Detection, identification and quantification of potential genotoxic compound in chlorhexidine drug substance

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Introduction

Potential genotoxic compounds can be generated in drug substances during storage or synthesis. Detection, identification and quantification of genotoxic compounds is time consuming but required by regulatory authorities. With advances in software tools, the detection of genotoxic compounds has become less time consuming and cost effective. The Agilent MassHunter Mass Profiler (MP) Software helps to analyze two sets of acquisition files and to determine significant differences. By comparing an unknown sample with a reference sample all impurities can be easily detected. Principal component analysis (PCA) plots within MP helps to compare if two groups of samples separate from each other. Significantly different compounds are those that exceeds values set for area fold change and abundance cutoff. The identified compounds are then searched against an in-house built accurate mass database. In this study, MS analysis of degraded chlorxhexidine samples showed compounds that are different than in the control sample. Among the degraded samples, 4-chloroaniline was detected and identified by accurate mass library matching and quantified using the same acquisition data file. The workflow used in this study is shown in figure 1.

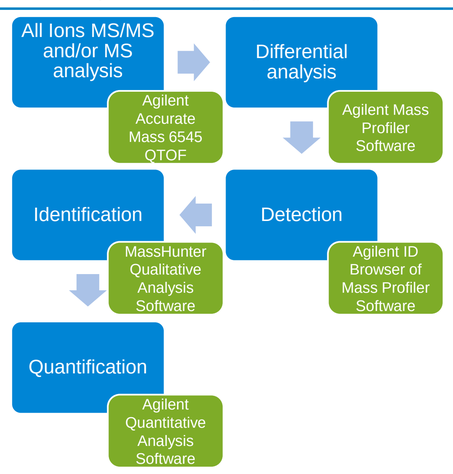

Figure 1. Workflow for genotoxic compound analysis.

Experimental

Experiments and methods

Instrumentation

Agilent 1290 Infinity LC System (binary)
Agilent 6545 Q-TOF
Agilent MassHunter data acquisition software (B.05.01), qualitative analysis software (B.07.00), Mass Profiler software and quantitative analysis software (B.07.00)

Sample preparation

Test samples: Chlorhexidine (Sigma Aldrich) was degraded by taking a 1000 ppm solution in methanol and adding an equal amount of 100% formic acid. The solution was heated to 80°C for 1 hr. The solution was diluted in 50-50 methanol-water solution to 150 ppm solution. During QTOF data acquisition, the chlorhexidine peak was diverted to waste via the integrated diverter valve. Four test samples were prepared.

Control sample: Chlorhexidine standard solution was not acid treated nor heated. Four control samples were used.

Stock solution: Chlorhexidine prepared in 100% methanol (1000 ppm). 4-chloroaniline prepared in 100% methanol (5000 ppm)

Dilution solvent: 1000 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. Each level was prepared in triplicates

Table 1. LC parameters. * All Ions MS/MS technique can also be used as a screening method

Parameter	Value	
Column	ZORBAX Eclipse Plus C18 RRHD, (3.0x50) 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) 6 sec	
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	Screening MS method*
	Time (min) %B 0.0 40 3.0 60 4.0 60 4.1 40 5.0 40 Stop time: 5.0 min Post time: 0.5 min	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 min

MS parameters

Agilent 6545 QTOF using Jet Stream Source, operating in positive mode, was tuned using Swarm Autotune. Swarm autotune uses Particle Swarm Optimization technology to optimize up to 21 parameters simultaneously. Tune was chosen specific to the desired mass range, 50-250 m/z when quantifying 4-chloroaniline.

