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APPLICATION OF THE STREAMING CURRENT DETECTOR TO THE ANALYSIS OF INDIVIDUAL BILE ACIDS

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SUMMARY

The streaming current detector for high-performance liquid chromatography has been applied successfully to the analysis of human bile acids. The detector showed almost equally high sensitivities for the detection of underivatized free and conjugated bile acids and their sulphates. The detection limits were about 100 ng and linear responses were obtained for amounts injected in the range 0.1–10 μg. Reversed-phase chromatographic conditions were employed with a chemically bonded C_{18} stationary phase and a water–methanol–acetone mobile phase containing ammonium carbonate. A synthetic serum sample with bile acids added was also successfully analysed.

INTRODUCTION

The fundamental characteristics of the streaming current detector for high-performance liquid chromatography (HPLC) under reversed-phase chromatographic conditions have already been reported[1]. The operating principle of the detector is based on the measurement of the streaming current, which is produced when a liquid is forced to flow through a capillary or a packed bed[2]. The streaming current detector, which has a cell volume of about 1 μl, is structurally simple, inexpensive, practically maintenance-free and selectively sensitive to ionizable compounds in spite of being a bulk-property detector in principle.

Many HPLC studies on the analysis of individual bile acids have been made[3–13] for the purpose of diagnosing liver diseases. Most of the studies have employed reversed-phase chromatographic conditions. The difficulty of bile acid analysis by HPLC lies in achieving a high sensitivity of detection because none of the acids possesses an effective UV absorption, although the glyco- and tauro-conjugated bile acids show moderate absorptions around 200 nm[4,11,12]. The sensitivity of the differential refractometer is too low to detect the bile acids in serum[7,14]. Therefore, UV[10,11,15] or fluorimetric[16] derivatizations at the carboxyl group have been conducted in order to obtain high sensitivities of detection. Tauro-conjugated bile acids, however, must be separated from free and glyco-conjugated bile acids and hydrolysed before the derivatization[15]. This kind of procedure seems rather time consuming and

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unfavourable for quantitative analysis. The most sensitive method for bile acids at present is to use the reaction detector in which NADH, produced from the reaction of a bile acid with 3α-hydroxysteroid dehydrogenase, is determined fluorimetrically[3,13,17]. The sulphated bile acids at the 3α-position, however, cannot be detected by this method.

The characteristic feature of the streaming current detector strongly suggests that it is promising for use in bile acid analysis. The purpose of the present study is to explore the applicability of the detector to the HPLC analysis of individual bile acids. The separation and detection of unconjugated bile acids, glyco- and tauro-conjugated bile acids and three 3-sulphates of free and conjugated lithocholic acids is described.

EXPERIMENTAL

Reagents and materials

Methanol of HPLC quality and the other chemicals of reagent grade were purchased from Wako (Osaka, Japan) and used without further purification. Water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). All of the bile acids investigated, cholic acid (C), chenodeoxycholic acid (CDC), deoxycholic acid (DC), lithocholic acid (LC), ursodeoxycholic acid (UDC), glyco(G)- and tauro(T)-conjugates of each bile acid and 3α-sulphates of LC (LC-3αS), GLC (GLC-3αS) and TLC (TLC-3αS), were kindly donated by Dr. K. Uchida of Shionogi Research Laboratory. A control serum (Control Serum I, lot no. 1890W004AA) was obtained from Hyland (Bannockburn, IL, U.S.A.).

Apparatus

The HPLC system consisted of a Jasco Tri Rotar high-pressure pump, a Jasco VL-611 sample injector and the streaming current detector described previously[1]. As the streaming current detector is very sensitive to flow-rate, a Glenco PD-3000 flow-through pulse damper and an air damper consisting of a stainless tube (50 cm × 8 mm I.D.) were used to reduce the pulsating flow, in addition to the pulse damper built in the pump. A Cecil CE212 variable-wavelength UV detector was connected in series with the streaming current detector to make a comparison of the two detectors when a human serum was analysed. The separation column (25 cm × 4.6 mm I.D.) and the pre-column (5 cm × 4.6 mm I.D.) were slurry-packed with Develosil ODS-5 (Nomura Chemical, Seto, Japan) by use of a Chemco Model 124 slurry packing apparatus.

