Screening, Identifying, and Quantifying Potential Genotoxic Compounds Using High Resolution LC/MS

Analysis of Chlorhexidine drug substance using an Agilent 6545 Accurate Mass Q-TOF System and MassHunter Mass Profiler Software

Application Note

Pharmaceutical

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Abstract

This study demonstrates a routine screening of drugs to identify and quantify potential genotoxic compounds. In this Application Note, we used an Agilent 6545 Q-TOF LC/MS system to acquire accurate mass data of samples containing chlorhexidine as the drug substance. Agilent MassHunter Mass Profiler software was used to mine the data and compare different samples to generate a differential list of compounds. An accurate mass database search against the differential list identified 4-chloroaniline, a potential genotoxic compound. All Ions MS/MS acquisition mode was used to confirm 4-chloroaniline by MS/MS library matching, and quantify it using external standards. This workflow is suitable for batch-to-batch sample analysis for detecting and quantifying known potential genotoxic compounds.

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Introduction

Drug substances may produce potential genotoxic compounds when they are stored for extending periods of time, or when they are stored inappropriately. Detection, identification, and quantification of genotoxic compounds is a time-consuming process. Regulatory authorities[1] require reporting the formation of genotoxic compounds. Recent advances in software tools enables the fast and cost-effective detection of potential genotoxic compounds in complex samples. Agilent MassHunter Mass Profiler (MP) software allows the comparison of two sets of samples, and the determination of any significant differences between them. Principal component analysis (PCA) tools within MP assists the classification of compounds based on identified differentiation markers. A differentiation marker is a compound that exceeds a defined concentration, when compared to a control sample. A custom-built accurate mass database was used to identify the differences between samples. In this study, MP analysis of degraded and nondegraded chlorhexidine samples gave a list of statistically different compounds between samples. Using an Agilent ID Browser feature within the MP software, these compounds were searched with a custom database containing potential genotoxic compounds. Compounds were further confirmed using accurate mass library matching, then quantified. Figure 1 shows the workflow used in this study.

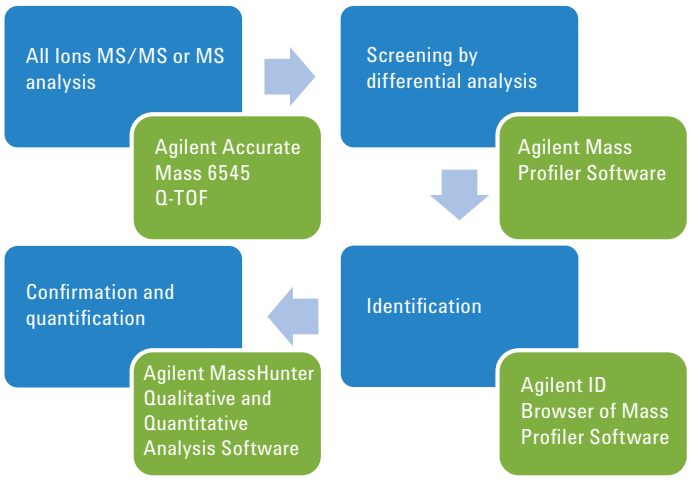

Figure 1. Workflow for genotoxic compound analysis.

Experimental

Reagents and materials

Chlorhexidine and 4-chloroaniline were purchased from Sigma-Aldrich (India). Methanol was LC/MS grade (Lab Scan, Bangkok). Purified water was from a Milli-Q water purification system (Millipore, USA).

Sample preparation

Test samples

Chlorhexidine was subjected to degradation by taking a 1,000 µg/mL solution in methanol and adding an equal amount of 100 % formic acid. This solution was heated to 80 °C for one hour. The solution was then diluted in a 50/50 methanol/water solution to a 150 µg/mL solution. During LC/MS analysis, the chlorhexidine peak was diverted to waste through the integrated diverter valve. Four test samples were prepared.

Control sample

The chlorhexidine standard solution was neither acid treated nor heated. Four control samples were used.

