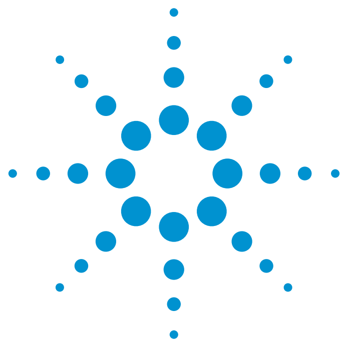

Agilent LC/Q-TOF and Mass MetaSite Software for Seamless Metabolite Identification

Application Note

Small Molecule Pharmaceuticals

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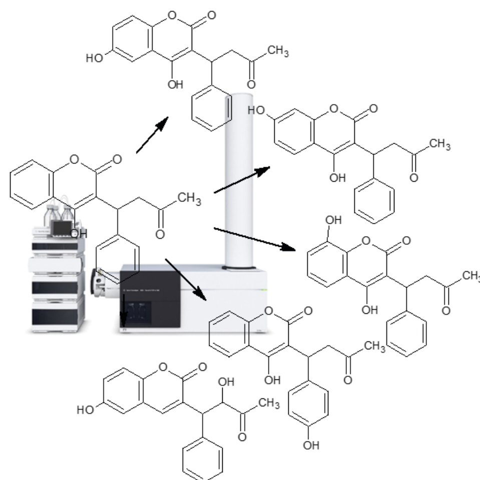
Abstract

The detection and identification of metabolites is a critical step in the early drug discovery phase. The identification of metabolites from complex biological samples is often challenging, as they may be present at trace levels. This Application Note describes the seamless integration of a high sensitivity Agilent LC/Q-TOF instrument with software tools (Mass MetaSite software) for the identification of drug metabolites. Mass MetaSite can predict metabolic transformations, estimate the primary site of metabolism, and assign the structures of metabolites from accurate mass MS/MS data. The workflow is demonstrated by predicting and identifying primary metabolites from rat hepatocyte incubation of warfarin for 24 hours.



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Introduction

Understanding drug metabolism is essential in drug discovery and development. Regulatory agencies require identification of all significant metabolites in the early phases of drug selection. Availability of metabolite reference standards are often limited, and this impacts confident identification. However, due to technological advancements in high sensitivity accurate mass measurements, MS/MS data can provide tremendous structural information for metabolite identification. Thus, high resolution accurate mass spectrometry is as an ideal technique for the identification of metabolites from a complex biological matrix. This Application Note describes the use of an Agilent LC/Q-TOF system and Mass-MetaSite software for the identification and structure elucidation of warfarin metabolites.

Experimental

Chemicals and reagents

Methanol was LC/MS grade, and formic acid was LC/MS eluent additive grade (Sigma-Aldrich, Bangalore, India). Milli-Q water was used in all experiments (Merck, Darmstadt, Germany). All other chemicals used for the study were purchased from Sigma-Aldrich (Bangalore, India).

Hepatocyte incubation sample

Samples derived from rat hepatocytes incubated for 24 hours with racemic warfarin were provided by a collaborator. Freshly isolated rat hepatocytes were plated on a 24-well collagen-coated plate at a seeding density of 0.2 million cells/well/0.5 mL in Williams E media, supplemented with fetal bovine serum (Life Technologies, Carlsbad, CA). The plating buffer was aspirated after 2 hours, and washed twice with serum-free Williams E media. Subsequently, 0.5 mL of serum-free Williams E media containing 30 µM of warfarin was added to each well, and the plate placed in a CO_2 (5 %) incubator at 37 °C for 24 hours. Then, the cells were scraped and quenched in two volumes of acetonitrile. The reaction mixture was then centrifuged at 12,000 rpm for 10 minutes, and the supernatant used for the LC/Q-TOF analysis.

Workflow

After the chromatographic separation of the metabolites using an Agilent 1290 Infinity LC system, accurate mass MS and MS/MS spectra were acquired using an Agilent 6550 Q-TOF system. The data acquisition files were processed using Mass-MetaSite software to identify metabolites. The workflow is illustrated in Figure 1.

Instrumentation and software

Reverse phase chromatographic analysis was carried out using an Agilent 1290 Infinity LC system consisting of:

- Agilent 1290 Infinity Binary Pump G4220A
- Agilent 1290 Infinity Autosampler G4226A
- Agilent 1290 Infinity Thermostat G1330B
- Agilent 1290 Infinity Thermostatted Column Compartment G1316C

The separation was achieved using an Agilent ZORBAX Eclipse Plus Phenyl Hexyl, 2.1 × 150 mm, 1.8 µm column. Agilent 1290 Infinity LC method parameters are tabulated in Table 1.

