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).

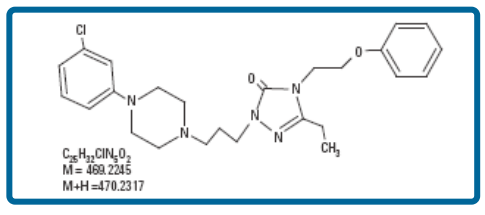

Figure 1: Structure of Nefazodone.

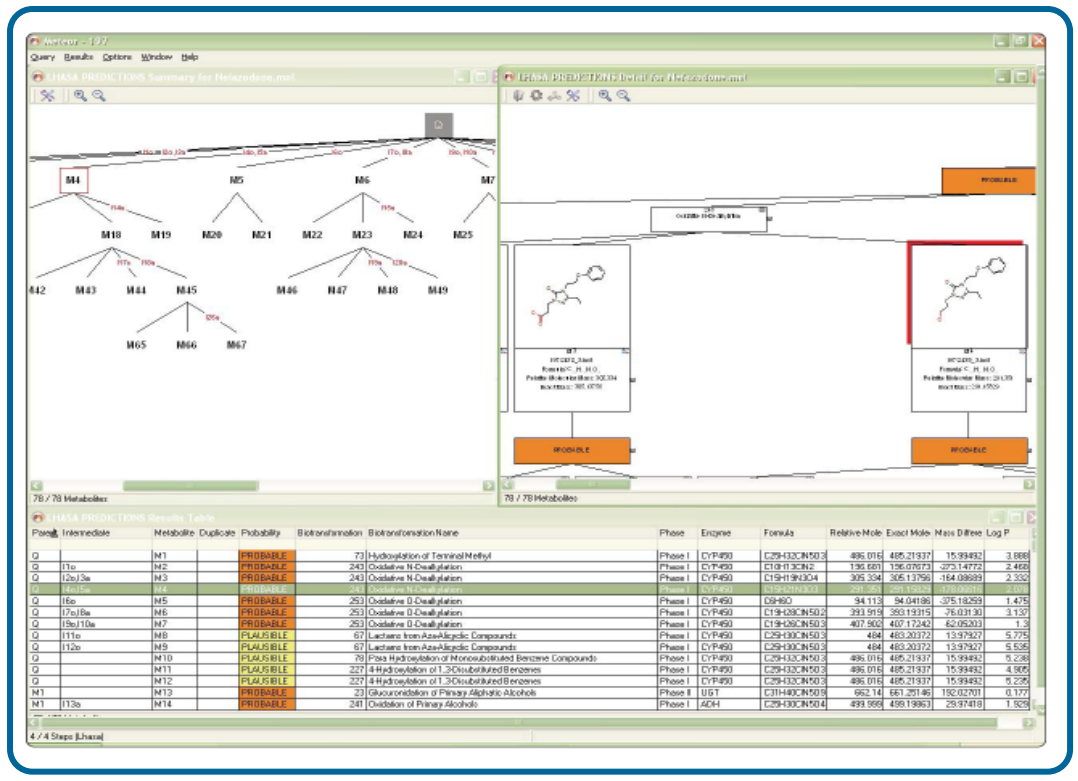

Figure 2: Meteor results display.

Experimental

Metabolite sample / Control sample preparation

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

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.*

LC method

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.

Q-TOF MS and MS/MS method

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 ºC

Dry gas: 7.0 L/min, Dry Temp: 300 ºC

Nebulizer: 45 psi

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

Data Analysis method

- *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.

