Software Assisted Structure Assignment Of Pharmaceutical Drug Metabolites Using UHPLC QTOF MSMS With Metabolite Prediction Software

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# Introduction

As a result of metabolism multiple metabolites can be found in both in vitro and in vivo metabolism samples. However, some metabolic processes can change the parent molecule significantly: for example, with a reaction which cleaves into different parts and initiates reactions with the corresponding fragments, it may be very difficult to predict and identify such products manually. Metabolism prediction software can provide a solution to this problem. We present the use of a combination of the expert prediction system Meteor (Lhasa Limited, Leeds, UK) with the Agilent MassHunter Metabolite Identification software to predict and subsequently identify metabolites of the drug Nefazodone (Figure 1).



Figure 1: S Nefazodone.

Structure

of



### Experimental

<u>Metabolite sample /Control sample preparation</u> Phosphate buffer 100 mM, pH 7.4; 5 mM MgCl<sub>2</sub> Nefazodone hydrochloride 250 µM in phosphate buffer NADPH solution, 10 mg/mL in phosphate buffer Microsmal S9 preparation (rat liver), 20 mg protein/mL

- 1. Dilute 25 μL nefazodone (Fig. 1) with 180 μL (210 μL) phosphate buffer in a 1.5 mL Eppendorf vial.
- 2. Add 15 μL S9 preparation and 30 μL NADPH (No 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.
- 5. Remove 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.

### <u>LC method</u>

Agilent 1290 Infinity LC system

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

Solvent A: Water + 0.1% formic acid (FA), Solvent B: AcN + 0.1 %FA.

Flow: 0.5 mL/min. Inj. vol: 5 μL. Sample cooler: 4 °C. 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.

*Needle wash: 50% methanol for 5 sec, TCC temperature: 60 °C.* 

### <u>**Q-TOF MS and MS/MS method**</u>

Agilent 6530 Accurate-Mass Q-TOF LC/MS system Agilent Jet Stream Technology in positive mode Reference masses:m/z 121.05087 and m/z 922.00979 2 GHz enlarged dynamic range Mass range: 100 Da-1000 Da Sheath gas: 11 L/min at 400 °C Dry gas: 7.0 L/min, Dry Temp: 300 °C Nebulizer: 45 psi

						Procedule.						WOOKELE	
													-5
						<b>B C C C</b>							
3778 M	etabolitex.					78 / 78 Metabolites							-
<b>CON</b>													
areat In	lemediale	Netabolike D	uplicate I	Probability	Biotransformation	Biotransformation Name	Phase	Enzyme	Formula	Relative Mole	Exact Mole	Nass Differe L	og P
		M1		PROBABLE	73	Hydraigilation of Terminal Methyl	Phase I	CYP450	C25H32CIN503	495.016	485.21937	15.99492	3
11	D	M2		PRODABLE	243	Oxidative N-Deallylation	Phase I	EVT-450	E10H13CIN2	195.551	196.07673	-273.14772	
12	oJ3a	M3		PROBABLE	243	Oxidative N-Deallylation	Phase I	CYP490	C19H19N304	305.334	305.13796	-164.08689	
14	oJ5a	144		PROBABLE	243	Oxidative N-Deall plation	Phase I	EYP490	E19H21N303	291.351	291 15829	478.08616	
16	0	M5		PROBABLE	253	Oxidative D-Deallylation	Phase I	CYP450	DSHIBO	94.113	94.04186	-375.18259	1
17	o/Be	MB		PROBABLE	253	Oxidative B-Deallylation	Phase I	CYP450	C19H28CIN582	393.919	393.19315	76.03130	1
19	b,I1Da	M7		PROBABLE	253	Oxidative B-Deallylation	Phase I	CYP490	C19H26CIN503	407.902	407.17242	-62.05203	_
11	10	MB		PLAUSIBLE	67	Lectens from Aze-Alicyclic Compounds	Phase I	CYP450	C29H30CIN503	484	483.20372	13.97927	-
11	2o	M9		PLAUSIBLE	67	Lecters from Aze-Alicyclic Compounds	Phase I	CYP490	C29H30CIN503	484	483.20372	13.97927	-
		M10		PLAUSIBLE	78	Para Hydroxylation of Monosubotituted Benzene Compounds	Phase I	CYP450	C25H32CIN503	495.015	485.21937	15.99492	1
		M11		PLAUSIBLE	227	4-Hydroxylation of 1.3-Disubstituted 8 enzenes	Phase I	CYP490	C25H32CIN503	495.015	485.21937	15.33432	
		M12		PLAUSIBLE	227	4-Hydroxylation of 1.3-Dioubstituted Benzenes	Phase I	CYP450	C25H32CIN503	495.015	485.21937	15.33432	1
		M13		PROBABLE	23	Glucuronidation of Prinkary Aliphatic Alcohols	Phase II	UGT	C31H40CIN509	652.14	661.20146	192.02701	(
11	36	N14		PROBABLE	241	Ovidation of Primary Alcoholo	Phase I	ADH	C25H30CIN504	433, 555	459.13963	29.97418	1
													-
													a.,

Figure 2: Meteor results display.

Fragmentor: 200 V, Skimmer: 60 V, Capillary: 3500 V, Collision energy: 30 V

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





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# **Results and Discussion**

#### <u>Data Analysis method</u>

Molecular feature extraction (MFE) for control and metabolite sample.

- 1. Extract all detectable mass signals
- 2. Isotopic pattern and adduct grouping
- 3. Noise removal

➤Criteria for compound list selection:

- New peaks
- Intensity doubled peaks

Metabolite Prediction (Reference1)

- Compound processed in Meteor, Version 11
- Processing Constraints

>Do not grow from phase 2 products

- Absolute reasoning 'plausible'
- ➢ Relative reasoning 'n = 2'
- > Metabolic tree (Figure 2).

