An interwoven, multi-algorithm approach for computer-assisted identification of drug metabolites

Rapid identification of drug metabolites from accurate QTOF MS and MS/MS data by Agilent MassHunter metabolite identification software

Application Note

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Agilent Equipment:

1200 Series Rapid Resolution LC system
6510 quadrupole time-of-flight LC/MS
ZORBAX RRLC column
MassHunter metabolite identification software

Application Area:

Metabolite identification in drug discovery and development

Abstract

This Application Note is based on scientific poster MOP 253 presented at the ASMS conference of mass spectrometry, June 4, 2007, in Indianapolis, Indiana, USA. The note describes:

- Rapid, computer-assisted identification of drug metabolites using a multi-algorithm approach.
- High resolution chromatographic separation of drug metabolites from an in vitro experiment.
- High mass accuracy measurement of drug metabolites by QTOF MS and MS/MS.

Agilent Technologies

Introduction

In modern pharmaceutical drug discovery it is of crucial importance to identify all possible metabolites of a new chemical entity because of possible toxic effects on humans and to evaluate its potential as new drug substance. Today, high resolution and high mass accuracy QTOF MS and MS/MS data[1], which are acquired from in vitro as well as in vivo metabolism experiments, are used for metabolite identification in the different stages of the drug discovery and development process. To make use of all potential information contained in such data, it is essential to use different and complementary computer algorithms for data analysis. Each of these algorithms provides an individual result based on the specific functionality and the analyzed part of the data. The real advantage of computer-assisted data analysis comes into being when these algorithms work together in an interwoven fashion and contribute to a overall result of higher confidence. To do so, all scores produced by the individual algorithms are combined and weighted with user-settable factors. This Application Note describes an example for the analysis of QTOF data from a metabolite identification experiment using the drug compound Nefazodone[2] by means of a the Agilent MassHunter metabolite identification software which uses the described approach.

Experimental

Equipment

- Agilent 1200 Series Rapid Resolution LC system, including degasser, binary pump SL, high performance autosampler SL with thermostat, thermostatted column compartment and diode array detector SL
- Agilent ZORBAX SB-C18 column, 2.1 x 150 mm, 1.8 µm particle size
- Agilent 6510 quadrupole time-of-flight LC/MS system

Sample preparation

- Stock solutions:
  20 mg/mL S9 liver homogenate preparation
  0.1 mg/mL nefazodone in water
  1.6 mg NADP in 1.6 mL
  0.1 M phosphate buffer at pH 7.4
  50 mM isocitrate/$MgCl_2$ (203 mg $MgCl_2.6H_2O$ + 258.1 mg isocitrate in 20 mL water)
  Isocitrate dehydrogenase 0.33 U/mL
- NADPH regeneration system:
  1.6 mL NADP solution + 1.6 mL isocitrate solution + 100 µL isocitrate dehydrogenase solution
- Incubation mixture:
  3.85 µL substrate + 200 µL NADPH regeneration system + 746.15 µL phosphate buffer + and 50 µL S9 liver homogenate

Incubation was carried out at 37 °C for 60 minutes, a 100 µL aliquot was taken at 0 and 60 min. The reaction was stopped by adding 6 µL perchloric acid and 100 µL acetonitrile to the aliquots followed by centrifugation for 15 min at 14.000 g. The supernatant was evaporated to dryness using a SpeedVac concentrator and reconstituted with water containing 0.1 % formic acid (FA) for LC/MS analysis as described below. Incubations stopped at 0 min were used as controls.

Figure 1
System configuration for the metabolite identification experiment, comprising the Agilent 1200 Series Rapid Resolution LC with 1.8 µm particle size column and Agilent 6510 QTOF LC/MS.

