Improving performance, data and results through expanded dynamic range and higher mass resolution

Identification of pharmaceutical metabolites using the Agilent 6520 Accurate-Mass Q-TOF LC/MS system and Agilent MassHunter Metabolite Identification software

Application Note

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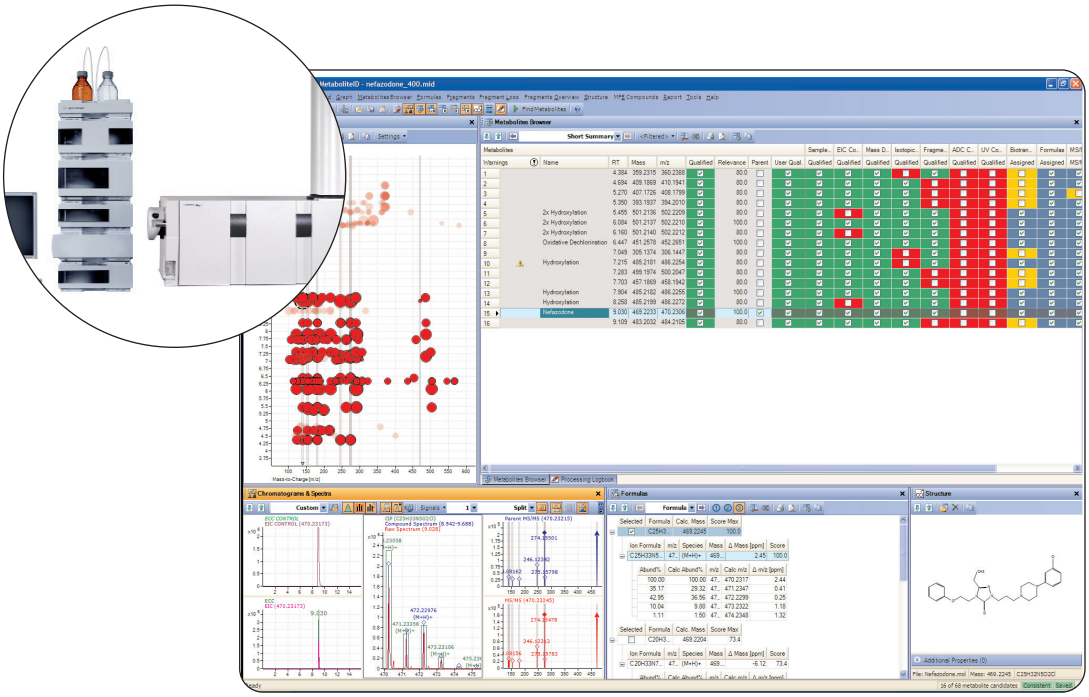

Abstract

This Application Note describes:

- Identification of pharmaceutical metabolites using the Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS system and Agilent MassHunter Metabolite Identification (MetID) software
- The effect of enlarged dynamic range for detection of low abundance metabolites in the presence of other high abundant compounds
- The effect of high mass resolution in MS/MS spectra on the structure elucidation of metabolites

Agilent equipment

- 1200 Series Rapid Resolution LC
- 6520 Accurate-Mass Q-TOF LC/MS system
- MassHunter Metabilite Identification software

Application area

Identification of metabolites of pharmaceutical drugs during drug discovery and development

Agilent Technologies

Introduction

In the development of modern pharmaceutical drugs, it is of crucial importance to identify as early as possible metabolites that occur during drug application because of their potential toxicity. For this task, modern LC/MS equipment is used on a routine basis for data acquisition, which is followed by computer-assisted analysis of the data for increased productivity. An increase in the quality of the acquired data has a tremendous impact on the results of the data analysis. The mass resolution of the mass spectrometry is of special importance. Higher mass resolution creates the possibility to distinguish between two molecules or molecule fragments that have isobaric mass but different molecular constitution, to elucidate the chemical structures.

