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

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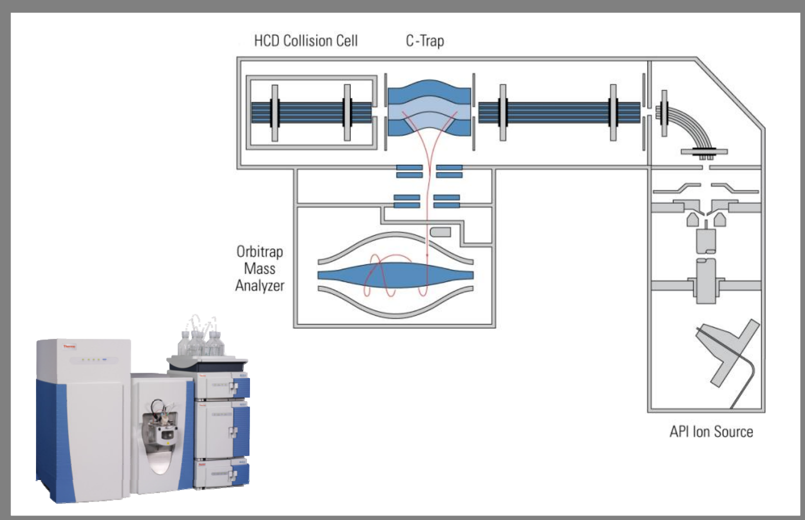
Overview: Current approaches to discovery stage drug metabolism studies (pharmacokinetics, hepatocyte stability, etc.) have focused on the use of targeted analysis (MRM) based approaches for quantitative analysis. This necessitates the optimization of parameters such as Q1 and Q3 *m/z* values, collision energy and interface voltages. These studies only detect the specified compound and 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 an in vitro rat hepatocyte study were analyzed using the Exactive™ non-hybrid Orbitrap LC-MS (see schematic in Figure1).

Introduction: 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 (see Figure 2).

Figure 1. Schematic Diagram of the Exactive Bench top Orbitrap LC-MS instrument



Rat Hepatocyte Incubations of Tolbutamide:

Initial concentration 2 µM: 0, 2, 4, 6 hr; 10 µM: 0, 2, 4, 6, and 24 hr; 50 µM: 24 hr 1.5mL each [sample:ACN:MeOH (2:1:1)] to be mixed 1:1 with internal standard [0.2 µM] chlorpropamide in H_2O:ACN:MeOH (2:1:1)]. This gave the final sample to be injected into the mass spectrometer. Details: Incubations of tolbutamide at a final incubation concentration of 2 and 10 µM (final organic concentration in the incubation: 0.1% DMSO) in cryopreserved suspension hepatocytes from Sprague-Dawley rat at 0.5 million viable cells/mL of incubation. Incubations performed in modified Williams E medium at a constant temperature of 37ºC under an atmosphere of 5% CO_2. Time point samples generated at 0, 2, 4, and 6 hours with a 24-hour time point taken for maximal conversion of tolbutamide. Incubates quenched with an equal volume of ACN:MeOH (1:1), well mixed, and centrifuged for 30 min at 19,000g to precipitate cells and proteins. Supernatant removed and stored at -20ºC until analysis.

Chromatography Conditions:

Thermo Scientific Accela LC and autosampler
Mobile Phase: (A) H_2O with 0.1% HCOOH, 10mM HCO_2NH_4
(B) ACN with 0.1% HCOOH
Flow rate: 600 µL/min
Column: Thermo Scientific Hypersil GOLD aQ
(100 X 2.1, 3µm particle size)
Injection volume: 5µL

Gradient:
5-min method

0 min	5% B
0.5	5
3.0	40
3.2	90
3.7	90
3.8	5
5.0	5

Mass Spectrometry

All samples were analyzed using the Exactive operating in alternating full MS and HCD modes, with a mass range of *m/z* 85-850 at 10,000 resolution, i.e.. 10 Hz scan rate.

