Application Note: 504

Full Scan Data Acquisition for Rapid Quantitative and Qualitative Analysis Using the Exactive Benchtop LC-MS High Resolution Mass Spectrometer

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Key Words

- Exactive
- Quan/Qual
- Drug Discovery
- Metabolite Identification
- Mass Frontier

Introduction

Current approaches to discovery stage drug metabolism studies, such as pharmacokinetics and hepatocyte stability, have focused on the use of targeted analysis-based approaches like multiple reaction monitoring (MRM) for quantitative analysis. This necessitates the optimization of parameters such as Q1 and Q3 *m/z* values, collision energy, and interface voltages. These studies detect only the specified compound; information about other components, such as metabolites, is lost. The ability to do full scan acquisition for quantitation eliminates the need for compound optimization, while enabling the detection of metabolites and other non-drug related endogenous components.

Samples from a tolbutamide *in vitro* rat hepatocyte incubation were analyzed using the Thermo Scientific Exactive benchtop liquid chromatography-Orbitrap mass spectrometry system to demonstrate its capability to produce high resolution, full scan MS and Higher Energy Collision Cell Dissociation (HCD) data. This data can be used for qualitative and quantitative assessment of compounds in early drug discovery studies. From the same data set, HCD can be used for structural elucidation as well as to obtain quantitative data to generate plots for the disappearance of the parent and the appearance of metabolites.

Tolbutamide is metabolized almost exclusively along a single pathway. Methyl hydroxylation to form hydroxytolbutamide is the initial and rate limiting step. Subsequent oxidation of hydroxytolbutamide by alcohol and aldehyde dehydrogenases results in carboxytolbutamide (Figure 1).

Goal

To demonstrate the applicability of using full scan data acquisition on the Exactive™ Orbitrap™ system to analyze samples from an *in vitro* rat hepatocyte incubation study.

Experimental Conditions

Reagents

All reagents were purchased from Sigma-Aldrich. Hepatocytes were obtained from CellzDirect.

Sample Preparation

Incubations of tolbutamide at final concentrations of 2 µM, 10 µM, and 50 µM were conducted in cryopreserved suspension hepatocytes from Sprague-Dawley rats at 0.5 million viable cells/mL of incubation. The final organic concentration in the incubation was 0.1% DMSO.

Incubations were performed in modified Williams E medium at a constant temperature of 37 °C under an atmosphere of 5% CO_2. Time point samples were generated at 0, 2, 4, and 6 hours with a 24-hour time point taken for maximal conversion of tolbutamide. The incubates were quenched with equal volumes of acetonitrile and methanol, mixed well, and centrifuged for 30 minutes at 19,000 g to precipitate cells and proteins. The supernatant was removed and stored at -20 °C until analysis. Prior to mass spectrometry analysis, 1.5 mL of each time point aliquot was mixed with an equal volume of the internal standard (0.2 µM chlorpropamide) prepared in water:acetonitrile:methanol [2:1:1 (v/v/v)].

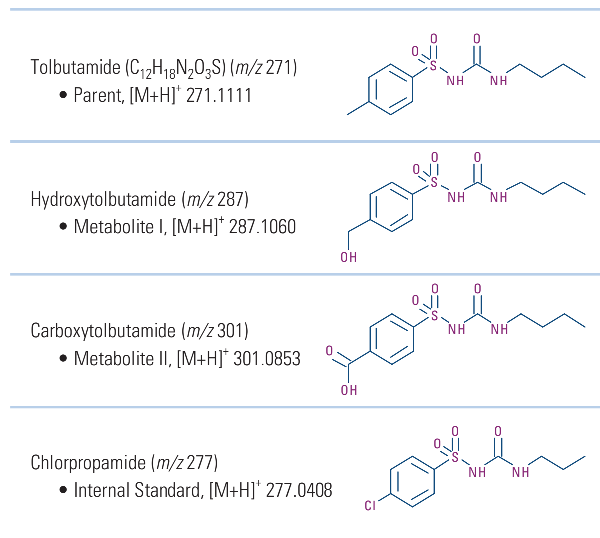

Figure 1. Structures of tolbutamide and its major metabolic products

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HPLC

Chromatographic analysis was performed using the Thermo Scientific Accela U-HPLC system. The chromatographic conditions were as follows:

