Simultaneous qualitative and quantitative analysis using the Agilent 6540 Accurate-Mass Q-TOF

Technical Overview

Authors

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Abstract

The utility of the Agilent 6540 Accurate-Mass Q-TOF LC/MS System for the determination of metabolic stability, profiling, and identification in a single experiment has been demonstrated in a buspirone metabolic study, thus maximizing the quantitative and qualitative information that can be obtained from one experiment. The validity of the quantitative data was confirmed by analysis using the Agilent 6460 Triple Quadrupole LC/MS System, operating in multiple reaction monitoring (MRM) mode.

Introduction

Determining metabolic stability is an important aspect of early drug discovery, due to its role in drug half-life and bioavailability. Metabolite identification is crucial in order to understand drug safety and efficacy. In early drug discovery, samples are typically analyzed by a variety of different LC/MS methods in order to generate both metabolic stability (quantitative) and metabolite identification (qualitative) data. The ability to obtain both quantitation and identification in a single analysis makes metabolic stability, profiling, and identification studies much more efficient. The study described here demonstrates the use of the high resolution and accurate mass determination capabilities of the Agilent 6540 QTOF LC/MS to perform both quantitation and identification of metabolites in the same experiment.

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Experimental

Instruments

Metabolic stability, profiling, and identification were performed using an Agilent 1290 Infinity LC System coupled to an Agilent 6540 Accurate-Mass Q-TOF System. The 6540 QTOF was operated in two modes: an initial MS-only mode at 5 Hz acquisition rate, and an automatic MS/MS mode, with the masses of the expected metabolites on the inclusion list. Quantification results were confirmed using the same LC system and MRM on the Agilent 6460 Triple Quadrupole LC/MS System. The same conditions (e.g. collision energy) were used for both instruments because they use the same hardware design from ion source to collision cell. The instrument conditions are given in **Tables 1** and **2**.

Sample preparation

Buspirone was chosen as a model compound for the metabolic stability study. This drug was incubated at 1 µM in a rat liver S9 preparation, using an NADPH regenerating system. The incubation was carried out at 37°C and 100 µL aliquots were taken at 0, 5, 10, 15, and 20 minutes. The reaction was stopped by adding acetonitrile; then the sample was centrifuged. The supernatant was evaporated to dryness using a gentle stream of nitrogen and reconstituted in the mobile phase for combined ultra high performance liquid chromatography (UHPLC) and MS.

UHPLC Run Conditions	
Column	Agilent ZORBAX Eclipse Plus C18, 2.1 mm x 100 mm, 1.8 µm
Injection volume	1 µL
Needle wash	Flush port (75:25 MeOH:H_2O, 0.1% formic acid ,10 sec)
Mobile phase	A = H_2O + 0.1% formic acid B = acetonitrile + 0.1% formic acid
Flow rate	1.0 mL/min
Gradient	5% B to 95% B
Stop time	5.5 min

Table 1. Ultra High Performance Liquid Chromatography (UHPLC) Conditions

QTOF and Triple Quadrupole MS Conditions	
Ion Source Settings for Both Instruments	
Ion mode	Positive, ESI
Drying gas temperature	350°C
Drying gas flow	10 L/min (nitrogen)
Sheath gas temperature	400°C
Sheath gas flow	12 L/min
Capillary voltage	4000 V
Nozzle voltage	0 V (+)
QTOF MS/MS Settings	
Acquisition type	Auto MS/MS (data-dependent)
Mass range	100–1000 *m/z* for both MS and MS/MS
Transients	1557/spectrum for both MS and MS/MS
Acquisition rate	5 spectra/sec (combined MS and MS/MS)
Precursors/cycle	1
Active exclusion	Exclude after 2 spectra, release after 0.05 min
Collision energy	28 EV (same for Triple Quad)
Charge state	1+ and unknown
Preferred list	All expected metabolites
Mode	Extended dynamic range (2 GHz)
Triple Quadrupole MS/MS Settings	
MS1/MS2 resolution	Unit
Delta EMV	200 V
Time filtering	0.03 min
MRM transitions	386.3 → 122.1 (Buspirone) 609.3 → 195.1 (Reserpine, internal standard)
Dwell time	100 ms per transition
Cycle time	203.5 ms

