Journal of Chromatography, 258 (1983) 125–134
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,518

MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS OF BILE ACIDS

TOYOHIDE TAKEUCHI*, SHINJI SAITO and DAIDO ISHII
Department of Applied Chemistry, Faculty of Engineering, Nagoya University, Chikusa-ku, Nagoya-shi 464 (Japan)
(Received November 12th, 1982)

SUMMARY

Separations of bile acids by micro high-performance liquid chromatography (micro-HPLC) using an immobilized 3α-hydroxysteroid dehydrogenase post-column and a spectrofluorimeter are described. The sensitivity of detection is greatly increased by pre-mixing β-nicotinamide adenine dinucleotide with the mobile phase, using a single pump. The detection limit is 0.04–0.7 ng, depending on the solute. Instruments suitable for the analysis of bile acids in micro-HPLC are discussed.

INTRODUCTION

The determination of bile acids in body fluids is important as the abnormal presence of bile acids in body fluids reflects a functional disorder of the liver. Many methods for analysing individual bile acids have been reported, *e.g.*, gas chromatography[1], gas chromatography–mass spectrometry[2,3], thin-layer chromatography[4] and high-performance liquid chromatography (HPLC)[5–14]. HPLC analysis of bile acids using 3α-hydroxysteroid dehydrogenase (3α-HSD) post-column derivatization[10,14] seems to be very promising compared with other methods with respect to resolution, sensitivity and quantitation.

By the use of immobilized enzyme, covalently bound to aminopropyl-controlled pore glass (CPG), the running costs are substantially reduced compared with the use of an enzyme solution for post-column derivatization. The 3α-hydroxy group in each bile acid is oxidized to a keto group by the enzyme reaction, while β-nicotinamide adenine dinucleotide (NAD) is reduced to NADH, which is subjected to fluorimetric detection. Kamada *et al.*[14] reported the reaction of phenazine methosulphate with the NADH produced by the enzyme reaction, followed by electrochemical detection.

In this paper, the micro-HPLC analysis of bile acids is examined and instruments suitable for micro-HPLC using a 3α-HSD-immobilized post-column and a spectrophotometer are discussed.

0021-9673/83/0000–0000/$03.00

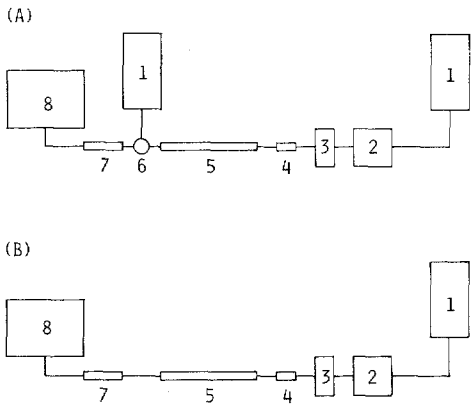

Fig. 1. Schematic diagrams of the systems: (A) post-column mixing system; (B) pre-mixing system. 1 = Pump (Micro Feeder); 2 = gradient equipment; 3 = micro valve injector; 4 = guard column; 5 = separation column; 6 = T-piece; 7 = immobilized enzyme column; 8 = spectrophotofluorimeter.

EXPERIMENTAL

Schematic diagrams of the systems examined are illustrated in Fig. 1. Fig. 1A shows the post-column mixing system, assembled from two Micro Feeder (Azuma-denki Kogyo, Tokyo, Japan) equipped with a gas-tight syringe for pumping the mobile phase and the reaction solution, a home-made gradient equipment, a micro valve injector (0.02 μl) (JASCO; Japan Spectroscopic, Tokyo, Japan), a guard column, a separation column, a T-piece, an immobilized enzyme column and an FP-110C spectrophotofluorimeter (JASCO). Fig. 1B shows the pre-mixing system in which NAD is mixed with the mobile phase and consequently a single pump is employed. The latter system is intended to reduce the noise arising from pulsation based on post-column mixing, leading to an increase in sensitivity.

Gradient elution is necessary to elute individual bile acids in a reasonable time. Recently, we have developed a simple continuous gradient elution technique for micro-HPLC[15], using a single pump and a mixing vessel, which is applicable to the separation of bile acids. The gradient profile is exponential, depending on the volume of the mixing vessel and the flow-rate of the mobile phase. The volume of the mixing vessel employed was 109 μl.

