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High sensitive analysis of rat serum bile acids by liquid chromatography/electrospray ionization tandem mass spectrometry

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Abstract

Sensitive liquid chromatography (LC)/electrospray ionization (ESI) tandem mass spectrometry (MS) can be used to analyze the bile acid composition of rat serum. This method can analyze eight common bile acids and their glycine and taurine conjugates in 100 μl rodent serum by gradient elution on a reversed-phase column using a mixture of 20 mM ammonium acetate buffer (pH 8.0), acetonitrile and methanol as a mobile phase. Selected reaction monitoring analysis under negative ion detection mode allowed the achievement of a high sensitive assay with a simple solid phase extraction using an ODS cartridge column. We used this method to investigate the effect of a one-day fast on the concentration and composition of serum bile acids in rats. The results suggested that the method described here is useful for the dynamic analysis of serum bile acids in rats.

Keywords: Bile acid; Rat serum; LC–MS/MS; Electrospray ionization; Simultaneous determination

1. Introduction

Bile acids, synthesized in the liver from cholesterol by the action of hepatic enzymes, are excreted into the small intestine via the bile duct as glycine and taurine conjugates. Within the intestinal lumen, bile acids assist lipolysis and fat absorption by forming mixed micelles. These molecules then return to liver following reabsorption in the ileum and proximal colon. Due to efficient hepatic uptake, bile acids are only present at low concentrations in the peripheral circulation. Recent studies have indicated that the nuclear farnesoid X receptor regulates the bile acid pool by repressing the transcription of genes encoding hepatocyte transporters [1] and cholesterol 7α-hydoxylase [2,3], the rate-limiting enzyme for bile acid biosynthesis.

Recently, we demonstrated the existence of three unconjugated bile acids, chenodeoxycholic acid (CDCA), cholic acid (CA) and deoxycholic acid (DCA) in the cytoplasmic fractions of rat brain [4]. As these brain bile acids have only been found in extracts following protein denaturation, these bile acids may tightly, but non-covalently, bind to proteins in the rat brain. We have also reported that CDCA can be synthesized by rat brain enzymes from 24-hydroxycholesterol via a 3β-hydroxy-5-cholenoic acid intermediate [5]. Recent observations have demonstrated the existence of a specific mechanism that allows the efflux of taurocholic acid from the bloodstream across the blood–brain barrier [6]. The organic-

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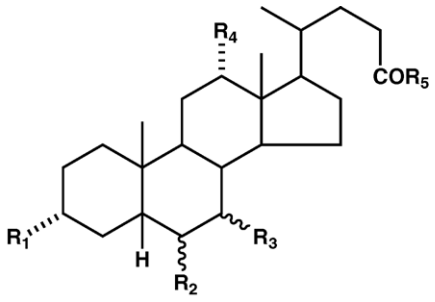
anion-transporting polypeptide, which functions as an anion exchanger that facilitates the transport of anions such as bile acids from the systematic portal blood into hepatocytes, is abundant in the human brain [7]. To clarify the origin of the brain bile acids, we first need to determine the composition of bile acids both in brain tissue and in serum.

High-performance liquid chromatography (HPLC) is the most suitable method for the simultaneous analysis of bile acids in biological samples [8]. Sakakura et al. [9] reported a fluorescent HPLC method coupled to a post-column enzymatic reaction to analyze bile acid composition in rat serum and bile. Although the method can separate and detect the major bile acids in rat bile, the analysis time is lengthy, measuring 180 min for a single sample. In addition, this technique is not so sufficiently sensitive to allow the analysis of serum bile acids. The presence of an acidic functional group at the C-24 position of bile acids makes these substances suitable for electrospray ionisation (ESI). Recently, we performed the sensitive and quantitative determination of bile acid sulfate [10] and glucuronide [11,12] composition in human urine by liquid chromatography/mass spectrometry (LC/MS) coupled with ESI. In this paper, we developed a sensitive liquid chromatographic method to determine bile acid composition in rat serum using tandem mass spectrometry (MS/MS).



	R_1	R_2	R_3	R_4
cholic acid	OH	H	α-OH	OH
chenodeoxycholic acid	OH	H	α-OH	H
deoxycholic acid	OH	H	H	OH
lithocholic acid	OH	H	H	H
ursodeoxycholic acid	OH	H	β-OH	H
α-muricholic acid	OH	β-OH	α-OH	H
β-muricholic acid	OH	β-OH	β-OH	H
hyodeoxycholic acid	OH	α-OH	H	H

	R_5
unconjugate	OH
glycine conjugate	$NHCH_2COOH$
taurine conjugate	$NH(CH_2)_2SO_3H$

Fig. 1. Structures of common rodent bile acids.