As an example in the MS domain, the two compounds butyl paraben and methyl acetyl salicylic acid with isobaric mass *m/z* 195 and 36.4 mDa distance were resolved with a resolution of more than 14,000 and their masses were measured with high accuracy for formula confirmation (figure 1). In the MS/MS domain, the two isobaric fragments of sulfadimethoxine with *m/z* 156 and 65.4 mDA distance were resolved with a resolution of more than 11,000 and formulas of the fragments were calculated with relative mass errors below 1 ppm (figure 2).

Another important factor in the acquisition of a mass spectrum is the dynamic range of the mass spectrometer. With an expanded

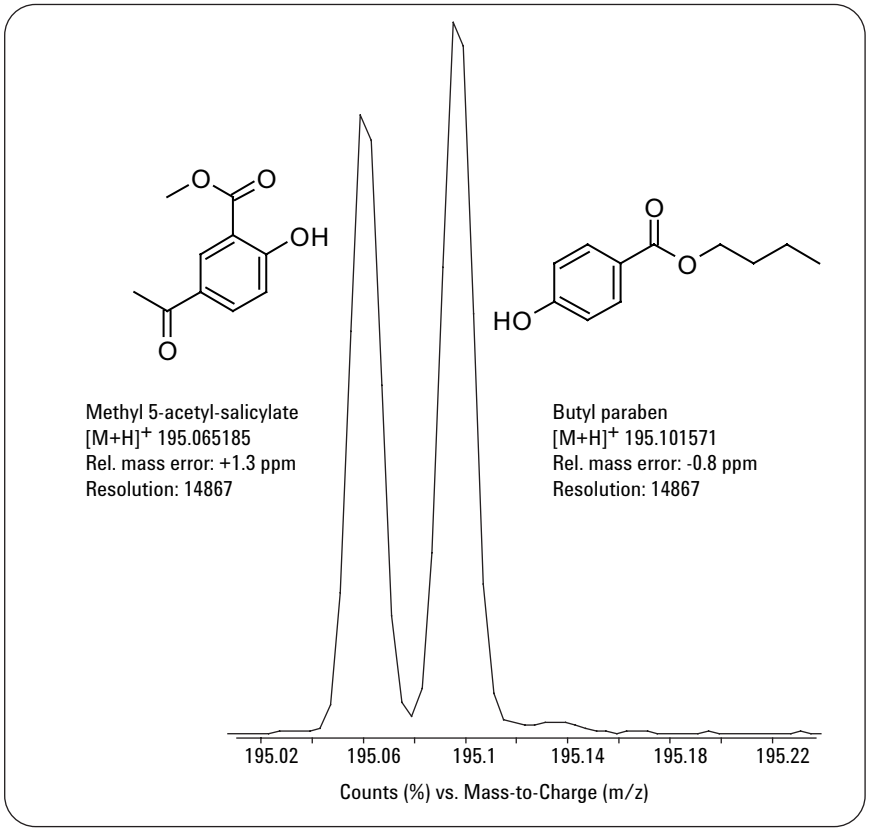

Figure 1
Enhanced resolving power for the separation of compounds with isobaric molecular mass and measured mass accuracies.