High resolution LC/MS method

- Agilent 1200 Series binary pump SL
  Solvent A: Water + 0.1 % 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
- Agilent 1200 Series autosampler SL
  Injection volumes:
  1-10 µL with needle wash
  Sample temperature: 4 °C
  Automated delay volume reduction
- Agilent 1200 Series diode array detector SL
  Detection wavelength:
  210 nm, (± 4 nm)
  Reference wavelength:
  360 nm (± 16 nm)
  Flow cell: 2 µL volume,
  3 mm path length
- Agilent 1200 Series thermostatted column compartment
  Column temperature: 60 °C

QTOF MS and MS/MS method

- Agilent 6510 QTOF LC/MS system
  Source: ESI, positive mode with dual spray for reference mass solution
  Dry gas: 12.0 L/min (m/z 121.005 and m/z 922.00)
  Dry temperature: 300 °C
  Nebulizer pressure: 60 psi
  Mass range:100-1000
  Fragmentor voltage: 200 V
  Skimmer: 60 V
  Capillary voltage: 4000 V
  Collision energy: 35 V
  Data dependent MS/MS: 2 compounds, 2 MS/MS spectra, exclusion for 0.03 min

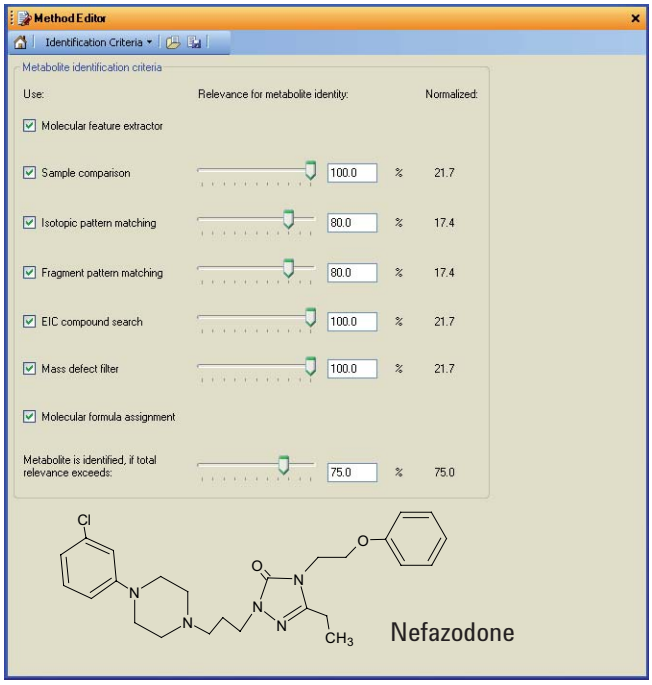

Figure 2
Setup of metabolite relevance score for each individual algorithm and weighted overall identification relevance score threshold.

Data analysis

The first step in the analysis of the data comprises the introduction of the compound structure formula and calculation of the molecular weight followed by the comparison between the data file that contains the metabolite compounds (sample, incubation time t>0) and the data file that contains only the parent drug (control, incubation time t=0). In this comparison, all detectable mass signals are extracted from the MS level data using the Molecular Feature Extraction (MFE) algorithm. 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 which are new or increased in the metabolized sample are considered potential metabolites and are subjected to further analysis by different algorithms, which can be specified by the user (figure 2). The algorithms can identify and qualify new metabolites or can just 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 collected in a results table and can be inspected at-a-glance.