Fused-silica micro-packed columns, 100–250 × 0.26 mm I.D., packed with Bilepak (5 μm, JASCO) or silica ODS SC-01 (5 μm, JASCO) were employed as separation columns. Fused-silica tubing is a good column material owing to its smooth and inert surface and mechanical strength, leading to high efficiency[16]. The columns were packed manually as described elsewhere[17]. A guard column composed of PTFE tubing, *ca.* 10 × 0.2 mm I.D., was packed with the same packing as the separation column.

Immobilized enzyme columns were prepared according to a method reported elsewhere[17]. A 0.2-g amount of Aminopropyl-CPG (200–400 mesh, mean pore diameter 515 Å) (Electro-Nucleronics, NJ, U.S.A.) was added to 2 ml of 2.5% glutaraldehyde in aqueous solution (Wako, Osaka, Japan). The solution was degassed at room temperature (*ca.* 25°C) for 30 min and left at room temperature for 1 h. After

washing with distilled water, 5 mg of hydroxysteroid dehydrogenase (Sigma; MO, U.S.A.) in 1 ml of 10 m*M* potassium dihydrogen orthophosphate solution (pH 7.8) were added and the mixture was degassed for 30 min in an ice-bath. The reaction was allowed to proceed at 1 atm for 30 min in the ice-bath. The products were washed with distilled water and stored in 10 m*M* potassium dihydrogen orthophosphate solution (pH 7.8) containing 0.05% of 2-mercaptoethanol (Wako) and 1 m*M* disodium ethylenediaminetetraacetate (EDTA) (Wako), cooled in a refrigerator. Portions were packed into 20 × 0.34–0.5 mm I.D. fused-silica or PTFE tubing.

Ursodeoxycholic acid, cholic acid, chenodeoxycholic acid, deoxycholic acid and lithocholic acid were obtained from Sigma and glycine conjugates and taurine conjugates were kindly supplied by JASCO and Nipponkayaku (Tokyo, Japan). Bile acids were dissolved in ethanol and 0.02 μl of the solution was injected by the micro valve injector with good reproducibility.

NAD (Sigma) was dissolved in 10 m*M* potassium dihydrogen orthophosphate solution (pH 7.0) containing 0.05% of 2-mercaptoethanol and 1 m*M* EDTA.

In micro-HPLC, band spreading of the solute in extra-column components, such as the injector, connecting parts, the mixing T-piece, the post-column and the detector should be controlled. Band broadening in the injector was negligibly small when using the micro valve injector[18]. Fused-silica tubing of 50–80 μm I.D. (Scientific Glass Engineering, Melbourne, Australia) was employed as connecting tubing together with stainless-steel tubing (0.13 mm I.D. and 0.31 mm O.D.) and PTFE tubing (0.2 mm I.D. and 2 mm O.D.).

The structure of the home-made mixing T-piece is illustrated in Fig. 2, broadening being minimized by the use of narrow-bore tubing.

The micro flow cell for the FP-110C that is commercially available is unsuitable for micro-HPLC using narrow-bore fused-silica separation columns. A micro flow cell suitable for this work was therefore prepared. Quartz tubing of *ca.* 0.2 mm I.D. and *ca.* 0.4 mm O.D. mounted on the cell holder was used as the flow cell and was connected to the post-column with narrow-bore fused silica tubing (50 μm).

Other reagents were obtained from Wako, unless stated otherwise.

