Streamlining the Metabolite Identification Workflow Using High-Resolution Q-TOF Data and Mass-MetaSite

Application Note

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Abstract

This application note describes the separation of metabolites from a pharmaceutical drug using an Agilent 1290 Infinity LC System and an Agilent 6530 Accurate Mass Q-TOF LC/MS System to generate mass spectral data. Mass-MetaSite can be used to identify the metabolites once Agilent MassHunter files are integrated into Mass-MetaSite. Finally, WebMetabase can be used as an automatic database and reporting system to streamline the metabolite identification process.

Introduction

The availability of high resolution accurate mass Q-TOF data has revolutionized the metabolite identification field. An expert can generate a large amount of accurate data for each chromatographic peak in order to assign the chemical structure of metabolites. Therefore, sophisticated data processing software is needed to help transform this huge volume of data into information that can be shared in an efficient manner.

This application note demonstrates the use of Mass-MetaSite integrated with the Agilent MassHunter file format to identify drug-related material peaks in the chromatogram, assign chemical structures for each of the peaks found, database the fragmentation analysis, and share the results with other team members in the drug discovery process.

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Experimental

Equipment

- Agilent 1290 Infinity LC System with an Agilent 1290 Infinity Binary Pump
- Agilent 1290 Infinity Autosampler
- Agilent 1290 Infinity Thermostatted Column Compartment (TCC)
- Agilent 6530 Accurate-Mass Q-TOF LC/MS

Columns

- Agilent ZORBAX Rapid Resolution
- High Definition (RRHD) SB-C18, 2.1 × 100 mm, 1.8 µm Sample preparation

Sample preparation

The following stock solutions were created for this analysis:

- NADPH-GS solution (for 1 mL of solution: 15 mg NADPH, 13.8 mg G-6-P, 6 µL G-6-P dehydrogenase, 1 mL of 48 mM $MgCl_2$)
- Human Microsomal Liver (HLM) preparation (UltraPool HLM 150 cod. 452117 from BD Bioscience)
- Human CYP3A4 + Reductase + b5 Supersomes–0.5 nmole (from BD Bioscience)
- Human CYP2C9 + Reductase + b5 Supersomes–0.5 nmole (from BD Bioscience)
- Human CYP2D6 + Reductase + b5 Supersomes–0.5 nmole (from BD Bioscience)

Metabolite sample

Metabolite samples were created by adding 0.1 M phosphate buffer at pH=7.4. (For HLM, 234 µL was added to a 1.4 mL tube, and for recombinant enzymes, 215 µL was added). Next, 6.3 µL of HLM or 25 µL of the recombinant-enzyme preparation were added to the tubes. Then 5 µL of the 0.25 mM sample solution in acetonitrile:water was followed by 5 µL of the NADPH-GS solution. The resulting solutions were vortexed and incubated at 37 °C for 0, 3, 7, 12, 20, and 30 minutes. The reactions were stopped by adding 250 µL of acetonitrile. Next, the tube was centrifuged at 10,000 rpm, for 10 minutes at 4 °C, and 200 µL of the supernatant was analyzed.

Data dependent MS/MS

Two compounds were run, generating three MS/MS spectra, exclusion for 0.25 minutes.

Agilent Jet Stream Technology was run in positive mode with reference mass solution. The mass list was generated using the list of potential metabolites generated by Mass-MetaSite from the chemical structure of the parent compound and the microsomal reactions from Mass-MetaSite and three generations of metabolites.