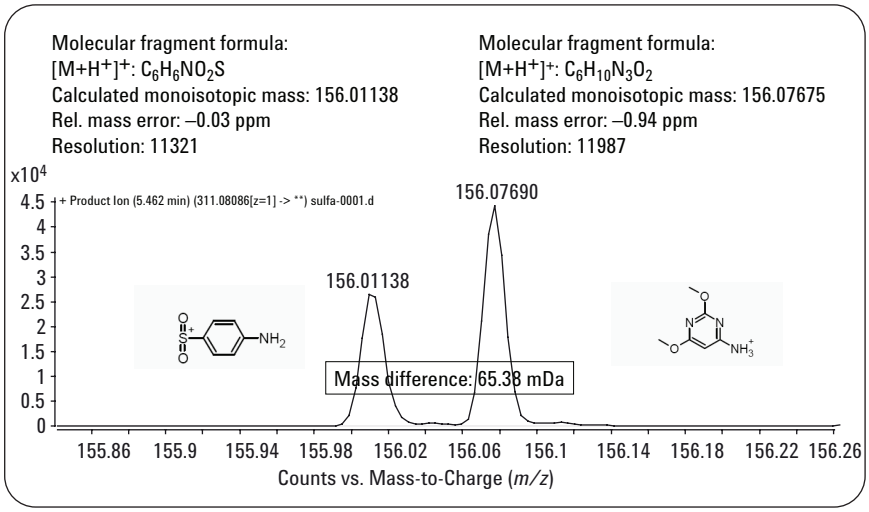

Figure 1
Enhanced resolving power for the separation of MS/MS fragments of the compound sulfadimethoxine with isobaric mass at *m/z* 156.0, measured mass accuracies and calculated formulas (Sulfdiamethoxine: molecular formula $[M+H^+]^+$, $C_{12}H_{15}N_4O_4S$; calculated monoisotopic mass 311.08085; measured mass 311.08086; relative mass error 0.01 ppm; resolution 15176).

dynamic range potential low abundant metabolites can be detected in the presence of other high abundant metabolites, parent drug or matrix component. As an example, a series of concentrations of the pharmaceutical drug nefazodone was measured. The detection began at a limit of detection (LOD) of 2 pg on-column and finished with detector saturation at a concentration of 100 ng on-column (figure 3). For all concentrations the formula was calculated from the measured mass with a relatively low mass error of below 2 ppm.

This Application Note describes the use of the expanded dynamic range of the Agilent 6520 mass-accurate Q-TOF LC/MS system to detect low abundant metabolites of a pharmaceutical drug. The high mass resolution of this system was used to elucidate the structures of two isomeric metabolites from their MS/MS spectra by differentiation between two fragments of isobaric mass.

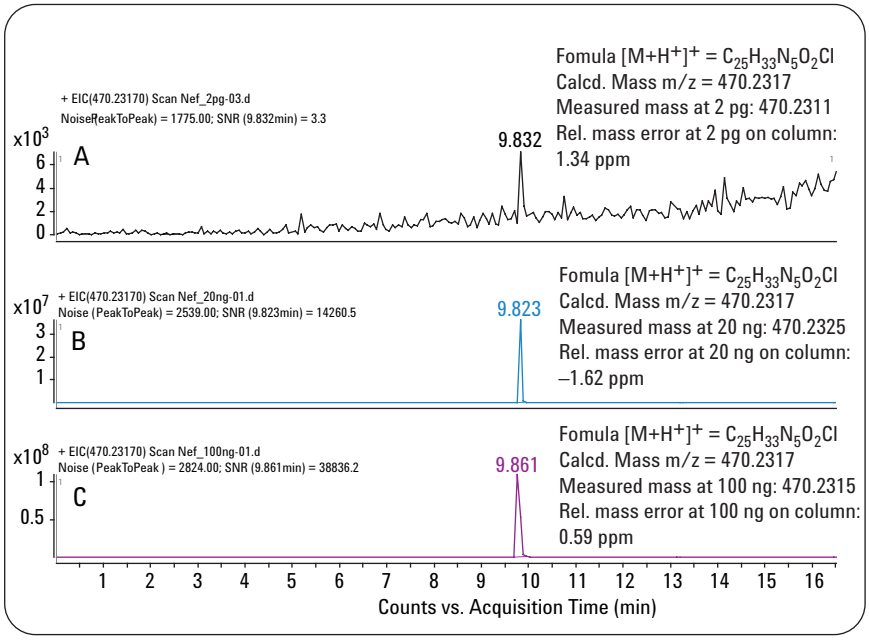

Figure 3
Dynamic range of nefazodone by QTOF analysis.
A) 2 pg on-column; S/N = 3.3 (LOD; relative mass error 1.34 ppm);
B) 20 ng on-column; S/N = 14,260.5 (4 orders of magnitude); relative mass error -1.62 ppm);
C) 100 ng on-column; S/N = 38,836.2 (4.5 orders of magnitude); relative mass error 0.59 ppm).

