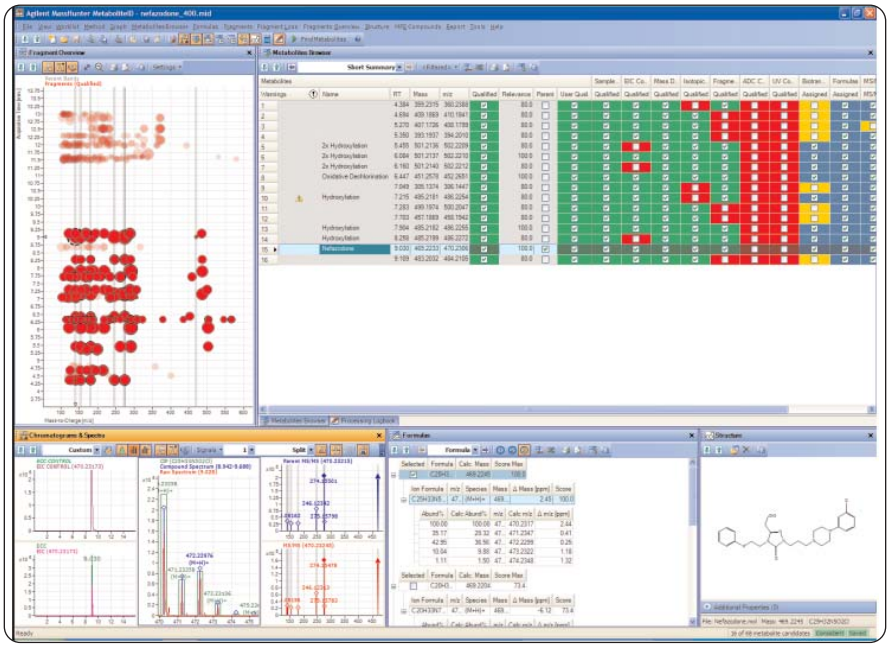
Computer Assisted Identification of Metabolites from Pharmaceutical Drugs

Part 1: Identification of Expected Metabolites of Nefazodone

Identification of metabolites by the MassHunter Metabolite ID software from RRLC – QTOF MS data

Application Note

Metabolite identification in drug discovery and drug development



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Abstract

This Application Note demonstrates:

- The use of the Agilent 1200 Rapid Resolution LC (RRLC) system for high resolution separation of metabolites from an in-vitro metabolism experiment.
- The use of the Agilent 6520 QTOF mass spectrometer for the acquisition of data for computer assisted metabolite identification.
- The use of the Agilent MassHunter Metabolite identification software for highly productive identification of expected metabolites.
- The results of the Metabolite ID data analysis for expected metabolites of the pharmaceutical drug nefazodone

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Introduction

The examination of the metabolism of new pharmaceutical drug candidates is an important step in the drug discovery and development process. For the evaluation of new technologies that improves the productivity in this important area, well known compounds are used as benchmarks. A compound that undergoes an extensive, well-documented metabolism and that can be used for this purpose is the pharmaceutical drug Nefazodone[1]. To confirm the utility of the MassHunter MetID for the identification of possible metabolites, it has been tested with this particular compound. The MassHunter MetID software uses the concept of multiple cooperative algorithms for the analysis of the QTOF MS and MS/MS data as a strategy to produce a confident overall result that normalizes the influence of a single algorithm2.
This Application note Demonstrates the use of the Agilent 1200 RRLC system and the Agilent 6500 Series Accurate Mass QTOF mass spectrometer for data acquisition from metabolism experiments, and the use of the MetID software for computer-assisted data analysis. The results of the data analysis are discussed in detail for examples of expected metabolites and unexpected metabolites[3] from the pharmaceutical drug Nefazodone.

Experimental

Equipment

- Agilent 1200 Series Rapid Resolution LC system with binary pump SL and degasser, high performance autosampler SL (ALS SL) with thermostat, thermostated column compartment (TCC) and Agilent 6500 Series Accurate Mass QTOF mass spectrometer.
- Column: ZORBAX SB-C18, 2.1 x 150 mm, 1.8 µm particle size.

Sample preparation

Stock solutions

- Phosphate buffer 100 mM, pH 7.4 (81.8 mL 0.1 M Na_2HPO_4 + 18.2 mL 0.1 M KH_2PO_4); 5 mM $MgCl_2$
- Nefazodone hydrochloride 250 µM in phosphate buffer
- NADPH solution, 10 mg/mL phosphate buffer
- Microsomal S9 preparation from rat liver, 20 mg protein/mL

Metabolite sample

Dilute 25 µL of Nefazodone in 180 µL phosphate buffer in a 1.5 mL Eppendorf vial. Add 15 µL S9 preparation and 30 µL NADPH solution. Vortex and incubate for 1 h at 37°C. Stop the reaction by adding 750 µL ice cold acetonitrile and centrifuge at 14,000 rpm for 15 minutes.

Remove the supernatant into a new 1.5 mL Eppendorf vial and evaporate to dryness in a speedvac. Dissolve the remaining pellet in 250 µL HPLC solvent A.

Control sample

Dilute 25 µL of Nefazodone in 210 µL phosphate buffer in a 1.5 mL Eppendorf vial. Add 15 µL S9 preparation. Vortex and incubate for 1 h at 37 °C. Add 750 µL ice cold acetonitrile and centrifuge at 14,000 rpm for 15 minutes.

Remove the supernatant to a new 1.5 mL Eppendorf vial and evaporate to dryness in a speedvac. Dissolve the remaining pellet in 250 µL HPLC solvent A.

All chemicals and bio-reagents were purchased from Sigma-Aldrich; HPLC solvents (acetonitrile) were purchased from Merck (Germany), and HPLC water from Mallinckrodt-Baker.

Methods

High resolution RR LC method

The Agilent 1200 Series binary pump SL was operated under the following conditions:

Solvent A:	Water + 0.1% formic acid (FA),
Solvent B:	ACN + 0.1% FA
Flow rate:	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

The Agilent 1200 autosampler SL was used to make injections of 1-10 µL sample with a 5 sec needle wash in 50% methanol and the samples were cooled to 4 °C. The TCC was operated at 60°C.