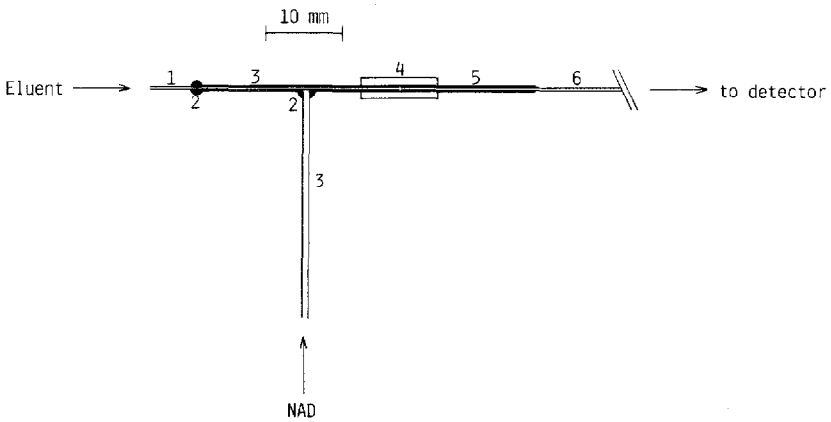

Fig. 2. Structure of a mixing T-piece. 1 = Stainless-steel tubing, 0.13 mm I.D. and 0.31 mm O.D.; 2 = silver solder; 3 = stainless-steel tubing, 0.33 mm I.D. and 0.63 mm O.D.; 4 = PTFE tubing, 0.5 mm I.D. and 2 mm O.D.; 5 = stainless-steel tubing, 0.33 mm I.D. and 0.63 mm O.D.; 6 = fused-silica tubing, 0.05 mm I.D. and 0.3 mm O.D.

RESULTS AND DISCUSSION

Post-column mixing system

The sensitivity of the spectrophotofluorimeter is dependent on the strength of the light source and the volume of the flow cell. The cell volume should be reduced so as not to cause additional band broadening in the flow cell. However, as the cell volume decreases, the sensitivity is also liable to decrease. Therefore, both the volume and the dimensions of the flow cell should be designed to take into account both the sensitivity and the additional band broadening. Quartz-tubing flow cells of various dimensions were prepared and their performances were examined. A flow cell of *ca.* 0.2 mm I.D. was suitable for micro-HPLC using fused-silica micro packed columns of 100–250 × *ca.* 0.25 mm I.D. The thickness of the tubing of the flow cell slightly affected the sensitivity, thick tubing having a tendency to give higher sensitivity.

Table I compares the dimensions and the performances of home-made and commercial flow cells. A 0.01% solution of fluoranthene in acetonitrile was filled in each flow cell set in the fluorimeter and the response was measured. The relative sensitivity increased with increasing cell volume, whereas the relative sensitivity to unit weight of fluoranthene was variable. The highest relative sensitivity to unit weight of solutes was achieved with the home-made flow cell, which suggests that the sensitivity will increase when using a home-made flow cell appropriate to the dimensions of the separation column.

As bile acids elute at higher concentrations from the separation column in micro-HPLC than in conventional HPLC, the concentration of NAD, which affects the post-column reaction, was studied. The dependence of peak height on the concentration of NAD and flow-rate of NAD solution is shown in Fig. 3. The flow-rate of the NAD solution was varied from 1 to 4 μl/min, that of the mobile phase being kept constant at 2.1 μl/min. As the flow-rate of the NAD solution was increased, the peak height was liable to decrease, owing to diffusion of the solute in the post-column. This suggests that the lower the flow-rate of the NAD solution, the higher is the observed response. However, a high ratio of the flow-rate of the NAD solution to that of the mobile phase led to non-uniform mixing, owing to pulsation of the pump. Thus, a desirable flow-rate of the NAD solution is around half of that of the mobile phase. The concentration of NAD that gives a maximum peak height was

TABLE I

COMPARISON OF THE DIMENSIONS AND PERFORMANCES OF HOME-MADE AND COMMERCIAL FLOW CELLS

Sample: 0.01% solution of fluoranthene in acetonitrile. Wavelength of detection: excitation 365 nm, emission 420 nm.

Flow cell	*Dimensions*				*Sensitivity**	
	I.D. (mm)	*O.D. (mm)*	*Length (mm)*	*Volume (μl)*	*A*	*B*
Home-made	0.18	0.4	3.3	0.084	100	100
For micro-HPLC	0.33	1.5	4.2	0.36	113	26
For HPLC	1.6	4.4	6.0	12	1560	11

* A, Relative sensitivity; B, relative sensitivity to unit weight of fluoranthene.

