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CCLII[a]. Separation and characterization of 3-oxobile acids in serum by high-performance liquid chromatography with fluorescence detection

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ABSTRACT

The separation of unconjugated and glycine- and taurine-conjugated bile acids with a C-3 oxo group has been carried out by high-performance liquid chromatography on a reversed-phase column. The chromatographic behaviour of these 3-oxobile acids was dependent on the number and positions of hydroxyl groups and the structure of the side-chain. The newly developed method has been applied to the characterization of 3-oxobile acids in biological fluids. The bile acid fraction was obtained from a serum specimen by passing it through a Sep-Pak C_{18} cartridge. 3-Oxobile acids were derivatized quantitatively to fluorescent oximes through the oxo group by treatment with O-(2-anthrylmethyl)hydroxylamine. The derivatives were separated into the unconjugated and glycine- and taurine-conjugated fractions by ion-exchange chromatography on a lipophilic gel, piperidinohydroxypropyl Sephadex LH-20. Subsequent resolution of each fraction into individual 3-oxobile acids was achieved by chromatography on a Nova-Pak Phenyl column using 3% methanol in 0.3% potassium phosphate buffer (pH 7.0)–acetonitrile (8:5, v/v) as the mobile phase. The derivatized 3-oxobile acids were monitored by fluorescence detection (excitation wavelength 260 nm and emission wavelength 405 nm), the limit of detection being 20 fmol. Glycine- and taurine-conjugated 7α,12α-dihydroxy- and 7α-hydroxy-3-oxo-5β-cholanoic acids in human serum were unambiguously identified on the basis of their chromatographic behaviour using mobile phases of different pH values.

INTRODUCTION

In recent years, considerable attention has been focused on the biosynthesis and metabolism of bile acids in humans in connection with the diagnosis of hepatobiliary diseases. It is well known that bile acids with a C-3 oxo group are reduced to 3α-hydroxylated metabolites in the liver and then excreted into the duodenum via the bile duct as the glycine- and taurine-conjugated forms. Recently, the existence of 3-oxobile acids in bile has been demonstrated in patients with

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liver diseases. Accordingly, the development of a reliable method is urgently required for the analysis of these bile acids in biological fluids.

The methods commonly used for the determination of bile acids in biological specimens involve prior solvolysis and/or hydrolysis, followed by chromatographic separation of deconjugated bile acids. This procedure, however, has inevitable disadvantages, such as the lack of reliability and the loss of information about the conjugated form. Moreover, 3-oxobile acids are unstable under the conditions of alkaline hydrolysis [1,2]. High-performance liquid chromatography (HPLC) with fluorescence detection is widely recognized as a powerful tool for the trace analysis of various compounds, in particular polar and thermally unstable biological substances. In the previous paper of this series, we described a new fluorescence precolumn derivatization reagent, O-(2-anthrylmethyl)hydroxylamine, and demonstrated its utility for selective and sensitive derivatization of bile acids through the 3-oxo group [3]. The present paper deals with a method for the separation and characterization of 3-oxobile acids in serum by HPLC with precolumn fluorescence labelling.

EXPERIMENTAL

Materials

Unconjugated and conjugated 3-oxobile acids were prepared in these laboratories. The Sep-Pak C_{18} cartridge (Millipore, Waters Chromatography Division, Milford, MA, U.S.A.) was washed successively with ethanol, water, 5% (w/v) aqueous bovine serum albumin solution and then water prior to use. All other chemicals employed were of analytical-reagent grade. Solvents were purified by distillation prior to use. O-(2-Anthrylmethyl)hydroxylamine [3] and piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) (acetate form, 0.6 mequiv./g) [4] were prepared in as previously reported. All glassware used was silanized with trimethylchlorosilane.

High-performance liquid chromatography

The apparatus was a 510 solvent-delivery system (Millipore) equipped with a 650-10LC fluorescence spectrophotometer (excitation wavelength 260 nm, emission wavelength 405 nm) (Hitachi, Tokyo, Japan). A Nova-Pak Phenyl (4 μm particle size, 150 mm × 4.6 mm I.D.) (Millipore) column was used at ambient temperature.