Procedure

Each bile acid was dissolved in methanol to give a concentration of 1 mg/ml. HPLC was carried out at room temperature. A pre-column was employed only for the analysis of serum samples.

Human serum samples were pre-treated according to the method of Uemura[18]. A 1-ml volume of human serum was deproteinized by the addition of 5 ml of ethanol and heating at 80°C. The precipitates were removed by centrifugation at 1800 *g* for 5 min and washed with 5 ml of ethanol three times. The collected supernatant of total *ca.* 21 ml was evaporated to dryness. The residue was dissolved in 100 μl of the mobile phase and an aliquot was introduced into the HPLC system. The serum

sample for the recovery test was prepared by adding known amounts of four bile acids in a control serum.

RESULTS AND DISCUSSION

Effect of salt concentration on the separation and sensitivity

The separation of the bile acids was difficult when a mixture of water and methanol containing no salt was employed as the mobile phase, because the peaks were broad and unsymmetrical. This low efficiency may be attributable to the partial ionization of the carboxyl group of the bile acid.

Nambara and co-workers[4,14] reported that an alkaline buffer solution of weakly basic ammonium carbonate or ammonium phosphate is effective as a mobile phase constituent in the reversed-phase chromatographic separation of bile acids. A weakly basic solution containing ammonium carbonate was chosen as a mobile phase in this study, as it is also advantageous for the highly sensitive detection of the bile acids by the streaming current detector as ionic species.

In order to determine the optimal concentration of ammonium carbonate in the mobile phase, the dependence of the plate number of the column and the peak height of the bile acid eluted at about 4 min on the salt concentration was investigated with the flow-rate and the amount injected kept constant. The results are illustrated in Fig. 1. The evaluation of the effect of the salt concentration required that the plate number and detector response be measured for the peak at 4 min instead of the peak of a particular bile acid, because not only the response and the plate number but also the retention time of a particular bile acid depended on the concentration of the salt. The characteristic of the streaming current detector that it shows substantially equal sensitivities to all the bile acids makes this evaluation reasonable.

Fig. 1 reveals that an increase in the ammonium carbonate concentration makes the efficiency of the column higher, but the peak height lower. A compromise between the resolution and the sensitivity led to an optimal concentration of about 0.1 m*M*.

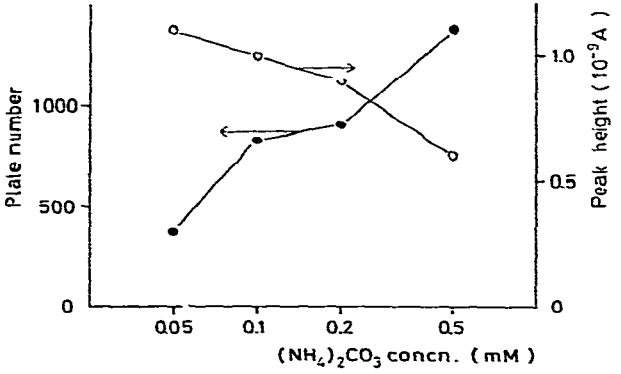

Fig. 1. Dependence of plate number and detector response of a bile acid on the concentration of ammonium carbonate in the mobile phase. The plate number and detector response were measured for a peak eluted at *ca.* 4 min. Column, Develosil ODS-5, 15 cm × 4.6 mm I.D.; mobile phase, water–methanol (30:70) containing ammonium carbonate; flow-rate, 1.0 ml/min; sample size, 2 μg.

Separation with water–methanol binary solvent

The chromatogram of ten individual bile acids shown in Fig. 2 was recorded with water–methanol containing 0.1 m*M* ammonium carbonate as the mobile phase. Each pair of glyco- and tauro-conjugated bile acids was not resolved completely under this condition.