QTOF MS and MS/MS method

The Agilent 6500 Series Accurate Mass QTOF was operated in the 2GHz extended dynamic range mode with the following acquisition parameters:

Source:	ESI in positive mode with dual spray for reference mass solution (m/z 121.05087 and m/z 922.00979)
Drying gas flow:	10.0 L/min
Dry gas temperature:	300 ºC
Nebulizer:	45 psi
Mass range:	100-1000
Fragmentor:	200 V
Skimmer:	60 V
Capillary:	3500 V
Collision energy:	30 V
Data depended MS/MS:	2 compounds 3 MS/MS spectra exclusion for 0.1 min

Data analysis method in the MetID software

The first step in the analysis of the data consists of a comparison between the data file that contains the metabolite compounds (metabolite sample) and the data file that contains

only the parent drug (control sample). In this analysis, all detectable mass signals are extracted from the MS level data using the Molecular Feature Extraction (MFE) algorithm. Then, related compound isotope masses and adduct masses are grouped together into discrete molecular features, and chemical noise is removed. The compound lists of the metabolized sample and the control are then compared. All compounds that are new or increased by at least 2-fold in the metabolized sample are considered potential metabolites and are subjected to further analysis by different algorithms, which can be specified by the user. The algorithms can identify and qualify new metabolites, or can simply qualify metabolites found by another algorithm. The results of all metabolite identification algorithms are weighted and combined into a final identification relevance score. Metabolites are qualified when their final score is above a defined relevance threshold. The results from all algorithms are populated in a results table and can be inspected at a glance[2].

Results and discussion

For the identification of possible metabolites, the basic information about isotope pattern, MS/MS fragmentation pattern and calculated formula of the parent drug nefazodone are taken from the control sample (Figures 1 and 2). The measured isotope pattern (blue lines, insert in Figure 1) clearly shows the pattern that is typical for a chlorinated compound, and is identical to the calculated isotope pattern (CIP, green box, insert in Figure 1) with the main ion $(M+H)^+$ at m/z 470.2319 ($C_{25}H_{33}N_5O_2Cl$). The

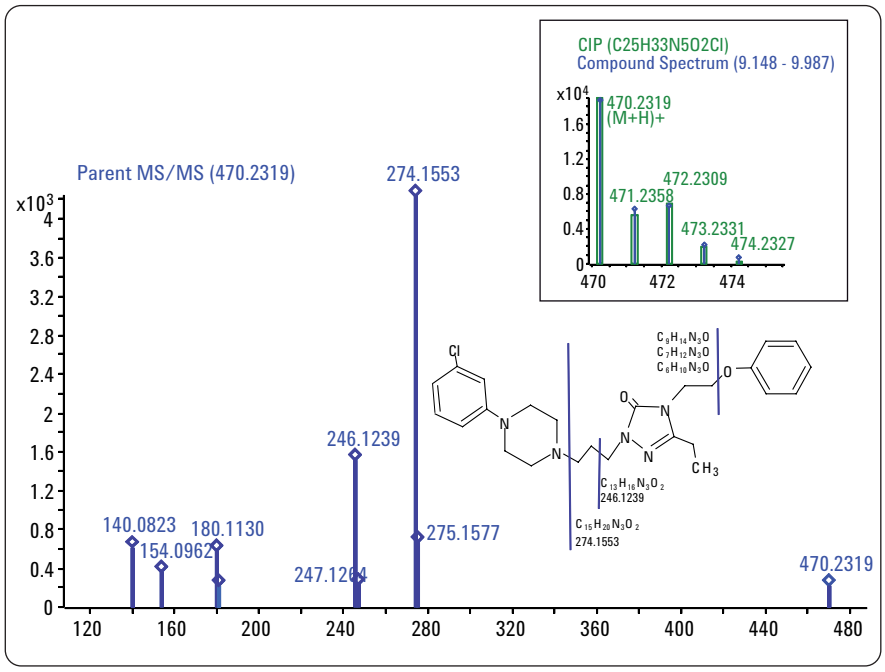

Figure 1
Mass spectrum, isotopic analysis and MS/MS spectrum for fragment assignment of the parent drug Nefazodone.

Formula	Calc. Mass	Mass	Δ Mass [mDa]	Δ Mass [ppm]	DBE	m/z	Species	Ion Formula
C25H32N5O2Cl	469.2245	469.2246	-0.16	-0.35	12	470.2319	(M+H)+	C25H33N5O2Cl

Isotopic pattern				
m/z	Calc. m/z	Δ Mass [ppm]	Abund %	Calc. Abund %
470.2319	470.2317	-0.35	100.00	100.00
471.2358	471.2347	-2.28	31.86	29.32
472.2309	472.2299	-2.24	33.67	36.56
473.2331	473.2322	-1.90	10.14	9.88
474.2327	474.2348	4.50	2.23	1.50

Figure 2
Calculated molecular formula and mass accuracies, and isotopic analysis of Nefazodone.

m/z	Mass	Formula	Calc. Mass	Δ Mass [mDa]	Δ Mass [ppm]	Loss Mass	Loss Formula	Neutral Loss
140.0823	139.0750	C6H9N3O	139.0746	-0.43	-3.12	330.1499	C19H23N2OCl	330.1482
154.0962	153.0890	C7H11N3O	153.0902	1.25	8.16	316.1342	C18H21N2OCl	316.1343
180.1130	179.1058	C9H13N3O	179.1059	0.11	0.62	290.1186	C16H19N2OCl	290.1175
246.1239	245.1167	C13H15N3O2	245.1164	-0.23	-0.94	224.1080	C12H17N2Cl	224.1066
274.1553	273.1481	C15H19N3O2	273.1477	-0.34	-1.26	196.0767	C10H13N2Cl	196.0751

Figure 3
Calculated MS/MS fragment formulas and neutral loss formulas for Nefazodone fragmentation.

