An HPLC-MS/MS Assay Method for the Determination of Digoxin in Human Plasma and Urine

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Background
Digoxin is a cardiac glycoside which has been used for many years in the management of congestive heart failure and other cardiac diseases. An HPLC-MS/MS method was developed and validated according to current industry guidelines for the determination of digoxin in human plasma and urine. The calibration range of the plasma method was 0.100 to 10.0 ng/mL and the range for the urine method was 1.00 to 100 ng/mL. Digitoxigenin was used as the internal standard. The analytes were extracted from plasma and urine using solid phase extraction cartridges.

Determination of Digoxin in Human Plasma and Urine

**Method**
Calibration curves and QC samples were prepared in blank human plasma and urine.

**Analyte:**
- HPLC-MS/MS
- Analysis Volume: 0.250 mL Human Plasma, 1.00 mL Human Urine
- Sample Extraction:
  - Plasma: Oasis HLB 3mL, 60mg SPE cartridge after addition of IS
  - Urine: Oasis HLB 1mL, 30mg 96 well plate SPE after addition of IS
- Mass Spectrometer:
  - ABSciex API 5000 (Plasma method) and ABSciex API 365 (Urine method)
- Calibration Range: Plasma 0.100 – 10.0 ng/mL Human plasma, 1.00 – 100 ng/mL Human urine

**Results**
Standard curves were produced by linear regression (weighted) using the peak area ratio of analyte to internal standard. Validation of the correlation coefficient of r was observed ≥0.9995 for all analytes.

**HPLC-MS/MS CHROMATOGRAMS FOR ANALYTES IN HUMAN PLASMA**
Six different lots of human plasma and urine were tested and no interference was detected in the plasma. Several lots of urine contained analyte at a maximum level <10.0% of LLOQ peak area.

**Accuracy and Precision**
- Intra- and inter-run accuracy and precision were established by analysing six replicate QC samples, at four different levels, in four primary runs in human plasma and urine. Back-calculated versus nominal concentrations were used to determine accuracy (%RE). Precision was expressed by the standard deviation (%CV).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Nominal Concentration</th>
<th>Inter-run (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/mL</td>
<td>%RE</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.100 (LLOQ)</td>
<td>10.8</td>
<td>12.6</td>
</tr>
<tr>
<td>0.300 (QC-L)</td>
<td>3.0</td>
<td>9.4</td>
</tr>
<tr>
<td>3.00 (QC-M)</td>
<td>-0.7</td>
<td>9.2</td>
</tr>
<tr>
<td>8.00 (QC-H)</td>
<td>-1.2</td>
<td>7.0</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00 (LLOQ)</td>
<td>1.1</td>
<td>9.7</td>
</tr>
<tr>
<td>3.00 (QC-L)</td>
<td>-2.2</td>
<td>7.7</td>
</tr>
<tr>
<td>30.0 (QC-M)</td>
<td>-2.4</td>
<td>8.7</td>
</tr>
<tr>
<td>80.0 (QC-H)</td>
<td>-6.3</td>
<td>6.4</td>
</tr>
</tbody>
</table>

**Sensitivity**
To establish the proposed LLOQ for digoxin in plasma and urine, six replicates at 0.100 and 1.00 ng/mL, respectively, were prepared and analysed as samples (independent from the calibration curve) over four separate runs. The intra-run accuracy for all 24 replicates of LLOQ samples was within ±20% RE of nominal concentration for the four primary runs. The mean accuracy and precision obtained for the LLOQ samples (n=24) was 9.2% RE and 10.8% CV for plasma and 1.1% RE and 9.7% CV for urine.

**Recovery and Extraction Efficiency From Spike Samples**
Recovery was calculated by comparing the peak area from the extracted QC sample analysis versus mean peak areas of the unextracted fortified reagent solutions at 3 different concentrations. Mean percent recovery for both digoxin and the internal standard from plasma and urine was 85.6% and 78.5%, respectively (n=18) from plasma. Mean percent recovery for both digoxin and the internal standard from urine was 77.3% and 82.2%, respectively (n=18 and 17, respectively). Extraction Efficiency was calculated by comparing the peak area from extracted QC sample analysis versus mean peak area of blank samples spiked after extraction at 3 different levels. Mean percent recovery for both digoxin and the internal standard from plasma was 91.6% and 83.1%, respectively (n=18) from plasma. Mean percent recovery for both digoxin and the internal standard from urine was 80.3% and 96.1%, respectively (n=18 and 17, respectively).

**Stability**
- The stability of digoxin in human plasma and urine was tested by analysing QC samples prepared at low and high concentrations. Autosampler stability, post extraction, was evaluated at one concentration (QC-medium). Analytes were considered stable if 95% of samples analysed at each concentration were within ±15% RE and ±15% CV. Stability was established for digoxin in human plasma and urine for:
  - 3 freeze / thaw cycles with storage at -20°C and thawing at ambient temperature.
  - Up to 24 hours at ambient temperature (bench top stability).
  - Up to 9 weeks for plasma and 9 weeks and 3 days for urine at -20°C (freezer storage stability).
  - Up to 13 hours for plasma and 57 hours for urine in sample extract (autosampler stability).
  - Up to 5 days at 4°C and 20°C for plasma and 1 week at 4°C and 20°C for urine (post-extract storage stability).
  - Re-rejection reproductibility.
- Stability was also established for digitoxin and digitoxygenin (IS) in reagent for:
  - Up to 14 weeks and 10 weeks and 4 days, respectively at -20°C (stock solution storage stability).
  - Up to 9 weeks and 4 days at −20°C (working solution storage stability).

**Conclusion**
Rapid and specific HPLC-MS/MS methods for the determination of digoxin in human plasma and urine have been successfully validated at Almac Sciences. The results obtained during the bioanalytical method validation proved the suitability of the HPLC-MS/MS assay for the determination of digoxin in the range of 0.100 to 10.0 ng/mL in human plasma and 1.00 to 100 ng/mL in human urine.

**References**