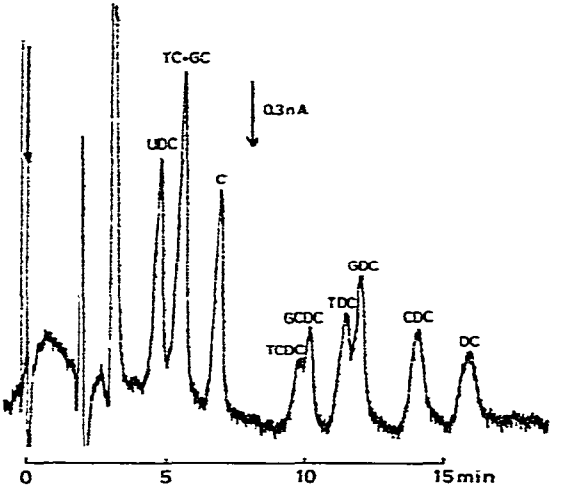

Fig. 2. Separation of individual bile acids with water–methanol (42:58) containing 0.1 m*M* ammonium carbonate as the mobile phase. Column, Develosil ODS-5, 25 cm × 4.6 mm I.D.; flow-rate, 0.8 ml/min; sample size, 1 μg each.

Periodic fluctuation observed in the baseline of the chromatogram, which is due mainly to noise from the detector, is associated with the pulsating flow by the reciprocating pump employed, because the streaming current detector is very sensitive to changes in flow-rate[1]. The baseline current was *ca.* $0.2 \cdot 10^{-7}$ A under the conditions indicated in Fig. 2. The downwards direction of the current scale drawn in Fig. 2. means that the current decreases at the peaks.

Separation with water–acetone binary solvent

Fig. 3 shows a chromatogram of individual bile acids obtained with water–acetone containing 0.1 m*M* ammonium carbonate as the mobile phase. In this chromatogram, the resolution of each pair of glyco- and tauro-conjugated bile acids was satisfactory. The glyco-conjugated acid was eluted faster than the corresponding tauro-conjugated acid, which was the reverse of the order of elution observed with water–methanol. The peaks of unconjugated acids tailed considerably and the peak of CDC partly covered that of DC. Moreover, TCDC and GDC were not separated completely.

The baseline current was *ca.* $0.4 \cdot 10^{-7}$ A under these conditions.

Separation with water–methanol–acetone ternary solvent

The results described above show that the unresolved peaks obtained with water–methanol could be separated successfully with water–acetone as the mobile

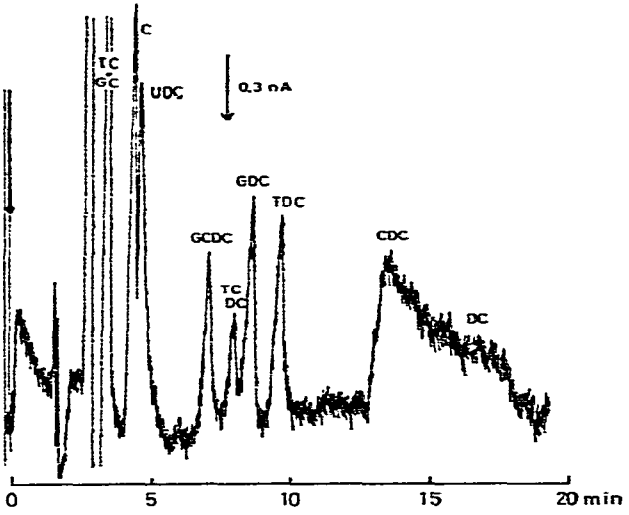

Fig. 3. Separation of individual bile acids with water–acetone (30:70) containing 0.1 m*M* ammonium carbonate as the mobile phase. Flow-rate, 0.9 ml/min; other conditions as in Fig. 2.

phase, and *vice versa*, suggesting that a water–methanol–acetone ternary solvent system might be effective for the complete separation of individual bile acids. This ternary solvent system gave successful results, as expected, but the retention times were so widely spread that the separation of all of the individual bile acids was impractical under isocratic elution conditions.