Experimental

Equipment

- Agilent 1200 Series Rapid Resolution LC system with binary pump SL and degasser, high performance autosampler SL with thermostat, thermostatted column compartment SL and diode array detector SL
- Agilent 6520 Accurate-Mass Q-TOF
- ZORBAX SB-C18 column, 2.1 x 150 mm, 1.8 µm

Sample preparation

The stock solutions contained:

- 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

The NADPH regeneration system consisted of 1.6 mL NADP solution + 1.6 mL isocitrate solution + 100 µL isocitrate dehydrogenase solution.

The incubation mixture consisted of 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 t=0 min and t=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 for LC/MS analysis. Incubation stopped at 0 min was used as control.

High resolution LC/MS method

Solvent A: Water + 0.1 % formic acid
Solvent B: ACN + 0.1 % formic acid
Flow rate: 0.5 mL/min
Gradient: 0 min, 5 %B; 15 min, 95 %B
Stop time: 15 min
Post time: 10 min
Inj. volume: 1-10 µL, needle wash, samples cooled to 4 °C, automated delay volume reduction function was used
Detection: 210 nm (±4), ref. 360 nm (±16), 2 µL flow cell, 3 mm path length
Column temp.: 60 °C

QTOF MS and MSMS method

Source: ESI in positive mode with dual spray for reference mass solution
Dry gas: 10.0 L/min
Dry Temp.: 300 °C
Nebulizer: 45 psi
Mass range: 100-1000
Fragmentor: 200 V
Skimmer: 60 V
Capillary: 4000 V
Collision energy: 35 V
Data depended MS/MS: 2 compounds, 3 MS/MS spectra, exclusion for 0.25 min

Data analysis with MassHunter MetID software

The first step in the analysis comprised a comparison between the data from the metabolite compounds (sample, incubation time t>0) and the data from the pure parent drug (control, incubation time t=0). All detectable mass signals were extracted from the MS level data using the molecular feature extraction (MFE) algorithm. Adduct masses of related compounds were grouped together into discrete molecular features and chemical noise was removed. The compound lists of the metabolite sample and the control were then compared. All new compounds or those which increased in amount in the metabolized sample were considered to be potential metabolites and subjected to further analysis by different user-specified algorithms. The algorithms were able to identify and qualify new metabolites or simply qualify impurity compounds found by another algorithm. The results of all compound identification algorithms were weighted and combined to a final identification relevance score. Metabolites were qualified when their final score was above a defined relevance threshold. The results from all algorithms were collated in a results table, which could be inspected at-a-glance.

Results and discussion

The pharmaceutical drug nefazodone is a good example of a drug that undergoes excessive metabolism. In the metabolic reaction, many expected metabolites such as hydroxylated compounds are formed. In addition, many unexpected low level metabolites of the drug such as modified fragments are created. To detect these low level metabolites, the injected amount of sample was increased. The expanded dynamic range of the Agilent 6520 Accurate-Mass Q-TOF LC/MS system facilitated easy detection of these metabolites as well as the main metabolites and the parent drug without overloading the detector or reducing the accuracy of the mass measurement (figure 4). The molecular

