Simple Quantitative Analysis of 40 Total and Fractionated Bile Acids in Serum Using LC-MS/MS for Clinical Research Use TP477

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ABSTRACT

Purpose: The function of bile acids is to facilitate the formation of micelles, which promote digestion and absorption of dietary fat and are increasingly being shown to have hormonal actions. The bile acids analyzed in this study include chenodeoxycholic acid, cholic acid, deoxycholic acid, glycochenodeoxycholic acid, glycocholic acid, glycodeoxycholic acid, glycolithocholic acid, glycoursodeoxycholic acid, lithocholic acid, taurochenodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, taurolithocholic acid, tauroursodeoxycholic acid, ursodeoxycholic acid, and their sulfated metabolites. A simple, sensitive and specific LC/MS/MS analytical method was developed for the quantitation of bile acids by triple quadrupole (QQQ) mass spectrometry using protein crash (PPT) sample preparation. This easy method achieved good analyte recovery and post-extraction cleanliness and is capable of the sensitivities to quantitate the bile acids in serum over their dynamic range.

Methods: A Thermo Scientific™ TSQ Quantis™ tandem mass spectrometer in negative electrospray mode and a Thermo Scientific™ Vanquish™ Horizon HPLC system were utilized. 100 µL of serum were used for the analysis of the relevant bile acids. Various columns were evaluated and a Thermo Scientific™ Hypersil GOLD™ C18 100 x 2.1 mm, 1.8 µm column with a water and acetonitrile/methanol mixture containing 0.1% formic acid achieved baseline chromatographic separation in a less than 10 minute run time for all compounds. Quantitative analysis was performed using selective reaction monitoring (SRM) transition pairs for each analyte and internal standard in negative mode, and accuracy of the method was verified using quality control materials and serum samples.

Results: Good linearity and reproducibility were obtained with the concentration range from 1 nM to 1000 nM for the respective free, unconjugated, conjugated, and sulfated bile acids with a coefficient of determination > 0.995 for both sample preparation. The lower limits of detection (LLOD) and lower limit of quantitation (LLOQ) were determined to range from 0.1 to 0.5 nM. Excellent reproducibility was observed for both compounds (CV < 10%) and all techniques and configurations. A sensitive, simple, specific, and accurate liquid chromatography QQQ mass spectrometry method was developed and verified for the simultaneous measurement of total and fractionated bile acids in serum. Due to the lack of fragmentation of unconjugated bile acids, the same mass was monitored for both parent and daughter ions. The *m/z* 80 (SO_3^-) was monitored as the daughter ion for all taurine-conjugated bile acids, and the *m/z* 74 ($NHCH_2COO^-$) was the major fragment for all glycine bile acids. Hydrophilicity, and therefore retention on a reversed phase C18 column, is influenced by both the bile acids nucleus and side chains structures, and the retention time is also determined by the position a stereochemistry of hydroxyl groups. The sample preparation techniques are quick and easily applied for high-throughput analysis and included protein precipitation and liquid-liquid extraction. Matrix effect caused by phospholipids was greatly reduced by forward flushing the column to maintain the integrity of the method.

INTRODUCTION

Bile acids are steroidic acids formed in the liver from cholesterol, stored and concentrated in the gall bladder, and excreted in the intestines in response to fatty food intake. The liver synthesizes the primary bile acids and bacterial action in the colon synthesizes secondary bile acids.

In this research study, we evaluated various columns and solvent combinations as well as simple and easy sample preparation techniques in order to develop an LC-MS/MS analytical method that can demonstrate the chromatographic separation, detection and quantification of 41 bile acids that include chenodeoxycholic acid, cholic acid, deoxycholic acid, glycochenodeoxycholic acid, glycocholic acid, glycodeoxycholic acid, glycolithocholic acid, glycoursodeoxycholicacid, lithocholic acid, taurochenodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, taurolithocholic acid, tauroursodeoxycholic acid, ursodeoxycholic acid, and their sulfated metabolites. The sample preparation choice was a simple protein crash with the methodologies being developed on a TSQ Quantis tandem mass spectrometer in negative electrospray ionization modes with a Vanquish Horizon HPLC system for a 10 minute analytical gradient.

