Introduction

Nicotine and its metabolites are para-symptomatichemical effects found in the nighthead family of plants.

Therefore in this case, we developed LC/MS/MS analytical methods and evaluated various columns and solvent combinations in order to demonstrate the chromatographic separation, detection and quantification of the Nicotine and its metabolites in Urine, Oral Fluid and Blood that included Nicotine, Cotinine, Nornicotine, Norcotinine, Anabasine, trans-3'-Hydroxycotinine, 4-(Methylthiomethylamino)-1-(3-pyridyl)-1-butanone (NNAL), N-Nitrosomonomocotinine (NNN), 4-(Methylthiostearic acid)-1-(3-pyridyl)-1-butanone (NNK), N-Nitrososabamine (NAB) and N-Nitrosoanatabine. The sample preparation choices were kept simple and included dilute and develop on an Agilent 1260 HPLC and 6460 Mass Spectrometer with a 6.5 minute analytical gradient method in positive ionization mode.

Experiment

Reagents, Standards, Calibrators and Controls

Standard/Calibrators- Corrionten:
Nicotine: 1 mg/mL
Cotinine: 1 mg/mL
Nornicotine: 1 mg/mL
Norcotinine: 1 mg/mL
Anabasine: 1 mg/mL
Trans-3'-Hydroxycotinine: 1 mg/mL
Trans-3'-Hydroxycotinine-D3: 100 µg/mL
Anabasine-D4: 100 µg/mL
Nornicotine-D4: 100 µg/mL
Norcotinine-D4: 100 µg/mL
N-Nitrosomonomocotinine (NNAL): 1 mg/mL
N-Nitrososabamine (NAB): 1 mg/mL
N-Nitrosoanatabine: 1 mg/mL

Sample Preparation- Urine

• 100 µL of urine sample, calibrators, controls was taken and 10 µL of ISTD at 1000 ng/mL were added to each and vortexed briefly

• 890 µL of HPLC grade water was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13800 rpm

• The supernatant was transferred to a MS vial

• All in-house calibrators and controls were prepared in drug-free urine (Golden West Biological, Inc)

Sample Preparation- Oral Fluid

• 100 µL of oral fluid sample (25 µL of oral fluid and 75 µL of buffer), calibrators, controls was taken and 2.5 µL of ISTD at 1000 ng/mL were added to each and vortexed briefly

• 397.5 µL of HPLC grade Water was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13800 rpm

• The supernatant was transferred to a MS vial

• All in-house calibrators and controls were prepared in negative oral fluid (Immunalysis, Ltd)

Sample Preparation- Blood

• 100 µL of blood sample, calibrators, controls was taken and 10 µL of ISTD at 1000 ng/mL were added to each and vortexed briefly

• 390 µL of HPLC grade Water was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 13800 rpm

• The supernatant was transferred to a MS vial

• All in-house calibrators were prepared in drug-free blood (Golden West Biological, Inc)

Experimental

Method

HPLC Conditions:
Agilent 1260 Infinity HPLC series binary pump, well plate, thermostated column compartment
Column: Agilent Technologies Poroshell 120 EC-C18, 2.1 x 100 mm, 2.7 µm
Column Temperature: 55 ºC
Injection Volume: 10 µL (Urine, Oral Fluid), 5 µL (Blood)
Autosampler Temperature: 4 ºC
Needle Wash: 100 µL (Honeywell/Methanol/50% Water) 10 seconds
Mobile Phase A: 0.1% Formic Acid + 5 mM Ammonium Formate in Water
Mobile Phase B: 0.1% Formic Acid in Methanol
Flow Rate: 0.45 mL/min
Gradient: 6 min - 95%A:5%B
3.0 min - 95%A:5%B
4.5 min - 5%A:95%B
Run/Stop time: 6.5 min/3.5 min

MS Conditions:
Agilent 6460 Triple Quadruple Mass Spectrometer Dynamic MRM
Ion mode: Agilent Jet Stream Positive Mode
Gas Temperature: 170 ºC
Gas Flow: 11 L/min
Nebulizer: 55 psi
Sheath Gas Temperature: 400 ºC
Sheath Gas Flow: 12 L/min
Capillary Voltage: 3800V
Nebulizer Voltage: 0V
Q1/Q2 Resolution: Wide/Unit
Delta EVM: +400V
Cell Accelerator Voltage: 2