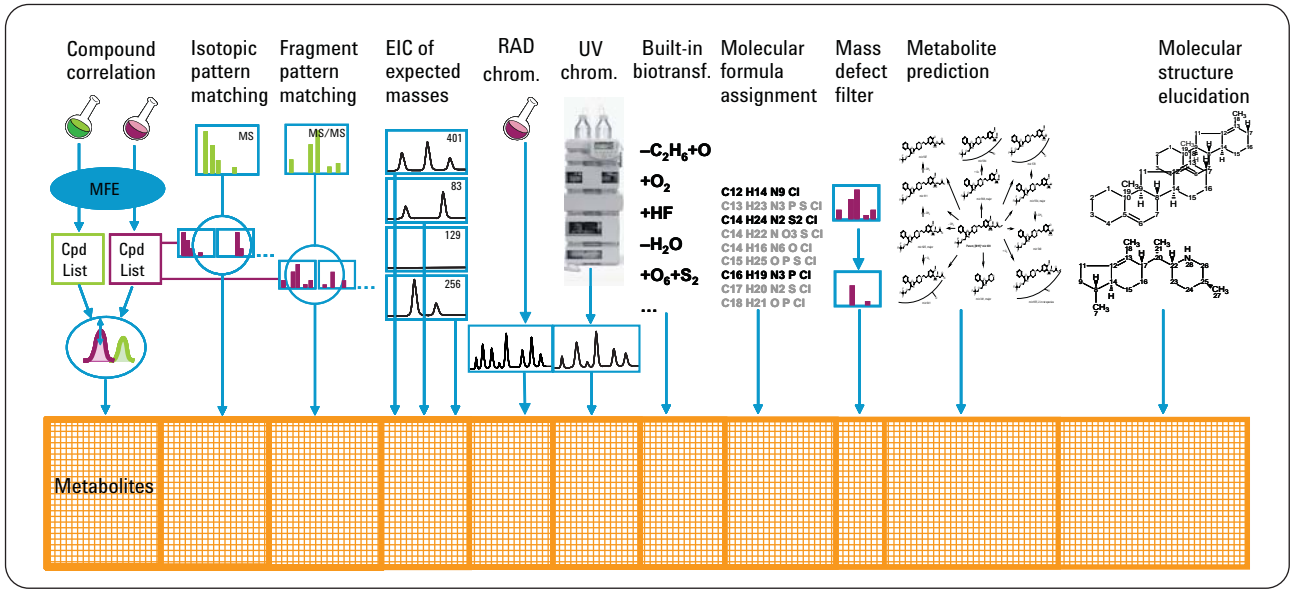

Figure 3
Metabolite identification software workflow, featuring different data analysis algorithms. Each time a new metabolite candidate is found, a new row is added to the table. Columns are added to the table to confirm an existing metabolite candidate and to show the result of the individual algorithm.

Results and discussion

The metabolite identification workflow (figure 3) used interwoven multiple algorithms to populate a results table with potential metabolites. A plurality of different procedures was used to identify the metabolites. Each time a new metabolite candidate was found, a new row was added to the table. For each individual algorithm that was used to find or qualify a potential metabolite, an additional column in the results table was used to display the results. The following points describe the individual algorithms and their interaction to generate the final result table.

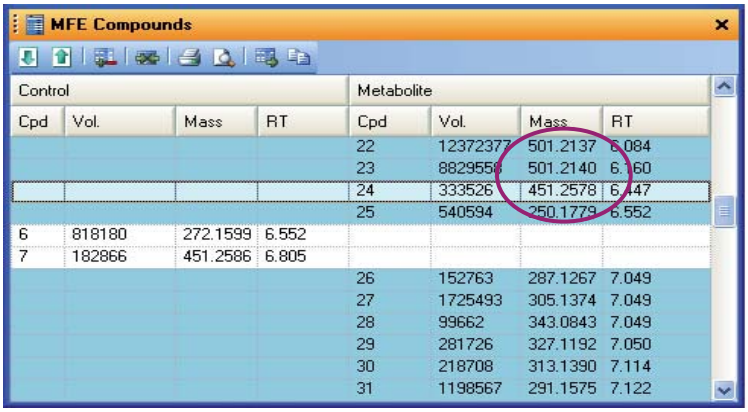

Figure 4
Comparison table between control sample and metabolite sample with e.g. di hydroxyl and dechlorination metabolites at mass 451.2578 and 501.2140 respectively.

1. Sample-control comparison (figure 4) – In a sample comparison table, the compounds found in the metabolite sample (time > 0, parent drug was metabolized) and the control (time = 0) are compared and aligned by mass and RT. This allowed detection of both expected and unexpected metabolites in the metabolized sample.

