Subject of testing	**Quantification of endogenous metabolites in mouse plasma, fecal, and tissue samples from a study on the gut-brain-axis using the MxP® Quant 500 assay.**	
Carried out by	**BIOCRATES Life Sciences AG** Eduard-Bodem-Gasse 8 6020 Innsbruck Austria Email: denise.sonntag@biocrates.com	
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Time table	Samples received: 2020-03-18 Analyses performed: 2020-03-24 – 2020-03-30 Test Report: 2020-04-27	
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ABBREVIATIONS

ESI	Electrospray ionization
FIA-MS/MS	Flow injection analysis-tandem mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
MRM	Multiple reaction monitoring
PITC	Phenyl isothiocyanate

1. QUALITY ASSURANCE STATEMENT

We declare that all pre-analytical and analytical procedures related to this project were performed, documented and reviewed according to our ISO 9001:2015 certified in-house quality management rules and guidelines.

2. TEST METHOD(S)

The analytical procedure for the determination of metabolites in murine brain samples is shown in the following chapters.

2.1. SAMPLING PRODECURE

The collection of samples was done by the client.

2.2. SAMPLE PREPARATION

All samples were stored immediately upon receipt at -80 °C.

For measuring metabolite concentrations in mouse plasma, the samples were centrifuged and the supernatant was used for further analysis.

For measuring metabolite levels in mouse brain tissue, mouse colon tissue, and mouse duodenum tissue, the samples were first suspended in 3 µL ethanol/phosphate buffer per mg tissue wet weight. Then, all samples were sonicated, vortexed and homogenized using a Precellys-24 instrument (Bertin Technologies, Montigny le Bretonneux, France), and the supernatant was used for further analysis. For measurement of some metabolites, it was necessary to dilute the mouse duodenum tissue samples 1:5 in buffer, before the samples were centrifuged and the supernatant was used for further analysis.

To extract metabolites from mouse duodenum content, mouse colon content, and mouse cecum content, samples were resuspended in extraction buffer (85% ethanol in phosphate buffer) and vortexed thoroughly until dissolution. After homogenization, the samples were put in a chilled ultrasonic bath for 5 min. Afterwards, samples were centrifuged. The supernatant was used for analysis. An additional 1:1000 dilution was prepared and measured for the analysis of highly concentrated bile acids.

2.3. MxP® Quant 500 Kit

Biocrates' commercially available MxP® Quant 500 kit was used for the quantification of several endogenous metabolites of various biochemical classes. Lipids and hexoses were measured by flow injection analysis-tandem mass spectrometry (FIA-MS/MS) using a 5500 QTRAP® instrument (AB Sciex, Darmstadt, Germany) with an electrospray ionization (ESI) source for the plasma samples and tissue samples, and a Xevo TQ-S (Waters, Vienna, Austria) instrument with an ESI source for the gut content samples. Small molecules were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS), also using a 5500 QTRAP® instrument for all samples. Gut tissue samples and gut content samples were partly also measured by LC-MS/MS on a Xevo TQ-S instrument. The experimental metabolomics measurement technique is described in detail by patents EP1897014B1 and EP1875401B1 (accessible online at https://patents.google.com/patent/EP1897014B1 and https://patents.google.com/patent/EP1875401B1). Briefly, a 96-well based sample preparation device was used to quantitatively analyze the metabolite profile in the samples. This device consists of inserts that have been impregnated with internal standards, and a predefined sample amount was added to the inserts. Next, a phenyl isothiocyanate (PITC) solution was added to derivatize some of the analytes (e.g. amino acids), and after the derivatization was completed, the target analytes were extracted with an organic solvent, followed by a dilution step. The obtained extracts were then analyzed by FIA-MS/MS and LC-MS/MS methods using multiple reaction monitoring (MRM) to detect the analytes. Data were quantified using appropriate mass spectrometry software (Sciex Analyst® and Waters MassLynx™) and imported into Biocrates Met*IDQ*™ software for further analysis.

3. PROJECT OUTLINE

A mass spectrometry-based metabolomics approach was used to determine the concentration of endogenous metabolites of various biochemical classes in samples of murine plasma, fecal, and tissue samples from a study on the gut brain axis using the MxP Quant 500 assay.