Separation and characterization of 3-oxobile acids in serum

To a serum specimen (500 μl), unconjugated and glycine- and taurine-conjugated 12-hydroxy-3-oxo-23-nor-5β-cholanic acids (3-dehydro-norDCA) (each 10 ng) were added as internal standards (I.S.), and the whole was diluted with 0.5 *M* sodium phosphate buffer (pH 7.0, 4 ml) and passed through a Sep-Pak C_{18} cartridge. The cartridge was washed with water (5 ml), and the bile acids were eluted

with 90% ethanol (5 ml). The eluate was concentrated under reduced pressure and mixed with O-(2-anthrylmethyl)hydroxylamine (100 μg) in methanol (100 μl). The resulting solution was heated at 60°C for 40 min, then applied to a column (18 mm × 6 mm I.D.) of PHP-LH-20 (100 mg). Elution was carried out at a flow-rate of 0.2 ml/min. After removal of neutral and basic compounds by washing with 90% ethanol (6 ml), unconjugated and glycine- and taurine-conjugated 3-oxobile acids were fractionally separated by stepwise elution with 0.1 *M* acetic acid in 90% ethanol (5 ml), 0.25 *M* formic acid in 90% ethanol (5 ml) and 0.3 *M* acetic acid–potassium acetate in 90% ethanol (pH 6.5, 5 ml). Each fraction was neutralized with 10% sodium hydroxide solution and evaporated to dryness, and the residue obtained was redissolved in methanol (100–200 μl). A 5–10 μl aliquot of the solution was injected into the HPLC system.

RESULTS AND DISCUSSION

Separation of 3-oxobile acid O-(2-anthrylmethyl)oxime derivatives

The analysis of 3-oxobile acids requires elimination of interferences due to common bile acids, which exist predominantly in biological fluids. In previous work, we prepared a new fluorescence labelling reagent, O-(2-anthrylmethyl)hydroxylamine, and demonstrated its utility for selective derivatization of bile acids through the 3-oxo group [3]. Accordingly, initial effort was directed towards the HPLC separation of derivatized 3-oxobile acids.

Various combinations of buffer solution and organic solvent on a Nova-Pak Phenyl column were examined for the suitable mobile phase. The use of potassium phosphate buffer–acetonitrile system appeared to be promising, because little resolution of *Z* and *E* isomers and no significant leading or tailing were observed on the chromatogram. The effect of the pH of the mobile phase on the capacity ratio (k') was, therefore, investigated with a 3% methanol in 0.3% potassium phosphate buffer–acetonitrile system. The k' values of unconjugated and glycine- and taurine-conjugated 3-oxobile acids relative to tauro-3-dehydro-norDCA were plotted against pH (Fig. 1). The k' values were markedly influenced by pH of the mobile phase. In the higher pH region, unconjugated and glycine- and taurine-conjugated 3-oxobile acids exhibited similar k' values. Conversely, the k' values of unconjugated and glycine-conjugated 3-oxobile acids increased as the pH decreased from 7.0 and 5.5, respectively. This chromatographic behaviour can be explained in terms of the dissociation of unconjugated (pK_a 6.0) and glycine- (pK_a 4.5) and taurine-conjugated (pK_a 1.5) bile acids [5].

The pH effect of the mobile phase on the resolution of two positional isomers, 7α-hydroxy-3-oxo-5β-cholanoic acid (3-dehydro-CDCA) and 12α-hydroxy-3-oxo-5β-cholanoic acid (3-dehydro-DCA), is illustrated in Fig. 2. Irrespective of the conjugated form at C-24, 3-dehydro-DCA exhibited a larger k' value than corresponding 3-dehydro-CDCA at pH 7.5. With decreasing pH, the k' values of unconjugated and glycine-conjugated 3-dehydro-DCA decreased, whereas tau-