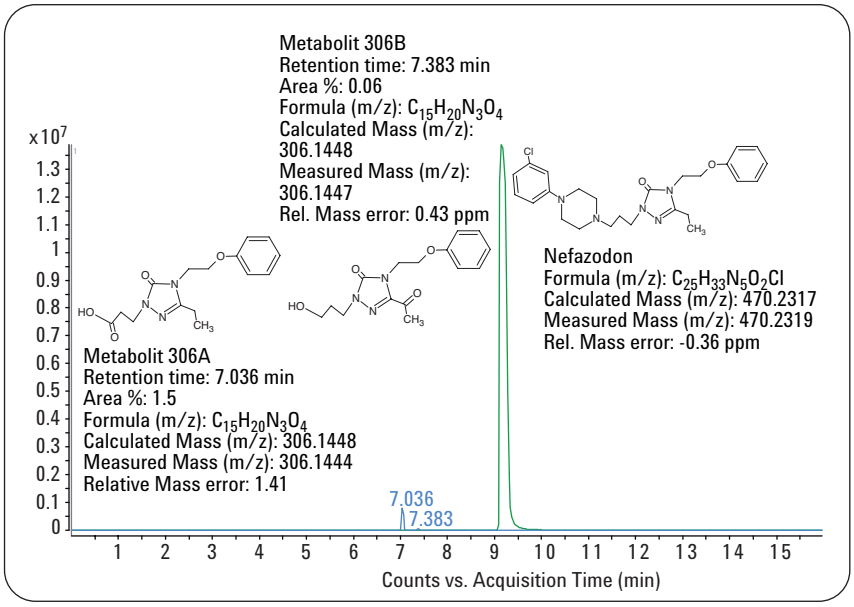

Figure 4
Extracted ion chromatogram (EIC) of the pharmaceutical drug nefazodon together with the isomeric low level metabolites at mass *m/z* 306.1448.

mass of the main compound nefazodone was measured with a mass error of -0.36 ppm relative to the calculated molecular formula. The MS/MS fragmentation of nefazodone is shown in figure 5. The mass of the metabolite 306A was measured with a mass error of 1.41 ppm relative to the calculated molecular formula and the mass of metabolite 306B was measured with a relative mass error of 0.43 ppm.

The metabolites 306A and 306B with an isobaric molecular mass of 305.1375 were distinguished by their MS/MS spectra and formulas could be assigned (figures 6 and 7). In comparison to the MS/MS spectrum of the parent drug, the spectra of the metabolites showed that they were derived from the nefazodone fragment at *m/z* 274.1553 by the addition of two oxygen atoms. The metabolite 306A showed a characteristic MS/MS fragment at *m/z* 234.1239 with the formula $C_{12}H_{16}N_3O_2$ and a relative mass error of -0.84 ppm (figure 6). Another fragment was observed at m/z 140.0823 with the formula $C_6H_{10}N_3O$ and a relative mass error of -3.30 ppm, which gave a definite structure assignment.

An alternative structure for this MS/MS fragment could be $C_5H_6N_3O_2$ at *m/z* 140.0454. The difference between both possibilities is only 36.4 mDa. Both possibilities can be differentiated easily as a result of mass resolution of the Agilent 6520 Accurate Mass Q-TOF LC/MS system, which is about 15,000 in the mass range between m/z 100 and 200. The mass error for the accurate mass of this fragment relative to the measured mass would be -263 ppm. With these considerations the proposed structure could be assigned to the metabolite 306A (figure 4).

The MS/MS fragment mass of metabolite 306B also indicates that this metabolite molecule is derived from the parent drug fragment at *m/z* 274.1553 by the addi-