2. Isotopic pattern matching (figure 5) – The isotopic pattern of a metabolite coming from an expected biotransformation was compared to the theoretical pattern of the biotransformed parent drug, while the pattern of an unexpected metabolite was compared to the theoretical isotope pattern of the parent drug.

3. Fragment pattern matching or MS/MS correlation (figure 6) – This procedure correlated the MS/MS spectrum of each potential metabolite with the MS/MS spectrum of the parent drug. Using this procedure mass shifts in the fragment ions due to biotransformations could be detected and visualized.

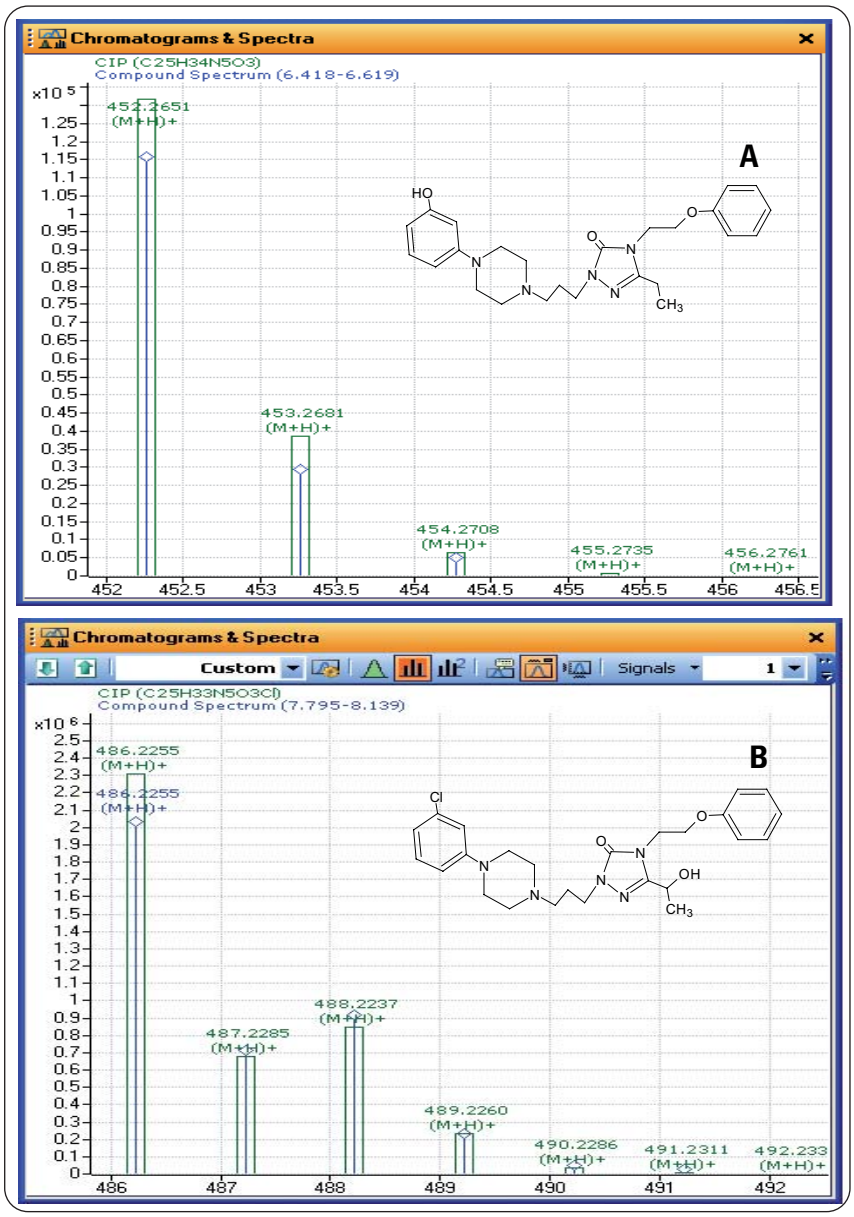

Figure 5
A) Isotopic pattern matching for dechlorinated metabolite by comparison with calculated isotopic pattern (CIP) after application of biotransformation to parent drug formula.
B) Isotopic pattern matching of chlorinated hydroxymetabolite by comparison to the calculated isotopic pattern of the chlorinated parent drug.

