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# Analysis of conjugated bile acids by packed-column supercritical fluid chromatography

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ABSTRACT

A rapid method has been developed for the simultaneous separation of the polar glycine- and taurine-conjugated bile acids by packed-column supercritical fluid chromatography. Samples were analysed on a cyanopropyl-bonded silica column with ultraviolet detection at 210 nm and carbon dioxide modified with methanol as the mobile phase. The influence of the stationary phase, modifier concentration, temperature, column pressure and modifier identity on retention was also studied. This new chromatographic method is applicable to the assay of conjugated bile acids in duodenal bile samples from patients with hepatobiliary diseases.

## INTRODUCTION

The naturally occurring bile acids in human fluids are present primarily as glycine and taurine conjugates [1–4]. The individual separation and quantification of these compounds are very important for the diagnosis of hepatobiliary and intestinal diseases and for clarification of pathophysiology [3,5,6].

The classical chromatographic techniques for the analysis of bile acid conjugates include thin-layer chromatography (TLC) [7–9] and gas chromatography (GC) after hydrolysis and derivatization [6,10]. At present, reversed-phase high-performance liquid chromatography (HPLC) is the method of choice [3,11] for the assay of conjugated bile acids in biological samples at elevated levels. A large number of publications have appeared concerning this particular application of HPLC (refs. 11 and 12 and references therein) with special emphasis on the use of octadecylsilica columns as the stationary phase.

Supercritical fluid chromatography (SFC) is complementary to both GC and HPLC for the analysis of organic compounds [13]. Unlike GC, it is not restricted by compound volatility and thermal lability and if capillary columns are used, chromatographic efficiency approaching that obtained by capillary GC is possible [13]. Packed-column SFC offers advantages over HPLC in terms of reduction of analysis time, rapidity of method development and higher transparency of the eluent at low UV wavelengths [13,14]. Furthermore interfacing of this technique with mass spectrometry is easier than with HPLC since the gas volumes generated from the mobile phase are lower [13]. Although SFC is widely perceived as a technique for the separation of low- to moderate-polarity molecules, the addition of polar modifiers to low-polarity supercritical fluids, like carbon dioxide, has extended the application of SFC to more polar solutes [15,16].

The present work was undertaken to investigate the potential use of this technique for the analysis of the polar conjugated bile acids. This

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study describes a method for the simultaneous separation of the glycine and taurine conjugates of the common bile acids by packed-column SFC. The application of this new procedure to the assay of conjugated bile acids in human bile is also demonstrated.

## EXPERIMENTAL

### *Reagents*

The sodium salts of glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA) and taurolithocholic acid (TLCA) were purchased from Sigma (St. Louis, MO, USA); the sodium salts of glycoursodeoxycholic acid (GUDCA) and tauroursodeoxycholic acid (TUDCA) were a gift from Gipharmex (Milan, Italy). Their purity was checked by HPLC prior to use. Instrument-grade liquid carbon dioxide supplied in cylinders with a dip tube was purchased from BOC (London, UK). HPLC-grade methanol, acetonitrile, propanol and water were from Fisons (Ipswich, UK). All other chemicals were of analytical grade (Sigma). Bakerbond $C_{18}$ cartridges were obtained from J. T. Baker (Phillipsburg, NJ, USA).

### *Chromatography*

For SFC a Hewlett-Packard 1084B high-performance liquid chromatograph (Hewlett-Packard, Avondale, PA, USA) modified for SFC operation [17] was used. The carbon dioxide was introduced directly into the "A" pump of the 1084B chromatograph and the solvent modifier (usually methanol) was placed in the "B" pump, which was operated in the HPLC mode. The liquid carbon dioxide and the pump heads of the chromatograph were cooled to −20°C using a Neslab RTE-4Z refrigerated bath (Neslab Instruments, Newington, NH, USA). To maintain supercritical conditions in the column, a Tescom mechanical back-pressure regulator was connected to the outlet of the flow cell. Samples were introduced onto the column via a Rheodyne 7125 injector fitted with a 10-μl sample loop (Rheodyne, Cotati, CA, USA). The column effluent was monitored by the built-in multiple-wavelength UV–VIS detector set at a wavelength of 210 nm and 0.05 a.u.f.s. Separations were performed on 250 mm × 4.6 mm I.D. stainless-steel columns packed with octadecyl-, octyl-, cyano- or amino-bonded 5-μm Zorbax phases (FSA Laboratory Supplies, Loughborough, UK). A 5-μm Deltabond cyano column (250 mm × 4.6 mm I.D.; Keystone Scientific, State College, PA, USA) was also used.