Table 2. 6540 QTOF and 6460 Triple Quadrupole LC/MS mass spectrometer conditions

Results and Discussion

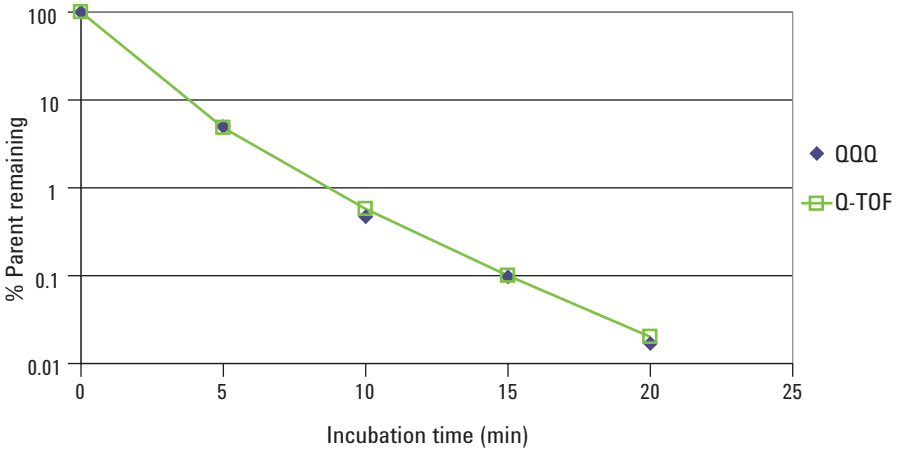
Reliable metabolic stability results

Metabolic stability results were plotted using the data from the QTOF MS only analysis and the Triple Quad MRM analysis. As shown in **Figure 1**, there was good correlation of the metabolic stability data obtained using the 6540 QTOF full-spectrum analysis, with the same data obtained using the 6460 Triple Quadrupole MRM analysis. The semi-log plot of percent parent versus time is a typical means used to determine and compare metabolite half lives. The 6540 QTOF is an extremely sensitive instrument capable of good quantitative measurements.

Simultaneous profiling of metabolites

The MS analysis using the 6540 QTOF provided full spectra through the entire run, allowing profiling of all metabolites, while the triple quadrupole analysis using the MRM mode provided analysis only of the parent drug and known, selected metabolites. **Figure 2** shows extracted ion chromatograms of the expected metabolites of buspirone, detected in the QTOF analysis. This sample was from a 5 min incubation time, and the QTOF was operated in high resolution MS scan mode.

QTOF delivers accurate metabolic stability data



Time	0 min	5 min	10 min	15 min	20 min
QTOF (%)	100	4.88	0.57	0.100	0.020
QQQ (%)	100	4.95	0.47	0.098	0.017

Figure 1. The 6540 QTOF gave metabolic stability results for buspirone that were in good agreement with those obtained using the 6460 Triple Quadrupole system. Results are plotted using a semi-log scale (log %Parent remaining) to provide a linear relationship to incubation time.

Simultaneous profiling of metabolites

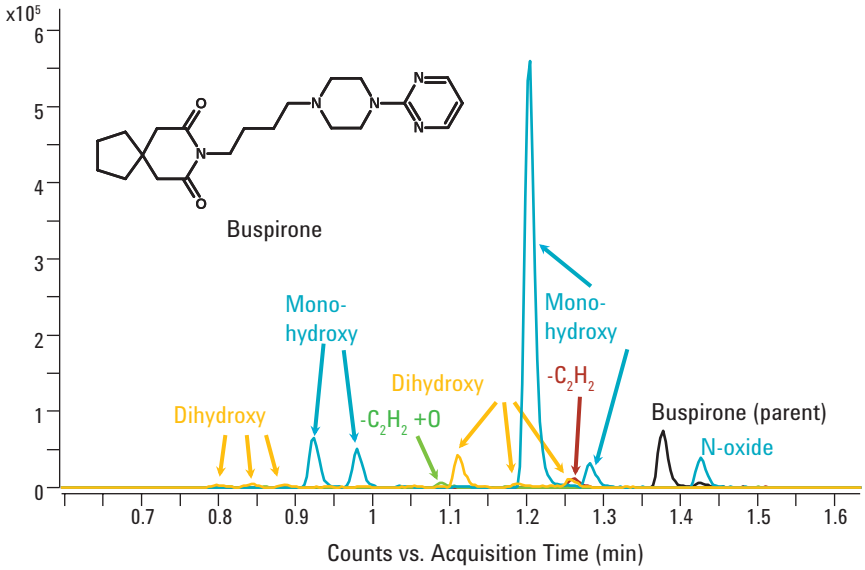


Figure 2. 6540 QTOF analysis EICs revealed the expected metabolites of buspirone.

