Identification of metabolites by triple quadrupole mass spectrometry

Detection and identification of pharmaceutical drug metabolites in DMPK by application of different scan modes of the Agilent 6410B Triple Quadrupole LC/MS system

Drug Discovery

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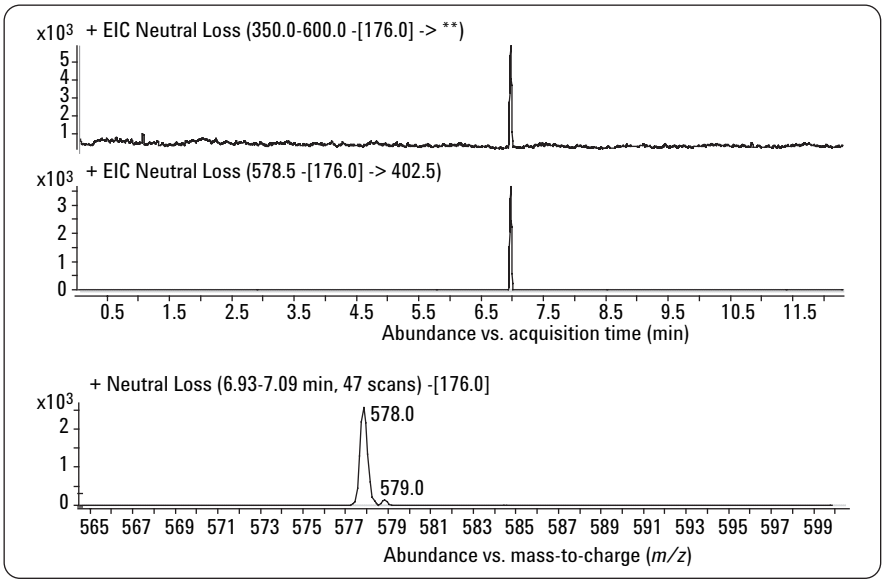

Abstract

This Application Note describes:

- Metabolite identification using the different scan modes available with the Agilent 6410B Triple Quadrupole LC/MS system
- Use of the Agilent 1200 Series Rapid Resolution LC (RRLC) system for separation of compounds in complex mixtures on Agilent ZORBAX Rapid Resolution High Throughput (RRHT) columns with 1.8 µm particle size
- The Agilent MassHunter Workstation software for data acquisition and qualitative data analysis

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Introduction

In the development of a new pharmaceutical drug, it is necessary to know all important ADME-Tox (adsorption, distribution, metabolism, excretion, and toxicity) parameters of a chemical compound because they give an indication about the capability of the new chemical entity (NCE) to act as a drug.[1] Today, mass spectrometry (MS) plays a major role in the DMPK (drug metabolism and pharmacokinetic) laboratory to measure these parameters.[2,3] In these specialized laboratories, an often-used MS is the triple quadruple (QQQ) mass spectrometer because of its capability to run a large variety of different experiments.[4] The QQQ instrument is comprised of three quadrupoles arranged in a linear row, where the first (Q1) and third (Q3) act as mass filters and the quadrupole in the middle (Q2) acts as a collision cell for molecular fragmentation and generation of MS/MS information.

The following types of experiments can be performed:

1. In the most simple full scan mode, the ions are passed through the first and second quadrupoles (Q1, Q2) and the last one (Q3) is scanned to generate a simple *m/z* versus abundance mass spectrum. To achieve selectivity, it is possible to adjust the last quadrupole (Q3) to pass only a single mass for selected ion monitoring (SIM).

When the QQQ instrument is used for metabolite identification, there are three special scan modes:

2. Product ion scan, where Q1 acts as a mass filter to selectively isolate a single *m/z*, which is fragmented in Q2. The fragments are scanned in Q3 for the generation of a fragment mass spectrum. This scan mode creates an MS/MS spectrum for the precursor ion selected by Q1.

3. Precursor ion scan, where Q1 scans the ion masses coming from the source, the ions are fragmented in Q2, and Q3 is configured as a static mass filter for a predefined ion. Within this operation mode, a predefined fragment ion can be detected and the corresponding precursor ion can be identified.

4. Neutral loss scan, where both Q1 and Q3 act as scanning analyzers and fragmentation takes place in Q2. The quadrupole analyzers Q1 and Q3 are linked and offset by a predefined neutral loss. A detected fragment mass is compared with the precursor molecular mass and only the spectra that show the predefined loss between precursor and product ion are indicated.

With these specialized scan modes, a workflow can be implemented using the QQQ to detect metabolites by precursor or neutral loss scan and to elucidate their identity by product ion scan.[5]

5. Another widely used QQQ scan mode is the multiple reaction monitoring (MRM) mode. In this scan mode, a single precursor ion is selectively filtered by Q1 and fragmented in Q2, and a unique fragment ion is selectively filtered by Q3. MRM provides minimal structural information, but is the method of choice for highest selectivity in quantification. This method is used, for instance, to measure a metabolic stability curve of a drug.[6]

This Application Note describes the use of the various scan modes available with the Agilent 6410B Triple Quadrupole LC/MS system for metabolite identification.

