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SIMPLE AND RAPID QUANTITATION OF 21 BILE ACIDS IN RAT SERUM AND LIVER BY UPLC-MS-MS: EFFECT OF HIGH FAT DIET ON GLYCINE CONJUGATES OF RAT BILE ACIDS

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ABSTRACT

In this report, we present a simple and rapid method for analysis of 21 kinds of bile acids and the conjugates in rat serum and liver samples by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS) in the negative ionization mode, using cholic-2, 2, 4, 4-d_4 acid as internal standard. After liquid-liguid extraction from serum and liver samples, specimens were analyzed by UPLC equipped with an Acquity TQD tandem quadrupole mass spectrometer. All of the 21 bile acids were sufficiently separated within 5 min. For most bile acids, calibration curves showed good linearities in the range of 0.25 to 5000 ng/mL for serum samples, 2.5 ng/g to 50 μg/g for liver samples. The limits of detection (LOD) were estimated to be less than 0.25 to 7.5 ng/mL in serum, less than 2.5 to 10 ng/g in liver samples. The present method was validated with respect to repeatability; the coefficient of variation (CV) values were less than 26.7% in the serum and 25.9% in the liver. In the animal study, we compared 21 bile acids in the serum and liver samples of the stroke-prone spontaneously hypertensive (SHRSP) rats fed with control (SP) diet or high-fat and high-cholesterol-containing (HFC) diet. By feeding with HFC diet, the glycine conjugates of some bile acids significantly increased and the taurine conjugate of ulsodeoxicolate (TUDC) decreased in serum and liver samples. Our results suggest that the change of bile acid profiles could be applied for the diagnosis of non-alcoholic fatty liver disease (NAFLD).

Key Words: UPLC, tandem MS, Bile acids, Glycine conjugates, Taurine conjugates

Abbreviations:

BA	bile acid
C	cholic acid
CDC	chenodeoxycholic acid
CV	coefficient of variation
DC	deoxycholic acid
DHC	dehydrocholic acid
GC	glycocholic acid

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GCDC	glycochenodeoxycholic acid
GDC	glycodeoxycholic acid
GDHC	glycodehydrocholic acid
GLC	glycolithocholic acid
GUDC	glycoursodeoxycholic acid
HDC	hyodeoxycholic acid
HPLC	high-performance liquid chromatography
IS	internal standard
LC	lithocholic acid
LOD	limit of detection
LOQ	limit of quantitation
α-MC	alpha-muricholic acid
β-MC	beta-muricholic acid
MRM	multiple reaction monitoring
MS-MS	tandem mass spectrometry
NAFLD	non-alcoholic fatty liver disease
SD	standard deviation of the mean
SHRSP rat	stroke-prone spontaneously hypertensive rat
TC	taurocholic acid
TCDC	taurochenodeoxycholic acid
TDC	taurodeoxycholic acid
TDHC	taurodehydrocholic acid
TLC	taurolithocholic acid
TUDC	tauroursodeoxycholic acid
UDC	ursodeoxycholic acid
UPLC	ultra-performance liquid chromatography

INTRODUCTION

Bile acids (BAs), which are synthesized from cholesterol in the liver, play different physiological functions. BAs help the absorption of lipophilic nuturients in the intestine and regulate cholesterol homeostasis, they control glucose, lipid and energy homeostatis.[1-3] There are two primary BAs, cholic acid (C), chenodeoxycholic acid (CDC) synthesized in the hepatocytes, and secondary bile acids, ursodeoxycholic acid (UDC), deoxycholic acid (DC), lithocholic acid (LC), are generated by the intestinal bacteria from primary BAs. BAs are regulated by different mechanisms; their kinetics are controlled by cholesterol 7α-hydroxylase involved in the rate-limiting step and other P450 isozymes, transporters such as bile salt excretion pump and apical bile salt transporter, receptors such as farnesoid X receptor, liver orphan receptor-α, and other molecules.[4-8]

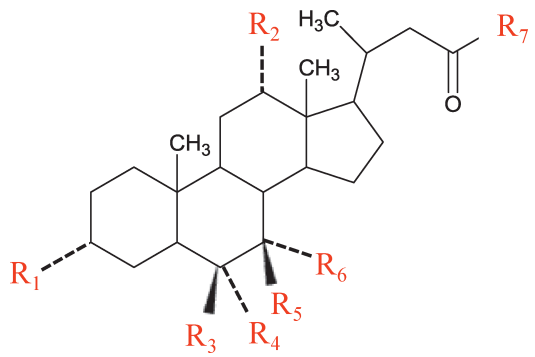
It is known that the amounts of total bile acids in body fluids are altered and reflect to hepatotoxicity after the intake of high fat diet, alcohol and hepatic toxicants.[6,9,10]

It is thus needed to develop a highly sensitive and rapid analysis method for detecting and measuring bile acids and their conjugates in biological specimen; high performance liquid chromatography-mass spectrometry (HPLC-MS) or -tandem mass spectrometry (HPLC-MS-MS) is most suitable for determination of bile acids in biological fluids and tissues.[11] Although several papers have been reported to analyze bile acids in biological fluids or tissues of human or other animals by HPLC-MS or HPLC-MS-MS,[12-20] it took more than 10 minutes for MS analyses or tedious procedures for the extraction of bile acids were needed. In this report, we have presented a simple analysis method of 21 kinds of bile acids and the metabolites by ultra-performance liquid chromatography (UPLC)-MS-MS, which has enabled the analysis rapidly. Also, we have found the change of bile acids profile in rat serum and liver samples by a high fat-cholesterol (HFC) diet.

MATERIAL AND METHODS

Chemicals and reagents

Cholic acid (C), chenodeoxycholic acid (CDC), ursodeoxycholic acid (UDC), lithocholic acid (LC), glycolithocholic acid (GLC), and dehydrocholic acid (DHC) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), taurocholic acid (TC), glycocholic acid (GC), taurochenodeoxycholic acid (TCDC), tauroursodeoxycholic acid (TUDC), deoxycholic acid (DC), taurodeoxycholic acid (TDC), and glycodeoxycholic acid (GDC) were purchased from Merck (Darmstadt, Germany), glycochenodeoxycholic acid (GCDC), and glycoursodeoxycholic acid (GUDC) from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA), taurolithocholic acid (TLC), hyodeoxycholic acid (HDC), ammonium sulfate, and charcoal activated from Sigma-Aldrich (St. Louis, MI), and taurodehydrocholic acid (TDHC), glycodehydrocholic acid (GDHC), alpha-muricholic acid (α-MC), and beta-muricholic acid (β-MC) from Steraloids, Inc. (Newport, RI). The chemical structures of the above 21 bile acids were shown in Fig. 1. Cholic-2, 2, 4,



BAs	M.W.	R_1	R_2	R_3	R_4	R_5	R_6
C	408	OH	OH	H	H	H	OH
CDC	392	OH	H	H	H	H	OH
UDC	392	OH	H	H	H	OH	H
DC	392	OH	OH	H	H	H	H
LC	376	OH	H	H	H	H	H
DHC	402	O	O	H	H	–	O
αMC	408	OH	H	OH	H	H	OH
βMC	408	OH	H	OH	H	OH	H
HDC	392	OH	H	H	OH	H	H

BAs	M.W.	R_7
unconjugate		OH
Taurine conjugate	+107	$NH(CH_2)_2SO_3H$
Glycine conjugate	+57	$NHCH_2COOH$

Fig. 1 Chemical structures of 9 bile acids and their glycine and taurine conjugates.