Figure 3 shows representative spectra from the QTOF analysis, acquired in MS mode with a 5 Hz acquisition rate. The spectra were taken from a sample at a 10 minute incubation time, when less than 1 percent of the parent drug remained. The QTOF spectra for buspirone and its monohydroxy and dihydroxy metabolites showed mass errors of less than 1 ppm, which enabled confident identifications.

Isotope ratios provide additional information that further increases confidence of identification. The insets in **Figure 3** show the measured isotope patterns versus theoretical (the latter shown in rectangles), based on the correct molecular formulae. The isotopic fidelity is excellent, due in part to the analog-to-digital converter (ADC) detector employed in Agilent TOF and QTOF systems. Time-to-digital converter (TDC) detectors used in some other TOF and QTOF systems are known to have problems with isotope ratios, which means that analysts cannot rely on this information for compound identification.

From these full scan MS mode analyses, abundances of buspirone and its metabolites were plotted as a function of incubation time (**Figure 4**). The graph demonstrates that the 6540 QTOF is capable of obtaining both metabolic stability and metabolic profiling in a single MS experiment.

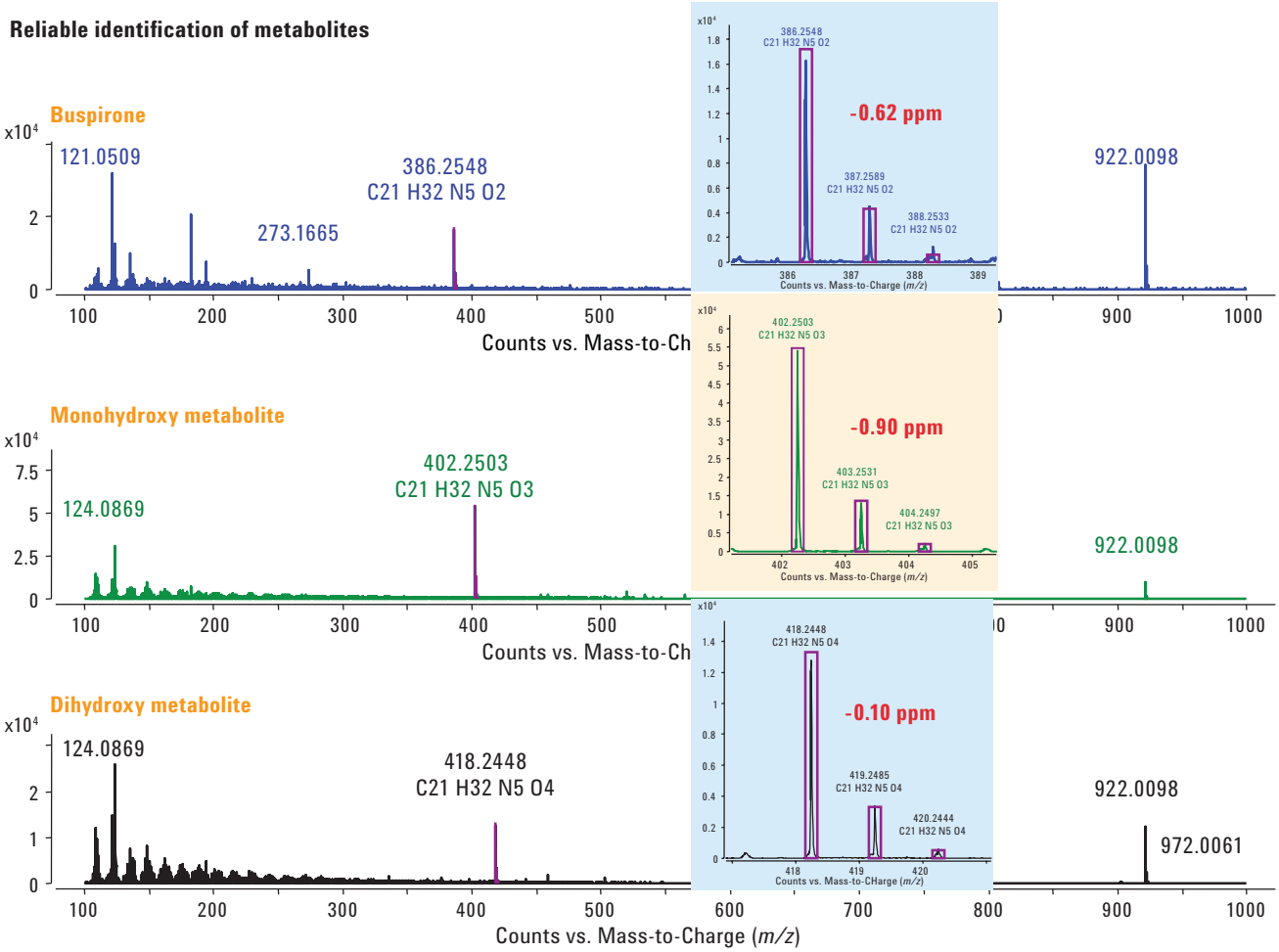


