate buffer and acetonitrile for better separation of bile acids (16). We found that acetonitrile severely damaged the 3 $\alpha$ -HSD column, and used ethanol in place of acetonitrile in the present system.

In the present study, the 3 $\alpha$ -HSD column was kept under well-controlled conditions: cooled to 4°C and washed thoroughly immediately after the analysis with the enzyme-column buffer. With these procedures, the 3 $\alpha$ -HSD column became stable and more than 400 biles could be analyzed.

The total bile acid amount calculated from the individual bile acid amounts determined by HPLC using a 3 $\alpha$ -HSD column was previously reported to be less than the actual bile acid amount (23). However, the total bile acid amount determined by the present system was larger than that determined directly by the Enzabile kit. We believe that the total amount determined by the present analytical system reflects the actual bile acid amount in human bile, because 1) as observed in the present studies, the known amount of standard bile acids analyzed was almost completely recovered after the passage through a Seppak C18 cartridge and a PHP-LH-20 column, and 2) bilirubin and/or unknown materials in bile, which might interfere with 3 $\alpha$ -HSD, were also removed

by the pretreatments and HPLC (24).

Thus all the results of the present study suggest that the method reported here is useful for the analysis of bile acids in many biological materials including human plasma.

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