LC Method	
Solvent A	Water + 0.1 % formic acid
Solvent B	Acetonitrile + 0.1 % formic acid
Flow	0.5 mL/min
Gradient	0 min, 5 % B
	15 min, 75 % B
	15.1 min, 95 % B
	16 min, 95 % B
Stop time	16 min
Post time	10 min
Injector volume	5 µL
Sample cooler	4 °C
Needle wash	50 % methanol for 5 s
TCC temperature	60 °C
Q-TOF MS and MS/MS Method	
The Agilent 6530 Q-TOF was operated in the 2 GHz enlarged dynamic range mode with the following acquisition parameters:	
Sheath gas	11 L/min at 400 °C
Dry gas	7.0 L/min
Dry temperature	300 °C
Nebulizer	45 psi
Mass range	100–1,000
Fragmentor	200 V
Skimmer	60 V
Capillary	3,500 V
Collision energy	30 V

Mass-MetaSite software

To integrate the MassHunter data, three MS/MS files (blank, substrate and incubation files) were input for each Mass-MetaSite run. A total of six runs were introduced in the Mass-MetaSite batch process that corresponds to each incubation time point under analysis using the settings detailed in Table 1.

Table 1. Mass-MetaSite batch process.

Hardware settings	
Application version	MetaSite CLI 3.1.4 Mass 1.3.0 alpha
Computation mode	DD-MS/MS
Mass spectrometer	Agilent Q-TOF
Ionization mode	[M+H]+
Prediction setting	
CYP(s)	LIVER
Import conditions	Protonation policy NEUTRALIZE; Protonation policy normalize pH 7; Maximum number of conformers: 20
Metabolite generation settings	
Minimum mass	50
Ignore metabolites stereochemistry enabled	true
Ignore redundant metabolites enabled	true
Ignore redundant metabolites fraction	30
Reaction mechanism enabled	Microsomal reaction set
Drug related material analysis	
Split computed DRM peaks	false
Max metabolite count enabled	true
Max metabolite limit	20
Peak area threshold	0.01
Rescue computed DRM peaks enabled	true
Same peak tolerance	10 mDa
Chromatogram automatic filtering threshold	0.95
MS automatic filtering threshold	0.9
MS/MS automatic filtering threshold	0.9
Fragmentation analysis	
Bond breaking limit	4
Number of metabolite generations	2
Bond breaking reorganization enabled, N-oxide	true
Bond breaking reorganization enabled, even electron	true
Bond breaking reorganization enabled, odd electron	true

Results and Discussion

The Mass-MetaSite process consists of two steps. The first step identifies drug related material using the chromatogram obtained from the Q-TOF MS and MS/MS methodology in order to find chromatographic peaks related to the parent compound (Figure 1). It reports two types of peaks. One type correlates to a mass shift from the parent corresponding to one (green peaks) or more (brown peaks) metabolic reactions (phase I and II) set prior to the calculation within the software. The second type are observed peaks from unknown metabolites (cyan peaks) that do not correspond to any of the list of known metabolic transformations.

The second step assigns the chemical structures that give rise to the peaks.

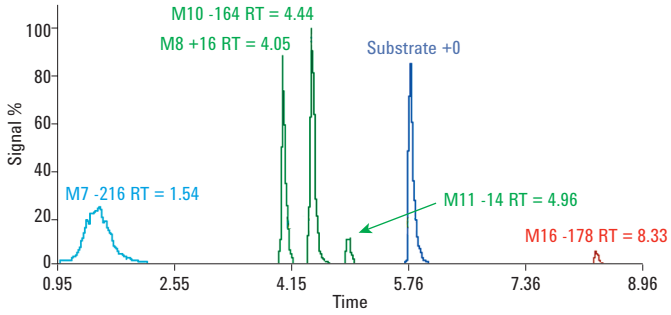

Figure 1. Extracted chromatogram after 20 minute incubation. Green peaks- first generation of metabolites, brown peak-second generation, cyan peak- unknown, and blue peak- parent compound.

In order to perform this operation, the software produces a set of theoretical fragments (structure and mass) for the parent and the metabolites. Next, the masses for that fragment list are compared with the actual fragment ions found in the MS and MS/MS spectra (Figure 2). From this comparison, the software can localize the region in the molecule where the metabolic reaction may take place. If the fragment analysis cannot specify a single atom in the parent that produces the metabolic reaction, a markush representation of the metabolites compatible with the mass spectra data is shown and the MetaSite Site of Metabolism (SoM) algorithm[1] is applied in order to prioritize the different potential structural solutions.