4-d_4 acid was obtained from CDN (Pointe-Claire, Quebec, Canada). Acetonitrile (HPLC grade), distilled water (LC-MS grade), disodium hydrogen phosphate 12-water, and sodium dihydrogen phosphate dehydrate were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Formic acid (abt. 99%) was obtained from Wako (Osaka, Japan). Other common chemicals used in this study were of analytical grade.

UPLC-MS-MS conditions

The LC instrument used in combination with an MS-MS detector was a Waters Acquity UPLC system, including an Acquity UPLC binary pump and a sample manager (Waters, Milford, MA). The column used for chromatographic separation was a Poroshell 120 EC-C8 column (2.1 x 50 mm, particle size 2.7 μm; Agilent Technologies, Santa Clara, CA). The column temperature was maintained at 30°C, and the gradient system was used with a mobile phase A (0.2% formic acid aqueous solution) and mobile phase B (0.2% formic acid in acetonitrile), at a total flow rate of 0.5 mL/min. The gradient program was started at 70% mobile phase A and 30% mobile phase B, increased linearly to 62% mobile phase A and 38% mobile phase B for 2.6 min, increased linearly to 2% mobile phase A and 98% mobile phase B for 6.0 min, held at 2% mobile phase A and 98% mobile phase B for 1.0 min, and brought back to 70% mobile phase A and 30% mobile phase B for 0.1 min followed by 0.9 min re-equilibration. The total run time for each sample analysis was 8.0 min. The samples for analysis were maintained at 4°C and injection volume to UPLC-MS-MS analysis was 10 μL.

The MS-MS detection was performed in the negative ionization modes on a tandem quadrupole mass spectrometer (Acquity TQD; Waters) equipped with an electrospray ionization interface. Quantitation was performed using multiple reaction monitoring (MRM) mode using the peak areas. The optimal MS parameters were as follows: capillary voltage, 1.9 kV; source temperature, 120°C; desolvation temperature, 400°C; nitrogen gas with flow rates of desolvation and cone gas, 1000 and 10 L/hr, respectively; argon was used as collision gas with flow rates of 0.14 mL/min. The MRM transitions for the analytes and IS, their optimal MS parameters, such as the cone voltages and collision energies, were summarized in Table 1.

Sample preparation

For serum samples, to a 100 μL sample of rat serum, mixed with 20 ng IS (2 μL of 10 μg/mL sample solution) and 200 μL of 0.5 M phosphate buffer (pH6.0), 700 μL of acetonitrile was added; the mixture was vortexed for 1 min, and centrifuged at 3,500 *g* for 1 min. The supernatant collected was evaporated under vacuum at room temperature, and the residue was reconstituted with 70 μL of 0.2% fomic acid and 30 μL of 0.2% formic acid in acetonitrile, and filtrated through a Millex®-GV 0.22 μm filter (Millipore, Billerica, MA) before subjecting to a UPLC-MS-MS analysis.

For liver samples, after approximately 100 mg of liver was homogenized with 9 volumes of 10 mM phosphate buffer (pH6.0), the homogenate was centrifuged at 3,500 *g* for 5 min. A 200-μL aliquot of the liver homogenate was mixed with 40 ng IS (4 μL of 10 μg/mL sample solution) and 20 μL saturated ammonium sulfate. To the homogenate, 800 μL of acetonitrile was added; the mixture was vortexed for 1 min, and centrifuged at 3,500 *g* for 1 min. The supernatant collected was evaporated under vacuum at room temperature, the residue was reconstituted with 70 μL of 0.2% fomic acid and 30 μL of 0.2% formic acid in acetonitrile, and filtrated through the 0.22 μm filter.

Method validation

For obtaining all calibration curves, intra-day and inter-day variations, matrix effects, and

Table 1 Multiple reaction monitoring (MRM) transitions and parameters for each detected compounds

Analyte	MRM transition	Cone voltage (V)	Collision energy (eV)
C	407 → 407	70	10
TC	514 → 514	70	20
GC	464 → 464	70	20
CDC	391 → 391	70	10
TCDC	498 → 498	70	20
GCDC	448 → 448	60	10
UDC	391 → 391	70	10
TUDC	498 → 498	70	20
GUDC	448 → 448	60	10
DC	391 → 391	70	10
TDC	498 → 498	70	20
GDC	448 → 448	60	10
LC	375 → 375	60	20
TLC	482 → 482	70	20
GLC	432 → 73.9	70	30
DHC	401 → 401	70	10
TDHC	508 → 508	70	20
GDHC	458 → 458	60	10
αMC	407 → 407	70	10
βMC	407 → 407	70	10
HDC	391 → 391	70	10
IS	411 → 411	70	10

recoveries, the matrices in which endogenous bile acids were deprived with the treatment using activated charcoal, were used; the procedure was based on the method previously described with slight modification.[18] In brief, a 1-mL aliquot of the serum or supernatant of liver homogenate sample was mixed with 50 mg of activated charcoal, and the mixture was shaken moderately on an orbital shaker overnight (for about 17 hr) at room temperature; after centrifugation at 3,500 *g* for 5 min, the supernatants were subjected for experiments of method validations. The calibration curves were drawn in the range of 0.25–7.5 ng/mL and 5 μg/mL for serum samples (8 to 14 points), and in the range of 2.5–25 ng/g liver and 50 μg/g liver for liver samples (10 to 14 points); at each concentration, triplicate samples were prepared. The value of limit of detection (LOD) and limit of quantitation (LOQ) for each bile acid in serum and liver samples were defined as the lowest concentrations which could provide a signal-to-noise ratio of 3:1 and 10:1,[21] respectively. The values of intra- and inter-day variations, matrix effects, and recoveries were validated at 3 quality control points that were 5, 50, 500 ng/mL for serum samples, and 50, 500 and 5000 ng/g liver for liver samples, respectively. Five or six replicates of each quality control point were analyzed each day to determine the intra- and inter-day accuracy and precision, as well as matrix effect and recovery. The precision of the assay was determined as a coefficient of variations (CV, %). The matrix effect and recovery were determined by the