Table 2. QTOF parameters

Parameters	Value
Gas temperature	175°C
Drying gas (nitrogen)	9 l/min
Nebulizer gas (nitrogen)	40 psig
Sheath gas temperature	200°C
Sheath gas flow	9 L/min
Capillary voltage	2500 V
Nozzle voltage	500 V
Fragmentor	120 V
Skimmer	40 V
Oct 1 RF Vpp	700 V
Reference mass	64.0158 and 922.0098
Acquisition	High sensitive slicer position 2GHz extended dynamic mode with 5 spectra/sec
Collision energies	0, 10 and 20 V

Results and Discussion

Differential Analysis

The data files from degraded and control samples were processed using recursive molecular feature extraction in Mass Profiler Software. Height filters of 4000 counts for extracted compound features, quality score 100 and fold change >4 were applied for statistical analysis. Figure 2 shows the statistical analysis results of feature plot of log abundance ratio vs retention time.

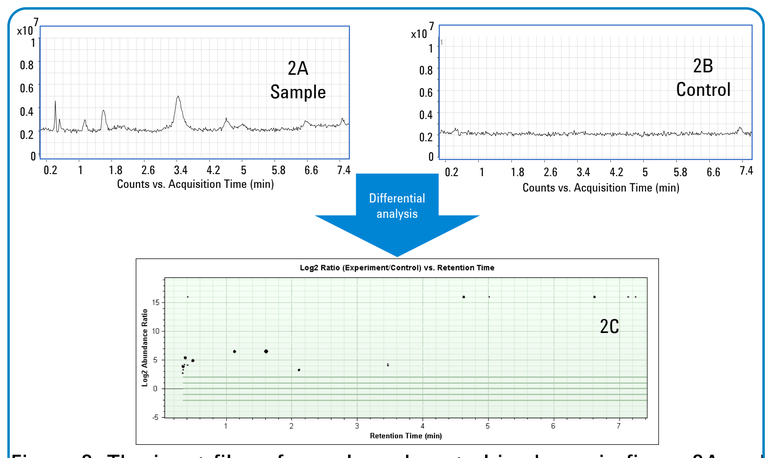

Figure 2. The input files of sample and control is shown in figure 2A and 2C respectively. The chlorhexidine peak elutes after 7.4 min and hence not shown on the plot. Figure 2C shows log abundance ratio vs retention time plot after differential analysis. The size of the bubble is proportional to the abundance value.

PCA plot

The PCA plot reveal that the test chlorhexidine sample is different and separates from the control sample (Figure 3). This indicates that the degraded chlorhexidine sample have features that are different than the control group. The four red dots represent four degraded samples under the same conditions showing minor differences within, while the control groups do not separate indicating no variation within.

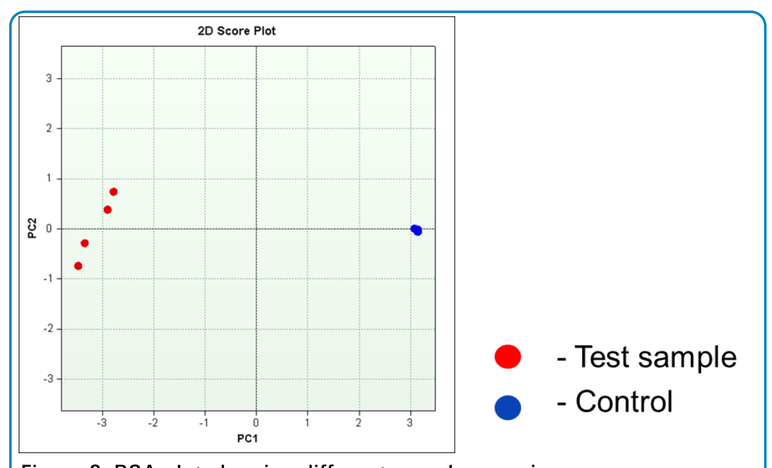

Figure 3. PCA plot showing different sample grouping

Detection

The accurate mass database and library was built in-house using ~~the~~ standards. The database also includes literature reported mass, formula and structures of chlorhexidine impurities. Post statistical analysis, the differential list of compounds were searched against accurate mass database using ID Browser feature of the Mass Profiler software. The results identified 4-chlorhexidine in degraded samples (Figure 4).