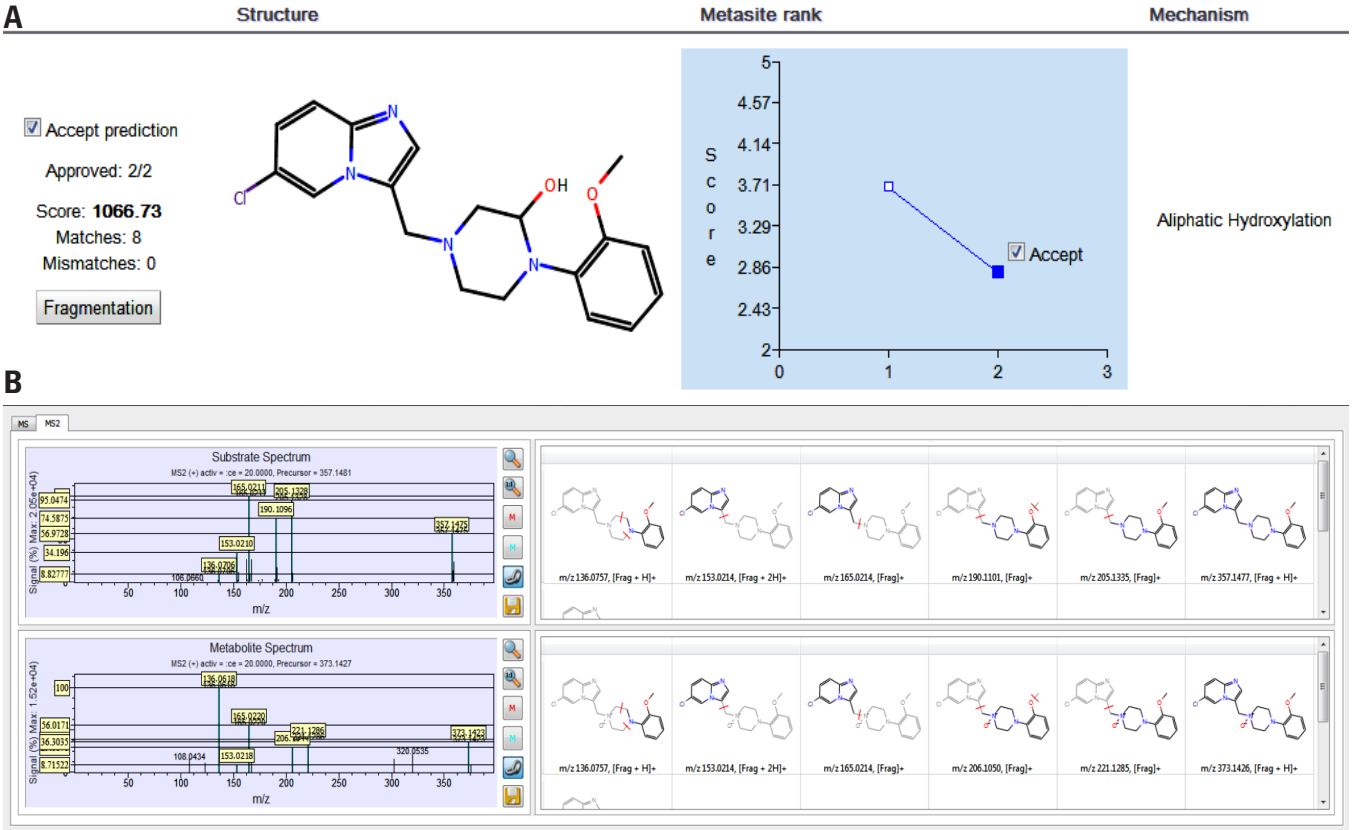

Figure 2. Metabolite structures were assigned to the 16+ metabolites observed in the chromatogram. The metabolite has a Mass-MetaSite score of 1066.73 (A) and it has two potential solutions (only one shown in the figure). The MetaSite rank is the most likely predicted SoM. The automatic fragment analysis displays the fragments that are used for the interpretation (B).

