Journal of Chromatography, 431 (1988) 27–36
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4298

RAPID AND SENSITIVE ON-LINE PRECOLUMN PURIFICATION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR BILE ACIDS IN SERUM

SHIGERU YOSHIDA*, HITOSHI MURAI and SHINGO HIROSE

Kyoto Pharmaceutical University, Nakauti-cho, Misasagi, Yamashina-ku, Kyoto 607 (Japan)

and

TATSUMORI TAKEDA

Ueno Civic Hospital, Shijuku-cho, Ueno-shi, Mie 518 (Japan)

(First received January 25th, 1988; received manuscript received May 2nd, 1988)

SUMMARY

A simple and rapid technique for the simultaneous isolation and analysis of fifteen kinds of bile acid was developed using reversed-phase high-performance liquid chromatography with an automatic dual-precolumn switching system. The serum samples were directly injected onto a first precolumn (hydroxyapatite), which was flushed with 1 m*M* phosphate buffer. Serum proteins were strongly retained on the hydroxyapatite column, but bile acids were unretained. The bile acids were adsorbed on a second precolumn (Serumout-25®) and eluted onto the analytical column with solvent B (acetonitrile–methanol–30 m*M* ammonium acetate, 20:20:60, v/v/v). For the separation of each bile acid, the gradient elution technique was used (solvent A was acetonitrile–methanol–30 m*M* ammonium acetate, 30:30:40). After separation of the bile acids, NADH was produced by use of immobilized 3α-hydroxysteroid dehydrogenase column and then determined fluorimetrically ($\lambda_{em} = 460$ nm, $\lambda_{ex} = 350$ nm). The recoveries of bile acids in serum generally approached 100%.

INTRODUCTION

The determination of individual bile acids in biological fluids is important in the study of their metabolism in hepatobility and other diseases [1]. In recent years, papers [2–4] on both the methodology and the clinical application of determinations of bile acids have reported contradictory results, primarily because of the complexity of the composition of serum bile acids and their low concentrations. Although the composition has been analysed by several techniques, high-performance liquid chromatography (HPLC) is clearly the method of choice [5,6], especially since fluorimetric detection methods have been introduced [7,8]. When

0378-4347/88/$03.50

gas chromatography was used, the glyco and tauro derivatives could not be identified or determined, and the results obtained always refer to cholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and lithocholic acids.

Although HPLC is well accepted as a method for the assay of bile acids, a sample pretreatment is necessary. Depending on the solute concentration and the detection limits of the method, a second objective may be the preconcentration of the bile acids to improve their detectability.

With the enzymatic methods involving the NAD^+-consuming 3α-hydroxysteroid dehydrogenase (3α-HSD, EC 1.1.1.50) followed by fluorimetric detection, the detection limit of each bile acid ranged from 0.8 to 1.5 ng at a signal-to-noise ratio of 2 [9]. This means that for normal serum, with concentrations of the total bile acids of ca. 1–2.5 μmol/l [1], a preconcentration step is always necessary.

Over the past ten years, the use of hot ethanol [9,10] has been by far the most popular method for the pretreatment of bile acids from body fluids. Good results have also been reported using thin-layer chromatography [4]. Until now, such sample pretreatment has mainly been carried out by off-line techniques, which are usually laborious and can give rise to a poor precision. On-line sample pretreatment is more convenient, while generally the precision of the method is improved. In principle, on-line sample pretreatment can be performed by the dual-precolumn switching system, in which adsorption of the serum proteins and concentration of the bile acids were effected on different columns. A precolumn packed with hydroxyapatite was used for the adsorption of various proteins. The retention behaviour of proteins in hydroxyapatite HPLC was related to their isoelectric points. On the hydroxyapatite column, adsorption of the bile acids does not occur. Dohji et al. [11] investigated the characteristics of a laboratory-synthesized reversed-phase resin (Serumout-25) for the on-line preconcentration of oestrogens and applied it to the assay of oestriol and oestradiol of a pregnant woman. The bile acids were also adsorbed and concentrated on the Serumout-25.