Experimental

Equipment

- Agilent 1200 Series Rapid Resolution LC (RRLC) system
- Agilent 6410B Triple Quadrupole LC/MS system
- Agilent ZORBAX SB-C18 RRHT column, 2.1 x 150 mm, 1.8 µm particle size
- Agilent MassHunter Workstation software for data acquisition and qualitative data analysis

RRLC method

- Solvent A: Water + 0.1 % formic acid (FA)
- Solvent B: Acetonitrile (ACN) + 0.1 % FA
- Flow rate: 0.5 mL/min
- Gradient: 0 min 5 %B, 0.2 min 5 %B, 17 min 85 %B, 17.1 min 95 %B, 20 min 95 %B
- Stop time: 20 min
- Post time: 10 min
- Sample injection volume: 1 µL with needle wash
- Thermostatted autosampler: 4 °C
- Automated delay volume reduction: ON
- Diode-array detector (DAD): 220 nm (+/-4), Ref. 360 nm (+/-8)
- Flow cell: 2 µL (10 mm path)
- Column oven: 50 °C

QQQ mass spectrometer method

- Source: Electrospray (ESI) in positive mode
- Nebulizer: 50 psi
- Dry gas: 12.0 L/min
- Dry temperature: 350 °C
- Capillary: 3000 V
- Fragmentor: 180 V
- Skimmer: 60 V
- Collision energy: 30 eV
- Mass range: 50-1000
- Data acquisition modes: MS2SIM (a SIM experiment where Q3 passes only a single *m/z* value), precursor ion scan, product ion scan, neutral loss scan

Sample preparation
The following stock solutions were prepared:

- 20 mg/mL S9 preparation from rat liver
- 0.1 mg/mL buspirone 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 $MgCl_2 \cdot 6H_2O$ + 258.1 mg isocitrate in 20 mL H_2O)
- Isocitrate dehydrogenase (IDH) 0.33 U/µL, where U is Units, a measure of enzymatic activity

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

The incubation mixture consisted of 3.85 µL substrate + 200 µL NADPH regeneration system + 746.15 µL phosphate buffer + 50 µL S9 preparation.

Incubation was carried out at 37 °C for 60 minutes. The reaction was stopped by adding 60 µL perchloric acid and 1000 µL acetonitrile, followed by centrifugation for 15 minutes at 14,000 rpm. The supernatant was removed and evaporated to dryness using a SpeedVac concentrator (Bachhover, Germany) and reconstituted with 250 µL water containing 0.1 % formic acid for LC/MS analysis as described above. For the control sample, incubation was stopped immediately at t=0 minutes by adding 60 µL perchloric acid and 1000 µL acetonitrile and processed as described.

Results and discussion

Analysis of the parent drug
In the first part of the experiment, the MS and MS/MS data of the parent drug buspirone were acquired. Therefore, a standard solution was analyzed first by MS2SIM on the mass of the protonated parent drug at *m/z* 386.25 ($C_{21}H_{32}N_5O_2$). See figure 1. The MS2SIM showed only the mass peak for the protonated ion of the parent drug (*m/z* 386.3) at a retention time of 9.1 minutes.

For the determination of the MS/MS fragmentation pattern of the parent drug, the QQQ was operated in the product ion scan mode to generate an MS/MS spectrum of the drug molecule (figure 2). With the ions obtained

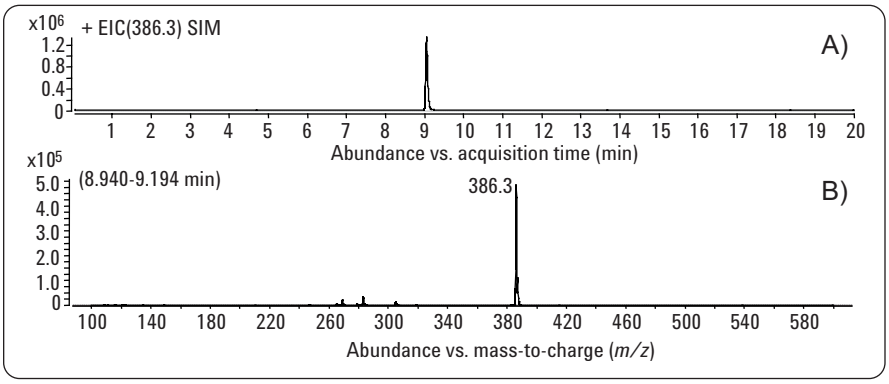

Figure 1
A) Extracted ion chromatogram (EIC) of buspirone (*m/z* = 386.3) from QQQ MS2SIM and B) corresponding MS spectrum.