Standard stock solution

Chlorhexidine prepared in 100 % methanol (1,000 µg/mL), and 4-chloroaniline prepared in 100 % methanol (5,000 µg/mL)

Calibration dilution solvent

1,000 ng/mL solution of chlorhexidine in 50/50 methanol/water solution

Calibration sample

Standard 4-chloroaniline was prepared in 0.12, 0.6, 1.2, 2.4, 3, 4, 5, 9, 15, 27, 54, 75, 150, and 300 ng/mL concentrations. The levels were chosen because they were low enough for genotoxic compounds to be detected. Each level was prepared in triplicate.

Instrumentation and software

LC parameters

An Agilent 1290 Infinity LC System (binary) was used for chromatographic separation of the analytes. A longer LC method (12 minutes) was used to screen samples for formation of new impurities. A shorter All Ions MS/MS (five minutes) quantification method was optimized for quantifying 4-cholorhexidine.

MS parameters

Agilent MassHunter data acquisition software (B.05.01), qualitative analysis software (B.07.00), Mass Profiler software, and quantitative analysis software (B.07.00) were used for data acquisition and analysis.

An Agilent 6545 Q-TOF using an Agilent Jet Stream Source, operating in positive mode, was tuned using Swarm Autotune. Swarm Autotune uses Particle Swarm Optimization technology, and allows up to 21 parameters to be adjusted simultaneously, resulting in more robust instrument tuning and optimization. Tune parameters were chosen specifically for the target mass range, *m/z* 50–250 when quantifying 4-chloroaniline (*m/z* 127.0189).

Table 1. LC parameters.

Parameter	Value	
Column	Agilent ZORBAX Eclipse Plus C18 RRHD, 3.0 × 50 mm, 1.8 µm (p/n 959757-302)	
Column temperature	40 °C	
Injection volume	5 µL	
Autosampler temperature	6 °C	
Needle wash	Flush port (100 % methanol) 5 seconds	
Mobile phase	A) 0.1 % formic acid in water B) 0.1 % formic acid in methanol	
Flow rate	0.5 mL/min	
Gradient	Quantitation All Ions MS/MS method Time (min) %B 0.0 40 3.0 60 4.0 60 4.1 40 5.0 40 Stop time: 5.0 minutes Post time: 0.5 minutes	Screening MS method Time (min) %B 0.0 20 1.0 20 7.0 40 8.0 95 10.0 95 11.0 20 Stop time: 12.0 minutes

Table 2. Q-TOF parameters.

Parameter	Value
Source conditions	
Gas temperature	250 °C
Drying gas (nitrogen)	11 L/min
Nebulizer gas (nitrogen)	40 psig
Sheath gas temperature	200 °C
Sheath gas flow	11 L/min
Capillary voltage	2,500 V
Nozzle voltage	500 V
Fragmentor	120 V
Skimmer	40 V
Oct 1 RF Vpp	700 V
Acquisition rate/time	5 spectra/sec
Reference mass	64.0158 and 922.0098
Tune	High sensitive slicer position 2 GHz extended dynamic mode
Collision energies	0, 10, and 20 eV

Results and Discussion

Screening by differential analysis

The data files from the LC/MS analysis of degraded and control samples were processed using recursive molecular feature extraction in Mass Profiler software. Height filters of 4,000 counts for extracted compound features, quality score 100 and >4 fold change were used for statistical analysis. A greater than 4-fold change was applied to detect those features that differed significantly from control samples. Figure 2 shows the statistical analysis results from a feature plot of log abundance ratio versus retention time using the screening method. The relative size of the dots in plot C is proportional to feature abundance. Individually, each dot can be identified using the database search, and visualized together with extracted ion chromatograms.

PCA plot

The PCA plot reveals that the degraded chlorhexidine samples are different and distinct from the control sample (Figure 3). This indicates that the degraded chlorhexidine sample contains features that are different from the control group. The control groups do not show significant separation, indicating no variation (blue dots) between samples.