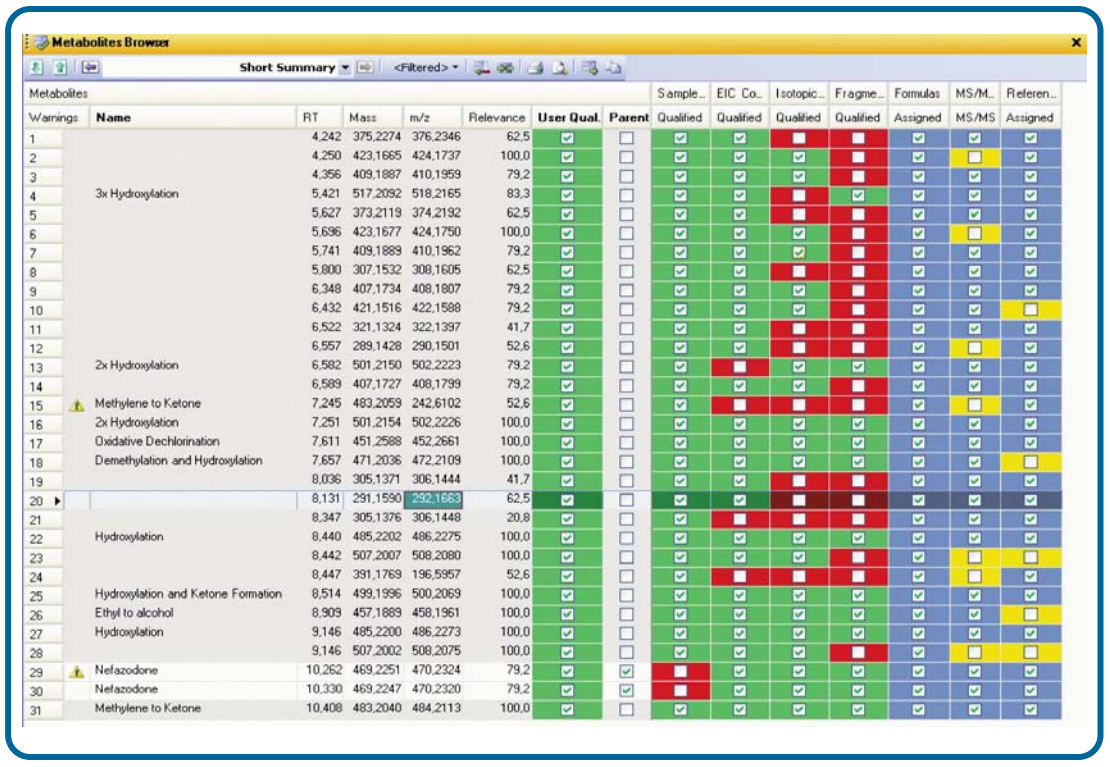

Figure 3: Metabolite ID 'At a glance' table.

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.

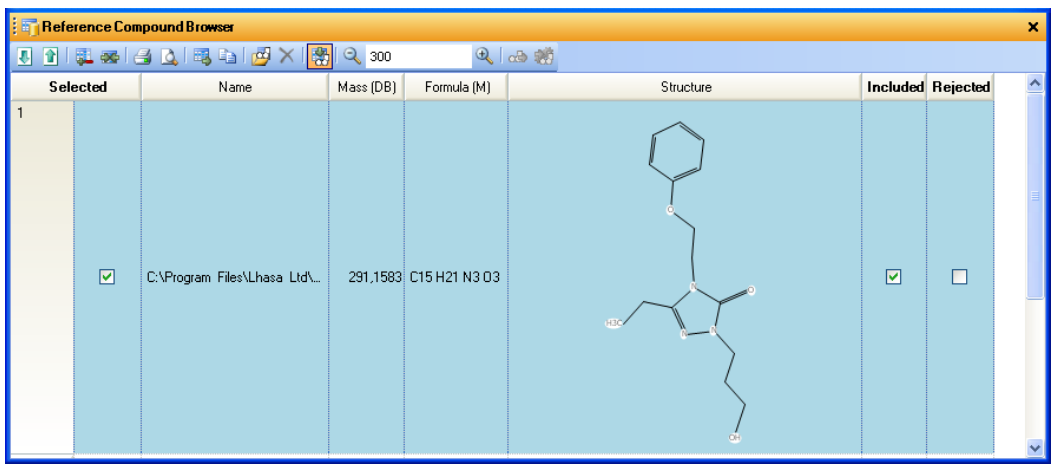

Figure 4: Result from the search of a Meteor metabolite prediction result file, which assigned a structure to an unexpected metabolite.

