Project: La Merrill WCMC Pilot & Feasibility Study

Summary Text for BILE ACID TISSUE EXTRACTION & ANALYSIS PROTOCOL

Conducted in the laboratory of Dr. John W. Newman

Bile Acid Extraction

A 15 mg liver sample (n=1 female/litter in 7 VEH and 8 DDT litters) was pulverized on dry ice, enriched with deuterated bile acid surrogates, butylated hydroxytoluene and ethylinediaminetetraacetic acid, and extracted twice with 500 µL methanol. The combined extract was dried, reconstituted in 100µL 50:50 methanol:acetonitrile with internal standards 1-phenyl-3-hexanoic acid urea (PHAU) and 1-cyclohexyl-3-dodecanoic acid urea (CUDA) and filtered at 0.1 µm. Extracts were stored at -20ºC until analysis by UPLC-MS/MS. The internal standard was used to quantify the recovery of surrogate standards. The internal standard was used to quantify the recovery of the deuterated extraction surrogates by ratio response

Bile Acid Analysis

Analytes in a 15 mg liver sample extract aliquot were separated with an Aquity C_{18} BEH 1.7µm 100mm x 2.1mm column utilizing a Waters Acquity UPLC (Waters, Milford, MA) with the solvent gradient described in Table 1, using modifications of previously published protocols (1,2). The autosampler was maintained at 10ºC. Resolved analytes were detected by negative mode electrospray ionization and multiple reaction monitoring on a API 4000 QTrap (AB Sciex, Framingham, MA) using the following operating parameters: Curtain gas = 10.0 psi, temperature = 600 °C, IonSpray voltage = -4500.00, collision gas = medium, ion source gas 1 & 2 = 40.0 psi, and entrance potential = -10.0 V. Analyte retention times, mass transitions, collision energies, cell exit and declustering potentials, dwell times, and analytical surrogate associations for each analyte are shown in Table 2. Analytes were quantified using isotope dilution and internal standard methodology with 5 to 7 point calibration curves ($r2 \geq 0.997$). Calibrants and internal standards were either synthesized [PHAU and CUDA] purchased from Steraloid Inc. (Newport, RI), Sigma-Aldrich (St. Louis, MO), and Medical Isotopes, Inc. (Pelham, NH). Data was processed utilizing AB Sciex Analyst version 1.6.2. Surrogate recoveries can be viewed in Table 3.

(1) Garcia-Cañaveras JC et al (2012). Targeted profiling of circulating and hepatic bile acids in human, mouse, and rat using a UPLC-MRM-MS validated method. *J Lipid Res*. 53:2231-41

(2) La Merrill MA et al (2014). Perinatal exposure of mice to the pesticide DDT impairs energy expenditure and metabolism in adult female offspring. PLOS ONE.

Table 1. UPLC parameters

Time (min)	A%	B%
0	90	10
0.5	90	10
1	75	25
11	60	40
12.5	5	95
14	5	95
14.5	90	10
16	90	10

Solvent A = 0.1% Formic Acid; Solvent B = 0.1% Formic Acid in Acetonitrile; flow rate = 0.4 mL/min, column 2.1 X 100mm, 1.7 µm BEH C18 (Waters, Milford, MA), column temp = 60 °C

Table 2. UPLC/Electrospray ionization QTRAP analyte and instrument parameters*

Analyte	Common Abbreviation	tR (min)	Transition (Da)	Declustering (V)	Collision (V)	Cell Exit (V)	Dwell (msec)	ISTD†
1-Phenyl 3-Hexadecanoic Acid Urea	PHAU	2.6	249.2 > 130.1	65	20	5	20	---
Taurodehydrocholic acid	TDHCA	2.82	508.2 > 80	155	110	2	20	TCDCA-d4
Tauro-ω-Muricholic acid	T-ω-MCA	3.33	514.3 >80	155	110	4	20	TCDCA-d4
Tauro-α-Muricholic acid	T-α-MCA	3.47	514.3 > 80	155	110	4	20	TCDCA-d4
Tauro-β-Muricholic acid	T-β-MCA	3.56	514.3 > 80	155	110	4	20	TCDCA-d4
Glycodehydrocholic acid screen††	GDHCA	3.74	458.3 > 74	110	65	7	20	GCDCA-d4
Taurohyocholic acid screen	THCA	4.56	514.3 > 80	155	110	10	20	TCDCA-d4
Tauroursodeoxycholic acid	TUDCA	5.48	498.3 >80	155	110	4	20	TCDCA-d4
Taurohyodeoxycholic acid screen	THDCA	5.58	498.3 > 80	145	110	4	20	TCDCA-d4
Taurocholic acid	TCA	5.91	514.3 > 80	185	115	4	20	TCDCA-d4
Dehydrocholic acid screen	DHCA	5.94	391.3 > 391.3	105	30	9	20	DCA-d4
Glycohyocholic acid screen	GHCA	6.51	464.3 > 74	110	70	10	20	GCDCA-d4
ω-Muricholic acid	ω-MCA	6.96	407.3 > 407.3	115	30	9	20	CA-d4
Glycoursodeoxycholic acid	GUDCA	7.12	448.3 > 74	115	70	4	20	GCA-d4
α-Muricholic acid	α-MCA	7.3	407.3 > 407.3	115	30	9	20	CA-d4
Glycohyodeoxycholic acid	GHDCA	7.32	448.3 > 74	120	70	4	20	GCA-d4
Glycocholic acid-d4	GCA-d4	7.4	468.3 > 74	125	70	4	20	PHAU
Glycocholic acid	GCA	7.41	464.3 > 74	125	70	4	20	GCA-d4
β-Muricholic acid	β-MCA	7.71	407.3 > 407.3	115	30	9	20	CA-d4

* - Analytes were separated under conditions described in Table I. Collision-induced dissociation was performed with nitrogen at a pressure of 2.3 mTorr. Dashed lines indicate separation between mass spectral multiple reaction monitoring functions.