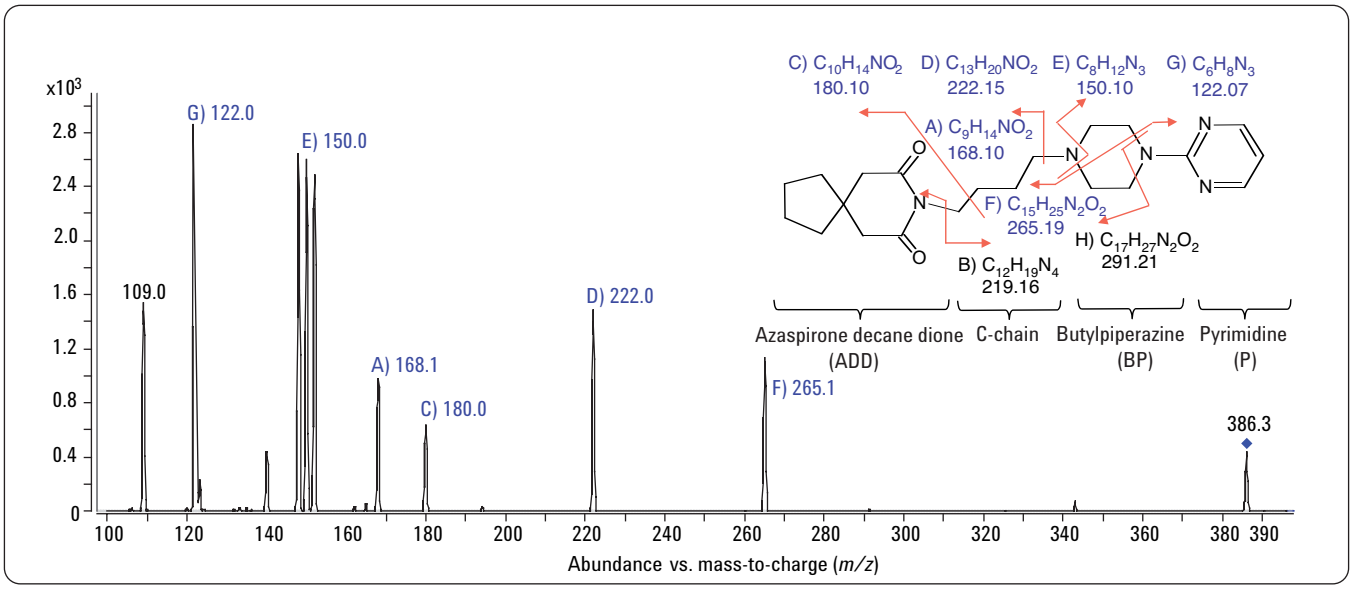

Figure 2
MS/MS spectrum of buspirone by QQQ product ion scan and interpretation of the fragmentation pattern.

in this MS/MS spectrum, the fragmentation behavior of the parent drug can be explained sufficiently. There are fragments that were very typical for different parts of the parent drug molecule. For example, the fragments G) at *m/z* 122.1 and E) at *m/z* 150.1 are typical for the butylpiperazine (BP) and pyrimidine (P) moieties, respectively. The fragments A) at *m/z* 168.1 and D) at *m/z* 222.1 are typical for the other side of the molecule – the azaspirone decane dione (ADD) and C-chain moieties of the molecule, respectively.

With this information from the MS/MS spectrum of the parent drug, the experiment for general metabolite detection could be set up in the precursor ion scan mode. Therefore, Q3 could be set to filter the typical fragment ions at *m/z* 122.1, 150.1, 168.1, and 222.1. As an example, the parent drug buspirone could be detected by the appearance of each fragment in the precursor ion scan (figure 3). Now, with the assumption that metabolite compounds of the parent drug are able to undergo similar fragmentation and produce some of the same mass fragments in the MS/MS spectrum, the samples of the metabolized drug could be examined.

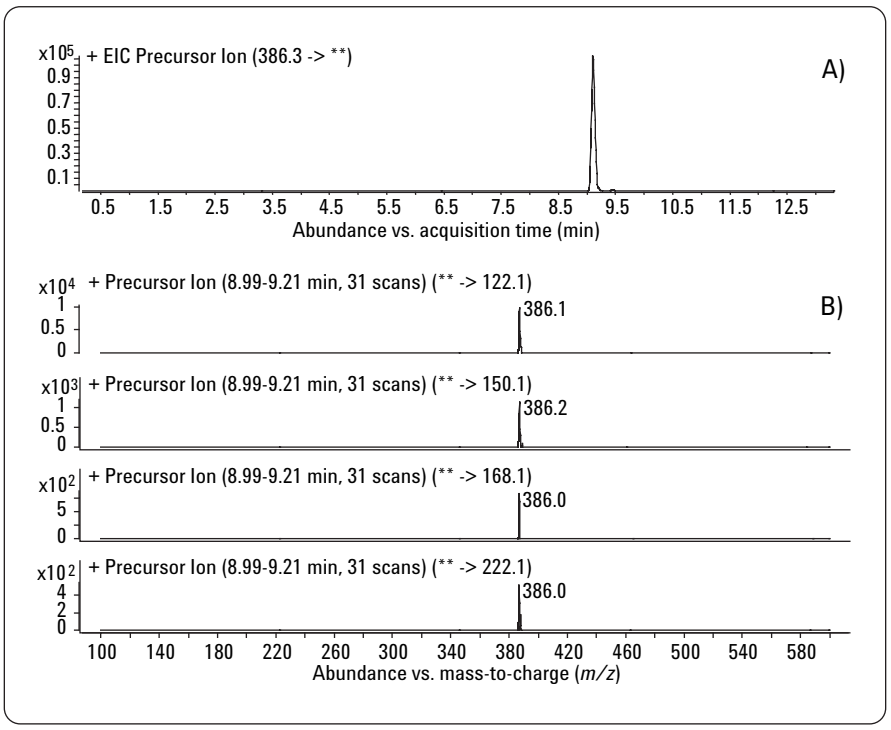

Figure 3
Identification of buspirone by QQQ precursor ion scan. A) Buspirone peak at a retention time (RT) of 8.9-9.2 minutes. B) Precursor ion spectra for the various fragment masses derived from buspirone (*m/z* 122.1, 150.1, 168.1, 222.1).

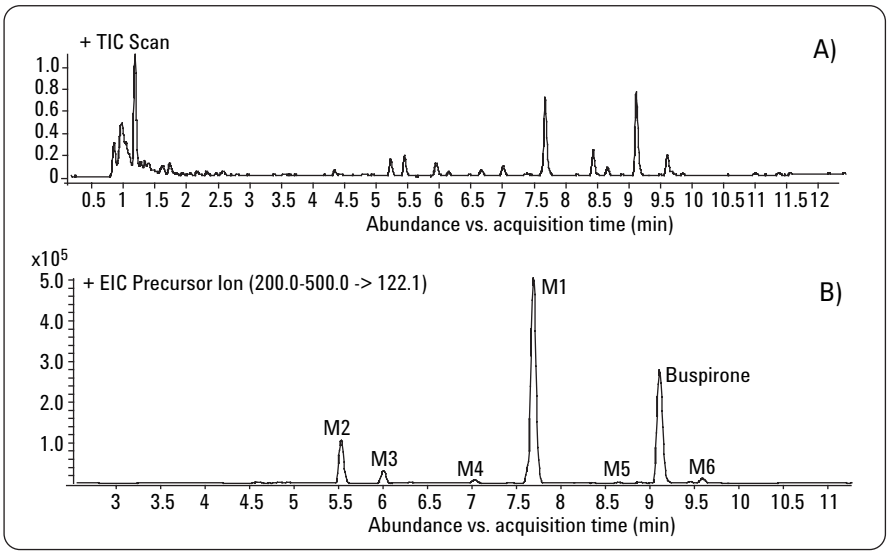

Figure 4
Detection of main metabolites from buspirone in a precursor ion scan experiment. A) Total ion chromatogram (TIC). B) Extracted ion chromatogram of the transition to the fragment ion at *m/z* 122.1. M = metabolite.

