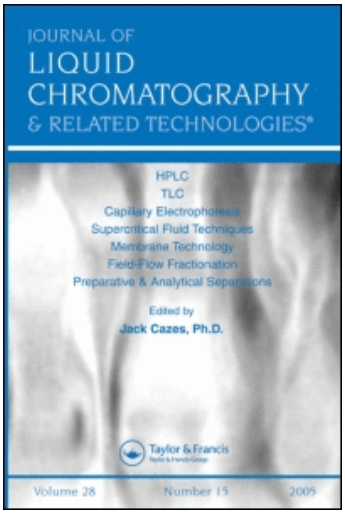

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High-Performance Liquid Chromatographic Determination of Conjugated and Unconjugated 3-Oxo-Δ^4- and 3-Oxo-$\Delta^{4,6}$-Bile Acids in Human Urine

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CONJUGATED AND UNCONJUGATED 3-OXO-Δ^4- AND 3-OXO-$\Delta^{4,6}$-BILE ACIDS IN HUMAN URINE

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ABSTRACT

A method for simultaneous determination of conjugated and unconjugated C_{24}-bile acids having 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-structures in human urine has been developed by high performance liquid chromatography coupled with dual wavelength UV detection. Satisfactory resolution was attained on a C_{18} reversed phase column by gradient elution with acetonitrile - sodium acetate within 50 min. The linearity of the calibration curve for each bile acid ranged from 10 pmol to 500 pmol per injection, and the detection limit was 2 - 4 pmol (SN = 5).

This method was used for direct analysis of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids present in the urine of healthy infants and patients with liver disease, after only a preliminary clean-up procedure using Bond Elut C18.

INTRODUCTION

Recently, a novel disorder in bile acid biosynthesis, named '3-oxo-Δ^4-steroid 5β-reductase deficiency, was described by Setchell et al.[1] and Clayton et al.[2] It is characterized by markedly elevated urinary levels of the amidates of 3-oxo-Δ^4-bile acids, 7α-hydroxy-3-oxo-4-cholenoic (CDCA-Δ^4-3-one) and 7α, 12α-dihydroxy-3-oxo-4-cholenoic (CA-Δ^4-3-one) acids (Fig. 1), and has been identified in more than twelve infants with liver diseases such as neonatal hepatitis and cholestasis.[1-5]

As an abnormal presence of these bile acids in urine reflects this disorder, their measurement in urine is important from a clinical point of view. We have also identified and determined these unusual bile acids in the urine from patients with severe cholestasis.[6,7]

The separation and quantification of these bile acids is carried out mainly by gas chromatography - mass spectrometry (GC-MS).[1-7] These procedures, however, have some disadvantages, such as tedious clean-up and insufficient information concerning the conjugation mode of the bile acids. Moreover, 3-oxo-Δ^4-bile acids are considered to be thermally unstable, and to be easily converted into their dehydrated products, 3-oxo-$\Delta^{4,6}$-bile acids, or into unknown degradation products, under the alkaline or acidic conditions usually used for deconjugation in the GC-MS method.

Therefore, high performance liquid chromatography (HPLC), which makes prior deconjugation unnecessary, and enables direct analysis of polar and thermally unstable biological substances, appears to be suitable for the separation and determination of bile acid conjugates.

This paper describes a simple method developed for the simultaneous quantitation of conjugated and unconjugated 3-oxo-Δ^4-bile acids and their dehydrated products, 3-oxo-$\Delta^{4,6}$-bile acids. The application of this method to the determination of these bile acids in urine from both healthy infants and patients with liver diseases is also discussed.

MATERIALS AND METHODS

Materials

CDCA-Δ^4-3-one, CA-Δ^4-3-one, 3-oxo-4,6-choladienoic (CDCA-$\Delta^{4,6}$-3-one) and 12α-hydroxy-3-oxo-4,6-choladienoic (CA-$\Delta^{4,6}$-3-one) acids were chemically synthesized as reported previously.[8-10] Glycine-conjugated bile acids and internal standards (IS) were synthesized according to the known methods using 2,2,2-trichloroethyl esters of the corresponding amino acids.[11-13]. Taurine-conjugated bile acids were prepared in the manner previously reported.[11] Bond Elut C18 cartridges were obtained from Varian (Harbor City, CA, U.S.A). All other reagents were of analytical grade.

