Bile acid Metabolism（Targeted metabolomics）

Method statement

V2.0

2021.8

Catalogue

1. Experimental Instruments and Softwares

Experimental Instruments：	
5500 QTRAP（AB SCIEX）	Waters ACQUITY UPLC I-Class (Waters)
low temperature Centrifuges（Eppendorf 5430R）	LC Waters, ACQUITY UPLC BEH Amide 1.7μm, 2.1 mm×100 mm column
Ultrasonic Liquid Processors （ Scientz JY92-II，Ningbo）	Concetrator plus/Vacufuge （ Eppendorf Concentrator Plus）
Vortex（QiTe QT-1，Shanghai）	MP Fastprep-24 Automated Homogenizer（MP Biomedicals)
Softwares：	
Multiquant	

2. Materials

Acetonirile (Merck，1499230-935)	formic acid(Honeywell,94318)
Methanol（Fisher Chemical,A452-4）	Metabolic standard (Sigma-Aldrich)
Negative ion mode	
HPLC Buffer A：0.1% formic acid in water	

HPLC Buffer B： 100% Methanol

3. Methods

3.1 Sample Preparation

The method was determined by the project proposal or preliminary experiment report.

i. methanol Lysis[1]：

Application: serum, plasma, urine, body fluid,

The samples were thawed at 4 °C and 100 µL aliquots were mixed with 10 µL of internal standard and 500 µL of cold methanol to remove the protein. The mixture was centrifuged for 20 min (14000g, 4 °C). The supernatant was dried in a vacuum centrifuge. For LC-MS analysis, the samples were re-dissolved in 100 µL methanol/water (1:1, v/v) and adequately vortexed, and then centrifuged (14000g, 4℃, 15 min). The supernatants were collected for LC-MS/MS analysis. Take the standard and dilute it to a series of gradient concentration standard working solution with methanol aqueous solution, prepare the standard curve solution according to the above method, and establish the standard curve by the isotope internal standard method.

ii. Homogenate Lysis：

Application: Tender tissues (brain, liver, muscle, etc), mollusk, microorganism, etc.

Samples(30 mg) were homogenized with 200 µl pre-cooled ultrapure water, add 800 µL of pre-cooled methanol and 10 µL of internal standard, vortex, and precipitate the protein for 20 min at − 20 °C; Centrifuge at 14,000 rcf for 15 min at 4 °C and take the supernatant to dry under vacuum;add 100 µL methanol–water (1:1, v/v) for reconstitution. Centrifuge at 14,000 rcf for 15 min at 4 °C and take the supernatant for analysis. Take the standard and dilute it to a series of gradient concentration standard working solution with methanol aqueous solution, prepare the standard curve solution according to the above method, and establish the standard curve by the isotope internal standard method.

3.2 HPLC-MS/MS analysis

Analyses were performed using an UHPLC (Waters Ltd.) coupled online to 5500 QTRAP Mass Spectrometry (AB SCIEX, USA).

i. HPLC analysis

Samples were analyzed using an cquity UPLC system (Waters Ltd.) coupled online to 5500 QTRAP Mass Spectrometry (AB SCIEX, USA).

The samples (2ul) were injected onto an ACQUITY UPLC BEH C18 1.7 μm, 2.1 mm × 100 mm column (Waters Ltd.). The samples were in the automatic sampler at 8 ℃, and the column temperatures were kept constant at 45℃, a 2 μL aliquot of each sample was injected. The samples were eluted at a flow rate of 300 μL/min with phase A (0.1% formic acid in water) and phase B (Methanol). The separation was performed as followed: linear gradient from 60 to 65% B (0–6 min), linear gradient from 65 to 80% B (6–13 min), linear gradient from 80 to 90% B (13–13.5 min), and isocratic at 90% B (13.5–15 min). The column temperature was 45 °C. A QC sample is set for each set of experimental samples in the sample queue to test and evaluate the stability and repeatability of the system of the method during its operation.MS/MS Analysis(MRM).

ii. MS/MS Analysis(MRM)

Mass spectrometry was performed as follows: source temperature: 550 °C; ion Source Gas1 (Gas1): 55; Ion Source Gas2 (Gas2): 55; Curtain gas (CUR): 40; ionSapary Voltage Floating (ISVF): − 4500 V; The MRM mode is used to detect the transitions to be measured

3.3 Data process

To construct the metabolite MRM library, each metabolite standard (100 mg/mL) was first analyzed in ESI negative mode via flow injection to get the optimal MRM transition parameters. Then the retention time of each metabolite was determined by measuring the corresponding MRM(Q1/Q3) transition individually on the column. A standard mixture sample (33 STD_mix) that contains all of the 33 metabolites was measured together with biological samples in each experiment.LC–MS quantification of bile acid metabolites achieved with six-point standard curves using isotope internal

standard diluted in a relevant matrix matched to the analytical sample(absolute quantification). Data acquisition and processing were accomplished using Multiquant software.

4.1 Boxplot analysis

Using Quartile (Quartile) calculation method, put the multiple sets of data drawing on the same coordinate, showing each group data of the distribution difference, and changes of the expression trends.

4.2 Hierarchical Clustering

The studied metabolites relative expression data was used to performing hierarchical clustering analysis. For this purpose, Cluster3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/ software.htm) and the Java Treeview software (http:// jtreeview.sourceforge.net) were used. Euclidean distance algorithm for similarity measure and average linkage clustering algorithm (clustering uses the centroids of the observations) for clustering were selected when performing hierarchical clustering. Heatmap is often presented as a visual aid in addition to the dendrogram.

5. Reference

[1] Cai Y., Weng K., Guo Y.et al. Metabolomics, 2015, 11 (6), 1575-1586 。