following methods. For the matrix effect, two sets of samples were prepared by directly spiking the analytes into the reconstituted solutions with or without the presence of the residue extracted from bile acids-free serum and liver samples. The matrix effect was calculated by using the following equation: matrix effect $=A_{ep}/A_{ns}$ x 100, where A_{ep} and A_{ns} represent the analyte peak area/IS peak area ratio of the extracted serum or liver sample, and the ratio of the peak areas of the neat solution, respectively.[16] The recovery value was calculated by using the equation: recovery $=A_{ex}/A_{ep}$ x 100, where A_{ex} and A_{ep} represent the analyte peak area/IS peak area ratio of the extracted serum or liver samples, and the ratio of the peak areas of the extracted blank serum or liver samples, spiked with the bile acids, respectively.

Animal study

The animal study was conducted according to the Guidelines for Animal Experiments of the Nagoya University Animal Center. In the experiments, the Stroke-prone spontaneously hypertensive (SHRSP) rats were used for the experiment; the rats had been fed with the SP and HFC diet (those compositions were shown in Table 2), for 2 and 8 weeks.[22]

Statistical analysis

In the method varidations and the animal studies, data of bile acids were shown as mean ± standard deviation of the mean (SD). Results from different groups were compared using Student's *t*-test. If the variance was heterogeneous, logarithm or square root transformation was performed before analysis. *P* values less than 0.05 were considered to be statistically significant. All analyses were performed with SPSS 17.0 software.

RESULTS AND DISCUSSION

Product ion mass spectra and a multiple reaction monitoring (MRM) chromatograms

After optimization, the protonated precursor molecular ions were chosen to produce product ions most efficiently. The conditions of MRM transition reactions for the bile acids and IS were shown in Table 1. In our conditions, the *m/z* values of the product ions were the same as those

Table 2 Nutrient components of SP and HFC diets (weight %)

	SP diet	HFC diet
Feed formulation rate		
SP diet	100	68
Palm oil		25
Cholesterol		5
Cholic acid		2
Interdients		
Crude protein	20.8	14.1
Crude lipid	4.8	35.3
Crude fiber	3.2	2.2
Crude ash	5	3.4
Moisture	8	5.4
Carbohydrate	58.2	39.6

of the precursor ions for most compounds; a similar phenomenon was observed in the analysis for amanitins.[23] As shown in Fig. 2, all the bile acids were sufficiently separated within 5 min. In the previous reports, the analyses took more than 10 min;[12-20] our present method has enabled high throughput analysis of different bile acids in biological specimens.

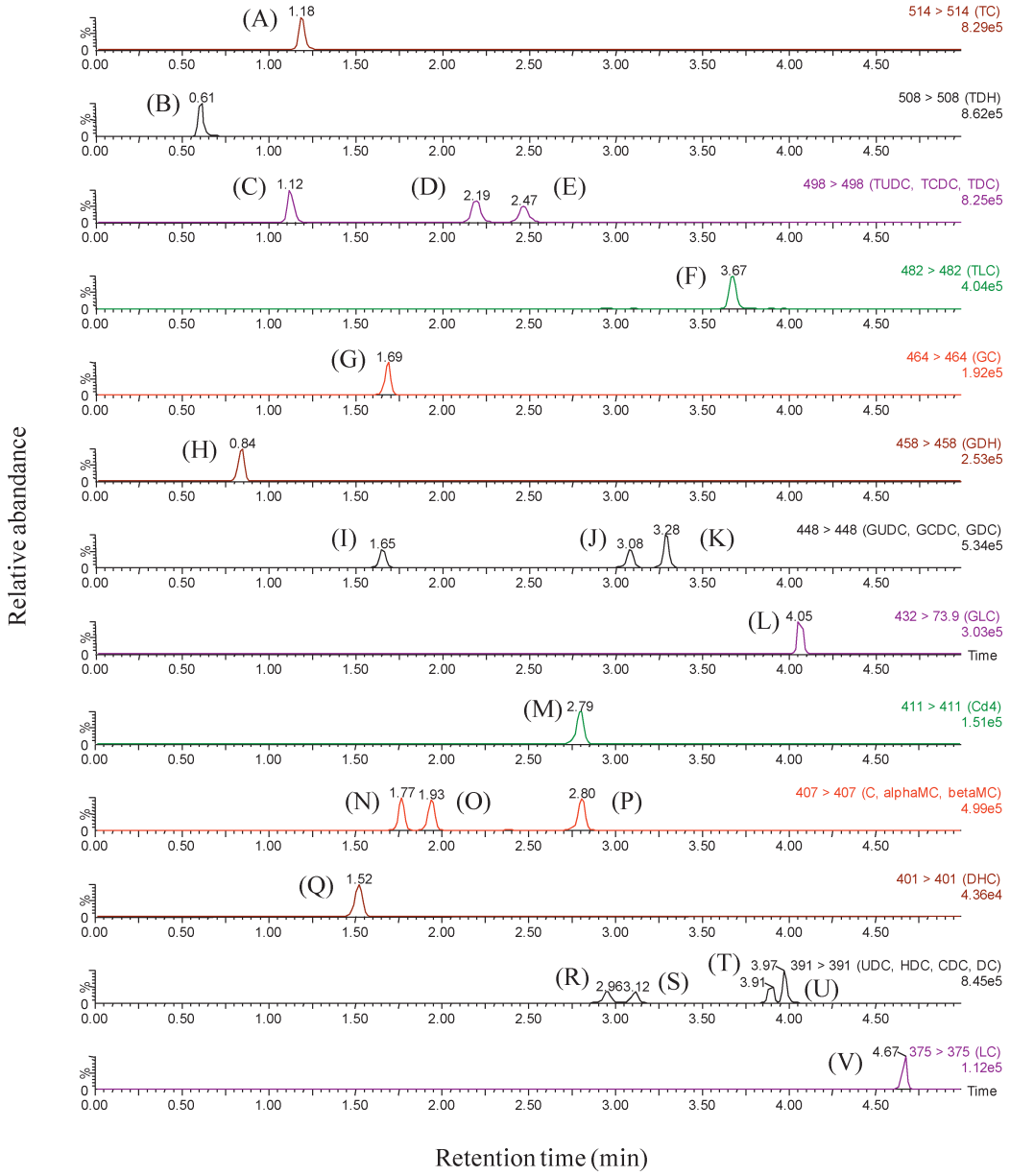


Fig. 2 Multiple reaction monitoring (MRM) chromatograms for **(A)** TC, **(B)** TDHC, **(C)** TUDC, **(D)** TCDC, **(E)** TDC, **(F)** TLC, **(G)** GC, **(H)** GDHC, **(I)** GUDC, **(J)** GCDC, **(K)** GDC, **(L)** GLC, **(M)** IS, **(N)** α-MC, **(O)** β-MC, **(P)** C, **(Q)** DHC, **(R)** UDC, **(S)** HDC, **(T)** CDC, **(U)** DC, and **(V)** LC, obtained by UPLC-MS-MS.

