INTRODUCTION

Here we present a UPLC-MS/MS method for analysis of methotrexate in human plasma and serum for research purposes. An analytically sensitive and precise method was developed using protein precipitation (PPE).

METHODS

Materials
- Certified methotrexate and methotrexate-2H3 reference materials were purchased from Cerilliant (Round Rock, TX, USA). Calibrators over the range 0.025-10 µmol/L and QC materials at 0.1, 2.5, 10 and 75 µmol/L were prepared in a surrogate matrix of pooled plasma purchased from Sera Laboratories International Ltd (West Sussex, UK).
- LGC (Teddington, UK) provided EQA samples in serum for accuracy testing.
- Department of Clinical Biochemistry, Manchester Royal Infirmary (Manchester, UK) provided anonymized plasma samples.
- Sera Laboratories International Ltd (West Sussex, UK) also provided matched sets for plasma and serum from 6 individuals.

Methods
- Samples (50 µL) were pre-treated with methanol containing the internal standard.
- Internal standard was added to 50 µL of sample. Following mixing and centrifugation, an aliquot of supernatant was diluted in water in a 96-well 2mL plate.
- Using a Waters ACQUITY UPLC® system, samples were injected onto a 2.1 x 30 mm Waters ACQUITY UPLC HSS C18 SB column using a water/methanol/ammonium acetate isocratic separation and analyzed with a Waters Xevo® TQD, using the MRM transitions in Table 1.
- The analysis time per sample was approximately 5.7 minutes injection-to-injection.

RESULTS

Chromatographic Selectivity
- The chosen chromatographic conditions separate Methotrexate from its principle metabolites 2,4-Diamino-N10-methylpteroyl acid (DAMPA) and 7-hydroxymethotrexate (see Figure 1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRM Transition (m/z)</th>
<th>Cone (V)</th>
<th>Collision (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate (Quantifier)</td>
<td>455.2 &gt; 175.1</td>
<td>36</td>
<td>42</td>
</tr>
<tr>
<td>Methotrexate (Qualifier)</td>
<td>455.2 &gt; 134.1</td>
<td>36</td>
<td>34</td>
</tr>
<tr>
<td>Methotrexate-2H3 (Internal Standard)</td>
<td>458.2 &gt; 175.1</td>
<td>36</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 1. MRM parameters used for the analysis of methotrexate and the internal standard.

Linearity and sensitivity
- Following CLSI-EP6-A, the method was shown to have a linear fit over the range of 0.0175–13.0 µmol/L.
- Analytical sensitivity investigations indicate the method would allow precise quantification (≤20%) at 0.0025 µmol/L.

Carryover
- No significant carryover was observed following analysis of plasma samples with methotrexate levels up to 100 µmol/L.

Precision
- Five replicates at each QC level in plasma were prepared daily over five days (n=25).
- Table 2 presents the results of these experiments.

<table>
<thead>
<tr>
<th>Nominal Concentration (µmol/L)</th>
<th>Total Precision (% RSD)</th>
<th>Repeatability (% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>5.1</td>
<td>4.0</td>
</tr>
<tr>
<td>2.5</td>
<td>4.6</td>
<td>1.8</td>
</tr>
<tr>
<td>10</td>
<td>5.4</td>
<td>2.9</td>
</tr>
<tr>
<td>75</td>
<td>5.5</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Table 2. Total precision and repeatability for the analysis of methotrexate.

Accuracy
- Accuracy was assessed by analysing serum samples spanning the concentration range 0.057-3.80 µmol/L from an EQA scheme (5 occasions, randomized order).
- Mean % deviations from absolute reference values ranged from –7.4% to 0.4%, with an overall mean of –5.7%.

Matrix Effects
- The normalized matrix factor (using analyte:internal standard response ratio) ranged from 1.01-1.12 and from 1.01-1.11 for low and high concentrations in plasma and serum respectively.

Matrix Equivalence
- Equivalence between plasma and serum samples was established by fortifying 6 individual matched sets with methotrexate concentrations listed in Table 2.
- Mean % differences between plasma and serum of –1.2% to 6.5% were observed when analysed.

Anonymized Plasma Samples
- Measurements were made on a plasma series drawn from an individual 27 hours pre– to 147 hours post-glucarpidase administration. Refer to Figure 2.
- UPLC-MS/MS data highlighted overestimation of immunoassay method post-glucarpidase administration and showed a ‘rebound’ phenomenon.

CONCLUSION

- An analytically sensitive and selective clinical research method has been developed for the analysis of plasma and serum methotrexate.
- Chromatographic separation from the metabolites of methotrexate is achieved.
- Benefits of selective detection provided by UPLC-MS/MS were shown in a plasma series pre– and post-glucarpidase administration.