As the streaming current detector is intrinsically a bulk property detector, gradient elution methods cannot be adopted. Consequently, fifteen bile acids were divided into four groups according to their retention behaviour and each group was analysed separately. This made a sufficient resolution in a short time possible. The bile acids of each group are listed below in order of elution, together with the composition of the mobile phase employed for respective groups. Ammonium carbonate was added to the mobile phase at the concentration of 0.1 m*M*.

(i) GUDC, TUDC, GC, TC; water–acetone (77:23);

(ii) UDC, C, GCDC, TCDC, GDC, TDC, CDC, DC; water-methanol–acetone (62:18:20);

(iii) GCDC, TCDC, GDC, TDC, CDC, DC, GLC, TLC; water–methanol–acetone (62:9:29);

(iv) LC; water–methanol–acetone (30:36:34).

Chromatograms of the four groups are shown in Figs. 4–7. No significant differences among the bile acids were noticed in the detector responses. The smaller peak areas of GCDC and TCDC arose from the low purities of the samples. The baseline current was *ca.* $0.3 \cdot 10^{-7}$ A under the conditions given in Fig. 5.

Separation and detection of sulphated bile acids

The separation of monosulphated bile acids by HPLC has already been reported by Goto *et al.*[19]. The 3-sulphated bile acids cannot be detected by the reaction detector with an enzyme[3,13,17] as mentioned above, because 3α-hydroxysteroid de-

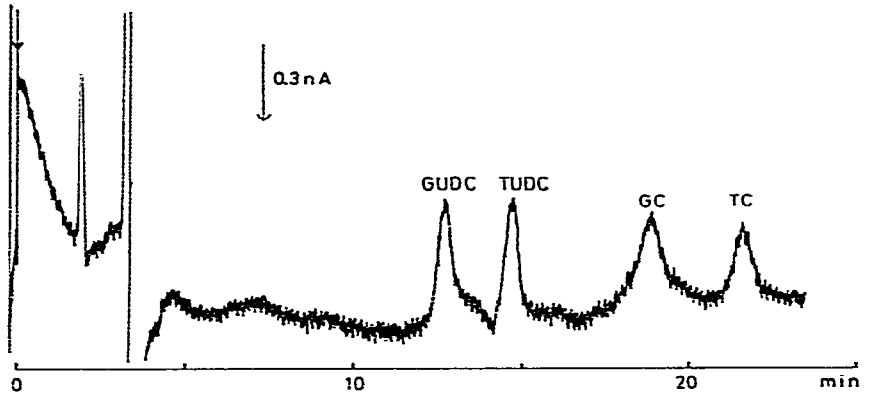

Fig. 4. Separation of four individual bile acids of group (i). Mobile phase, water–acetone (77:23) containing 0.1 m*M* ammonium carbonate; other conditions as in Fig. 2.

hydrogenase cannot oxidize these bile acids. As the streaming current detector was expected to be useful for detecting both sulphated and unsulphated bile acids, the applicability of this detector was examined for some sulphated bile acids.

Three sulphated lithocholic acids (LC) at the 3α-position, LC-3αS, GLC-3αS and TLC-3αS, were employed as samples. The optimal concentration of ammonium carbonate in the mobile phase was found to be 0.2 m*M* for the sulphated bile acids instead of 0.1 m*M* for the unsulphated acids. Concentrations of the salt lower than 0.2 m*M* gave broad, unsymmetrical peaks. A chromatogram obtained with water–methanol–acetone as the mobile phase is shown in Fig. 8. The slightly larger fluctuation in the baseline in Fig. 8 than in those in Figs. 2–7 is due to a residual pulsating