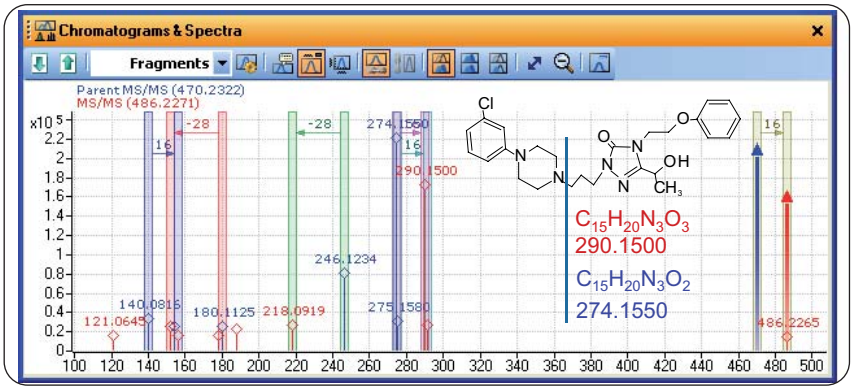

Figure 6
MS/MS fragment pattern matching between protonated parent drug (m/z 470.2323) and protonated hydroxy metabolite (m/z 486.2265) with biotransformation mass shift assignment. The MS/MS fragments at m/z 274.1550 from the parent drug and at m/z 290.1500 are related by a shift of 15.9999 for the metabolic hydroxylation reaction.

4. Extraction of chromatograms (figure 7) – This included generation of extracted ion chromatograms (EIC) directly from the data, and generation of extracted compound chromatograms (ECC) from extracted molecular features.

5. Compound search in RAD (radioactivity detection) chromatograms (not presented here).

6. Compound search in UV (ultraviolet) chromatograms or other detection methods (not presented here).

7. Biotransformation labeling (figures 6 and 10) – Expected metabolites were confirmed by comparison of parent ion mass shifts with a table of known biotransformations and these compounds were labeled with the name of biotransformation reaction in the result table (figure 10).

8. Molecular formula assignment (figures 8 and 9) – Molecular formula assignment was based on the assumption that only one elemental composition fits to the measured accurate mass of the product and that subsets of the same elemental composition must explain the product fragment masses and their neutral losses in the MS/MS spectrum.

9. Mass defect filter – Potential metabolites with a mass defect outside a defined mass defect window around the parent drug were filtered out.