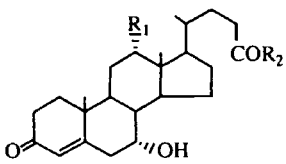
Urine Samples

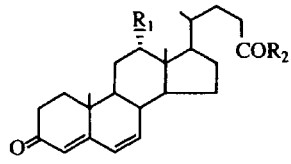
Urine samples were collected without preservatives from four patients (1 - 2 months old) with liver disease, who showed an abnormality in 3-oxo-Δ^4-steroid 5β-reductase by GC-MS analysis, and from ten healthy infants (1 - 2 months old) as a control. All specimens were stored at -25°C until analysis.

Apparatus and Chromatographic Conditions

The HPLC apparatus consisted of a Shimadzu LC-10A system equipped with an SPD-10A spectrophotometer, which was set at a dual wavelength mode (Shimadzu, Kyoto, Japan). The chromatographic separation was performed on an Inertsil ODS2 (250 x 4.6 mm i.d., 5 μm, GL Sciences, Tokyo, Japan) column using a gradient elution mode at a flow rate of 1.0 mL / min. The column temperature was ambient. Mobile phase A was 30 mM sodium acetate - acetonitrile (80 : 20, v/v) adjusted to an apparent pH 4.2 with phosphoric acid, and mobile phase B was acetonitrile.

The gradient program was as follows: isocratic elution with mobile phase A for 4 min, then a linear gradient to 50 % of mobile phase B over a period of 31 min, and a discontinuous gradient to 65 % of mobile phase B. The absorbance of the eluent was monitored with dual wavelengths at 245 nm for 3-oxo-Δ^4-bile acids and at 280 nm for 3-oxo-$\Delta^{4,6}$-bile acids. The void volume (t_0) was measured with sodium nitrate (UV 210 nm).[14]





CDCA-Δ^4-3-one	: R_1 = H,	R_2 = OH	CDCA-$\Delta^{4,6}$-3-one	: R_1 = H,	R_2 = OH
CA-Δ^4-3-one	: R_1 = OH,	R_2 = OH	CA-$\Delta^{4,6}$-3-one	: R_1 = OH,	R_2 = OH
G-CDCA-Δ^4-3-one	: R_1 = H,	R_2 = $NHCH_2CO_2H$	G-CDCA-$\Delta^{4,6}$-3-one	: R_1 = H,	R_2 = $NHCH_2CO_2H$
G-CA-Δ^4-3-one	: R_1 = OH,	R_2 = $NHCH_2CO_2H$	G-CA-$\Delta^{4,6}$-3-one	: R_1 = OH,	R_2 = $NHCH_2CO_2H$
T-CDCA-Δ^4-3-one	: R_1 = H,	R_2 = $NH(CH_2)_2SO_3K$	T-CDCA-$\Delta^{4,6}$-3-one	: R_1 = H,	R_2 = $NH(CH_2)_2SO_3K$
T-CDCA-Δ^4-3-one	: R_1 = OH,	R_2 = $NH(CH_2)_2SO_3K$	T-CDCA-$\Delta^{4,6}$-3-one	: R_1 = OH,	R_2 = $NH(CH_2)_2SO_3K$
IS1	: R_1 = OH,	R_2 = $NH(CH_2)_2CO_2H$	IS2	: R_1 = OH,	R_2 = $NH(CH_2)_2CO_2H$

Figure 1. Structures of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids.