The HPLC apparatus consisted of a Jasco chromatographic system (Model 880-PU pump, Model 880-02 ternary gradient unit and Model 875 UV–VIS detector; Jasco, Tokyo, Japan) linked to an injection valve with a 20-μl sample loop (Rheodyne) and a chromatographic data processor (Chromatopac C-R3A, Shimadzu, Kyoto, Japan). The detector was set at 210 nm and 0.08 a.u.f.s. Separations were performed according to the method described earlier [11], using a 5-μm Ultrasphere ODS column (Beckman, Berkeley, CA, USA) eluted with methanol–0.02 *M* aqueous sodium acetate (70:30, v/v) adjusted to pH 4.3 with phosphoric acid. The mobile phase was filtered through HVLP-type filters (0.45 μm; Millipore, Molsheim, France) and degassed on-line by a Model ERC-3311 automatic solvent degasser (Erma, Tokyo, Japan). Chromatography was performed at ambient temperature, at a flow-rate of 1.0 ml/min.

The identity of the separated compounds was assigned by co-chromatography with the authentic substances. Peak heights were quantified using the integrator, which was calibrated with standard solutions of pure bile acids.

### *Sample processing*

Duodenal bile was obtained from patients with various hepatobiliary diseases. The specimens were stored at −20°C until required for analysis and the conjugated bile acids extracted according to the method described in a previous study [18] with minor modifications. In brief, after thawing each sample was homogenized and centrifuged (1000 *g* for 3 min). A 0.5-ml aliquot of the supernatant was diluted with 5.0 ml of 0.1 *M* phosphate buffer (pH 7.5) and mixed in a vortex mixer. This solution was passed through a pre-conditioned (5 ml of methanol and then 5 ml of water)

Bakerbond $C_{18}$ cartridge (sorbent weight, 200 mg) and eluted successively with 5 ml of 40% (v/v) methanol in acetate buffer (0.05 *M*, pH 4.3), 2 ml of water and 2 ml of methanol. The last fraction, which contained the conjugated bile acids, was evaporated to dryness under a nitrogen stream, and the residue was dissolved in 0.5 ml of methanol and subjected to chromatographic analysis.

## RESULTS AND DISCUSSION

### *Chromatography*

Initially carbon dioxide was examined as a mobile phase for the separation of bile acid conjugates by packed-column SFC. None of the compounds investigated was eluted from any of the columns used at 300 bar and 40°C. This is due to the interactions between the polar conjugates and active sites on the column packing materials and also to carbon dioxide which is not a particularly good solvent for polar solutes.

The foregoing bile acids were completely adsorbed on the amino column even when a mobile phase containing up to 30% (v/v) methanol in carbon dioxide was employed.

Carbon dioxide modified with methanol was found to elute all the compounds examined from the non-polar octadecyl and octyl stationary phases. However, both the glycine and taurine conjugates merged as broad unresolved peaks close to the solvent front using mobile phases containing 5 or 15% methanol in carbon dioxide, respectively. At lower methanol concentration, no elution of the above steroids was observed. Therefore, although optimal resolution for the bile acid conjugates is achieved in HPLC by octadecyl or octyl packings [11], these stationary phases exhibit unsatisfactory retention and selectivity in SFC.

The analysis of conjugated bile acids on the cyanopropyl column showed a marked improvement in selectivity. In order to obtain reasonable elution times, methanol concentrations in carbon dioxide higher than 10% (v/v) were required. The effect of different amounts of the polar modifier, at 250 atm and 40°C, on the retention of the glycine- and taurine-conjugated bile acids on the cyano column is illustrated in Fig. 1. The addition of methanol to carbon dioxide not only reduced the elution times but also improved the peak shape considerably. This is due to a combination of the deactivation of the stationary phase and to an enhancement of the solvation power of the mobile phase [19]. Optimal chromatographic efficiency was observed after the column was purged with the methanol–carbon dioxide mixture for a few hours. Using carbon dioxide modified with 20 or 28% (v/v) methanol, the influence of the column pressure and temperature on the separation of conjugated bile acids was studied in the ranges 150–300 bar and 40–60°C, respectively. At 300 bar the resolution of several component peaks was lost. On the other hand, when the pressure was decreased below 250 bar, relatively long elution times, and broad and asymmetric peaks were observed, particularly for the taurine derivatives. Increasing the temperature from 40 to 60°C at constant pressure increased the capacity factors for all bile acids, with no significant effect on resolution. Moreover, greater baseline

