A validated UPLC/ESI-MS/MS bioanalytical method for the quantification of Perindopril and Amlodipine in human plasma

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Scope of the Method Validation

Encompasses all possible well-characterized and fully validated bioanalytical method to yield reliable results that can be satisfactorily interpreted.
A primary concern in biopharmaceutics is the bioavailability of drugs.

Bioavailability

refers to the measurement of the rate and extent of active drug that reaches the systemic circulation.

means access to the bloodstream
METHODOLOGY

Selection of drugs combination and collection based on literature survey

Study of physicochemical properties of drug molecule. Find out solubility of combination in solvent

Tuning of the molecule of interest, source parameters, MS scanning and optimization.

Selection of chromatographic method (based on solubility study, retention of compound)

Concentration range of compounds in sample of interest and Method validation

Report the final results and discussion
OPTIMIZED UPLC-ESI-MS/MS ACQUISITION CONDITIONS FOR PERINDOPRIL & AMLODIPINE

UPLC Conditions

- Mobile phase:
  - Solvent A: 0.1% Formic acid in MilliQ water (V/V)
  - Solvent B: 0.1% Formic acid in Acetonitrile (V/V)
- Injection Volume: 10 µL

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>0.2mL/min</td>
</tr>
<tr>
<td>Run time</td>
<td>5.5 min</td>
</tr>
<tr>
<td>TCC</td>
<td>25°C ± 2°C</td>
</tr>
<tr>
<td>Column</td>
<td>AQUITY UPLC BEHC18 , 2.1 x 100 mm, 1.7 µm</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
</tr>
<tr>
<td>3.5</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>5.5</td>
<td>2</td>
</tr>
</tbody>
</table>
## MS/MS Conditions

<table>
<thead>
<tr>
<th>Ion polarity</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data storage</td>
<td>Continuum</td>
</tr>
</tbody>
</table>

### Source

<table>
<thead>
<tr>
<th>Source</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source temperature (°C)</td>
<td>150</td>
</tr>
<tr>
<td>Gas flow (L/H)</td>
<td>300</td>
</tr>
<tr>
<td>Desolvation temperature (°C)</td>
<td>500</td>
</tr>
<tr>
<td>Capillary Voltage (KV)</td>
<td>3.5</td>
</tr>
<tr>
<td>Cone</td>
<td>35</td>
</tr>
</tbody>
</table>

### MRM

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent m/z</th>
<th>Product m/z</th>
<th>Cone (V)</th>
<th>Collision (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PER</td>
<td>369.58</td>
<td>172</td>
<td>35</td>
<td>28</td>
</tr>
<tr>
<td>AMD</td>
<td>408.97</td>
<td>238</td>
<td>35</td>
<td>18</td>
</tr>
<tr>
<td>LID</td>
<td>612.79</td>
<td>280</td>
<td>35</td>
<td>16</td>
</tr>
</tbody>
</table>

### Mass

<table>
<thead>
<tr>
<th>Mass</th>
<th>Min range</th>
<th>Max range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 m/z</td>
<td>700 m/z</td>
</tr>
</tbody>
</table>
Fifty microlitres of internal standard (50ng/ml of Licarnidipine in 50% methanol) was added to each 200 μl of human plasma samples

vortexed for 10 s

Ethyl acetate (2.5 ml) was added, followed by vortexing at 2500rpm for 15 min

centrifuged at 4000rpm, 10°C for 10 min

Organic layer was transferred to another set of labeled test tubes

Organic layer was evaporated under nitrogen

Residue was dissolved in 200μL of Reconstitution solution (1:1, Mobile phase A:B)
## CALIBRATION STANDARDS AND QC SAMPLE CONCENTRATION

<table>
<thead>
<tr>
<th>Final Conc. of perindopril (ng/mL)</th>
<th>Final Conc. of amlodipine (ng/mL)</th>
<th>Final Conc. of lercanidipine (IS)(ng/mL)</th>
<th>Standard ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.951</td>
<td>0.761</td>
<td>50.686</td>
<td>AQ.STD1</td>
</tr>
<tr>
<td>19.011</td>
<td>15.224</td>
<td>50.686</td>
<td>AQ.STD2</td>
</tr>
<tr>
<td>63.370</td>
<td>50.745</td>
<td>50.686</td>
<td>AQ.STD3</td>
</tr>
<tr>
<td>90.529</td>
<td>72.493</td>
<td>50.686</td>
<td>AQ.STD4</td>
</tr>
<tr>
<td>125.734</td>
<td>100.685</td>
<td>50.686</td>
<td>AQ.STD5</td>
</tr>
<tr>
<td>150.580</td>
<td>120.580</td>
<td>50.686</td>
<td>AQ.STD6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>QC Samples ID</th>
<th>Final Conc. of perindopril (ng/mL)</th>
<th>Final Conc. of amlodipine (ng/mL)</th>
<th>Final Conc. of lercanidipine (IS)(ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLOQ.QC</td>
<td>0.943</td>
<td>0.707</td>
<td>50.686</td>
</tr>
<tr>
<td>LQC</td>
<td>26.195</td>
<td>19.636</td>
<td>50.686</td>
</tr>
<tr>
<td>MQC</td>
<td>65.486</td>
<td>49.089</td>
<td>50.686</td>
</tr>
<tr>
<td>HQC</td>
<td>145.525</td>
<td>109.087</td>
<td>50.686</td>
</tr>
</tbody>
</table>
Bio Analytical Method validation Results