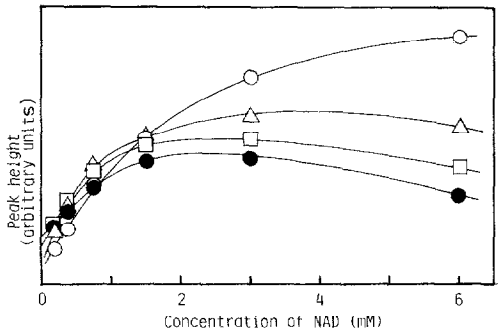

Fig. 3. Dependence of peak height on concentration of NAD and flow-rate of NAD solution. Mobile phase: acetonitrile–10 m*M* potassium dihydrogen orthophosphate (pH 7.8) (3:7). Flow-rate of mobile phase: 2.1 μl/min. Immobilized enzyme column: 22 × 0.5 mm I.D. Sample: 47 ng of cholic acid. Flow-rates of NAD solutions: ○, 1 μl/min; △, 2 μl/min; □, 3 μl/min; ●, 4 μl/min. Wavelength of detection: excitation 365 nm, emission 470 nm.

dependent on flow-rate. The higher the flow-rate, the lower was the concentration giving the maximum peak height. When using 12 m*M* NAD the peak height was considerably reduced, the reason for which was uncertain. Operating conditions of 6 m*M* and 1 μl/min give the highest peak.

The dimensions of the post-column and the particle diameter of the immobilized enzyme packings also affected the peak height. Post-columns of 0.34 and 0.5 mm I.D. packed with immobilized enzyme packings of 200–400 and 120–200 mesh were examined, and 0.34 mm I.D. and 200–400 mesh gave the highest sensitivity. If CPG glass with a smaller particle diameter (10–20 μm) were to be used the sensitivity would be even higher. However, 200–400 mesh is the smallest particle size that is commercially available.

A typical separation of bile acids is shown in Fig. 4. The flow-rates of the mobile phase and NAD solution were 1.4 and 0.69 μl/min, respectively. The resolution of some bile acids was unsatisfactory, which could be improved by investigating the gradient profile and the mobile phase itself. Drift of the baseline due to pulsation was observed.

Pre-mixing system

As the NAD solution contains 2-mercaptoethanol and EDTA and its pH is 7.0. the addition of NAD solution to the mobile phase affects the retention of bile acids. The retention of bile acids is strongly dependent on the type and composition of the organic solvent, the type and concentration of buffers, pH, concentration of salts, etc.

When using methanol and ethanol as the mobile phase, the proportion of organic solvent is too high (*ca.* 50%) to elute the solutes in a reasonable time, leading to a decrease in peak height. This is probably due to a decrease in the reaction efficiency and deactivation of enzyme packings. Acetonitrile overcame this drawback as its smaller proportion in the mobile phase led to elution of the bile acids in a reasonable time.

The effects of addition of a salt to the mobile phase on retention and peak area were examined by using an ODS column. The results are shown in Table II. The