MS/MS spectrum shows the main fragment $(M+H)^+$ at m/z 274.1553 with the formula $C_{15}H_{20}N_3O_2$ (Figure 1). The mass of nefazodone ($C_{25}H_{32}N_5O_2Cl$), which was calculated from the measured $(M+H)^+$ ion, shows a low relative mass error of -0.35 ppm (Figure 2) and the MS/MS fragment at a mass of 273.1481 ($C_{15}H_{19}N_3O_2$) of -1.26 ppm (Figure 3). This fragment formula, together with the assigned loss formula $C_{10}H_{13}N_2Cl$ calculated for this MS/MS fragment, fits to the parent drug formula $C_{25}H_{32}N5O2Cl$ (Figure 3). Other MS/MS fragments are assigned to the structural formula of nefazodone (Figure 1).

The following metabolites are clearly identified by several algorithms in the software such as isotope pattern matching and MS/MS fragmentation pattern matching to identify compounds whose pattern matches the patterns of the parent drug. There is also a metabolism reaction assignment for biotranformations that belong to the expected phase I metabolic reactions. The first example comes from a hydroxylation reaction. The mono-hydroxyl metabolite 1 of nefazodone, eluting at a retention time around 7.5 minutes, with m/z 486.2267 shows the measured isotope pattern (blue lines, insert in Figure 4) of a chlorinated compound similar to the calculated isotope pattern (CIP, green box, insert in Figure 4), and shows a relative mass error of -0.68 ppm for the calculated formula (Figure 5). The MS/MS fragmentation pattern is identical to the pattern of the parent drug, and the molecular ion is shifted by the mass of an oxygen atom (Figure 4). This indicates that the reaction takes place at the part of the molecule that is eliminated as the neutral fragment during MS/MS fragmentation (Figure 6). The fragment ion at m/z 274.1545 has the same formula as the fragment of the parent, with relative mass error of 1.70 ppm, and the neutral loss has the formula $C_{10}H_{13}N_2OCl$, which fits to

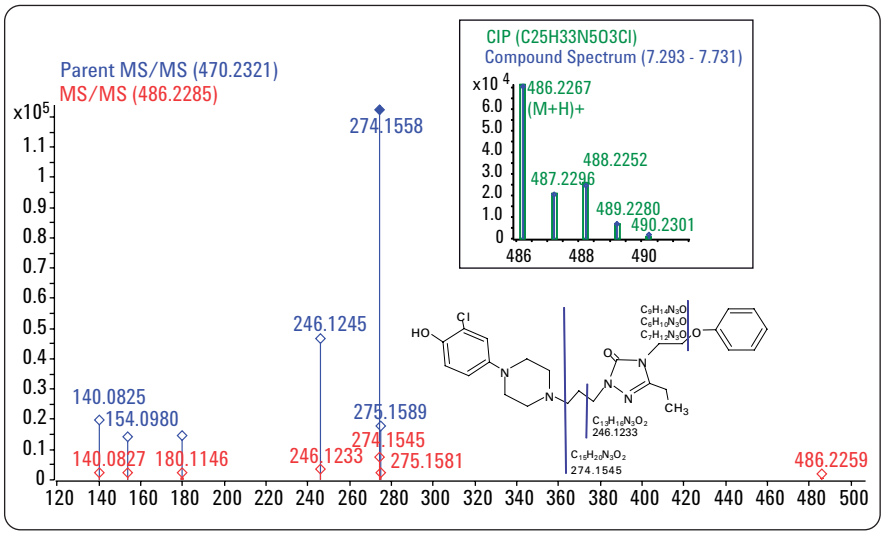

Figure 4
Mass spectrum, isotopic analysis and MS/MS spectrum with fragment assignment of the Nefazodone mono-hydroxy metabolite 1.

Formula	Calc. Mass	Mass	Δ Mass [mDa]	Δ Mass [ppm]	DBE	m/z	Species	Ion Formula
C25H32N5O3Cl	485.2194	485.2197	-0.33	-0.68	12	486.2270	(M+H)+	C25H33N5O3Cl

Isotopic pattern				
m/z	Calc. m/z	Δ Mass [ppm]	Abund%	Calc. Abund%
486.2267	486.2266	-0.09	100.00	100.00
487.2296	487.2296	0.01	26.32	29.36
488.2252	488.2248	-0.76	31.76	36.77
489.2280	489.2272	-1.64	8.80	9.95

Figure 5
Calculated molecular formula and mass accuracies, and isotopic analysis of the Nefazodone mono-hydroxy metabolite 1.

m/z	Mass	Formula	Calc Mass	Δ Mass [mDa]	Δ Mass [ppm]	Loss Mass	Loss Formula	Neutral Loss	FPM m/z	Shift m/z	Δ Shift [mDa]	Shift Formula
140.0827	139.0754	C6H9N3O	139.0746	-0.87	-6.27	346.1448	C19H23N2O2Cl	346.1458	140.0825		0.26	
154.0971	153.0899	C7H11N3O	153.0902	0.35	2.32	332.1292	C18H21N2O2Cl	332.1313	154.0980		0.82	
180.1146	179.1073	C9H13N3O	179.1077	0.36	2.00	306.1135	C16H19N2O2Cl	306.1139	180.1135		1.14	
246.1233	245.1160	C13H15N3O2	245.1164	0.44	1.79	240.1029	C12H17N2OCl	240.1052	246.1245		1.20	
274.1545	273.1473	C15H19N3O2	273.1477	0.46	1.70	212.0716	C10H13N2OCl	212.0739	274.1558		1.25	
486.2259									470.2321	15.9949	1.11	+O

Figure 6
Calculated MS/MS fragment formulas and neutral loss formulas for the Nefazodone mono-hydroxy metabolite 1 fragmentation.

the parent formula of the mono-hydroxylated metabolite 1 ($C_{25}H_{32}N_5O_3Cl$). Another MS/MS fragment that gives the same result is the MS/MS fragment at m/z 246.1233 for the fragment formula $C_{13}H_{15}N_3O_2$ with 0.44 ppm (assigned MS/MS fragments in Figure 4 and Figure 6).

