Determination of Clarithromycin in Human Plasma by LC-EI Tandem Mass Spectrometry: Application to Bioequivalence Study

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BABE- 29 Sept. 2014, Baltimore, USA
**Clarithromycin - Background**

- **Chemical Name:** 6-O-Methylerythromycin
- **Molecular Formula:** $C_{38}H_{69}NO_{13}$ (FW: 747.95)
- **Solubility:** Highly - Acetone; Slightly - Methanol, Ethanol
  Practically insoluble in water.

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Pharmacokinetic Study

- Bioavailability: About 50%
- Peak plasma concentration: 2.41-2.85 µg/ml after single 500 mg dose.
- Time Max: 2-3 hrs.
- Half life: 3.5 - 4.5 hrs.
Brand & Generic Names

- Active ingredient: Clarithromycin
- Brand Names: APO-Clarithromycin, Biaxin, Chemmart, Clarac, Clarithexal, Clarithromycin AN, Clarithromycin-PS, Prevpac, Klacid
- Generic Names: Claritt, Clarimac and Clarex (Saudi Arabia)
References:

The objective of the study:

- To develop and validate a rapid, sensitive, and reproducible method to determine clarithromycin levels in small volumes of human plasma by LC-MS/MS.
- Assess the stability of clarithromycin under various conditions.
- Application of method in Bioavailable and Bioequivalence study.
Methodology:

Material/Reagents & Equipment

- Clarithromycin, erythromycin – Certified Purity ≥ 99%, from USP, Rockville, MD, USA.

- Acetonitrile, methanol (HPLC Grade), Triethylamine, Phosphoric acid (AR-Grade) (All from Fisher Scientific, NJ, USA).

- HPLC grade water prepared by reverse osmosis and further purified by passing through a Synergy Water Purification System (Millipore, Bedford, MA, USA)

- Drug free human plasma from Blood Bank King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia.

- Instrument: MS/MS Micromass, Triple quadruple, HPLC, Alliance 2695, (Waters Associates Inc. Milford, MA, USA)
Standards and Quality Control Preparations

- Stock & Working Solutions:
  - Clarithromycin & Erythromycin (0.1 mg/ml, methanol)

- Working Solutions:
  Clarithromycin : 10 µg/ml in drug free human plasma
  Erythromycin : µg/ml in methanol

- Calibration Curve (10 concentrations)
  Range: 5 ng/ml - 4.0 µg/ml plasma

- Quality Control Samples
  1: LLQ (5 ng/ml),
  2: 3xLLQ (1.5 ng/ml)
  3: 0.5 HLQ (2.0 µg/ml)
  4: 0.9 HLQ (3.6 µg/ml)

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Analytical Conditions

Liquid Chromatograph:
- Column: Atlantis dC18 (2.1 x 100 mm, 3 µm)
- Guard Column: Symmetry C18 (2.1 x 100 mm, 5 µm)
- Mobile Phase: Acetonitrile and 0.5% Triethylamine (PH=4, with phosphoric acid), (65: 35, V/V)
- Flow rate: 0.25 ml/min.

Mass Spectrometric System:
Spray: Electrospray ionization (positive)
Voltages: Capillary 4.0 kV, Cone 30 V
Temperature: Source 125°C, Desolvation 350°C
Cone Gas Flow: 600 L/hr.
Collision Energy ; 25 eV
Mass Spectra of Clarithromycin

Transition: 749 → 158.4

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Mass Spectra of Erythromycin

Transition: 719.3 → 158.2

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Plasma Sample Preparation

- Human plasma (0.2 ml) + Erythromycin, (Internal standard) 
  (50 µl of 1.0 µg/ml, methanol )
- Add 4.0 ml- Tert. Butylmethylether
- Vertex 5 minutes then centrifuge at 6000 rpm for 10 minutes.
- Separate organic layer and evaporate solvent at 40 °C
- Reconstitute in mobile phase, inject volume 5.0 µL
- Run Time: 4 Minutes
Representative MRM Chromatogram of blank human plasma

Clarithromycin

Erythromycin (IS)

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Representative MRM Chromatogram of plasma spiked with IS

Clarithromycin

Erythromycin (IS)

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Representative MRM Chromatograms of plasma spiked with Clarithromycin and IS