Figure 3. Metabolite spectra taken with the 6540 QTOF demonstrated excellent mass accuracy and isotopic fidelity, which provided assurance of correct identifications.

Augmented metabolite identification with QTOF MS/MS mode analysis

After successfully determining metabolic stability and metabolic profiling using the 6540 QTOF, the possibility of including MS/MS for more comprehensive metabolite identification and structural characterization was investigated. For this targeted MS/MS analysis, the masses of the expected metabolites were specified on an inclusion list, to ensure that they would be analyzed.

With the combined MS and MS/MS acquisition, the MS acquisition rate was 2.5 spectra/second and provided sufficient data points across the peaks to generate semi-quantitative results. In fact, the resulting graph of drug and metabolite concentrations over time, shown in **Figure 5**, is almost identical to that in **Figure 4**. When operated in this mode, the Agilent 6540 QTOF used half of the time to carry out full scan MS data acquisition, and half of the time for MS/MS data acquisition, which was used for metabolite identification, thus providing quantitative and qualitative results in a single analytical experiment.

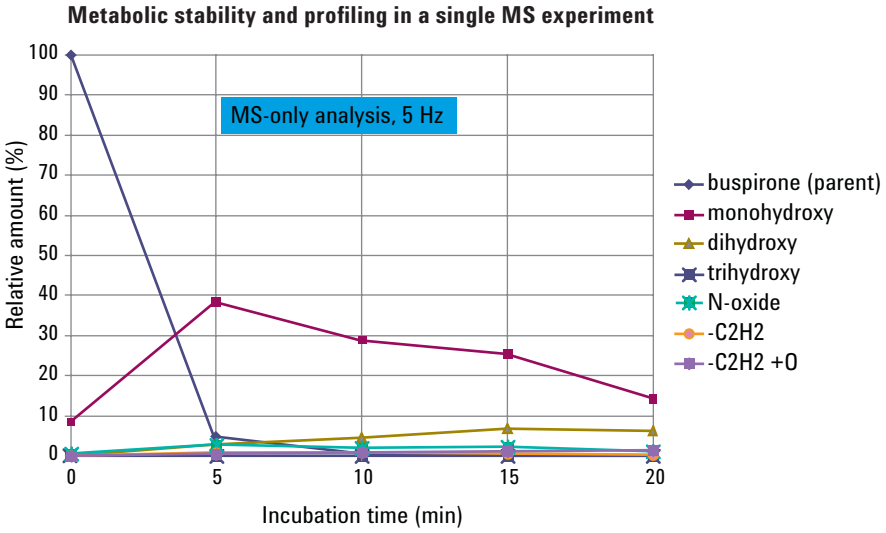


Figure 4. Metabolic stability and metabolic profiling from the same experiment (MS-only, 5 Hz acquisition rate) with the Agilent 6540 QTOF. Graph assumes all response factors were the same as for the parent buspirone.

