**Supplementary Information**

*Materials and Methods*

To 50 µL of sample, calibrator (SRM 914a, NIST, Gaithersburg, USA) or QC was added 50 µL of internal standard solution (160 µM, creatinine-d3, C/D/N Isotopes Inc, Quebec, Canada). Protein precipitation took place by the addition of 1 mL of ice cold ethanol. The sample was vortexed, gently shaken for 5 min and centrifuged at 21,500g for 10 min at 5°C. 200 µL of the supernatant was transferred into a clean tube and evaporated to dryness at 60°C resulting in a white/yellow pellet. After reconstitution in 1 mL of milliQ water, the sample was vortexed for 10 seconds and centrifuged shortly before being offered to the LC-MS/MS system.

Chromatography was performed using an ACQUITY UPLC® system (Waters Corporation, Etten-Leur, The Netherlands). Separation was achieved by a 5 μL injection of the prepared sample on an Acquity BEH C18 column (2.1 mm× 100 mm, 1.8 μm) with a column temperature of 27 °C. A gradient elution was used, utilizing 10 mM NH4Ac and methanol. Mass spectrometry was performed using a Quattro Premier XE (Waters Corporation, Etten-Leur, The Netherlands) tandem mass spectrometer. Creatinine was measured by electrospray ionization (ESI) in positive ionization mode with the following selected reaction monitoring mass transitions: *m/z* 113.8 > 44.0 and 116.8 > 47.0 for the natural and deuterated compounds, respectively. Other mass spectrometer settings were: capillary voltage 0.5 kV, cone voltage 30 V, desolvation temperature 350 °C at a gas flow of 750 L/h and cone gas flow 100 L/h. Argon was used as the collision gas at a flowrate of 0.20 mL/min.

Quantification of the LC-MS/MS analyses was performed using the peak area ratio of creatinine to the internal standard. Calibration lines were calculated using MassLynx™ software version 4.1 (Waters Corporation, Etten-Leur, The Netherlands). Linear extrapolation was used to quantify samples whose concentrations were below the lowest calibrator value.

*Validation of creatinine assay on LC-MS/MS*

Before the creatinine concentration in the icteric samples was measured with LC-MS/MS, the assay was subjected to a thorough validation. The linearity, lower limit of quantification (LLOQ), precision, matrix effect and carry-over were determined according to procedures similar to those described in another LC-MS/MS validation paper of our laboratory [Roelofsen-de Beer 2017]. The results are summarized in Table 3 and 4. The acceptance criteria of CV <5% for repeatability and between-day precision have been met on all concentration levels. Also, all other before mentioned performance characteristics are satisfactory.

**Table 3.** Precision results during validation

|  |  |  |  |
| --- | --- | --- | --- |
| Mean concentration (µmol/L) | Repeatability (%) | Between-day precision (CV%) | Total precision (CV%) |
| 80 | 1.3 | 3.7 | 3.9 |
| 237 | 1.2 | 2.8 | 3.0 |
| 394 | 1.3 | 4.1 | 4.3 |

**Table 4.** Summary of performance characteristics during validation

|  |  |  |  |
| --- | --- | --- | --- |
| Performance characteristic | Acceptance criterium | Results |  |
| linearity | Lack-of-fit <3.29 | 2-2400 µmol/L | Lack-of-fit = 1.32 |
| LLOQ  | VC <20%, S/N >10 | 2 µmol/L | VC 2% S/N 140 |
| matrix effect absolute | n. a. | 93% |  |
| matrix effect relative | >95% | 98% |  |
| carry-over | <0.5% | 0.00% |  |

The trueness of the method was established by measuring 12 serum samples (SKML, Nijmegen, The Netherlands) in which the creatinine concentration was targeted with the reference method of Siekmann [Siekmann 1985]. A %-difference plot was used to determine the trueness of the method expressed as bias. The LC-MS/MS method showed a positive bias of 1.4% [95%CI 0.8-2.1%] compared to the targeted samples, which falls within the acceptance criterium of <5%.

A method comparison study between the LC-MS/MS method and our automated enzymatic creatinine method was performed using 38 non-icteric samples (I-index between 4 – 33) with a broad range and equal distribution of creatinine concentrations (16 – 618 µmol/L). Passing & Bablok analysis yielded the following equation: creatinine enzymatic = -1.12 [-3.23 to 1.20] + 0.96 [0.94 to 0.97] × creatinine LCMS/MS, resulting in a bias of -5% [-9.7 to -0.2]. The LC-MS/MS method gives higher results than the enzymatic method, although both methods are traceable to a certified standard and reference testing.

Additionally, the extent of interference by bilirubin on the LC-MS/MS method was investigated. Theoretically, no icteric interference is expected, since the structure and molecular mass of bilirubin and creatinine are completely different. However, in this particular setting, it is good to experimentally rule out interference by bilirubin. To two different serum samples, commercially obtained unconjugated bilirubin (Sigma Aldrich, Zwijndrecht, The Netherlands) was added in increasing concentrations (maximal 4000 µmol/L). Then, the creatinine concentration was measured and appeared to remain unchanged, irrespective of the bilirubin concentration.

*References*

Roelofsen-de Beer RJAC, Zelst BD van, Rijke YB de. Simultaneous measurement of whole blood vitamin B1 & vitamin B6 using LC-ESI-MS/MS. J Chrom B 2017;1063:67-73

Siekmann L. Determination of Creatinine in Human Serum by Isotope Dilution-Mass Spectrometry: Definitive Methods in Clinical Chemistry, IV. J. Clin Chem Clin Biochem 1985;23:137-144