Software assisted identification of metabolites from pharmaceutical drugs using the Agilent 1290 Infinity LC System with an Agilent 6530 Q-TOF MS System and the expert prediction system Meteor

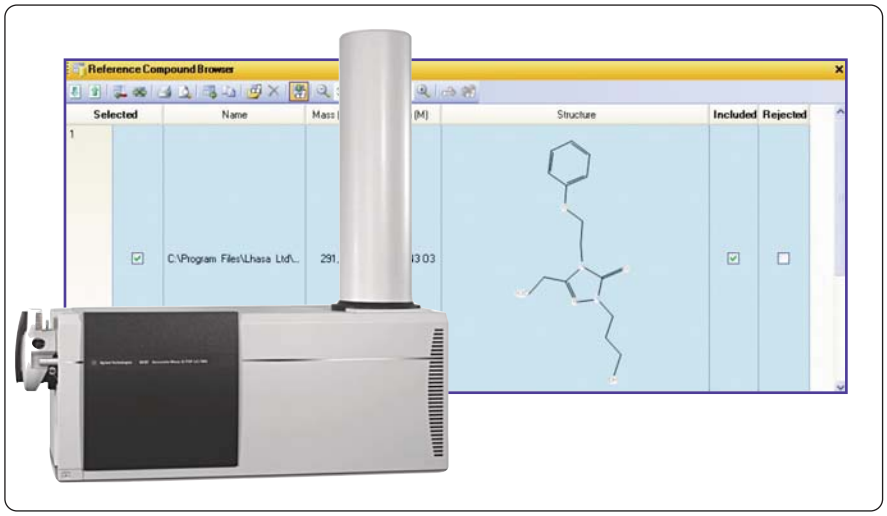
Application Note

Pharmaceutical, Drug Discovery

Authors

Edgar Naegele, Horst Lehmann
Agilent Technologies GmbH
Hewlett-Packard St. 8
76337 Waldbronn
Germany

Sian E. Ives, Kate Langton,
Lhasa Limited,
22-23 Blenheim Terrace
Woodhouse Lane
Leeds LS2 9HD United Kingdom
www.lhasalimited.org/meteor



Abstract

This Application Note describes:

- The separation of metabolites from a pharmaceutical drug on an Agilent 1290 Infinity LC System
- The generation of mass spectral data on an Agilent 6530 Accurate-Mass Q-TOF liquid chromatograph/mass spectrometer (LC/MS)
- The introduction of results from the expert metabolism prediction software Meteor (Lhasa Limited, Leeds, UK) into the Agilent MassHunter Metabolite ID software
- The software assisted identification of metabolites by the Agilent MassHunter Metabolite ID software

Agilent Technologies

Introduction

During the metabolism of pharmaceutical drugs, a large number of metabolites can be generated. Some of these metabolites are easy to predict and identify because they result from a single minor reaction. However, some metabolic reactions can change the pharmaceutical molecule significantly by a reaction which breaks the molecule into different parts and initiates reactions with the fragments. It can be very difficult to predict and identify such products manually. Metabolism prediction software provides a solution to this problem.

This Application Note demonstrates the use of the software package Meteor, (Lhasa Limited, Leeds, UK) with the Agilent MassHunter Metabolite ID software to predict and subsequently identify metabolites of a pharmaceutical drug.

Experimental

Equipment

Agilent 1290 Infinity LC system with 1290 Infinity pump with integrated degasser

Agilent 1290 Infinity Autosampler with thermostat

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

Stock solutions

Phosphate buffer 100 mM, pH 7.4; 5 mM $MgCl_2$

Nefazodone hydrochloride 250 µM in phosphate buffer (Figure 1)

NADPH solution, 10 mg/mL in phosphate buffer

Microsmal S9 preparation from rat liver, 20 mg protein/mL

Metabolite sample

1. Dilute 25 µL of nefazodone (Figure 1) with 180 µL phosphate buffer in a 1.5 mL Eppendorf vial.
2. Add 15 µL S9 preparation and 30 µL NADPH solution.
3. Vortex and incubate for 1 h at 37 °C.
4. Stop the reaction by adding 750 µL ice cold acetonitrile and centrifuge at 14,000 rpm for 15 minutes.
6. Remove the supernatant into a new 1.5 mL Eppendorf vial and evaporate to dryness in a speedvac.
6. Dissolve the remaining pellet in 250 µL HPLC solvent A.