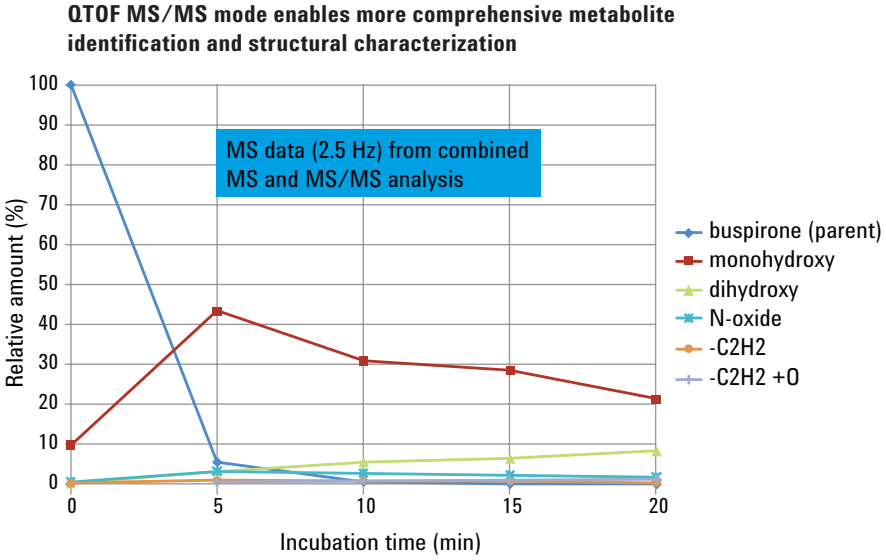


Figure 5. Metabolic stability and metabolic profiling from the same experiment (combined MS and MS/MS, MS at 2.5 Hz acquisition rate) with the Agilent 6540 QTOF. Graph assumes all response factors were the same as for the parent buspirone.

The quality of the MS and MS/MS spectra for metabolite identification was excellent, as shown in the example for the buspirone monohydroxy metabolite (**Figure 6**). The spectra are labeled with the results produced by molecular formula generation (MFG), which show an excellent match between the experimental data and the calculated formula.

In the MS spectrum at the top of **Figure 6**, the measured mass matches the calculated mass within 0.07 ppm, and the isotope abundances show excellent agreement with the theoretical values (rectangles). Other QTOF instruments that use a TDC detector have difficulty achieving these accurate isotope ratios, and due to TDC dead time, may suffer from poor mass accuracy for chromatographic peaks with high abundance. The larger mass errors in TDC-based instruments negatively affect both identification and quantitation. The ADC detector in the 6540 QTOF does not have these problems.

In the MS/MS spectrum at the bottom of **Figure 6**, the masses of the fragment ions agree very well with the theoretical values. The collision cell in the Agilent 6540 QTOF has a unique design feature that utilizes high pressure cooling, which means both product and precursor ions have the same relative energy. Therefore, the same tuning parameters can be used for both MS and MS/MS analysis, resulting in excellent MS/MS mass accuracy. Given the quality of the MS and MS/MS data in **Figure 6**, the identification of a metabolite can be made with a high degree of confidence.