Figure 2. Structure of Tolbutamide and its Major Metabolic Products

- Tolbutamide ($C_{12}H_{18}N_2O_3S$) (*m/z* 271)
  - Parent, MH+ 271.1111
- Hydroxytolbutamide (*m/z* 287)
  - Metabolite I, MH+ 287.1060
- Carboxytolbutamide (*m/z* 301)
  - Metabolite II, MH+ 301.0853
- Chlorpropamide (*m/z* 277)
  - Internal Standard, MH+ 277.0408

Results

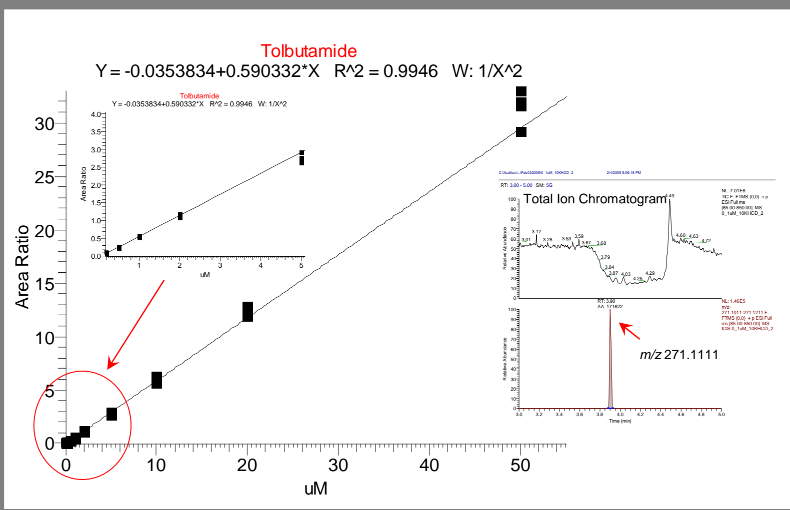
Samples from a tolbutamide *in vitro* rat hepatocyte incubation were analyzed using the Exactive in order to demonstrate the capability of the instrument to produce high resolution full scan MS and HCD data which can be used for qualitative and quantitative assessment of compounds in early drug discovery studies. From the same data set, it was possible to use HCD for structural elucidation as well as to obtain quantitative data to generate plots for the disappearance of drug and appearance of metabolites.

In the HCD experiment, ions are passed from the C-trap into a multipole collision cell where they are fragmented and stored. After that, the HCD cell voltages are ramped and ions are transferred back into the C-trap from where they are injected into the Orbitrap 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 <3 ppm.

Figure 3 displays the standard curve for tolbutamide over the concentration range 0.1 to 50µM. Also shown is the 0.1 to 5µM range. The internal standard used was chlorpropamide.

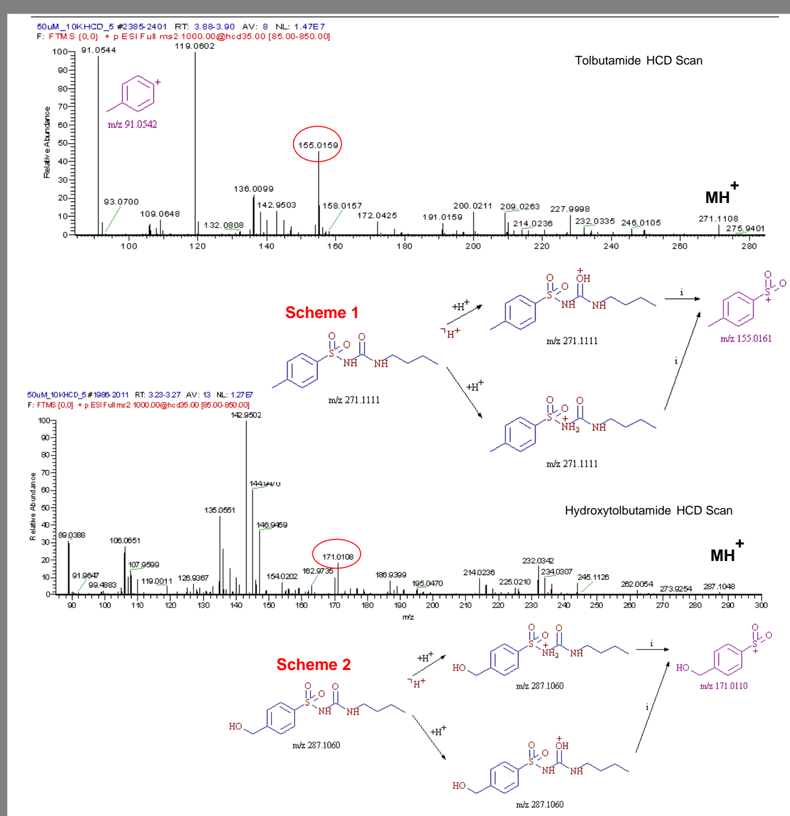
Mass Frontier 6.0 was used to generate fragmentation schemes for tolbutamide and the hydroxytolbutamide metabolite (and hydroxytolbutamide), as illustrated Scheme 1 and 2 in Figure 4.

