**Identification and validation of small molecule analytes in mouse plasma by liquid chromatography–tandem mass spectrometry: a case study of misidentification of a short-chain fatty acid with a ketone body**

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Abbreviations

3NPH: 3-Nitrophenyl Hydrazine, AA: Acetic Acid, AcAA: Acetoacetic acid, ACN: Acetonitrile, A.U.: Arbitrary Units, BA: Butyric Acid, EDC: N-(3-Dimethylaminopropyl)-N’-Ethylcarbodiimide, ESI: Electrospray Ionization, GC: Gas Chromatography, H2O: Water, HA: Hexanoic Acid, HBA: 3-Hydroxybutyric Acid, HMB: 3-Hydroxy-3-Methylbutyric Acid, IBA: Isobutyric Acid, ISTD: Internal Standard, IVA: Isovaleric Acid, LC: Liquid Chromatography, MBA: 2-Methylbutyric Acid, MRM: Multi-Reaction Monitoring, MS: Mass Spectrometry, PA: Propanoic Acid, Qn: Quadrupole *n*, RP: Reverse Phase, RT: Retention Time, SCFA: Short-Chain Fatty Acids, UPLC: Ultra Performance Liquid Chromatography, VA: Valeric Acid.

Table S1. Q1/Q3 transitions for all analytical ISTDs

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Compound | Precursor ion *m/z* | RT modified gradient (min) | Product ion *m/z* | Collision Energy (V) |
| d3-AA-3NPH | 197.054 | 8.4 | 153 | -14 |
| 154 | -14 |
| 137 | -19 |
| d5-PA-3NPH | 213.138 | 10.7 | 170 | -14 |
| 153 | -16 |
| 137 | -21 |
| d7-BA-3NPH | 229.129 | 12.9 | 153 | -17 |
| 186 | -14 |
| 137 | -21 |
| d9-VA-3NPH | 245.179 | 15.0 | 153 | -18 |
| 202 | -14 |
| 137 | -22 |
| d11-HA-3NPH | 261.225 | 17.0 | 153 | -20 |
| 218 | -15 |
| 137 | -22 |
| d3-AA-3NPH: d3-acetic acid derivative, d5-PA-3NPH: d5-propanoic acid derivative, d7-BA-3NPH: d7-butyric acid derivative, d9-VA-3NPH: d9-valeric acid derivative, d11-HA-3NPH: d11-hexanoic acid derivative |

Partial method validation

The proposed method was partially validated according to the EMA guidelines for bioanalytical method validation [1]. Peak areas of the metabolites of interest were first normalized to the following heavy labelled internal standards (ISTD): d3-AA for AA and HBA; d5-PA for PA and HMB; d7-BA for IBA and BA; d9-VA for MBA, IVA and VA; and d11-HA for HA and AcAA. Calibration curves spiked into commercial mouse plasma were used for the calculation of concentration accuracy and precision (Table S2). Background (endogenous) levels of metabolites found in the original plasma were first subtracted before the analysis. Linearity was then assessed by evaluating the R2 of the subsequent calibration curves. Lower limits of quantification (LLOQs) and upper limits of quantification (ULOQs) were set as the lowest and highest calibration points of each compound’s linear range, respectively. LLOQs had to be equal or higher than 5 x mean peak area of six process blank samples [1].

Accuracy and precision were monitored using three levels of spiked standards in quality control (QC) samples [1]: low (QC\_L), medium (QC\_M) and high (QC\_H) concentration (Table S2). For AA and HBA: QC\_L = 111.11 µM, QC\_M = 333.33 µM, QC\_H = 1,000.00 µM. For the rest of the compounds: QC\_L = 13.89 µM, QC\_M = 41.67 µM, QC\_H = 125.00 µM.

Matrix effects were evaluated by calculating a matrix factor (MF = level in plasma/level in solvent) for measured intensities of low (MF\_L), medium (MF\_M) and high (MF\_H) concentrations of the standards spiked into plasma and into solvent (50 % ACN:H2O). For AA and HBA: MF\_L = 49.38 µM, MF\_M = 444.44 µM, MF\_H = 1333.33 µM. For the rest of the compounds: MF\_L = 6.17 µM, MF\_M = 55.56 µM, MF\_H = 166.67 µM the matrix factor was calculated.