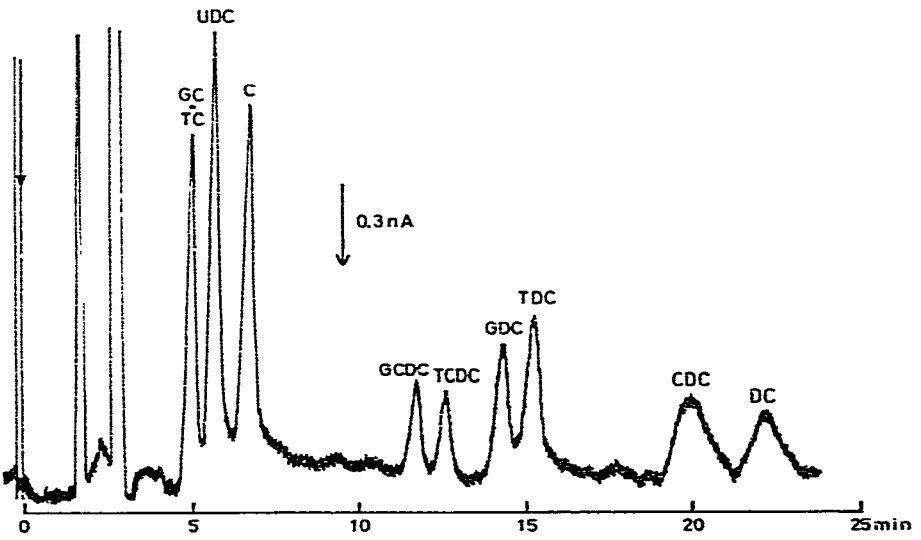

Fig. 5. Separation of eight individual bile acids of group (ii). Mobile phase, water–methanol–acetone (62:18:20) containing 0.1 m*M* ammonium carbonate; other conditions as in Fig. 3.

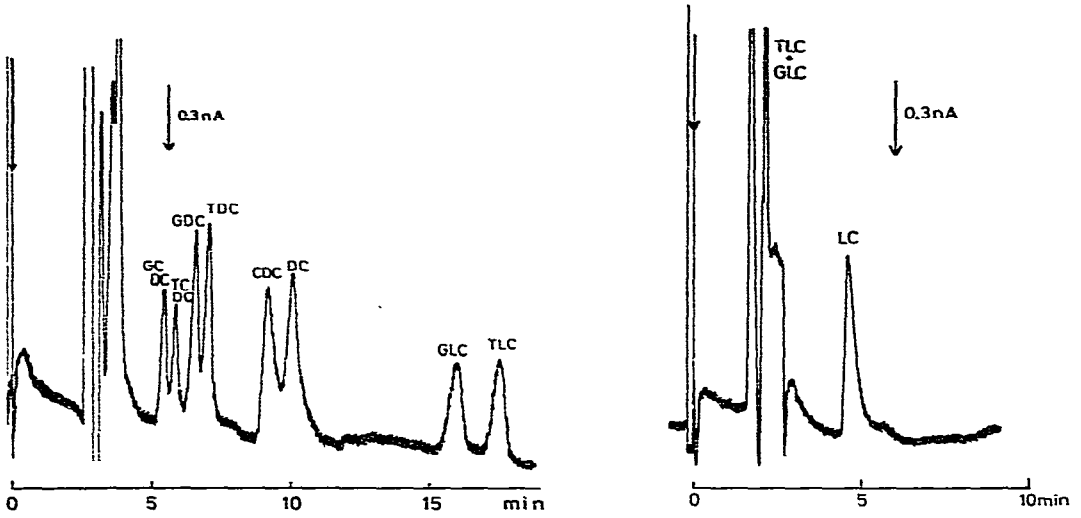

Fig. 6. Separation of eight individual bile acids of group (iii). Mobile phase, water–methanol–acetone (62:9:29) containing 0.1 m*M* ammonium carbonate; other conditions as in Fig. 3.