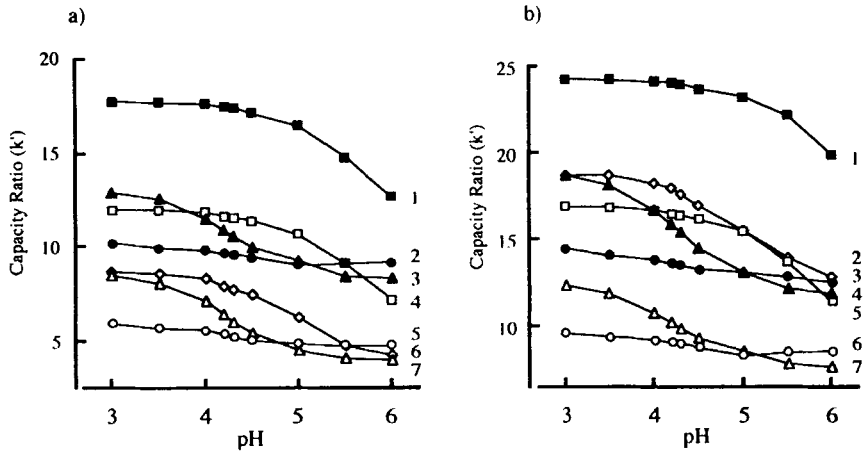


Figure 2. Effect of pH of mobile phase on capacity ratios (k') of 3-oxo-Δ^4- (a) and 3-oxo-$\Delta^{4,6}$- (b) bile acids. (a) 1 : CDCA-Δ^4-3-one, 2 : T-CDCA-Δ^4-3-one, 3 : G-CDCA-Δ^4- 3-one, 4 : CA-Δ^4-3-one, 5 : T-CA-Δ^4-3-one, 6 : IS1, 7 : G-CA-Δ^4-3-one, (b) 1 : CDCA-$\Delta^{4,6}$-3-one, 2 : IS2, 3 : T-CDCA-$\Delta^{4,6}$-3-one, 4 : G-CDCA-$\Delta^{4,6}$-3-one, 5 : CA-$\Delta^{4,6}$-3-one, 6 : T-CA-$\Delta^{4,6}$-3-one, 7 : G-CA-$\Delta^{4,6}$-3-one. IS1 : N-(7α, 12α-Dihydroxy-3-oxo-4-cholen-24-oyl)-3-aminopropionic acid. IS2 : N-(3-Oxo-4,6-choladien-24-oyl)-3-aminopropionic acid.

Analysis of 3-Oxo-Δ^4- and 3-Oxo-$\Delta^{4,6}$- Bile Acids

To a urine sample (0.1 - 4.0 mL), appropriate amounts of the internal standards were added, and the solution was loaded on a Bond Elut C18 cartridge. The cartridge was washed with 4 mL of water, and the bile acids were eluted with 4 mL of methanol. After evaporation of the solvent under reduced pressure at less than 30°C, the residue was dissolved in methanol, and subjected to HPLC analysis.

RESULTS AND DISCUSSION

Separation of 3-Oxo-Δ^4- and 3-Oxo-$\Delta^{4,6}$-Bile Acids

The chemical structures of the 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids examined and their abbreviations are shown in Fig. 1. 3-Aminopropionic acid amidates of CA-Δ^4-3-one and CDCA-$\Delta^{4,6}$-3-one, which were not present in human biological fluids and exhibited the same absorption maxima and molar absorptivity, were used as internal standards.

Exact control of pH in the mobile phase is essential for high resolution. Therefore, the effect of the mobile phase pH on the capacity ratio (k') values of unconjugated and glycine- and taurine-conjugated bile acids was initially examined on a reversed phase C_{18} column with a mixture of acetonitrile and sodium acetate. Acetonitrile gradient elution was employed in order to obtain sharp and symmetrical peaks, and complete separation of the fourteen 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acid conjugates including internal standards within a shorter period of time. Changes in the k' values in response to changes in the apparent pH of the initial mobile phase are shown in Fig. 2. As suggested by previous findings,[15-17] pH greatly influences the k' values of unconjugated and glycine-conjugated bile acids. In the lower pH region, a better separation of all the bile acids can be achieved, although the bile acids except for taurine conjugates, exhibit long retention times. The result of various combinations of pH and the acetonitrile gradient program showed that an apparent pH of 4.2 for the initial mobile phase provided good resolution of all the bile acids. The concentration of sodium acetate also affected on the k' values of glycine and taurine conjugates as was shown in previous reports,[15,17,18] although it did not affect the retention time of unconjugates or the peak shape of each bile acid. A higher resolution of all the bile acids was obtained with a salt concentration of over 30 mM.