2. Experimental

2.1. Chemicals

CA, CDCA, DCA, ursodeoxycholic acid (UDCA), lithocholic acid (LCA) and hyodeoxycholic acid (HDCA) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). α-Muricholic acid (α-MCA) and β-muricholic acid (β-MCA) were synthesized in our laboratory as previously reported [13]. The stable isotope-labeled internal standards (ISs), 3,7-[^{18}O, ^{2}H]$_2$-CDCA and 3-[^{18}O, ^{2}H]-LCA, were synthesized in our laboratory as reported [14]. Glycine- and taurine-conjugated bile acids were prepared by the carbodiimide method [15]. A Sep-Pak C_{18} cartridge, purchased from Waters (Milford, MA, USA), was washed successively in ethanol (20 ml), water (10 ml), 5% BSA aqueous solution (5 ml) and water (10 ml) prior to use. All glassware was silanized. All other chemicals and solvents were of analytical grade.

2.2. LC/MS analysis

LC/MS analysis was performed using a Quattro II triple-quadruple tandem mass spectrometer (Micromass, Manchester, UK) equipped with an ESI probe under negative ion detection. A capillary voltage of −3600 V and a source temperature

Table 1
Summary of calibration curves for all bile acids

Bile acid	Range (ng/ml)	*y*-intercept	Slope	Correlation coefficient
Unconjugates				
CA	5–2000	0.0770	0.0185	0.9991
α-MCA	5–2000	0.0073	0.0064	0.9961
β-MCA	5–2000	0.0142	0.0104	0.9973
CDCA	5–2000	0.0949	0.0333	0.9995
DCA	5–2000	0.0180	0.0359	0.9998
HDCA	5–2000	0.0753	0.0164	0.9991
UDCA	5–2000	0.0364	0.0102	0.9984
LCA	5–500	0.1381	0.0593	0.9997
Glycine conjugates				
CA	5–2000	0.0237	0.0054	0.9953
α-MCA	5–2000	0.0088	0.0052	0.9984
β-MCA	5–2000	0.0056	0.0069	0.9920
CDCA	5–2000	0.0175	0.0084	0.9995
DCA	5–2000	0.0412	0.0197	0.9996
HDCA	5–2000	0.0384	0.0077	0.9941
UDCA	5–2000	0.0317	0.0062	0.9968
LCA	5–500	0.0241	0.0559	0.9997
Taurine conjugates				
CA	5–2000	0.0226	0.0257	0.9994
α-MCA	5–2000	−0.0043	0.0107	0.9992
β-MCA	5–2000	0.0055	0.0107	0.9983
CDCA	5–2000	0.0654	0.0370	0.9993
DCA	5–2000	0.0465	0.0449	0.9994
HDCA	5–2000	0.0385	0.0153	0.9952
UDCA	5–2000	0.0325	0.0121	0.9976
LCA	5–500	0.0609	0.0554	0.9996

