Fast identification of main drug metabolites by quadrupole time-of-flight LC/MS

Measuring accurate MS and MS/MS data with the Agilent 6510 Q-TOF LC/MS and identification of main meta-bolites by comparison of samples and controls with Agilent MassHunter profiling software

Application Note

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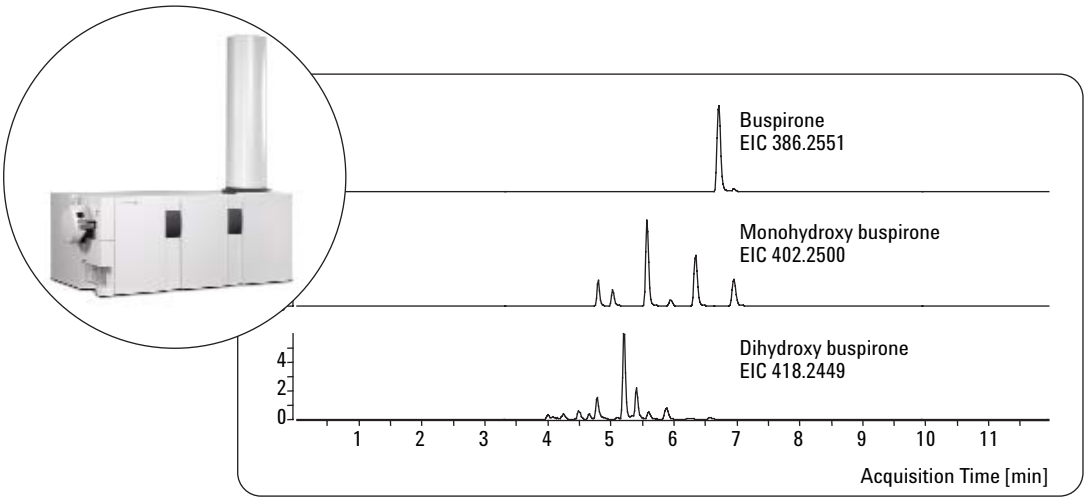

Abstract

This Application Note presents a workflow for the identification of main drug metabolites by sample comparison as well as by structure elucidation of the identified metabolites based on accurate MS and MS/MS data acquired with a quadrupole time-of-flight (Q-TOF) LC/MS.

Agilent Equipment

- 1200 Series Rapid Resolution LC system
- ZORBAX RRHT columns
- 6510 Q-TOF LC/MS
- MassHunter workstation software
- MassHunter profiling software

Application Area

- Metabolite identification in early ADME

In pharmaceutical drug development the main metabolites of a new drug candidate must be identified as fast as possible and with high confidence. Software for this task is described, which analyzes the metabolized drug samples and visualizes the differences with non-metabolized drug samples for manual investigation. The structures of possible metabolites are elucidated by examination of the original highly-accurate Q-TOF mass data based on the differences between the metabolized and non-metabolized drug samples.

Agilent Technologies

Introduction

In modern pharmaceutical drug development it is crucially important to know the adsorption, distribution, metabolism and excretion (ADME) behavior of new drug substances. The confident identification of the main metabolites derived from a drug substance is highly important because of the potential to cause a toxic reaction in humans. Therefore, a recent trend in the drug discovery and development process is to shift the starting point of drug metabolism studies to a time as early as possible in the development chain to address potential issues in parallel with the optimization of the drug's lead structure[1]. To meet this challenge, it is necessary to use general computer assisted methods to identify potential main metabolites as fast and as early as possible. For this purpose the instrument of choice is a Q-TOF LC/MS that combines the capability to measure accurate molecular mass and to acquire MS/MS data[2]. This Application Note describes a workflow for fast identification of main drug metabolites by comparison of Q-TOF data of samples and controls as well as structure elucidation of the identified metabolites from accurate MS and MS/MS data.