Column:	Thermo Scientific Hypersil GOLD aQ (100 mm × 2.1 mm 3 µm)		
Injection volume:	5 µL		
Mobile phase A:	0.1% formic acid in water		
Mobile phase B:	0.1% formic acid acid in acetonitrile		
Flow rate:	600 µL/min		
Gradient:	**Time (min)**	**A%**	**B%**
	0.0	95	5
	0.5	95	5
	3.0	60	40
	3.2	10	90
	3.7	10	90
	3.8	95	5
	5.0	95	5

Mass Spectrometry

MS analysis was carried out on an Exactive benchtop Orbitrap mass spectrometer, operating in alternating full MS and Higher Energy Collisional Dissociation (HCD) modes over a mass range of *m/z* 100–850 at 25,000 resolving power (4 Hz scan rate).

Results and Discussion

In the HCD experiment, ions were passed from the C-trap into a multipole collision cell where they were fragmented and stored. The HCD cell voltages were then ramped and ions were transferred back into the C-trap and injected into the Orbitrap mass analyzer for detection. The instrument design allows high efficiency All-Ions MS/MS experiments by means of HCD. The HCD cell has high fragmentation efficiency, and fragment ions are detected with mass accuracies less than 5 ppm.

The standard calibration curve for tolbutamide over the concentration range of 0.1 to 50 µM is shown in Figure 2. The calibration curve was linear over this entire range with a correlation coefficient greater than 0.99.

The HCD spectra of tolbutamide and hydroxytolbutamide are shown in Figures 3 and 4, respectively. The fragmentation schemes for tolbutamide and the two metabolites hydroxytolbutamide and carboxytolbutamide were generated by Thermo Scientific Mass Frontier 6.0 software. The presence of the fragment ion at *m/z* 155.0159 in the tolbutamide and *m/z* 171.0108 in the hydroxytolbutamide spectra confirm the location of the hydroxyl group within hydroxytolbutamide. The absence of a peak at *m/z* 91.0544 within the hydroxytolbutamide spectrum also confirms the location of this hydroxyl group. These data, along with high resolution accurate mass data for carboxytolbutamide in the same fashion, illustrate that accurate mass fragmentation data from HCD spectra can be used for structural elucidation.

The peak areas from the high resolution extraction ion chromatogram for tolbutamide (Figure 5) and those for the two metabolites can be used to determine concentration levels over the course of the *in vitro* incubation. The summary results are plotted in Figure 6 and reveal the conversion of tolbutamide to hydroxytolbutamide and carboxytolbutamide within the rat hepatocytes over time.

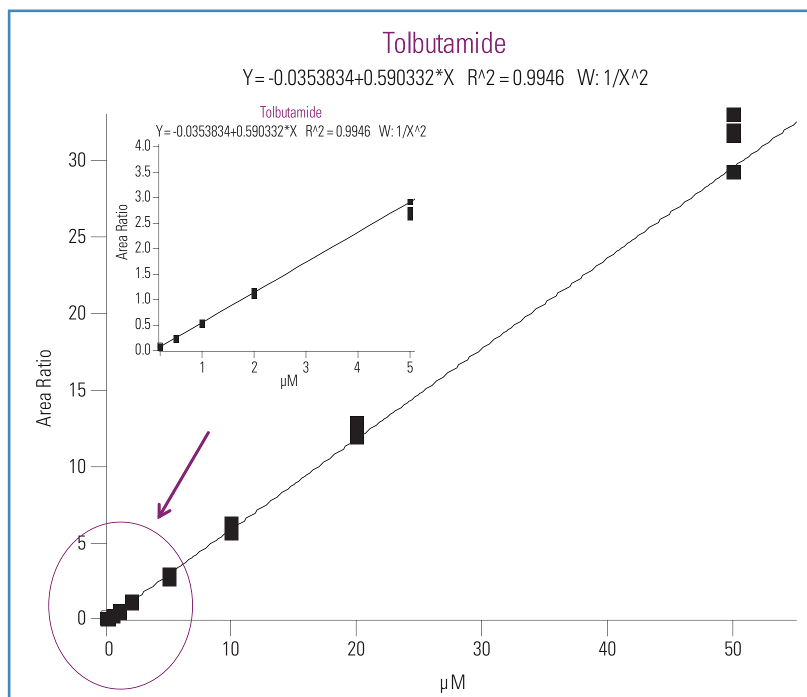

Figure 2. Standard curve for tolbutamide over the range 0.1 to 50 µM. Chlorpropamide was used as an internal standard.