Detection of metabolites by a precursor ion scan experiment

To identify possible metabolites of the drug buspirone, a typical precursor ion scan experiment was performed with adjustment of the third quadrupole to filter the masses of fragments G) at *m/z* 122.1, E) at *m/z* 150.2, A) at *m/z* 168.1, and D) at *m/z* 222.1. From the obtained data, a total ion chromatogram (TIC) was created (figure 4A). An extracted ion chromatogram (EIC) was created for the transition of the fragment ion at *m/z* 122.1, which showed the detection of six possible main metabolites (figure 4B). Examination of the mass spectra revealed that the precursors of this transition were the metabolites at *m/z* 402.25 ($C_{21}H_{32}N_5O_3$) derived from a metabolic hydroxylation reaction (figure 5A). By examining the individual transitions in more detail, it was possible to make rough decisions about the structure of the possible metabolites (figures 5B through 5D).

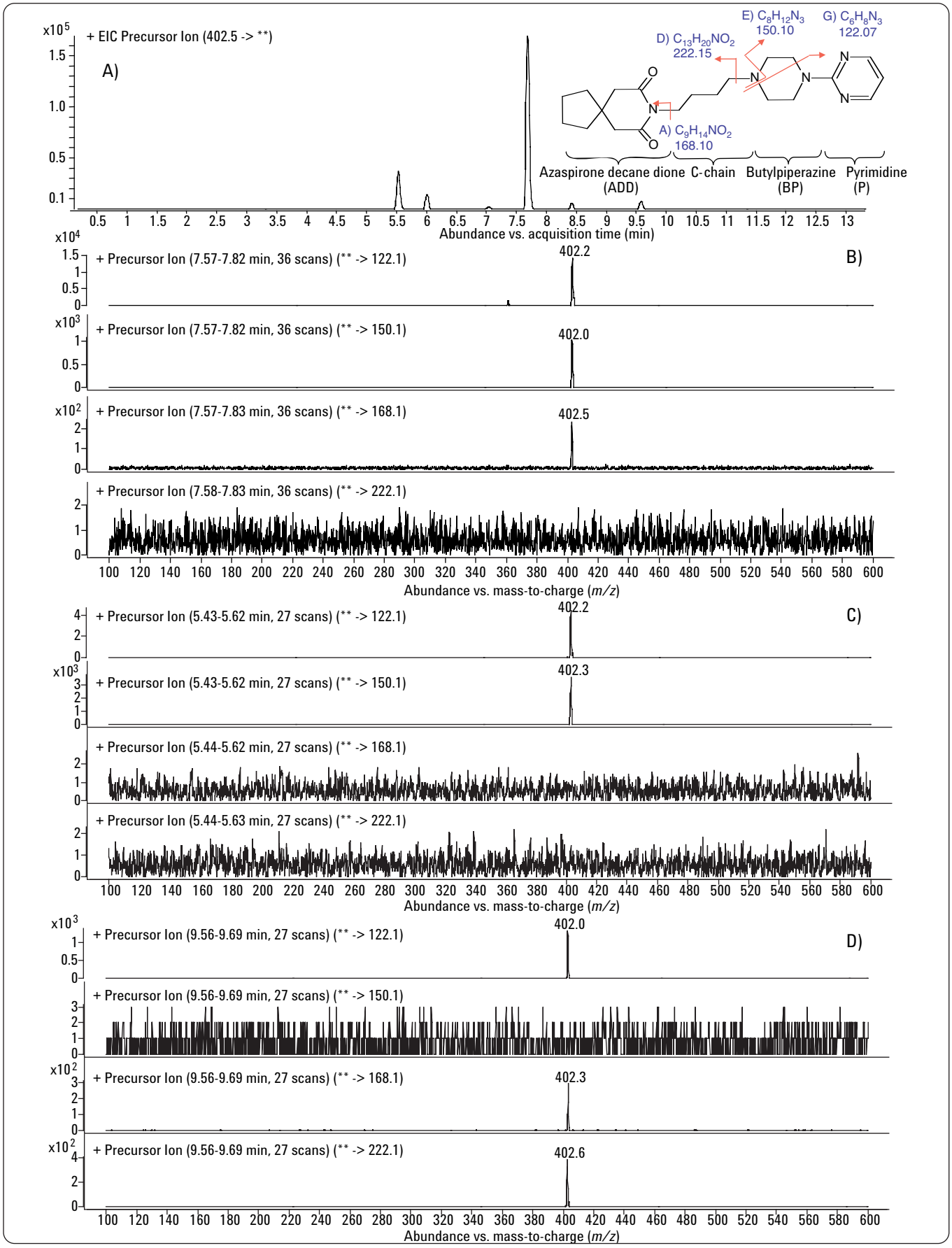

Figure 5

Detection of metabolites by precursor ion scan mode. A) Extracted ion chromatogram of metabolites at *m/z* 402.5, showing the product ions G) at *m/z* 122.1, E) at *m/z* 150.1, A) at *m/z* 168.1, and D) at *m/z* 222.1. B-D) Precursor ion scan spectra of various metabolites.

