Quantification of 4-Chloroaniline in Chlorhexidine using the Agilent 1200 Series Rapid Resolution LC System coupled with the Agilent 6410B Triple Quadrupole LC/MS System

Application Note

Pharmaceutical R&D

Author
Siji Joseph
Agilent Technologies
Bangalore, India

Abstract
Genotoxic impurities are of major concern in the pharmaceutical industry and obtaining sufficient sensitivity is the major challenge for quantification. Tandem MS/MS (QQQ) is the technique of choice for quantification of such impurities due to multiple reaction monitoring (MRM) sensitivity and selectivity. High throughput and shorter analysis times are desirable, so by utilizing fast chromatography this can be realized with increased chromatographic resolution.

This study demonstrates the quantification of 4-Chloroaniline, (a degradation product) in Chlorhexidine using Agilent 6410 QQQ and 1200 Series RRLC. The linearity plot covers a wide concentration range of 0.3 ng/mL to 1000 ng/mL with a correlation coefficient of > 0.9998. The observed limit of detection (LOD) is 0.2 ng/mL and the limit of quantification (LOQ) is 0.3 ng/mL.
**Introduction**

Aromatic amines are believed to cause mutations since they are typically electrophilic and can form strong covalent bonds with DNA so that the exact replication can be prevented. 4-Chloroaniline is a degradation product of Chlorhexidine (1, 1’-(Hexane-1, 6-diyl) bis [5-(4-chlorophenyl) biguanide]), which is widely used as an active ingredient in dentistry. The formation of 4-Chloroaniline can be stimulated by heat. The compound is highly toxic and can cause hemolysis and methemoglobinemia\(^2,3\). This explains the importance of developing a robust and sensitive method for the quantification of 4-Chloroaniline in Chlorhexidine.

This application note describes a simple and sensitive LC/ESI/MSMS method that can detect 4-Chloroaniline at a concentration level of 0.2 ng/mL in Chlorhexidine. The molecular structures of Chlorhexidine and 4-Chloroaniline are shown in Figure 1.

**Experimental**

**Chemicals**

Chlorhexidine and 4-Chloroaniline were obtained from Sigma-Aldrich. All solvents were of HPLC grade. Methanol was purchased from Merck, Formic acid from Fluka, and Millipore water was used.

**Instrumentation and chromatographic condition**

All analyses were performed using the Agilent 6410B Triple Quadrupole MS coupled with a 1200 Series RRLC system. The RRLC system components included an Agilent 1200 Series binary pump SL with degasser, an Agilent 1200 Series autosampler SL, and an Agilent 1200 Series thermostatted column compartment SL. The Agilent Mass Hunter Workstation software (version: B.01.04) was used for system control and data acquisition.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Set value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nebulizing gas flow-rate</td>
<td>12 L/min</td>
</tr>
<tr>
<td>Nebulizer pressure</td>
<td>40 psi</td>
</tr>
<tr>
<td>Drying gas temperature</td>
<td>325 °C</td>
</tr>
<tr>
<td>Capillary voltage</td>
<td>1500 V</td>
</tr>
<tr>
<td>Ionization mode</td>
<td>Electrospray positive</td>
</tr>
<tr>
<td>Fragmentor voltage for Chloroaniline</td>
<td>110 V</td>
</tr>
<tr>
<td>Collision energy for Chloroaniline</td>
<td>18 V</td>
</tr>
<tr>
<td>Fragmentor voltage for Chlorhexidine</td>
<td>150 V</td>
</tr>
<tr>
<td>Collision energy for Chlorhexidine</td>
<td>24 V</td>
</tr>
</tbody>
</table>

**Sample preparation**

Stock solutions of 4-Chloroaniline of concentration 500 µg/mL were prepared in diluent and diluted further to get the desired concentrations. The prepared linearity levels are 0.2 (LOD), 0.3 (LOQ), 0.4, 0.5, 0.6, 0.7, 0.75, 0.8, 0.9, 1.0, 2.5, 5.0, 7.5, 10, 25, 50, 75, 100, 250, 500, 750, 1000 ng/mL of 4-Chloroaniline. Three replicates were injected for each level and the response was used to plot the linearity curve.
Results and discussion

Short run times of about 4 minutes were achieved in which 4-Chloroaniline eluted close to 2.8 minutes and Chlorhexidine at 3.5 minutes. The peaks of interests were free from interfering peaks at their respective retention times. The total ion chromatogram (TIC) for the test mix including Chlorhexidine and 4-Chloroaniline is shown in Figure 2.

The fragmentation patterns for Chlorhexidine and 4-Chloroaniline are shown in Figures 3 and 4, respectively.

The calibration curve for 4-Chloroaniline shows excellent linearity $R^2 > 0.9998$ over a wide concentration range of 0.3 to 1000 ng/mL (21 levels, three injections each, see Figure 5). The observed LOD is 0.2 ng/mL ($S/N \sim 7$) and LOQ ($S/N \sim 11$) is 0.3 ng/mL.
The concentration accuracy in percent over the linearity range showed an excellent average value of 102.2 ± 9.7. The accuracy values for all the levels are tabulated in Table 2.

**Conclusion**

A sensitive and robust LC/ESI/MSMS method for the quantification of 4-Chloroaniline in Chlorhexidine was developed. The method is linear over a wide range of 0.3 ng/mL (LOQ) to 1000 ng/mL with a correlation coefficient of >0.9998. The observed LOD is 0.2 ng/mL. The concentration accuracies for the linearity levels were found to be about 102.2 ± 9.7%. This experiment demonstrates the capability of Agilent 6410B Triple Quadrupole LC/MS to deliver excellent and accurate results in trace-level quantification of the genotoxic impurity 4-Chloroaniline.

**References**