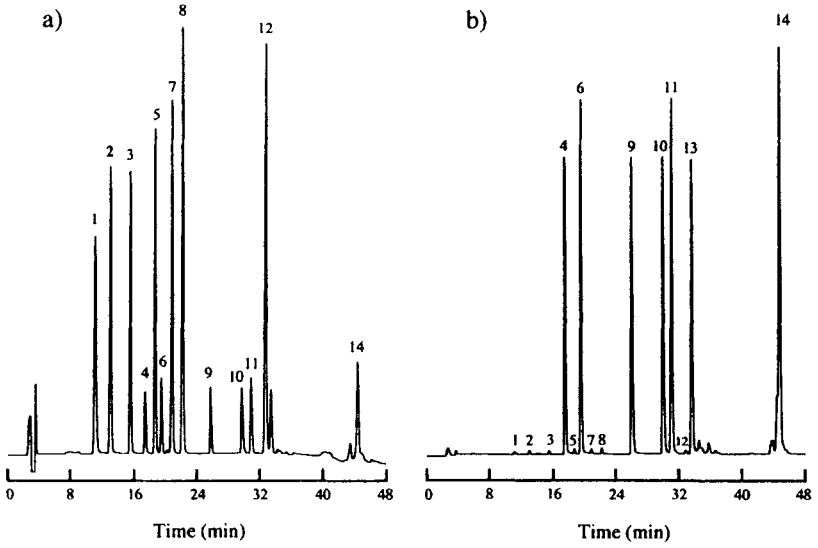


Figure 3. Typical high-performance liquid chromatograms of the standard 3-oxo-Δ^4- (a) and 3-oxo-$\Delta^{4,6}$- (b) bile acids. (a) 1 : T-CA-Δ^4-3-one, 2 : G-CA-Δ^4-3-one, 3 : IS1, 4 : T-CA-$\Delta^{4,6}$-3-one, 5 : T-CDCA-Δ^4-3-one, 6 : G-CA-$\Delta^{4,6}$-3-one, 7 : G-CDCA-Δ^4-3-one, 8 : CA-Δ^4-3-one, 9 : T-CDCA-$\Delta^{4,6}$-3-one, 10 : G-CDCA-$\Delta^{4,6}$-3-one, 11 : CA-$\Delta^{4,6}$-3-one, 12 : CDCA-Δ^4-3-one, 13 : IS2, 14 : CDCA-$\Delta^{4,6}$-3-one.

From these results, a gradient of acetonitrile in the starting eluent consisting of 30 mM sodium acetate - acetonitrile (80 : 20) adjusted to apparent pH 4.2 was used in the present study. The optimal analytical conditions are as described in **MATERIALS AND METHODS**.

Typical chromatograms obtained using authentic specimens of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids were recorded simultaneously at wavelengths of 245 nm and 280 nm (Fig. 3). All the bile acids were completely separated from each other without noticeable peak asymmetry within 50 min. The retention of the bile acid species increased in the following order : CA-Δ^4-3-one < CA-$\Delta^{4,6}$-3-one < CDCA-Δ^4-3-one < CDCA-$\Delta^{4,6}$-3-one regardless of whether the bile acids were conjugated or not. When the bile acids were conjugated, the ordering of the k' values was taurine conjugates < glycine conjugates < unconjugates. Reproducible retention times were obtained for all the bile acids under the present HPLC conditions. The calibration curves were constructed by plotting the relative peak area of each bile acid to the internal standard against the amounts of the corresponding bile acid. A good linear relationship to each

