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# Simultaneous determination of bile acids in rat liver tissue by high-performance liquid chromatography

Hiroo Sakakura[a,*], Noriyuki Kimura[b], Haruki Takeda[b], Hiroshi Komatsu[b], Kaoru Ishizaki[b], Sachiko Nagata[b]

[a]*Product Planning and Development Department, Consumer Products Marketing Division, Tokyo Tanabe Co. Ltd., 2-6, Nihonbashi-honcho 2-Chome, Chuo-ku, Tokyo 103-8405, Japan*

[b]*Kazusa Research Laboratories, Tokyo Tanabe Co. Ltd., 100-5 Yana Kisarazu-shi, Chiba 292-0812, Japan*

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**Abstract**

A method for the simultaneous determination of bile acids in rat liver tissue by high-performance liquid chromatography was developed. Without prior fractionation and alkaline hydrolysis, 30 unconjugated, glycine- and taurine-conjugated bile acids were detected by post-column enzymatic reaction and fluorescence detection. They were separated on a reversed-phase column using a linear gradient solvent system of 10 m*M* tribasic ammonium phosphate–acetonitrile–methanol (44:12:5, v/v/v) and 20 m*M* dibasic ammonium phosphate–acetonitrile–methanol (2:1:2, v/v/v). The limits of detection were 1–5 pmol, and calibration curves were linear for concentrations ranging between 10 and 4000 pmol per 10 μl injection. This rapid and reliable method is effective for measuring bile acid levels in liver tissue not only of rats but also of patients with hepatobiliary and other diseases. 

*Keywords:* Bile acids

## 1. Introduction

Bile acids are synthesized in the liver and circulate through the bile, intestine, and the portal vein. Therefore, determination of the bile acid composition in the liver as well as that in the serum and bile is important in clarifying the synthesis and enterohepatic circulation of bile acids and studying their metabolism in hepatobiliary diseases.

The following methods of determining bile acids in the liver have been reported. Liver tissue is homogenized with an organic solvent or alkaline solution and dissolved by heating for extraction of bile acids. After prior fractionation of bile acids into unconjugated, glycine- and taurine-conjugated groups, deconjugation (such as alkaline and/or enzymatic hydrolysis), or conversion into derivatives, bile acids are determined by radioimmunoassay [1,2], gas chromatography, gas chromatography–mass spectrometry [3–15] or high-performance liquid chromatography (HPLC) [16]. In HPLC for determination of bile acids, refractive index [17–19], ultraviolet absorbance [20–24], and the post- or pre-column fluorescence detection methods [25–30] are widely used.

Experimental studies on bile acid metabolism in hepatobiliary diseases are generally performed using rats. In rats, in addition to cholic acid (CA;

*Corresponding author.

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3α,7α,12α-trihydroxy-5β-cholan-24-oic acid), chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid), deoxycholic acid (DCA; 3α,12α-dihydroxy-5β-cholan-24-oic acid), lithocholic acid (LCA; 3α-hydroxy-5β-cholan-24-oic acid), the following specific bile acids are present in the unconjugated or glycine- or taurine-conjugated form: α-muricholic acid (α-MCA; 3α,6β,7α-trihydroxy-5β-cholan-24-oic acid), β-muricholic acid (β-MCA; 3α,6β,7β-trihydroxy-5β-cholan-24-oic acid), ω-muricholic acid (ω-MCA; 3α,6α,7β-trihydroxy-5β-cholan-24-oic acid), and hyodeoxycholic acid (HDCA; 3α,6α-dihydroxy-5β-cholan-24-oic acid).

Simultaneous analysis of these bile acids by the above methods is difficult, and complicated procedures such as prior fractionation or deconjugation, or conversion into derivatives are necessary.

Therefore, we evaluated our previously reported method of simultaneously determining 26 bile acids by HPLC [31] and the method of extracting bile acids in rat liver tissue. This paper describes a sensitive method developed for the determination simultaneously of 30 bile acids in the rat liver by HPLC that requires only a simple pretreatment method and no complicated pretreatment such as prior fractionation, deconjugation, or conversion into derivatives. This method is sensitive and simple in practice, which makes it suitable for routine analysis.

## 2. Experimental

### *2.1. Reagents*

β-Nicotinamide adenine dinucleotide (β-NAD) was obtained from Sigma (St. Louis, MO, USA). Methanol and acetonitrile were of HPLC-grade and the other reagents were of analytical grade. Bond Elut C18 cartridges (200 mg) were obtained from Varian Sample Preparation Products (Harbor City, CA, USA).