Control sample

1. Dilute 25 µL of nefazodone with 210 µL phosphate buffer in a 1.5 mL Eppendorf vial.
2. Add 15 µL S9 preparation (without the NADPH, the metabolic reaction will not start, only enzymatic degradation will occur).
3. Vortex and incubate for 1 h at 37 °C.
4. Add 750 µL ice-cold acetonitrile and centrifuge at 14,000 rpm for 15 minutes.
5. Remove the supernatant to a new 1.5-mL Eppendorf vial and evaporate to dryness in a speedvac.
6. Dissolve the remaining pellet in 250 µL HPLC solvent A.

LC method

Solvent A:	Water + 0.1% formic acid (FA)	
Solvent B:	AcN + 0.1 %FA.	
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 sec.	
TCC temperature:	60 °C.	

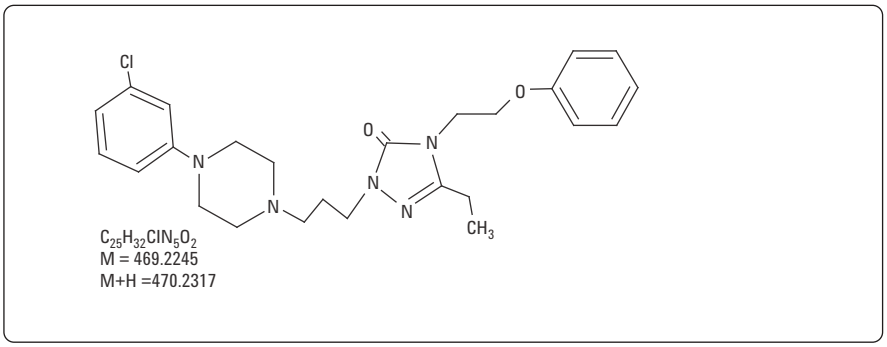


Figure 1
Formula of the pharmaceutical compound nefazodone.

QTOF 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:

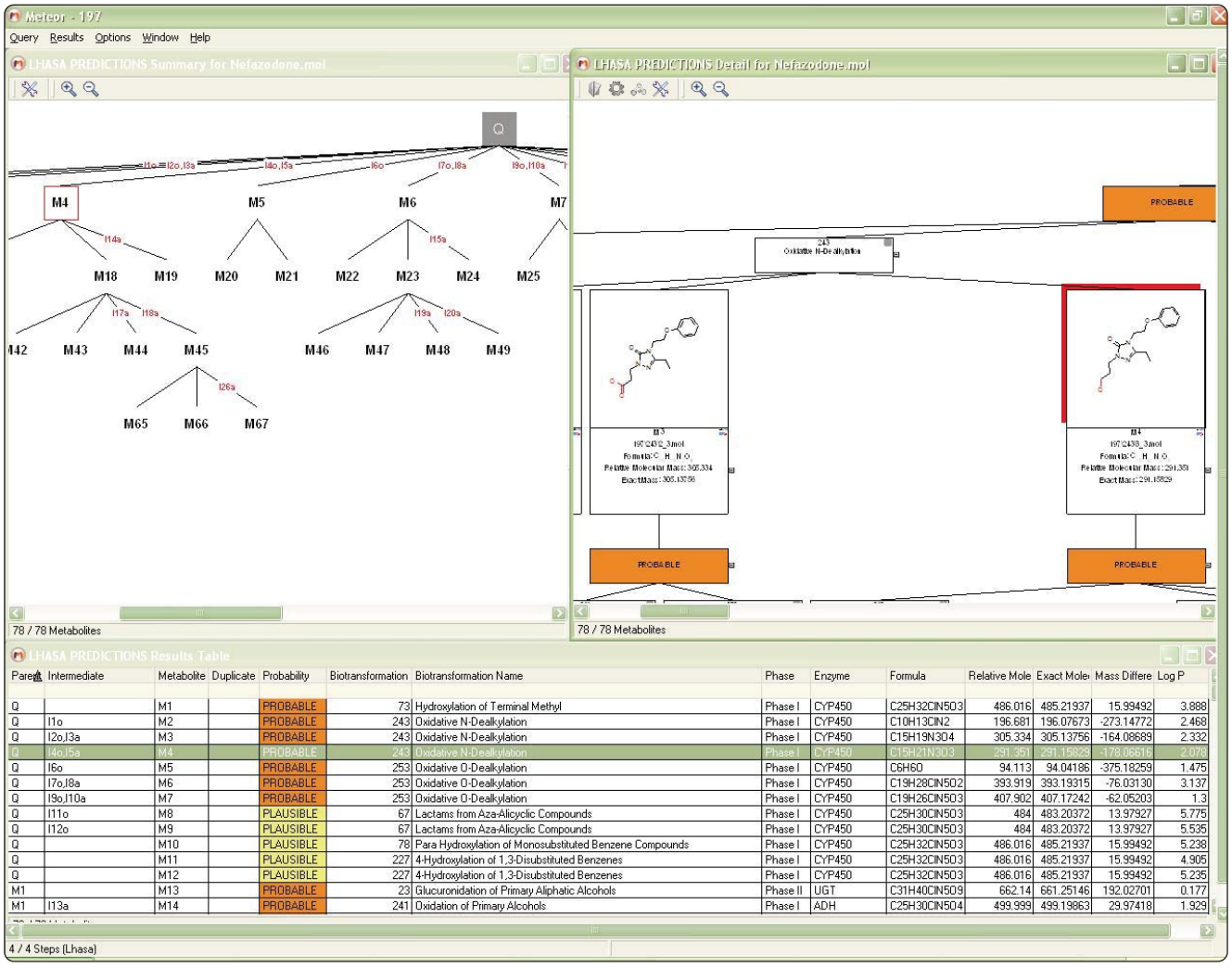
Sheath gas:	11 L/min at 400 °C
Dry gas:	7.0 L/min
Dry Temp:	300 °C
Nebulizer:	45 psi
Mass range:	100-1000
Fragmentor:	200 V
Skimmer:	60 V
Capillary:	3500 V
Collision energy:	30 V

Data dependent MS/MS: 2 compounds, 3 MS/MS spectra, exclusion for 0.25 min.

Agilent Jet Stream Technology in positive mode with reference mass solution (*m/z* 121.05087 and *m/z* 922.00979).

Data analysis method in the Metabolite ID software

A comparison was made between the metabolite compounds (metabolite sample) data file and the parent drug (control sample) data file. All detectable mass signals were extracted from the MS level data using the Molecular Feature Extraction (MFE) algorithm. Related compound isotope masses and adduct masses were then grouped together into discrete molecular features and the chemical noise was removed. The compound lists of the metabolized sample and the control were then compared. All compounds that were new or had doubled signal intensity in the metabolized sample were considered potential metabolites and subjected to further analysis by different algorithms. The algorithms can either identify and qualify new metabolites or simply qualify metabolites found by another algorithm.