Additionally, there is a second mono-hydroxyl metabolite of nefazodone, 2, eluting at a retention time around 8.3 minutes, with the same mass and isotope pattern as 1. The difference in elution behavior indicates a different structure for 2 compared with 1. This can be seen by comparing the fragmentation pattern of the MS/MS spectrum of metabolite 2 (red spectrum) with the MS/MS fragmentation pattern of the parent drug (blue spectrum) (Figure 7). The MS/MS spectrum of 2 shows a shift from the parent drug at m/z 470.2319 by the mass of an oxygen atom to m/z 486.2279, with a deviation of -2.55 ppm for the calculated formula (Figure 8), and a similar shift for the fragment mass at m/z 274.1582 to m/z 290.1508 with a deviation of -2.91 ppm for the calculated fragment formula (Figure 9). This fragment has the formula $C_{15}H_{19}N_3O_3$ and the corresponding loss formula $C_{10}H_{13}N_2Cl$, which is identical to the parent drug's MS/MS (Figure 9). Other MS/MS fragments are assigned to the structural formula of metabolite 2, and support the proposed structure (Figure 7).

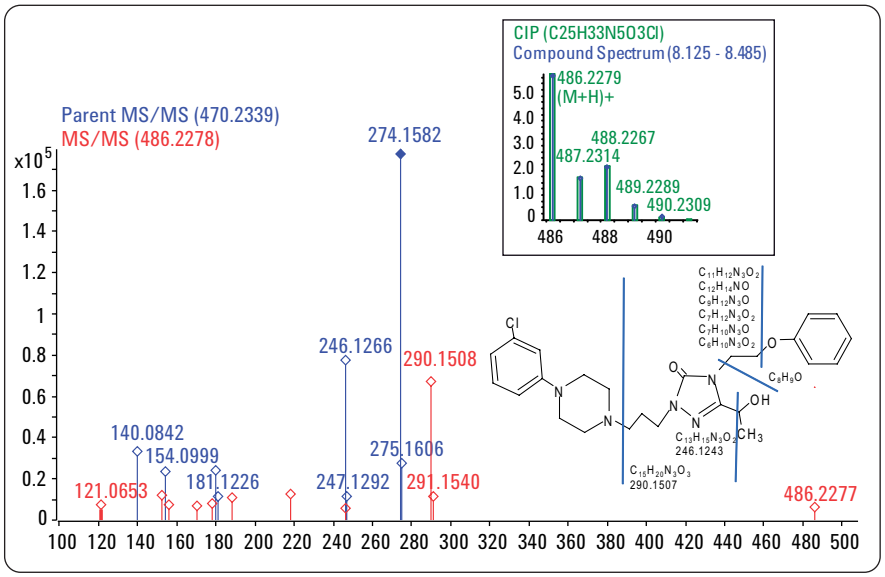

Figure 7
Mass spectrum, isotopic analysis and MS/MS spectrum with fragment assignment of the Nefazodone mono-hydroxy metabolite 2.

Formula	Calc. Mass	Mass	Δ Mass [mDa]	Δ Mass [ppm]	DBE	m/z	Species	Ion Formula
C25H32N5O3Cl	485.2194	485.2206	-1.24	-2.55	12	486.2279	(M+H)+	C25H33N5O3Cl

Isotopic pattern				
m/z	Calc. m/z	Δ Mass [ppm]	Abund%	Calc. Abund%
486.2279	486.2266	-2.55	100.00	100.00
487.2314	487.2296	-3.66	27.42	29.36
488.2267	488.2248	-3.81	34.81	36.77
489.2289	489.2272	-3.52	7.99	9.95
490.2309	490.2297	-2.35	1.24	1.58

Figure 8
Calculated molecular formula and mass accuracies, and isotopic analysis of the Nefazodone mono-hydroxy metabolite 2.

m/z	Mass	Formula	Calc. Mass	Δ Mass [mDa]	Δ Mass [ppm]	Loss Mass	Loss Formula	Neutral Loss	FPM m/z	Shift m/z	Δ Shift [mDa]	Shift Formula
121.0653	120.0580	C8H8O	120.0575	-0.48	-3.96	365.1619	C17H24N5O2Cl	365.1625				
152.0824	151.0751	C7H9N3O	151.0746	-0.56	-3.70	334.1448	C18H23N2O2Cl	334.1454	180.1154	-28.0313	1.72	-C2-H4
156.0776	155.0703	C6H9N3O2	155.0695	-0.83	-5.35	330.1499	C19H23N2OCl	330.1502	140.0842	15.9949	1.51	+O
170.0931	169.0859	C7H11N3O2	169.0851	-0.73	-4.33	316.1342	C18H21N2OCl	316.1347				
178.0982	177.0909	C9H11N3O	177.0902	-0.68	-3.83	308.1292	C16H21N2O2Cl	308.1296				
188.1077	187.1004	C12H13NO	187.0997	-0.71	-3.78	298.1197	C13H19N4O2Cl	298.1201				
218.0930	217.0857	C11H11N3O2	217.0851	-0.61	-2.83	268.1342	C14H21N2OCl	268.1348				
246.1243	245.1170	C13H15N3O2	245.1164	-0.55	-2.24	240.1029	C12H17N2OCl	240.1036				
290.1508	289.1435	C15H19N3O3	289.1426	-0.84	-2.91	196.0767	C10H13N2Cl	196.0770	274.1582	15.9949	2.33	+O
486.2277									470.2339	15.9949	1.18	+O

Figure 9
Calculated MS/MS fragment formulas and neutral loss formulas for the Nefazodone mono-hydroxy metabolite 2 fragmentation.

An additional metabolic reaction oxidizes the hydroxyl metabolite 2 to the oxo metabolite 3 at m/z 484.2111 with a deviation of -0.26 ppm for the calculated formula (Figures 10 and 11). The MS/MS fragment pattern matching (FPM) indicates several fragments that are transformed by the hydroxylation followed by the oxidation (Figure 10). The main fragment at m/z 288.1350 has the formula $C_{15}H_{17}N_3O_3$ calculated with a deviation of -2.72 ppm, and some other shifted fragments are also indicated (Figure 12, formula in Figure 10).