MATERIALS AND METHODS

Standards
The following analytical reference standards and internal standards were used:

Obtained from Isosciences, Inc., King of Prussia, PA

Cholic Acid (CA)	Cholic Acid-D4
Cholic Acid Sulfate (CA-S)	
Chenodeocycholic Acid (CDCA)	Chenodeoxycholic Acid-D4
Chenodeoxycholic Acid Sulfate (CDCA-S)	
Deoxycholic Acid (DCA)	Deoxycholic Acid-D4
Deoxycholic Acid Sulfate (DCA-S)	
Glycochenodeoxycholic Acid (GCDA)	Glycochenodeoxycholic Acid-D4
Glychenodeoxycholic Acid Sulfate (GCDA-S)	
Glycocholic Acid (GCA)	Glycocholic Acid-D4
Glycocholic Acid (GCA-S)	
Glycodeoxycholic Acid (GDCA)	Glycodeoxycholic Acid-D4
Glycodeoxycholic Acid Sulfate (GDCA-S)	
Glycolithocholic Acid (GLCA)	Glycolithocholic Acid-D4
Glycotithicholic Acid Sulfate (GLCA-S)	
Glycoursodeoxycholic Acid (GUDCA)	Glycoursodeoxycholic Acid-D4
Glycoursodeoxycholic Acid Sulfate (GUDCA-S)	
Lithocholic Acid (LCA)	Lithocholic Acid-D4
Lithocholic Acid Sulfate (LCA-S)	
Taurocholic Acid (TCA)	Taurocholic Acid-D4
Taurocholic Acid Sulfate (TCA-S)	
Taurochenodeoxycholic Acid (TCDCA)	Taurochenodeoxycholic Acid-D4
Taurochenodeoxycholic Acid Sulfate (TCDCA-S)	
Taurodeoxycholic Acid (TDCA)	Taurodeoxycholic Acid-D4
Taurodeoxycholic Acid Sulfate (TDCA-S)	
Taurolithocholic Acid (TLCA)	Taurolithocholic Acid-D4
Taurolithocholic Acid Sulfate (TLCA-S)	
Tauroursodeoxycholic Acid (TUDCA)	Tauroursodeoxycholic Acid-D4
Tauroursodeoxycholic Acid Sulfate (TUDCA-S)	
Ursodeoxycholic Acid (UDCA)	Ursodeoxycholic Acid-D4
Ursodeoxycholic Acid Sulfate (UDCA-S)	

Obtained from Cayman Chemicals, Inc., Ann Arbor, MI

Glycohyocholic Acid (GHCA)	Glycohyodeoxycholic Acid (GHDCA)
Hyocholic Acid (HCA)	Hyodeoxycholic Acid (HDCA)
Alpha-Muricholic Acid	Beta-Muricholic Acid
Murideoxycholic Acid (MDCA)	
Taurohyocholic Acid (HCA)	Taurohyodeoxycholic Acid (THDCA)
Tauro-Alpha-Muricholic Acid (TAMCA)	Tauro-Beta-Muricholic Acid (TBMCA)

Reagents
The following Fisher Scientific™ acids, reagents, and solvents were used:

HPLC Grade Water	Formic Acid
Methanol	Acetonitrile

The standards and internal standards were made up in in methanol.

Sample Preparation- Protein Crash

- 200 µL of bile acid depleted serum calibrators, controls, and serum sample were added to 1.5 mL Eppendorf tubes and 20 µL of bile ISTD mixture at 1000 ng/mL were added to each tube and vortexed briefly.
- 200 µL of acetonitrile was added to each tube and vortexed for 1 min prior to centrifugation for 10 min at 13,000 rpm.
- The supernatant was transferred to an MS vial containing 200 µL of water and capped.
- All in-house calibrators were prepared in bile acid depleted serum and water (Golden West Biological, Inc, Temecula, CA)

The calibration curves ranged from 1 ng/mL to 1000 ng/mL, and various pooled donor samples were used as control material.

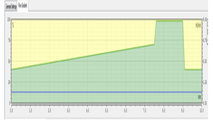
Data Analysis

The software used for this method included Thermo Scientific™ Xcalibur™ 3.1 software, Thermo Scientific™ TSQ Quantiva™ Tune™ 2.1 software and Thermo Scientific™ TraceFinder™ 4.1 software.