Parent	Intermediate	Metabolite	Duplicate	Probability	Biotransformation	Biotransformation Name	Phase	Enzyme	Formula	Relative Mole	Exact Mole	Mass Differe	Log P
Q		M1		PROBABLE	73	Hydroxylation of Terminal Methyl	Phase I	CYP450	C25H32ClN5O3	486.016	485.21937	15.99492	3.888
Q	I1o	M2		PROBABLE	243	Oxidative N-Dealkylation	Phase I	CYP450	C10H13ClN2	196.681	196.07673	-273.14772	2.468
Q	I2o,I3a	M3		PROBABLE	243	Oxidative N-Dealkylation	Phase I	CYP450	C15H19N3O4	305.334	305.13756	-164.08689	2.332
Q	I4o,I5a	M4		PROBABLE	243	Oxidative N-Dealkylation	Phase I	CYP450	C15H21N3O3	291.351	291.15829	-178.06616	2.076
Q	I6o	M5		PROBABLE	253	Oxidative O-Dealkylation	Phase I	CYP450	C6H6O	94.113	94.04186	-375.18259	1.475
Q	I7o,I8a	M6		PROBABLE	253	Oxidative O-Dealkylation	Phase I	CYP450	C19H28ClN5O2	393.919	393.19315	-76.03130	3.137
Q	I9o,I10a	M7		PROBABLE	253	Oxidative O-Dealkylation	Phase I	CYP450	C19H26ClN5O3	407.902	407.17242	-62.05203	1.3
Q	I11o	M8		PLAUSIBLE	67	Lactams from Aza-Alicyclic Compounds	Phase I	CYP450	C25H30ClN5O3	484	483.20372	13.97927	5.775
Q	I12o	M9		PLAUSIBLE	67	Lactams from Aza-Alicyclic Compounds	Phase I	CYP450	C25H30ClN5O3	484	483.20372	13.97927	5.535
Q		M10		PLAUSIBLE	78	Para Hydroxylation of Monosubstituted Benzene Compounds	Phase I	CYP450	C25H32ClN5O3	486.016	485.21937	15.99492	5.238
Q		M11		PLAUSIBLE	227	4-Hydroxylation of 1,3-Disubstituted Benzenes	Phase I	CYP450	C25H32ClN5O3	486.016	485.21937	15.99492	4.905
Q		M12		PLAUSIBLE	227	4-Hydroxylation of 1,3-Disubstituted Benzenes	Phase I	CYP450	C25H32ClN5O3	486.016	485.21937	15.99492	5.235
M1		M13		PROBABLE	23	Glucuronidation of Primary Aliphatic Alcohols	Phase II	UGT	C31H40ClN5O9	662.14	661.25146	192.02701	0.177
M1	I13a	M14		PROBABLE	241	Oxidation of Primary Alcohols	Phase I	ADH	C25H30ClN5O4	499.999	499.19863	29.97418	1.929

Figure 2
Meteor results display.

Simultaneously, nefazodone was run through Meteor (Version 11) on the following processing constraints:

- 'Do not grow from phase 2 products'
- Absolute reasoning 'plausible'
- Relative reasoning 'n = 2'

A metabolic tree was produced (Figure 2). The metabolites were saved as an SDfile (structure-data file, which contains the structural information and associated data items for one or more compounds), and input to the Metabolite ID software.

In Metabolite ID, the Meteor SD file was searched against all compounds and matching masses were assigned with the corresponding structure.

Metabolites can be qualified by the user or qualified automatically when their final score is above the stringently defined relevance threshold. The results from all algorithms are populated in a results table that can be inspected "At-a-glance" and reported.

Results and discussion

The results table generated in the Metabolite ID software shows all relevant metabolites at-a-glance (Figure 3).

The left side of the table shows information about the retention time, the molecular and ion mass, the metabolic reaction and the overall acceptance level for each individual metabolite.

In the middle of the table, the results from the individual comparison algorithms are displayed in a red-green pattern. These algorithms compare the potential metabolite compound to the parent drug. If an algorithm exceeds its defined threshold it is marked as "relevant" in green and identifies a potential metabolite.

On the right side of the table, additional information is given in a yellow-blue pattern. If special additional information, for example, MS/MS spectra or reference structures, which are not calculated by an algorithm are available then the availability is coded in blue (for example, the availability of an assigned structure from Meteor in the final column).