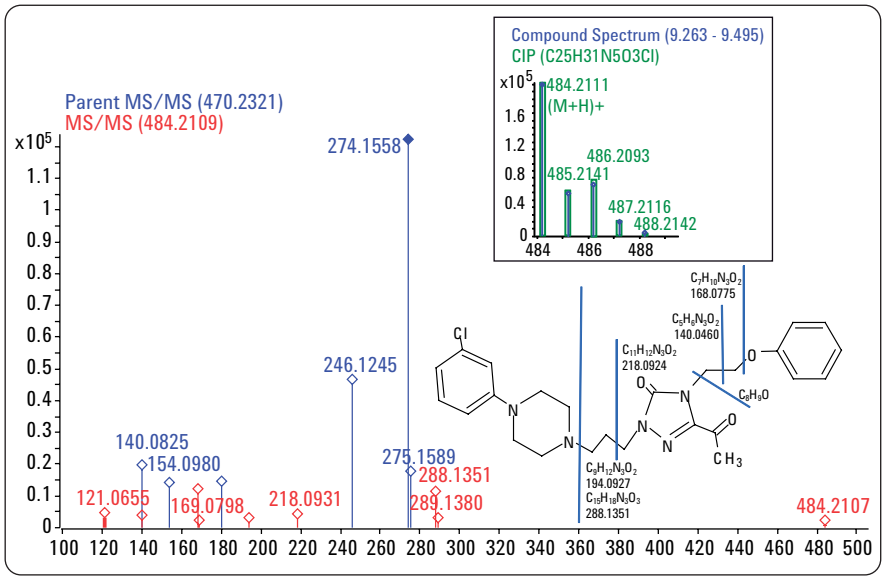

Figure 10
Mass spectrum, isotopic analysis and MS/MS spectrum with fragment assignment of the efazodone mono-oxo metabolite 3.

Formula	Calc. Mass	Mass	Δ Mass [mDa]	Δ Mass [ppm]	DBE	m/z	Species	Ion Formula	Score
C25H30N5O3Cl	483.20371	483.20384	-0.125	-0.26	13	484.21112	(M+H)+	C25H31N5O3Cl	100

Isotopic pattern				
m/z	Calc. m/z	Δ Mass [ppm]	Abund%	Calc. Abund%
484.2111	484.2110	-0.26	100.00	100.00
485.2139	485.2140	0.22	26.66	29.34
486.2092	486.2092	0.01	32.31	36.77
487.2117	487.2115	-0.29	7.83	9.95
488.2169	488.2141	-5.73	1.4	1.58

Figure 11
Calculated molecular formula and mass accuracies, and isotopic analysis of the Nefazodone mono-oxo metabolite 3.

m/z	Mass	Formula	Calc. Mass	Δ Mass [mDa]	Δ Mass [ppm]	Loss Mass	Loss Formula	Neutral Loss	FPM m/z	Shift m/z	Δ Shift [mDa]	Shift Formula
121.06550	120.05822	C8H8O	120.05751	-0.70	-5.86	363.14620	C17H22N5O2Cl	363.14537				
140.04608	139.03880	C5H5N3O2	139.03818	-0.63	-4.51	344.16554	C20H25N2OCl	344.16479				
168.07747	167.07019	C7H9N3O2	167.06948	-0.71	-4.27	316.13424	C18H21N2OCl	316.13340	154.09795	13.97926	0.25	-H2+O
194.09267	193.08539	C9H11N3O2	193.08513	-0.27	-1.38	290.11859	C16H19N2OCl	290.11820	180.11347	13.97926	0.07	-H2+O
218.09308	217.08580	C11H11N3O2	217.08513	-0.67	-3.11	266.11859	C14H19N2OCl	266.11779				
288.13505	287.12777	C15H17N3O3	287.12699	-0.78	-2.72	196.07673	C10H13N2Cl	196.07582	274.15579	13.97926	0.00	-H2+O
484.21073									470.23212	13.97926	0.65	-H2+O

Figure 12
Calculated MS/MS fragment formulas and neutral loss formulas for the Nefazodone mono-oxo metabolite 3 fragmentation.

In the metabolite that elutes at a retention time around 5.5 minutes, both hydroxylations have taken place to produce the dihydroxy metabolite 4 (Figure 13). The fragment pattern matching (FPM) shows the shift of the parent drug mass by the mass of two oxygen atoms from m/z 470.2319 to m/z 502.2211, with a relative mass error of 0.88 ppm for the calculated molecular formula $C_{25}H_{32}N_5O_4Cl$ (Figure 14). One of the oxidations shifts the parent drug MS/MS fragment ion from m/z 274.1557 to m/z 290.1504, with the formula $C_{15}H_{19}N_3O_3$, which indicates the same structural modification as for the monohydroxy metabolite 2 (Figure 15 and Figure 9 on page 5). The corresponding loss formula $C_{10}H_{13}N_2OCl$ indicates the site of the second hydroxylation reaction in the molecule comparable to metabolite 1 (Figure 15 and Figure 6 on page 4). This is additionally supported by another MS/MS fragment ion at m/z 262.1188, which is also shifted by the mass of an oxygen atom from the parent drug's MS/MS fragment at m/z 246.1244 (Figure 15 and Figure 3 on page 3).This fragment has the formula $C_{13}H_{15}N_3O_3$ compared to $C_{13}H_{15}N_3O_2$ for the parent drug.