### *2.2. Bile acids*

Ursodeoxycholic acid (UDCA; 3α,7β-dihydroxy-5β-cholan-24-oic acid) and CDCA were obtained from Tokyo Tanabe (Tokyo, Japan). The following unconjugated bile acids were purchased from Steraloids (Wilton, NH, USA): α-MCA, β-MCA, hyocholic acid (HCA; 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid), HDCA, CA, DCA, LCA and 23-nordeoxycholic acid (3α,12α-dihydroxy-23-nor-5β-cholan-24-oic acid). ω-MCA was synthesized in our laboratory according to the method reported by Hsia [32].

The glycine (G)- and taurine (T)-conjugated bile acids were synthesized from the following respective unconjugated bile acids in our laboratory according to the method reported by Kanazawa et al. [33]: G-α-MCA, T-α-MCA, G-β-MCA,T-β-MCA, G-ω-MCA, T-ω-MCA, G-HCA, T-HCA, G-UDCA, T-UDCA, G-HDCA, T-HDCA, G-CA, T-CA,G-CDCA, T-CDCA, G-DCA, T-DCA, G-LCA,T-LCA and tauro-23-nordeoxycholic acid.

### *2.3. Bile acids standard solution and internal standard solution*

The bile acids standard solution containing 30 bile acids was prepared at a concentrations of 30 nmol for each bile acid per 1 ml of ethanol. Internal standard (I.S.) solution of tauro-23-nordeoxycholic acid was prepared in ethanol at a concentration of 80 nmol/ml. Bile acids standard and I.S. solutions were stable for at least three years when stored at 5°C.

### *2.4. Chromatographic apparatus*

The HPLC system was an LC-10A equipped with an LC RF-550 spectrofluorometer (Shimadzu Seisakusho Co., Kyoto, Japan). STR ODS-II (150× 4.6 mm I.D., particle diameter 5 μm, pore size 120 Å; Shinwa Chemical Industries, Kyoto, Japan) was used for the separation of bile acids. For the immobilized 3α-hydroxysteroid dehydrogenase (3α-HSD) column, an E-3α-HSD column (20×4.0 mm I.D.; Sekisui Chemical, Osaka, Japan) was used.

### *2.5. Chromatographic conditions*

Individual bile acids were eluted on reversed-phase HPLC with a linear gradient at a flow-rate of 1.0 ml/min. Mobile phase A was 10 m*M* tribasic ammonium phosphate–acetonitrile–methanol (44:12:5, v/v/v) and mobile phase B was 20 m*M*

dibasic ammonium phosphate–acetonitrile–methanol (2:1:2, v/v/v).

The samples were eluted with mobile phase A for an initial 60 min after injection, then with a linear gradient of mobile phase B from 0% to 100% over 40 min, followed by continued elution with mobile phase B for 40 min. Before injection of the next sample, the column was equilibrated with 100% mobile phase A for 30 min.

Bile acids separated on a reversed-phase column, were mixed with β-NAD reagent pumped at a flow-rate of 1.0 ml/min. The mixture migrated through the 3α-HSD immobilized enzymatic column, forming β-NADH and the corresponding 3-keto bile acids [25]. The separating and enzymatic columns were maintained at 20°C. The β-NADH generated by the enzymatic reaction was determined by fluorescence detection (excitation at 340 nm, emission at 460 nm). The β-NAD reagent for the enzymatic reaction contained 0.3 m*M* β-NAD, 1 m*M* disodium EDTA and 0.05 v/v % 2-mercaptoethanol in 10 m*M* monobasic potassium phosphate, adjusted to pH 8.0 with 6 *M* KOH. Quantification was based on peak-height measurements.

### *2.6. Procedure for determination of bile acids in rat liver tissue*

Eleven-week-old male Wistar rats (Japan SLC Co., Hamamatsu, Japan) weighing 290–330 g, were housed in an air-conditioned room (25±1°C, 50–60% humidity) under a light–dark cycle (8:00 a.m. to 8:00 p.m.) and fed a commercial balanced stock diet (CLEA CE-2, Clea Japan, Tokyo, Japan) and water ad libitum. Rats were abdominally dissected under ether anesthesia. After the rats were killed by decapitation, the livers were removed, perfused with ice-cold saline to remove blood, dried on filter paper, weighed, and kept frozen at −20°C until analysis.