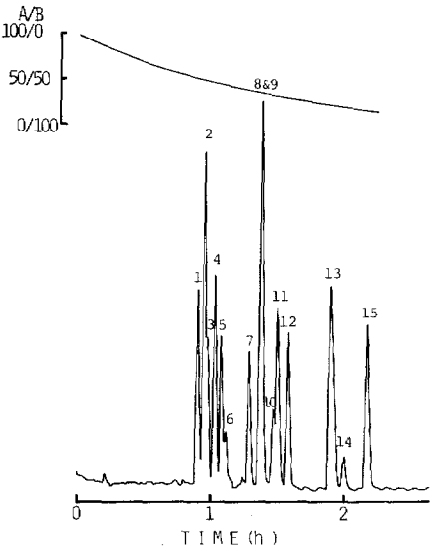

Fig. 4. Separation of bile acids by the post-column mixing system. Separation column: Bilepak, 250 × 0.26 mm I.D. Mobile phase: gradient profile as indicated of acetonitrile–30 m*M* (A) or 10 m*M* (B) potassium dihydrogen orthophosphate (pH 7.8) in the ratios (A) 18:82 and (B) 35:65. Flow-rate of the mobile phase: 1.4 μl/min. Immobilized column: 3α-HSD, 20 × 0.34 mm I.D. Reaction solution: 10 m*M* potassium dihydrogen orthophosphate containing 6 m*M* NAD, 0.05% 2-mercaptoethanol and 1 m*M* EDTA (pH 7.0). Flow-rate of the reaction solution: 0.69 μl/min. Samples: 1 = 40 ng of ursodeoxycholic acid (UDC); 2 = 44 ng of cholic acid (C); 3 = 40 ng of glycoursodeoxycholic acid (GUDC); 4 = 40 ng of glycocholic acid (GC); 5 = 44 ng of tauroursodeoxycholic acid (TUDC); 6 = 32 ng of taurocholic acid (TC); 7 = 48 ng of chenodeoxycholic acid (CDC); 8 = 40 ng of deoxycholic acid (DC); 9 = 48 ng of glycochenodeoxycholic acid (GCDC); 10 = 32 ng of glycodeoxycholic acid (GDC); 11 = 48 ng of taurochenodeoxycholic acid (TCDC); 12 = 44 ng of taurodeoxycholic acid (TDC); 13 = 44 ng of lithocholic acid (LC); 14 = 44 ng of glycolithocholic acid (GLC); 15 = 44 ng of taurolithocholic acid (TLC). Wavelength of detection: excitation 365 nm, emission 470 nm.

retention of bile acids is not dependent on the type of salt but on pH. The retention of lithocholic acid decreases with increasing pH. Table II also indicates that the peak area increases with increasing pH, which may be due to an increase in the efficiency of the post-column reaction. The addition of a salt to the mobile phase affected the response.

The selectivity of bile acids was improved by addition of ammonium carbonate to the mobile phase. Table III shows the variation of retention with pH. The retention of free bile acids decreases with increasing pH, whereas that of conjugate bile acids increases slightly with increasing pH. This suggests that the selectivity can be improved by the selection of a suitable pH.

Fig. 5 demonstrates separations of free, glycine-conjugate and taurine-conjugate bile acids. The sensitivity is greatly increased, 5 ng of each solute being detectable. The detection limit was 0.04–0.2 ng for a signal-to-noise ratio of 2, depending on the solute. This is mainly due to the absence of pulsation. The reaction efficiency was dependent on the structure of bile acids. Although each type of ursodeoxycholic acid and cholic acid is not resolved, they will be resolved by gradient elution.

Figs. 6 and 7 demonstrate separations of fifteen bile acids on a phenylethyl

TABLE II

EFFECT OF SALTS ON RETENTION AND SENSITIVITY

Column: silica ODS SC-01, 100 × 0.26 mm I.D. Mobile phase: acetonitrile–10 m*M* potassium dihydrogen orthophosphate (pH 7.8)–NAD solution (32:38:30) plus the salt indicated. [NAD solution = 10 m*M* potassium dihydrogen orthophosphate containing 6 m*M* NAD, 0.05% 2-mercaptoethanol and 1 m*M* EDTA (pH 7.0)]. Sample: lithocholic acid.

Salt	*Concentration*	*pH of eluent*	*Retention volume (µl)*	*Peak area (arbitrary units)*
Ammonium carbonate	0.096%	9.14	28.9	29
Sodium hydrogen carbonate	10 m*M*	7.89	41.5	27
No addition	–	7.52	61.7	16
Ammonium acetate	20 m*M*	7.33	94.4	22
Ammonium oxalate	10 m*M*	7.30	94.4	22

