# USE OF HIGH RESOLUTION QTOF WITH ENHANCED ION MOBILITY CAPABILITIES FOR FACILE METABOLITE IDENTIFICATION AND CHARACTERIZATION USING UPLC/IMS/MS$^E$

*Stephen McDonald[1], Joseph Marini[2], Andrew Baker[3]*
*[1]Waters Corp., Beverly, MA; [2]Covance, Madison, WI; [3]Waters Corp., Pleasanton, CA;*

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## INTRODUCTION

**The challenges of metabolite identification are well understood. For *in vivo* studies the need to completely characterize each sample is key and often is difficult due to the nature of the sample matrix complexity. Chromatographic resolution is essential in this type of analysis in order to resolve the compounds of interest from the endogenous background, as is high resolution spectral data for similar reasons. For many analyses it has been shown that ion mobility coupled with these techniques provides an additional degree of specificity. However, the duty-cycle, resolution and dynamic range of these applications have often been insufficient to be beneficial in metabolite identification studies. The method presented here utilizes QuanTof[1] detector technology and HDMS™ capabilities of the SYNAPT® G2 mass spectrometer to overcome these limitations and combine them into a single analysis.**

## METHODS

**Chemicals and reagents**
Buspirone hydrochloride, verapamil hydrochloride and polyethylene glycol (PEG) 400 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Pooled Sprague Dawley (SD) rat liver microsomes (RLM) containing 20 mg protein/mL was purchased from Cellz Direct, Inc. (Austin, TX, USA). Control SD rat urine was collected in-house. HPLC-grade acetonitrile was from Sigma-Aldrich.

***In vitro* formation of metabolites**
Buspirone hydrochloride and verapamil hydrochloride at 10mM were separately incubated with pooled SD RLM (1 mg/mL) at 37°C in 1.0 mL of 0.1 M potassium phosphate buffer (pH 7.4) containing 1mM ethylenediaminetetraacetic acid (EDTA) and 250 mL of 5mM nicotinamide adenine dinucleotide phosphate (NADPH). The 20-min incubation was terminated by the addition of 1.25 mL of ice-cold acetonitrile to precipitate microsomal proteins. The mixture was vortex-mixed, centrifuged and the supernatant was removed. A 0-min incubation was also generated by adding ice-cold acetonitrile to the incubation mix before NADPH addition. In order to evaluate the performance of the SYNAPT G2 for *in vivo* metabolite identification studies, the above *in vitro* samples were diluted 10-fold with SD rat urine containing 0.1% PEG 400 by volume.

**LC-MS Methodology**

**ACQUITY UPLC®**
Column: ACQUITY HSS T3 1.8µM 2.1 x 100 mm
Flow Rate: 0.7 mL/min
Column Temperature: 40°C
A: Water + 0.1% formic acid
B: Acetonitrile + 0.1% formic acid

*In vitro:* 0 min at 0.0% B; 8.5 min, curve 7, 100.0% B; 9 min, curve 11, 0.0% B.
*In vivo:* 0 min at 0.0% B; 18 min, curve 7, 100.0% B; 19 min, curve 11, 0.0% B.

The mass spectrometer, a **SYNAPT G2 HDMS**, was operated in positive ion electrospray mode and set to resolution mode, which is specified at 20,000 resolution (FWHM). Capillary was set at 0.5KV, cone voltage 25V, desolvation temperate 450°C, desolvation gas flow 800L/Hr.

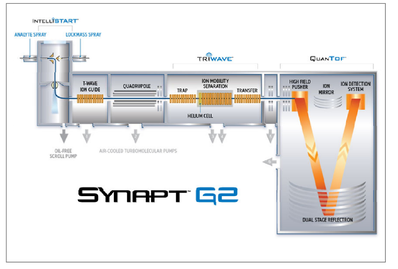


*Figure 1. Instrument Schematic of the SYNAPT G2 HDMS.*

The MS$^E$ data acquisition technique was used to acquire all data. In this mode the instrument alternates between a low and high collision energy state on alternate scans (Fig. 2) This allows collection of precursor and fragment ion information for all species in an analysis without the sampling bias introduced with other common methods, such as DDA; where a specific *m/z* must be isolated before fragmentation.