Metabolites Browser

Short Summary <Filtered>

Metabolites								Sample...	EIC Co...	Isotopic...	Fragme...	Formulas	MS/M...	Referen...
Warnings	Name	RT	Mass	m/z	Relevance	User Qual.	Parent	Qualified	Qualified	Qualified	Qualified	Assigned	MS/MS	Assigned
1		4,242	375,2274	376,2346	62,5	☑	☐	☑	☑	☐	☐	☑	☑	☑
2		4,250	423,1665	424,1737	100,0	☑	☐	☑	☑	☑	☐	☑	☐	☑
3		4,356	409,1887	410,1959	79,2	☑	☐	☑	☑	☑	☐	☑	☑	☑
4	3x Hydroxylation	5,421	517,2092	518,2165	83,3	☑	☐	☑	☑	☐	☑	☑	☑	☑
5		5,627	373,2119	374,2192	62,5	☑	☐	☑	☑	☐	☐	☑	☑	☑
6		5,696	423,1677	424,1750	100,0	☑	☐	☑	☑	☑	☐	☑	☐	☑
7		5,741	409,1889	410,1962	79,2	☑	☐	☑	☑	☑	☐	☑	☑	☑
8		5,800	307,1532	308,1605	62,5	☑	☐	☑	☑	☐	☐	☑	☑	☑
9		6,348	407,1734	408,1807	79,2	☑	☐	☑	☑	☑	☐	☑	☑	☑
10		6,432	421,1516	422,1588	79,2	☑	☐	☑	☑	☑	☐	☑	☑	☐
11		6,522	321,1324	322,1397	41,7	☑	☐	☑	☑	☐	☐	☑	☑	☑
12		6,557	289,1428	290,1501	52,6	☑	☐	☑	☑	☐	☐	☑	☐	☑
13	2x Hydroxylation	6,582	501,2150	502,2223	79,2	☑	☐	☑	☐	☑	☑	☑	☑	☑
14		6,589	407,1727	408,1799	79,2	☑	☐	☑	☑	☑	☐	☑	☑	☑
15	Methylene to Ketone	7,245	483,2059	242,6102	52,6	☑	☐	☑	☐	☐	☐	☑	☐	☑
16	2x Hydroxylation	7,251	501,2154	502,2226	100,0	☑	☐	☑	☑	☑	☑	☑	☑	☑
17	Oxidative Dechlorination	7,611	451,2588	452,2661	100,0	☑	☐	☑	☑	☑	☑	☑	☑	☑
18	Demethylation and Hydroxylation	7,657	471,2036	472,2109	100,0	☑	☐	☑	☑	☑	☑	☑	☑	☐
19		8,036	305,1371	306,1444	41,7	☑	☐	☑	☑	☐	☐	☑	☑	☑
20		8,131	291,1590	292,1663	62,5	☑	☐	☑	☑	☐	☐	☑	☑	☑
21		8,347	305,1376	306,1448	20,8	☑	☐	☑	☐	☐	☐	☑	☑	☑
22	Hydroxylation	8,440	485,2202	486,2275	100,0	☑	☐	☑	☑	☑	☑	☑	☑	☑
23		8,442	507,2007	508,2080	100,0	☑	☐	☑	☑	☑	☐	☑	☐	☐
24		8,447	391,1769	196,5957	52,6	☑	☐	☑	☐	☐	☐	☑	☐	☑
25	Hydroxylation and Ketone Formation	8,514	499,1996	500,2069	100,0	☑	☐	☑	☑	☑	☑	☑	☑	☑
26	Ethyl to alcohol	8,909	457,1889	458,1961	100,0	☑	☐	☑	☑	☑	☑	☑	☑	☐
27	Hydroxylation	9,146	485,2200	486,2273	100,0	☑	☐	☑	☑	☑	☑	☑	☑	☑
28		9,146	507,2002	508,2075	100,0	☑	☐	☑	☑	☑	☐	☑	☐	☐
29	Nefazodone	10,262	469,2251	470,2324	79,2	☑	☑	☐	☑	☑	☑	☑	☑	☑
30	Nefazodone	10,330	469,2247	470,2320	79,2	☑	☑	☐	☑	☑	☑	☑	☑	☑
31	Methylene to Ketone	10,408	483,2040	484,2113	100,0	☑	☐	☑	☑	☑	☑	☑	☑	☑

Figure 3
At-a-glance table of identified metabolites.