Methods used for quantitative measurement of analytes in any given biological matrix must be **reliable and reproducible for the intended use**…

- Selectivity
- Carry-over
- Calibration curve
- Accuracy & Precession
- Recovery
- Matrix effect
- Dilution integrity
- Suitability for the assay

\[
C_{\text{max}} \ (\text{ULOQ}) \\
\frac{\text{AUC}_t}{\text{AUC}_\infty} \geq 80\% \ (\text{LLOQ}) \\
\text{Carry-over (LLOQ} \leq 5\% \ C_{\text{max}}) \\
15–20\% \text{ Bias / Precision}
\]
1. Screening and Selectivity

**Specificity**: for an analyte

**Selectivity**: for a matrix

**Experimental Design:**
- Matrix blanks: 8 lots, n=1 for each lot
- Matrix blank fortified with IS: 8 lots, n=1 for each lot
- LLOQ Selectivity Sample: 6 lots, fortified with analyte at LLOQ level and IS. n=3 for each lot

**Result:** 7 out of the 8 lots meet the following criteria:
- Response for the analyte in matrix blanks and matrix blank fortified with IS were ≤20% of the mean analyte response in the acceptable LLOQ.
- Selectivity LLOQ replicates for each lot meets accuracy acceptance limit, and the mean accuracy was within ±20.0% of the nominal concentration.
2. ASCOT (AUTO SAMPLER CARRIES OVER TEST)

**Sequence:**
- Aqueous blank (without spiked drug)-1
- Highest aqueous concentration
- Aqueous blank (without spiked drug)-2
- Lowest aqueous concentration.
- Blank matrix without drug-1
- Extracted Highest concentration
- Blank matrix without drug -2
- Extracted Lowest concentration

\[
\% \text{Carry Over for Aqueous samples} = \left\{ \frac{\text{Area of Aq.Blank-2} - \text{Aq.Blank-1}}{\text{Area of Aq.LLOQ}} \right\} * 100
\]

\[
\% \text{CarryOver for Extracted samples} = \left\{ \frac{\text{Area of Ex.Blank-2} - \text{Ex.Blank-1}}{\text{Area of Ext STD8}} \right\} * 100
\]

**Result:** Calculated the % carryover at the RT of analyte/ISTD in both unextracted and Extracted samples. The %Carry over for RT of analyte & ISTD not more than 5%.
3. Linearity

Experimental Design: A calibration curve consist of

- A blank sample (matrix sample processed without analyte or internal standard),
- A zero sample (matrix sample processed without analyte but with internal standard), and
- Six non-zero samples (matrix samples processed with analyte and internal standard) covering the expected range, including LLOQ.
- Four concentrations (including LLOQ, low, medium, and high), each concentration n=3

Results:

- Standards were not more than 15% of nominal concentrations, except at LLOQ where the standard was not more than 20%.
- The analyte response at the LLOQ was five times the response compared to blank response.
- Correlation coefficient: r² was 0.9889 to 0.9985 for both drugs.
4. Accuracy and precision

Experimental Design: For both the inter-run and intra-run experiments, as followed and used the linearity data to calculate the accuracy and precision.

Result:
✓ For Accuracy, the mean values for both PER and AMD were within 15% of the nominal value, except at LLOQ, where it was not more than 20%.
✓ The precision determined at each concentration level and it were not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it show was not exceed 20% of the CV.
Bio Analytical Method validation Results (cond..,)

5. Recovery

Experimental Design: Analyte at LQC and HQC levels, and IS at the level of use: pre extraction spiked samples (n=6) are compared with mean response of post extraction spiked matrix samples (n=6)

\[
\text{Extracted sample Response} \\
\% \text{ Recovery} = \frac{\text{Extracted sample Response}}{\text{Un-extracted sample response}} \times 100
\]

Result: Recovery of the analyte were not be 100%, but the extent of recovery of an analyte and of the internal standard was observed consistent, precise, and reproducible.
Bio Analytical Method validation Results (cond..,)

6. Dilution (Parallelism)

- Dilution of samples should not affect the accuracy and precision. If applicable, dilution integrity should be demonstrated by spiking the matrix with an analyte concentration above the ULOQ and diluting this sample with blank matrix. Accuracy and precision should be within the set criteria, i.e. within ±15%.

Experimental Design: Two level at ULOQ concentration (2 fold and 4fold dilution); each dilution, n=6.

Result:

Mean accuracy was within ±15.0% RE of nominal; precision was ≤15.0% RSD.
Bio Analytical Method validation Results (cond..,)

7. Matrix Effect

Experimental Design: It was evaluated by processing post extracted spiked samples at six replicates of LQC and HQC concentration and analyzed with aqueous LQC and HQC concentration and difference of response is calculated.

Calculation:

Matrix factor = B/A

% Matrix effect = [(B-A)/A] * 100

where, A, is the response of the aqueous sample and B is response for the post extracted spiked samples.

Result:

Both QC samples MF was within 0.85 to 1.15 and %CV for each set of LQC and HQC were not more than 15%.
References

Indian Pharmacopoeia, 2007, Volume-II, the Indian Pharmacopoeia Commission, Ghaziabad, Govt. of India, Ministry of Health and Family Welfare, pp 741.

United state pharmacopoeia, 30- National formulary 25.


ICH Q2A, Text on Validation of Analytical Procedure, 1995


Shaalan R A, Belal T S, “Simultaneous Spectrofluorimetric determination of amlodipine besylate and valsartan in their combined tablets” Drugs Test Anal. 2010 2(10), 489-93.


Thank you for your attention