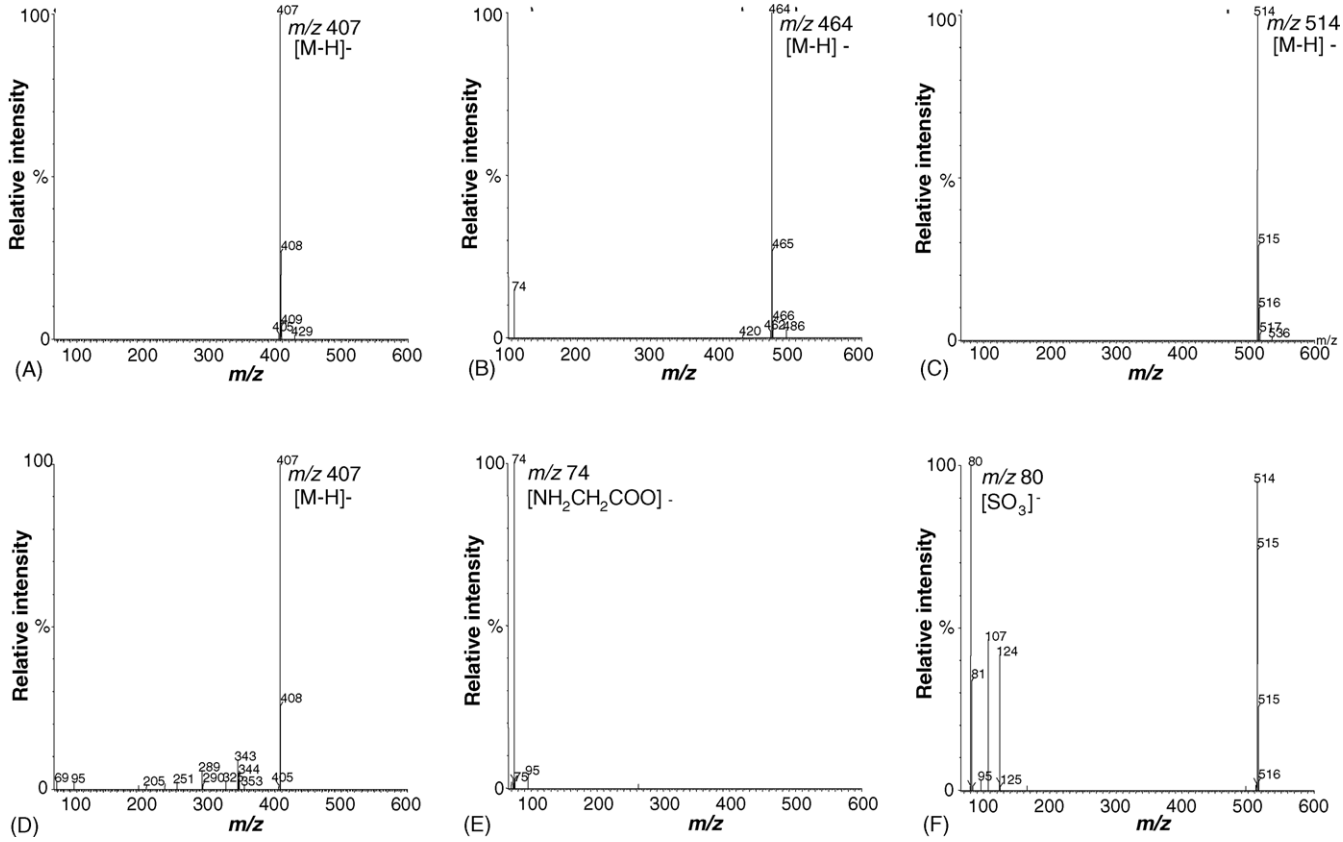


Fig. 2. Typical ESI mass spectra (A–C) and product ion mass spectra (D–F) using each deprotonated molecule as a precursor ion for (A, D) cholic acid (B, E) glycocholic acid and (C, F) taurocholic acid. Mass spectrometric conditions: introducing solvent, methanol/water (1:1, v/v); capillary voltage, −3600 V; cone voltage, −40 V; source temperature, 130 °C, collision gas, argon; collision energy, 50 eV.

of 160 °C were used. The cone voltage was 80, 70 and 90 V for unconjugated, glycine-conjugated and taurine-conjugated bile acids, respectively. The collision gas (argon) pressure was 1.5×10^{-3} mbar with a collision energy of 5, 40 and 75 eV for unconjugated, glycine-conjugated and taurine-conjugated bile acids, respectively. Selected reaction monitoring (SRM) was performed by examination of the transition reactions from precursor ions to product ions for the deprotonated molecules of each bile acid. For unconjugated bile acids, mono-, di- and tri-hydroxylated bile acids with *m/z* of 375, 391 and 407, respectively, were selected as precursor ions. The precursor ions themselves were also selected as product ions, because no characteristic product ions could be identified. For glycine-conjugated bile acids, *m/z* 432, 448 and 464, representing mono-, di- and tri-hydroxylated bile acids, respectively, were selected as precursor ions, and *m/z* 74 (a fragment ion of the glycine moiety) was selected as a product ion. For taurine-conjugated bile acids, *m/z* 482, 498 and 514, the mono-, di- and tri-hydroxylated bile acids, respectively, were selected as precursor ions, and *m/z* 80 (an SO_3 anion from the taurine moiety) was selected as a product ion.