4. SAMPLE INFORMATION

The study included a total of 226 murine samples, of which 23 were plasma samples, 69 were gut content samples (23 cecum content samples, 23 colon content samples, and 23 duodenum content samples), and 133 were tissue samples (88 brain samples, 23 colon tissue samples, and 23 duodenum tissue samples). Originally, 161 mouse tissue samples had been anticipated. The samples were received in a frozen state on 2020-03-18. Sample labels were properly attached and readable. The receipt of the samples was confirmed by email to Rima Kaddurah-Daouk on 2020-03-18.

5. RESULTS

Metabolite concentrations of the samples were determined and the results are provided in the following Excel file:

5370_Gut brain axis study_Sub1_Pilot study_Data_2020-04-27.xlsx

The first tab includes data on plasma and tissue samples, while the second tab comprises data on intestinal gut content samples. Concentration values for all metabolites are given in µM in these two tabs. The file contains all obtained values, and values below the limit of detection (LOD) were replaced by "< LOD". The occasional additional dilutions of 1:5 or 1:1000 have been taken into account. In addition, all concentrations (except for the plasma samples) are displayed in pmol/mg tissue in the third tab or in pmol/mg gut content in the fourth tab, respectively.

Two metabolites, TMAO and indole, could not be quantified for technical reasons when gut content samples were measured on the TQ-S instrument, and are displayed as "NA". When measuring the concentration of abscisic acid in duodenal content samples, there was a interfering substance present in the matrix that prevented peak quantitation; the concentration values are also displayed as "NA" in these samples.

Concentrations of taurine, arginine, and TMCA sometimes substantially exceeded the calibration range and could not be absolutely quantified. In these cases, the values are displayed in bold gray italic numbers and represent estimated concentrations.

Details and further information on the analytes can be found in the tab "Metabolite information".

6. DISCUSSION

The accuracy of the measurements, determined with the accuracy of the calibrators, was in the normal range of the method for all analytes. Quality control samples were within the predefined tolerances of the method.

Please note that concentrations of taurine, arginine, and TMCA in several samples substantially exceeded the calibration range and could not be absolutely quantified. The concentration values outside the quantitative range are marked in bold gray italic and represent estimated concentrations. Especially Taurine concentrations were often so high that detector saturation was reached; the true concentrations are therefore higher (probably markedly higher) than the displayed estimated concentrations. Similarly, TMCA concentrations in the duodenum tissue samples, and GABA concentrations in the brain tissue

samples were very high occasionally, inducing detector saturation. Also in these samples, actual concentrations may be higher than the displayed values.

The concentrations of TMCA in the duodenal content samples were calculated from measurements of samples diluted 1:1000, while the TMCA concentrations in duodenal tissue samples were calculated from samples diluted 1:5. For this reason, there are some duodenal tissue samples in which the TMCA concentration exceeds the quantitative range even though the concentrations are noticeably lower than valid quantitative concentrations in the duodenal content samples.

Hexoses had a much higher LOD threshold when measured with the Waters TQ-S instrument than on the Sciex 5500 QTRAP® instrument, as had been observed before. As a consequence, the hexose concentration would have fallen below the threshold in some samples measured on the TQ-S, while being still higher than in valid samples measured on the 5500 QTRAP® instrument. In addition, there was still a marked gap between the hexose concentrations in the zero samples and the customer samples when measuring on the TQ-S instrument. The overall quality of the measurement allowed the conclusion that the hexose concentration in all samples may be interpreted as “above the LOD threshold”. The hexose concentration in those samples that would have fallen below the higher LOD threshold on the TQ-S instrument were thus defined as valid in the accompanying data file.

7. SUMMARY

Validated analytical methods were used for sample analysis. Absolute concentrations in murine plasma samples, tissue samples, and gut content samples were obtained for metabolites from various biochemical classes.

8. DATA STORAGE

The following data will be stored by BIOCRATES Life Sciences AG until the end of the project:
(i) standard operating procedures, (ii) raw data, (iii) processed data, and (iv) duplicates of this report
The data, complete with back-up media (CD-R’s or adequate), are stored for at least five years in the archives of BIOCRATES Life Sciences AG and then processed according to the costumer’s decision.

9. NOTES

The present results refer only to the samples measured.
This test report shall not be reproduced except in full, without the written approval of the laboratory.

End of Test Report

10. APPENDIX

Results for project 5370_Gut brain axis study_Sub1_Pilot study:

5370_Gut brain axis study_Sub1_Pilot study_Data_2020-04-27.xlsx