In this paper, we present the results of an investigation of the application of a dual-precolumn packed with hydroxyapatite and Serumout-25, respectively, to the on-line pretreatment of bile acids in serum samples prior to their determination by HPLC with fluorimetric detection.

EXPERIMENTAL

Apparatus

A Model LC-6A liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Model RF-535 spectrofluorimeter (excitation and emission wavelengths set at 350 and 460 nm, respectively), a Model 7125 sample injector (sample loop 20 μl) (Rheodyne, Cotati, CA, U.S.A.), a Model FCV-2AH switching valve (Shimadzu) connected to a Model LC-6A for sample enrichment and a Model FCV-3AL switching valve for sample purification were used for HPLC analysis. The switching system was controlled by a Model SCL-6A control module (Shimadzu). A Model 1003-A pump (Eldex Labs., San Carlos, CA, U.S.A.) was used to mix the enzymatic reagents. Sample purifiction and preconcentration were performed by a hydroxyapatite HP-10, 5-μm precolumn [$CA_{10}(PO_4)_6(OH)_2$, 5

cm × 4.5 mm I.D.] (Pentax, Tokyo, Japan) and by a Serumout-25, 5-μm reversed-phase precolumn (3.5 cm × 4 mm I.D.) (Sekisui Kagaku, Tokyo, Japan), respectively. The analytical column was packed with Bilepack II, 5 μm (12 cm × 4.5 mm I.D.) (JASCO, Tokyo, Japan). Immobilized 3α-HSD, Enzyme-pak (3.5 cm × 4.6 mm I.D.) was purchased from JASCO.

Chemicals and reagents

The different concentrations of bile acid standards were prepared in methanol and normal human serum. Each contained cholic (CA), deoxycholic (DCA), chenodeoxycholic (CDCA), lithocholic (LCA), ursodeoxycholic (UDCA), glycocholic (GCA), glycodeoxycholic (GDCA), glycochenodeoxycholic (GCDCA), glycolithocholic (GLCA), glycoursodeoxycholic (GUDCA), taurocholic (TCA), taurodeoxycholic (TDCA), taurochenodeoxycholic (TCDCA), taurolithocholic (TLCA) and tauroursodeoxycholic (TUDCA) acids (all from Techno Chemical Corporation, Tokyo, Japan). These bile acids were more than 99% pure. All other chemicals were of analytical-reagent or reagent grade and were used without further purification. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.).

Human sera were collected from normal fasting volunteers and patients with various diseases who were admitted to Ueno Civic Hospital.

Stock solutions of bile acids were prepared at concentrations of 220 μg/ml in methanol.

Mobile phases

The mobile phase was filtered and vacuum-degassed before use. Mobile phase I was 1 m*M* phosphate buffer (pH 6.8) used at a flow-rate of 0.8 ml/min. Mobile phase II was solvent A [acetonitrile–methanol–30 m*M* ammonium acetate (pH 6.8), 30:30:40, v/v/v] and solvent B [acetonitrile–methanol–30 m*M* ammonium acetate (pH 6.8), 20:20:60, v/v/v], run as a gradient from 100% B to 100% A in 37 min (linear), and 100% A for 23 min (total analysis time was 60 min). The flow-rate was 1.0 ml/min, the column pressure was 10.78 MPa, and the column temperature was ambient.

Enzymatic reagents

The β-NAD^+ solution introduced in this method was prepared as follows: 0.3 m*M* β-NAD^+ was dissolved in 10 m*M* potassium phosphate buffer (pH 6.8) containing ethylenediaminetetraacetic acid (EDTA) solution (1 m*M*) and 2-mercaptoethanol (0.05%). The flow-rate of the β-NAD^+ solution was 1.0 ml/min. NADH produced was monitored fluorimetrically at 460 nm emission and 350 nm excitation.