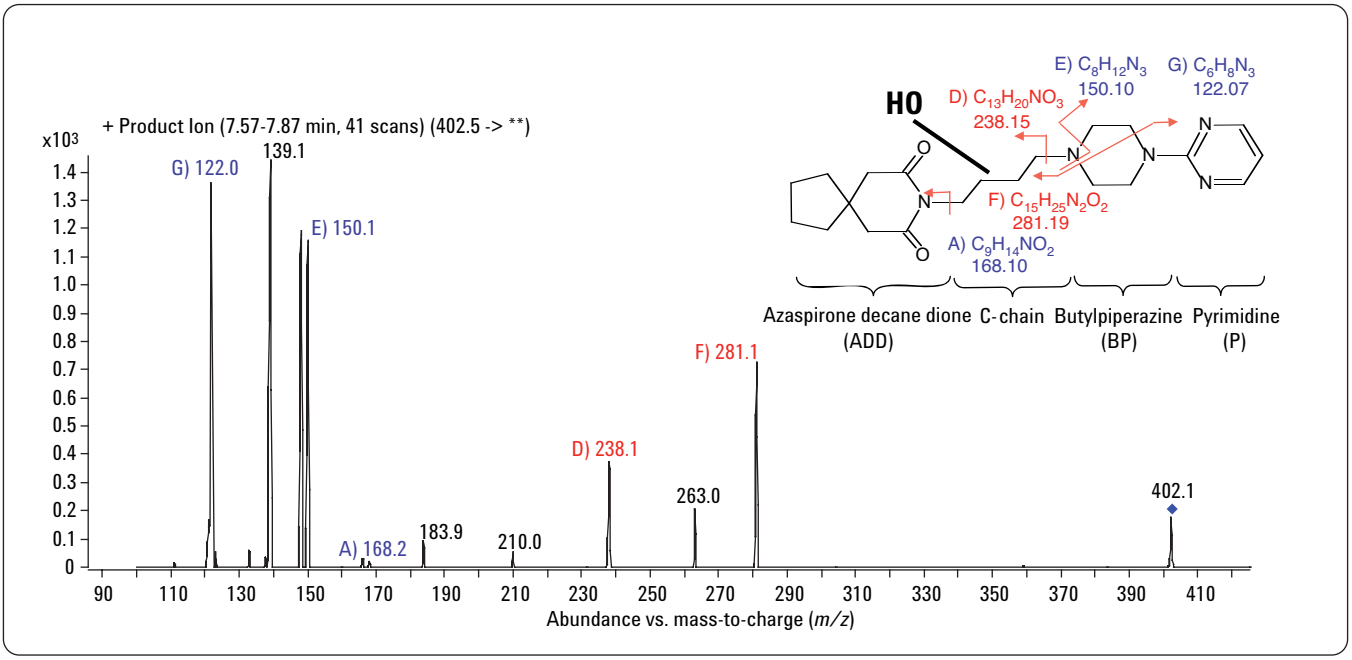

Figure 6
MS/MS spectrum of hydroxy metabolite of buspirone, retention time 7.57-7.87 minutes, with hydroxy modification at the C-chain.

For the first investigated hydroxy metabolite, which was eluted at a retention time of 7.57-7.82 minutes, there was no fragment D) at *m/z* 222.1 (figure 5B). This evidence, together with the transitions that were detected for the remaining fragments G) at *m/z* 122.1, E) at *m/z* 150.1 and A) at *m/z* 168.1, indicates that the compound had a hydroxylation modification at the C-chain of buspirone (figure insert). The hydroxy metabolite that was eluted at 5.43-5.62 minutes could have the hydroxyl modification at the azaspirone decane dione (ADD) moiety (figure 5C), because only the fragments E) and G) were detected. And the metabolite that was eluted at 9.56-9.69 minutes should have the modification at the butylpiperazine (BP) moiety because only the fragment G) at *m/z* 150.1 was not detected (figure 5D).

For a more detailed structure elucidation, an MS/MS spectrum of the metabolite eluting at 7.57-7.87 minutes was acquired in the product ion scan mode (figure 6). The MS/MS spectrum clearly showed that the fragment masses D) and F) were shifted by the addition of an oxygen atom. The masses of the fragments A), E), and G) were retained, which gave evidence for a hydroxylation at a carbon atom in the C-chain.

Additionally, as shown in figure 7A, lower-level metabolites showing the same fragment masses in the precursor ion scan were detected and identified as dihydroxy metabolites of buspirone at *m/z* 418.25 ($[M+H]^+$ = $C_{21}H_{32}N_5O_4$). The metabolite eluting at 7.35 minutes was probably modified by the hydroxylation in the ADD and the BP moiety of the original drug molecule because only the fragment D) at *m/z* 122.1 was retained (figure 7B).

Detection of metabolites by neutral loss scan

In the course of the metabolism of drug compounds, they first undergo simple phase I reactions, for example, hydroxylation. To make these modified drug compounds more soluble and more easily excreted via the renal pathway, they are conjugated (for example, with glucuronic acid) in phase II metabolism. Under fragmentation conditions during MS/MS analysis of such compounds, they preferably lose exactly these conjugated parts of the molecule.