Experimental

Equipment

- Agilent 1200 Series Rapid Resolution LC system
- Agilent 6510 quadrupole time-of-flight (Q-TOF) LC/MS
- ZORBAX Rapid Resolution High Throughput (RRHT) SB C18 Column, 2.1 x 150 mm, 1.8 µm particle size
- MassHunter Workstation software for data acquisition and qualitative data analysis
- MassHunter Profiling software for differential analysis of the mass data

Method for RRLC

- Solvent A: Water + 0.1 % FA (formic acid)
  Solvent B: ACN + 0.1 % FA (formic acid)
- 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. The samples were cooled to 4 °C in the injector. Automated delay volume reduction was used.
- Diode array detection: 220 nm ±4 nm, Ref. 360 nm ±8 nm with 2 µL flow cell, 10 mm path length
- Column temperature: 50 °C

Method for Q-TOF LC/MS

- Source: ESI in positive mode with two sprayers for ion-suppression-free introduction of reference mass solution (m/z 121.05087 and m/z 922.00980)
- Nebulizer: 50 psi
- Dry gas: 12.0 L/min
- Dry temperature: 350 °C
- Capillary: 3000 V
- Fragmentor: 200 V
- Skimmer: 60 V
- Mass range: 50–1000
- Data acquisition: Data dependent, one MS spectrum per second and two MS/MS spectra per second, two selected precursors for three spectra and subsequent exclusion for 0.5 minutes.

Sample preparation

- Stock solutions: 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 $MgCl_2 \times 6H_2O$ + 258.1 mg isocitrate in 20 mL H_2O); isocitrate dehydrogenase (IDH) 0.33 U/mL
- NADPH regeneration system: 1.6 mL NADP solution + 1.6 mL isocitrate solution + 100 µL IDH solution
- Incubation mixture: 3.85 µL substrate + 200 µL NADPH regeneration system + 746.15 µL phosphate buffer + 50 µL S9
- 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 min at 14,000 g. The supernatant was evaporated to dryness using a SpeedVac concentrator (Bachhover, Germany) and reconstituted with water containing 0.1 % formic acid for LC/MS analysis as described above. For the control sample, incubation was stopped immediately at t = 0 min.

Results and discussion

The acquired Q-TOF data of the in-vitro metabolized drug compound sample were compared to the control sample where the metabolism reaction was sopped immediately after incubation with the S9-preparation. In this study the anxiolytic pharmaceutical drug buspirone ($C_{21}H_{31}N_5O_2$, M=385.2478) was used as a model drug compound. Both samples were injected five times so that the software-assisted data analysis could provide rugged statistical data. The obtained data files were grouped accordingly for differential analysis by the Agilent MassHunter Profiling software. For the differential analysis of both groups the molecular features of each group (the grouped molecular masses of isotopes and the adducts belonging to a single compound at a specific retention time) are displayed in a $\log_2$ abundance plot that shows the abundance ratio of the metabolized buspirone sample compared to the buspirone control sample (figure 1).

The plot has five lines for selected levels of abundance differences in the two sample groups. Molecular features lying on the center line (labeled 1x) are equal in both groups, molecular features within the margins labeled 2x are present up to a twofold abundance in one group, and molecular features within the margins labeled 4x up to fourfold. Beyond these margins a molecular feature is nearly unique or exclusively present in one group. The plot clearly shows a few molecular features that have higher abundance in the metabolized sample. Examination of the molecular feature data shows that the molecular masses of six compounds at M=401.2427 and of two compounds at M=417.2326 are the molecular masses for the mono- and dihydroxy-buspirone metabolites with the respective empirical formulas $C_{21}H_{31}N_5O_3$ and $C_{21}H_{31}N_5O_4$. As an example, the abundance of the mono-hydroxyl metabolite at retention time 5.5 minutes in the sample group with the complete metabolism reaction exceeds the abundance of this compound in the control samples by about 400 times (table 1).