Chromatographic system

The experimental set-up as used for on-line sample pretreatment, HPLC analysis of the bile acids and enzymatic reactions is shown in Fig. 1. At time $t = 0$ the sample was injected via valve V_1 and transported to the precolumn C_1 (hydroxyapatite) by pump P_1, which pumped mobile phase I (preconcentration and clean-

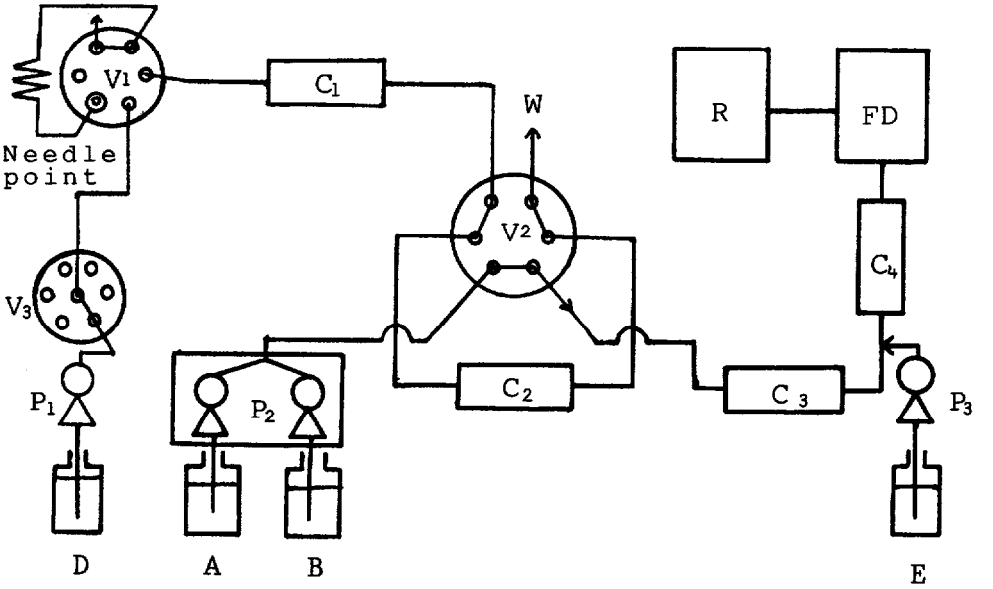

Fig. 1. Configuration of the on-line pre-concentration system. P_1, P_2 and P_3 = pumps; V_1, V_2 and V_3 = switching valves; C_1 = precolumn (hydroxyapatite, on which serum constituents such as proteins were retained strongly); C_2 = precolumn (Serumout-25, on which bile acids were retained and condensed); C_3 = separation column (bile pack); C_4 = immobilized enzyme column (3α-HSD); FD = fluorescence detector; A, B and D = carrier reservoirs; E = enzymatic reagents; R = recorder; W = waste.

up mobile phase) at a flow-rate W_1. On the precolumn C_1, serum proteins were retained very strongly but the bile acids were unretained. The purification and the concentration of the bile acids were performed on the precolumn C_2 (Serumout-25). After a washing step, at time t_1 valve V_2 was switched, and the concentrated bile acids were desorbed from C_2 and injected on the analytical column C_3, where separation was effected; this was achieved with mobile phase II (desorption and separation mobile phase), which was pumped by P_2 at a flow-rate W_2. At time t_2 valve V_2 was switched back in order to recondition C_2 for pretreatment of the next sample. The immobilized 3α-HSD column was connected to the HPLC system as shown in Fig. 1, and the enzymatic reagent was introduced by P_3.

RESULTS AND DISCUSSION

Adsorption and desorption characteristics of serum proteins on hydroxyapatite

A first objective of this study was to obtain an insight into the retention behaviour of serum proteins on the hydroxyapatite column as a function of ion strength. The retention of the proteins on the column was measured by injection of the control serum containing bile acids on a 50 mm × 4.5 mm I.D. column followed by the use of 1–30 m*M* phosphate buffer and UV detection (280 nm) of the column effluent (Fig. 2). At low concentrations of phosphate buffer (1–6 m*M*) proteins were retained very strongly but bile acids were unretained. At 10