By analysis of phase II metabolites in the neutral loss mode of a triple quadrupole instrument, such specific losses are easily detectable and hence the phase II metabolite can be identified. The neutral loss experiment from a phase II metabolism sample of

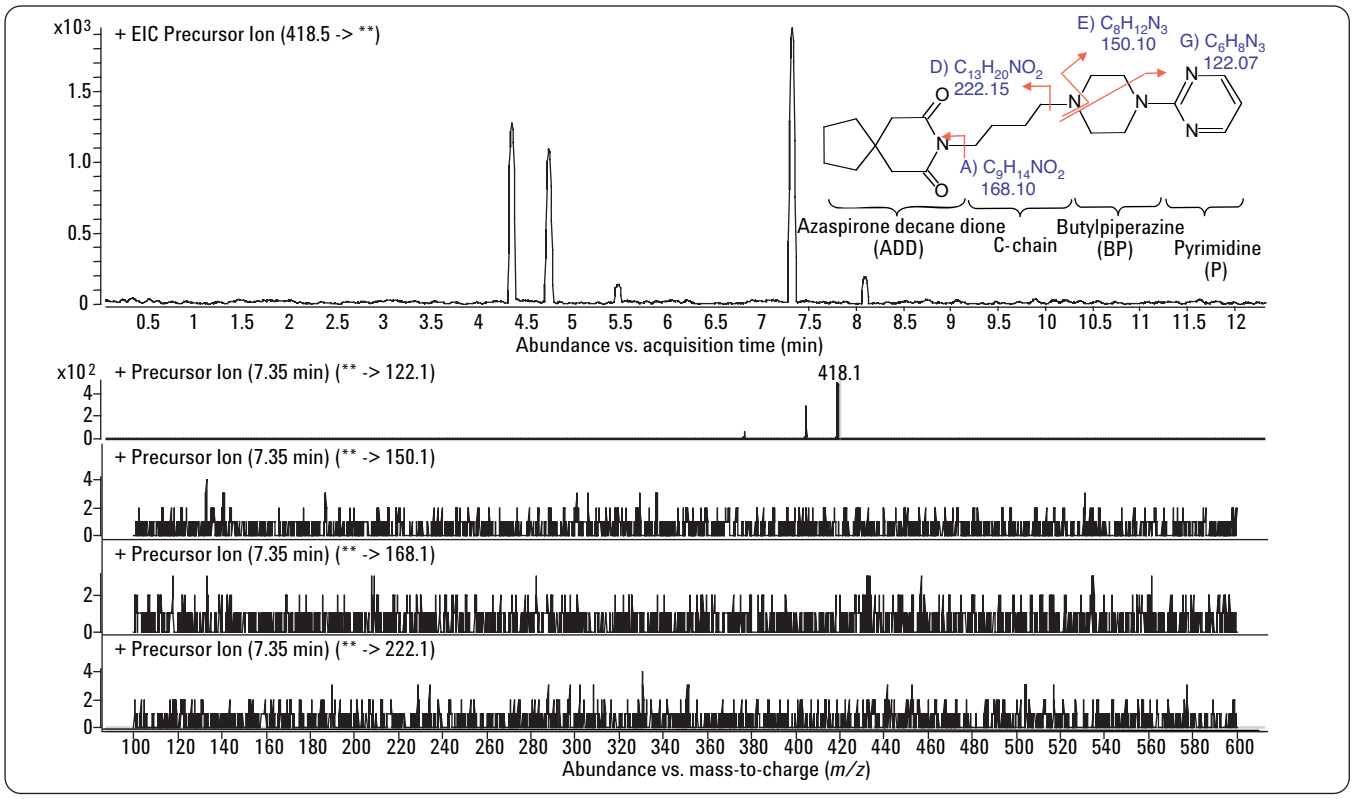

Figure 7
A) EIC of dihydroxy metabolites at *m/z* 418.25. B) Precursor ion spectra of dihydroxy metabolite at a retention time of 7.35 minutes.

the drug buspirone gave a single peak in the EIC at a retention time of 6.93-7.09 minutes. The peak was for the neutral loss of a fragment with mass 176.0 in the compound mass range from 300 to 600. This is characteristic for the loss of a glucuronic acid fragment from a hydroxy metabolite (figure 8A). The EIC for the neutral loss transition for the hydroxy buspirone glucuronic acid conjugate at *m/z* 578.5 to the hydroxy buspirone precursor at *m/z* 402.5 showed the metabolite peak at 6.93-7.09 minutes (figure 8B). The MS spectrum gave basic mass information (figure 8C).

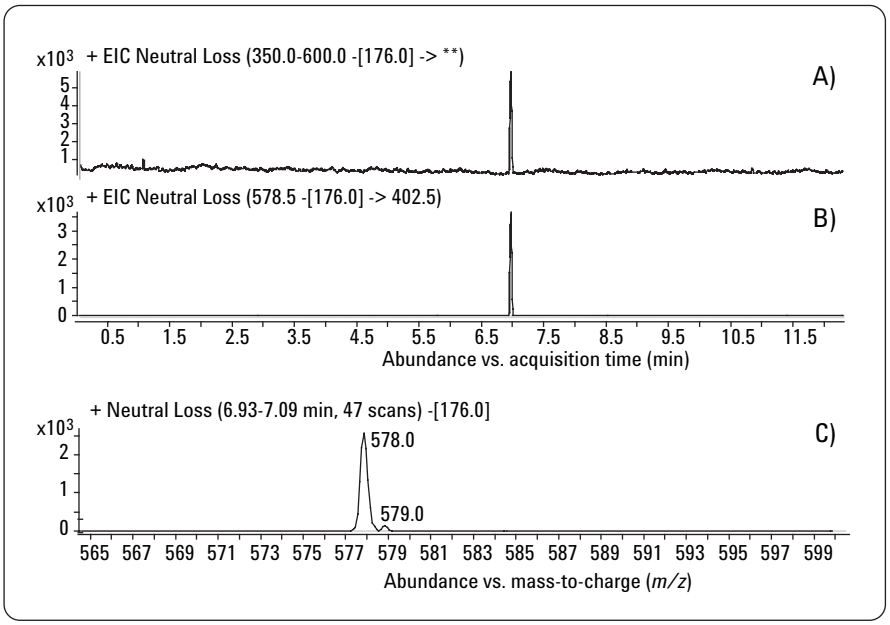

Figure 8
Triple quadrupole neutral loss experiment. A) Detection of phase II metabolites by neutral loss of 176. B) EIC of neutral loss from the hydroxy buspirone glucuronic acid conjugate at *m/z* 578.5 to the monohydroxylated compound. C) MS spectrum of glucuronide conjugate.