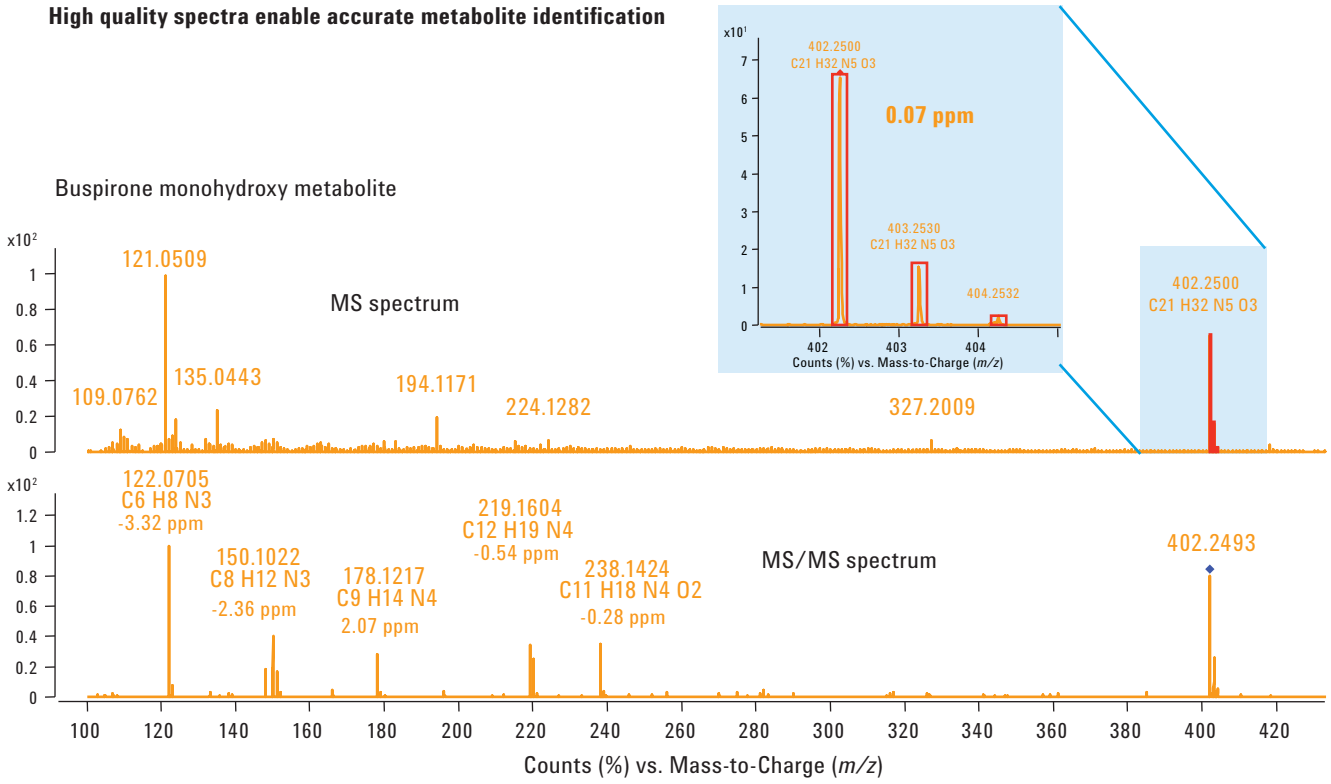


Figure 6. The molecular formula generation algorithm identified the metabolites by taking advantage of both the excellent match of the precursor isotope pattern and the mass agreement for precursor and product ions.

Identification of unexpected metabolites with QTOF MS/MS analysis

A compelling reason to use an accurate-mass MS/MS instrument for this workflow is to identify un-targeted metabolites in addition to the expected metabolites. This may be done using the standard data dependent acquisition parameters in combination with appropriate data mining tools. The Find by Auto MS/MS algorithm in the Agilent MassHunter Qualitative Analysis software was used to automatically discover metabolites which may be present in the sample. This processing feature revealed all the known phase I metabolites detected in this sample, which were automatically extracted for verification, without prior specification. This experiment demonstrates the feasibility of discovering unexpected metabolites using this QTOF MS/MS procedure.

Conclusions

This study clearly demonstrated the feasibility of using the 6540 QTOF for metabolic stability, profiling, and identification in a single experiment. It gave quantitation results that were comparable to those obtained with the triple-quadrupole instrument. The Agilent Triple Quad and QTOF employ common hardware from source to collision cell, which makes it easy to transfer methods and compare results. While the QTOF does not provide the ultimate detection limits of the most advanced triple-quadrupole systems, the sensitivity is more than sufficient for early compound assessment in drug discovery.

When operated in targeted MS/MS mode, the 6540 QTOF enabled metabolite identification, a semi-quantitative metabolic stability study, and metabolic profiling—all within the same experiment. The 6540 QTOF produced very high quality accurate mass MS and MS/MS spectra for confident metabolite identification, including accurate isotope ratios. The Agilent QTOF provided excellent data quality in all dimensions, simultaneously, for combined quantitative and qualitative analyses.

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