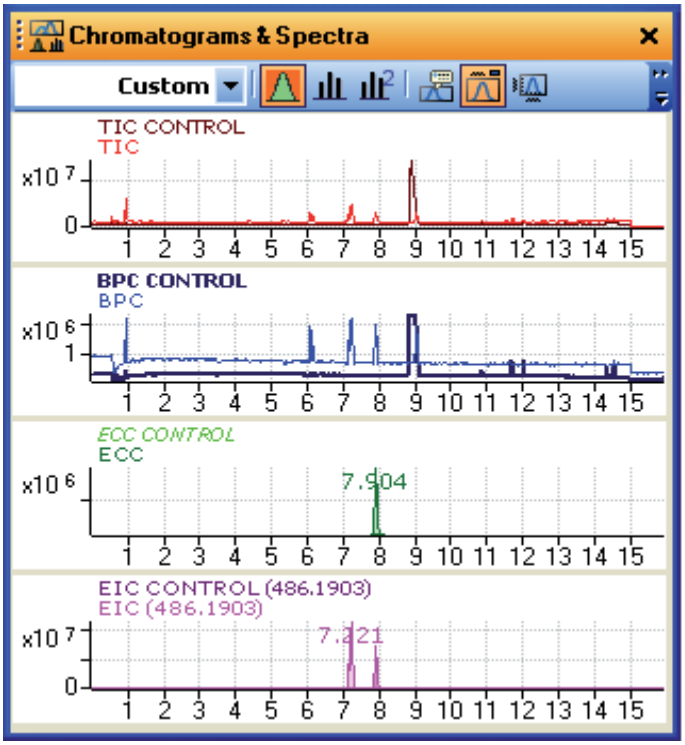

Figure 7
The extracted ion chromatograms (EIC) were directly obtained from the measured data for the control as well as metabolite sample, and the extracted compound chromatograms (ECC) were obtained from extracted molecular features – shown here shown for two different hydroxy metabolites at m/z 486.1903 at RT 7.2 and 7.9 min. The metabolites were not present in the control sample.

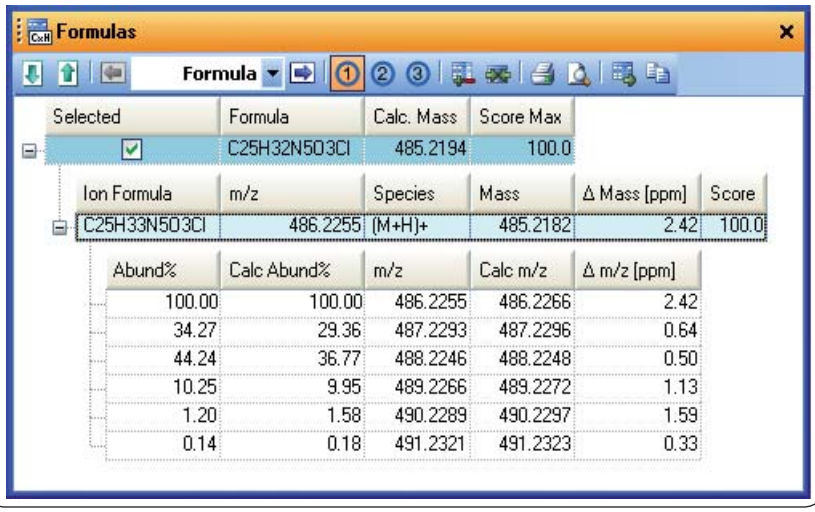

Figure 8
Calculated formula for highest score hydroxyl metabolite and abundance for isotopic pattern and masses. The relative mass error was calculated to be 2.42 ppm.

10. Metabolite prediction – Structures from manual or computer-assisted metabolite prediction were assigned to the identified compounds in the result table.

11. Molecular structure elucidation – All data were consolidated for structure elucidation and structure formula assignment.

12. Population of the final metabolite result table (Figure 10): The identified metabolites were collected in a result table, which shows the major information about the compounds and the qualification from each individual algorithm as well as additional available information. There is the possibility to produce and to report more detailed result tables.

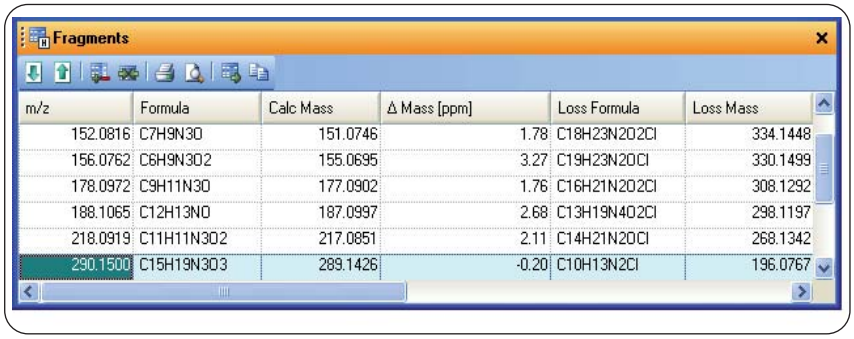

Figure 9
Measured masses of MS/MS fragments and calculated fragment formulae for the the protonated hydroxy.metabolite at m/z 486.2255. The loss masses as well as the calculated fragment formula of the loss are displayed. The relative mass error of fragment m/z 290.1500 was calculated to be -0.20 ppm.