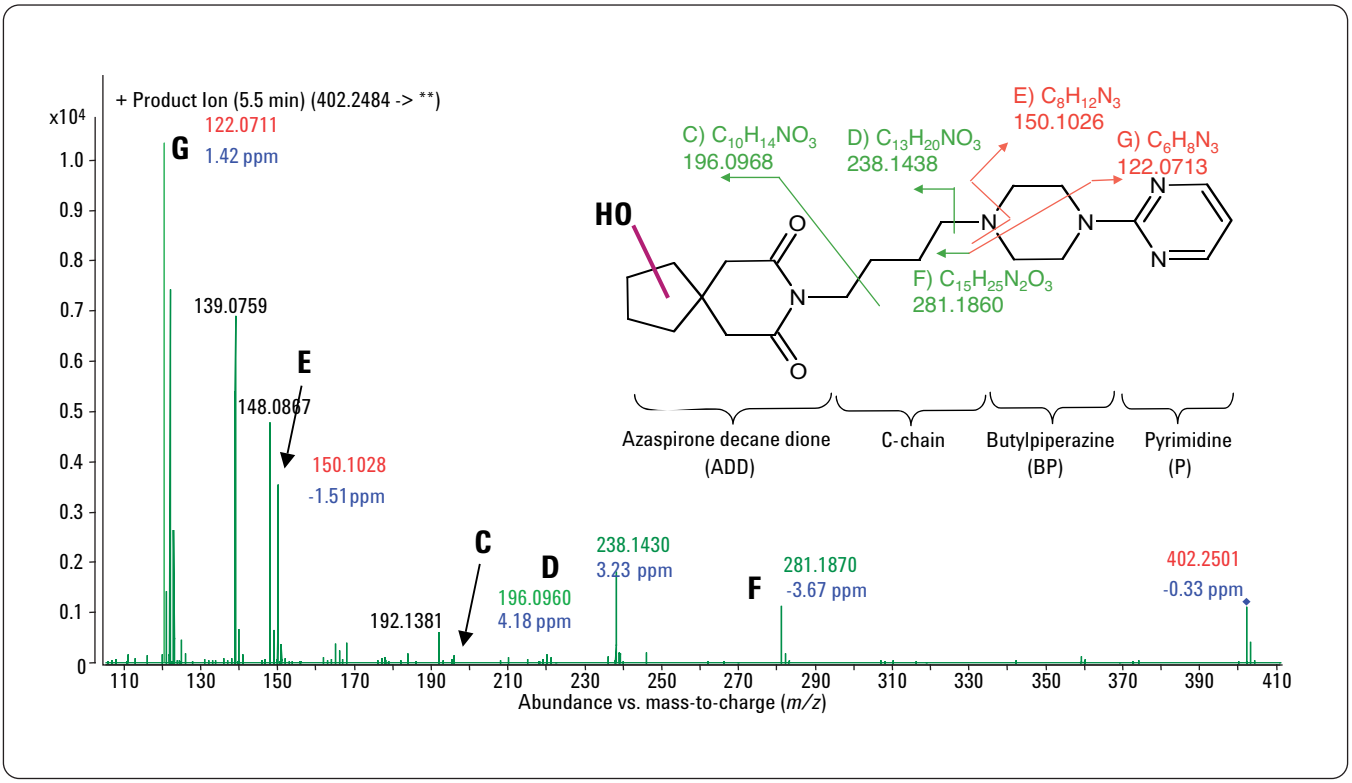

Figure 9
Q-TOF MS/MS spectrum of the monohydroxy metabolite of buspirone at a retention time of 5.5 minutes. Comparison with the MS/MS spectrum of buspirone indicates the hydroxylation at the ADD group. Fragments that show mass shifts have green labels, while those that do not are in red. Calculated relative mass accuracies are annotated in blue.

Additional information from other MS techniques

Detection and identification of metabolites by other MS techniques like accurate mass measurement and MS^n fragmentation yields additional information. The measurement of MS and MS/MS spectra of metabolite compounds by quadrupole time-of-flight (Q-TOF) mass spectrometry not only gives additional structural information from tandem mass spectrometry, but also provides accurate mass information for the calculation of empirical formulas for the metabolites and their MS/MS fragments. The fragmentation of a hydroxy buspirone metabolite by Q-TOF MS/MS delivered the molecular formula of the parent compound and the formulae of the fragments of the metabolite (figure 9). With the fragment and formula information, it is easily possible to allocate a possible structure.

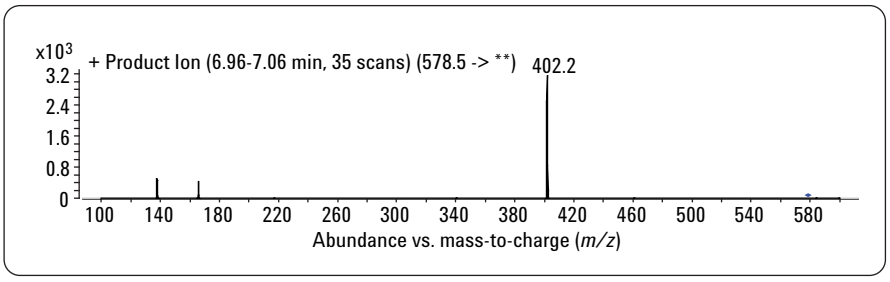

Figure 10
Triple quadrupole MS/MS spectrum of phase II metabolite at *m/z* 578.5. The metabolite is a hydroxy buspirone glucuronic acid conjugate.