As one can see, there are some metabolites which are assigned to a known metabolic reaction. These metabolites are "expected metabolites". For some other metabolites, not all identifying algorithms exceed the defined threshold. These are the "unexpected metabolites". One example is compound number 20 (highlighted in Figure 3) which elutes at a retention time of 8.13 min and at an *m/z* of 292.1663. This compound did not exceed the threshold for the isotopic pattern-identifying algorithm and the MS/MS fragment pattern-identifying algorithm. A search of the Meteor result file found a predicted metabolite with a calculated masss of 291.1583; therefore a structure (Figure 4) and formula - $C_{15}H_{21}N_3O_3$ - could be assigned to this unexpected metabolite. The extracted ion chromatogram (EIC) and the extracted compound chromatogram (ECC) of this compound are shown in Figure 5A and 5B, respectively.

The comparison of the isotopic pattern of the metabolite compound and the parent drug shows that there is a significant difference (Figure 5C) in the measured isotopic pattern of the metabolite (blue) and the calculated isotopic pattern of the parent drug (green, CIP). The reason for this is the loss of the part of the parent drug nefazodone which contains a chlorinated phenyl ring (Figure 1, Figure 4).

The formula $C_{15}H_{21}N_3O_3$ of the metabolite compound is confirmed by accurate mass measurement with a mass accuracy of 2.54 ppm (Figure 6). The measured isotopic pattern of the metabolite compound is also confirmed by accurate mass measurement with high accuracy (Figure 6).

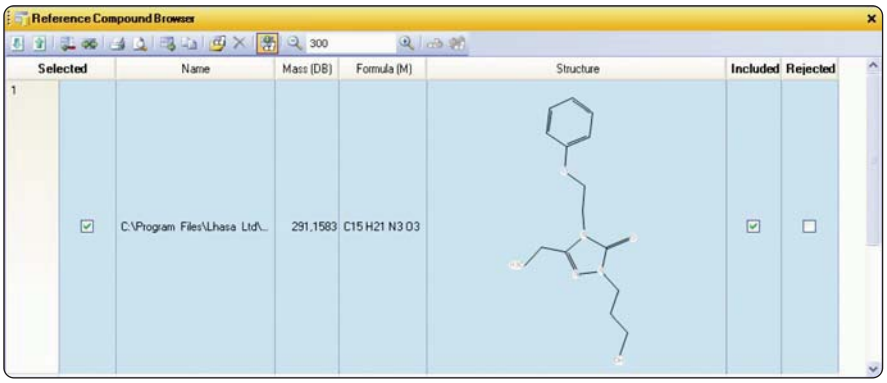


Figure 4
Result from the search of a Meteor metabolite prediction result file, which assigned a structure to the unexpected metabolite.