Liver tissue (100 mg), minced using scissors, was

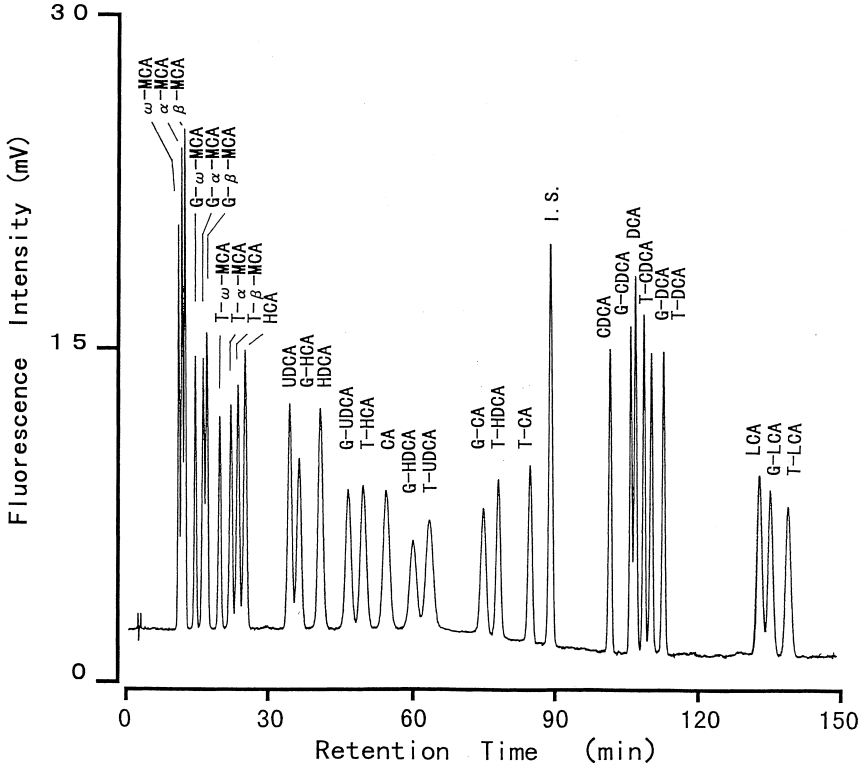


Fig. 1. Typical elution profile of the 30 bile acids. Conditions were as described in Section 2. Aliquots of 150 pmol of individual bile acids were injected.

mixed with 100 μl of I.S. solution and 1 ml of 0.2 *M* NaOH that had been maintained at 80°C. The mixture was immediately heated at 80°C for 20 min to dissolve the liver tissue, mixed with 3 ml water, and left for cooling to room temperature. To remove *n*-hexane-soluble material such as neutral lipids and basic compounds, the mixture was shaken with 3 ml of *n*-hexane for 10 min under alkaline conditions, followed by centrifugation at 1600×*g* for 20 min at 25°C to separate the layers and the upper organic layer was discarded. This extraction procedure with *n*-hexane was performed twice. The remaining aqueous phase was passed through a Bond Elut C18 cartridge, washed successively with methanol (3 ml×2) and water (3 ml×2) prior to use, and 10 ml of air were forced through the cartridge. Bile acids were eluted from the cartridge with 3 ml of methanol. The eluate was evaporated under reduced pressure. The residue was reconstituted in 200 μl of mobile phase B. This solution was centrifuged at 1600×*g* for 20 min at room temperature, and 10 μl of the supernatant were injected onto the HPLC system.

### *2.7. Analytical recovery*

To determine the analytical recoveries and reproducibilities of the 30 bile acids, aliquots of 1 or 40 nmol of standard bile acids were added to 100 mg of liver tissue. These samples were subjected to the clean-up procedures described above.

## 3. Results and discussion

### *3.1. Chromatographic conditions*

The best chromatographic conditions were achieved by optimizing the separation column and pH (salt), salt concentration, percentage of organic solvents in the mobile phase and column temperature as described in Section 2.

The bile acids were efficiently separated in basic mobile phase rather than in an acidic or neutral mobile phase. Consequently, among the various salts added to the mobile phase, the combination of tribasic and dibasic ammonium phosphate (pH 8–9) improved both the separation of the bile acids and the shape of the eluted peaks by reducing tailing. This basification (pH 8–9) of the mobile phase increased the sensitivity of the enzymatic reaction in the detection system more than under acidic or neutral conditions.