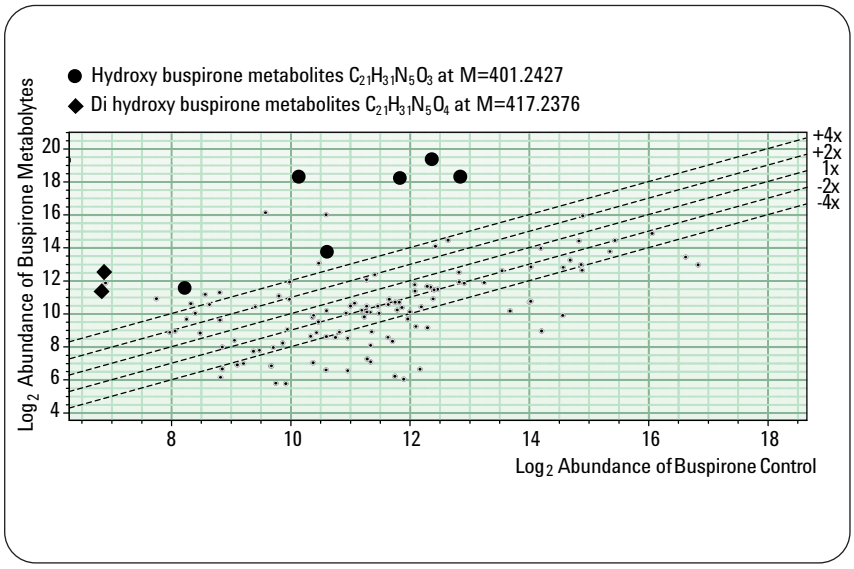

Figure 1
Differential analysis of the metabolized drug buspirone ($C_{21}H_{31}N_5O_2$, M=385.2478) compared to the control sample where the metabolism was stopped immediately after incubation.

ID	Name	RT	Mass	Abundance	Mass error [mDa]	Mass error [ppm]
1	Buspirone_metabolized-1	5.531	401.2421	409,387	-0.6	1.47
2	Buspirone_metabolized-2	5.524	401.2426	408,933	-0.1	0.22
3	Buspirone_metabolized-3	5.523	401.2426	410,559	-0.1	0.22
4	Buspirone_metabolized-4	5.523	401.2423	414,209	-0.4	0.97
5	Buspirone_metabolized-5	5.517	401.2425	409,787	-0.2	0.47
6	Buspirone_control-1	5.521	401.2426	911	-0.1	0.22
7	Buspirone_control-2	5.513	401.2421	1,008	-0.6	1.47
8	Buspirone_control-3	5.522	401.2415	887	-1.2	2.96
9	Buspirone_control-4	5.524	401.2430	934	-0.3	0.77
10	Buspirone_control-5	5.527	401.2412	1,083	-1.5	3.71

Table 1
Comparison of the abundance of the hydroxylated buspirone metabolite $C_{21}H_{31}N_5O_3$ with M=401.2427 at RT=5.55 min.

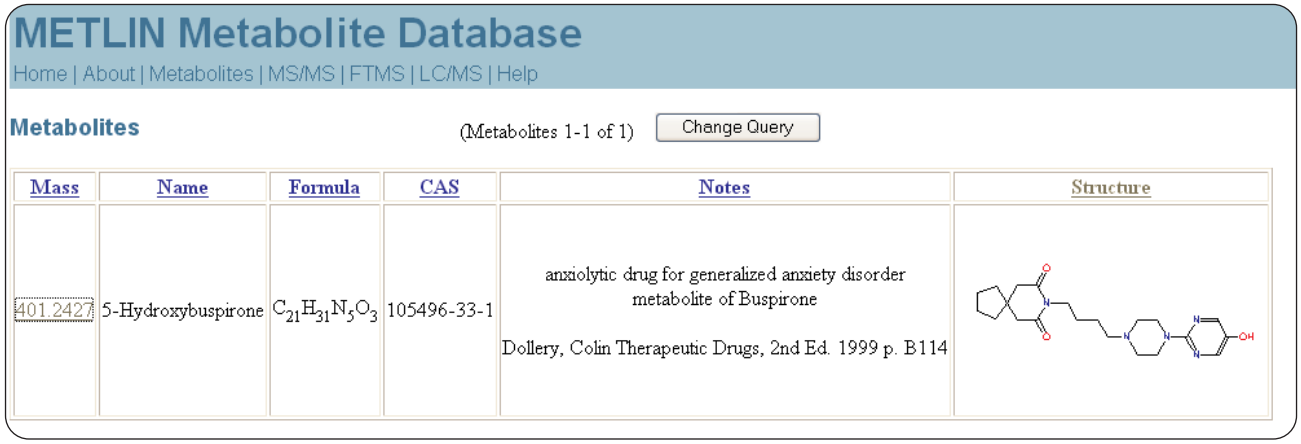

Figure 2
Search results from the METLIN metabolite database for potential buspirone metabolites using the measured accurate molecular mass.

The average relative mass accuracy for this particular compound at retention time 5.5 minutes is 0.67 ppm, which confirms the calculated empirical formula $C_{21}H_{31}N_5O_3$ for the monohydroxy metabolite. For identification of potentially known metabolites, it is possible to submit the measured accurate mass data directly to a metabolite database such as METLIN (figure 2). For the submitted mass M=401.2426 measured for the compound at retention time 5.5 minutes, the compound 5-hydroxy buspirone was identified as a possible metabolite.