Table 1: MRM Acquisition Table- * Quantifier Ion

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rt Min</th>
<th>MRMO Transitions</th>
<th>Fragmentation Voltage</th>
<th>Collision Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>1.96</td>
<td>163.1-&gt;132/130</td>
<td>67</td>
<td>12/20</td>
</tr>
<tr>
<td>N-Nitrosomonomocotinine</td>
<td>1.96</td>
<td>167.1-&gt;136</td>
<td>67</td>
<td>12</td>
</tr>
<tr>
<td>Cotinine</td>
<td>2.64</td>
<td>177.1-&gt;98.1/180.1</td>
<td>77</td>
<td>20/24</td>
</tr>
<tr>
<td>Nornicotine DL</td>
<td>2.62</td>
<td>180.1-&gt;80.1</td>
<td>77</td>
<td>24</td>
</tr>
<tr>
<td>Trans-3'-Hydroxycotinine</td>
<td>1.76</td>
<td>193.1-&gt;134/80.1</td>
<td>77</td>
<td>32/36</td>
</tr>
<tr>
<td>Trans-3'-Hydroxycotinine-D3</td>
<td>1.74</td>
<td>196.1-&gt;80.1</td>
<td>77</td>
<td>36</td>
</tr>
<tr>
<td>Anabasine</td>
<td>3.29</td>
<td>163.1-&gt;120/80.1</td>
<td>77</td>
<td>12/24</td>
</tr>
<tr>
<td>Nornicotine</td>
<td>3.19</td>
<td>167.1-&gt;84.1</td>
<td>77</td>
<td>36</td>
</tr>
<tr>
<td>Nornicotine-D4</td>
<td>1.8</td>
<td>149.1-&gt;132/80.1</td>
<td>77</td>
<td>12/24</td>
</tr>
<tr>
<td>Nornicotine-D4</td>
<td>1.77</td>
<td>153.1-&gt;84.1</td>
<td>77</td>
<td>32</td>
</tr>
<tr>
<td>Nornicotine-13C3</td>
<td>1.77</td>
<td>166.1-&gt;80.1</td>
<td>77</td>
<td>28</td>
</tr>
<tr>
<td>NNAL</td>
<td>3.2</td>
<td>210.1-&gt;180/93.1</td>
<td>62</td>
<td>4/20</td>
</tr>
<tr>
<td>NNN</td>
<td>4.04</td>
<td>178.4-&gt;148/120</td>
<td>57</td>
<td>8/16</td>
</tr>
<tr>
<td>NAB</td>
<td>5.71</td>
<td>208.1-&gt;122/79.1</td>
<td>62</td>
<td>8/48</td>
</tr>
<tr>
<td>N-Nitrosoanatabine</td>
<td>5.62</td>
<td>190.1-&gt;79/160*</td>
<td>57</td>
<td>4/36</td>
</tr>
</tbody>
</table>

Table 2: Sensitivity

<table>
<thead>
<tr>
<th>Compound (ng/mL)</th>
<th>LOD Urine</th>
<th>LOD Oral Fluid</th>
<th>LOD Blood</th>
<th>LOD Urine</th>
<th>LOD Oral Fluid</th>
<th>LOD Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>0.5</td>
<td>1</td>
<td>0.25</td>
<td>1</td>
<td>2.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Cotinine</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>Trans-3'-Hydroxycotinine</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>Anabasine</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>2.5</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>Nornicotine</td>
<td>2.5</td>
<td>1</td>
<td>5 (Interference)</td>
<td>2.5</td>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td>Norcotinine</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>2.5</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>NNAL</td>
<td>1</td>
<td>2.5</td>
<td>0.5</td>
<td>2.5</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>NNN</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>NAB</td>
<td>10</td>
<td>0.25</td>
<td>0.25</td>
<td>25</td>
<td>0.5</td>
<td>0.5</td>
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<tr>
<td>N-Nitrosoanatabine</td>
<td>0.1</td>
<td>1</td>
<td>0.25</td>
<td>2.5</td>
<td>2.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Results and Discussion

Accuracy

The accuracy was determined by the analysis of the Tri-level Recipe QC and In house control material as the percentage deviation from the target mean and the results were <10% for all levels in each matrix. Therefore, the analytical method in positive mode can achieve the required levels for the analysis of Nicotine in Urine, Oral Fluid and Blood.

Precision/Specificity

The intra-assay precision (%CV) of the Nicotine's in each matrix were determined by extracting and quantifying five replicates of a Tri-level QC material from Reciproc resulting in a mean of 168.5 ng/ml, 921 ng/mL and 4080 ng/mL for Urine for Nicotine, Cotinine and Trans-3'-Hydroxycotinine. The inter-assay precision was determined over 5 consecutive days and was found to have a %CV <10% for each Nicotine's for Level 1, 2 and 3 of 170, 1000 and 4000 ng/mL respectively in Urine. In house controls of the other Nicotine's and controls for Oral Fluid and Blood at 25, 150 and 750 ng/mL and resulted in comparable %CV results where possible.

Table 3: Chromatograms

Urine 100 ng/mL
Oral Fluid 100 ng/mL
Blood ng/mL

Conclusions

• Baseline separation of the Nicotine’s in 6.5 minutes with good LOD/LOQ in positive mode was achieved in three different matrix types except for NNN.
• Simple sample preparation in three matrices (Urine, Oral Fluid and Blood) achieved desirable LOD/LOQ but Oral Fluid may require better sample cleanup to achieve consistent sensitivity and to avert the effect of the blue buffer contained in the oral fluid collection device.
• Excellent linearity of calibration curves with acceptable accuracy, precision and reproducibility in positive mode was achieved in all matrices <10% for %CV.
• Further evaluate different sample preparation techniques to determine which gives the best results while maintaining low cost and ease of use.