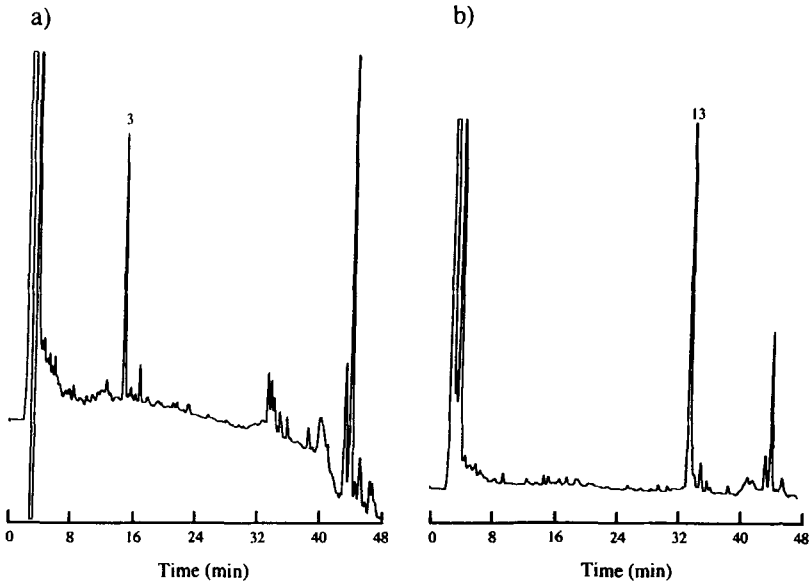


Figure 4. High performance liquid chromatograms of 3-oxo-Δ^4- (a) and 3-oxo-$\Delta^{4,6}$- (b) bile acids in urine from a healthy infant. Peak identity is the same as in Fig. 3.

Table 1

Recoveries of Conjugated Bile Acids Added to Urine

Bile Acid[a]	Relative Recoveries (%, n=5)		
	Unconjugate	Glycine	Taurine
CA-Δ^4-3-one	97.5 ± 2.4[b]	103.4 ± 1.3	99.0 ± 1.8
CDCA-Δ^4-3-one	102.2 ± 0.9	98.8 ± 1.3	100.2 ± 1.6
CA-$\Delta^{4,6}$-3-one	98.2 ± 2.5	101.3 ± 1.8	99.5 ± 2.8
CDCA-$\Delta^{4,6}$-3-one	101.3 ± 3.4	97.2 ± 3.2	98.3 ± 3.3

[a] See Fig. 1 for abbreviations.

[b] Each value represents Mean ± S.D. of the recovery to that of IS.

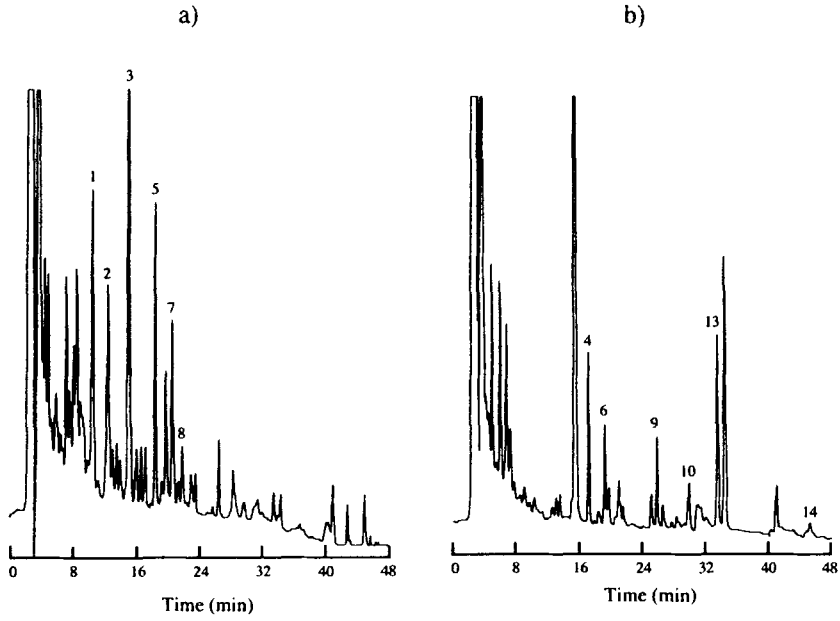


Figure 5. High performance liquid chromatograms of 3-oxo-Δ^4- (a) and 3-oxo-$\Delta^{4,6}$- (b) bile acids in urine from a patient with liver disease. Peak identity is the same as in Fig. 3.

bile acid was obtained over the range of 10 - 500 pmol with linear correlation coefficients of more than 0.999 for all the bile acids. The coefficients of variation for the measurement of 20 pmol of each standard bile acid were less than 3 %. The detection limits of the 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids were estimated to be *ca.*4 and 2 pmol (signal-to-noise ratio = 5), respectively.