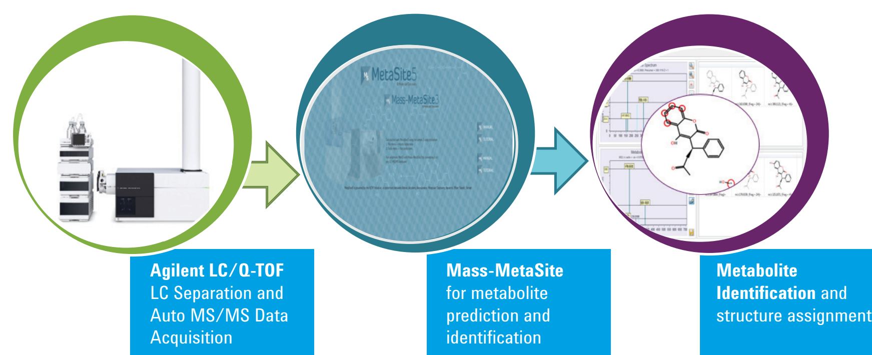

Figure 1. Metabolite identification workflow using an Agilent LC/Q-TOF instrument with Mass MetaSite software.

Table 1. LC Method parameters.

Agilent 1290 Infinity LC Chromatographic Run Conditions	
Mobile phases	A) 0.05 % formic acid in water B) 0.05 % formic acid in methanol
Column	Agilent ZORBAX Eclipse Plus Phenyl Hexyl, 2.1 × 150 mm, 1.8 µm
Column temperature	55 °C
Injection volume	3 µL flush port for 10 seconds
Needle wash	Methanol
Flow rate	0.24 mL/min
Gradient	Time (min) %B 0 55 12 98 18 98 21 55 25 55

The eluent from the LC separation was detected using an Agilent 6550 Q-TOF Mass Spectrometer. The electrospray source was equipped with Agilent Jet Stream (AJS) thermal gradient focusing technology to enhance sensitivity, and was operated in positive mode. Agilent MassHunter Workstation (Version B.05.01) Software was used for the acquisition and data analysis. Compounds with *m/z*, 121.0505 and 922.0098 were used as internal reference masses, and reference mass correction was enabled in the software to achieve simultaneous accurate mass calibration during acquisition. Table 2 shows the method parameters of the 6550 Q-TOF. This instrument setup was used to collect data-dependent MS/MS spectra of blank (acetonitrile), substrate (warfarin in acetonitrile), and hepatocyte samples incubated with warfarin for 24 hours. Data were processed using Mass MetaSite software (Version: 3.2.0) using the parameters listed in Table 3.

Table 2. Agilent 6550 Q-TOF method parameters.

Agilent 6550 Q-TOF acquisition parameters	
Acquisition mode	Auto MS/MS
Ion polarity	Positive
Gas temperature	275 °C
Gas flow	8 L/min
Nebulizer	25 psi
Sheath gas	325 °C
Sheath gas flow	10 L/min
Nozzle voltage	500 V
Fragmentor	150 V
Skimmer	65 V
Collision energy	Formula, Charge: 1, Slope: 3.6, Offset: –4.8
V Cap	3,500
Ref mass	121.0505 and 922.00979
MS range	100–1,000 *m/z* at 6 spectra/s rate
MS/MS range	50–2,000 *m/z* at 5 spectra/s rate

Table 3. MassMetaSite parameters.

Mass-MetaSite parameters	
Computation mode	DD-MS/MS
Mass spectrometer	Agilent 6550 Q-TOF
Ionization mode	$[M+H]^+$
CYP(s)	Liver
Number of metabolite generations	2
Substrate Bond breakage limit	4
Bond breaking recognition, even electron	Enabled
Bond breaking recognition, odd electron	Enabled
Bond breaking recognition, N-oxide	Enabled
Reaction mechanism	Microsomal reaction set
Same peak tolerance (amu)	0.01
Chromatogram automatic filtering threshold	0.97
MS automatic filtering threshold	0.97
MS/MS automatic filtering threshold	0.9
Signal filtering	Automatic
Scan filtering	Automatic

Results and Discussion

Mass MetaSite for site of metabolism and metabolite prediction

Mass MetaSite was used to predict the most probable metabolic sites in the drug using the *mol* file for warfarin structure. The liver enzymatic metabolism model was selected, and the software predicted a list of metabolites, as shown in Figure 2. These are phase 1 metabolites, and mainly include hydroxylated metabolites. The probability of formation of a specific metabolite was derived from the site of metabolism prediction, and the results are shown as bar graphs (Figure 3). The exact monoisotopic mass and the RRT (based on related lipophilicity of each metabolite) were also predicted.