The salt concentration of the mobile phase also played an important role in separation. With 20 m*M* dibasic ammonium phosphate (pH 8), all bile acids except HCA, UDCA and their conjugates were satisfactorily separated. Because the initial mobile phase was 10 m*M* tribasic ammonium phosphate (pH 9), the HCA, UDCA and their conjugates became more symmetrical, confirming the efficient separation of these bile acids.

Table 1
The linear regression equations and the correlation coefficients

| Bile acid | Linear regression equations | Correlation coefficients |
|---|---|---|
| *Unconjugated bile acid* | | |
| β-MCA | $7.50\times10^{-3}\ X+0.044$ | 1.000 |
| α-MCA | $7.37\times10^{-3}\ X+0.055$ | 1.000 |
| ω-MCA | $6.40\times10^{-3}\ X+0.048$ | 1.000 |
| DCA | $5.84\times10^{-3}\ X+0.026$ | 1.000 |
| CDCA | $4.80\times10^{-3}\ X+0.030$ | 1.000 |
| HCA | $4.26\times10^{-3}\ X+0.045$ | 1.000 |
| UDCA | $3.51\times10^{-3}\ X+0.028$ | 1.000 |
| HDCA | $3.32\times10^{-3}\ X+0.031$ | 1.000 |
| LCA | $2.78\times10^{-3}\ X+0.028$ | 1.000 |
| CA | $2.15\times10^{-3}\ X+0.018$ | 1.000 |
| *Glycine conjugate* | | |
| CDCA | $5.02\times10^{-3}\ X+0.032$ | 1.000 |
| DCA | $4.59\times10^{-3}\ X+0.049$ | 1.000 |
| β-MCA | $4.55\times10^{-3}\ X+0.024$ | 1.000 |
| α-MCA | $4.43\times10^{-3}\ X+0.015$ | 1.000 |
| ω-MCA | $4.42\times10^{-3}\ X+0.026$ | 1.000 |
| HCA | $2.66\times10^{-3}\ X+0.023$ | 1.000 |
| LCA | $2.54\times10^{-3}\ X+0.024$ | 1.000 |
| UDCA | $2.19\times10^{-3}\ X+0.015$ | 1.000 |
| CA | $1.92\times10^{-3}\ X+0.022$ | 1.000 |
| HDCA | $1.40\times10^{-3}\ X+0.009$ | 1.000 |
| *Taurine conjugate* | | |
| CDCA | $5.29\times10^{-3}\ X+0.027$ | 1.000 |
| DCA | $4.56\times10^{-3}\ X+0.023$ | 1.000 |
| β-MCA | $3.83\times10^{-3}\ X+0.021$ | 1.000 |
| α-MCA | $3.63\times10^{-3}\ X+0.017$ | 1.000 |
| ω-MCA | $3.46\times10^{-3}\ X+0.022$ | 1.000 |
| CA | $2.68\times10^{-3}\ X+0.033$ | 1.000 |
| HDCA | $2.37\times10^{-3}\ X+0.023$ | 1.000 |
| LCA | $2.30\times10^{-3}\ X+0.022$ | 1.000 |
| HCA | $2.20\times10^{-3}\ X+0.035$ | 1.000 |
| UDCA | $1.62\times10^{-3}\ X+0.022$ | 1.000 |

### 3.2. Separation of bile acids

In Fig. 1 is shown a typical elution profile of the 30 bile acids and I.S. representing 150 pmol of individual bile acids per 10 μl injection.

All bile acids and I.S. were separated into 31 peaks within about 2.5 h in a gradient elution run. With this method, the bile acids were eluted in a definite order depending upon the number, position and configuration of the hydroxyl groups on the steroid nucleus [34–36]. That is, trihydroxylated (α-MCA, β-MCA, ω-MCA, HCA and CA), dihydroxylated(UDCA, HDCA, CDCA and DCA) and monohydroxylated (LCA) bile acids were eluted in order of decreasing hydrophilicity. The trihydroxylated bile acids, i.e. ω-MCA, α-MCA, β-MCA, HCA and CA were also eluted in this order. However, the dihydroxylated bile acids such as HDCA and UDCA had elution times similar to those of trihydroxylated bile acids (CA and HCA) because HDCA and UDCA, with a 6α- and a 7β-hydroxyl group, respectively, were more hydrophilic than other dihydroxy-