Liquid chromatographic separation was performed using a Nanospace SI-1 HPLC system (Shiseido, Tokyo, Japan) with gradient elution from a Luna C18(2) column, maintained at 40 °C (3 μm, 2.0 mm I.D. × 150 mm, Phenomenex, CA, USA), at a flow rate of 200 μl/min. A mixture of 20 mM ammonium acetate buffer (pH 8.0), acetonitrile and methanol (12:3:1, v/v/v) was used as the initial mobile phase; the solvent was gradually changed to a 6:3:1 (v/v/v) mixture over 30 min, then to a 12:21:7 (v/v/v) mixture over 15 min, which was then kept constant for five additional minutes. Column effluent was introduced into the mass spectrometer using a switching valve from 10 to 50 min after the injection.

2.3. Sample preparation

Male Wistar rats (200–250 g) were fed a commercial pellet diet and water ad libitum. After collection, venous blood was kept at room temperature for 30 min, then centrifuged to obtain serum. Rat serum (100 μl) was diluted in 5 ml 0.1 M potassium phosphate buffer (pH 7.0), to which 2 ng of each IS was added. The mixture was passed through a Sep-Pak C_{18} cartridge, which was subsequently washed with 5 ml water. Target molecules were eluted in 8 ml of water/ethanol (1:9, v/v). The eluate was evaporated in vacuo, then redissolved in 100 μl of 20 mM ammonium acetate buffer (pH 8.0)/methanol (1:1, v/v). A 10 μl aliquot of each sample solution was then injected into LC/ESI–MS/MS system for analysis.