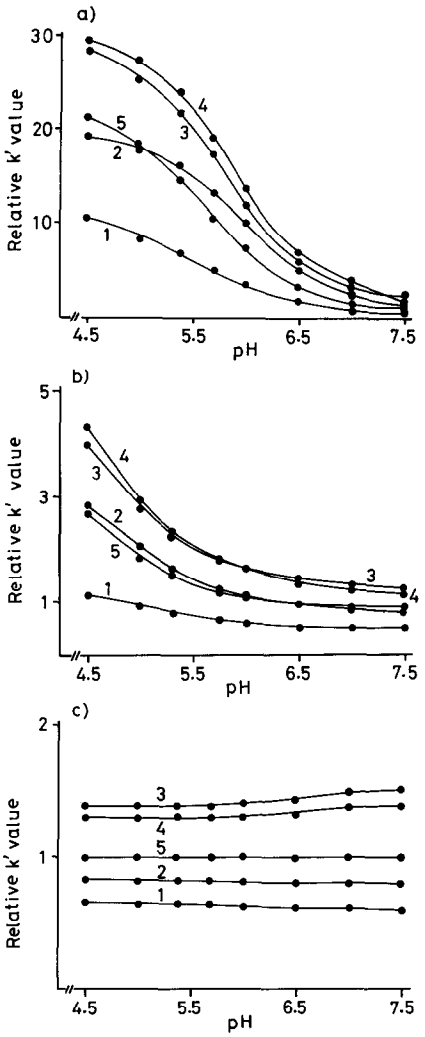

Fig. 1. Effect of pH of mobile phase on k' values of 3-oxobile acids relative to tauro-3-dehydro-norDCA. Curves: 1 = 3-dehydro-CA; 2 = 3-dehydro-UDCA; 3 = 3-dehydro-DCA; 4 = 3-dehydro-CDCA; 5 = 3-dehydro-norDCA. (a) Unconjugated, (b) glycine-conjugated, and (c) taurine-conjugated 3-oxobile acids. Conditions: column, Nova-Pak Phenyl; mobile phase, 3% methanol in 0.3% potassium phosphate buffer–acetonitrile, 1.5 ml/min.

rine-conjugated 3-dehydro-DCA exhibited a larger k' value than the corresponding 3-dehydro-CDCA in the pH range 4.5–7.5. It has been reported that steric interaction between the carboxylic acid or sulphonic acid residue on the side-chain and the hydroxyl group at C-12 may occur in the pH region where the acidic moiety is dissociated, resulting in larger retention values for bile acids with a 12α-hydroxyl group [6]. The resolution of 3-dehydro-CDCA and 3-dehydro-DCA could be due to the same steric interaction .

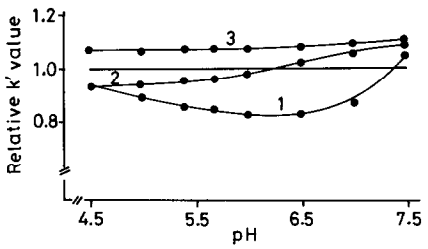

Fig. 2. Effect of pH of mobile phase on k' values of 3-dehydro-DCA relative to 3-dehydro-CDCA. Curves: 1 = unconjugated; 2 = glycine-conjugated; 3 = taurine-conjugated. Conditions as in Fig. 1.

On the basis of these data, 3% methanol in 0.3% potassium phosphate buffer (pH 7.0)–acetonitrile (8:5, v/v) was chosen as a suitable mobile phase. Typical chromatograms of glycine- and taurine-conjugated 3-oxobile acids are shown in Fig. 3. Each 3-oxobile acid exhibited a single sharp peak, except 7β-hydroxy-3-oxo-5β-cholanoic acid (3-hydroxy-UDCA), which gave a broad peak owing to Z and E isomers. The resulting oximes were monitored by fluorescence detection (excitation wavelength 260 nm, emission wavelength 405 nm), the limit of detection being 20 fmol (at a signal-to-noise ratio of 5).

Separation and characterization of 3-oxobile acids in serum

The separation and characterization of 3-oxobile acids in serum were carried out according to the scheme shown in Fig. 4. After addition of I.S., a serum sample from a patient with obstructive jaundice was extracted with a Sep-Pak C_{18} cartridge. The extract was then subjected to derivatization with O-(2-anthrylmethyl)hydroxylamine, according to the procedure described above. The fluorescent derivatives of 3-oxobile acids were separated into the unconjugated and glycine- and taurine-conjugated fractions by ion-exchange chromatography on a lipophilic gel, PHP-LH-20 [4], and each fraction was then subjected to HPLC on a Nova-Pak Phenyl column. As illustrated in Fig. 5, peaks with k' values identical with those of glycine- or taurine-conjugated 7α,12α-dihydroxy-3-oxo-5β-cholanoic acids (3-dehydro-CA) and 3-dehydro-CDCA were distinctly separated.

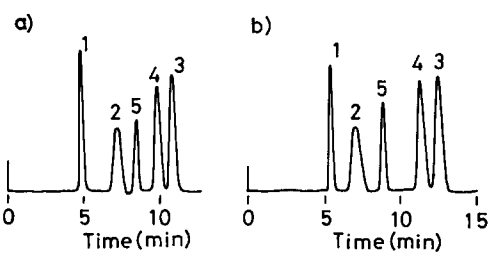

Fig. 3. Separation of O-(2-anthrylmethyl)oximes of 3-oxobile acids. Peaks: 1 = 3-dehydro-CA; 2 = 3-dehydro-UDCA; 3 = 3-dehydro-DCA; 4 = 3-dehydro-CDCA; 5 = 3-dehydro-nor-DCA. (a) Glycine-conjugated, (b) taurine-conjugated 3-oxobile acids. Conditions; column, Nova-Pak Phenyl; mobile phase, 3% methanol in 0.3% potassium phosphate buffer (pH 7.0)–acetonitrile (8:5, v/v), 1.5 ml/min.

