**TIMP-2 and IGFBP7 are promising biomarkers for acute kidney injury**

Urinary levels of tissue inhibitor of metalloprotease-2 (TIMP-2) and insulin-like growth factor binding protein 7 (IGFBP7) are promising biomarkers for acute kidney injury (AKI) in patients who receive chemotherapy or other nephrotoxic medication, or who undergo major surgery (e.g., cardiac surgery). TIMP-2 and IGFBP7 are not yet routinely implemented in medical labs. So far, research-use only immunoasays and a CE-marked NephriCheck™ test have been developed.

We here aim to develop an in-house LC-MRM-MS test with high specificity and multiplexing capability. As urinary matrixes are highly variable among patients and diseases, we have investigated the effect of different urinary protein concentrations and urinary proteases on quantitation of urinary TIMP-2 and IGFBP7 using a bottom-up proteomics approach.

**LC-MRM-MS method development**

An Agilent 1290 infinity LC system coupled to an Agilent 6495 QQQ-MS was used to develop a targeted LC-MRM method. 3-4 proteotypic peptides were selected based on theoretical characteristics and intensity in an MS2 scan of reciprocant TIMP-2 and IGFBP7 tryptic digests. Specific fragments were identified and the collision energies were optimized to obtain MRM transitions for each peptide. (Table 1). Urine samples (150 µL) were spiked with 0.1 mM SI peptides ([13C6,15N2]-lysine or [13C6,15N2]-arginine) as internal standard. The LC gradient was optimized for fast but specific quantitation (Figure 1).

**Table 1.** Proteotypic peptides and transitions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide</th>
<th>Rt (min)</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP-2</td>
<td>HEVTGWVLVSPLSK</td>
<td>2.2</td>
<td>531.3</td>
<td>530.3</td>
</tr>
<tr>
<td></td>
<td>FFACcamIK</td>
<td>2.5</td>
<td>530.3</td>
<td>531.3</td>
</tr>
<tr>
<td></td>
<td>EYLIAGK</td>
<td>3.5</td>
<td>491.0</td>
<td>490.4</td>
</tr>
<tr>
<td>IGFBP7</td>
<td>YQKEQ</td>
<td>2.3</td>
<td>568.3</td>
<td>567.7</td>
</tr>
<tr>
<td></td>
<td>FFACcamIK</td>
<td>2.8</td>
<td>464.2</td>
<td>463.6</td>
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<tr>
<td></td>
<td>EYLIAGK</td>
<td>5.1</td>
<td>517.9</td>
<td>516.9</td>
</tr>
</tbody>
</table>

**Tryptic digestion of TIMP-2 and IGFBP7 is less efficient in urines with high total protein content**

Figure 2. The completeness of digestion was evaluated by varying the incubation time with constant amount of trypsin. Digestion was considered complete (curve reaching plateau) when 6 h for peptide EYLIA (2AB), IQYEIK and ITYVEQ. In urine with high total protein and target protein content the formation of EYLIA is still ongoing after 24 h (2C & 2D).

**Ion suppression in high protein urine samples**

Figure 3. A relationship was observed between the total urinary protein in 14 samples and the MS-signal of the internal standard (Figure 3A, SIL-EYLIA). Ion suppression diminished 10-50 times the peptide MS response in samples with high protein content (> 0.5 g/L). To study whether this effect is indeed (mainly) due to the amount of non-target proteins, a mixture of synthetic peptides was added to 6 concentrations of digested albumin. Non-target peptides from albumin suppress peptide MS response and therefore assay sensitivity (Figure 3B, synthetic EYLIA). As the relative response remains constant (CV < 1%), the internal standard properly corrects for ion suppression.

**Urinary proteases inactivated by thermal denaturation or inhibited by protease inhibitors**

Figure 4. Sample-specific variance in abundance of internal standard was observed, while equal amounts were added to four urine samples (Figure 4A). Thermal denaturation (95 °C for 10 min) prior to the addition of internal standard resulted in a signal increase (up to 8x) and reduction of sample-specific variance (Figure 4B). To verify whether this effect was due to naturally occurring protease activity in urine, protease inhibitors (Complete™ Protease Inhibitor Cocktail, Roche) were added during sample preparation. Although peptide signal abundance was increased, the inhibition effect was less consistent compared to protease inactivation by thermal denaturation (Figure 4C).

**Preliminary method comparison**

Figure 5. For preliminary method comparison, urinary TIMP-2 was measured with current LC-MS method and an enzyme linked immunosorbsent assay (ELISA) in urines of patients who underwent major surgery (cardiac surgery or kidney transplantation). The two methods correlate poorly in the quantification of urinary TIMP-2 (Figure 5A, all samples, Pearson’s r = 0.29, n = 18).

However, when excluding all urines with proteinuria (total protein ≥ 0.5 g/L, n= 4) the LC-MS method correlates with ELISA (Figure 5B, Pearson’s r = 0.84, n=14). The relative response (RR), the ratio trypsinic peptide and internal standard, was used for quantification in the LC-MS method. Results shown of a single ELISA and one LC-MS experiment performed in duplicate a 3D with linear regression with 95% CI interval.

**Conclusions**

We aimed to develop a robust LC-MRM-MS method for quantification of urinary kidney injury markers TIMP-2 and IGFBP7. To that end we studied the effect of urine matrix (protein content and urine proteases) on trypsin digestion kinetics and MS-signal development. Sample preparation for quantification of urinary TIMP-2 and IGFBP7 using LC-MRM-MS is highly affected by total protein content and by urine protease activity.

1. Thermal denaturation of urinary proteases prevents non-specific protein degradation during tryptic digestion whereas the addition of protease inhibitors partially reduces confounding by proteases.
2. Although SI-peak-based internal standards correct for ion suppression from eluting compounds in ELISA, urinary non-target proteins rapidly diminish MS-signals and analytical sensitivity of bottom-up proteomics tests. This is further demonstrated for TIMP-2 in a preliminary method comparison of the LC-MRM-MS method against an ELISA method.

Due to urine matrix effects, the sample preparation in LC-MRM-MS test development of urinary biomarkers is very challenging and confounds test accuracy. To overcome urine matrix effects, direct sample preparation should be abandoned and immunocapture-based isolations of the measureds of interest will be considered.

**References**