The Importance of Stable Isotope Internal Standards for the LC-MS/MS Analysis of Immunosuppressant Drugs in Whole Blood

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Introduction
External quality assurance programs (QAP) have demonstrated marked inter-laboratory imprecision of immunosuppressant assays by LC-MS/MS in whole blood. The problem was most probably due to the use of non-isotopic internal standards. We assessed the impact of now readily available stable isotope internal standards on the performance of LC-MS/MS assay for Cyclosporine, Tacrolimus, Sirolimus and Everolimus.

Method
Whole blood patient samples and external QAP materials (RCPA and UK NEQAS) were analysed by LC-MS/MS in Multiple Reaction Monitoring mode with 2 forms of internal standards:
1) ClinMass®- non-isotopic Internal Standard Ascomycin (Tacrolimus, Sirolimus, Everolimus) and Cyclosporine D (Cyclosporine)
2) UTK Laboratories deuterated standards: IS immunosuppressants 1 Mix – (d12 Cyclosporin A, 2C13, d4 Everolimus, C13, d3 Sirolimus and C13, d2 Tacrolimus).

Addition C13, d2Tacrolimus was added to the working internal standard to overcome isotopic contamination of +3 Da Tacrolimus. (see arrow on Figure 1)

Figure 1: Isotopic contribution of Tacrolimus
Tacrolimus – C42H65NO12

Results
Table 1: Improvements in imprecision by the use of stable isotope internal standards
Stable isotope internal standards reduced between-batch imprecision in whole blood samples measured for all four immunosuppressants. These improvements were still present but to a far lesser degree in quality control (QC) material measured for the four compounds.

<table>
<thead>
<tr>
<th>Target Analyte</th>
<th>Cyclosporine</th>
<th>Everolimus</th>
<th>Sirolimus</th>
<th>Tacrolimus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal Standard</td>
<td>CYC d0</td>
<td>CYC d12</td>
<td>Ascomycin</td>
<td>Everolimus d4</td>
</tr>
<tr>
<td>Low QC (UTK LO)</td>
<td>7.0%</td>
<td>3.9%</td>
<td>9.4%</td>
<td>7.6%</td>
</tr>
<tr>
<td>High QC (UTK HI)</td>
<td>6.5%</td>
<td>3.4%</td>
<td>7.3%</td>
<td>5.9%</td>
</tr>
<tr>
<td>Low patient sample pool</td>
<td>6.7%</td>
<td>6.4%</td>
<td>13.6%</td>
<td>5.2%</td>
</tr>
<tr>
<td>Mid patient sample pool</td>
<td>7.3%</td>
<td>3.8%</td>
<td>10.6%</td>
<td>6.3%</td>
</tr>
<tr>
<td>High patient sample pool</td>
<td>10.7%</td>
<td>5.0%</td>
<td>13.7%</td>
<td>4.9%</td>
</tr>
</tbody>
</table>

Discussion and Conclusion
Ascomycin and Cyclosporine D used in non-isotopic standards do not demonstrate the same ionisation effects as the target analytes and thus are inferior to stable isotopes for internal standardisation. Pseudo-matrix calibrators, quality control and QAP samples do not demonstrate the same matrix effects as that of whole blood, thus caution should be applied before interpreting these results for trueness and imprecision testing.

Matrix effects investigation showed that Ascomycin displayed signal suppression in whole blood, versus signal enhancement in QAP material which may explain some of the differences observed in this study.

To assess between batch imprecision and uncertainty of measurement, a whole blood quality control material should be introduced.