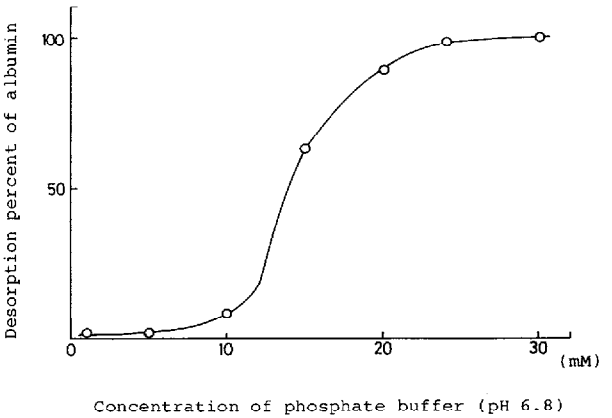

Fig. 2. Desorption ratio of proteins on hydroxyapatite column. Column, 50 mm×4.5 mm I.D.; flow-rate, 0.8 ml/min; UV detector, 280 nm; sample, control serum; sample volume, 2 μl. Serum globulin is desorbed with 100 m*M* phosphate buffer (pH 6.8).

m*M* neither proteins nor bile acids were retained. The results show that 1 m*M* phosphate buffer is the best choice for sample clean-up of the hydroxyapatite column.

Preconcentration of the bile acids

From the large difference in the retention times between the bile acids and serum proteins (1 m*M* phosphate buffer as the mobile phase), the bile acids were eluted from the hydroxyapatite column as very broad tailing peaks. In the preconcentration column (Serumout-25), the bile acids and other hydrophobic components were adsorbed on the porous beads, whereas other serum components such as carboxyhydrates and water-soluble polar compounds were not.

Fig. 3 shows the effectiveness of the preconcentration on the Serumout-25 precolumn as a function of pretreatment time; when the washing volume was 5 ml (flow-rate 1 ml/min) all bile acids were adsorbed quantitatively, so the excellent selectivity of Serumout-25 was demonstrated. However, when the washing volume was very large, e.g., 16 ml, GCA and TCA were eluted from the precolumn.

In order to minimize the contribution of the preconcentration process to peak broadening, it is very important to elute the bile acids from the precolumn in as small a volume as possible. This implies that after valve-switching (V_2) the polarity of the carrier in the precolumn should change instantaneously from 1 m*M* phosphate buffer to mobile phase II, solvent A (acetonitrile–methanol–30 m*M* ammonium acetate, 30:30:40, v/v/v). This was favoured by performing the preconcentration step with a relatively low buffer concentration and the desorption step with a more concentrated buffer containing acetonitrile and methanol.

Three different liquid phases are needed: one for clean-up and preconcentration of the sample, a second for desorption of the bile acids from the precolumn and a third for their separation, for the on-line preconcentration system as described here. Solvent B was used for the desorption. For the separation of individual bile acids, the gradient elution technique was used, with solvent A and solvent B as eluent. A comparison of on-line preconcentration with direct injec-