Table 2
Recovery of bile acids added to rat liver tissue

| Bile acid | Recovery (%)[a] | | | |
|---|---|---|---|---|
| | 1 nmol/100 mg[b] | R.S.D. (%) | 40 nmol/100 mg[b] | R.S.D.(%) |
| *Unconjugated bile acid* | | | | |
| ω-MCA | 99.5±1.00 | 1.0 | 100.5±1.31 | 1.3 |
| α-MCA | 99.3±2.81 | 2.8 | 103.1±2.21 | 2.1 |
| β-MCA | 99.8±2.80 | 2.8 | 103.6±2.46 | 2.4 |
| HCA | 98.1±3.55 | 3.6 | 103.3±1.86 | 1.8 |
| UDCA | 94.1±3.40 | 3.6 | 102.8±1.87 | 1.8 |
| HDCA | 98.3±3.20 | 3.3 | 102.8±2.07 | 2.0 |
| CA | 95.4±3.96 | 3.2 | 102.4±1.41 | 1.4 |
| CDCA | 98.4±3.00 | 3.0 | 101.7±1.07 | 1.1 |
| DCA | 99.7±3.30 | 3.3 | 101.7±1.32 | 1.3 |
| LCA | 96.8±3.35 | 3.5 | 101.1±2.26 | 2.2 |
| *Glycine conjugate* | | | | |
| ω-MCA | 99.5±1.00 | 1.0 | 102.8±1.29 | 1.3 |
| α-MCA | 105.0±4.35 | 4.1 | 105.8±2.29 | 2.2 |
| β-MCA | 103.6±3.86 | 3.7 | 104.5±2.46 | 2.4 |
| HCA | 97.7±2.34 | 2.4 | 103.5±1.53 | 1.5 |
| UDCA | 97.2±3.22 | 3.3 | 102.5±1.80 | 1.8 |
| HDCA | 100.2±2.71 | 2.7 | 103.4±2.01 | 1.9 |
| CA | 97.5±4.13 | 4.2 | 101.2±0.70 | 0.7 |
| CDCA | 98.5±2.09 | 2.1 | 101.8±1.37 | 1.3 |
| DCA | 96.6±4.40 | 4.6 | 100.3±1.57 | 1.6 |
| LCA | 102.0±3.85 | 3.8 | 104.4±3.48 | 3.3 |
| *Taurine conjugate* | | | | |
| ω-MCA | 99.5±1.20 | 1.2 | 98.5±1.31 | 1.3 |
| α-MCA | 98.6±3.57 | 3.6 | 102.3±1.85 | 1.8 |
| β-MCA | 99.1±4.11 | 4.1 | 103.1±2.02 | 2.0 |
| HCA | 99.5±1.00 | 1.0 | 98.5±1.31 | 1.3 |
| UDCA | 100.4±3.95 | 1.8 | 103.4±1.00 | 1.0 |
| HDCA | 96.3±1.31 | 3.9 | 101.3±4.42 | 4.4 |
| CA | 100.4±1.89 | 1.4 | 100.6±0.92 | 0.9 |
| CDCA | 94.7±2.55 | 2.7 | 99.8±1.22 | 1.2 |
| DCA | 99.3±2.40 | 2.8 | 100.4±1.04 | 1.0 |
| LCA | 103.9±2.49 | 2.4 | 103.5±3.55 | 3.4 |

[a] Mean±S.D. ($n$=3).
[b] Amount of standard bile acids added to 100 mg of liver tissue.

lated bile acids such as CDCA and DCA. The unconjugated form of each bile acid eluted before the glycine conjugate, which in turn eluted before the taurine conjugate.

### 3.3. Assay evaluation

#### 3.3.1. Reproducibility

The reproducibility of the retention times and the relative peak heights of each bile acid to that of the I.S. for 150 pmol of the individual bile acids per 10 μl injection (3 nmol per 100 mg of liver tissue) were determined.

The relative standard deviation (R.S.D.) for the retention times and the relative peak heights were less than 0.3 and 2.4%, respectively.

#### 3.3.2. Linearity and sensitivity

The linear regression equations and the correlation coefficients for each bile acid are listed in Table 1. Between the relative peak heights and the concentrations from 10 to 4000 pmol per 10 μl injection (from 0.2 to 80 nmol per 100 mg of liver tissue), the correlation coefficients were all over 0.995. The limits of detection ranged from 1 to 5 pmol per 10 μl injection (from 0.02 to 0.1 nmol per 100 mg of liver tissue), with a signal-to-noise ratio of 2.