METHOD

HPLC Conditions
Vanquish Horizon HPLC binary pump, well plate, thermostatted column compartment

Column:	Hypersil GOLD C18, 100 x 2.1 mm, 1.9 µm
Column Temperature:	50 °C
Injection Volume:	10 µL
Sampler Temperature:	4 °C
Needle Wash:	Flush port (50% methanol/50% water) 10 s
Mobile Phase A:	0.1% Formic acid
Mobile Phase B:	Methanol/acetonitrile (1:1)
Flow Rate:	0.65 mL/min
Gradient:	0.0 min- 60% A:40% B
	7.5 min- 30% A:70% B
	7.6 min- 2% A:98% B
	9.0 min- 2% A:98% B
	9.1 min- 60% A:40% B
Run time:	10 min



MS and Ion Source Conditions
TSQ Quantis triple quadrupole mass spectrometer

Ion Mode:	Negative Electrospray (H-ESI) Mode
Vaporizer Temperature:	350 °C
Ion Transfer Tube Temperature:	300 °C
Sheath Gas:	75
Aux Gas:	10
Sweep Gas:	0
Spray Voltage:	Negative Ion: 3500 V
Q1/Q2 Resolution:	0.7/0.7 (FWHM)
Cycle Time:	0.6 s
CID Gas (mTorr):	2
Chromatographic Peak Width:	6 s

Table 1- Scan Parameters- SRM table

Compound	Rt (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energies (V)	Rf Lens (V)
T-A/B-MCA	2.20	Negative	514.28	124.1/79.9	54/54	299
THCA	3.03	Negative	514.37	123.9/79.9	54/54	246
TUDCA	3.27	Negative	498.41	124.1/79.9	52/55	201
TUDCA-S	1.30	Negative	578.25	498.3/123.9	24/55	165
THDCA	3.46	Negative	498.38	124.1/79.9	51/55	299
TCA	3.84	Negative	514.39	124.1/79.9	53/55	299
TCA-S	1.65	Negative	594.23	514.3/124.1	25/55	162
GHCA	4.61	Negative	464.26	400.3/74.1	38/36	299
GUDCA	4.84	Negative	448.28	386.4/74.1	34/34	299
GUDCA-S	2.54	Negative	528.28	448.3/74.1	30/55	130
GHDCA	5.12	Negative	448.26	386.4/74	34/34	299
TCDCA	5.16	Negative	498.36	124.1/79.6	52/54	299
TCDCA-S	2.44	Negative	578.23	498.3/129.9	25/55	168
B-MCA	5.21	Negative	407.23	407.23	8	299
A-MCA	5.44	Negative	407.26	407.26	8	299
TDCA	5.46	Negative	498.37	124.1/79.9	51/55	211
TDCA-S	2.61	Negative	578.18	498.3/124.1	25/53	171
GCA	5.51	Negative	464.28	402.3/74.1	34/36	299
GCA-S	3.12	Negative	544.29	464.3/74.1	31/43	146
MDCA	5.90	Negative	391.24	391.24	8	299
HCA	6.28	Negative	407.58	407.58	8	299
UDCA	6.44	Negative	391.28	391.28	8	299
UDCA-S	3.81	Negative	471.18	391.3/79.9	42/55	299
TLCA	6.71	Negative	482.58	124.1/79.6	50/55	299
TLCA-S	3.49	Negative	562.34	482.32/124.1	34/55	178
HDCA	6.90	Negative	319.27	391.27	8	299
CA	6.94	Negative	407.28	407.28	8	299
CA-S	3.73	Negative	487.08	457.08	5.41	130
GCDCA	7.03	Negative	448.33	386.3/74.1	34/34	299
GCDCA-S	4.23	Negative	528.32	448.3/74.1	30/42	133
GDCA	7.35	Negative	448.32	404.4/74.1	32/35	299
GDCA-S	4.43	Negative	528.26	448.3/74.1	31/43	132
CDCA	8.55	Negative	391.25	391.25	8	299
CDCA-S	5.55	Negative	471.28	471.2/79.9	34/41	299
DCA	8.72	Negative	391.26	391.26	8	299
DCA-S	5.67	Negative	471.23	391.3/79.9	34/41	299
GLCA	8.70	Negative	432.28	388.3/74.1	32/33	299
GLCA-S	5.46	Negative	512.24	432.3/74.1	30/41	224
LCA	9.10	Negative	375.28	375.28	8	299
LCA-S	6.81	Negative	455.23	455.23	8	171