Reliability of the method

The peak area ratio of each bile acid to IS obtained from each MRM chromatogram was used method validation and quantitation of the samples. Calibration curves were plotted at different concentrations in the range of 0.25 to 5000 ng/ml for each bile acid in rat serum samples, except for CDC, GCDC, UDC, GUDC, DC, LC, DHC TDHC, GDHC, α-MC, β-MC and HDC; the LOQ values were estimated to be 0.5 ng/mL for CDC, UDC, GUDC, DC, TDHC, α-MC, β-MC and HDC, 0.75 ng/mL for LC and GDHC, and 7.5 ng/mL for DHC, respectively. Calibration curves were drawn in the range of 2.5 ng to 50 μg/g liver in rat liver samples, except for UDC, LC, DHC and GDHC; the LOQ values were estimated to be 7.5 ng/g for UDC and LC, 10 ng/g for GDHC, and 25 ng/g for DHC, respectively. In the calibration ranges of the compounds, good linearities were obtained for all bile acids with correlation coefficients (r^2) more than 0.990 as shown in Table 3. The limits of detection, defined as the concentration giving the signal-to-noise ratio of 3, were estimated to be in the range of less than 0.25 to 2.5 ng/mL for rat serum samples, and in the range of less than 2.5 to 10 ng/g for rat liver samples, respectively. The sensitivities obtained were poorest for LC and DHC; this phenomenon could be explained because of their hydrophobicity and consequent difficulties in the ionization. In previous methods by HPLC-MS-MS, the detection limits for bile acids were approximately 5 ng/ml[14] and 0.5 to 2.0 ng/ml[16] for rat serum, 0.001 to 0.008 nmol/ml for human plasma,[18] 0.2 to 0.5 ng/ml for the

Table 3 Linearity data, limits of quantitation (LOQ) and limits of detection (LOD) in serum and liver samples

Calibration curves of serum samples

BAs	calibration range	correlation coefficient	LOQ (S/N=10)	LOD (S/N=3)
	ng/mL	r^2	ng/mL	ng/mL
C	0.25-5000	0.999	0.25	< 0.25
TC	0.25-5000	0.999	0.25	< 0.25
GC	0.25-5000	0.999	0.25	< 0.25
CDC	0.5-5000	0.998	0.5	0.25
TCDC	0.25-5000	0.998	0.25	< 0.25
GCDC	0.25-5000	0.999	0.25	< 0.25
UDC	0.5-5000	0.999	0.5	0.25
TUDC	0.25-5000	0.999	0.25	< 0.25
GUDC	0.5-5000	0.999	0.5	0.25
DC	0.5-5000	0.999	0.5	0.25
TDC	0.25-5000	0.998	0.25	< 0.25
GDC	0.25-5000	0.999	0.25	< 0.25
LC	0.75-5000	0.996	0.75	0.5
TLC	0.25-5000	0.996	0.25	< 0.25
GLC	0.25-5000	0.999	0.25	< 0.25
DHC	7.5-5000	0.993	7.5	2.5
TDHC	0.5-5000	0.999	0.5	0.25
GDHC	0.75-5000	0.999	0.75	0.5
αMC	0.5-5000	0.999	0.5	2.5
βMC	0.5-5000	0.999	0.5	0.25
HDC	0.5-5000	0.999	0.5	0.25

Calibration curves of liver samples

BAs	calibration range	correlation coefficient	LOQ (S/N=10)	LOD (S/N=3)
	ng/g liver	r^2	ng/g liver	ng/g liver
C	2.5-50000	0.999	2.5	< 2.5
TC	2.5-50000	0.999	2.5	< 2.5
GC	2.5-50000	0.999	2.5	< 2.5
CDC	2.5-50000	0.999	2.5	< 2.5
TCDC	2.5-50000	0.999	2.5	< 2.5
GCDC	2.5-50000	0.999	2.5	< 2.5
UDC	7.5-50000	0.999	7.5	5
TUDC	2.5-50000	0.99	2.5	< 2.5
GUDC	2.5-50000	0.996	2.5	< 2.5
DC	2.5-50000	0.999	2.5	< 2.5
TDC	2.5-50000	0.999	2.5	< 2.5
GDC	2.5-50000	0.999	2.5	< 2.5
LC	7.5-50000	0.999	7.5	5
TLC	2.5-50000	0.998	2.5	< 2.5
GLC	2.5-50000	0.999	2.5	< 2.5
DHC	25-50000	0.99	25	10
TDHC	2.5-50000	0.998	2.5	< 2.5
GDHC	10-50000	0.999	10	5
αMC	2.5-50000	0.999	2.5	< 2.5
βMC	2.5-50000	0.999	2.5	< 2.5
HDC	2.5-50000	0.996	2.5	< 2.5

biological fluids of mice.[20] The detection limits of our present method seems almost comparable to those in the previous reports.

Table 4.1 and 4.2 show the values of coefficient of variation (CV), accuracy in intra- and inter-day variations (n=5–6), matrix effect, and recovery for each bile acid in rat serum and liver samples, respectively. Using liquid-liquid extraction, bile acids were recovered more than 65.3% in the serum, more than 69.0% in the liver. The CV values were not greater than 26.7% in the serum and 25.9% in the liver. The values of accuracy ranged from 53.1 to 186% in serum, and from 78.6 to 180% in liver. The CV values in rat live samples were much better than those of rat serum samples. Especially the values for DHC and its conjugates in rat serum samples were poor, due to their low sensitivities; the problem remains to be solved in the further research. The matrix effects were observed in some specimen; the values were 52.9 to 330% for the serum, and 28.5 to 368% for the liver. Altough most values ranged from approximately 80 to 130%, highly positive matrix effects were observed in TLC of rat serum and liver, and negative matrix effect observed in DHC of rat liver at 50 ng/g; we observed a similar phenomenon in the detection of amanitins in human or rat urine samples using UPLC-MS-MS.[23] We thus recommend that the calibration curves for bile acids should be prepared using the same biological samples.