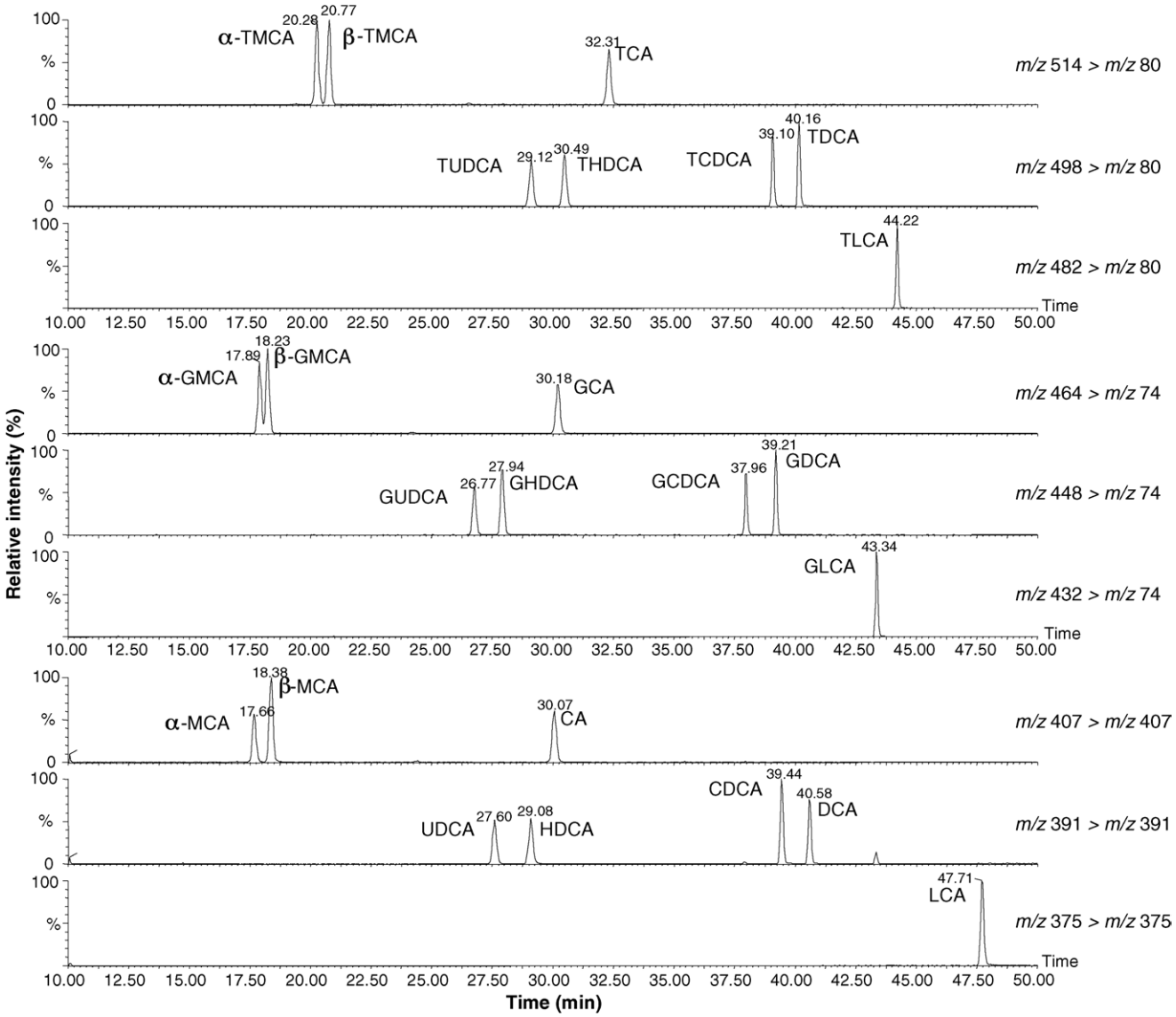


Fig. 3. Typical SRM chromatograms of authentic standards of 24 bile acids. Chromatographic conditions and mass spectrometric conditions are detailed in Section 2.

2.4. Accuracy and precision of the assay

Analytical errors were investigated using blank serum, which was prepared as follows. An equal volume of rat serum (6 ml) was added to 6 ml 0.1 M potassium phosphate buffer (pH 7.0) with 3.0 g activated charcoal to remove endogenous bile acids. After centrifugation of the mixture at 700 g for 10 min, the supernatant (4.2 ml) was collected for use as blank serum.

To evaluate accuracy and precision, we prepared and analyzed six samples, each containing three different concentrations of bile acids (30, 300 and 1000 ng/ml). The relative error (RE%) was calculated as [(determined concentration − theoretical concentration)/added concentration] × 100 (%). The precision was obtained as the coefficient of variation (relative standard deviation (R.S.D., %)).

3. Results and discussion

3.1. Mass spectrometry of unconjugated, glycine-conjugated and taurine-conjugated bile acids

In humans, bile acids are mainly two primary bile acids, CA and CDCA, three secondary bile acids, DCA, LCA and UDCA, and their glycine and taurine conjugates. These 15 bile acids are also present in rats. Rat hepatocytes possess 7α-hydroxylation activity to produce CA and CDCA from DCA and LCA [16,17]. In addition, CDCA can be converted into α-MCA and β-MCA in rats [17,18]. α-MCA is a precursor of β-MCA; in rats, β-MCA can also be formed from UDCA. Therefore, CA and β-MCA also are considered to be major bile acids in rats, requiring us to analyze eight bile acids and their glycine and taurine conjugates (Fig. 1).

Typical ESI mass spectra of unconjugated, glycine-conjugated and taurine-conjugated CA are shown in Fig. 2A–C. All bile acids possess an acidic group on their side chains, which can efficiently produce deprotonated molecules in ESI. The product ion mass spectra of unconjugated, glycine-conjugated and taurine-conjugated CA using the deprotonated molecules as precursor ions are illustrated in Fig. 2D–F. As unconjugated bile acids are quite stable in the usual collision-induced dissociation process, the *m*/*z* the precursor ion was also used as the SRM monitoring ion to reduce background noise. For glycine and taurine conjugates, we observed the formation of the characteristic product ions, *m*/*z* 74 and *m*/*z* 80, respectively, in each product ion mass spectrum. For these analyses, *m*/*z* 74 and 80, obtained from the precursor ions, were used as the SRM monitoring ions, respectively. Although MS/MS can separate different mass substances, bile acids contain multiple isomers based on the position and/or configuration of hydroxyl groups. These isomers require chromatographic separation.

3.2. HPLC method development and validation

We previously investigated the chromatographic behavior of bile acids on reversed-phase chromatography [8]. Mobile phase pH greatly influenced the retention of bile acids by affecting the side chain acidic group. The dissociated side chain acidic group can then bind the 12α-hydroxy group, changing the hydrophobicity of the molecule. Organic modifiers can also affect chromatographic behavior. The addition of methanol or ethanol to the mobile phase can sharpen many bile acid peaks, potentially by contributing to the solubility of the bile acids. Using these separation conditions, optimized for column, pH, organic modifiers in the mobile phase and the gradient program, we achieved the simultaneous separation of all bile acids (Fig. 3). The method described here has a sufficient dynamic range of 100-fold (50 pg to 5 ng/injection) for unconjugated, glycine-conjugated and taurine-conjugated LCA and 400-fold (50 pg to 20 ng/injection) for other bile acids, which all exhibited excellent correlations (0.9920–0.9998) (Table 1). The accuracy and precision of this method were also investigated for 30, 300 and 1000 ng/ml bile acid in serum (Table 2). For 30 ng/ml samples, the relative standard deviation for all bile acids ranged from 1.6 to 12.5%, while the relative errors varied from −15.3 to 10.7%. For 300 ng/ml samples, the relative standard deviations ranged from 1.5 to 12.0%, with relative errors from −14.8 to 16.3%. For 1000 ng/ml samples, in which unconjugated, glycine-conjugated and taurine-conjugated LCA analyzed, the relative standard deviations