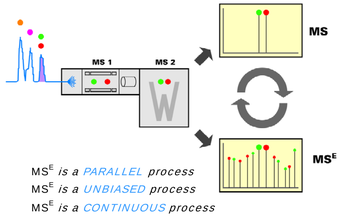


*Figure 2. The MS$^E$ acquisition technique.*

## RESULTS AND DISCUSSION

### UPLC/IMS/MS analysis

Analysis of the *in vitro* microsomal incubation of buspirone reveals that the drug undergoes extensive phase 1 metabolism. The most abundant metabolites formed are +16 Da corresponding to addition of oxygen through hydroxylation and other routes[2]. Also seen are several +32 Da metabolites, although at a lower relative intensity. After UPLC-MS analysis MetaboLynx XS™ automatically detected 11 of these +32 Da metabolites (Fig. 3).

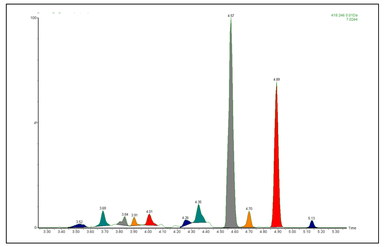

*Figure 3. 11 components identified in the extracted ion chromatogram of m/z 418.246.*

UPLC/IMS/MS analysis was also performed (Fig. 4). The compounds, initially chromatographically separated, now undergo a second stage of separation in the IMS T-wave region of the Triwave Device[3]. After the ion mobility separation (IMS) the ions are measured in four dimensions; Retention time (rt), *m/z*, intensity and drift-time (dt). This information may then be visualized in Driftscope™ a software package that works with the 4D data and allows facile visualization of each sample in several 2D plots[4] (Fig. 5).

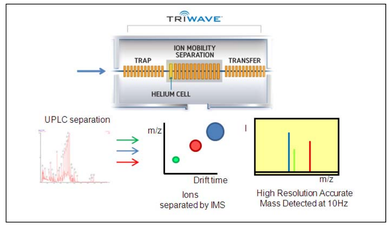


*Figure 4. Data collection using LC/IMS/MS.*

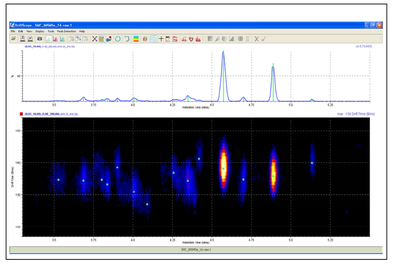

*Figure 5. Driftscope visualization of m/z 418.246. 14 +32 Da metabolites of buspirone can be identified.*

Driftscope not only allows for visualization of the HDMS data but also peak detection, export and extraction. Apex 4D, the algorithm used in this process, detects every component as a function of rt, m/z, intensity and drift time; highlighting them on the chromatographic trace with a green line and on the 2D map with a single green spot (Fig. 5).
The 2D plot is a heat map with intensity represented by color intensity. Drift time is displayed on the *y*-axis with retention time on the *x*-axis. It can be seen that while 11 metabolites at *m/z* 418.246 were detected in the LC-MS trace (Fig. 3), 14 distinct metabolites are identified in Driftscope owing to the extra dimension of separation. Furthermore, due to the nature of the separation, the collisional cross-section of each metabolite may be inferred leading to possible structural identification.

### UPLC/IMS/MS$^E$ extraction from *in vivo* matrices

Metabolites can often be obscured in the presence of matrix. Here the verapamil incubation is spiked into rat urine + 0.1% PEG400 for the purpose of observing these effects (Fig. 6). As in the previous example, the ions are separated using the Triwave device. However, using the MS$^E$ principle, the ions are now dissociated in the TRANSFER T-wave collision cell, *after* the IMS T-wave. Using this approach co-eluting molecular ions are further separated by IMS prior to generation of the fragment ions. By extracting data over a characteristic drift time window one can obtain very clean, independent fragment ion spectra for each molecular ion of interest, which simplifies interpretation and increases confidence in identification.

