Assay of free and glycine- and taurine-conjugated bile acids in serum by high-pressure liquid chromatography by using post-column reaction after group separation

Shoju ONISHI,* †Susumu ITOH* and Yasuo ISHIDA‡

*Department of Pediatrics, Kagawa Medical School, Mikicho, Kitagun, Kagawa 760-07, Japan, and

‡Shimadzu Seisakusho Ltd., Nakagyoku, Kyoto 604, Japan

(Received 3 November 1981/Accepted 11 January 1982)

An accurate and sensitive method that involves the group separations of serum bile acids (i.e. free and glycine- and taurine-conjugated bile acid fractions) by ion-exchange chromatography on piperidinoxypropyl-Sephadex LH-20 is described. Each group was then analysed by high-pressure liquid chromatography by using the post-column reaction technique with immobilized 3 α -hydroxy steroid dehydrogenase. The bile acid patterns in the umbilical venous serum samples were analysed by this method. Taurochenodeoxycholate predominated in the umbilical blood.

The study of the bile acids in biological fluids of patients exhibiting hepatobiliary disease has increased in importance with the availability of newer methods. In recent years considerable attention has been focused on the correlation between the serum concentration of bile acids and liver function. The most commonly used methods for serum bile acid patterns involve the hydrolytic cleavage of conjugates and subsequent analysis by g.l.c. Such procedures have inevitable disadvantages, such as the loss of information about the conjugated forms (Eneroth & Sjoevall, 1971; Alme *et al.*, 1977). In 1976, an h.p.l.c. method for assay of individual bile acids was reported (Okuyama *et al.*, 1976; Shaw & Elliott, 1976). Since then various attempts have been made to improve the h.p.l.c. method (Baba *et al.*, 1978; Okuyama *et al.*, 1979; Arisue *et al.*, 1980). The present paper describes an accurate and sensitive method for determination of free and conjugated bile acids in serum that involves separation into three groups (free and glycine- and taurine-conjugated bile acid fractions) by ion-exchange chromatography on PHP-Sephadex LH-20 and the subsequent h.p.l.c. separation of each group using post-column reaction technique by immobilized 3 α -hydroxy steroid dehydrogenase.

Materials and methods

Apparatus and principle

The flow diagram used is shown in Fig. 1. A Shimadzu LC-3A high-pressure liquid chromato-

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; PHP, piperidinoxypropyl.

† To whom reprint requests should be sent.

graph was used as pump 1 and a Shimadzu PRP-1 pump as pump 2. The sample injector used was a Shimadzu SIL-1A model. A DuPont Zorbax C₈ column (25 cm $\times$ 4.6 mm) was used in a Shimadzu CTO-2A oven and kept at 40°C. The mixing coil and the enzyme column was placed in a water bath at 20°C. The detector was a Shimadzu RF-500 LCA fluorescence spectromonitor, which has a flow-through cell with an inner volume of 14 μ l. The excitation wavelength was set at 350 nm and the emission wavelength at 460 nm. The data processor used was a Chromatopac C-R1A model. The immobilized 3 α -hydroxy steroid dehydrogenase (100 mg) was packed in a stainless-steel column (5 cm $\times$ 2.1 mm). The concentration of each bile acid was estimated by the fluorescence intensity of NADH that was generated within 10 s from NAD⁺ in the presence of immobilized 3 α -hydroxy steroid dehydrogenase in proportion to the amounts of bile acid. The fluorescence intensity of NADH was measured by the aforementioned fluorescence spectromonitor. The concentration of each bile acid was calculated by the measurement of peak height by using a Shimadzu Chromatopac C-R1A and by the recovery of each bile acid.

Except when in use, the enzyme column should be disconnected and sealed at both ends of the column with screws and preserved at 4°C. Before the disconnection of the enzyme column, it should be filled only with the reaction reagent, i.e. the NAD⁺ solution, to protect the enzyme from inactivation.

Chromatographic operation

The mobile phase of h.p.l.c. used for separation of each fraction of free and glycine- and taurine-

Fig. 1. Schematic flow diagram of the system (a) and outline of procedure for analysis of bile acid profiles in serum (b)

conjugated bile acids was 100 mM-potassium phosphate buffer (pH 7.5)/ethanol/methanol (5:4:1, by vol.). The flow rate of pump 1 was 0.6 ml/min. Column pressure was 50–60 kg/cm². Column temperature was 40°C. The reaction reagent was 1 mM-NAD⁺ in 0.1 M-Tris/HCl buffer (pH 8.5), containing 6.5 mM-dithiothreitol. NAD⁺ should be dissolved in the buffer just before use. The flow rate of pump 2 was 1.2 ml/min.