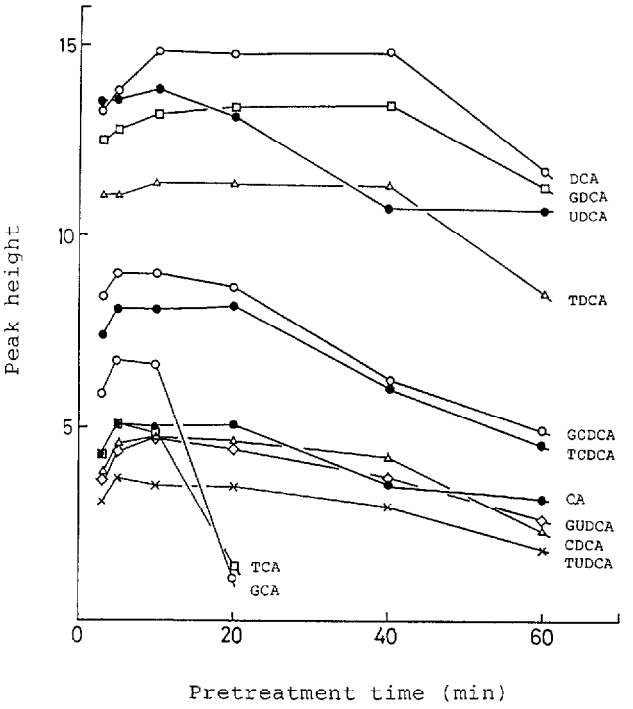

Fig. 3. Effect of variation of the washing volume on Serumout-25. Injection of 2 μl of serum sample spiked with bile acids. Flow-rate, 1 ml/min; range, 32.

tion of the same amount (440 ng) of each compound in a small volume (2 μl) showed that there is no significant extra band broadening and that the peak heights decreased by ca. 2.4% (mean value). In a patient serum sample, the concentration effect on the dual-pretreatment column is shown in Fig. 4.

Preliminary results indicated that the on-line sample pretreatment method is also suitable for the determination of bile acids in serum. A blank serum was spiked with 220 μg/ml each of the fifteen bile acids, and the chromatogram of the bile acids after direct injection of the standards and the same amount of spiked serum showed good agreement. For twelve of the bile acids the peak heights and chromatographic efficiencies are similar if 2 μl are introduced directly or via the dual precolumn.

When the immobilized 3α-HSD column was washed with the enzymatic reagent solution for 15 min before storing (4°C), the immobilized 3α-HSD retained 80% or more of its initial activity for 50 days when ten serum samples were assayed every day.

When the hydroxyapatite column was washed with 1 *M* phosphate buffer (pH 6.8) for 20 min before storing, up to 500 samples were applied on to the precolumn before it was necessary to change the precolumn, which was easy to pack (dry packing).

The number of injections on Serumout-25 varied, depending on the injection volume. The total serum volume injected before the column had to be changed,

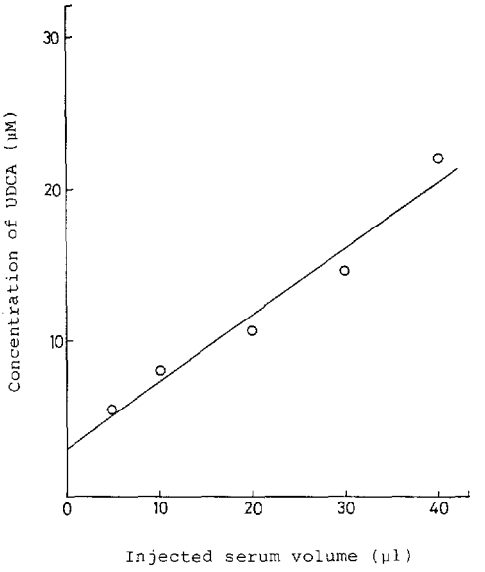

Fig. 4. Concentration effect in a patient serum sample put through the on-line sample pretreatment. Chromatographic conditions: flow-rate (W_1), 1.0 ml/min; flow-rate (W_2), 1.0 ml/min; pretreatment time, 10 min; range, 4.

either because of increasing back-pressure or declining sensitivity, was 5–10 ml. The precolumn was not regenerated.

Quantitation

The linearity of the method was investigated by standard additions of the bile acids to serum. For all fifteen compounds plots of peak height versus amount injected were linear in the range 14–500 ng.

Recovery

The recovery tests of the bile acids after dual-precolumn treatment were carried out by determining pooled serum samples spiked with known amounts of fifteen components. The recoveries were calculated by comparison of the peak heights obtained by preconcentration of 10-μl portions and direct injection of 2-μl portions containing the same amount of the bile acids. As summarized in Table I, the recoveries generally approach 100%. However, for GLCA, TLCA and LCA the recovery is lower, ca. 93.6, 95.5 and 98.8%, respectively.

Serum levels of UDCA

Serum levels of UDCA after an oral dose of 300 mg to three healthy volunteers are shown in Fig. 5. The UDCA peaked in the serum (4–8 μM) within 30–40 min after dosing, and thereafter decreased slowly.

