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

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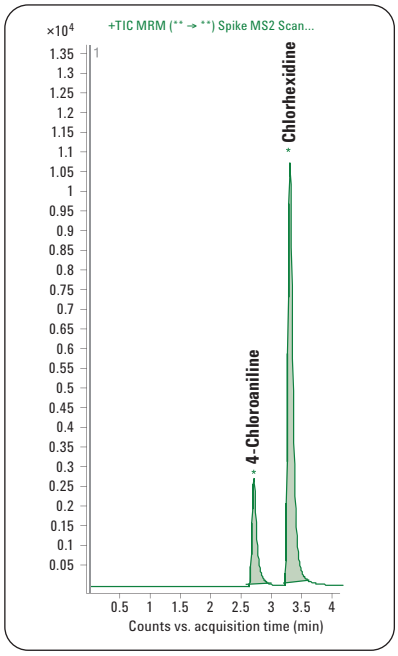
Abstract

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.



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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[1]. 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 MassHunter Workstation software (version: B.01.04) was used for system control and data acquisition.

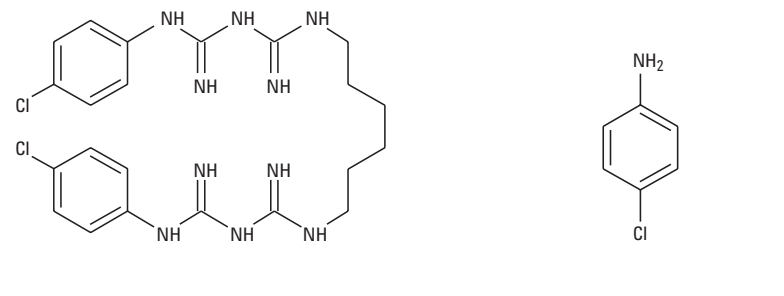

Figure 1
Molecular structures of Chlorhexidine and 4-Chloroaniline.

An Agilent ZORBAX Eclipse Plus C18, 4.6 mm × 100 mm, 1.8 µm column was used for all chromatographic separations. Methanol with 0.1% Formic acid was used as the organic and water containing 0.1% Formic acid was the aqueous mobile phase. The linear gradient starts with 50% of organic mobile phase and increased the percentage to 70% in three minutes' time. The percentage of B remained constant for one more minute. Post-time for the gradient was 3 minutes. The autosampler was operated at 6 µL injection volume with flush port activated using a rinse time of 10 seconds. The sample temperature was set at 15 °C. The LC binary pump was operated at 700 µL/min. The column thermostat was set at 40 °C. The diluent was a premixed 1:1 solution of aqueous and organic mobile phase. The MS parameters are tabulated in Table 1.

The ions monitored for 4-Chloroaniline were *m/z*: 128 → 93 (quantifier), *m/z*: 128 → 111 (qualifier), and for Chlorhexidine is *m/z*: 505.4 → 184.3. All ions had a dwell time of 200 ms ion^{-1}.

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.

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

Table 1
MS parameters.

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.

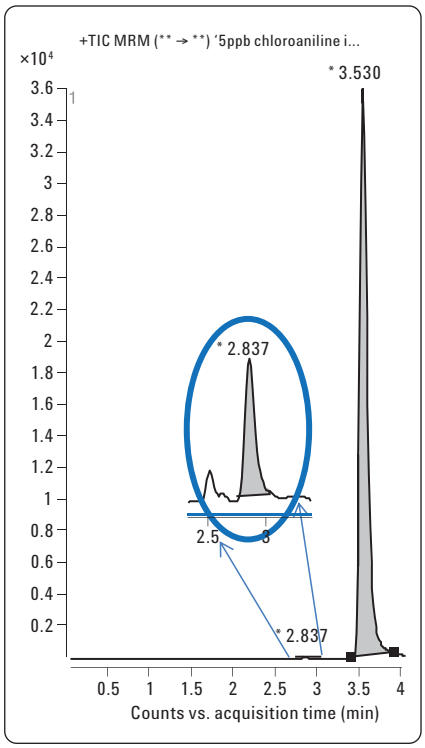

Figure 2
TIC of spiked 0.5 ppb 4-Chloroaniline in 1000 ppb Chlorhexidine.

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 ~ 7) and LOQ (S/N ~ 11) is 0.3 ng/mL.