Conc. 0.015 µg/ml

Conc. 2.0 µg/ml

Conc. 3.6 µg/ml

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## Method Validation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Acceptable limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td>Blank plasma (6) Commonly used drugs</td>
</tr>
<tr>
<td>Recovery</td>
<td>Consistent</td>
</tr>
<tr>
<td>Linearity</td>
<td>Analyte Conc./Response (6-8, Zero and Blank)</td>
</tr>
<tr>
<td>Accuracy &amp; Precision</td>
<td>±15% (3 levels)</td>
</tr>
<tr>
<td>Stability</td>
<td>±20% for LLQ</td>
</tr>
</tbody>
</table>

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Specificity

- 6 different batches of human plasma screened
- Eight commonly used medications: Acetaminophen, Ibuprofen, Aspirin, Omeprazole, Nicotinic acid, Ascorbic acid, Ranitidine and Caffeine.
## Recovery from human plasma

<table>
<thead>
<tr>
<th>Clarithromycin (µg/ml)</th>
<th>Mobile Phase</th>
<th>Human Plasma</th>
<th>Recovery (%)</th>
<th>Mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Height</td>
<td>SD</td>
<td>Mean Height</td>
<td>SD</td>
</tr>
<tr>
<td>0.005</td>
<td>122</td>
<td>4.36</td>
<td>120</td>
<td>12.16</td>
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<tr>
<td>0.015</td>
<td>322</td>
<td>4.58</td>
<td>325</td>
<td>8.02</td>
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<tr>
<td>2.0</td>
<td>38439</td>
<td>1188</td>
<td>34299</td>
<td>1028</td>
</tr>
<tr>
<td>3.6</td>
<td>67845</td>
<td>1601</td>
<td>58253</td>
<td>997</td>
</tr>
<tr>
<td>IS (1.0)</td>
<td>17260</td>
<td>838</td>
<td>17024</td>
<td>453</td>
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</tbody>
</table>

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## Linearity

<table>
<thead>
<tr>
<th>Nominal Conc. (µg/ml)</th>
<th>CLA-PH</th>
<th>IS-PH</th>
<th>Ratio</th>
<th>Measured Conc. (µg/ml)</th>
<th>Acc. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>766</td>
<td>43847</td>
<td>0.0175</td>
<td>0.006</td>
<td>116</td>
</tr>
<tr>
<td>0.010</td>
<td>991</td>
<td>39553</td>
<td>0.0251</td>
<td>0.009</td>
<td>92</td>
</tr>
<tr>
<td>0.020</td>
<td>2097</td>
<td>43542</td>
<td>0.0482</td>
<td>0.020</td>
<td>98</td>
</tr>
<tr>
<td>0.080</td>
<td>6907</td>
<td>38113</td>
<td>0.1812</td>
<td>0.079</td>
<td>99</td>
</tr>
<tr>
<td>0.200</td>
<td>25597</td>
<td>58360</td>
<td>0.4386</td>
<td>0.194</td>
<td>97</td>
</tr>
<tr>
<td>0.500</td>
<td>97180</td>
<td>85465</td>
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<td>0.507</td>
<td>101</td>
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<tr>
<td>1.000</td>
<td>149208</td>
<td>66571</td>
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<td>100</td>
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<td>2.000</td>
<td>231682</td>
<td>51170</td>
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<td>3.000</td>
<td>326101</td>
<td>50037</td>
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<td>97</td>
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<tr>
<td>4.000</td>
<td>456860</td>
<td>50136</td>
<td>9.0439</td>
<td>4.046</td>
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</tbody>
</table>

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Representative Standard Calibration Curve

**SUMMARY OUTPUT**

<table>
<thead>
<tr>
<th>Regression Statistics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple R</td>
<td>0.9997</td>
</tr>
<tr>
<td>R Square</td>
<td>0.9994</td>
</tr>
<tr>
<td>Adjusted R Square</td>
<td>0.9994</td>
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<tr>
<td>Standard Error</td>
<td>0.0789</td>
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<tr>
<td>Observations</td>
<td>10</td>
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<tr>
<td>Intercept</td>
<td>0.0020</td>
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<tr>
<td>X Variable 1</td>
<td>2.2337</td>
</tr>
</tbody>
</table>