† - Internal Standards (ISTD) - Analytes were corrected for recoveries of listed surrogates. 1-Cyclohexylureido,3-dodecanoic acid (CUDA) and 1-Phenyl 3-Hexadecanoic Acid Urea (PHAU) were introduced immediately prior to analysis and used to quantify surrogate recoveries.

†† - Compounds labeled as "screen" are compounds for which we did not have calibration standards. The compounds were identified based on retention time and transition (Da) and produced qualitative data.

Table 2. UPLC/Electrospray ionization QTRAP analyte and instrument parameters (continued)*

Analyte	Common Abbreviation	tR (min)	Transition (Da)	Declustering (V)	Collision (V)	Cell Exit (V)	Dwell (msec)	ISTD†
Taurochenodeoxycholic acid-d4	TCDCA-d4	8.63	502.3 > 80	175	110	4	20	PHAU
Taurochenodeoxycholic acid	TCDCA	8.66	498.3 > 80	145	110	4	20	TCDCA-d4
Taurodeoxycholic acid	TDCA	9.4	498.3 > 80	140	110	4	20	TCDCA-d4
Murocholic acid screen	MCA	9.54	391.3 > 391.3	105	30	9	20	DCA-d4
Hyocholic acid screen	HCA	9.56	407.3 > 407.3	140	30	10	20	DCA-d4
Cholic acid-d4	CA-d4	10.13	411.4 > 411.4	120	30	9	20	CUDA
Cholic acid	CA	10.16	407.3 > 407.3	125	30	9	20	CA-d4
Ursodeoxycholic acid	UDCA	10.26	391.3 > 391.3	125	30	9	20	DCA-d4
Hyodeoxycholic acid screen	HDCA	10.33	391.3 > 391.3	125	30	9	20	DCA-d4
Glychochenodeoxycholic acid-d4	GCDCA-d4	10.81	452.3 > 74	120	65	4	20	CUDA
Glychochenodeoxycholic acid	GCDCA	10.83	448.3 > 74	125	65	4	20	GCDCA-d4
Glycodeoxycholic acid	GDCA	11.54	448.3 > 74	125	65	4	20	GCDCA-d4
1-Cyclohexyl Urea 3-Dodecanoic Acid	CUDA	12.06	341.3 > 216.2	65	35	3	20	---
Taurolithocholic acid	TLCA	12.3	482.3 > 80	150	110	4	20	LCA-d4
Chenodeoxycholic acid-d4	CDCA-d4	12.42	395.3 > 395.3	125	25	9	20	CUDA
Chenodeoxycholic acid	CDCA	12.42	391.3 > 391.3	130	30	9	20	CDCA-d4
Deoxycholic acid-d4	DCA-d4	12.49	395.3 > 395.3	125	30	9	20	CUDA
Deoxycholic acid	DCA	12.5	391.3 > 391.3	130	30	9	20	DCA-d4
Glycolithocholic acid	GLCA	12.56	432.3 > 74	120	65	4	20	GCDCA-d4
Trihydroxycholestanoic acid screen	TriHCA	12.74	449.3 > 449.3	140	30	10	20	LCA-d5
Lithocholic acid-d5	LCA-d5	13.03	380.3 > 380.3	135	30	10	20	CUDA
Lithocholic acid	LCA	13.03	375.3 > 375.3	130	35	8	20	LCA-d5
Dihydroxycholestanoic acid screen	DiHCA	13.05	433.3 > 433.3	140	30	10	20	LCA-d5

* - Analytes were separated under conditions described in Table I. Collision-induced dissociation was performed with nitrogen at a pressure of 2.3 mTorr. Dashed lines indicate separation between mass spectral multiple reaction monitoring functions.

† - Internal Standards (ISTD) - Analytes were corrected for recoveries of listed surrogates. 1-Cyclohexylureido,3-dodecanoic acid (CUDA) and 1-Phenyl 3-Hexadecanoic Acid Urea (PHAU) were introduced immediately prior to analysis and used to quantify surrogate recoveries.

†† - Compounds labeled as "screen" are compounds for which we did not have calibration standards. The compounds were identified based on retention time and transition (Da) and produced qualitative data.

Table 3. Analytical surrogate recoveries

Chemical class	Compound	Mean ± SD	%RSD
Primary bile acid	CA-d4	96.7 ± 10.2%	10.5%
Primary bile acid	CDCA-d4	79.9 ± 11.9%	14.9%
Secondary bile acid	DCA-d4	74.6 ± 14.9%	20.0%
Tertiary bile acid	LCA-d4	13.5 ± 3.0%	22.5%
Glycine-conjugate	GCA-d4	93.2 ± 10.8%	11.6%
Glycine-conjugate	GCDCA-d4	96.4 ± 9.5%	9.8%
Taurine-conjugate	TCDCA-d4	91.2 ± 7.2%	7.9%

† -Relative standard deviation (standard deviation divided by the mean) x 100