Chromatogram of a serum sample

Fig. 6 shows a typical chromatogram of a normal serum sample obtained from a 63-year-old male. The peaks for CA, GCA, TCA, DCA, GDCA, TDCA, CDCA,

TABLE I

RECOVERIES OF BILE ACIDS AFTER ON-LINE DUAL-PRECOLUMN TREATMENT

The recoveries were calculated by comparison of peak heights obtained with standard solution and spiked serum samples. Chromatographic conditions as in Fig. 4. The concentrations of bile acids in normal human serum are negligibly low under these conditions.

Bile acid	Peak height (cm)		Recovery (%)
	Standard bile acid*	Bile acid added to serum sample**	
GUDCA	8.6	8.9	103.5
TUDCA	8.9	8.9	100.0
UDCA	14.9	15.3	102.7
GCA	2.5	2.7	108.0
TCA	2.9	3.1	106.9
CA	3.7	3.8	102.7
GCDCA	6.9	6.9	100.0
TCDCA	4.5	4.4	97.8
GDCA	13.6	13.8	100.7
TDCA	11.2	11.4	101.7
CDCA	6.0	5.9	98.3
DCA	14.6	15.0	102.7
GLCA	11.0	10.3	93.6
TLCA	10.6	10.1	95.5
LCA	8.3	8.0	98.8

*Direct injection on analytical column of 2 μl of mixture containing 220 ng/μl of each component in methanol solution ($n=5$).

**Injection via the precolumn (50 mm × 4.5 mm I.D.) of 10 μl of mixture containing 44 ng/μl of each component in normal human serum (44 ng/μl × 2 μl injected; $n=5$).

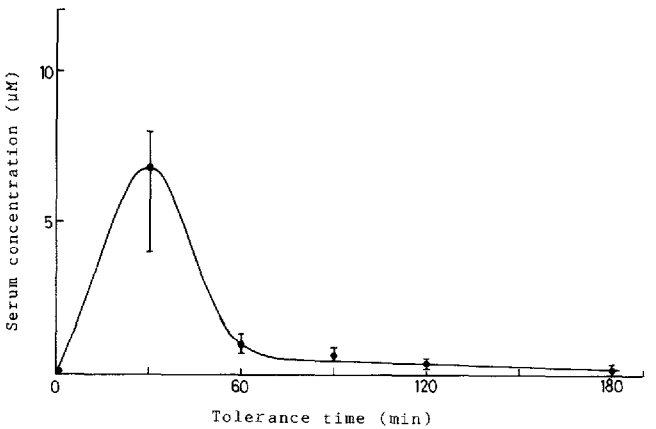

Fig. 5. Concentration–time curve after a single oral dose of 300 mg of UDCA administered to three healthy volunteers. Chromatographic conditions as in Fig. 4. Range, 4; sample volume 10 μl.

GCDCA, TCDCA, LCA, GLCA, TLCA, UDCA, GUDCA and TUDCA were clearly separated and identified by comparison with the authentic samples. A chromatogram of bile acids in the serum of patient with acute hepatitis is shown in Fig. 7. In acute hepatitis, increased concentrations of GCA, GCDCA, TCDCA and GDCA were observed.