Sample preparation for h.p.l.c.

The sample preparation was performed by the procedure modified from the method of Kimura *et al.* (1979). A serum sample (0.5 ml) was diluted by 0.1 M-NaOH/0.9% NaCl (4.5 ml), mixed gently for 30 s and applied to a Sep-pak C₁₈ column. After washing with water (30 ml), the bile acid fraction was eluted by 90% ethanol (4 ml). The solution was evaporated and the residue was dissolved in 90% ethanol (2 ml). The solution was applied to a column of PHP-Sephadex LH-20 (acetate form; 100 mg; 18 mm × 6 mm) for group separation. After washing with 90% ethanol (2 ml), stepwise elution for group separation was carried out at a flow rate of 6 ml/min. The free and glycine- and taurine-conjugated bile acid fractions were eluted with 0.1 M-acetic

acid in 90% ethanol (4 ml), 0.2 M-formic acid in 90% ethanol (4 ml) and 0.3 M-acetic acid/potassium acetate (pH 6.5) in 90% ethanol (4 ml) respectively. The only taurine-conjugated bile acid fraction was evaporated and the residue was dissolved in water (2 ml), applied to Sep-pak C₁₈ column rewash with water (5 ml) and then eluted by ethanol (5 ml). Each fraction was evaporated *in vacuo* at 30°C and the residue was dissolved in the mobile phase (200 μ l) and then 100 μ l of it was taken for h.p.l.c. as shown in Fig. 1.

Ageing of Sep-pak C₁₈

Sep-pak C₁₈ was immersed in methanol overnight. Semi-transparent Sep-pack C₁₈ was successively washed with ethanol (100 ml), chloroform (10 ml), ethanol (10 ml) and water (10 ml).

Preparation of PHP-Sephadex LH-20

The preparation of PHP-Sephadex LH-20 was performed by the method of Goto *et al.* (1978). A suspension of chlorohydroxypropyl-Sephadex (27.2 g) in piperidine (100.5 ml) was stirred at room temperature for 30 min. After addition of KOH (5.74 g) in methanol (302 ml) the mixture was heated at 50–60°C for 3 h with occasional shaking. The resulting product was collected by filtration, washed with aq. 50% ethanol and 90% ethanol successively, until the washing became neutral. PHP-Sephadex LH-20 thus prepared showed an exchange capacity of 0.5 m-equiv./g and was stored in 90% ethanol in the acetate form.

Immobilization

The 3 α -hydroxy steroid dehydrogenase was immobilized with glutaraldehyde on aminated porous glass beads made by Sanyo Chemical Inc., Higashiyamaku, Kyoto, Japan by the method of Weetall (1976). The glass beads were about 100 mesh in size, 200 m²/g in surface area, and 10 nm in pore size. The enzyme was immobilized at a ratio of 46 mg/g of glass beads with a yield of 69.5%.

Recovery test

The test samples were prepared by adding approx. 1.5 or 10 nmol each of free and glycine- and taurine-conjugated cholate, chenodeoxycholate and deoxycholate to human serum (0.5 ml). The recovery of bile acids was estimated by the standard procedure.

Reagents and chemicals

Free and glycine- and taurine-conjugated cholate, chenodeoxycholate, deoxycholate and lithocholate were purchased from Sigma Chemical Co. and purified before use. Sephadex LH-20, Sep-pak C₁₈ and immobilized-enzyme column were purchased from Pharmacia Fine Chemicals, Waters Asso-

Fig. 2. H.p.l.c. scan of each group of authentic bile acids

(a) shows the chromatographic scan of free bile acids; (b) and (c) show the scans of glycine- and taurine-conjugated bile acids respectively. Abbreviations used: CA, cholate; CDCA, chenodeoxycholate; DCA, deoxycholate; LCA, lithocholate; a G or T prefix before the aforementioned abbreviations indicates the glycine or taurine conjugate respectively.

Fig. 3. Linearity of detection for individual bile acids
For definition of the abbreviations, see the legend to Fig. 2.

ciates and Shimadzu respectively. Other chemicals were obtained from commercial sources and were of the highest purity available.