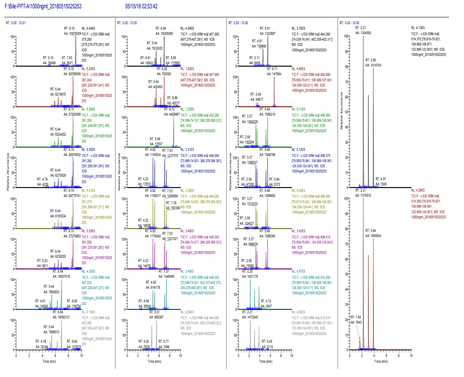
RESULTS

Linearity/Sensitivity
The assay was linear over the calibration curve for the bile acids in serum as shown in the table with their mean of coefficient of determinations (R^2) for negative mode and for the sample preparation techniques used. The linearity of each extraction was determined in triplicate over 3 days, and the results are shown with the LOQ being determined as 10:1 of signal to noise. The mean coefficient of determination (R^2) > 98 for each sample extraction technique, and the %CV for each calibration point were all < 10% in order to be accepted.

Precision/Specificity
The inter-assay precision and accuracy for bile acids was determined by extracting and quantifying five replicates of the in-house control material resulting in mean %CV of < 10% deviation form the targeted mean. Therefore, this analytical method meets the laboratory required accuracy for the analysis of bile acids in serum in negative mode.

Figure 1. Chromatograms Negative Mode

Bile Acids



Sulfated Bile Acids

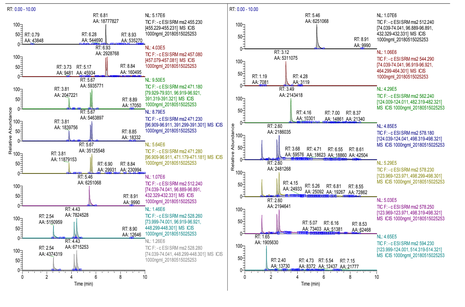


Table 2. Linearity and Sensitivity

Compound	LOD (ng/mL)	LOQ (ng/mL)	Compound	LOD (ng/mL)	LOQ (ng/mL)
T-AB-MCA	2.5	5	HCA	50	50
THCA	5	10	UDCA	2.5	5
TUDCA	5	10	UDCA-S	1	2.5
TUDCA-S	2.5	5	TLCA	2.5	5
THDCA	10	25	TLCA-S	2.5	5
TCA	5	10	HDCA	5	10
TCA-S	2.5	5	CA	1	2.5
GHCA	5	10	CA-S	2.5	5
GUDCA	2.5	5	GCDCA	1	2.5
GUDCA-S	1	2.5	GCDCA-S	1	2.5
GHDCA	5	10	GDCA	2.5	5
TCDCA	2.5	5	GDCA-S	5	10
TCDCA-S	1	2.5	CDCA	2.5	5
B-MCA	5	10	CDCA-S	5	10
A-MCA	2.5	5	DCA	1	2.5
TDCA	2.5	5	DCA-S	2.5	5
TDCA-S	2.5	5	GLCA	2.5	5
GCA	1	2.5	GLCA-S	5	10
GCA-S	5	10	LCA	1	2.5
MDCA	2.5	5	LCA-S	2.5	5

CONCLUSIONS

- Baseline separation of 40 bile acids and their metabolites in 10 minutes with good LOD/LOQ negative mode was achieved.
- A clean serum matrix is required to achieve the desired calibration curve and LOQ as the bile acids, which are steroid derivatives, are found in serum products tested.
- Excellent linearity of calibration curves with acceptable accuracy, precision, and reproducibility in negative mode was achieved in all matrices analyzed with <10% %CV.
- Mobile phases and column used were chosen to ensure complete baseline separation of all bile acids, metabolites, and interferences, which can be considerable.
- Bile acids have different pKa moieties so better sample preparation techniques will be further evaluated to determine which give the best results while removing the interferences.

For Research Use Only. Not for use in diagnostic procedures.

REFERENCES

1. Determination of bile acids in biological fluids by liquid chromatography-electrospray tandem mass spectrometry, *Journal of Lipid Research*. **2001**; *42*, 114 –119.
2. Bile Acid Profiling and Quantification in Biofluids Using Ultra-Performance Liquid Chromatography, *Tandem Mass Spectrometry Anal Chem*, **2016**; *87* (19), 966 –9670.

TRADEMARKS/LICENSING

PO65274-EN 0518S