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## Precision & Accuracy

<table>
<thead>
<tr>
<th>Nominal (µg/ml)</th>
<th>Measured (µg/ml)</th>
<th>SD</th>
<th>CV (%)</th>
<th>Bias (%)</th>
<th>Measured (µg/ml)</th>
<th>SD</th>
<th>CV (%)</th>
<th>Bias (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>0.0053</td>
<td>0.0007</td>
<td>13.1</td>
<td>5.1</td>
<td>0.0047</td>
<td>0.0004</td>
<td>9.5</td>
<td>-5.9</td>
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<tr>
<td>0.150</td>
<td>0.0164</td>
<td>0.0018</td>
<td>10.7</td>
<td>9.0</td>
<td>0.0171</td>
<td>0.0016</td>
<td>9.6</td>
<td>12.2</td>
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<td>2.000</td>
<td>1.8219</td>
<td>0.0647</td>
<td>3.6</td>
<td>-9.0</td>
<td>1.8699</td>
<td>0.0529</td>
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<td>-7.0</td>
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<td>3.600</td>
<td>3.3252</td>
<td>0.0953</td>
<td>2.9</td>
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<td>3.3878</td>
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<td>-6.3</td>
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</table>

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# Stability: Processed & unprocessed samples

<table>
<thead>
<tr>
<th>Storage Condition</th>
<th>Nominal (µg/ml)</th>
<th>Measured (µg/ml)</th>
<th>SD</th>
<th>Stability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base line/None</td>
<td>0.015 3.600</td>
<td>0.015 3.394</td>
<td>0.002</td>
<td>100 98 97</td>
</tr>
<tr>
<td>Processed</td>
<td>0.015 3.600</td>
<td>0.015 3.343</td>
<td>0.002</td>
<td>104 97</td>
</tr>
<tr>
<td>24 h. RT</td>
<td>0.015 3.600</td>
<td>0.015 3.291</td>
<td>0.002</td>
<td>104 97</td>
</tr>
<tr>
<td>48 h. (-20°)</td>
<td>0.015 3.600</td>
<td>0.015 3.342</td>
<td>0.001</td>
<td>94 93</td>
</tr>
<tr>
<td>Unprocessed</td>
<td>0.015 3.600</td>
<td>0.012 3.421</td>
<td>0.001</td>
<td>94 93</td>
</tr>
<tr>
<td>24 h. RT</td>
<td>0.015 3.600</td>
<td>0.013 3.342</td>
<td>0.001</td>
<td>94 93</td>
</tr>
<tr>
<td>14 wks (-20°)</td>
<td>0.015 3.600</td>
<td>0.013 3.342</td>
<td>0.002</td>
<td>94 93</td>
</tr>
<tr>
<td>FT: Cycle-1</td>
<td>0.015 3.600</td>
<td>0.012 3.306</td>
<td>0.001</td>
<td>83 99</td>
</tr>
<tr>
<td>FT: Cycle-2</td>
<td>0.015 3.600</td>
<td>0.015 3.163</td>
<td>0.002</td>
<td>104 95</td>
</tr>
<tr>
<td>FT: Cycle-3</td>
<td>0.015 3.600</td>
<td>0.013 3.403</td>
<td>0.001</td>
<td>87 102</td>
</tr>
</tbody>
</table>

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Ruggedness & Robustness

Ruggedness: Mobile Phase:
- Altering Strength of Triethyleamine
- Proportion of Acetonitrile

Robustness: Analyst
- Split Analysis
Method Application

- No. of Samples collected: 18 within 24 hrs.
- Processed: According to method
- Analyzed: LC-MS/MS
Typical MRM chromatograms of plasma sample obtained from healthy volunteer before and 1 & 2 hrs. after oral a single 500 mg Clarithromycin dose.

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Measured levels

- Samples collected from a health volunteer before and after ingestion of a single oral dose of 500 mg clarithromycin analyzed according method.

- Measured concentration:
  Range 0 - 2.03 µg/ml.
Conclusions

- A simple, precise, and accurate assay for the measurement of clarithromycin in human plasma was developed and fully validated.
- The assay was successfully applied to monitor stability of clarithromycin under various condition routinely encountered by the laboratory.
- The assay was applied to determine the level of clarithromycin in 0.2 ml plasma sample obtained from a healthy volunteer.
Acknowledgements

- This work was funded by a grant from the King Abdul-Aziz City for Science and Technology, under National Comprehensive Plan for Science and Technology, Riyadh, Saudi Arabia, (Biotech:10-BIO96).

- Dr. Muhammad M Hammami, MD. PhD, Chairman, Department Clinical Studies & Empirical Ethics, KFSHRC

- Staff members of Clinical & Bio-analysis Laboratories CSEED, KFSHRC

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Thanks for your Attention