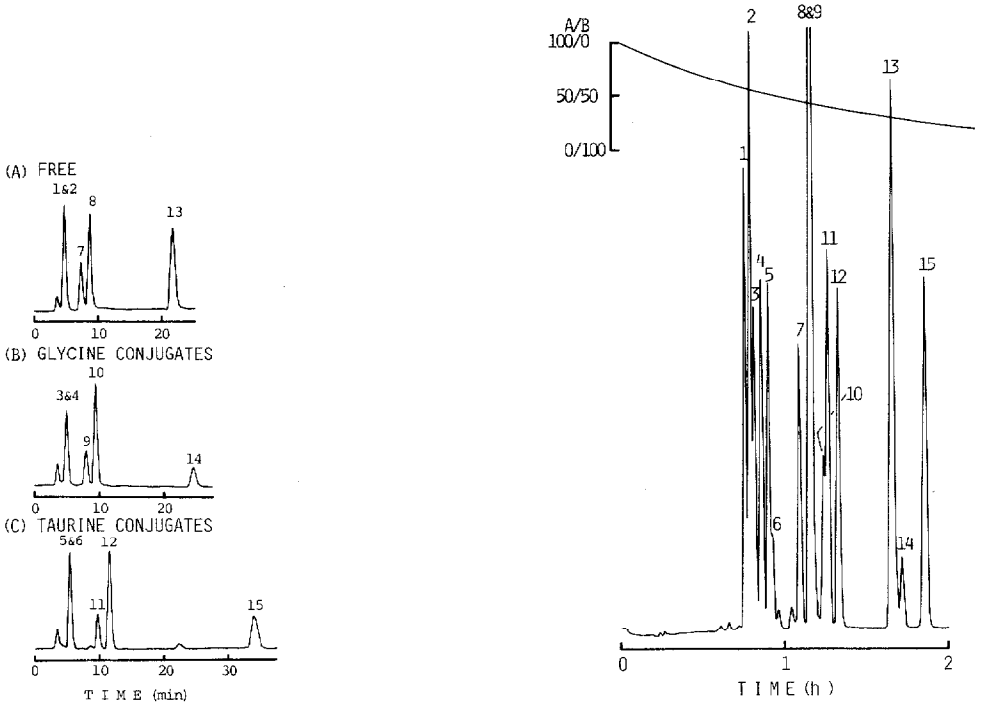

Fig. 5. Separations of bile acids by the pre-mixing system. Column: Silica ODS SC-01, 100 × 0.26 mm I.D. Mobile phase: acetonitrile–10 m*M* potassium dihydrogen orthophosphate (pH 7.8)–10 m*M* potassium dihydrogen orthophosphate (pH 7.0) containing 6 m*M* NAD, 0.05% 2-mercaptoethanol and 1 m*M* EDTA (32:38:30); pH adjusted to 8.1 with ammonium carbonate. Flow-rate: 2.1 µl/min. Immobilized column: 3α-HSD, 20 × 0.34 mm I.D. Sample: 5 ng each; sample numbers as in Fig. 4. Wavelength of detection: excitation 365 nm, emission 470 nm.

Fig. 6. Separation of bile acids by the pre-mixing system. Column: Bilepak, 250 × 0.26 mm I.D. Mobile phase: gradient profile as indicated of acetonitrile–30 m*M* (A) or 10 m*M* (B) potassium dihydrogen orthophosphate (pH 7.8)–10 m*M* potassium dihydrogen orthophosphate (pH 7.0) containing 6 m*M* NAD, 0.05% 2-mercaptoethanol and 1 m*M* EDTA, in the proportions (A) 18:52:30 and (B) 35:35:30, each containing 0.1% ammonium carbonate.

TABLE III

DEPENDENCE OF RETENTION ON pH

Column: silica ODS SC-01, 100 × 0.26 mm I.D. Mobile phase: acetonitrile–10 m*M* potassium dihydrogen orthophosphate (pH 7.8)–NAD solution (32:38:30) [NAD solution: 10 mM potassium dihydrogen orthophosphate containing 6 m*M* NAD, 0.05% 2-mercaptoethanol and 1 m*M* EDTA (pH 7.0)]; the pH was adjusted with ammonium carbonate.

pH	*Retention volume (μl)*											
	*Free**				*Glycosine-conjūgate**				*Taurine-conjugate**			
	UDC + C	*CDC*	*DC*	*LC*	*GUDC + GC*	*GCDC*	*GDC*	*GLC*	*TUDC + TC*	*TCDC*	*TDC*	*TLC*
8.97	8.9	12.2	13.8	30.3	9.0	13.8	16.0	38.7	9.9	16.1	18.7	51.7
8.48	9.0	12.9	14.4	33.6	9.4	13.7	15.6	38.7	9.6	15.5	18.7	51.0
7.92	10.4	14.2	15.2	45.6	8.2	12.9	14.9	39.7	10.1	16.2	18.7	51.7
7.52	10.1	15.9	17.3	59.9	9.1	12.7	14.4	32.6	9.9	14.8	16.8	43.2

* For abbreviations, see Fig. 4.