Fig. 7. Separation of lithocholic acid from other bile acids. Mobile phase, water–methanol–acetone (30:36:34) containing 0.1 m*M* ammonium carbonate; other conditions as in Fig. 3.

flow because of the additional flow-through damper not being used in recording the chromatogram shown in Fig. 8. These larger noises made the apparent sensitivity for sulphated bile acids lower than that for the unsulphated acids, although the actual detector response was virtually the same in spite of the increased salt concentration.

On the basis of the argument made by Goto *et al.*[19] that the order of elution of 3-sulphated bile acids is identical with that of unsulphated bile acids, LC-3αS may be

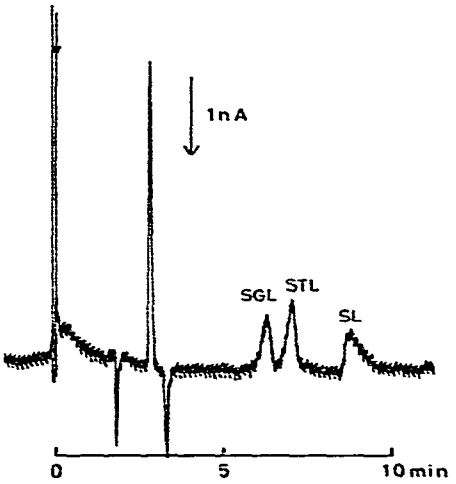

Fig. 8. Separation of three 3α-sulphates of free and conjugated lithocholic acids. Mobile phase, water–methanol–acetone (66:20:14) containing 0.2 m*M* ammonium carbonate; other conditions as in Fig. 3.

regarded as the last species eluted among all 3-sulphated bile acids under the conditions employed in this study. The retention time of GUDC, which was eluted first among the unsulphated bile acids, was 14 min and longer than that of LC-3αS by 4 min under the conditions indicated in Fig. 8. This suggests that the sulphated bile acids can be separated easily from the unsulphated bile acids.

Detector response and detection limits

An example of the dependence of the detector response on the amount of a bile acid injected is illustrated in Fig. 9. The slope of the calibration graph in Fig. 9 is nearly unity in the examined range of amounts injected of 0.1–10 μg.

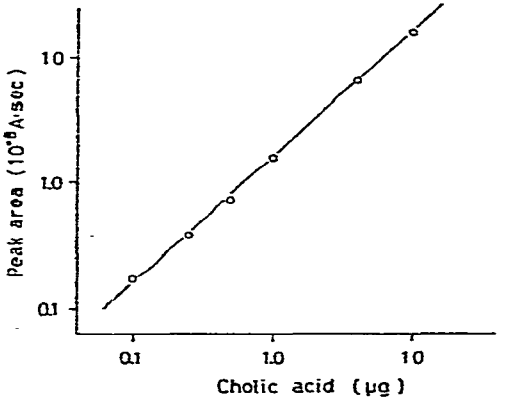

Fig. 9. Dependence of peak area on the amount of cholic acid injected. Flow-rate, 0.7 ml/min; other conditions as in Fig. 5.

The detection limit of the streaming current detector was 100 ng for the individual bile acids eluted at $k' \approx 1$. The detection limit is, as noted above, largely dependent on the concentration of ammonium carbonate in the mobile phase and the above value was determined at a concentration of 0.1 m*M*. The relationship between the concentration of ammonium carbonate and detection limit was investigated for the peak at $k' \approx 1$: for concentrations of 0, 0.1, 1 and 4 m*M*, the detection limits were 20, 100, 500 and 5000 ng, respectively. No significant differences in detector response were observed among free, glyco-conjugated, tauro-conjugated and sulphated bile acids under the condition as described above.

Application to human serum

Blank test. A control serum was deproteinized as described under Experimental and subjected to HPLC to give the chromatogram shown in Fig. 10. The only peak observed was a major one at 2–4 min, which may be assigned to inorganic ions and other ionic species such as amino acids because they are hardly retained under the reversed-phase condition employed. This result implies that the bile acids expected to be originally contained in a control serum could not be detected. That no peak was observed in the region of the bile acids means that no contaminant will interfere in the analysis of the bile acids in serum from a patient with a liver disease.