The SoM prediction is used to indicate the regioselectivity of the metabolic reaction only for phase I metabolism. It is based on an analysis of the potential interactions of the compounds in the cytochrome P450 cavity and the chemical reactivity towards oxidation.

Once the LC/MS/MS data has been automatically analyzed, it is submitted to a web server (WebMetabase). The metabolite ID expert can connect through a web browser, such as Internet Explorer and inspect the results, including the peak finding and fragmentation analysis, to elucidate the metabolite structure. The expert at this point can accept, reject, or edit the proposed results. In addition, the expert can customize the data view that other members of the discovery team have on that particular experiment (Figure 3). When the experiment has been approved by the expert, its state is changed and it can be viewed by other members of the discovery team, making the report of the metabolite identification straightforward from the interpretation of the data. Additionally, the WebMetabase application can follow metabolites across different samples of the same compound, for example analyzing kinetic data for both disappearance of the parent compound and appearance of the metabolites.

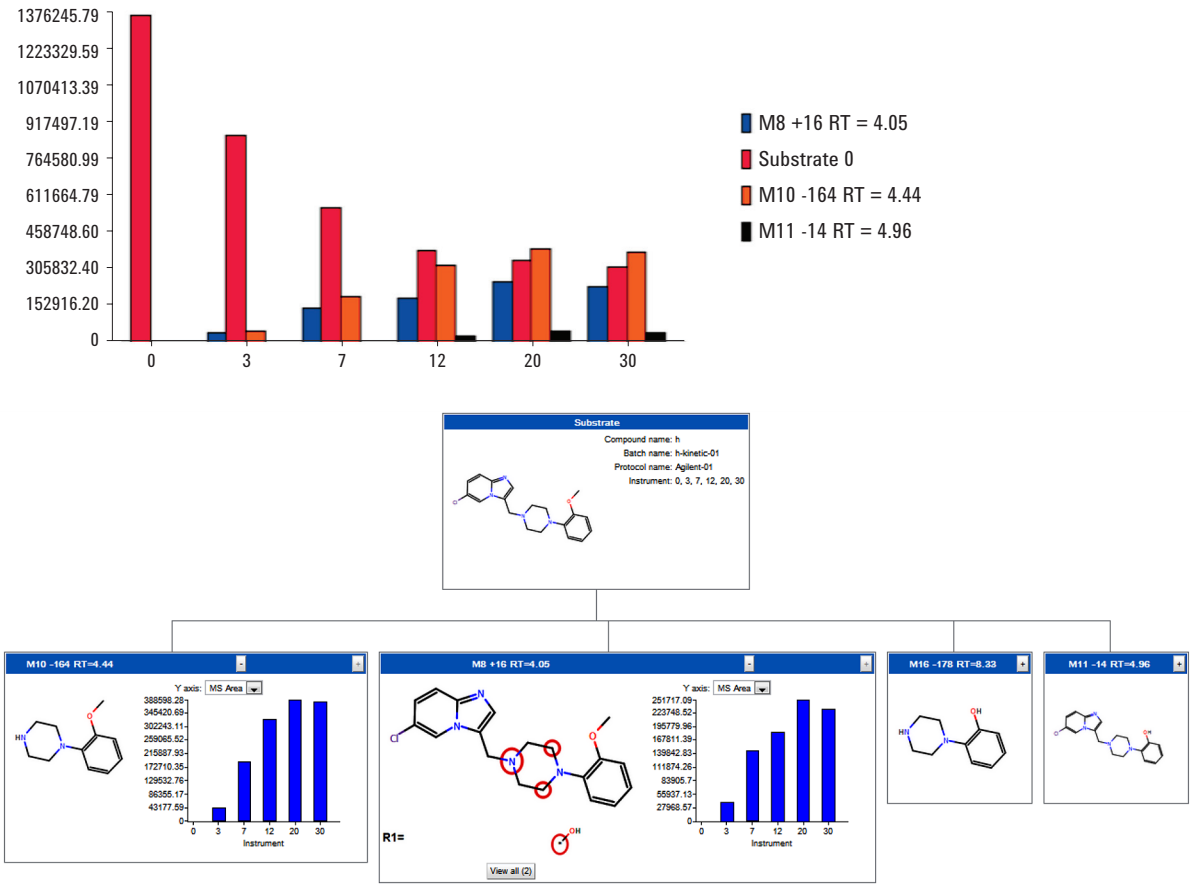

Figure 3. The disappearance or the appearance of the parent or metabolites can be plot together (A). The automatic report displays the information that the metabolite ID expert shows to the rest of the discovery team (B).