Results

The chromatograms of standard samples of each free and glycine- and taurine-conjugated bile acid are shown in Fig. 2. The individual bile acids in each fraction were easily separated into the components by reversed-phase h.p.l.c. with the isocratic mobile phase. The fluorescence intensity of enzymically generated NADH had a tendency to decrease with higher ethanol content in the buffer. Under our chromatographic conditions, the influences of reagent pH and enzymic reaction temperature on fluorescence intensity were examined, showing the maximum value at pH 8.5 and 25°C respectively. The time required for the reaction to be completed in the immobilized-enzyme column was about 10 s. The detection limit was about 10 ng. The recovery of the free and glycine- and taurine-conjugated bile acids was 73–97% (Table 1). The reproducibility of our method was satisfactory with a standard deviation of 0.8–6.1% in healthy serum. Linear relationships were found between peak heights and quantities of bile acids up to 2 μg (Fig. 3). The method was applied to normal human samples from umbilical-cord blood. As shown in Fig. 4, our h.p.l.c. method showed the presence of taurochenodeoxycholate, glycochenodeoxycholate, taurocholate, glyco-

Table 1. *Recovery test for free and conjugated bile acids added to serum*
The values represent mean recovery rates (%) ($n = 3$).

Compound	Recovery rate (%)		
	Free	Glycoconjugate	Tauroconjugate
Cholate	90	92	93
Chenodeoxycholate	93	97	93
Deoxycholate	93	94	90
Lithocholate	86	77	73

Fig. 4. *H.p.l.c. scan of each group of bile acids in umbilical venous serum*

(a) shows the chromatographic scan of free bile acids. (b) and (c) show the scans of glycine- and taurine-conjugated bile acids respectively. For definition of the abbreviations, see the legend to Fig. 2.

cholate, chenodeoxycholate, cholate and deoxycholate in normal umbilical serum in the order of concentrations. The pattern agreed well with those previously reported in the literature (Laatikainen, 1977; Barbara *et al.*, 1980).

Discussion

The lack of unsaturation or aromaticity in the bile acid structure, complicates its detection considerably in h.p.l.c. Baba *et al.* (1978) described h.p.l.c. analysis of individual bile acids, using

3 α -hydroxy steroid dehydrogenase in solution. This method is suitable with respect to specificity, but consumes considerable amounts of enzyme. Okuyama *et al.* (1979) attempted to use immobilized enzyme. Their system has advantages when compared with the method using soluble enzyme. A linear-gradient elution was used to separate individual bile acids. Since the enzymic reaction in immobilized enzyme proceeds in the mobile phase, which changes with time, reliability and reproducibility become serious problems with such a technique. Resolution of the common bile acids

glycodeoxycholate and taurochenodeoxycholate by the gradient-elution techniques is difficult, and the span of life of the immobilized enzyme column is short. In contrast, although the sample preparation is more complicated, these problems were eliminated in the present method. It is noteworthy, however, that the 3 β -hydroxy-5-cholanoate, the important bile acid in the perinatal period (Alme *et al.*, 1977), is not detected by the present method.

This work was supported in part by Research Grant nos. 487097 and 480193 from the Ministry of Education of Japan.

References

- Alme, B., Bremmelgaard, A., Sjoevall, J. & Thomassen, P. (1977) *J. Lipid Res.* **18**, 339–362
- Arisue, K., Ogawa, Z., Kohda, K., Hayashi, C. & Ishida, Y. (1980) *Rinsho Kagaku* **9**, 104–110
- Baba, S., Suminoe, K., Uenoyama, R. & Kamenno, Y. (1978) *Med. Biol.* **97**, 219–223
- Barbara, L., Lazzari, R., Roda, A., Aldini, R., Festi, D., Sama, C., Moeselli, A. M., Collina, A., Bazzolli, F., Mazzella, G. & Roda, E. (1980) *Pediatr. Res.* **14**, 1222–1225
- Eneroth, P. & Sjoevall, J. (1971) in *The Bile Acids* (Nair, P. P. & Kritchevsky, D., eds.), vol. 1, pp. 121–171, Plenum Press, New York
- Goto, J., Hasegawa, M., Kato, H. & Nambara, T. (1978) *Clin. Chim. Acta* **87**, 141–147
- Kimura, H., Suzuki, N., Sato, T., Goto, J. & Nambara, T. (1979) *Rinsho Kagaku* **8**, 126–130
- Laatikainen, T. (1977) *Scand. J. Clin. Lab. Invest.* **37**, 605–608
- Okuyama, S., Uemura, D. & Hirata, Y. (1976) *Chem. Lett.* 679–682
- Okuyama, S., Kokubun, N., Higashidate, S., Uemura, D. & Hirata, Y. (1979) *Chem. Lett.* 1443–1446
- Shaw, R. & Elliott, W. H. (1976) *Anal. Biochem.* **74**, 273–281
- Weetall, H. H. (1976) *Methods Enzymol.* **44**, 134–148