Table 2
Accuracy and precision in analysis of bile acids in rat serum

Bile acid	Blank	30 ng/ml			300 ng/ml			1000 ng/ml		
		Found (ng/ml)	R.S.D. (%)	RE (%)	Found (ng/ml)	R.S.D. (%)	RE (%)	Found (ng/ml)	R.S.D. (%)	RE (%)
Unconjugates										
CA	NQ	32.2	2.5	7.3	274.8	3.9	−8.4	996.6	3.8	−0.3
α-MCA	NQ	32.2	3.1	7.3	255.7	3.3	−14.8	1175.3	1.3	17.5
β-MCA	NQ	31.4	3.5	4.7	276.0	2.5	−8.0	1123.7	3.2	12.4
CDCA	7.3 ± 1.3	37.2	1.6	−0.3	304.6	1.5	−0.9	984.0	1.9	−2.3
DCA	12.7 ± 1.4	39.2	2.8	−11.7	297.2	1.8	−5.2	993.3	2.0	−1.9
HDCA	NQ	28.6	4.2	−4.7	338.3	8.7	12.8	1097.8	4.2	9.8
UDCA	NQ	29.6	5.4	−1.3	333.9	12.0	11.3	1099.9	3.2	10.0
LCA	NQ	33.2	4.2	10.7	312.1	6.6	4.0	NT		
Glycine conjugates										
CA	NQ	29.6	3.7	−1.3	319.2	7.9	6.4	970.7	3.0	−2.9
α-MCA	NQ	31.5	3.5	5.0	258.4	5.1	−13.9	1018.5	1.3	1.9
β-MCA	NQ	27.3	4.8	−9.0	259.8	6.9	−13.4	955.0	1.8	−4.5
CDCA	NQ	31.2	5.1	4.0	317.5	1.4	5.8	1118.4	2.0	11.8
DCA	NQ	31.2	2.6	4.0	321.5	3.6	7.2	1015.9	1.8	1.6
HDCA	NQ	28.1	9.6	−6.3	313.0	11.8	4.3	990.3	2.8	−1.0
UDCA	NQ	29.6	6.4	−1.3	309.7	9.4	3.2	987.7	2.8	−1.2
LCA	NQ	32.4	4.6	8.0	317.7	6.5	5.9	NT		
Taurine conjugates										
CA	NQ	31.6	8.9	5.3	298.8	5.0	−0.4	1154.7	4.8	15.5
α-MCA	NQ	27.5	10.2	−8.3	293.7	3.8	−2.1	1004.4	4.1	0.4
β-MCA	NQ	26.2	6.9	−12.7	290.4	3.3	−3.2	984.2	3.8	−1.6
CDCA	NQ	31.5	4.1	5.0	348.8	4.8	16.3	1140.3	5.4	14.0
DCA	NQ	32.0	5.9	6.7	318.1	2.8	6.0	1065.5	1.3	6.6
HDCA	NQ	25.4	8.3	−15.3	333.2	7.0	11.1	984.1	5.0	−1.6
UDCA	NQ	26.5	12.5	−11.7	328.9	10.3	9.6	1000.7	4.0	0.1
LCA	NQ	33.0	5.5	10.0	307.8	4.3	2.6	NT		

NQ: less than limit of quantification. NT: not tested.