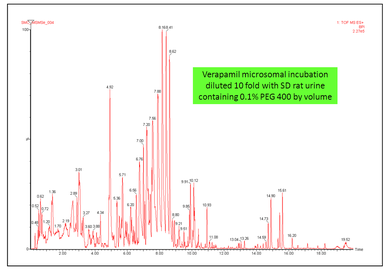


*Figure 6. UPLC/IMS/MS$^E$ chromatogram of verapamil microsomal incubation at 1µM spiked into rat urine +0.1% PEG400*

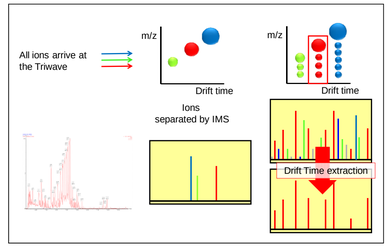


*Figure 7. Drift time extraction of IMS/MS$^E$ data.*

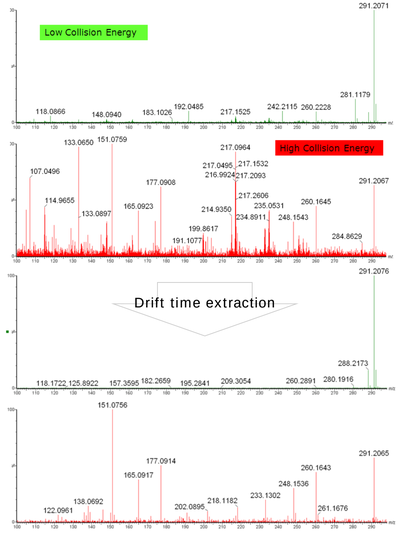


*Figure 8. Ion mobility clean-up of precursor and product ion information.*

Figure 8 shows the data extracted in the standard LC-MS data. Many interferences exist due to the high background typical in *in vivo* analysis. Filtering the data to show only ions with a drift time of 87 ms ± 4 ms removes ions that are present in the analysis at the same retention time but differ in size, shape or charge. Performing elemental composition analysis of the parent ion yields the formula $C_{17}H_{27}N_2O_2$ with an accuracy of 0.3 mDa (1.0 ppm) and is consistent with the proposed metabolite.

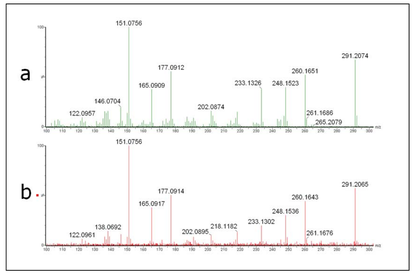


*Figure 9. MS$^E$ data from verapamil dealkylation metabolite at a) 10µM in solvent; b) 1µM in rat urine + 0.1% PEG 400 post drift time extraction.*

When the drift time extracted high collision energy data is compared with standard data acquired in solvent, minimal differences between the two spectra can be seen (Fig. 9).
Finally, fragment analysis of the high collision energy drift time extracted data was performed using MassFragment. MassFragment employs a systematic bond disconnection approach to assign fragment ions to proposed structures giving a score for the most probable[5]. The results show mass measurement accuracy of 0.76 mDa RMS on the nine most intense fragments for the drift time extracted data.

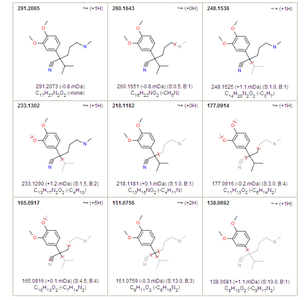

*Figure 10. MassFragment analysis of the drift time extracted product ions of verapamil metabolite at m/z 291.2076.*

## CONCLUSION

- **Additional selectivity and specificity by incorporating ion mobility separation into metabolite identification workflow**
- **Fidelity of dynamic range and accurate mass with multidimensional LC-IMS-MS separation enabled by QuanTof technology**
- **Use of ion mobility to clean data through removal of dosing vehicle and endogenous compounds**
- **Enhancement of DriftScope visualization with APEX4D for automated, comprehensive peak detection from UPLC/IMS/MS data.**
- **Comprehensive *in vivo* IMS-MS$^E$ datasets that leaverage mobility separation for improved correlation of precursor and fragment ions**

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