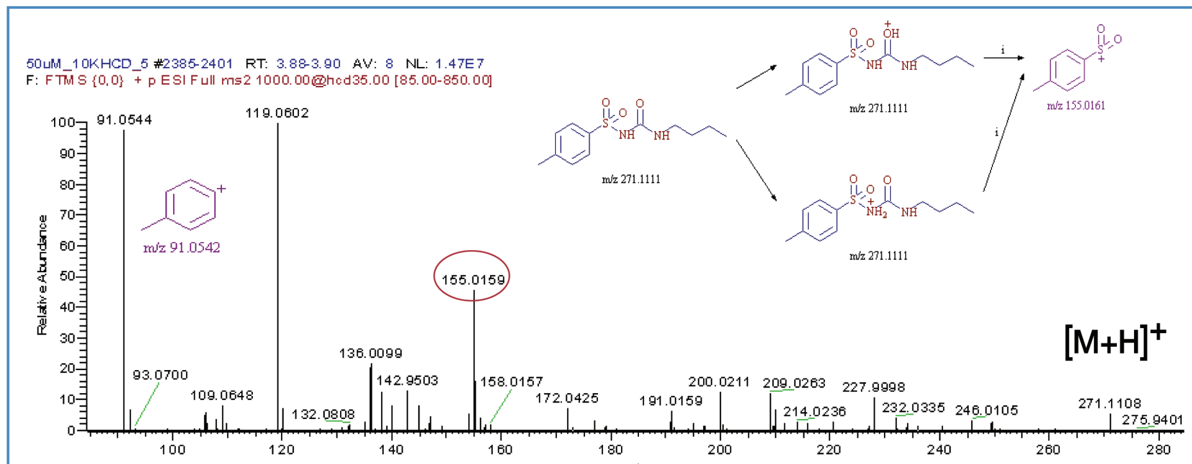

Figure 3. HCD spectrum of tolbutamide. The fragmentation scheme (using Mass Frontier 6.0) is shown for the formation of the ion at *m/z* 155, reflecting the nature and site of metabolic modification when compared to the HCD spectrum of hydroxytolbutamide in Figure 4.

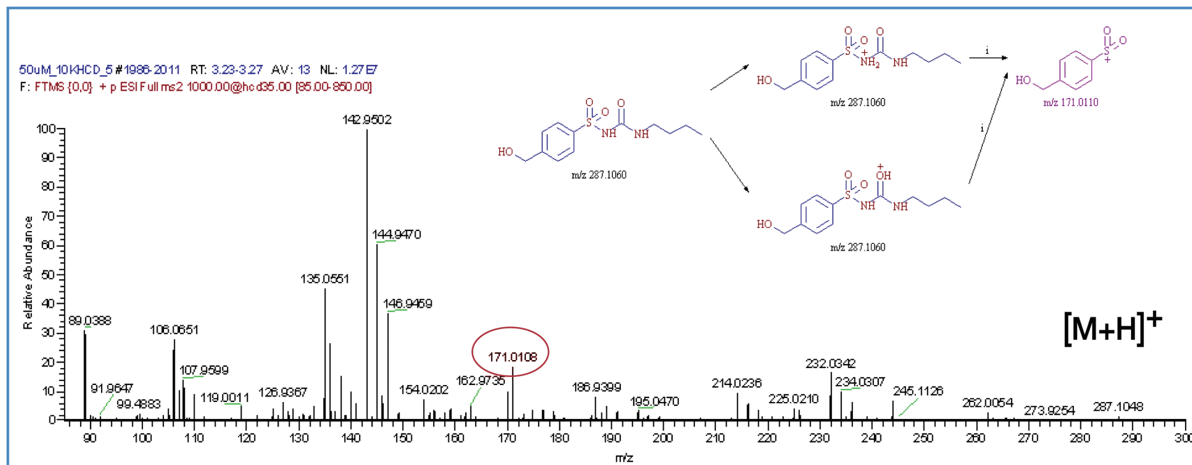

Figure 4. HCD spectrum of hydroxytolbutamide. The fragmentation scheme (using Mass Frontier 6.0) is shown for the formation of the ion at *m/z* 171, reflecting the nature and site of metabolic modification when compared to the HCD spectrum of tolbutamide in Figure 3.