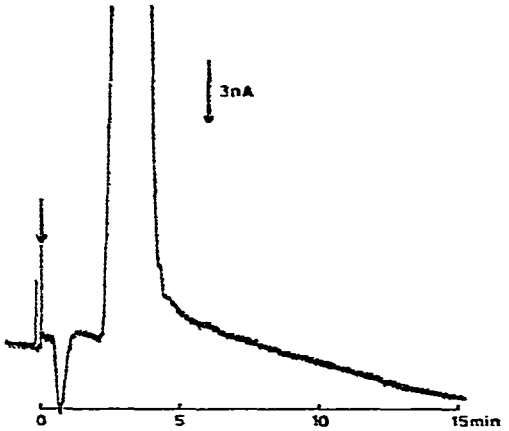

Fig. 10. Blank test of a human serum. A control serum of 1 ml was deproteinized, concentrated to 100 μl and introduced into the HPLC system. Column, Develosil ODS-5, 25 cm × 4.6 mm I.D.; pre-column, Develosil ODS-5, 5 cm × 4.6 mm I.D.; mobile phase, water–methanol–acetone (3:1:1) containing 0.1 m*M* ammonium carbonate; flow-rate, 0.8 ml/min; sample size, 25 μl.

Recovery of bile acids in serum samples. A synthetic mixture was prepared by the addition of four bile acids, 10.8 μg of GC, 38.6 μg of TC, 12.8 μg of GCDC and 36.1 μg of TCDC, to 1 ml of a control serum. It was pre-treated as described above and analysed by HPLC. A chromatogram of the synthetic serum sample is shown in Fig. 11. The recoveries were 83, 74, 97 and 100% for GC, TC, GCDC and TCDC, respectively. The recovery of cholic acid added to serum was found to be 90% by the same procedure in a separate test.

Retention behaviour of bile acids

The structure *versus* mobility relationship of bile acids in reversed-phase HPLC at pH 7.0 has been discussed in terms of the contributions of substituents to mobili-

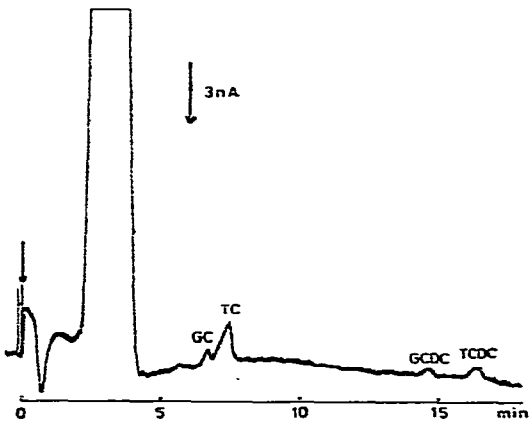

Fig. 11. Chromatogram of a synthetic serum sample. The sample, prepared by adding 10.8 μg of GC, 38.6 μg of TC, 12.8 μg of GCDC and 36.1 μg of TCDC to 1 ml of control serum, was pre-treated as described in Fig. 10. Sample size, 10 μl; other conditions as in Fig. 10.

ty[20]. Bloch and Watkins[7] found that the separation factors between glyco- and tauro-conjugates in reversed-phase HPLC at pH 4.7 are independent of the core bile acids and, therefore, the separation factors associated with differences in the parent structure are independent of the amino acid moiety. The relationship between selectivity and structure mentioned by them was confirmed for the three solvent systems employed in the present study. However, the selectivities between free and either glyco- or tauro-conjugated bile acids appear not to follow this relationship, as judged from the present results.

Five different bile acids having identical amino acid moieties were eluted in the order UDC, C, CDC, DC and LC irrespective of the carboxyl group at C-24 being unconjugated, glyco-conjugated or tauro-conjugated. This order seems general for reversed-phase HPLC regardless of pH and salt concentration in the mobile phase[3,4,7,11,12].

