**Laboratory Analytic Protocols**

Urinary lactulose and mannitol concentrations were determined by HPLC platforms running a harmonized protocol. Five sites (BRF, NEB, PKN, SAV, and TZH) sent samples to the University of Ceara, in Fortaleza, Brazil, for L:M determination by HPLC-PAD. One site (PEL) sent samples to an LC-MSMS lab (Oregon Analytics), and two sites (INV and BGD) each analyzed their own samples by HPLC-PAD (Christian Medical College Vellore, India, and the International Centre for Diarrhoeal Disease Research (icddr,b), Bangladesh, respectively).

***University of Ceara, Fortaleza, Brazil***

For HPLC testing, 50 μL of each urine sample was diluted with 50 μL of melibiose solution (3.6 mM) and completed with distilled and deionized water up to 2.9 ml. All samples were filtered (0.22 μL) and 50 μL from each sample was automatically injected into the HPLC column system.

The ICS3000 carbohydrate analyzer HPLC system (Dionex Co., Sunnyvale, CA, USA) was composed of the following modules: AS3000 Automated Sampler for injections, SP3000 Gradient Pump, DC Detector/Chromatography module with column and ED40 Electrochemical Detector. A CarboPac MA-1 anion-exchange column (250 x 4.0 mm i.d., pellicular resin) with an associated guard column was also from Dionex. Elution of the sugar alcohols, monosaccharides and disaccharides was achieved with an isocratic eluent of 480 mM NaOH at a flow-rate of 0.4 ml/min using the SP3000 and MA1 CarboPac column. Column temperature was set at 30 °C. Detection used the ED3000 detector with a waveform consisting of the following potential-duration profile: sampling = 0.1 V, 200 ms; oxidation = 0.10 V, 400 ms; reduction = -2 V, 410 ms and -2 V, 420 ms; oxidation = 0.6 V, 430 ms; reduction = -0.1 V, 440 ms and -0.1 V, 500 ms. Output range of the detector was set at 1.0 mA with integration response time of 3 seconds as previously reported. A 50 μL volume of each sample was injected automatically using the AS3000 Automated Sampler. Sugars were identified and measured using Chromeleon 6.8 software package (Dionex Co., Sunnyvale, CA, USA).

***Oregon Analytics, Eugene, OR***

*Reagents and Calibrators*

Calibrator solutions were prepared via serial dilutions of 10 mg/ml mannitol and lactulose (Sigma-Aldrich, St. Louis MI) diluted in HPLC grade water. Internal standards contained 0.01 mg/ml of mannitol 13C6 and lactulose 13C12 (Sigma-Aldrich). Pooled blank urine (urine collected prior to the administration of lactulose and mannitol) was spiked with independently prepared solutions containing serial dilutions of 10 mg/ml for assay validation and for quality control on all runs. Linear responses in the calibration equation were observed with an r>0.999 in the range of 10-4000 μg/ml for both lactulose and mannitol with 95% confidence intervals of 2%. A second transition was monitored for each analyte to confirm identity and required to be within 20% of the relative peak area of the first transition used for quantification.

*Sample Preparation*

Each urine sample was thawed and vortexed for 10 seconds. A volume of 20μl of sample was diluted in a mix of 70% acetonitrile containing internal standard, 0.1% sodium acetate and diluted 1:4 in MS grade water, transferred to the auto sampler vial and vortexed for 10 seconds.

*LC-MSMS*

The LC-MSMS system was composed of a LC- Shimadzu prominence LC20AD (Shimadzu) with an ultramino 3 um 150 x 2.1 mm 100A aminopropylsilane Restek column (Restek, Bellafonte, PA) with a flow rate of 0.30 ml/min with degasser and autosampler SIL-20AC HT (Shimadzu Scientific Instruments) and column oven CTO-20A (Shimadzu) run at 40˚C. Mobile phase A was HPLC grade water with 0.1% formic acid, and phase B was acetonitrile with 0.1% formic acid. Elution was programmed to start at 70% phase B for 0.5 min, then fall to 5% B at 2 minutes, return to 70%B at 2.5 min and equilibrate for two minutes prior to the next sample injection.

The MS/MS-API 5500 (AB Sciex, US) with turbo-ion probe (ESI) operated at 600C.