Endogenous concentrations in unspiked commercial mouse plasma were calculated using background-removed calibration curves.

Table S2. Method validation parameters for each SCFA and ketone body

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Compound | LLOQ (µM) | ULOQ (µM) | R2 | Accuracy % (n=6) | Precision % (n=6) | Matrix factor (MF)\* |
| QC\_L | QC\_M | QC\_H | QC\_L | QC\_M | QC\_H | MF\_L | MF\_M | MF\_H |
| AA | 0.61 | 3000.00 | 0.9956 | 86.75 | 113.17 | 110.68 | 12.83 | 12.54 | 12.92 | 0.90 | 1.17 | 1.26 |
| AcAA | 0.69 | 166.67 | 0.9996 | 89.88 | 96.51 | 84.69 | 6.41 | 9.41 | 9.22 | 0.80 | 0.81 | 0.78 |
| BA | 0.08 | 166.67 | 0.9999 | 90.77 | 107.33 | 94.10 | 7.63 | 7.06 | 9.29 | 1.13 | 1.03 | 1.09 |
| HA | 0.23 | 166.67 | 0.9941 | 91.69 | 112.05 | 88.56 | 17.74 | 7.79 | 4.51 | 0.87 | 0.98 | 0.82 |
| HBA | 49.38 | 1333.33 | 0.9970 | 80.98 | 105.60 | 95.07 | 14.63 | 9.88 | 13.66 | 1.59 | 1.68 | 1.73 |
| HMB | 2.06 | 166.67 | 0.9995 | 90.09 | 97.74 | 85.96 | 4.19 | 6.98 | 9.06 | 0.94 | 0.96 | 1.07 |
| IBA | 0.08 | 166.67 | 0.9963 | 85.49 | 109.80 | 100.67 | 7.03 | 4.46 | 3.05 | 0.99 | 1.06 | 1.02 |
| IVA | 0.23 | 166.67 | 0.9991 | 89.08 | 111.73 | 96.88 | 7.32 | 2.33 | 6.24 | 0.89 | 1.00 | 1.05 |
| MBA | 0.08 | 166.67 | 1.0000 | 96.29 | 106.13 | 94.51 | 8.65 | 6.29 | 7.81 | 0.93 | 1.03 | 1.21 |
| PA | 0.08 | 500.00 | 0.9953 | 90.22 | 95.45 | 84.98 | 7.16 | 8.51 | 11.37 | 1.03 | 1.01 | 1.08 |
| VA | 0.23 | 166.67 | 0.9999 | 94.52 | 108.19 | 96.73 | 7.21 | 6.46 | 6.38 | 0.90 | 0.99 | 1.13 |
| \* Acceptable ranges for MF: 0.85 – 1.15 according to the EMA guidelines |

Table S3. Estimated concentration in mouse plasma

|  |  |
| --- | --- |
| Compound | Estimated concentration in mouse plasma (µM) |
| AA | 192.37 |
| AcAA | 0.31 |
| BA | 3.69 |
| HA | 12.35 |
| HBA | 65.45 |
| HMB | 1.82 |
| IBA | 1.43 |
| IVA | 2.76 |
| MBA | 2.44 |
| PA | 7.86 |
| VA | 1.92 |

Table S4. Peak areas of AA-3NPH signal (transition 194 🡪 152) in commercial mouse plasma

|  |  |  |  |
| --- | --- | --- | --- |
|  | AA-3NPH | in HBA-3NPH | Sum |
| RT (min) | 8.6 | 9 | Range: 8.2-9.2 |
| Mean | 4.05E+06 | 1.01E+06 | 5.08E+06 |
| SD | 2.34E+05 | 2.04E+04 | 2.40E+05 |



Figure S1. Mass spectrum in positive mode ionization for AA-3NPH (upper panel) and HBA-3NPH (lower panel) showing common m/z signals. Neither the protonated molecular ion with m/z = 196 for AA-3NPH nor m/z = 240 for HBA-3NPH were found.



Figure S2. Chromatogram of pure derivatized HBA using the optimized gradient. The transitions for HBA-3NPH appear at RT = 9.0 min., while there is no signal for the AA-3NPH at RT = 8.6, as an indication that the HBA standard was not contaminated with AA.



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References:

1. European Medicines Agency, *Guideline on bioanalytical method validation.* 2011.