#### 3.3.3. Analytical recovery

The recoveries of the 30 bile acids added to rat liver tissue shown in Table 2 were in the range of 94.1–105.8% (R.S.D.: 0.7–4.4%, $n=3$). This indicated that the bile acids were effectively extracted by the Bond Elut C18 cartridges. These results were more satisfactory than those of previous methods requiring prior fractionation and deconjugation [3,8,12].

#### 3.3.4. Column performance

When the 3α-HSD column was used repeatedly, the peak heights of the bile acids gradually decreased, owing to deactivation of the enzyme. The

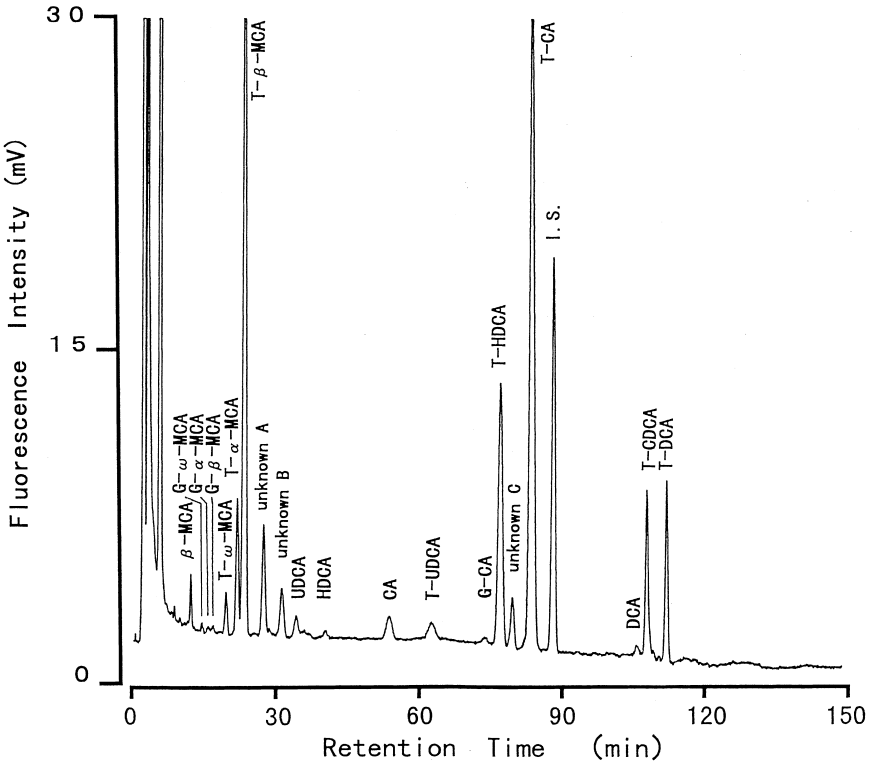


Fig. 2. Representative separation profile of bile acids in rat liver tissue. Peaks A, B and C are unknown.

decrease in peak heights was marked, especially among bile acids possessing a 7α-hydroxyl group such as i.e. α-MCA, HCA, CA and CDCA. Under the present conditions, however, the 3α-HSD column and separation column were stable for at least 1 month, enabling more than 200 analyses to be performed. The separation column was washed with mobile phase B for 3 h or more immediately after analysis, completely replaced by mobile phase B, and stored at room temperature. The 3α-HSD column was washed with the β-NAD reagent for 3 h or more, completely replaced by the β-NAD reagent, and stored at 5°C or less.

### *3.4. Sample analysis*

Six normal rat liver tissue samples were analysed by HPLC and a representative chromatogram is shown in Fig. 2. The composition and the total amounts of bile acids are presented in Table 3.