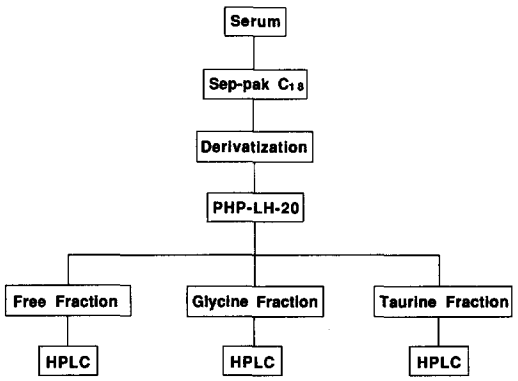

Fig. 4. General scheme for separation of 3-oxobile acids in human serum.

Previously, we investigated the chromatographic behaviour of bile acids with mobile phases of various pH values, and found that the retention values were dependent on the number and positions of hydroxyl groups on the steroid nucleus as well as the conjugated form at C-24. This finding was used in the structural characterization of 3-oxobile acids in serum. The eluate corresponding to each peak on the chromatogram (Fig. 5) was collected and, after addition of I.S., subjected to HPLC on the Nova-Pak Phenyl column with mobile phases of different pH values. It is evident from the data in Table I that the relative k' values of 3-oxobile acids in serum were identical with those of the authentic samples. Moreover, peak-area ratios of 3-oxobile acids to the I.S. were almost constant.

It is obvious from these results that 3-dehydro-CA and 3-dehydro-CDCA are present in human serum as the glycine and taurine conjugates. When 10 ng each of glycine- and taurine-conjugated 3-dehydro-CA were added to serum of a healthy volunteer, the recoveries were 92.5 ± 7.2 and 91.8 ± 6.5% (n = 8),

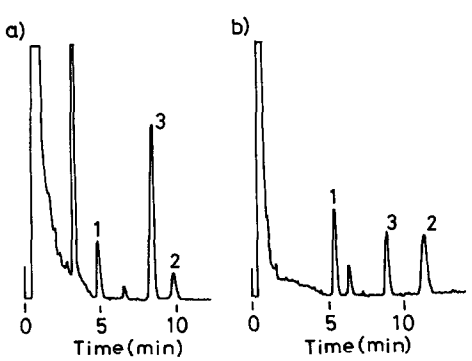

Fig. 5. Chromatograms of 3-oxobile acids in serum from a patient with obstructive jaundice as O-(2-anthrylmethyl)oxime derivatives. Peaks: 1 = 3-dehydro-CA; 2 = 3-dehydro-CDCA; 3 = 3-dehydro-nor-DCA (I.S.). (a) Glycine-conjugated, (b) taurine-conjugated fraction. Conditions as in Fig. 3.

TABLE I

RELATIVE k' VALUES OF O-(2-ANTHRYLMETHYL)OXIMES OF 3-OXOBILE ACIDS IN HUMAN SERUM AT VARIOUS pH VALUES

The figures are k' values relative to the internal standard. Conditions: column, Nova-Pak Phenyl; mobile phase, 3% methanol in 0.3% potassium phosphate buffer–acetonitrile, 1.5 ml/min.

3-Oxobile acid	Internal standard	k'				
		pH 7.5	pH 7.0	pH 6.5	pH 5.5	pH 4.5
Tauro-3-dehydro-CA	Tauro-3-dehydro-CDCA		0.41		0.45	0.46
Serum			0.41		0.45	0.46
Tauro-3-dehydro-CDCA	Tauro-3-dehydro-CA		2.44		2.17	2.23
Serum			2.45		2.17	2.22
Glyco-3-dehydro-CA	Glyco-3-dehydro-CDCA	0.40	0.39	0.36	0.34	
Serum		0.40	0.39	0.36	0.34	
Glyco-3-dehydro-CDCA	Glyco-3-dehydro-CA	2.48	2.51	2.80	2.94	
Serum		2.48	2.51	2.80	2.94	

respectively. The total amount of these 3-oxobile acids was *ca.* 100 pmol/ml, corresponding to 0.1% of the common bile acids, and the ratio of glycine- and taurine-conjugated bile acids was found to be *ca.* 1:3. It is sufficiently substantiated that the serum levels of glycine- and taurine-conjugated bile acids in patients with obstructive jaundice are nearly equal. Therefore, it should be noted that 3-oxobile acids in human serum are present mainly as the taurine conjugates.

Further studies on the metabolism of 3-oxobile acids in hepatobiliary diseases are being conducted in these laboratories, and the results will be reported elsewhere.

ACKNOWLEDGEMENTS

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