Conclusion

Metabolites from the drug compound Nefazodone were automatically identified by means of a computer-assisted approach, which applied several interleaved algorithms to QTOF MS and MS/MS data. The comparison of control and metabolized sample was based on molecular feature extraction (MFE) to extract metabolites. High mass accuracy MS and MS/MS data were acquired with low single digit relative mass error and used for molecular formula generation (MFG). In the final at-a-glance result table, an overall relevance score was created for the identified metabolites, which was calculated from the weighted relevance score from each algorithm.

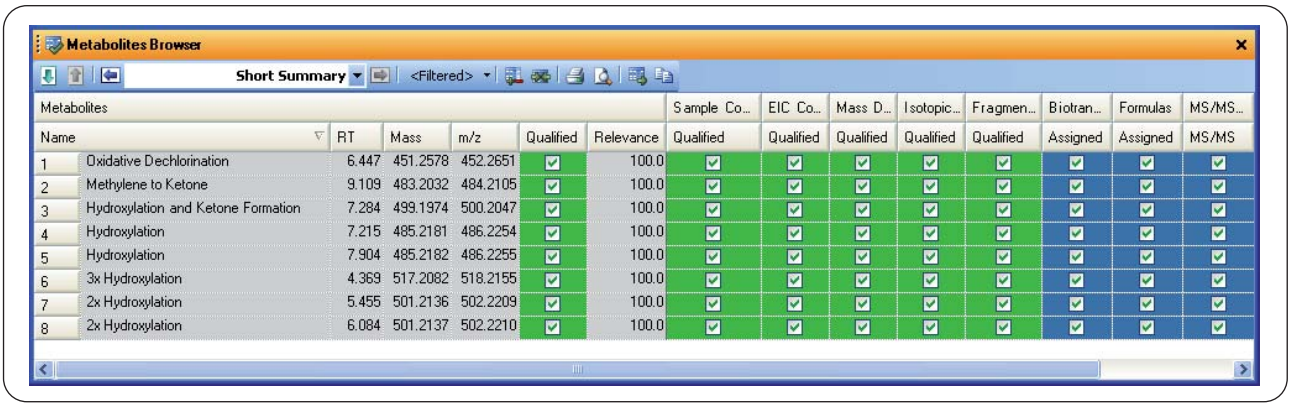

Figure 10
Final result table for overall qualified metabolites, which are related to a known biochemical metabolic reaction. Qualified results form the algorithms "sample control comparison", "isotopic pattern matching", "fragment pattern matching" and "mass defect filter" are marked in green. Additional information such as "assigned biotransformation2", "calculated formula" and "available MS/MS" spectra are marked in blue. Structures can be assigned manually. Metabolites not related to a known biotransformation can also be extracted from the data by the same algorithms.

References

1.
Hopfgartner G., Chernushevich I. V., Covey T., Plomley J. B., Bonner R., "Exact mass measurement of product ions for the structural elucidation of drug metabolites with a tandem quadrupole orthogonal acceleration time-of-flight mass spectrometer", *J. Am. Soc. Mass Spectrom.*, *10*, *1305-1314*, **1999.**

2.
Peterman S. M., Duczak J. N., Kalgutkar A. S., Lame M. E., Soglia J. R., "Application of a linear ion trap/orbitrap mass spectrometer in metabolite characterization studies: Examination of the human liver microsomal metabolism of the non-tricyclic antidepressant nefazodone using data-dependent accurate mass measurement", *J. Am. Soc. Mass Spectrom.*, *17*, *363-375*, **2006.**

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