In liver tissue, the total amounts of bile acids were 32.74±3.852 nmol/100 mg of liver tissue. More than 95% of the total amount of bile acids consisted of taurine conjugates. Glycine conjugates and unconjugated bile acids were present at very low levels in the six samples. The major components were T-β-MCA (36.2%), T-CA (31.8%) and T-HDCA

Table 3
Composition and total amount of bile acids in rat liver tissue

| Bile acid[a] | | Bile acid composition (%) | | | | | | Mean±S.D. |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | |
| ω-MCA | F | –[b] | – | – | – | – | – | – |
| | G | 0.7 | 0.7 | 0.4 | 0.6 | 0.4 | – | 0.5±0.27 |
| | T | 2.6 | 2.5 | 1.8 | 2.0 | 2.2 | 2.9 | 2.3±0.41 |
| α-MCA | F | – | – | – | – | – | – | – |
| | G | – | 0.3 | 0.2 | 0.2 | 0.2 | – | 0.2±0.12 |
| | T | 4.7 | 5.5 | 5.1 | 6.0 | 3.5 | 5.4 | 5.0±0.87 |
| β-MCA | F | – | 0.3 | 0.9 | – | 0.1 | – | 0.2±0.35 |
| | G | – | 0.3 | 0.2 | 0.2 | 0.3 | – | 0.2±0.14 |
| | T | 37.0 | 37.9 | 28.8 | 37.0 | 35.8 | 40.4 | 36.2±3.92 |
| HCA | F | – | – | – | – | – | – | – |
| | G | – | – | – | – | – | – | – |
| | T | – | – | – | – | – | – | – |
| UDCA | F | – | 0.9 | 0.9 | 0.7 | 0.9 | – | 0.6±0.45 |
| | G | – | – | – | – | – | – | – |
| | T | – | 1.2 | 1.3 | – | 0.9 | – | 0.6±0.63 |
| HDCA | F | – | 0.1 | 0.3 | – | – | – | 0.1±0.12 |
| | G | – | – | – | – | – | – | – |
| | T | 16.5 | 10.3 | 14.4 | 11.7 | 13.3 | 15.8 | 13.7±2.39 |
| CA | F | – | 0.4 | 1.5 | – | 0.8 | – | 0.5±0.61 |
| | G | – | – | 0.6 | 0.4 | 0.9 | – | 0.3±0.38 |
| | T | 29.5 | 31.5 | 33.7 | 32.5 | 35.3 | 28.1 | 31.8±2.66 |
| CDCA | F | – | – | – | – | – | – | – |
| | G | – | – | – | – | – | – | – |
| | T | 4.4 | 4.1 | 4.3 | 4.9 | 2.9 | 3.7 | 4.1±0.69 |
| DCA | F | – | 0.2 | 0.2 | 0.2 | 0.2 | – | 0.1±0.10 |
| | G | – | – | – | – | – | – | – |
| | T | 4.6 | 3.8 | 5.2 | 3.7 | 2.3 | 3.7 | 3.9±0.98 |
| LCA | F | – | – | – | – | – | – | – |
| | G | – | – | – | – | – | – | – |
| | T | – | – | – | – | – | – | – |
| Total[c] | | 31.91 | 28.42 | 36.62 | 30.69 | 30.50 | 38.27 | 32.74±3.852 |

[a] F, unconjugated; G, glycine conjugated; T, taurine conjugated.
[b] Not detected.
[c] Total amount of bile acids is given in nmol per 100 mg of liver tissue.

(13.7%). T-α-MCA, T-CDCA, T-DCA and T-ω-MCA were minor components, but UDCA was found only at a low level, HCA and LCA was not found in any sample. Appreciable inter-sample variations were observed in total amount and composition of the bile acids.

In liver tissue, the unidentified peaks A, B and C appeared at 26.7, 30.5 and 78.8 min, as shown in Fig. 2. These may have been due to 3α-hydroxy-steroids, since none of them appeared when the samples were analysed without β-NAD in the enzymatic reaction. These may correspond to taurine conjugates of 3α-hydroxyketobile acids, other bile acids and $\Delta^{22}$-β-muricholic acid ($\Delta^{22}$-β-MCA: 3α, 6β, 7β-trihydroxy-5β, 22-cholen-24-oic acid) in consideration of the results described by Rodrigues et al. [16] and Thompson et al. [30].

This method was shown here to provide reproducible, accurate and sensitive results, allowing the 30 bile acids in rat liver tissue to be simultaneously determined using a simple extraction procedure. This rapid and reliable method should provide more precise knowledge on the metabolic profile of bile acids in liver tissue not only of rat but also of human, mouse, rabbit, hamster, guinea pig, dog and pig. This HPLC method may be applicable to the analysis of 30 bile acids in serum and bile, treated with the clean-up procedure as previously reported [31]. Moreover, this method will be useful in diagnosing hepatobiliary diseases such as primary biliary cirrhosis and liver cirrhosis.

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