Determination of 3-Oxo-Δ^4- and 3-Oxo-$\Delta^{4,6}$ Bile Acids in Infant Urine

The HPLC method was applied to the determination of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids in urine samples from healthy infants and from patients with liver disease, in which an abnormality in 3-oxo-Δ^4-steroid 5β-reductase was suggested.[6,7] Urine samples were submitted to a preliminary clean-up procedure using conventional C_{18} reversed phase extraction prior to HPLC analysis. The recovery rates through the clean-up procedure described in **MATERIALS AND METHODS** were tested by adding pre-determined

Table 2

Concentration of 3-Oxo-Δ^4- and 3-Oxo-$\Delta^{4,6}$-Bile Acids in Urine from Normal Children and Patients with Liver Disease

	Concentration (nmol/mL)				
Bile Acid[a]	**P1[b]**	**P2**	**P3**	**P4**	**Normal[c]**
CA-Δ^4-3-one	8.0	3.14	4.47	1.54	0.03 ± 0.03
G-CA-Δ^4-3-one	29.0	6.39	7.42	4.16	0.08 ± 0.12
T-CA-Δ^4-3-one	31.4	7.17	1.58	0.34	0.05 ± 0.03
CDCA-Δ^4-3-one	n.d.	0.51	1.18	0.08	n.d.[d]
G-CDCA-Δ^4-3-one	25.1	6.85	15.10	0.47	n.d.
T-CDCA-Δ^4-3-one	23.0	13.50	6.23	0.53	n.d.
CA-$\Delta^{4,6}$-3-one	2.61	0.08	0.20	0.20	n.d.
G-CA-$\Delta^{4,6}$-3-one	5.03	0.54	0.50	0.23	0.04 ± 0.02
T-CA-$\Delta^{4,6}$-3-one	7.56	1.18	0.31	0.24	0.02 ± 0.02
CDCA-$\Delta^{4,6}$-3-one	n.d.	0.18	0.15	0.13	n.d.
G-CDCA-$\Delta^{4,6}$-3-one	3.47	0.46	0.71	0.16	n.d.
T-CDCA-$\Delta^{4,6}$-3-one	4.04	3.15	1.02	0.07	n.d.
Total	139.2	43.15	38.87	8.15	0.22

[a] See Fig. 1 for abbreviations.
[b] Number of patients, P: Patient.
[c] Each value represents Mean ± S.D. (n=10).
[d] Not detected.

amounts of conjugated and unconjugated 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids (each 200 μg) to steroid-free urine (1.0 mL) prepared by charcoal extraction.[19] As shown in Table 1, all the bile acids were recovered at a rate of more than 97 %, and the coefficients of variation were less than 4 % (n=5). Decomposition or dehydration of the 7α-hydroxy group were not observed in the clean-up procedure.

Figures 4 and 5 show typical chromatograms obtained from the urine of a normal infant and a patient with liver disease, respectively. A marked difference in the bile acid profiles was observed between the normal infant and

liver disease patient. The peaks of almost all of the 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids in Fig. 5 were identified on the basis of their retention times. The results obtained from four liver disease patients and ten normal infants are summarized in Table 2. The means of the total concentrations of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids in urine of the normal 1 - 2 month-old infants were estimated to be 0.16 and 0.06 nmol / mL, respectively.

On the other hand, all the bile acid levels in urine from the liver disease patients were significantly higher than those in normal subjects, accounting for 7.12 - 116.5 nmol / mL for 3-oxo-Δ^4-bile acids and 1.03 - 22.71 nmol / mL for 3-oxo-$\Delta^{4,6}$-bile acids. The glycine and taurine amidates of CA-Δ^4-3-one and CDCA-Δ^4-3-one were the predominant components in liver disease patients, accounting for more than *ca.* 70 % of the total amounts of the 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids. The proportion of 3-oxo-$\Delta^{4,6}$-bile acids to total bile acids ranged from 7 to 16 %.

The proposed method, coupled with dual wavelength UV detection, is suitable for the simultaneous determination of 3-oxo-Δ^4- and 3-oxo-$\Delta^{4,6}$-bile acids in routine clinical analysis. Further studies on the urinary excretion of these bile acids in patients with hepatobiliary disease using this method are in progress.

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