T2309. Development of an LC-MS/MS Method for the Determination of the Antifungal Luliconazole in Human Toenails

M. K. Leahy1, L. Geisler1, J. Wang1, K. Morton2, N. Nakamura2 and M. Wolter2
1Covance Laboratories Inc., Madison, WI; 2Topica Pharmaceuticals, Inc., Los Altos, CA

Background
Luliconazole is a novel antifungal agent, in the class of imidazoles, being developed for the treatment of onychomycosis. Currently approved therapies are either ineffective or are complicated by the potential of liver toxicity requiring hepatic monitoring. Topical application of luliconazole has demonstrated rapid penetration and therapeutically effective levels in the nail bed. (Innes T and Tawakol A, AAC 2013 Jun; 57(6):2684-6)

A sensitive and selective LC-MS/MS method was developed to determine the concentrations of luliconazole in human toenails to support clinical trials. Challenges encountered during method development included separation of isomeric forms of the drug, and the homogenization and processing of a solid matrix (i.e. toenails).

Sample Preparation
Published methods for toenail sample preparation typically include acid or alkaline digestion. These methods are not practical for luliconazole due to compound degradation, therefore mechanical homogenization was evaluated. Different homogenizing mills (Figure 1) and sample vial/crusher systems (Figure 2) were tested. The optimized sample processing procedure is listed below.

1. Transfer the entire toenail sample into a reinforced polypropylene vial and add a 1.00 mL of Methanol:DMSO (1:1, v:v) to each vial. Leave the stainless steel beads in the vials.
2. Vortex-mix for 10 minutes, then sonicate the samples for 30 minutes.
3. Centrifuge the samples at a minimum of 8320 x g for 5 minutes.
4. Transfer 50.0 μL aliquots of the supernatant to polypropylene tubes. Note: After storage frozen for possible reassay.
5. Add 50.0 μL of Working Internal Standard Solution to the samples. Vortex-mix for 1 minute.
6. Dilute the samples with Methanol:Water (1:1, v:v) and transfer into a 96-well collection plate.
7. Store at refrigerated conditions if needed prior to injection.

Chromatography
It is known that luliconazole can convert to a Z,luliconazole metabolite, both of which share the same precursor and product ions or MRM transitions. Baseline chromatographic separation was therefore required to resolve luliconazole from Z,luliconazole. The best resolution was obtained using a Kinetex 2.6μm PFP column (Phenomenex).

Chromatograms of extracted toenails are shown in Figure 4. A sensitive and selective LC-MS/MS method was developed to determine the concentrations of luliconazole in human toenails to support clinical trials. Challenges encountered during method development included separation of isomeric forms of the drug, and the homogenization and processing of a solid matrix (i.e. toenails).

Extraction
1. Remove the samples from the Auto-Mill and add 1.00 mL of Methanol:DMSO (1:1, v:v) to each vial. Leave the stainless steel beads in the vials.
2. Vortex-mix for 10 minutes, then sonicate the samples for 30 minutes.
3. Centrifuge the samples at a minimum of 8320 x g for 5 minutes.
4. Transfer 50.0 μL aliquots of the supernatant to polypropylene tubes. Note: After storage frozen for possible reassay.
5. Add 50.0 μL of Working Internal Standard Solution to the samples. Vortex-mix for 1 minute.
6. Dilute the samples with Methanol:Water (1:1, v:v) and transfer into a 96-well collection plate.
7. Store at refrigerated conditions if needed prior to injection.

Results
The calibration curves were linear in the range of 100 to 10000 μg/g based on a 5 mg toenail sample size for calibration standards. The overall precision and accuracy values, based upon relative standard deviation (RSD) and relative error (RE) of quality control (QC) samples, were ≤5.1% and ≤4.5%, respectively. Luliconazole was stable in homogenized nail clippings subjected to 6 freeze/thaw cycles, at least 24 hour storage at room temperature, and at least 29 days stored at -20°C. Luliconazole was in fortified nail clippings (prior to homogenization) for at least 48 days stored at -20°C. Processed samples are stable in injection solvent for up to 4 days. Toenail clippings from 6 individual healthy donors not currently undergoing an antifungal regimen were obtained from Bionelization for selectivity testing. No significant interference was found in the MRM chromatograms of the six lots of nail clippings tested. Additionally, no matrix effect was detected in these six lots of nail clippings. An estimated recovery is performed since it is not known whether the blank matrix is penetrated by the analyte after spiking. Recoveries were determined by comparing the mean peak area of processed samples with the mean peak area of recovery samples prepared by adding luliconazole and lanoconazole (internal standard) to blank human toenail extracts. The overall recoveries for luliconazole and lanoconazole were 91.6 and 98.7%, respectively.

Chromatograms of extracted toenails are shown in Figure 4. A sensitive and selective LC-MS/MS method was developed to determine the concentrations of luliconazole in human toenails to support clinical trials. Challenges encountered during method development included separation of isomeric forms of the drug, and the homogenization and processing of a solid matrix (i.e. toenails).

Table 1. Mass Spectrometry Parameters

<table>
<thead>
<tr>
<th>Component</th>
<th>MRM Transition</th>
<th>Approximate RF (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luliconazole</td>
<td>354.1 → 150.3</td>
<td>2.7</td>
</tr>
<tr>
<td>Z,Luliconazole</td>
<td>354.1 → 150.3</td>
<td>2.4</td>
</tr>
<tr>
<td>Lanoconazole (Internal Standard)</td>
<td>320.1 → 150.3</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Table 2. Quality Control Data for Luliconazole in Human Toenails

<table>
<thead>
<tr>
<th>Component</th>
<th>LLOQ QC</th>
<th>LQC</th>
<th>MQC</th>
<th>HQC</th>
<th>DQC (10X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luliconazole</td>
<td>100 μg/g</td>
<td>500 μg/g</td>
<td>1500 μg/g</td>
<td>5000 μg/g</td>
<td>50000 μg/g</td>
</tr>
<tr>
<td>Lanoconazole (Internal Standard)</td>
<td>500 μg/g</td>
<td>1500 μg/g</td>
<td>5000 μg/g</td>
<td>25000 μg/g</td>
<td></td>
</tr>
</tbody>
</table>

Mean Measured Concentration (μg/g)
- Luliconazole: 976 ± 288 (n=6)
- Lanoconazole: 4.96 ± 1.06 (n=6)

Inter-run SD
- Luliconazole: 3.2 ± 1.3 (n=6)
- Lanoconazole: 2.4 ± 0.7 (n=6)

Conclusion
Method development for the quantification of luliconazole in human toenails using LC-MS/MS presented unique challenges in sample preparation due to an atypical matrix and in chromatography due to the requirement for baseline separation of luliconazole and Z,luliconazole. The method has been successfully validated and used in the analysis of unknown samples.
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