Conclusions

This application note describes the integration of Agilent Q-TOF high-resolution accurate-mass measurements with software that processes the data to suggest peaks and assign them to the metabolite identification expert through the automatic Mass-MetaSite batch process and reporting system based on the WebMetabase application. The computational tool performs a systematic analysis of the fragmentation by breaking bonds to elucidate the structure of the metabolite and report the suggested structural fragments to the expert. In addition, when the mass spectral data is not enough to localize a definitive structure of the metabolite, the MetaSite SoM prediction algorithm is applied to suggest a potential metabolite that is in agreement with the spectral data.

The WebMetabase tool collects the data from the automatic procedure in Mass-MetaSite and presents the data to the expert for approval, keeping all the fragments in a database and allowing the user to approve, remove, or edit the proposed results. WebMetabase also allows the analysis of experiments with multiple samples, such as when performing a kinetic analysis and maintaining both qualitative and quantitative data in a single system. The entire process is summarized in Figure 4.

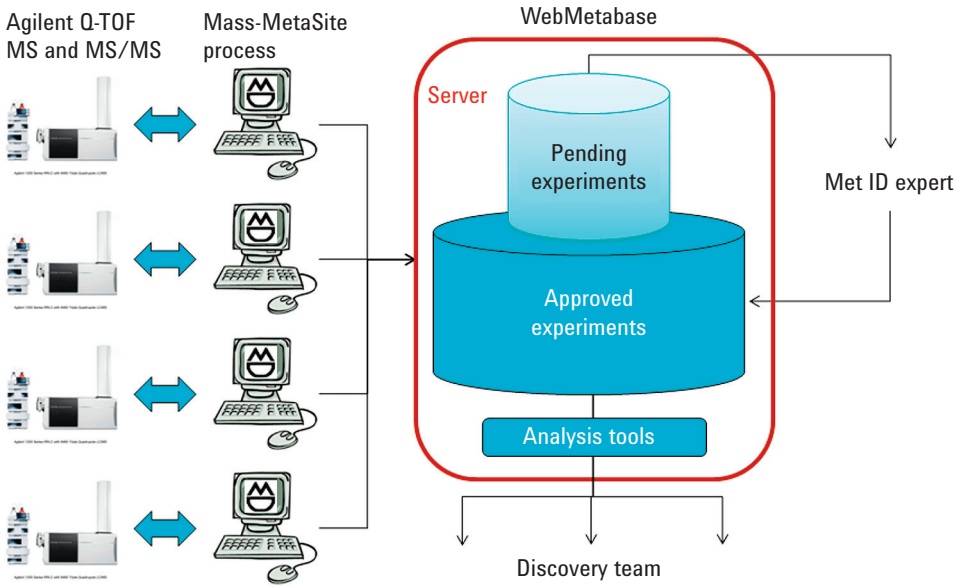

Figure 4. The entire work flow reduces the time for analysis of metabolite identification, making the process more efficient.

References

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