LC/Q-TOF Analysis

The LC method using an Agilent ZORBAX Eclipse Plus Phenyl Hexyl column was able to give a commendable separation of the peaks from the complex incubated sample matrix. Figure 4 shows the Q-TOF Total Ion Chromatogram (TIC) of the elution profile.

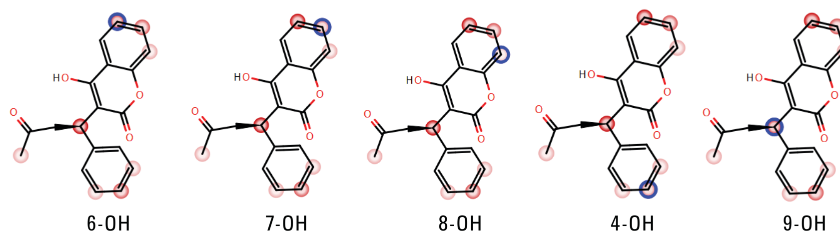

Figure 2. Predicted hydroxywarfarin metabolite structures.

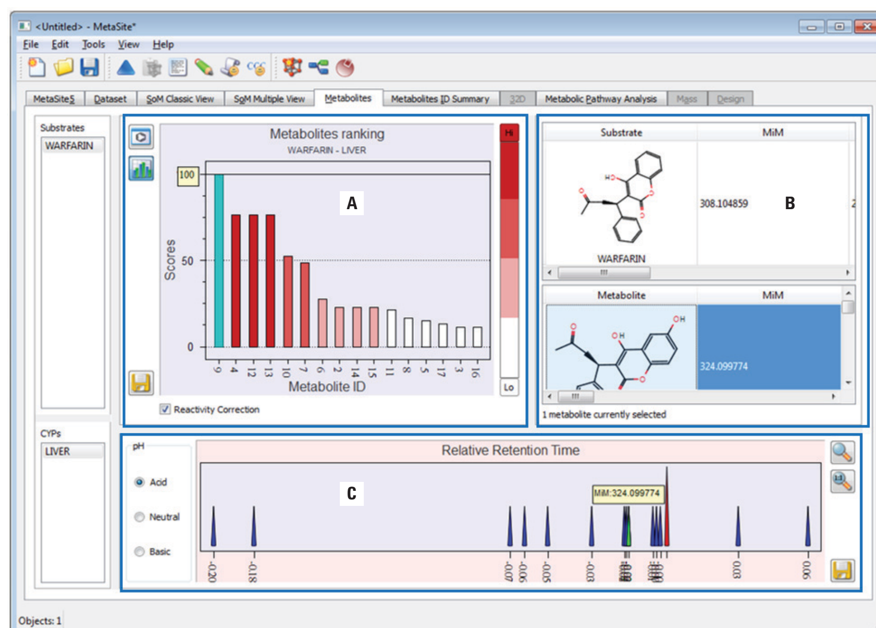

Figure 3. A) Probability of formation of metabolites are displayed as bar chart. The blue bar in panel A is the most probable metabolite. By clicking each bar in panel A, the details of each predicted metabolite can be displayed in panel B. C) The relative retention time compared to substrate in acidic mobile phase condition is displayed along with accurate mass of each metabolite.

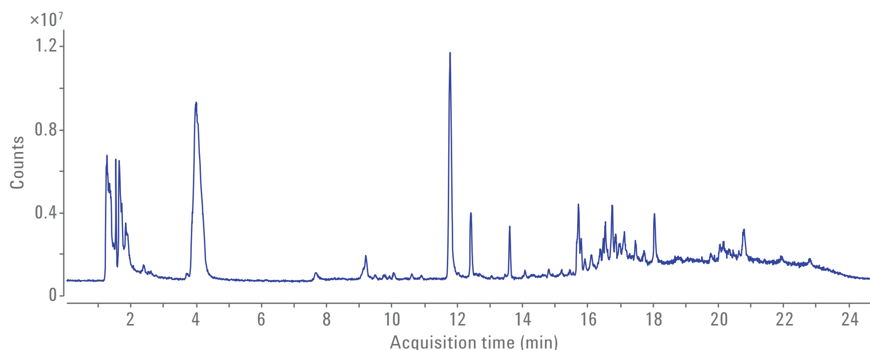

Figure 4. TIC Scan of rat hepatocyte incubated sample with warfarin. The complexity of the sample is evident from many peaks in TIC.