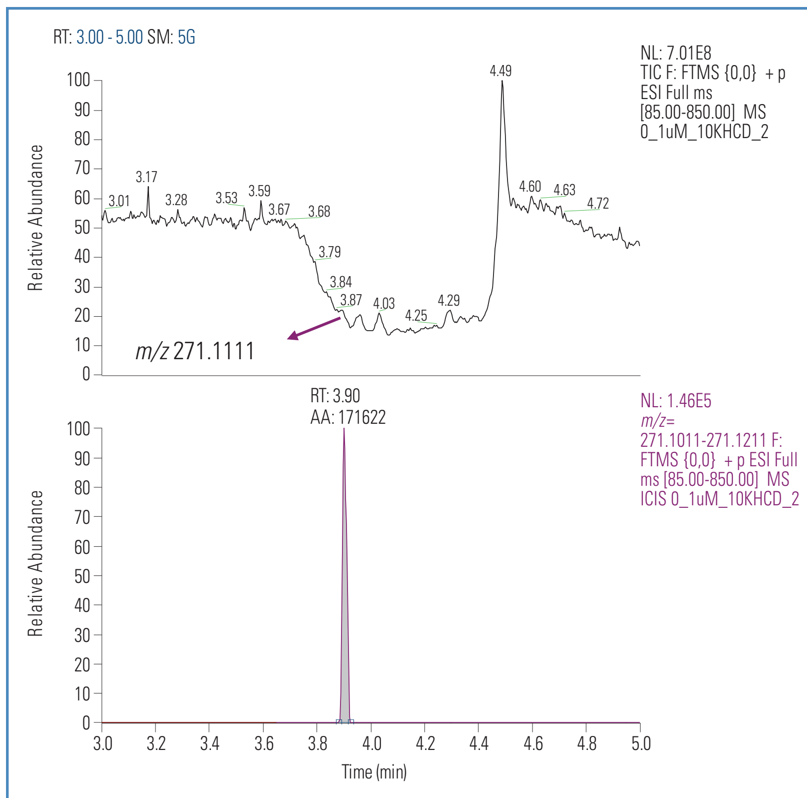

Figure 5. Total ion chromatogram of the LC/MS data from 1 µM incubation sample (top), and extracted ion chromatogram for tolbutamide (bottom) from the same data file at *m/z* 271.1111 with a mass tolerance window of 5 ppm (resolution 25,000).

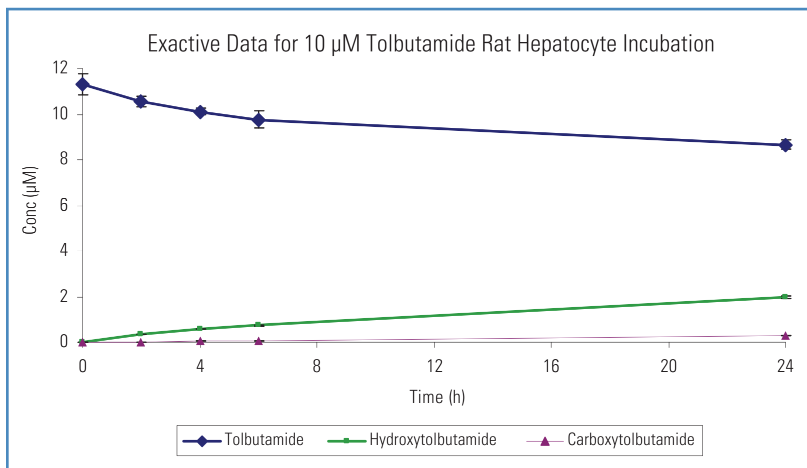

Figure 6. Plot showing the disappearance of tolbutamide (blue trace) following incubation with rat hepatocytes and the appearance of metabolites (shown by the purple and green traces).

Conclusion

The acquisition of full scan MS data enables flexibility in post-acquisition data processing that is not available when doing targeted analysis. This study has illustrated that full scan MS data can be used to provide quantitative information about the parent drug and its metabolites without *a priori* knowledge of the metabolites. In addition, high resolution accurate mass data from HCD scans can be used for structural elucidation.

Acknowledgements

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Reference

Bateman, K.P.; Kellmann, M.; Muenster, H.; Papp, R.; Taylor, L. "Quantitative-qualitative data acquisition using a benchtop Orbitrap mass spectrometer" J Am Soc Mass Spectrom. 2009, 20(8):1441-50.

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