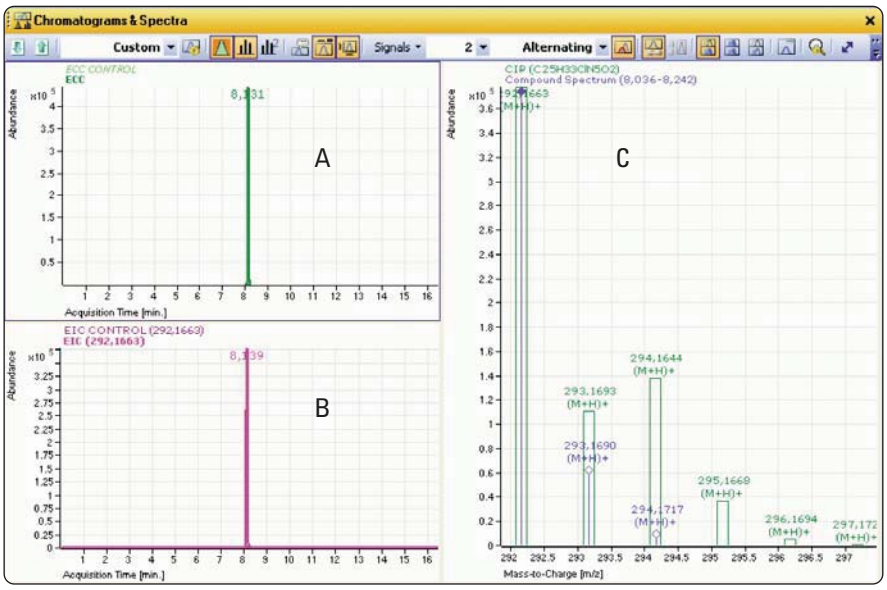


Figure 5
Chromatograms and spectra of unexpected metabolite at *m/z* 292.1663. A) Extracted compound chromatogram (ECC). B) Extracted ion Chromatogram (EIC). C) Measured isotopic pattern (blue) in comparison to the calculated isotopic pattern (green, CIP) of the parent drug .

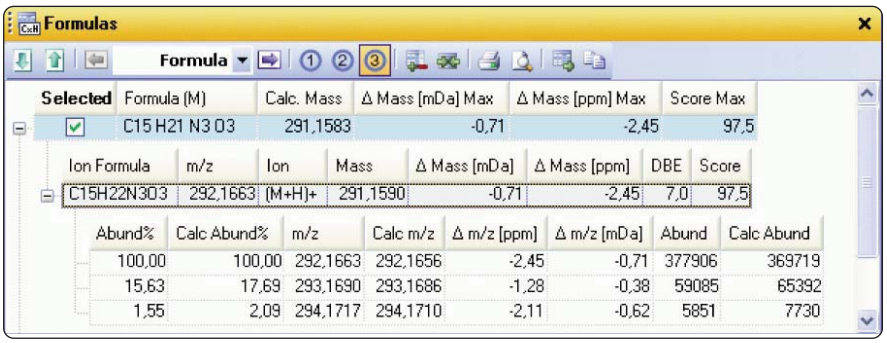


Figure 6
Calculated formula of the unexpected metabolite compound with calculated mass accuracy and isotopic pattern.

The Meteor-assigned metabolite structure can be confirmed by the interpretation of the obtained MS/MS spectrum (Figure 7). For all ions in the MS/MS spectrum a formula can be calculated (Table 1) and assigned to a structure fragment (insert in Figure 7).

Comparison of this metabolite spectrum (Figure 7, red) to the MS/MS spectrum of the parent drug nefazodone (Figure 7, blue) shows only a minor overlap, which is too insignificant for automatic detection.