Figure 3. Standard curve for Tolbutamide over the range 0.1 to 50 µM. Chlorpropamide was used as an internal standard (detail shows range 0.1 to 5 µM). Inset shows the extracted ion chromatogram for Tolbutamide at *m/z* 271.1111 with a mass tolerance window of 5 ppm (resolution 10,000)



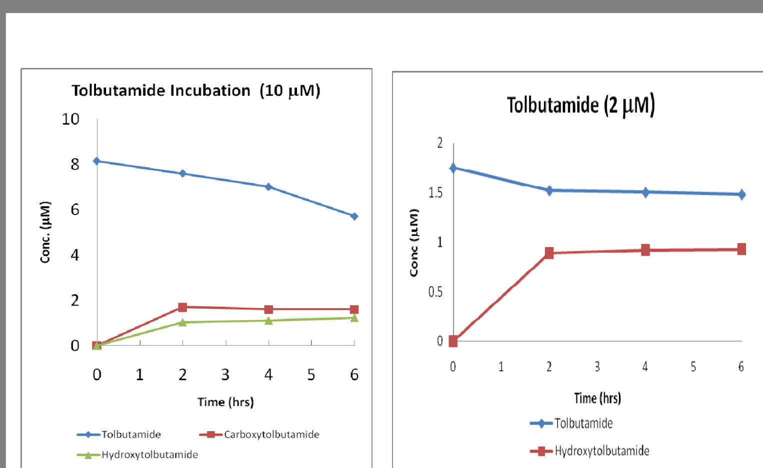
Shown in Figure 4 are the HCD spectra of tolbutamide and hydroxytolbutamide together with the fragmentation pathways for the ions at *m/z* 155.0161 and 171.0110 which confirms the site of hydroxylation (note the absence of the ion at *m/z* 91 for the metabolite). This and similar high resolution accurate mass data for the carboxytolbutamide demonstrates that accurate mass fragmentation data from HCD spectra can be used for structural elucidation.

Figure 4. HCD spectra of Tolbutamide (upper panel) and Hydroxytolbutamide (lower panel). Also shown are the fragmentation schemes (using Mass Frontier 6.0) for formation of the ions at *m/z* 155 and 171, reflecting the nature and site of metabolic modification.



The peak areas from high resolution extraction ion chromatogram for tolbutamide (Figure 3) 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 5.

Figure 5. Plots showing the disappearance of Tolbutamide (blue trace) following incubation with rat hepatocytes (at initial concentrations of 10 and 2 µM), and the appearance of metabolites.



Conclusions

The acquisition of full scan MS data enables flexibility in post acquisition data processing which is not available when doing targeted analysis. This includes, for example, the ability to acquire data without the need to develop SRM methods for quantitation, and the use of HCD data for metabolite identification.

We have illustrated that full scan MS data can be used to provide quantitative information about parent drug and metabolites without *a priori* knowledge of the metabolites.

In addition, high resolution accurate mass data from HCD scans can be used for structural elucidation.

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References:

1. K. P. Bateman, M. Kellmann, H. Muenster, R. Papp, L. Taylor, JASMS 2009 in press

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