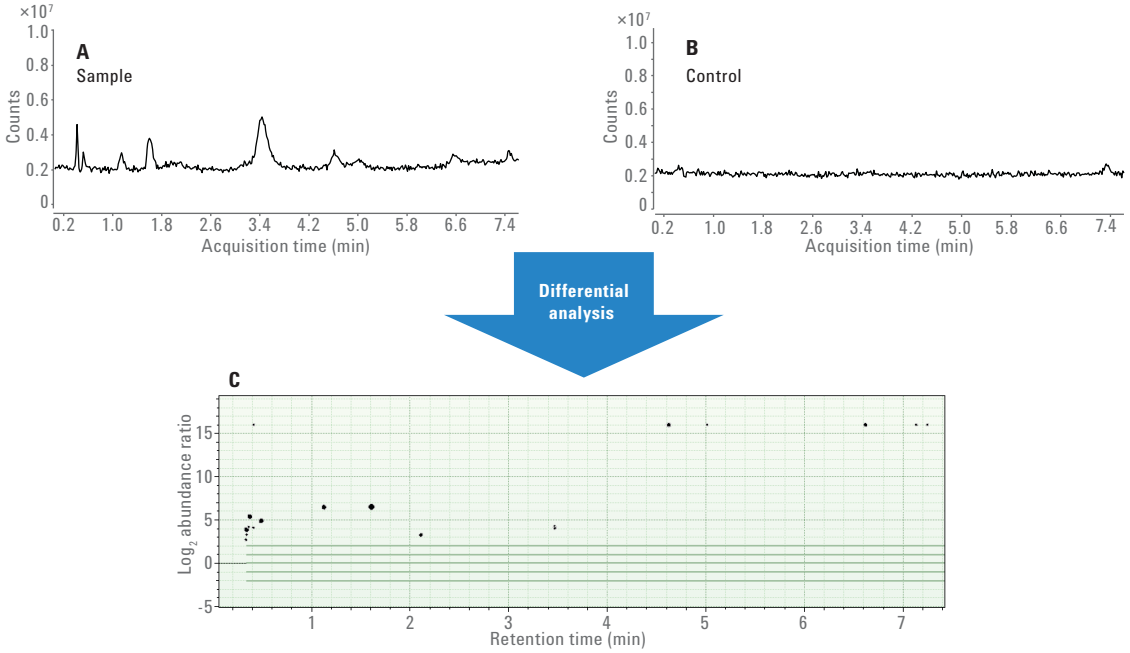

Figure 2. The input files for sample and control are shown in Figure A and B, respectively. The chlorhexidine peak elutes after 7.4 minutes and, hence, not shown on the plot. Figure C shows log abundance ratio versus retention time plot after differential analysis.

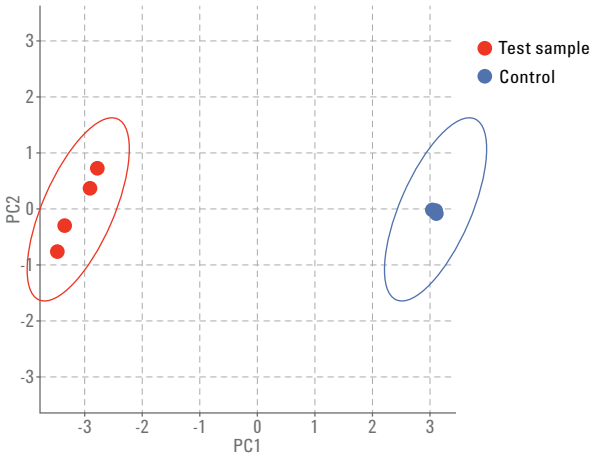

Figure 3. PCA plot showing different sample grouping. Red dots represent test samples and blue bots represent control samples.

Compound identification

A customized accurate mass database and library was created using standard compounds. The database also included literature reported mass, formula, and structures of chlorhexidine impurities. Post-statistical analysis, the differential list of compounds was searched against the accurate mass database using the ID Browser feature within Mass Profiler. The results indicated the presence of a potential genotoxic, 4-chlorhexidine in the degraded samples (Figure 4).