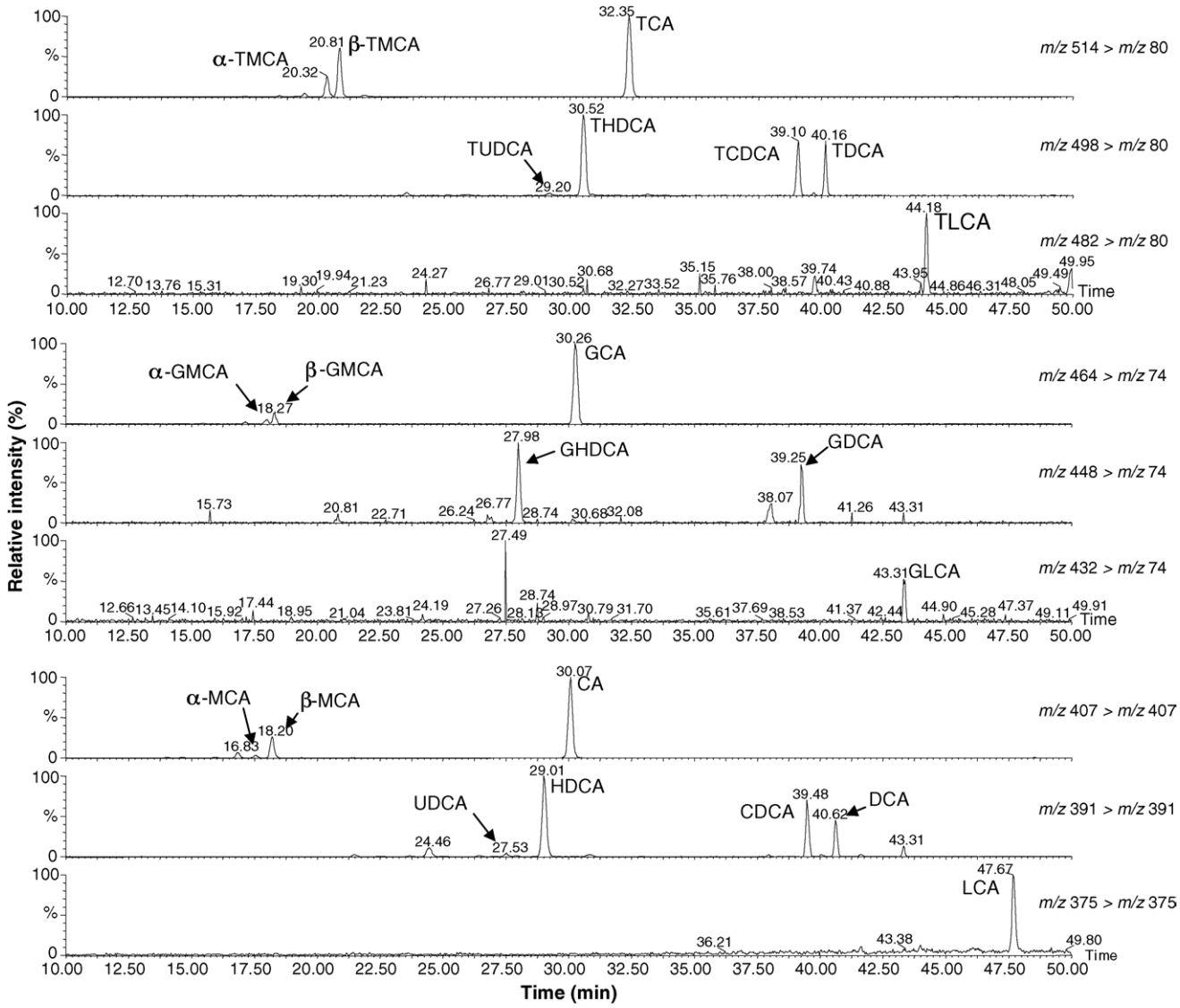


Fig. 4. Typical SRM chromatograms of rat serum bile acids. Chromatographic conditions and mass spectrometric conditions are detailed in Section 2.

varied from 1.3 to 5.4%, with relative errors from −4.5 to 17.5%.

3.3. Analysis of rat serum bile acids

Next, we applied this method to the analysis of bile acids in rat serum. Typical SRM chromatograms of rat serum bile acids are illustrated in Fig. 4. All bile acid peaks were clearly detected without any influence of contaminants, indicating that the method was suitable for the high sensitivity quantification of rat serum bile acids. To demonstrate the utility of this method, we examined the sera of five rats that had fasted for a day (fast model) and five rats that were fed freely for a day following one-day fast (control model). Determination of serum bile acid concentrations (Table 3) revealed that the concentrations of glycine-conjugated bile acids were lower than the other conjugates in all animals. Only four bile acids, glyco-CA, glyco-β-MCA, glyco-CDCA and glyco-HDCA could be readily detected. Rodent livers have a higher content of taurine than human liver, as human liver has a low taurine biosynthetic activity. In addition, the *N*-acyltransferase in rat hepatocytes may have a higher activity for taurine conjugation than for glycine [19], as the mouse *N*-acyltransferase, which is highly homologous to the rat enzyme, only forms taurine-conjugated bile acids [20]. In fasting rats, the total serum bile acid concentration, 2764 ng/ml, was reduced to less than half that seen in control rats (6322 ng/ml). CA concentrations were also influenced by fasting. In fasting rats, the uptake of bile acids from the portal blood into hepatocytes may increase, due to a decrease in fecal excretion that results in a reduction of the biosynthesis of bile acids in the liver. In addition, 6-hydroxylated bile acids, α- and β-MCA, HDCA and their taurine conjugates, were abundant in rat serum, while CDCA, the precursor of 6β-hydroxylated bile acids [17,18], only existed at low concentrations.