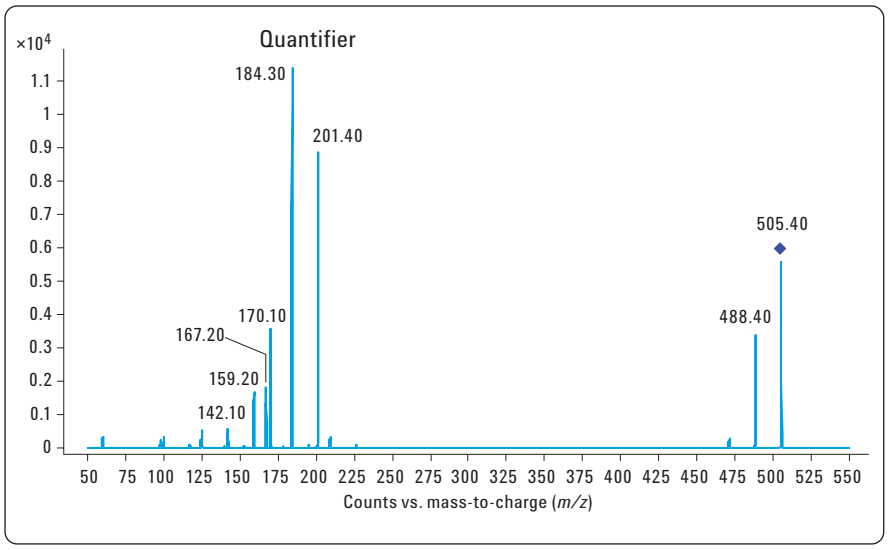

Figure 3
Fragmentation pattern for Chlorhexidine.

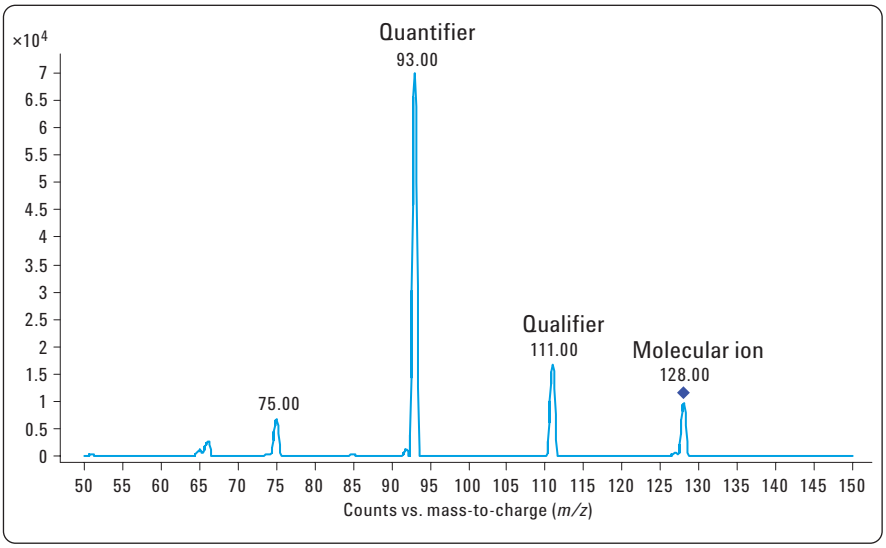

Figure 4
Fragmentation pattern for 4-Chloroaniline.

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

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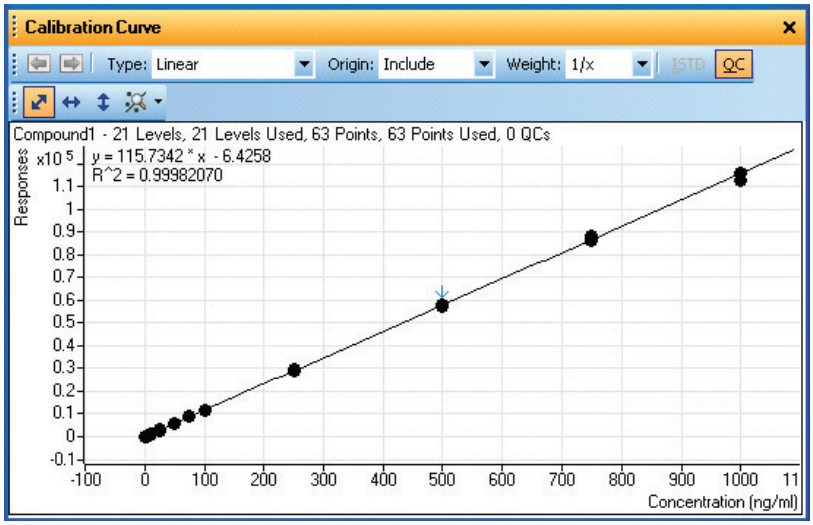

Figure 5
Calibration curve for 4-Chloroaniline.

Linearity sample	Concentration (ng/mL)	Accuracy
Level: 1 (LOQ)	0.30	111.9
Level: 2	0.40	107.1
Level: 3	0.50	92.5
Level: 4	0.60	101.9
Level: 5	0.70	97.7
Level: 6	0.75	104.0
Level: 7	0.80	93.7
Level: 8	0.90	97.9
Level: 9	1.00	95.3
Level: 10	2.50	97.9
Level: 11	5.00	97.6
Level: 12	7.50	100.6
Level: 13	10.00	99.6
Level: 14	25.00	101.0
Level: 15	50.00	97.8
Level: 16	75.00	100.6
Level: 17	100.00	99.9
Level: 18	250.00	101.9
Level: 19	500.00	99.7
Level: 20	750.00	100.6
Level: 21	1000.00	99.3

Table 2
Accuracy for all linearity level samples.

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