Feature summary of compounds

Table 3 shows a summary of differential analysis and database search results. The differential score was calculated using the Student's t-test. A value between 0 and 100 indicates whether the data groups are significantly different. A larger value indicates, with higher confidence, that the data sets in the two groups are different. The concentration of 4-chloroaniline, which was also present in minor amounts in control samples, was significantly lower than the concentration found in the degraded sample. A built-in formula generator within the ID Browser was used to generate formulas for all compounds. Since 4-chloroaniline is a potential genotoxic compound, it was further confirmed by library matching, and quantified. Some other compounds, such as compound 1, were detected at higher concentration, but were not found in the customized genotoxic database. For such compounds, formulas were calculated.

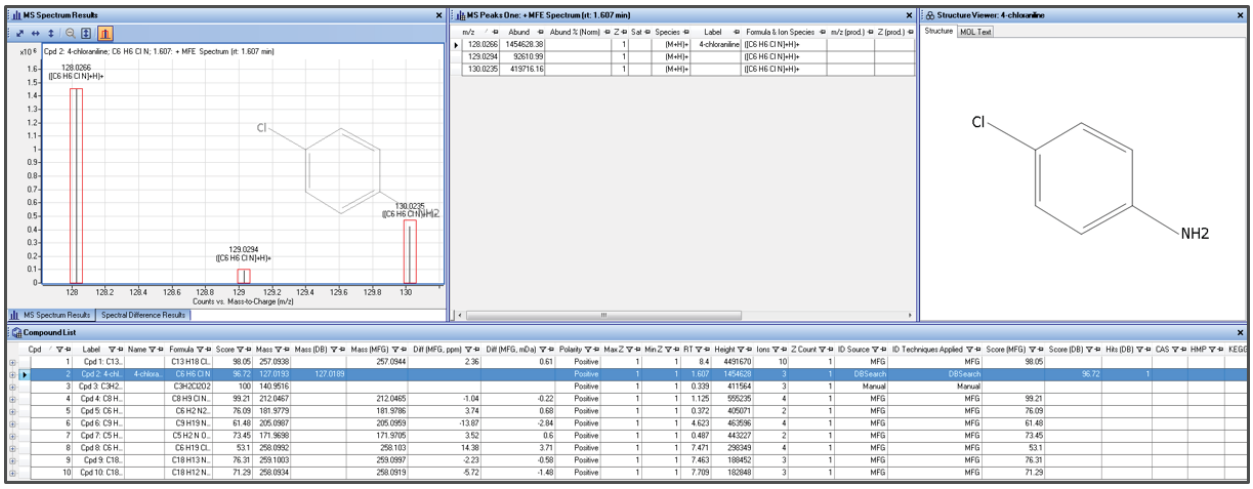

Figure 4. Identification of potential genotoxic compounds using the database/library.

Table 3. Feature summary of differential analysis showing compounds which have significantly increased between sample and control.

MassProfiler Feature Summary									
ID	Formula	Name	RT	Mass	Abundance	Q Score	Log2 (A1/A2)	Expression	Diff. score
1	$C_{13}H_{18}ClO_3$		8.4	257.0942	4664291	100	7.86	up	100
2	$C_6H_6Cl\ N$	4-chloroaniline	1.61	127.0192	1480334	100	6.51	up	100
3	$C_3H_2Cl_2O_2$		0.34	140.9516	592521	100	3.89	up	99.9
4	$C_8H_9ClN_4O$		1.13	212.0464	551298	100	6.48	up	100
5	$C_6H_2N_2O_3S$		0.37	181.9781	543519	100	5.4	up	99.9
6	$C_9H_{19}NS_2$		4.62	205.098	456724	100	16	up	100
7	$C_5H_2NO_4S$		0.49	171.9698	429576	100	4.89	up	100
8	$C_8H_{19}ClN_6OS$		7.47	258.1016	299708	100	5.68	up	100
9	$C_{18}H_{13}NO$		7.46	259.1006	186491	100	6.35	up	100
10	$C_{18}H_{12}NO$		7.71	258.0938	184274	100	16	up	100

Confirmation and quantification of potential genotoxic compounds

A shorter data-independent acquisition method was used for the targeted confirmation and quantification of 4-chloroaniline. In data-independent acquisition (All Ions MS/MS) of drug samples, both MS and MS/MS information are generated. The fragment ions in the MS/MS spectra of the personnel data compound library (PCDL) were used to extract ion chromatograms from the high energy channel. The extracted ion chromatogram (EIC) of the precursors from the low energy channel were aligned with fragment/product ion EICs to obtain the coelution score (Figure 5). The 4-chloraniline was confirmed based on accurate mass fragment matching and coelution of the precursor and product ions. 4-Chloroaniline was found with three qualified spectra in the library MS/MS spectrum where the fragments are selected from high energy MS analysis. The selected spectra were used with the qualifier and quantifier ions for the quantification method.