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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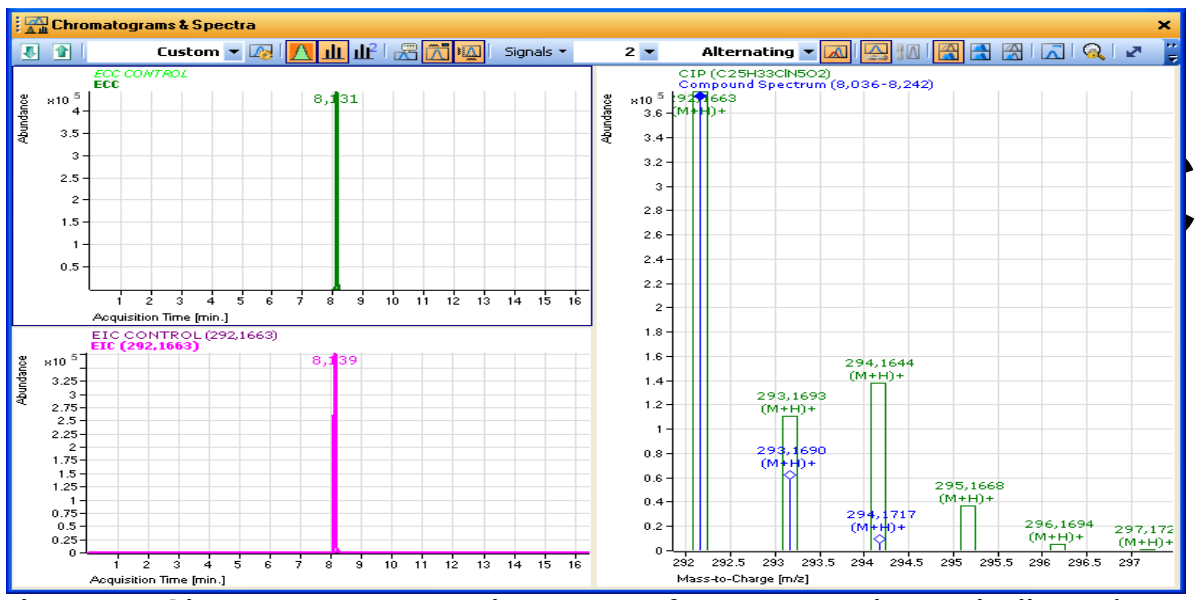

Figure 5: Chromatograms and spectra of unexpected metabolite m/z 292.1663. A) Extracted compound chromatogram (ECC). B) Extracted Ion Chromatogram (EIC). C) Measured isotopic pattern (blue) in comparison to the calculated isotopic pattern (green, CIP) of the parent drug.

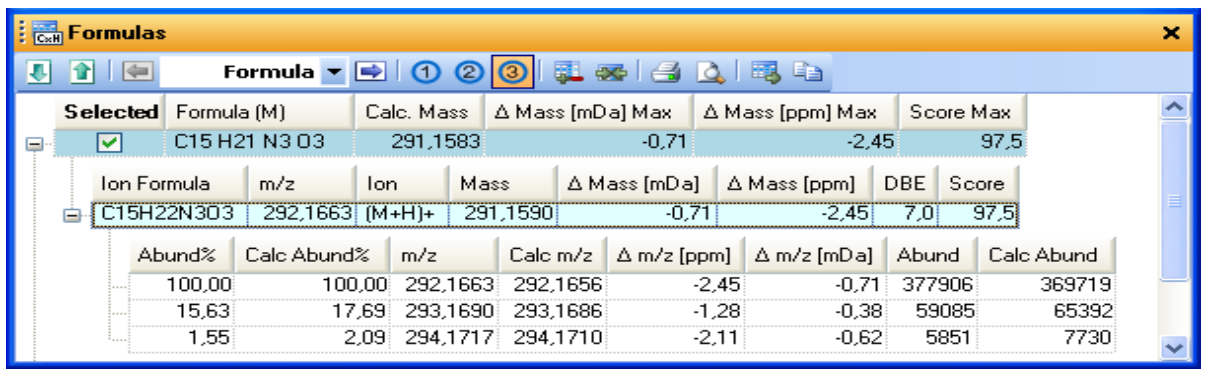

Figure 6: Calculated formula of the unexpected metabolite compound with calculated mass accuracy and isotopic pattern.