Table 3
Effect of one-day fast on serum bile acid concentrations

Bile acid	Found (ng/ml)											
	Rat 1[a]	Rat 2[a]	Rat 3[a]	Rat 4[a]	Rat 5[a]	Mean ± S.D.[a]	Rat 6[b]	Rat 7[b]	Rat 8[b]	Rat 9[b]	Rat 10[b]	Mean ± S.D.[b]
Unconjugates												
CA	6	835	283	553	492	434 ± 310	163	67	3423	8432	1051	2627 ± 3515
α-MCA	6	12	10	11	25	13 ± 7	NQ	NQ	36	223	13	55 ± 96
β-MCA	11	109	54	128	217	104 ± 79	NQ	NQ	259	1887	125	454 ± 808
CDCA	NQ	42	15	29	73	32 ± 28	25	19	118	394	58	123 ± 157
DCA	32	77	44	52	62	53 ± 17	27	46	93	119	51	67 ± 37
HDCA	200	366	242	227	277	262 ± 64	198	173	256	1197	204	405 ± 443
UDCA	NQ	8	6	11	32	11 ± 12	NQ	NQ	14	101	11	42 ± 51
LCA	NQ	NQ	NQ	NQ	6	–	NQ	5	5	6	NQ	3 ± 3
Glycine conjugates												
CA	NQ	51	54	73	18	39 ± 29	NQ	NQ	37	237	10	57 ± 102
α-MCA	NQ	NQ	NQ	NQ	NQ	–	NQ	NQ	NQ	NQ	NQ	–
β-MCA	NQ	NQ	9	12	NQ	–	NQ	NQ	11	54	NQ	–
CDCA	NQ	NQ	NQ	NQ	NQ	–	NQ	NQ	NQ	9	NQ	–
DCA	NQ	NQ	NQ	NQ	NQ	–	NQ	NQ	NQ	NQ	NQ	–
HDCA	NQ	NQ	8	8	NQ	–	NQ	NQ	NQ	26	NQ	–
UDCA	NQ	NQ	NQ	NQ	NQ	–	NQ	NQ	NQ	NQ	NQ	–
LCA	NQ	NQ	NQ	NQ	NQ	–	NQ	NQ	NQ	NQ	NQ	–
Taurine conjugates												
CA	267	764	1325	1234	418	801 ± 473	357	653	1000	1958	672	928 ± 619
α-MCA	37	48	79	87	82	67 ± 23	56	89	142	451	105	169 ± 161
β-MCA	104	257	489	559	150	312 ± 203	152	232	435	1451	285	511 ± 535
CDCA	56	111	127	143	98	107 ± 33	79	145	144	313	98	156 ± 93
DCA	31	81	89	59	70	66 ± 23	23	79	89	89	64	69 ± 28
HDCA	287	520	589	570	266	446 ± 157	334	502	532	1510	338	643 ± 493
UDCA	5	8	12	8	6	8 ± 3	NQ	7	6	30	6	10 ± 11
LCA	NQ	NQ	NQ	NQ	NQ	–	NQ	NQ	NQ	NQ	NQ	–
Total	1041	3289	3435	3764	2291	2764 ± 1109	1417	2017	6599	18485	3091	6322 ± 7090

NQ: less than limit of quantification.
[a] Fast.
[b] Control.

4. Conclusion

We reported the development of a sensitive assay measuring rat serum bile acids. The method consists of a simple solid phase extraction using an ODS cartridge column and gradient LC/ESI–MS/MS. This procedure can simultaneously analyze eight bile acids, including 6-hydroxylated bile acids and their conjugates. We analyzed rat serum samples obtained after different treatments, such as fasting. The results clearly suggested that the method is suitable for the separation and analysis of rat serum bile acids. This method should be useful for the dynamic analysis of serum bile acids in rats. In addition, we can determine and compare the composition of bile acids both in brain tissue [4] and in serum, leading to clarify the origin of the brain bile acids.

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