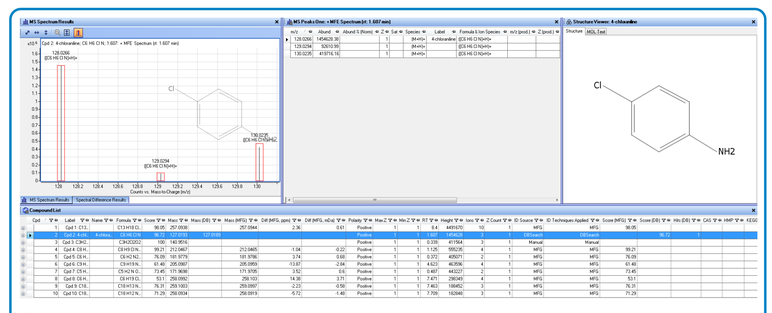

Figure 4. Identification of potential genotoxic compounds using in-house database/library

Feature summary of the compounds

The summary of differential analysis and database search results are shown in Table 3. 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. The differential score was calculated using the Student's t-test. A value between 0 and 100 represents whether the data groups are significantly different. A larger value indicates with higher confidence that the data sets in the two groups are different.

Table 3. Feature summary of differential analysis showing compounds which "up" fold change

MassProfiler Feature Summary									
ID	Formula	Name	RT	Mass	Abundance	Q Score	Log2(A1/A2)	Expression	Diff.Score
1	C13 H18 Cl O3		8.4	257.0942	4664291	100	7.86	up	100
2	C6 H6 Cl N	4-chloroaniline	1.61	127.0192	1480334	100	6.51	up	100
3	C3H2Cl2O2		0.34	140.9516	592521	100	3.89	up	99.9
4	C8 H9 Cl N4 O		1.13	212.0464	551298	100	6.48	up	100
5	C6 H2 N2 O3 S		0.37	181.9781	543519	100	5.4	up	99.9
6	C9 H19 N S2		4.62	205.098	456724	100	16	up	100
7	C5 H2 N O4 S		0.49	171.9698	429576	100	4.89	up	100
8	C6 H19 Cl N6 O S		7.47	258.1016	299708	100	5.68	up	100
9	C18 H13 N O		7.46	259.1006	186491	100	6.35	up	100
10	C18 H12 N O		7.70	258.0938	184274	100	16	up	100

Results and Discussion

Identification

In data independent acquisition (All Ions MS/MS) of drug samples, both MS and MS/MS information was available. The product ions formed for 4-chloroaniline in degraded samples were searched against the library spectra. Additionally, the co-elution score plots of extracted precursor and matched fragments chromatograms are shown in Figure 5. 4-chloraniline was identified based on accurate mass fragment matching and co-elution of the precursor and product ions. The qualified spectra were used as qualifier and quantifier ions for the quantification method.

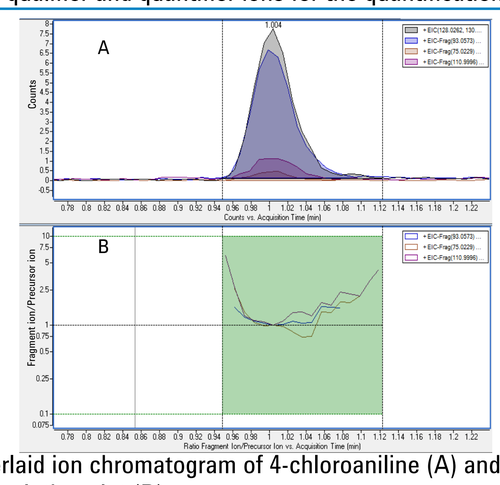

Figure 5. Overlaid ion chromatogram of 4-chloroaniline (A) and the calculated co-elution plot (B)

Quantification of potential genotoxic compound

4-chloroaniline was found with three qualified spectra from the library MS/MS spectrum where the fragments are selected from high energy channel. The qualifiers and quantifier fragments contains compound names, retention time, precursor ion, fragment ion, collision energies and relative abundances were exported to MassHunter Quantitative Analysis software to setup a quantitative method as shown in Figure 6.