Quantitative analysis of rat serum and liver samples

Using the present method, we have measured and compared bile acid profiles of the rats fed with SP and HFC diet. The comparisons of the rat serum samples among four groups were shown in Fig. 3, those of liver samples, shown in Fig. 4. For the primary bile acids, C and CDC, in both serum and liver samples, no differences were found between SP and HFC diet groups. However, the total amount of C and its conjugates (C group), as well as that of CDC and its conjugates (CDC group), also increased in the serum samples. The increase of C and CDC groups must be due to the increase of cholesterol by HFC diet. Moreover, their glycine conjugates (GC and GCDC) obviously increased by the HFC diet groups, compared to the taurine conjugates. Conjugation of glycine and taurine is catalyzed only by amino acid *N*-acetyltransferase (BAAT) and BAAT is strictly located in peroxisome.[8,9,24] For explaining the discrepancy of both conjugates, a possibility that intracellular taurine could be easily exhausted compared to glycine, may exist; it remains for further investigation. Recently, Kitamori *et al.* reported that the feeding of HFC diet for 8 weeks induced steathohepatitis and the severe fibrosis progression in a new rat strain SHRSP5/Dmcr, which is a model of non-alcoholic fatty liver disease (NAFLD).[22] In our experiments, no fibrosis was observed in SHRSP rats after feeding HFC diet for 8 weeks. Interestingly, the change of bile acid profile in the serum by HFC diet seems similar to that in the liver, except LC and TLC; this finding suggests that the change of bile acids in the serum would reflect the pathological change in the liver. Taken together, the change of some bile acids and the conjugates could be applied to diagnose NAFLD in the early stage.

CONCLUSIONS

We have developed a simple and rapid method to determine the 21 kinds of bile acids in rat serum and liver samples. This method includes a simple solvent extraction procedure followed by UPLC-MS-MS detection. Also, we have shown some bile acids and the conjugates in the serum and liver of SHRSP rats, changed after HFC diet; this method could be utilized for early diagnosis of NAFLD.

Table 4.1 Precision, accuracy, matrix effect, and recovery data of 21 bile acids in rat serum samples

BAs	concentration	intra-day (n=6)		inter-day (n=5-6)		matrix effect	recovery
	ng/mL	CV (%)	accuracy (%)	CV (%)	accuracy (%)	(n=6, %)	(n=6, %)
C	5	16.4	95.3	3.9	82.0	105±14.9	109±8.4
	50	4.3	95.9	4.7	94.3	97.2±3.2	109±1.4
	500	5.2	103	4.2	104	91.7±1.6	110±4.1
TC	5	22.8	102	26.4	63.2	110±30.2	141±24.2
	50	4.5	104	24.2	91.4	126±12.7	113±9.8
	500	3.3	100	17.2	87.1	132±9.0	110±13.5
GC	5	16.3	122	20.8	80.2	220±35.4	65.3±10.4
	50	7.4	94.2	22.8	125	122±22.1	94.0±24.7
	500	12.0	89.9	27.5	125	81.1±12.1	148±14.1
CDC	5	11.8	88.8	11.7	100	88.4±11.3	129±15.5
	50	14.5	108	3.1	94.5	79.6±4.2	101±8.9
	500	9.4	102	9.5	95.7	110±9.5	91.1±17.7
TCDC	5	6.4	83.7	18.3	77.6	128±21.6	141±24.2
	50	5.2	98.4	11.3	88.0	143±18.9	121±16.6
	500	3.6	100	13.4	86.9	171±25.6	116±20.5
GCDC	5	8.4	90.7	14.7	82.4	88.6±7.1	116±10.0
	50	6.1	104	12.4	98.5	106±4.9	106±6.4
	500	4.3	101	7.3	96.7	101±8.1	114±7.6
UDC	5	2.7	88.5	4.6	83.1	80.0±6.9	130±12.0
	50	5.2	110	6.4	94.9	89.3±4.6	111±7.6
	500	3.9	97.3	6.8	87.9	102±7.4	102±6.9
TUDC	5	10.2	103	26.7	69.2	117±19.9	146±32.8
	50	4.1	105	21.2	90.4	133±15.7	113±9.8
	500	3.5	99.9	16.1	87.8	142±10.8	111±14.3
GUDC	5	7.0	95.3	6.2	74.8	106±9.6	117±15.4
	50	4.1	106	12.5	93.3	99.4±8.3	110±14.1
	500	4.3	108	9.0	108	94.8±19.6	140±28.2
DC	5	18.7	106	8.9	103	85.5±9.5	116±31.0
	50	8.9	97.2	7.5	98.6	85.1±4.8	97.3±11.1
	500	9.0	98.8	12.1	98.9	66.6±6.0	121±11.8
TDC	5	4.5	89.3	16.7	76.0	145±19.8	138±24.4
	50	5.8	110	13.5	88.2	154±21.2	121±18.4
	500	8.4	103	13.5	95.7	189±22.7	114±14.4
GDC	5	3.6	93.5	14.0	71.6	97.2±8.3	116±11.0
	50	6.2	99.5	9.0	95.8	102±5.0	107±9.2
	500	5.6	102	8.9	99.4	103±5.0	114±8.1
LC	5	7.0	175	17.9	186	68.0±4.1	94.1±10.7
	50	11.9	116	15.5	109	62.8±8.2	85.5±19.4
	500	17.2	106	29.5	98.3	89.7±17.4	88.9±19.7
TLC	5	7.1	84.1	25.5	66.6	241±53.7	131±36.2
	50	6.9	104	10.1	83.9	261±23.4	110±10.1
	500	4.4	97.6	11.3	83.9	330±30.0	100±11.8
GLC	5	17.8	103	17.8	107	107±6.8	97.7±7.6