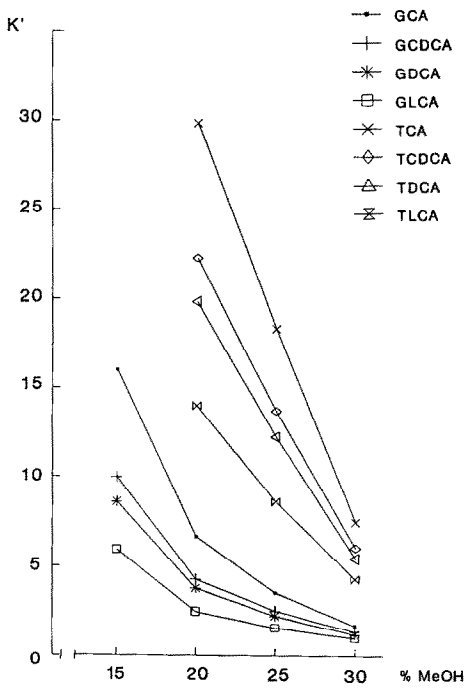


Fig. 1. Effect of methanol concentration in carbon dioxide on the capacity factors ($k'$) of glycine- and taurine-conjugated bile acids. Operating conditions: column, cyanopropyl Zorbax; flow-rate, 4 ml/min; inlet pressure, 250 atm; temperature, 40°C; UV detection, 210 nm.

instability was observed at higher temperatures because of the low UV wavelength (210 nm) used for the detection of these compounds lacking a strong chromophore. Consequently, subsequent analyses were carried out at 40°C and 250 bar. Fig. 1 suggests that a methanol gradient might resolve all the solutes in a moderate analysis time. The separation of the commonly occurring bile acids (*i.e.*, GLCA, GDCA, GCDCA, GCA, TLCA, TDCA, TCDCA and TCA) on the cyanopropyl column using a mobile phase containing 20% methanol in carbon dioxide increasing after 4.5 min to 28% methanol, over a period of 1.5 min, is shown in Fig. 2. GUDCA and TUDCA, which are of primary interest during ursodeoxycholic acid (UDCA) treatment [20,21], are also separated, but not yet as efficiently as the other conjugates, using the system described here (retention times: GCDCA, 3.5 min; GUDCA, 3.8 min; TCDCA, 9.2 min; TUDCA, 9.5 min). Although a modifier gradient was used, the equilibration of the column with the initial mobile phase composition was rapid (less than 5 min) and reproducibility in retention times satisfactory (relative standard deviation values $\leq$ 0.88%). Under the conditions used (Fig. 2), the mobile phase is not supercritical. However, as reported in the literature (ref. 16 and references therein), chromatographic results indicate that such a differentiation is irrelevant since there is no difference in the solvent properties of a just supercritical dense gas or a just subcritical dense fluid, provided the densities of the two fluids are similar.

Fig. 2 indicates that probably a normal-phase separation mechanism is operating since the solutes elute in order of increasing polarity; the glycine conjugates ($pK_a \approx 4$) elute before the taurine derivatives ($pK_a \approx 2$) and the elution order of bile acids within each conjugates group follows the number of hydroxyl groups on the steroid backbone (*i.e.*, monohydroxy < dihydroxy < trihydroxy). Although normal-phase systems for the HPLC separation of conjugated bile acids have been reported [22,23], they have distinct disadvantages such as rapid column deterioration [22], inadequate resolution of the isomeric dihydroxy bile acid conjugates [22,23] and the need for several columns connected in series [22]. The