Mass MetaSite for metabolite identification

Typically, the analysis of Q-TOF data with Mass-MetaSite consists of two steps:

- Identification of drug and related metabolites as chromatographic peaks from the complex TIC trace
- Assignment of chemical structures for each identified metabolite

While assigning the structures, the software predicts the theoretical fragments for the parent drug and metabolites, and compares them with experimentally obtained MS and MS/MS results. In this experiment, Mass MetaSite identified three hydroxywarfarin metabolites from Q-TOF data (Figure 5). The green color of these peaks confirmed that the metabolites originated from phase 1 metabolic reactions. The mass values of the identified metabolites were matched with the predicted values.

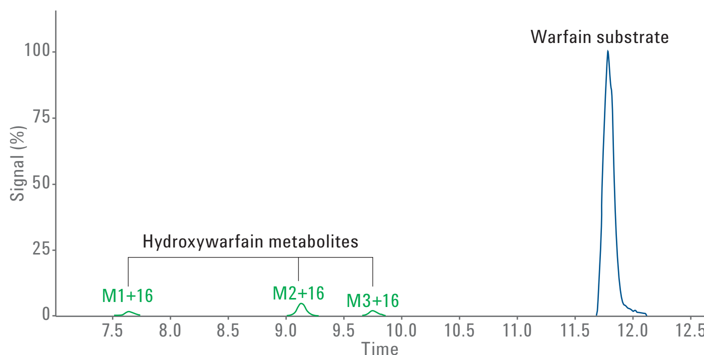

Figure 5. Elution profile of warfarin and hydroxy metabolites originated from hepatocyte incubation. The three hydroxy metabolites are labeled M1+16, M2+16, and M3+16, along with the substrate warfarin peak.

Structural identification of hydroxywarfarin regio-isomers based on fragmentation pattern

Oxidative metabolism of warfarin can lead to the formation of five major regio-isomers of hydroxyl metabolites[1]. While 4′- and 10-hydroxywarfarin have characteristic MS/MS fragments, the other three hydroxyl metabolites of warfarin, namely 6-, 7-, and 8-hydroxywarfarin, have a similar fragmentation pattern with a signature fragment at *m/z* 179, and cannot be differentiated from each other by MS/MS spectra[2,3].