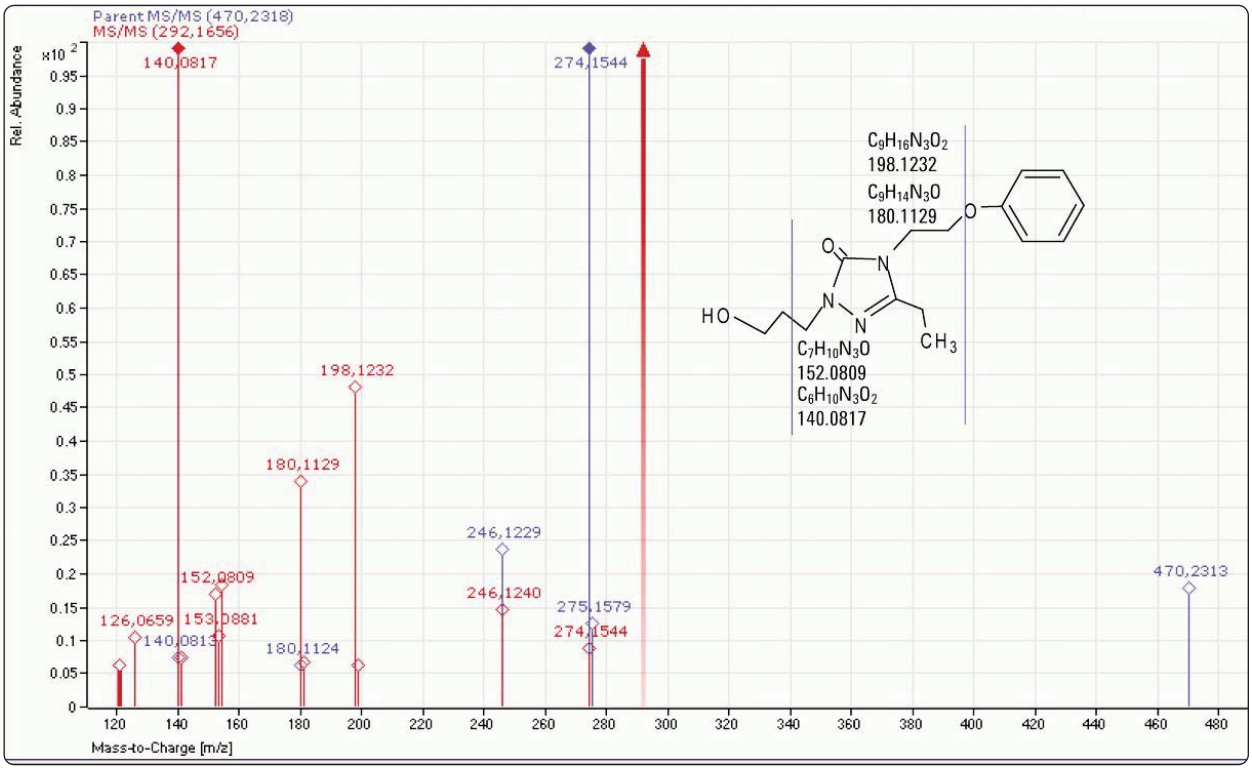


Figure 7
MS/MS spectrum with fragment assignment of the unexpected nefazodone metabolite identified by Meteor.

m/z	Ion formula	Calc. m/z	Δ *m/z* [mDa]	Δ *m/z* [ppm]	Neutral loss	Loss formula	Loss mass
121.0647	C_8H_9O	121.0648	0.09	0.71	171.1009	$C_7H_{13}N_3O_2$	171.1008
126.0659	$C_5H_8N_3O$	126.0662	0.31	2.46	166.0998	$C_{10}H_{14}O_2$	166.0994
140.0817	$C_6H_{10}N_3O$	140.0818	0.16	1.15	152.0840	$C_9H_{12}O_2$	152.0837
152.0809	$C_7H_{10}N_3O$	152.0818	0.89	5.86	140.0847	$C_8H_{12}O_2$	140.0837
180.1129	$C_9H_{14}N_3O$	180.1131	0.25	1.36	112.0528	$C_6H_8O_2$	112.0524
198.1232	$C_9H_{16}N_3O_2$	198.1237	0.48	2.45	94.0424	C_6H_6O	94.0419
246.1240	$C_{13}H_{16}0N_3O_2$	246.1237	–0.26	–1.06	46.0417	C_2H_6O	46.0419
274.1544	$C_{15}H_{20}N_3O_2$	274.1550	0.64	2.32	18.0113	H_2O	18.0106

Table 1
Calculated MS/MS fragment formulas and loss formulas for unexpected nefazodone metabolite fragmentation pattern.

Conclusion

This Application Note demonstrates how the use of a rule-based metabolite prediction software package (Meteor, Lhasa Limited, Leeds, UK) can be beneficial for the inclusion and source of metabolite structures within the MassHunter Metabolite id software. In this note, it has been demonstrated that when a structure cannot be identified or qualified based on known metabolic reactions, these unexpected metabolites can be assigned structures by Meteor based on mass and formula information. This can further be confirmed by accurate mass measurement.

www.agilent.com/chem/metid

© Agilent Technologies, Inc., 2010, 2015
August 21, 2015
Publication Number 5990-4583EN
For Research Use Only. Not for use in diagnostic procedures.

Agilent Technologies