However, this compound is only one possible isomer of the six identified compounds with an accurate mass of M=401.2427. Therefore, after the described review of the sample, a more detailed data analysis at the MS/MS level in combination with accurate mass is necessary. The first step in the manual metabolite identification is the extraction of the molecular masses of possible expected metabolites and of the parent drug from the MS data (figure 3).

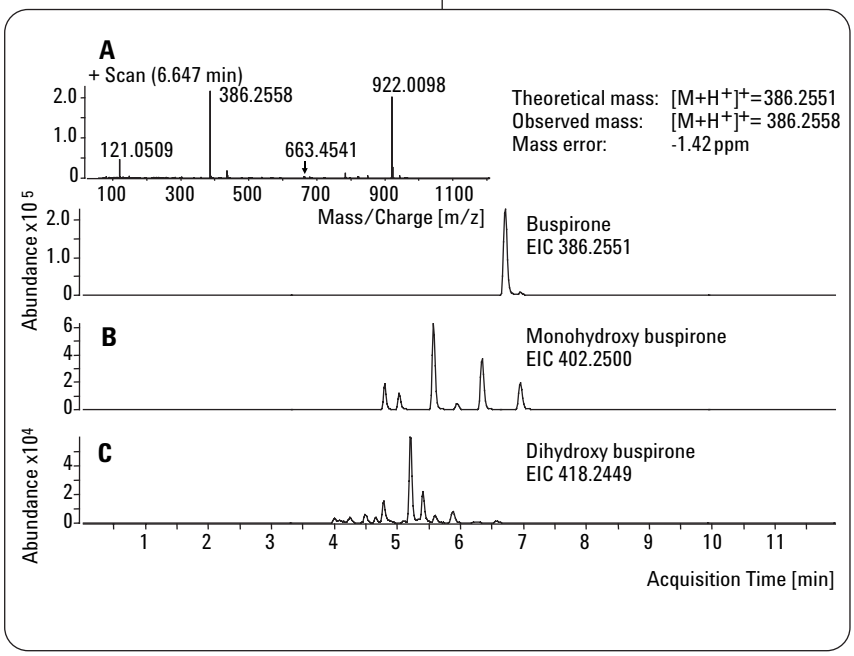

Figure 3
Manual metabolite identification approach by extraction of the $[M+H]^+$ mass of expected metabolites from QTOF data.
A) Extracted ion chromatogram (EIC) and mass spectrum of the protonated parent drug compound.
B) EIC of accurate mass of protonated mono-hydroxy metabolites.
C) EIC of accurate mass of protonated dihydroxy metabolites.

In the example used for this study, the drug buspirone was found with an excellent mass accuracy of -1.42 ppm for the protonated parent ion in the control sample. As expected metabolites, the mono and dihydroxylated species were extracted. The EIC traces show that there is more than one mono and dihydroxylated compound, which are chromatographically separated. The different individual compounds are produced by oxidizing reactions that take place at different parts of the parent drug, which leads to chromatographically different and separable compounds. To elucidate the structure of the metabolites a MS/MS spectrum of buspirone was extracted from the obtained Q-TOF data for comparison with the MS/MS spectra of the metabolites (figure 4).

For all assigned fragments the calculated relative mass accuracies were sufficiently low in the single digit range to be able to calculate correct empirical formulas of the MS/MS fragments of buspirone. Similar fragmentation patterns were found for the MS/MS fragmentation of the hydroxy metabolites and the mass shifts of some fragments gave information about the real structure and the modification. The MS/MS spectrum of the mono-hydroxy buspirone metabolite eluting at a retention time of 5.5 minutes from the column was examined in more detail (figure 5).