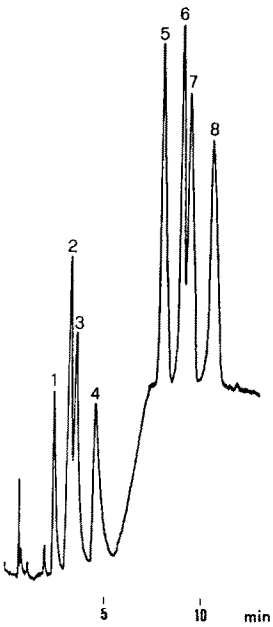


Fig. 2. SFC separation of a synthetic mixture of conjugated bile acids. The mobile phase was initially carbon dioxide modified with 20% methanol. After 4.5 min the concentration of methanol was increased to 28% over a period of 1.5 min. Other operating conditions as in Fig. 1. Peaks: 1 = GLCA; 2 = GDCA; 3 = GCDCA; 4 = GCA; 5 = TLCA; 6 = TDCA; 7 = TCDCA; 8 = TCA.

retention sequence reported above (Fig. 2) is the reverse of that obtained by reversed-phase HPLC systems [11], with the exception of the conjugates of UDCA. In fact, although UDCA (3α,7β-dihydroxy) contains fewer hydroxyl groups than cholic acid (3α,7α,12α-trihydroxy), TUDCA and GUDCA are more hydrophilic than the corresponding conjugates of cholic acid on octadecyl columns [24,25]. This unexpected chromatographic behaviour has been traced to the presence in UDCA of a β-hydroxyl group which reduces the affinity of the hydrophobic surface of the steroid nucleus for the stationary phase [24].

In addition to the resolution of individual bile acids, the SFC method developed in this study also affords the separation of the glycine and taurine conjugates into the two groups (Fig. 2). The chromatographic profile of the two separate fractions allows quantification in the presence of much larger amounts of one of the conjugated forms. By contrast, none of the existing reversed-phase HPLC methods for the analysis of bile

acids [11,12] are able to elute the compounds as two distinct groups (*i.e.*, glycine and taurine derivatives).

The effect of different modifiers on the forcgoing bile acid retention was also examined. Under the same experimental conditions, *n*-propanol produced increased analysis times compared to methanol accompanied by tailing peaks and unsatisfactory resolution. Acetonitrile (30%, v/v, in carbon dioxide) failed to elute the conjugates from the cyano column, thus acting as a weak solvent. Accordingly, acetonitrile allows larger injection volumes (>10 μl) compared to methanol [19]. However, samples were dissolved in methanol, because of the lower solubility of the bile acids in acetonitrile [4].

Other authors [16] have reported that small amounts of very polar additives (*e.g.*, trifluoroacetic acid) dissolved in methanol and mixed with carbon dioxide improve the peak shape of polar solutes such as benzene polycarboxylic acids. This was not found to be the case; the addition of trifluoroacetic acid (0.1%, v/v) to the methanol modifier did not affect the chromatography of the glycine conjugates significantly, whereas increased retention and poor separation were observed for the taurine derivatives. Moreover trifluoroacetic acid causes instability and a severe drift of the baseline during the modifier gradient at the detection wavelength used (210 nm).

A cyano column with a deactivated surface consisting of polymer-coated silica (Deltabond cyano) was also examined for the SFC separation of conjugated bile acids, using the same experimental conditions reported above. Although in comparison with the conventional cyano column, a lower percentage of methanol in carbon dioxide was required to elute the conjugates in similar retention times (10% for the glycine derivatives; 17% for the taurine derivatives), no improvement in the separation efficiency and a deterioration of the taurine conjugate resolution were observed.

*Application*

The SFC system devised in this study was applied to the assay of conjugated bile acids in human duodenal bile from patients with various hepatobiliary diseases. The conjugated bile acids were extracted from the samples by Bakerbond $C_{18}$ cartridges and analysed by SFC. Representative chromatograms of duodenal bile samples from a patient with chronic hepatitis and from a subject with liver cirrhosis are shown in Figs. 3 and 4, respectively. The conjugates are separated within a shorter analysis time (11 min) compared to the published HPLC methods [3,4,11]. The detection limits at 210 nm ranged from 0.30 to 0.45 μg on column, with a signal-to-noise ratio of 3. There was a linear correlation between peak heights and concentration up to 58 μg (GLCA, $r=0.997$, $a=0.112$, $b=0.084$; GDCA, $r=0.998$, $a=0.199$, $b=0.050$; GCDCA, $r=0.999$, $a=0.144$, $b=0.053$; GCA, $r=0.997$, $a=0.094$, $b=0.038$; TLCA, $r=0.999$, $a=0.226$, $b=0.114$; TDCA, $r=0.999$, $a=0.236$, $b=0.074$; TCDCA, $r=0.999$, $a=0.182$, $b=0.144$; TCA, $r=0.999$, $a=0.134$, $b=0.104$). The reproducibility of the method was evaluated by repeated ($n=10$) SFC analyses of the same duodenal bile sample. The relative standard deviation for each bile acid concentration ranged from 2.8 to 6.6%.

