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 C18 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 ≥ 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.

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| **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 | |  |  |

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| **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. | | | | | | | | | | |  |  |

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| **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. | | | | | | |  |  |

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| **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 |  |  |  |