On the other hand, the order of elution of unconjugated, glyco-conjugated and tauro-conjugated bile acids having identical parent structures has been reported to depend on pH and salt concentration[4,14,19]. In the present study, the elution of these three types was in the order tauro-conjugated, glyco-conjugated and unconjugated bile acids with water–methanol as the mobile phase, and in the order glyco-conjugated, tauro-conjugated and unconjugated bile acids with water–acetone. A similar retention behaviour was observed between water–methanol and water–tetrahydrofuran and between water–acetone and water–acetonitrile binary mixtures containing 0.1 m*M* ammonium carbonate in a preliminary experiment.

The relative retention between unconjugated and glyco- and tauro-conjugated bile acids mentioned above was found to be reversed with concentrations of ammonium carbonate higher than 0.5 m*M* in water–methanol. No attempt was made to clarify the effect of pH on the retention behaviour of the bile acids because of the very low concentration of the salt employed. The pH measured for 0.1 m*M* ammonium carbonate was about 9.0. The life of the column was longer than 6 months, no significant decrease in efficiency being observed despite the high pH of the mobile phase.

Comparison between the streaming current detector and the UV detector

An outstanding characteristic of the streaming current detector is that it shows almost equal sensitivities to all kinds of bile acids. The sensitivity of the UV detector at about 200 nm may be slightly superior or equivalent to that of the streaming current detector for detecting glyco- and tauro-conjugated bile acids[4,12]. For the detection of unconjugated bile acids, however, the latter is about five times more sensitive than the former.

Chromatograms recorded simultaneously with both detectors are illustrated in Fig. 12 for comparison. In these chromatograms, glyco- and tauro-conjugated bile acids were not resolved because of the use of water–methanol as the mobile phase. It is evident that no appreciable unknown peaks were revealed by the streaming current detector and that in contrast, some extra peaks could seriously interfere in the detection of bile acids in serum samples using the UV detector.

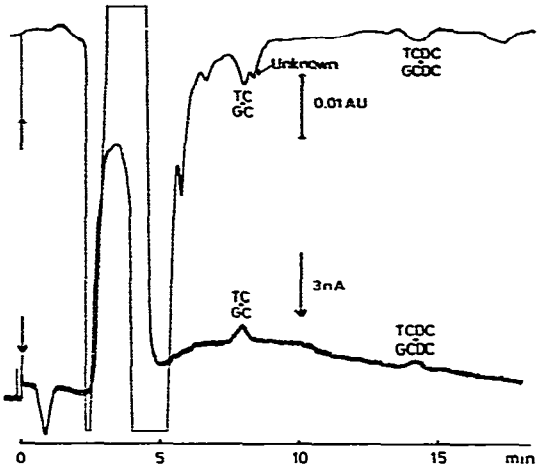

Fig. 12. Comparison of the streaming current detector (lower trace) and the UV detector (upper trace). A synthetic serum sample was prepared and pre-treated as described in Fig. 11. Mobile phase, water–methanol (42:58) containing 0.1 m*M* ammonium carbonate; detection (UV), 210 nm; sample size, 10 μl; other conditions as in Fig. 10.

CONCLUSION

Fifteen individual bile acids were separated successfully under reversed-phase chromatographic conditions, but it required the use of four mobile phases, which is impractical for routine analysis. It is desirable for the individual bile acids to be resolved completely with at most two isocratic conditions in clinical analysis. The use of a fourth organic modifier, *i.e.*, the use of a quaternary solvent system, may improve this undesirable situation, as the change of the salt concentration is fairly limited for the streaming current detector.

It is concluded that the streaming current detector is capable of detecting bile acids in amounts as low as 0.1 μg under reversed-phase chromatographic conditions. At present it is not sensitive enough to detect the bile acids contained in a control serum, but sensitive enough to detect the bile acids in bile or in serum samples from patients suffering from various hepatobiliary diseases.

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