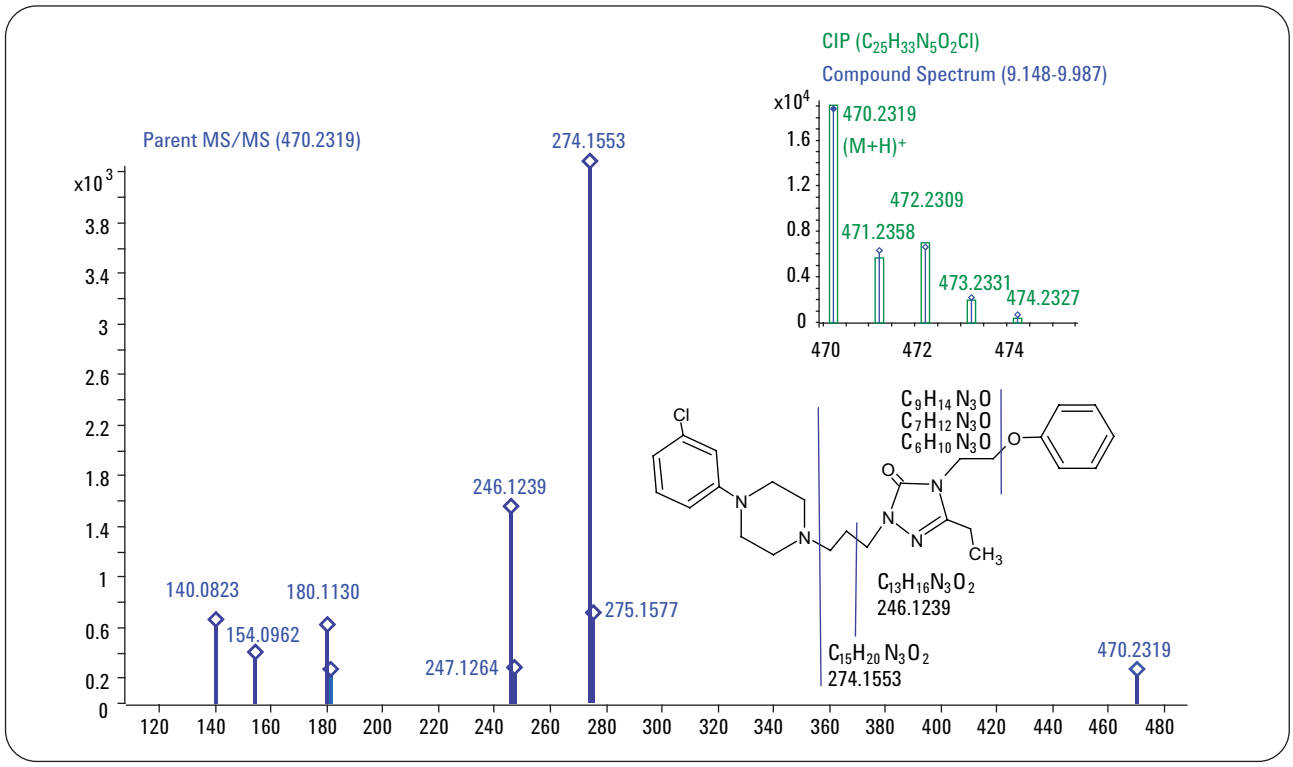

Figure 5
Mass spectrum, isotopic analysis and MS/MS spectrum for fragment assignment of parent drug nefazodone.