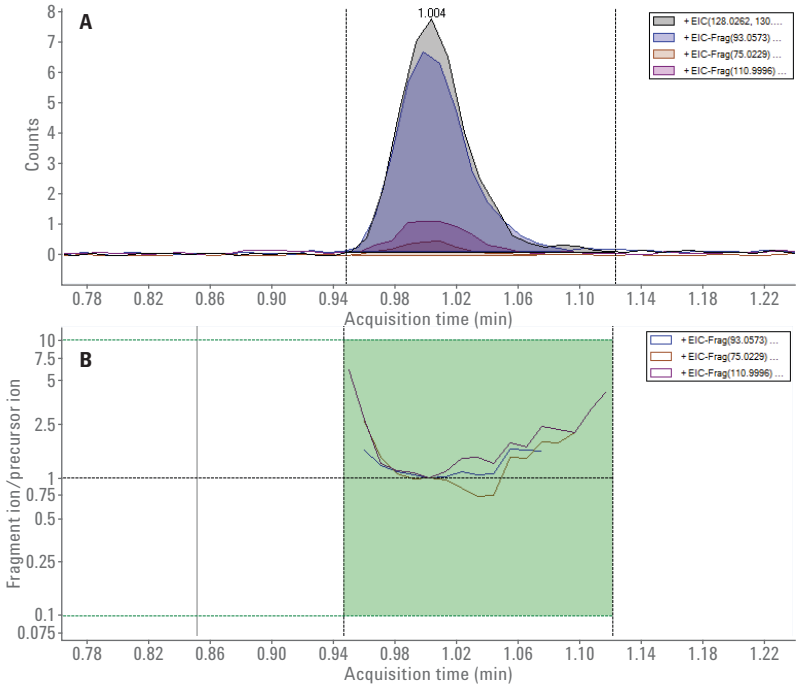

Figure 5. Overlay of extracted ion traces within the chromatographic peak which are above a selected threshold in the ion chromatogram (A). The coelution plot (B) shows a peak purity > 98 %.

The qualifier and quantifier fragment ions, together with compound names, retention time, precursor ion, fragment ion, collision energies, and relative abundances were exported to MassHunter Quantitative Analysis software to set up a quantitative method, as shown in Figure 6. The most intense ion was used as a quantifier trace, while the less intense and unique fragment ions were used as qualifiers.

A calibration curve with >3 orders of magnitude was plotted from 0.1 to 300 ng/mL (Figure 7). The 6545 was calibrated and tuned in high sensitivity mode. In addition, tuning for low mass (50–250 *m/z*) using Swarm autotune was enabled since some of the product ions for 4-chloroaniline were of low mass. The results of sample analysis showed an average value of 29 ng/mL in the degraded sample. Potential genotoxic compounds typically have a limit for reporting of 0.05 %. When 1 mg chlorhexidine is dissolved in 10 mL solution, a 0.05 % limit would require quantitation down to 50 ng/mL. Therefore, any assay must be capable of a lower LOQ. The method developed in this study can detect impurities present at a concentration <1 ng/mL.