The present SFC method for the simultaneous determination of the glycine- and taurine-conju-

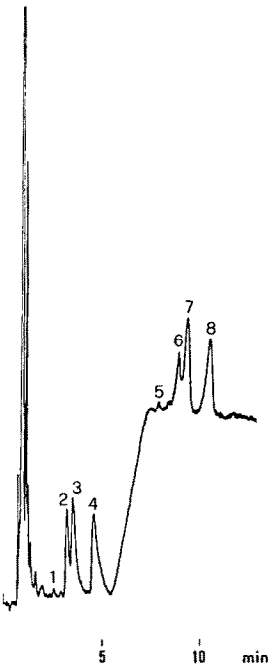


Fig. 3. Chromatogram of the separation of conjugated bile acids in a sample of duodenal bile from a patient with chronic hepatitis. Conditions and peak identification as in Fig. 2.

TABLE I

COMPARISON OF BILE ACID COMPOSITION IN DUODENAL BILE OF PATIENTS WITH LIVER DISEASE DETERMINED BY SFC AND REVERSED-PHASE HPLC

| Bile acid | Percentage of total bile acids | | | | | |
|---|---|---|---|---|---|---|
| | Patient 1 | | Patient 2 | | Patient 3 | |
| | SFC | HPLC | SFC | HPLC | SFC | HPLC |
| GLCA | | | | | 0.9 | 0.8 |
| GDCA | 15.5 | 16.9 | 4.9 | 4.4 | 14.7 | 14.3 |
| GCDCA | 14.1 | 13.5 | 40.0 | 40.8 | 23.1 | 24.1 |
| GCA | 39.3 | 37.5 | 46.0 | 45.1 | 26.7 | 26.6 |
| TLCA | | | | | 0.1 | 0.1 |
| TDCA | 7.6 | 8.2 | | | 6.4 | 5.9 |
| TCDCA | 5.9 | 6.3 | 3.4 | 3.6 | 12.7 | 11.6 |
| TCA | 17.6 | 17.6 | 5.5 | 6.1 | 15.1 | 16.5 |
| TBA[a] (mmol/l) | 5.8 | 6.3 | 5.5 | 5.0 | 6.5 | 6.2 |

[a] TBA, total bile acid concentration.

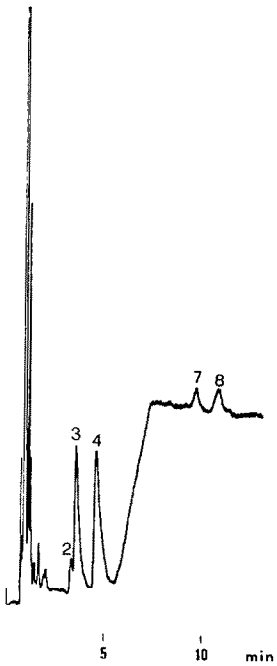


Fig. 4. SFC separation of conjugated bile acids from the duodenal bile of a patient with liver cirrhosis. Conditions and peak identification as in Fig. 2.

gated bile acids was validated by comparison with the previously adopted reversed-phase HPLC procedure [11] on the same biological sample. Three different duodenal bile specimens were assayed. The two methods produced consistent results (see Table I), thereby proving the validity of the SFC determination of conjugated bile acids.

## CONCLUSIONS

The first SFC method has been developed for the simultaneous determination of the glycine and taurine conjugates of bile acids. The procedure offers an alternative separation selectivity to the reversed-phase HPLC techniques used currently. Because of its rapidity and reproducibility, the method is suitable for routine clinical analyses. Work is in progress in this laboratory to interface this SFC technique to mass spectrometry.

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