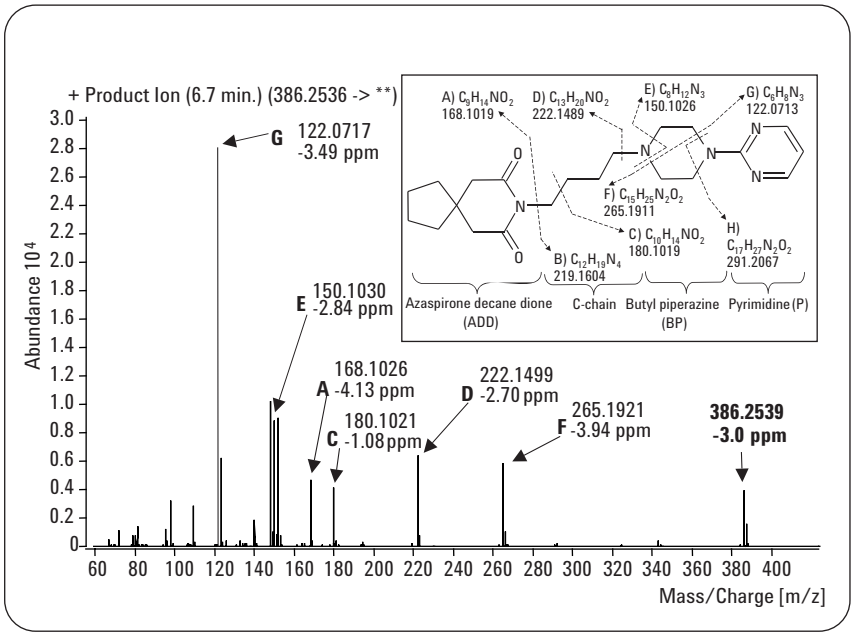

Figure 4
Q-TOF MS/MS spectrum of buspirone showing measured accurate masses of the fragments and calculated relative mass accuracies. The insert shows the MS/MS fragments of buspirone with calculated $[M+H+]^+$ and empirical formulas.

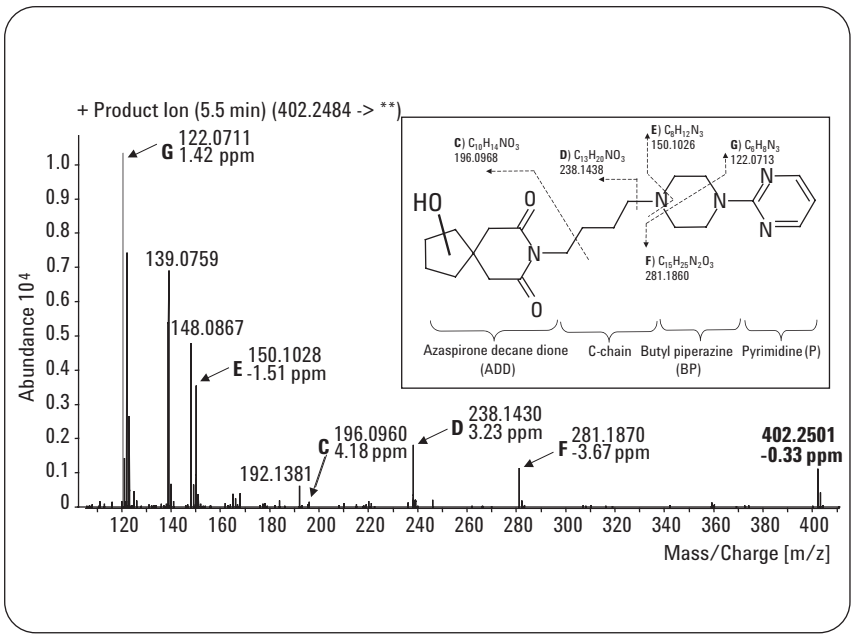

Figure 5
MS/MS spectrum of the mono-hydroxy metabolite of buspirone, which elutes at retention time 5.5 minutes. The mass shifts of the fragments (upper values for C, D and F) in comparison to the MS/MS spectrum of buspirone and the comparable fragments (upper values for E and G) indicate the hydroxylation at the ADD Group (lower values are calculated relative mass accuracies).

The mass shifted fragments indicate hydroxylation and a comparison to fragments with a mass identical to the fragments of the original drug molecule indicates the position of the metabolic reaction. This identifies clearly the oxygen modification by the shift of 16 for the assigned fragments and the location of the modification from the metabolism reaction on the azaspiro decane dion moiety of the molecule.

Conclusion

This Application Note demonstrates the use of the MassHunter Profiling software, which is part of the MassHunter Workstation software, for the analysis of complex samples. As an example a metabolism sample was created and rapidly analyzed for newly emerging compounds in comparison to a non-metabolized parent drug control sample. From the measured highly accurate Q-TOF data with relative mass errors in the low single digit ppm range, the molecular features were extracted and newly emerging compounds were identified by software-assisted differential analysis. After identification of the new compounds, their empirical formulas were calculated from the highly-accurate measured masses. The highly-accurate measured masses were used to search a database for known metabolites with the identified masses. The structures of unknown compounds were elucidated from the Q-TOF MS/MS spectra by using the accurate mass data to calculate the empirical formula of fragments. Metabolism reactions were identified by mass shifts in the MS/MS spectra.

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