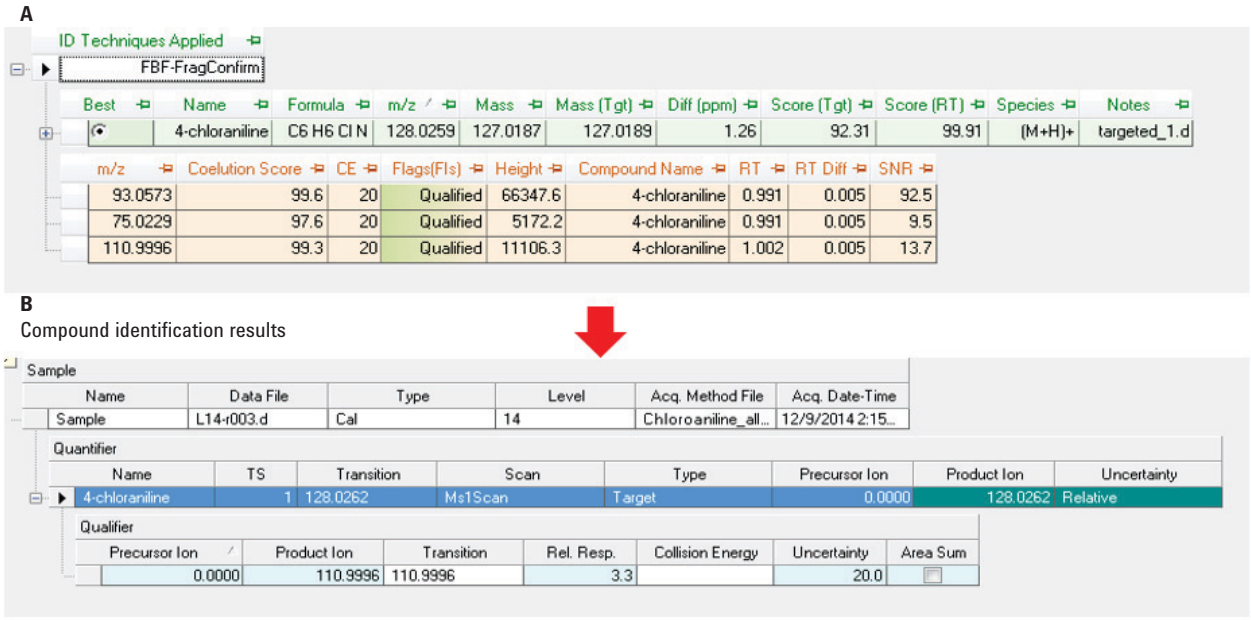

Figure 6. Quantitative method setup using compound identification results. A) Screenshot of qualitative analysis software; (B) illustrates method creation.

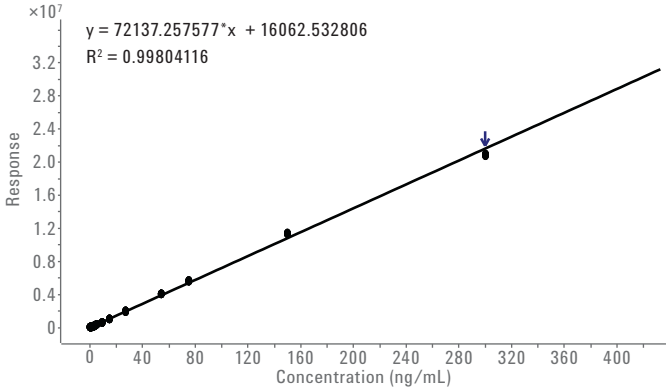

Figure 7. Calibration curve of 4-chloroaniline calculated using All Ions MS/MS.

Conclusions

This Application Note demonstrates that potentially genotoxic compounds can be screened, identified, and quantified using high resolution LC/MS. A streamlined workflow was achieved by combining All Ions MS/MS data with Agilent MassHunter Mass Profiler software (Rev. 7.0). Automated differential marker analysis revealed significant differences between sample and control sets. The workflow also included the automated detection and identification of potential genotoxic impurities as target compounds using a PCDL. The All Ions MS/MS methodology was used to generate both quantifier and qualifier ions. This enabled the quantification of the target compound. The test sample processed with this technique was determined to be at a concentration of ~29 ng/mL or 0.02 % of 4-chloraniline (assay linear range from 0.1–300 ng/mL). This workflow can be used as part of routine drug sample analysis for the identification and reporting of potentially genotoxic compounds.

Reference

1. EMA Guidance on the limits of genotoxic impurities, EMEA/CHMP/QWP/251344/2006 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002903.pdf

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Published in the USA, November 1, 2015
5991-6378EN

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