	50	15.5	125	14.8	139	147±17.4	96.7±24.2
	500	7.9	111	17.5	137	117±9.2	85.6±18.7
DHC	5	n.d.*	n.d.*	n.d.*	n.d.*	n.a.†	n.a.†
	50	7.0	90.5	22.2	112	98.6±13.8	82.8±16.4
	500	11.6	82.8	26.7	93.9	71.8±5.3	116±18.6
TDHC	5	15.2	106	17.2	53.4	52.9±5.3	133±20.2
	50	10.1	100	17.7	53.1	58.0±2.3	107±9.5
	500	8.6	104	15.1	59.2	65.1±7.5	127±22.1
GDHC	5	10.2	87.8	7.7	56.8	80.0±9.8	116±19.4
	50	8.7	94.1	16.3	69.2	68.1±5.4	105±10.6
	500	4.7	82.8	9.5	69.5	59.5±3.7	118±4.6
αMC	5	9.0	84.6	7.0	69.4	87.2±5.9	104±8.0
	50	2.6	96.6	2.6	92.6	77.0±13.9	113±29.9
	500	6.3	97.9	4.5	84.4	79.9±14.1	122±23.0
βMC	5	6.4	121	7.3	78.7	82.2±8.7	132±14.8
	50	3.8	104	8.8	89.0	79.2±14.7	122±30.7
	500	3.7	99.3	6.7	88.4	93.9±14.5	112±20.6
HDC	5	9.0	115	5.8	105	81.9±9.6	123±10.8
	50	5.9	112	7.7	99.5	91.4±7.1	111±12.0
	500	2.6	97.4	7.7	101	103±9.9	104±8.9

*: Not detected
†: Not applicable

Table 4.2 Precision, accuracy, matrix effect, and recovery data of 21 bile acids in rat liver samples

BAs	concentration	intra-day (n=6)		inter-day (n=6)		matrix effect	recovery
	ng/g liver	CV (%)	accuracy (%)	CV (%)	accuracy (%)	(n=6, %)	(n=6, %)
C	50	2.6	99.1	3.3	98.8	103±5.0	98.6±6.5
	500	2.5	91.0	5.6	91.0	93.8±5.7	106±6.4
	5000	1.1	103	1.8	102	103±12.5	97.7±11.4
TC	50	8.0	104	5.6	111	105±9.0	113±9.3
	500	2.2	112	6.3	121	123±5.6	98.6±6.2
	5000	2.0	114	3.5	117	130±13.8	99.2±12.0
GC	50	3.1	92.1	7.0	111	87.3±9.3	91.7±9.2
	500	4.4	101	8.8	116	81.0±5.0	104±8.4
	5000	4.0	78.5	5.4	85.7	98.7±13.6	96.0±11.2
CDC	50	14.0	100	14.2	107	65.0±11.4	123±14.4
	500	4.4	78.6	9.5	87.7	111±10.6	95.1±13.1
	5000	3.3	94.9	4.6	90.9	94.3±19.3	102±16.9
TCDC	50	4.0	106	3.4	106	172±6.4	97.3±4.1
	500	3.3	119	4.8	120	150±3.3	104±3.7
	5000	1.0	107	2.7	110	144±14.7	100±13.1
GCDC	50	3.7	91.6	3.2	94.8	108±5.9	96.1±9.4
	500	2.9	102	5.9	106	99.7±4.2	105±5.6
	5000	2.9	85.1	1.9	109	104±16.3	98.9±13.8
UDC	50	9.0	102	7.6	103	68.5±8.1	134±11.6

	500	2.9	111	7.1	109	99.2±6.4	101±8.3
	5000	2.2	105	3.6	105	106±9.3	93.6±9.7
TUDC	50	6.7	95.8	4.4	106	114±6.1	97.9±8.2
	500	4.1	112	6.4	118	125±5.9	106±9.7
	5000	2.6	111	2.4	89.7	131±15.2	99.5±12.5
GUDC	50	2.2	91.3	7.7	98.8	81.6±8.7	102±7.7
	500	3.5	109	7.2	118	85.3±6.9	107±8.1
	5000	4.5	101	4.0	105	104±14.3	101±11.0
DC	50	3.5	84.7	8.4	97.2	97.1±16.4	86.0±19.8
	500	6.0	107	14.1	108	95.4±8.3	99.7±5.2
	5000	6.4	83.0	13.3	107	105±6.0	83.1±7.6
TDC	50	3.7	113	6.5	106	170±6.5	103±4.2
	500	2.8	113	5.9	120	163±3.1	101±4.4
	5000	1.4	110	2.5	113	153±15.7	102±13.3
GDC	50	3.9	101	2.8	97.4	112±19.0	101±20.5
	500	3.0	110	4.8	87.5	106±5.4	107±6.3
	5000	1.9	101	3.2	103	114±15.1	99.5±12.8
LC	50	15.3	90.3	11.2	84.8	67.4±10.0	108±17.8
	500	11.9	91.8	16.7	96.1	102±15.1	70.8±14.5
	5000	6.9	100	6.9	97.9	98.6±11.6	69.0±16.7
TLC	50	5.6	116	9.3	103	368±7.6	84.2±12.3
	500	5.4	103	5.7	113	260±4.9	97.1±6.0
	5000	3.4	89.9	5.8	95.9	242±16.5	91.8±15.5
GLC	50	12.5	90.1	8.6	96.0	166±14.0	74.3±11.7
	500	8.1	109	14.2	108	93.5±17.4	104±13.0
	5000	8.4	99.6	9.5	106	114±12.3	81.6±9.6
DHC	50	19.9	91.0	25.9	180	28.5±34.8	191±28.3
	500	3.0	115	4.0	117	84.4±19.5	88.0±21.8
	5000	7.1	130	7.0	133	89.1±3.7	76.7±6.9
TDHC	50	18.0	119	9.9	114	43.7±15.2	121±25.5
	500	5.9	140	7.1	140	75.1±11.3	103±14.9
	5000	5.4	83.2	9.0	88.3	91.7±6.7	88.4±8.6
GDHC	50	14.3	92.2	6.2	101	41.5±13.6	94.9±24.9
	500	10.7	101	7.5	109	48.4±10.0	94.9±18.2
	5000	11.0	110	3.0	87.8	56.8±14.6	86.5±15.1
αMC	50	4.0	94.2	3.5	94.6	77.7±6.3	105±9.4
	500	2.4	110	6.4	111	85.1±5.5	105±6.9
	5000	3.2	94.0	2.4	93.5	89.9±12.8	97.8±11.0
βMC	50	3.4	99.5	3.5	96.7	83.6±6.1	114±8.2
	500	2.0	111	6.4	109	89.9±7.4	104±8.2
	5000	3.1	104	2.8	102	98.6±10.7	95.8±9.7
HDC	50	8.0	108	7.6	101	58.3±13.2	150±13.1
	500	1.5	112	5.0	108	99.1±8.1	98.8±8.6
	5000	1.9	93.0	3.2	95.7	109±9.7	93.5±9.1