Metabolites saved as Structure data format (SDF)

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 automatically when their final score is above the stringently defined relevance threshold. The results from all algorithms were populated in a results table that could be inspected "At a glance" and reported.

Figure 3 shows the 'At a glance' table. The left hand side columns show retention time, m/z, and biotransformation assignment. The middle columns show individual comparison algorithms as results:

predefined threshold exceeded: green predefined threshold missed: red

The right hand columns show additional information such as formula assignment, MS/MS spectra availability and reference structure availability.

8 8 8	🐖 Short Su	ummary •	•	-iltered> *	1 32 00 0	4018	43							
Metabolites	1	_						Sample	EIC Co.	Isotopic	Fragme_	Formulas	MS/M_	Referen
Warnings	Name	BT	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	~		~		~		~	~	2
4	3x Hydroxylation	5,421	517,2092	518,2165	83,3							~	<b>v</b>	
5		5,627	373,2119	374,2192	62,5							<b>V</b>		
6		5,696	423,1677	424,1750	100,0									
7		5,741	409,1889	410,1962	79,2		ā							
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								2	
14		6,589	407,1727	408,1799	79,2			~	~	~				<b>V</b>
15	Methylene to Ketone	7,245	483,2059	242,6102	52,6									
16	2x Hydroxylation	7,251	501,2154	502,2226	100,0						<b>V</b>			
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 1		8,131	291,1590	292,1663	62,5							<b>V</b>	~	
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	2		~						~
25	Hydroxylation and Ketone Formation	8,514	499,1996	500,2069	100,0	<b>V</b>		2	2	~	<b>V</b>	~	~	
26	Ethyl to alcohol	8,909	457,1889	458,1961	100,0			<b>V</b>						
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: Metabolite ID 'At a glance' table.

Expected metabolites can directly be assigned to a known metabolic reaction. For other metabolites, not all identifying algorithms may exceed the defined threshold. These may be "unexpected metabolites", e.g. Compound 20 (highlighted in Figure 3) which elutes at a 8.13 min with 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.



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

A search of the Meteor result file found a predicted metabolite with a calculated mass of 291.1583; therefore a *structure* (Figure 4, top right) and formula -  $C_{15}H_{21}N_3O_3$  - could be assigned to this unexpected metabolite.





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Figure 5: Chromatograms and spectra of unexpected metabolite 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.

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	Sele	cted	Formul	a (M)	Ca	lc. Ma:	ss 1	∆ Mas	s [mD	a] Max	ΔM	lass (ppm) M	ах	Score	Max		^
<b>.</b>		2	C15 H2	21 N3 O3		291,15	583			-0,71		-	2,45	i .	97,5		
	le	on For	mula	m/z	lor	1	Mas	s	ΔМ	ass (mDa	]	Mass [ppm]		BE S	core		
		15H2	2N3O3	292,1663	(M·	+H)+	291	,1590		-0,	71	-2,4	15	7,0	97,5		
		AЬ	und%	Calc Abund	1%	m/z		Calc	m/z	∆ m/z [p	opm]	∆ m/z [mD	a]	Abund	Calo	Abund	
			100,00	100	0,00	292,1	663	292,	1656		-2,45	i -0	,71	377906	3	369719	_
		••••	15,63	17	,69	293,1	690	293,	1686		-1,28	-0	,38	5908	5	65392	
	1		1,55	2	2,09	294,1	717	294,	1710		-2,11	-0	,62	585	1	7730	~

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



### **Results and Discussion**

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 (Figure 5C) showed that there was a significant difference in the measured isotopic pattern of the metabolite (blue) and the calculated isotopic pattern (CIP) of the parent drug (green), due to the loss of the part of the nefazodone which contains a chlorinated phenyl ring (Figures 1 and 4).

### Conclusions

This work demonstrates the use of the combination of a rule-based metabolite prediction software (Meteor, Lhasa Limited, Leeds, UK) with automated LC/MS/MS data processing for assignment of potential drug structures. Especially metabolites for unexpected metabolites, the introduction and assignment of predicted structures, which may derive from various sources, significantly speeds up the process of metabolite identification. The predicted structures can be readily verified based on accurate mass MS/MS and isotopic patterns. A detailed Application Note is available from the Authors on request (Reference 2).

#### References:

1. In Silico Tools for Sharing Data and Knowledge on Toxicity and Metabolism: Derek for Windows, Meteor, and Vitic. Marchant CA, Briggs KA and Long A.

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

m/z	Ion Formula	Calc m/z	∆ m/z [mDa]	∆ m/z [ppm]	Neutral Loss	Loss Formula	Loss Mass
121.0647	C8 H9 O	121.0648	0.09	0.71	171.1009	C7 H13 N3 O2	171.1008
126.0659	C5 H8 N3 O	126.0662	0.31	2.46	166.0998	C10 H14 O2	166.0994
140.0817	C6 H10 N3 O	140.0818	0.16	1.15	152.0840	C9 H12 O2	152.0837
152.0809	C7 H10 N3 O	152.0818	0.89	5.86	140.0847	C8 H12 O2	140.0837
180.1129	C9 H14 N3 O	180.1131	0.25	1.36	112.0528	C6 H8 O2	112.0524
198.1232	C9 H16 N3 O2	198.1237	0.48	2.45	94.0424	C6 H6 O	94.0419
246.1240	C13 H16 N3 O2	246.1237	-0.26	-1.06	46.0417	C2 H6 O	46.0419
274.1544	C15 H20 N3 O2	274.1550	0.64	2.32	18.0113	H2 O	18.0106

Toxicology Mechanisms and Methods, (2008), 18, 177-187

2. Application note: 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, Agilent pub number: 5990-4583EN