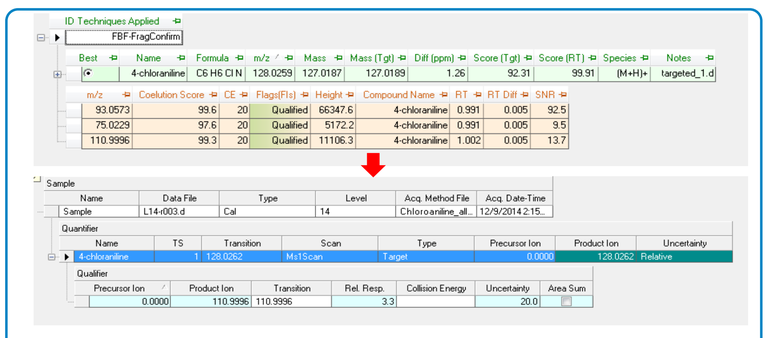

Figure 6. Quantitative method setup from compound identification results

The All Ions MS/MS acquisition was used to draw a calibration curve of 4-chloroaniline. A calibration curve of >3 orders of magnitude was observed from 0.1 to 300 ng/mL. The Agilent 6545 QTOF which was calibrated in high sensitivity mode helped to achieve lower limit of detection thereby enabling sensitive analysis. In addition, tuning for low mass (50-250 m/z) using Swarm autotune was also applied since some of the product ions of 4-chloroaniline were of low mass. The results of sample analysis showed average value of 29 ng/mL in the degraded sample. Potential genotoxic compounds typically have a limit of 0.05% quantitation limit. If 1 mg chlorhexidine is dissolved in 10 mL solution, a 0.05% limit would be require quantitation down to 50 ng/mL

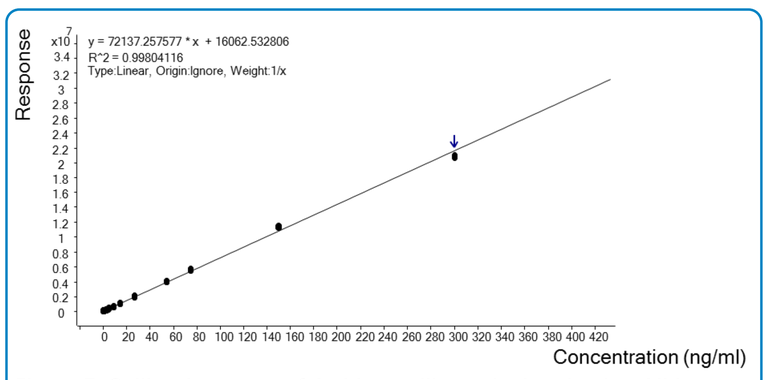

Figure 7. Calibration curve of 4-chloroaniline calculated using All Ions MS/MS acquisition

Conclusions

- The workflow demonstrated here involves screening of samples for potential genotoxic compounds using differential analysis, identification and quantitation.
- A screening method uses MS or All Ions MS/MS acquisition files to directly process in Mass Profiler (rev 7.0) software.
- Differential analysis can rapidly distinguish componet differences between sample sets.
- A differential compound list has been created to facilitate indentification of target compounds.
- The compounds in the differential list are identified using an in-house build database containing potential genotoxic compounds.
- The potential genotoxic compounds were confirmed by library fragment matching and are exported for quantitation.
- If detected, additional experiments are performed for quantitation
- The test sample processed with this technique had a concentration of ~29 ng/mL (assay linear range from 0.1 – 300 ng/mL).
- With the application of threshold setting, degradation due to storage and large batch of QC samples can be routinely monitored.