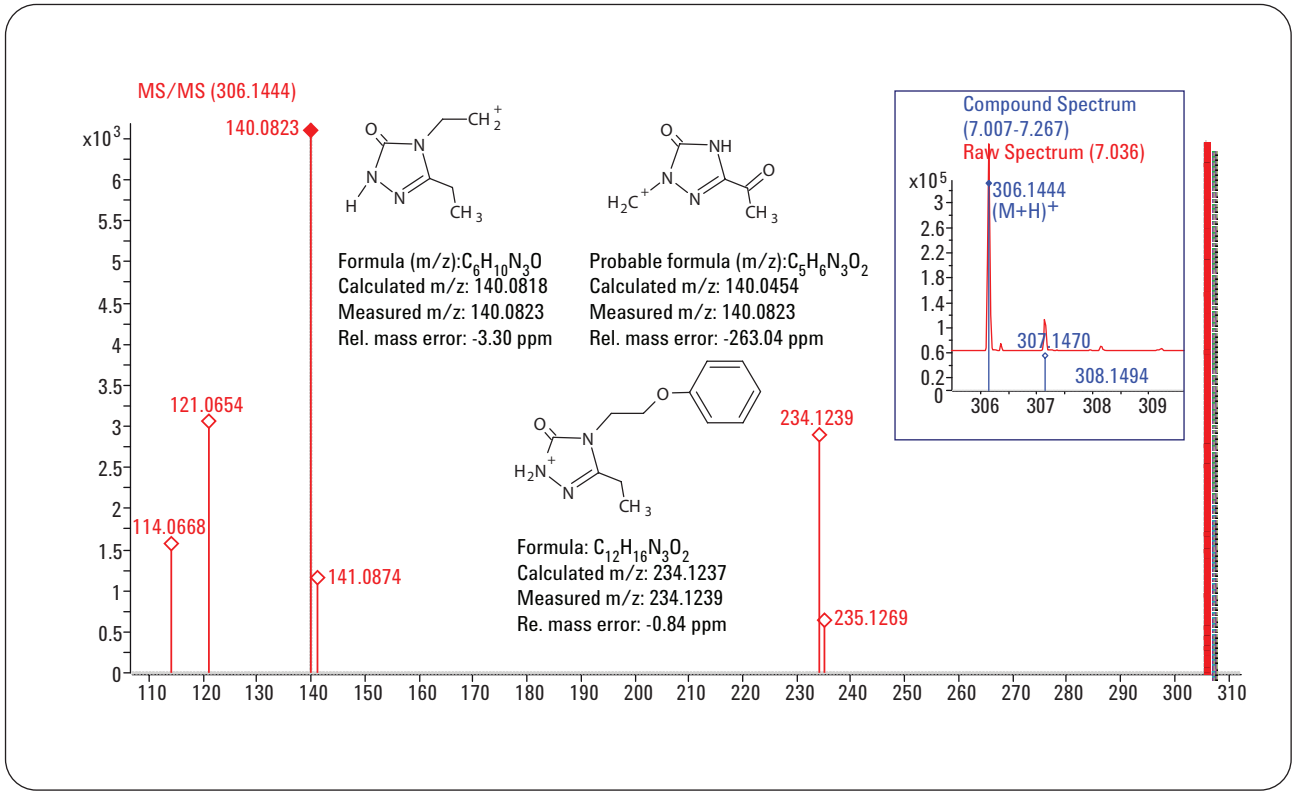

Figure 6
MS/MS spectrum of the metabolite 306A with its characteristic fragmentation. The insert shows the MS spectrum and the isotopic pattern of the metabolite 306A.

tional mass of two oxygen atoms (figure 7). But compared to metabolite 306A the reversed situation was found for the typical MS/MS fragment at *m/z* 140 in the MS/MS spectrum of metabolite 306B. The MS/MS spectrum was different from the previous one and indicates a different structure for this metabolite. The fragment at *m/z* 212.1039 with the formula $C_9H_{14}N_3O_3$ indicated that two additional oxygen atoms were present in the left part of the molecule, which remained after fragmentation of the phenyl moiety (C_6H_6O). The fragment at m/z 194.0932 with the formula $C_9H_{12}N_3O_2$ indicated the loss of a water molecule. This led to the conclusion that one of the oxygen atoms was in a hydroxyl group at the left end of the molecule. The MS/MS fragment at *m/z* 140.0460 with the formula $C_5H_6N_3O_2$ (-3.91 ppm) indicated oxidation of metabolite 306B to a keto group when compared to the measured mass for this fragment in metabolite 306A and nefazodone at *m/z* 140.0823 with the formula $C_6H_{10}N_3O$.