*: Not detected

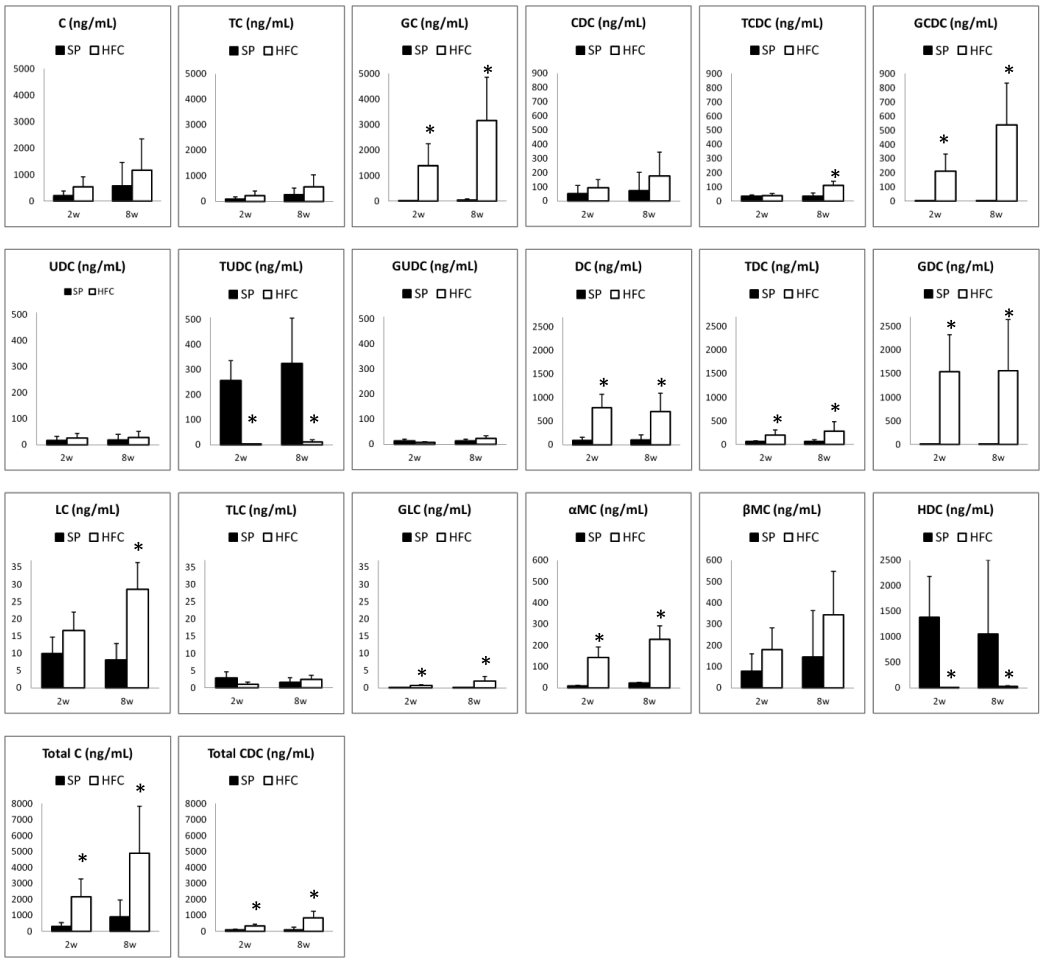


Fig. 3 The values of BAs in serum samples of SHRSP rats fed with control (SP) diet (shown in filled bars) and high fat and cholesterol-containing (HFC) diet (shown in open bars). The C or CDC group represents the sums of C, TC and GC, or CDC, TCDC and GCDC, respectively. The values of DHC, TDHC, and GDHC were below the LOQ values (data not shown).
*: significant differences between each of the samples fed with SP and each of those fed with HFC diet (p <0.05).

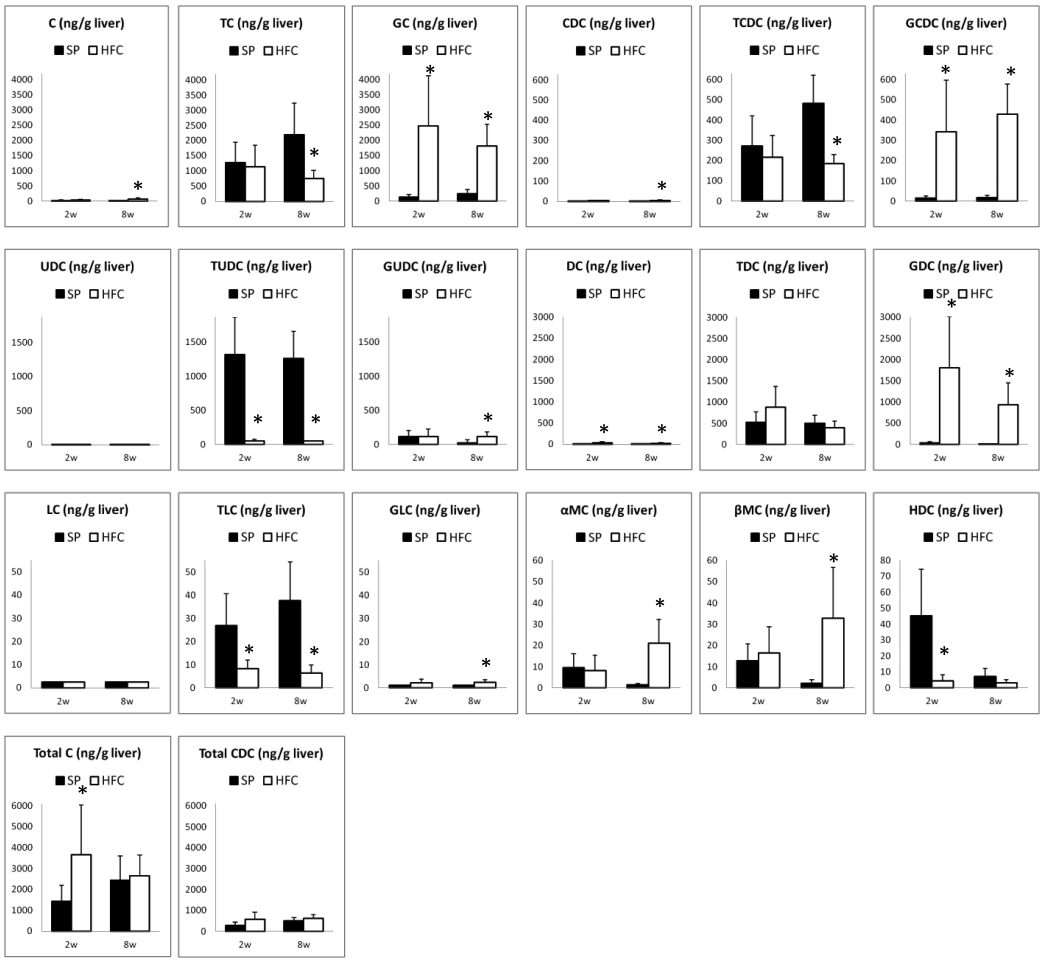


Fig. 4 The values of BAs in liver samples of SHRSP rats fed with control (SP) diet (shown in filled bars) and high fat and cholesterol-containing (HFC) diet (shown in open bars). The C or CDC group represents the sums of C, TC and GC, or CDC, TCDC and GCDC, respectively. The values of DHC, TDHC, and GDHC were below the LOQ values (data not shown).
*: significant differences between each of the samples fed with SP and each of those fed with HFC diet (p <0.05). not shown).