The Q-TOF mass spectrometer with its advantageous combination of tandem mass spectrometry and accurate mass measurement is able to provide a high gain in productivity for the identification of metabolites, especially in combination with a dedicated metabolite identification software.[7]

With the product ion scan on the triple quadrupole instrument, the MS/MS spectrum of the buspirone glucuronic

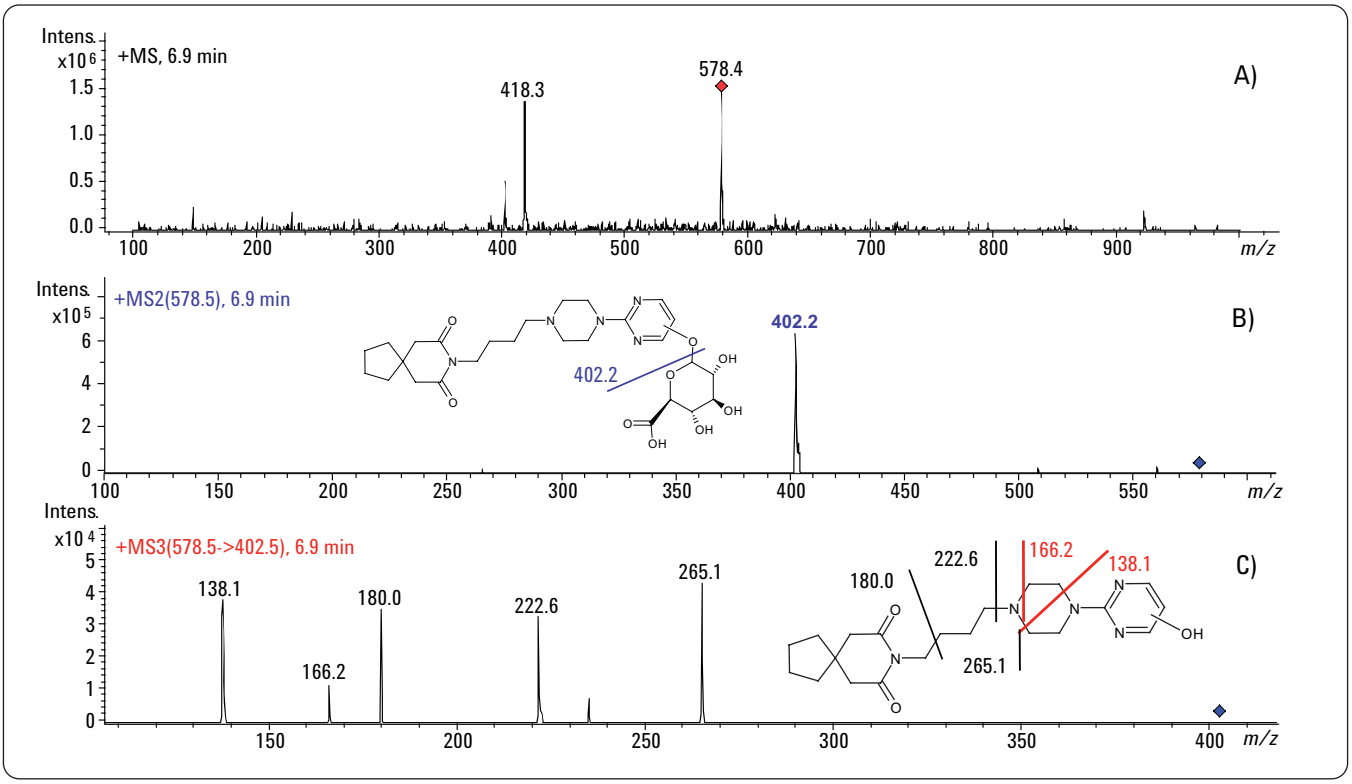

Figure 11
A) Parent mass spectrum for hydroxy buspirone glucuronic acid conjugate at *m/z* 578.4. B) MS/MS spectrum of conjugate at *m/z* 578.5 cleaves off the glucoronic acid and leaves the hydroxylated product. C) MS^3 fragmentation of the isolated hydroxy metabolite provides detailed structure elucidation.

acid conjugate showed only the loss of the glucuronic acid fragment and the ion of the remaining hydroxy buspirone at *m/z* 402.2 (figure 10). To elucidate the structure of the glucuronic acid conjugate and to locate the point of conjugation, it is advantageous to use an ion trap mass spectrometer with its capability of controlled MS^n fragmentation. The ion trap MS spectrum showed the compound at *m/z* 578.5 that was isolated for the MS/MS fragmentation (figure 11A). In the MS/MS fragmentation, the glucuronic acid conjugate was cleaved off and released the intermediate hydroxylated compound (figure 11B). The final MS^3 fragmentation gave enough fragment information to elucidate the structure of the phase II metabolite and to determine the position of the modification to be the pyrimidine ring moiety of buspirone (figure 11C).

Conclusion

This Application Note shows the use of the Agilent 6410B Triple Quadrupole LC/MS system for qualitative metabolite identification. For the detection of phase I metabolites from the *in vitro* incubation of the pharmaceutical buspirone, the triple quadrupole was operated in precursor ion scan mode, with adjustment of the third quadrupole to the typical MS/MS fragment masses of buspirone. These fragments were determined in a preliminary MS/MS experiment of the parent drug in product ion scan mode. More complex phase II metabolism products, for example, the glucuronic acid conjugate of buspirone, were detected by the neutral loss scan mode. The obtained MS/MS information from the precursor and product ion scan experiments was used for structure elucidation.

Additional compound information was generated by other MS instruments. TOF and Q-TOF gave accurate mass measurement and molecular formula confirmation, and ion trap MS^n experiments provided structure elucidation.

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