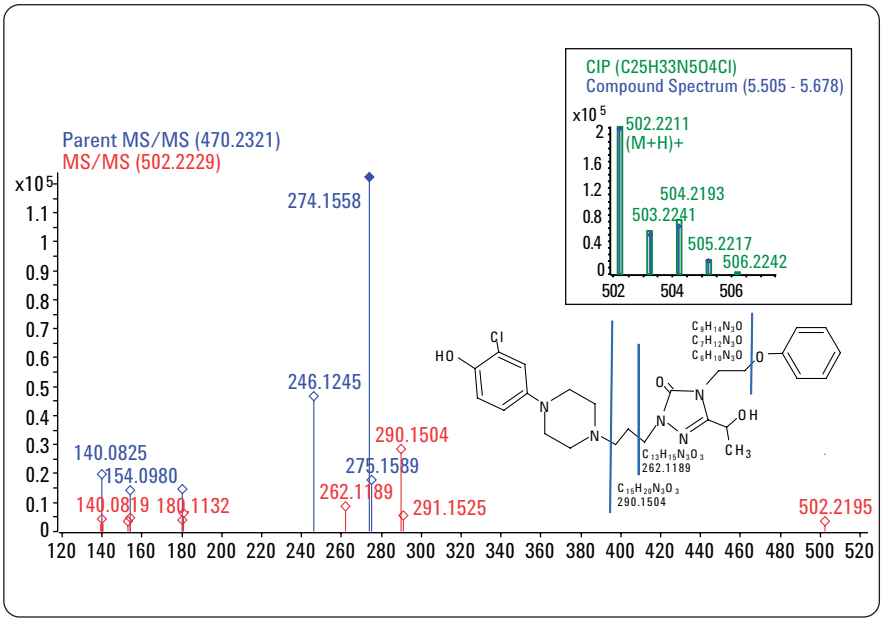

Figure 13
Mass spectrum, isotopic analysis and MS/MS spectrum with fragment assignment of the Nefazodone dihydroxy metabolite 4.

Formula	Calc. Mass	Mass	Δ Mass [mDa]	Δ Mass [ppm]	DBE	m/z	Species	Ion Formula	Score
C25H32N5O4Cl	501.21428	501.21383	0.44	0.88	12	502.22111	(M+H)+	C25H33N5O4Cl	100

Isotopic pattern				
m/z	Calc. m/z	Δ Mass [ppm]	Abund %	Calc. Abund%
502.2211	502.2216	0.89	100.00	100.00
503.2240	503.2246	1.08	25.72	29.40
504.2194	504.2198	0.77	31.64	36.99
505.2222	505.2221	-0.16	7.83	10.03

Figure 14
Calculated molecular formula and mass accuracies, and isotopic analysis of the Nefazodone dihydroxy metabolite 4.

m/z	Mass	Formula	Calc. Mass	Δ Mass [mDa]	Δ Mass [ppm]	Loss Mass	Loss Formula	Neutral Loss	FPM m/z	Shift m/z	Δ Shift [mDa]	Shift Formula
140.08188	139.07461	C6H9N3O	139.07456	-0.04	-0.31	362.13972	C19H23N2O3Cl	362.14100				
154.09701	153.08973	C7H11N3O	153.09021	0.48	3.15	348.12407	C18H21N2O3Cl	348.12588				
180.11324	179.10596	C9H13N3O	179.10586	-0.10	-0.54	322.10842	C16H19N2O3Cl	322.10965				
262.11887	261.11160	C13H15N3O3	261.11134	-0.26	-0.98	240.10294	C12H17N2OCl	240.10401	246.12446	15.99	0.50	+O
290.15040	289.14312	C15H19N3O3	289.14264	-0.48	-1.66	212.07164	C10H13N2OCl	212.07249	274.15579	15.99	0.31	+O
502.21955									470.23212	31.99	2.41	+O2

Figure 15
Calculated MS/MS fragment formulas and neutral loss formulas for the Nefazodone dihydroxy metabolite 4 fragmentation.

As a following metabolism step, the dihydroxy metabolite 4 undergoes an additional oxidation to form the hydroxy-oxo metabolite 5 of nefazodone. This metabolite, with the mass at m/z 500.2058, has the formula $C_{25}H_{31}N_5O_4Cl$, calculated with a relative mass accuracy of 0.13 ppm (Figures 16 and 17).

The calculated isotope pattern (CIP) calculated for the metabolite formula $C_{25}H_{31}N_5O_4Cl$ shows an excellent fit to the measured isotope pattern (see insert in Figure 16). The fragment pattern matching (FPM) shows a similar fragmentation pattern to metabolite 3 with the exception that the original fragment at m/z 274.1557 with the formula $C_{15}H_{19}N_3O_2$ is not shifted by the mass of the oxygen only but instead by the combination of the hydroxylation reaction followed by oxidation to m/z 288.1344, with the formula $C_{15}H_{17}N_3O_3$, calculated with -0.70 ppm relative mass accuracy (Figure 18).

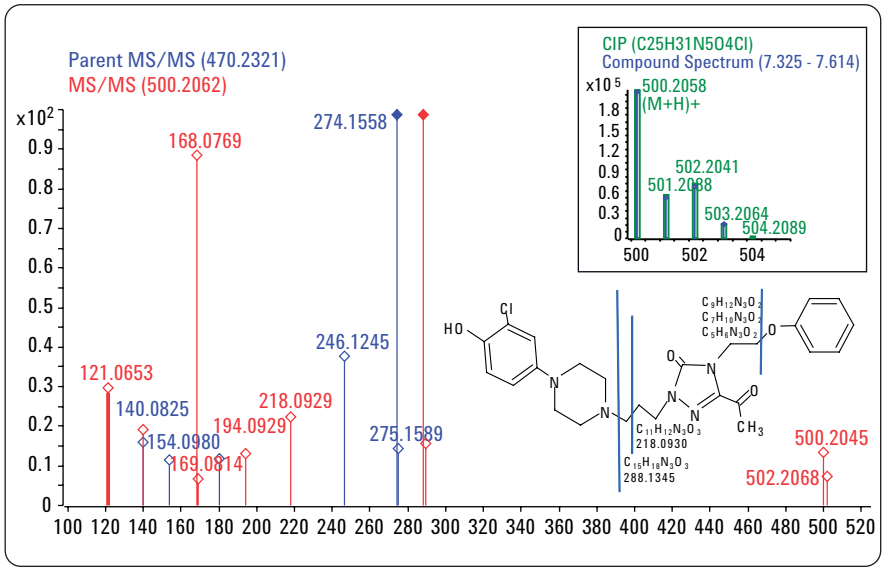

Figure 16
Mass spectrum, isotopic analysis and MS/MS spectrum with fragment assignment of the efazodone hydroxy oxo metabolite 5.