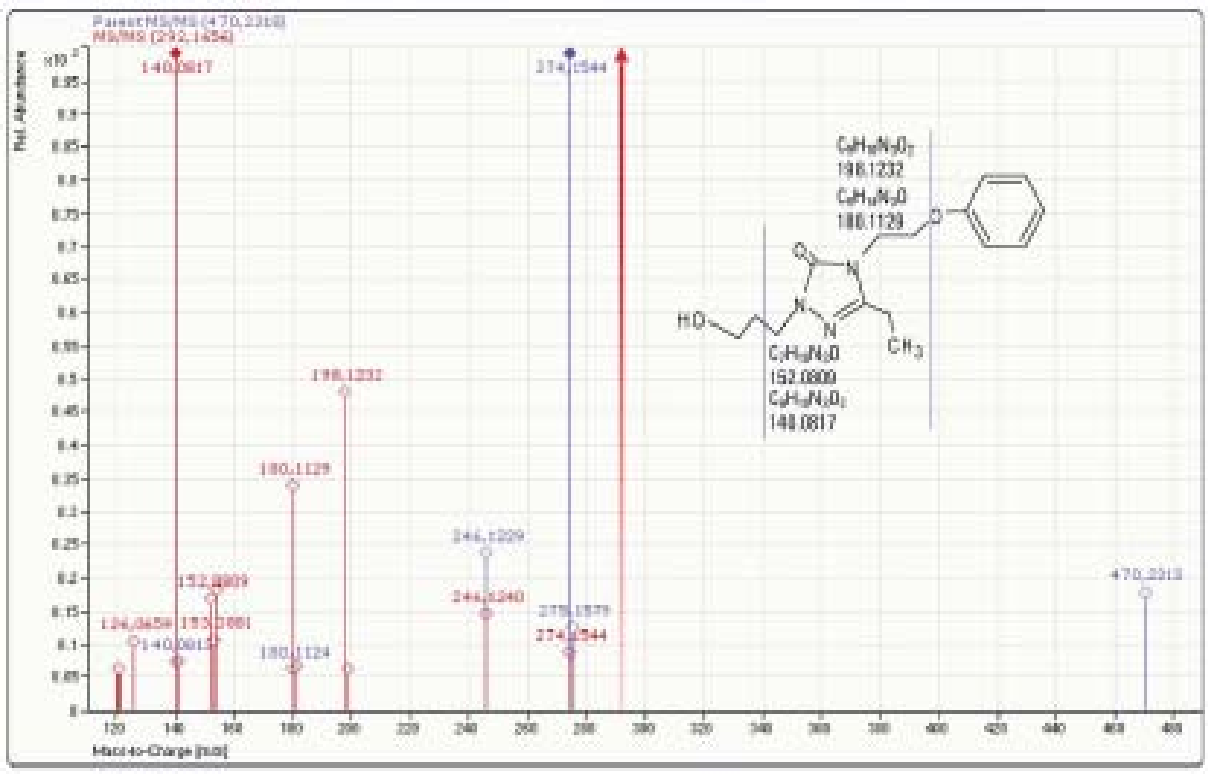

Figure 7: MS/MS spectrum with fragment assignment of the unexpected nefazodone metabolite by Meteor.

m/z	Ion Formula	Calc m/z	Δ m/z [mDa]	Δ m/z [ppm]	Neutral Loss	Loss Formula	Loss Mass
121.0647	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

Conclusions

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).

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 metabolites structures. Especially 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. 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*