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REFERENCES

1) Li T, Chiang JYL. Bile acid signaling in liver metabolism and diseases. *J Lipid Res*, 2012; 2012: 754067.
2) Monte MJ, Marin JJG, Antelo A, Varquez-Tato J. Bile acids: Chemistry, physiology, and pathophysiology. *World J Gastroenterol*, 2009; 15: 804–816.
3) Hoffmann AF. The enterohepatic circulation of bile acids in mammals: form and functions. *Frontier Biosci*,

2009; 14: 2584–2598.
4) Chiang JYL. Bile acids: regulation of synthesis. *J Lipid Res*, 2009; 50: 1955–1966.
5) Chiang JYL. III. Bile acids and nuclear receptors. *Am J Physiol Gastrointest Liver Physiol*, 2003; 284: G349–G356.
6) Hofmann AF. The continuing importance of bile acids in liver and intestinal desease. *Arch Intern Med*, 1999; 159: 2647–2658.
7) Ridlon JM, Kang D-J, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res*, 2006; 47: 241–259.
8) Falany CN, Johnson MR, Barnes S, Diasio RB. Glycine and taurin conjugation of bile acids by a single enzyme. *J Biol Chem*, 1994; 269: 19375–19379.
9) Pellicoro A, Faber KN. Review article: the function and regulation of proteins involved in bile salt biosynthesis and transport. *Alimentary Pharmacol Therap*, 2007; 26: 149–160.
10) Pauli-Magnus C, Meier PJ. Hepatobiliary transporters and drug-induced cholestasis. *Hepatology*, 2006; 44: 778–787.
11) Griffiths WJ, Sjövall J. Bile acids: analysis in biological fluids and tissues. *J Lipid Res*, 2010; 51: 23–41.
12) Perwaiz S, Tuchweber B, Mignault D, Gilat T, Yousef IM. Determination of bile acids in biological fluids by liquid chromatography-electrospray mass spectrometry. *J Lipid Res*, 2001; 42: 114–119.
13) Burkard I, von Eckardstein A, Rentsch. Differentiated quantitation of human bile acids in serum by high-performance liquid chromatography-tandem mass spectrometry. *J Chromatogr B*, 2005; 826: 147–159.
14) Ando M, Kaneko T, Watanabe R, Kikuchi S, Goto T, Iida T, Hishinuma T, Mano N, Goto J. High sensitive analysis of rat serum bile acids by liquid chromatography/electrospray ionization tandem mass spectrometry. *J Phamaceut Biomed Anal*, 2006; 40: 1179–1186.
15) Alnouti Y, Csanaky IL, Klaassen CD. Quantitative-profiling of bile acids and their conjugates in mouse liver, bile, plasma, and urine using LC-MS/MS. *J Chromatogr B*, 2008; 873: 209–217.
16) Yang L, Xiong A, He Y, Wang Z, Wang C, Wang Z, Li W, Yang L, Hu Z. Bile acids metabonomic study on the CCl_4- and α-Naphthylisothiocyanate- induced animal models: quantitative analysis of 22 bile acids by ultraperformance liquid chromatography-mass spectrometry. *Chem Res Toxicol*, 2008; 21: 2280–2288.
17) Bobeldijk I, Maarten H, de Vries-van der Weij J, Coulier L, Ramaker R, Kleemann R, Kooistra T, Rubingh C, Freidig A, Verheij E. Quantitative profiling of bile acids in biofluids and tissues based on accurate mass high resolution LC-FT-MS: Compound class targeting in a metabolomics workflow. *J Chromatogr B*, 2008; 871: 306–313.
18) Xiang X, Han Y, Neuvonen M, Laitila J, Neuvonen PJ, Niemi M. High performance liquid chromatography-tandem mass spectrometry for the determination of bile acid concentrations in human plasma. *J Chromatogr B*, 2010; 878: 51–60.
19) Want EJ, Coen M, Masson P, Keun HC, Pearce JTM, Reily MD, Robertson DG, Rohde CM, Holmes E, Lindon JC, Plumb RS, Nicholson JK. Ultra performance liquid chromatography-mass spectrometry profiling of bile acid metabolites in biofluids: application to experimental toxicology studies. *Anal Chem*, 2010; 82: 5282–5289.
20) Huang J, Bathena SPR, Csanaky IL, Alnouti Y. Simultaneous characterization of bile acids and their sulfate metabolites in mouse liver, plasma, bile, and urine using LC-MS/MS. *J Pharmaceut Biomed Anal*, 2010; 55: 1111–1119.
21) International Conference on Harmonisation of Technical Requirements for Registartion of Pharmaceutical for Human Use. Varidation of Analytical Procedures: Text and Methodology Q2 (R1) 2005.
22) Kitamori K, Naito H, Tamada H, Kobayashi M, Miyazawa D, Yasui Y, Sonoda K, Tsuchikura S, Yasui N, Ikeda K, T Moriya, Yamori Y, Nakajima T. Development of novel rat model for high-fat and high-cholesterol diet-induced steatohepatitis and severe fibrosis progression in SHRSP5/Dmcr. *Environ Health Prev Med*, 2012; 17: 173–182.
23) Nomura M, Suzuki Y, Kaneko R, Ogawa T, Hattori H, Seno H, Ishii A. Simple and rapid analysis of amatoxins using UPLC-MS-MS. *Forensic Toxicol*, 2012; 30: 185–192.
24) Pellicolo A, van den Heuvel FAJ, Geuken M, Moshage H, Jansen PLM, Faber KN. Human and rat bile acid-CoA: Amino acid *N*-acetyltransferase are liver specific peroxisomal enzymes: Implications for intracellular bile salt transport. *Hepatology*, 2007; 45: 340–348.