Formula	Calc. Mass	Mass	Δ Mass [mDa]	Δ Mass [ppm]	DBE	m/z	Species	Ion Formula	Score
C25H30N5O4Cl	499.19863	499.19856	0.06	0.13	13	500.20584	(M+H)+	C25H31N5O4Cl	100

Isotopic pattern				
m/z	Calc. m/z	Δ Mass [ppm]	Abund %	Calc. Abund %
502.2211	502.2216	0.89	100.00	100.00
503.2240	503.2246	1.08	25.72	29.40
504.2194	504.2198	0.77	31.64	36.99
505.2222	505.2221	-0.16	7.83	10.03

Figure 17
Calculated molecular formula and mass accuracies, and isotopic analysis of the Nefazodone hydroxy oxo metabolite 5.

m/z	Mass	Formula	Calc. Mass	Δ Mass [mDa]	Δ Mass [ppm]	Loss Mass	Loss Formula	Neutral Loss	FPM m/z	Shift m/z	Δ Shift [mDa]	Shift Formula
121.06526	120.05798	C8H8O	120.05751	-0.46	-3.87	379.14112	C17H22N5O3Cl	379.14091				
140.04633	139.03906	C5H5N3O2	139.03818	-0.88	-6.33	360.16046	C20H25N2O2Cl	360.15983				
168.07685	167.06958	C7H9N3O2	167.06948	-0.10	-0.60	332.12916	C18H21N2O2Cl	332.12931	154.09795	13.97926	0.36	-H2+O
194.09291	193.08564	C9H11N3O2	193.08513	-0.51	-2.65	306.11351	C16H19N2O2Cl	306.11325	180.11347	13.97926	0.18	-H2+O
218.09295	217.08567	C11H11N3O2	217.08513	-0.55	-2.52	282.11351	C14H19N2O2Cl	282.11321				
288.13447	287.12719	C15H17N3O3	287.12699	-0.20	-0.70	212.07164	C10H13N2OCl	212.07169	274.15579	13.97926	0.59	-H2+O
500.20445									470.23212	29.97418	1.85	-H2+O2

Figure 18
Calculated MS/MS fragment formulas and neutral loss formulas for the Nefazodone hydroxy oxo metabolite 5 fragmentation.

The final expected metabolite 6 that was identified with assignment of a biotransformation reaction is produced by a dechlorination reaction. Here, the measured isotope pattern is changed dramatically by the dechlorination (blue lines, insert in Figure 19). But there is a clear accordance with the calculated isotope pattern (CIP) for this biotransformation (green boxes, insert in Figure 19). The formula $C_{25}H_{33}N_5O_3$ for this metabolite was calculated for the mass at m/z 452.2617 with 0.98 ppm relative mass accuracy (Figure 20). The fragment pattern matching (FPM) shows clear identity of the metabolite's fragmentation pattern compared to the parent's fragmentation pattern. The shift +O+H-Cl is assigned only to the parent (Figure 19). This means the dechlorination takes place in the part of the molecule that is lost as a neutral loss during MS/MS fragmentation. The difference formula of the neutral loss from the fragment at m/z 274.1554 and the molecular ion at m/z 452.2651 is $C_{10}H_{14}N_2O$, which, compared to the parent drug (Figure 3 on page 5), contains no chlorine but instead contains an additional oxygen and hydrogen atom (Figure 21).

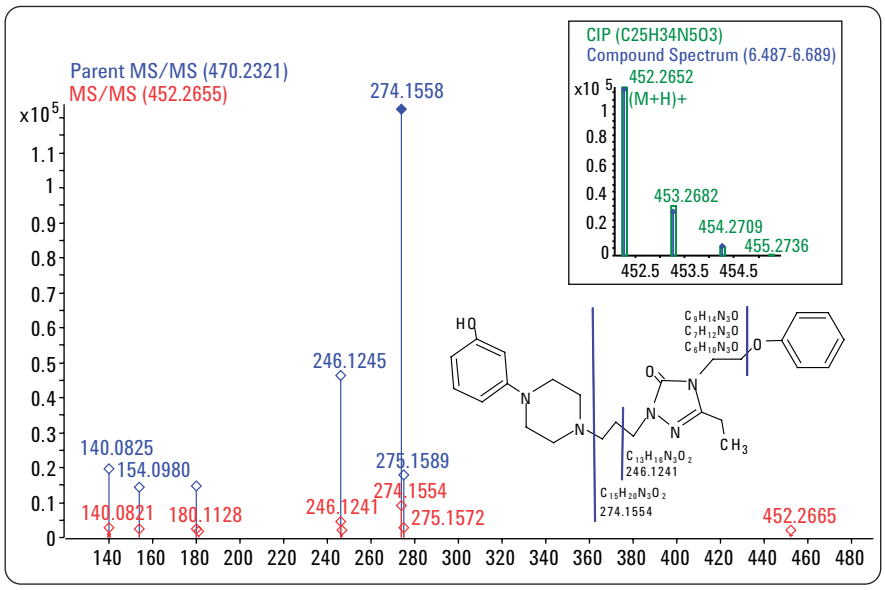

Figure 19
Mass spectrum, isotopic analysis and MS/MS spectrum with fragment assignment of the Nefazodone oxidative dechlorinated metabolite 6.

Formula	Calc. Mass	Mass	Δ Mass [mDa]	Δ Mass [ppm]	DBE	m/z	Species	Ion Formula	Score
C25H33N5O3	451.25833	451.25789	0.44	0.98	12	452.26517	(M+H)+	C25H34N5O3	100

Isotopic pattern

m/z	Calc. m/z	Δ Mass [ppm]	Abund %	Calc. Abund %
452.2652	452.2656	0.98	100.00	100.00
453.2682	453.2686	0.93	24.82	29.37
454.2694	454.2714	4.39	4.16	4.78

Figure 20
Calculated molecular formula and mass accuracies, and isotopic analysis of the Nefazodone oxidative dechlorinated metabolite 6.