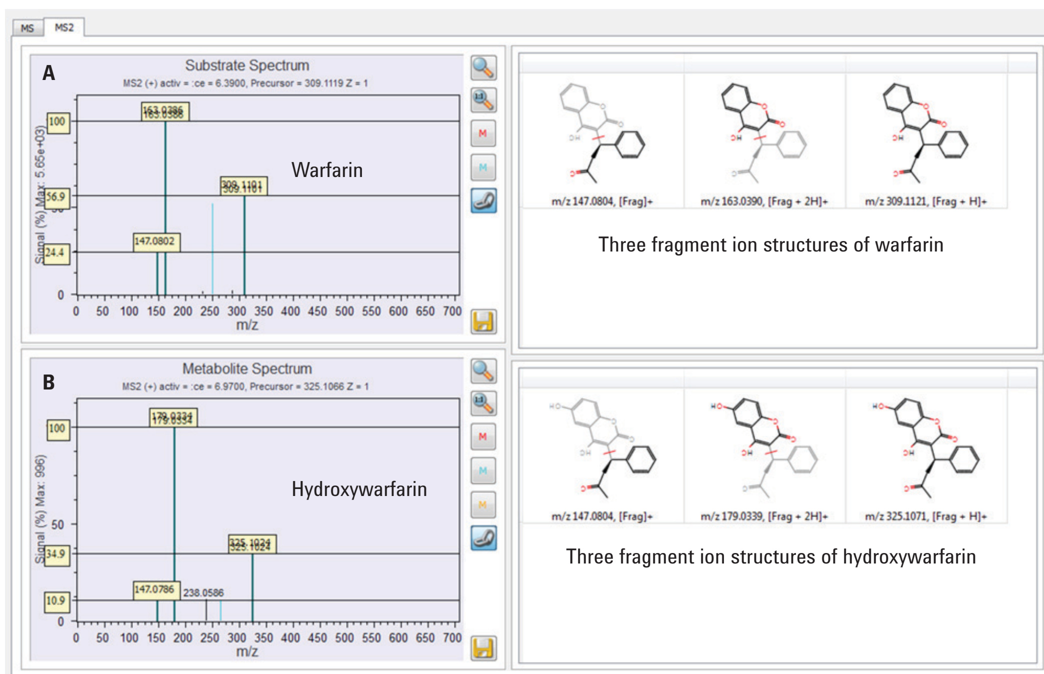

Figure 6. Structure assignment for 6-hydroxywarfarin metabolite (M+16). The automatic fragment analysis displays the substructures used for the interpretation. A shows the fragmentation pattern and structure elucidation of substrate (warfarin) and B shows the M+16 metabolite. The predicted structure of *m/z* 179 is theoretically possible for 6-, 7-, or 8-hydroxywarfarin.

Q-TOF MS/MS data of hepatocyte incubation was processed using Mass MetaSite software to assign chemical structures to the regio-isomers of hydroxywarfarins. Using unique MS/MS fragmentation patterns, the software identified one of the metabolite peak as 4-hydroxywarfarin. However, two other metabolite peaks give similar fragmentation patterns with a signature fragment of *m/z* 179. Thus, it is evident that the two other metabolite peaks can be assigned to either 6-, 7-, or 8-hydroxywarfarin. Figure 6 shows, as an example, results from structure assignment of the third hydroxywarfarin (M+16) metabolite peak which elutes at 9.7 minutes. Here, Mass-MetaSite software identified the peak as 6-hydroxywarfarin. Further observation of mass accuracy values from MassHunter Qual software is shown in Figure 7.

Conclusion

This study describes an easy and reliable method to identify drug metabolites from biological samples. An Agilent 1290 Infinity LC system gave excellent chromatographic separation and an Agilent 6550 Q-TOF provided high resolution accurate mass MS/MS data. Furthermore, the use of Mass MetaSite software was useful for the confident identification of metabolite structures in a systematic way. The experimental workflow effortlessly identified three regio-isomers of hydroxy warfarin metabolites formed from incubation of warfarin with rat hepatocytes for 24 hours. Understanding structures of metabolites derived from the parent drug compound increases the confidence of their identification and facilitates better drug design and discovery.

Spectrum Identification Results: + Scan (rt: 9.065-9.209 min)

Automatically Show Columns

Best	ID Source	m/z	Formula	Species	Score	Diff (ppm)	Score (MFG)
	MFG	325.1069	C19 H16 O5	(M+H)+	98.31	0.79	98.31

Species	m/z	Score (iso. abund)	Score (mass)	Score (MS)	Score (MFG)	Score (iso. spacing)	Ion Formula	Height	Score (MFG
(M+H)+	325.1069	96.62	99.55	98.31	98.31	97.84	C19 H17 O5	53740.8	

Height (Calc)	Height Sum% (Calc)	Height % (Calc)	m/z (Calc)	Diff (mDa)	Height	Height %	Height Sum %	m/z	Diff (ppm)
52716	80.4	100	325.1071	0.2	53740.8	100	82	325.1069	0.56
11036.5	16.8	20.9	326.1104	0.8	10128.3	18.8	15.4	326.1097	2.41
1638.3	2.5	3.1	327.113	-0.7	1529	2.8	2.3	327.1137	-2.24
182	0.3	0.3	328.1156	1.1	174.8	0.3	0.3	328.1145	3.49

Figure 7. Screenshot from Agilent MassHunter Qualitative Analysis Software, showing mass accuracy values for hydroxywarfarin metabolite [$(M+H)^+$, $C_{19}H_{17}O_5$, *m/z* 325.1069]. Unique design features of Agilent Q-TOF offer high mass accuracy values which helps to confirm the elemental compositions and, thus, identify metabolites with high confidence level. Low mass error values (< 3.5 ppm) for isotopes are highlighted.

Acknowledgement

We thank Dr. Murali Subramanian, BMS-Biocon Research Center, Syngene Ltd., Bangalore, India for his immense support for generously providing us hepatocyte incubated samples.

References

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Published in the USA, September 1, 2015
5991-6129EN

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