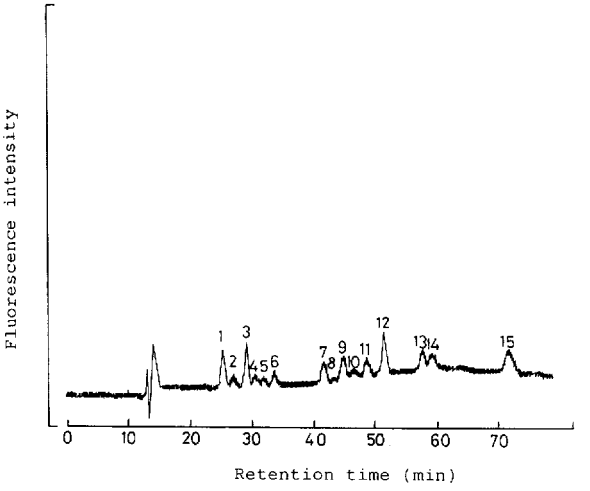

Fig. 6. Typical chromatogram of a normal human serum (male; age 63). Chromatographic conditions as in Fig. 5. Peaks: 1 = GUDCA; 2 = TUDCA; 3 = UDCA; 4 = GCA; 5 = TCA; 6 = CA; 7 = GCDCA; 8 = TCDCA; 9 = GDCA; 10 = TDCA; 11 = CDCA; 12 = DCA; 13 = GLCA; 14 = TLCA; 15 = LCA. Sample volume, 40 μl.

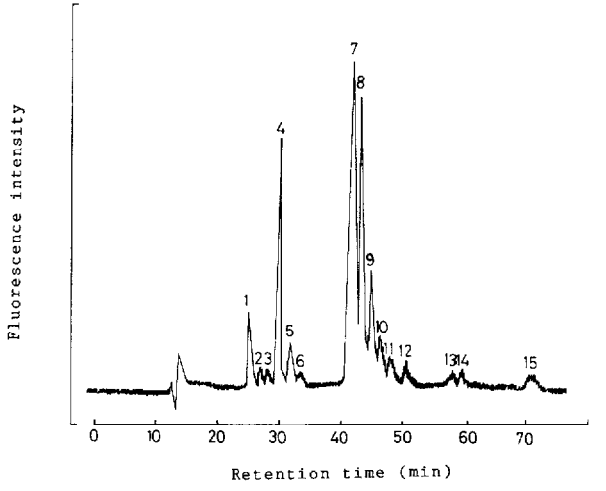

Fig. 7. Chromatogram of a serum sample from a patient with acute hepatitis. Chromatographic conditions as in Fig. 5. Peaks: 1 = GUDCA; 2 = TUDCA; 3 = UDCA; 4 = GCA; 5 = TCA; 6 = CA; 7 = GCDCA; 8 = TCDCA; 9 = GDCA; 10 = TDCA; 11 = CDCA; 12 = DCA; 13 = GLCA; 14 = TLCA; 15 = LCA. Sample volume, 40 μl.

CONCLUSIONS

The dual-precolumn technique with clean-up and preconcentration is a very powerful technique for the work-up of serum samples. The precolumn technique has several advantages over the extraction technique: (1) loss due to binding to glassware is avoided; (2) the recovery is near 100%; (3) it is simple; (4) it is fast; (5) very large sample volumes can be injected.

REFERENCES

1 A. Roda, S. Girotti, S. Ghini, B. Grigolo, G. Carrea and R. Bovara, Clin. Chem., 30 (1984) 206.
2 F. Mashige, K. Imai and T. Osuga, Clin. Chim. Acta, 70 (1976) 79.
3 P.S. Tietz, J.L. Thistle, L.J. Miller and N.F. LaRusso, J. Chromatogr., 336 (1984) 249.
4 L.R. Ferguson, G.W. Rewcastle, J.M. Lello, P.G. Alley and R.N. Seelye, Anal. Biochem., 143 (1984) 325.
5 R. Shaw and W.H. Elliott, Anal. Biochem., 74 (1976) 273.
6 M.S. Sian and A.J.H. Rains, Clin. Chim. Acta, 98 (1979) 243.
7 S. Kamada, M. Maeda and A. Tsuji, J. Chromatogr., 272 (1983) 29.
8 T. Kawasaki, M. Maeda and A. Tsuji, J. Chromatogr., 272 (1983) 261.
9 M. Hayashi, Y. Imai, Y. Minami, S. Kawata, Y. Matsuzawa and S. Tarui, J. Chromatogr., 338 (1985) 195.
10 B. Johansson, J. Chromatogr., 378 (1986) 419.
11 T. Dohji, M. Fushimi, T. Kawabe, F. Kamiyama, M. Mori, N. Sugita and O. Tanizawa, J. Chromatogr., 311 (1984) 249.