m/z	Mass	Formula	Calc. Mass	Δ Mass [mDa]	Δ Mass [ppm]	Loss Mass	Loss Formula	Neutral Loss	FPM m/z	Shift m/z	Δ Shift [mDa]	Shift Formula
140.08205	139.07478	C6H9N3O	139.07456	-0.22	-1.55	312.18378	C19H24N2O2	312.18348	140.08245	0	0.40	
154.09777	153.09049	C7H11N3O	153.09021	-0.28	-1.84	298.16813	C18H22N2O2	298.16776	154.09795	0	0.18	
180.11275	179.10548	C9H13N3O	179.10586	0.39	2.15	272.15248	C16H20N2O2	272.15278	180.11347	0	0.72	
246.12411	245.11684	C13H15N3O2	245.11643	-0.41	-1.68	206.14191	C12H18N2O	206.14142	246.12446	0	0.35	
274.15542	273.14815	C15H19N3O2	273.14773	-0.42	-1.54	178.11061	C10H14N2O	178.11011	274.15579	0	0.36	
452.26651								-0.00097	470.23212	-17.9661	0.50	+O+H-Cl

Figure 21
Calculated MS/MS fragment formulas and neutral loss formulas for the Nefazodone oxidative dechlorinated metabolite 6 fragmentation.

Expected Metabolic Pathways of Nefazodone

The pharmaceutical drug nefazodone undergoes extensive metabolism in the human body and yields not only the typical expected metabolites but also several unexpected metabolites initiated by a cleavage of the original drug molecule.

The metabolites that were identified in this work as previously discussed are displayed in Figure 22 as simplified proposed metabolic pathways for the expected metabolites.

As outlined in Figure 22, there are a few metabolic reactions that do not change the skeleton of the molecule, and lead to the expected metabolites by simple modification. The monohydroxylation of nefazodone can take place either at the chlorinated phenyl ring, leading to the monohydroxyl metabolite 1, or at the ethyl group that is connected to the central triazole-3-one ring, leading to monohydroxyl metabolite 2. A further metabolic oxidation reaction, which oxidizes the hydroxyl group present in 2 into a keto group, leads to the oxo metabolite 3. The metabolic hydroxylation reaction can take place in both parts of the molecule, leading to the dihydroxy metabolite 4. After dihydroxylation, the following metabolic oxidation leads to the oxy-hydroxyl metabolite 5. A metabolic oxidative dechlorination reaction leads to the loss of the chlorine atom from nefazodone and to the substitution of the chlorine by a hydroxyl group, yielding the dechlorinated metabolite 6. and accurate-mass-based formulae calculation. In the third step, the elucidated sample was compared with a new sample obtained from another ginseng species, and new compounds and those that increased in amount were identified.

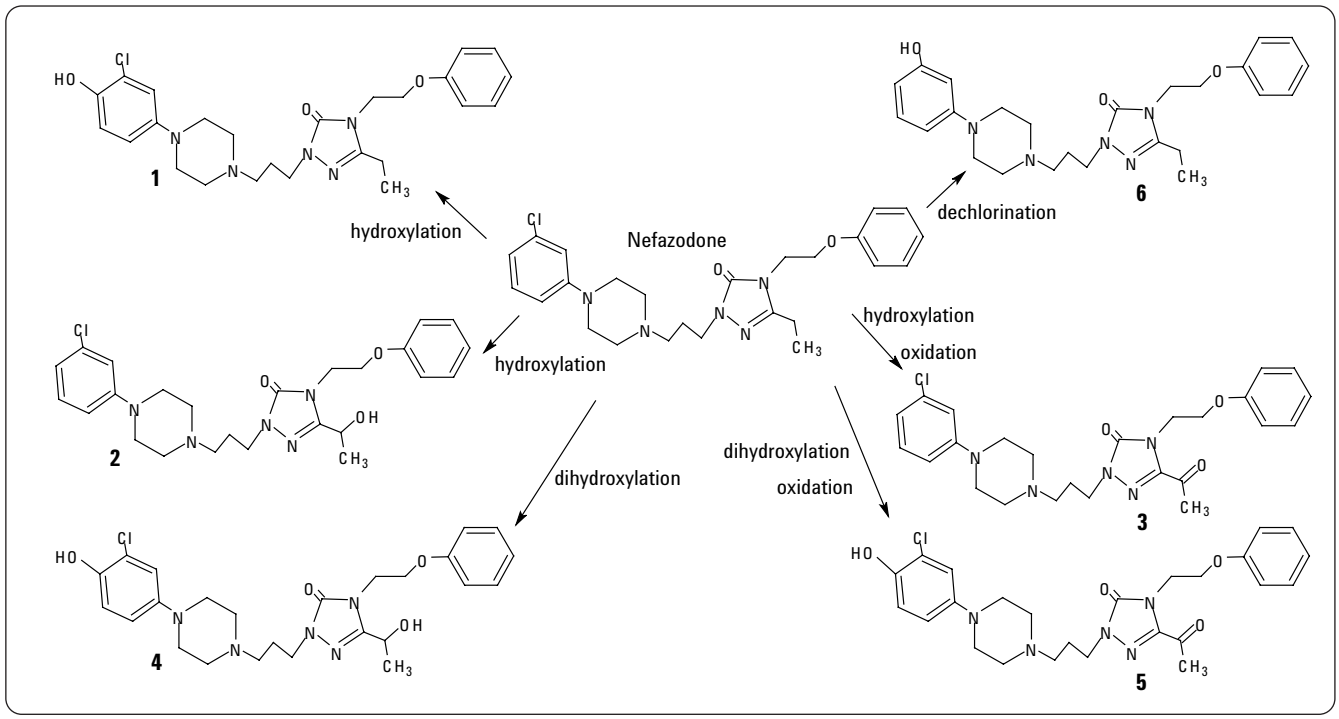

Figure 22
Proposed metabolic pathways for the expected metabolites of Nefazodone.

Conclusion

This work demonstrates the interpretation of the results produced by the MassHunter Metabolite Identification (MetID) software for the identification of metabolites created by expected biotransformations. The assignment of metabolite structures by interpretation of information created from QTOF mass spectrometry data by various algorithms like isotope pattern matching, MS/MS fragment pattern matching and formula calculation based on accurate mass measurement for MS and MS/MS is demonstrated. This work is an example of the gain in productivity that can be achieved by using the MetID software for the interpretation of QTOF data from metabolite identification experiments.

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