Conclusion

This Application Note described the value of improved performance in dynamic range and mass resolution of the Agilent 6520 Accurate Mass Q-TOF LC/MS system in combination with the specialized Agilent MassHunter Metabolite ID (MetID) software for the identification of metabolites of pharmaceutical drugs. The enlarged dynamic range facilitated the detection of low level metabolites in the presence of other high

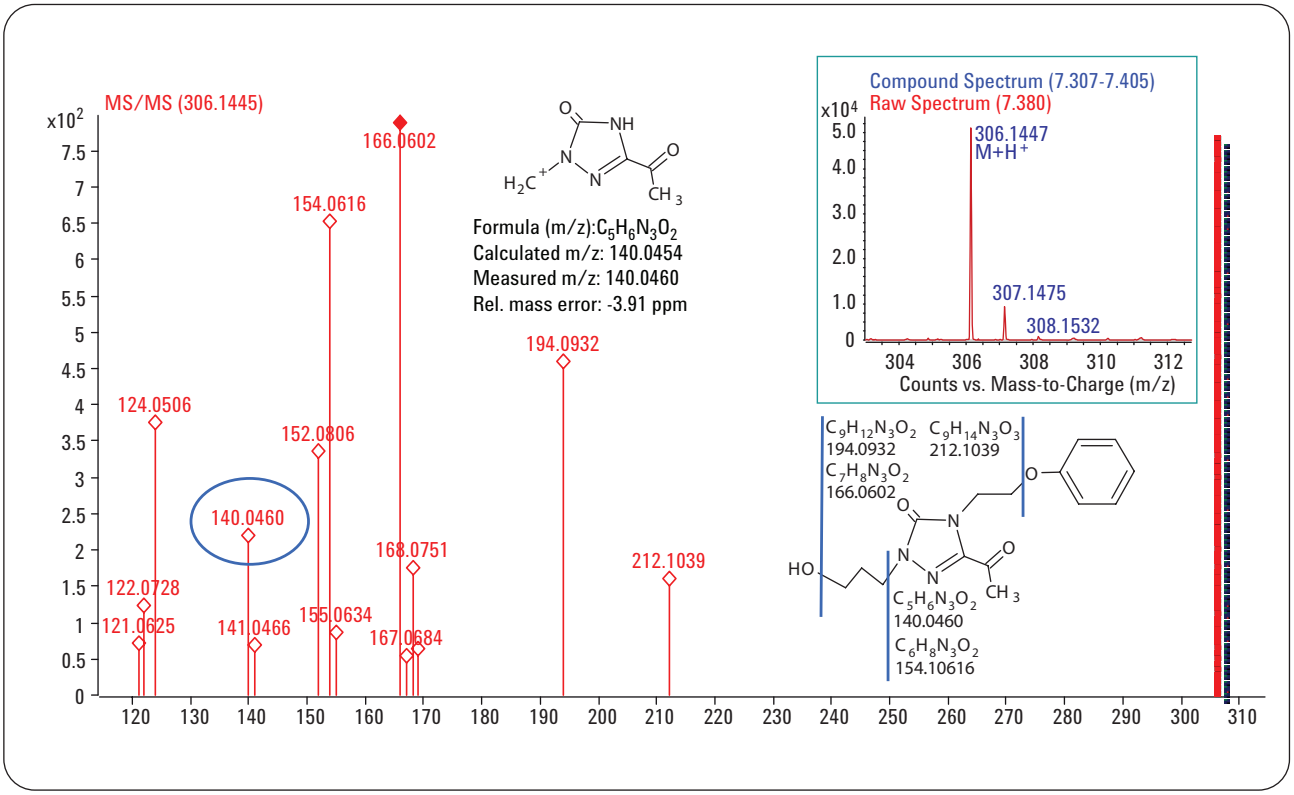

Figure 7
MS/MS spectrum of the metabolite 306B with its characteristic fragmentation. The insert shows the MS spectrum and the isotopic pattern of the metabolite 306B.

abundant compounds such as the highly concentrated drug, high level metabolites or matrix components, without influencing the accuracies of measured masses for the calculation of molecular formulas. The improved resolution allowed reliable differentiation between possible molecular and fragment formulas and thereby more reliable structure elucidation.

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