***Christian Medical College Vellore, India***

*Reagents and Calibrators*

Stock solution of lactulose (Fluka Analytical) and mannitol (SD Fine Chemical) was prepared at concentrations of 100mM in 1ml deionized water (MilliQ water purification system-Millipore). For each standard curve 10 µl from 100mM stock solution was made up to 1 ml using de ionized water to obtain a final concentration of 1mM. A standard curve was obtained by injecting different volumes namely 5, 10, and 15 and 20µl corresponding to a concentration of 5 to 20 µmoles. Melibiose (Sigma-Aldrich) which is used as an internal control was also run at the above described dilutions to serve as an additional control. The R2 value obtained for the standard curves generated for lactulose, mannitol, and melibiose standards was above 0.99. HPLC grade acetonitrile was used for mobile phase.

*Sample Preparation*

Frozen urine samples were brought to ambient temperature just before analysis. The urine was vortexed and centrifuged in microcentrifuge at 10000 rpm for 10 minutes at 4°C. 500µl of urine supernatant, 50µl of melibiose (3.6mM) internal standard and 450µl of deionised water were added to 0.1g washed Duolite resin (BDH) and thoroughly vortexed. It was centrifuged again as described above and supernatant was filtered using 0.45 micron filter. 20µl of filtered supernatant was injected into the HPLC column using an auto injector.

*HPLC*

*HPLC instrument details*

The HPLC instrument used for separation and quantitation includes UFLC Prominence (Shimadzu) composed of following modules: LC-20AD: Pump, CBM-20A: Communication Bus Module to communicate instrument to the LC solution software, SIL-20AD: Auto injector and CTO-20AC Column Oven. Separation column was Luna 5u NH2 100A (250X460mm with a diameter of 5µl) with an associated guard column from Phenomenxex.

The chromatographic separation was performed in 10 minutes at a flow rate of 2.0ml/min, using HPLC grade acetonitrile: water (80: 20) as mobile phase and keeping the column temperature at 40°C. The Varian ELSD (Evaporator Light Scattering Detector) with a evaporator temperature set at 90°C, Nebiliser at 40°C and the carrier gas flow rate set at 1 SLM (standard liter per min) was used.

Sugars were identified and measured using LC solution (Shimadzu) software. The concentration of lactulose and mannitol were calculated from the peak area and recovery of the internal standard and expressed as the ratio of lactulose:mannitol. Recovery of internal standard was always more than 95%.

***International Centre for Diarrhoeal Disease Research (icddr,b), Bangladesh***

For HPIC testing, 100µL of each urine sample and 50µL of a solution containing melibiose (3.0 mM) diluted in 2.85 mL of distilled and deionized water. The final concentration of melibiose is 50 µM. The mobile phase should be filtered through a 0.45-µM millipore filter. All samples (500 µL each) were transferred to sample vial with optional 20 µm filter cap and placed to the auto sampler from where samples were automatically injected to the HPIC system. A 25 µL volume of each sample was injected automatically using the AS-DV sampler. Five levels of calibration standards were run with each batch to generate a standard curve using Chromeleon software. The Dionex ICS-5000 carbohydrate analyzer HPIC system (Dionex Co., Sunnyvale, CA, USA) was composed of the following modules: AS-DV Automated Sampler for injections, SP5000 Gradient Pump, DC Detector/Chromatography module with column and ED Electrochemical Detector with integrated Pulse Amperometry Detector. A Dionex CarboPac MA1 anion-exchange column (250 x 4.0 mm, Thermo Scientific,BioLC) with an associated CarboPac MA1 guard column (50 x 4.0mm) was also from Dionex. Elution of the monosaccharides and disaccharides was achieved with an isocratic eluent of 450 mM NaOH at a flow-rate of 0.4 ml/min using the Dionex ICS5000 SP and MA1CarboPac column. Column temperature was set at 26 °C. Detection used the ED5000 detector with a waveform Carbohydrate (standard Quad) consisting of the following potential-duration profile: sampling = 0.1 V, 200 ms; oxidation = 0.10 V, 400 ms; reduction = -2 V, 410 ms and -2 V, 420 ms; oxidation = 0.6 V, 430 ms; reduction = -0.1 V, 440 ms and -0.1 V, 500 ms. Output range of the detector was set at 1.0 mA with integration response time of 3 seconds as previously reported. Sugars were identified and measured using Chromeleon 6.8 software package (Dionex Co., Sunnyvale, CA, USA).