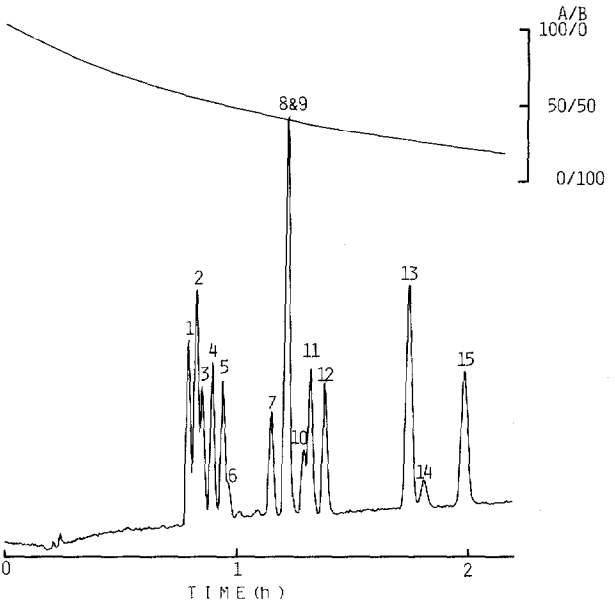

Fig. 7. Separation of bile acids by the pre-mixing system. Operating conditions as in Fig. 6 except the amounts of solutes were one-eighth of those in Fig. 6 (each around 5 ng).

column (Bilepak). The selectivity and sensitivity were improved by the addition of ammonium carbonate, despite the fact that NAD was present in the mobile phase. The detection limit was 0.09–0.7 ng for a signal-to-noise ratio of 2, depending on the solute.

CONCLUSION

By pre-mixing NAD with the mobile phase, using a single pump, the sensitivity of detection in micro-HPLC was increased 10–100-fold compared with that in conventional HPLC using an 3α-HSD immobilized column and a spectrophotofluorimeter. Unsatisfactory resolution of some bile acids will be improved by investigating the mobile phase composition. This technique will be useful for the analysis of bile acids in human body fluids.

ACKNOWLEDGEMENTS

We thank Mr. S. Higashidate (JASCO) and Mr. S. Mizuguchi (Research Laboratories, Pharmaceutical Division, Nipponkayaku) for helpful discussion.

REFERENCES

1 P. P. Nair and C. Garcia, *Anal. Biochem.*, 29 (1969) 164.
2 G. Karlaganis and G. Paumgartner, *Clin. Chim. Acta*, 92 (1979) 19.
3 J. Yanagisawa, M. Itoh, M. Ishibashi, H. Miyazaki and F. Nakayama, *Anal. Biochem.*, 104 (1980) 75.
4 A. K. Batta, G. Salen and S. Shefer, *J. Chromatogr.*, 168 (1979) 557.

5 K. Shimada, M. Hasegawa, J. Goto and T. Nambara, *J. Chromatogr.*, 152 (1978) 431.
6 J. Goto, M. Hasegawa, H. Kato and T. Nambara, *Clin. Chim. Acta*, 87 (1978) 141.
7 S. Okuyama, D. Umemura and Y. Hirata, *Bull. Chem. Soc. Jap.*, 52 (1979) 124.
8 D. P. Matthees and W. C. Purdy, *Anal. Chim. Acta*, 109 (1979) 161.
9 S. Okuyama, D. Umemura and Y. Hirata, *Chem. Lett.*, (1979) 461.
10 S. Okuyama, N. Kokubun, S. Higashidate, D. Umemura and Y. Hirata, *Chem. Lett.*, (1979) 1443.
11 S. Miyazaki, H. Tanaka, R. Horikawa, H. Tsuchiya and K. Imai, *J. Chromatogr.*, 181 (1980) 177.
12 G. Mingrone, A. V. Greco and S. Passi, *J. Chromatogr.*, 183 (1980) 277.
13 F. Nakayama and M. Nakagaki, *J. Chromatogr.*, 183 (1980) 287.
14 S. Kamada, M. Maeda, A. Tsuji, Y. Umezawa and T. Kurahashi, *J. Chromatogr.*, 239 (1982) 773.
15 T. Takeuchi and D. Ishii, *J. Chromatogr.*, 253 (1982) 41.
16 T. Takeuchi and D. Ishii, *J. Chromatogr.*, 213 (1981) 25.
17 H. H. Weetall, *Immobilized Enzymes, Antigens, Antibodies and Peptides*, Marcel Dekker, New York, 1975.
18 T. Takeuchi and D. Ishii, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 469.