TOWARDS IN-VITRO DIAGNOSTIC IMAGING OF HEPA-RG SPHEROIDS BY DESI TANDEM QUADRUPOLE MS

Mark Towers[1], *Lisa Reid*[1], *Alicia Rosell-Hidalgo*[2], *Kirsten Headspith*[2]

[1] Waters Corporation, Wilmslow, Cheshire, UK [2] Cyprotex Ltd., Alderley Park, Cheshire, UK

INTRODUCTION

During the development stages for novel pharmaceutical compounds there is the requirement for in-vivo dosing for efficacy and safety assessment. With the introduction of the 3R's concept, scientists have been constantly improving in-vitro tests, allowing for a more informed candidate selection and elimination process prior to animal study requirement.

Until recently in vitro studies have focused primarily on 2D cell cultures, however there is a growing interest in 3D models that better mimic the micro-environment within an organism. In particular, 3D liver models such as HepaRG spheroids have been developed to investigate drug-induced liver injury (DILI). Here we show targeted high sensitivity DESI imaging analysis for mapping analyte localization with the HepaRG spheroids.

METHODS

HepaRG spheroids were cultured for 7 days, after which they were dosed at typical therapeutic levels, with either; perhexiline, carbamazepine or warfarin.

Perhexiline was dosed at between 10 µM and 0.004 µM, carbamazepine and warfarin were dosed at 1000 µM and 0.4 µM. The compounds were dissolved in DMSO with a final media concentration of 0.5% DSMO. An additional DMSO control vehicle was also prepared. ~72 hrs. after dosing the spheroids were moved to storage at 4 ºC.

Prior to analysis the spheriods were washed by transfer into 50 µL of 1XPBS. Using a wide bore pipette tip, the spheriod was allowed to fall to the end of the tip, and the tip touched to the surface of the PBS solution allowing the spheroid to migrate into the PBS solution whilst minimising cell media transfer.

Spheriods were spotted onto polylysine coated glass slides by again pipetting with a wide bore pipette tip, allowing the spheriod to drop to the end of the tip and gently touching the meniscus against the glass slide minimising PBS transfer. The slides were dried for 5 mins in a desiccator prior to analysis. A 1 µL PBS blank was also spotted.

A dilution series of each compound was prepared, perhexaline dissolved in ethanol, carbamazepine and warfarin dissolved in acetonitrile. 1 µL of each concentration was spotted on a Teflon spotted glass slide.

Samples were analyzed using a Waters™ Xevo™ TQ Absolute triple quad mass analyzer fitted with a Waters DESI XS source using the high performance sprayer.

DESI conditions

Flow rate— 1.5 µL/min

Solvent—95% Methanol

Gas pressure—10 PSI

Capillary voltage— 0.66kV Pos, 0.6kV Neg

Pixel size—100 x 100 µm

Scan rate—5 HZ dilution series 10HZ spheroid analysis

Experiment optimization

The compounds were screened by SIR in positive and negative mode to determine optimum polarity cone voltage and adduct for MRM analysis.

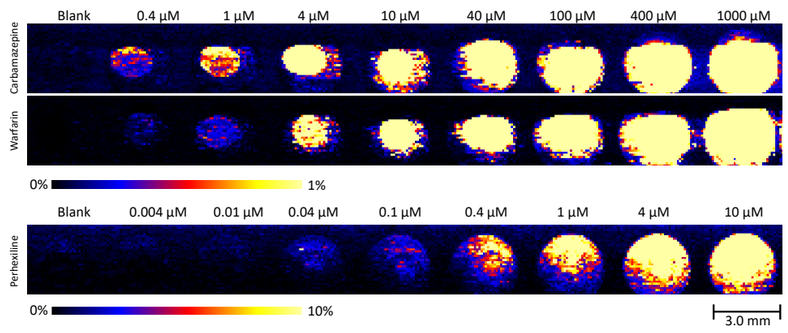
Perhexiline and carbamazepine were found to have higher sensitivity in positive ionization mode. Warfarin was found to preferentially ionize in negative ionization mode.

MSMS acquisitions of each standard were performed to determine optimum collision energy and to select MRM transitions. The main metabolites were determined from literature (1,2,3). For perhexiline, cis-hydroxyperhexiline was selected, for carbamazepine, carbamazepine-10,11-epoxide and for warfarin, 10-OH warfarin and 7-OH warfarin. MRM product ions were also taken from literature (1,2,3). In addition; two lipids one for positive ionization mode (m/z 782.6) and one for negative ionization mode (m/z 682.6) were imaged. Acquisition was performed without collision energy and the transitions were pseudo. MRM transition settings can be seen in ***Table 1***.

Table 1. Transitions for each spheroid dosed sample

Compound	Precursor	Product	Cone V	CeV	Dwell (MS)
Perhexiline	278.3	82.9	30	25	29.3
Cis hydroxyperhexiline	294.3	95.2	30	25	29.3
Carbamazepine	237.1	194.0	30	25	29.3
Carbamazepine 10,11-epoxide	253.09	180.04	30	25	29.3
Warfarin	307.1	160.8	20	15	21
10 OH Warfarin	323.1	250.0	20	15	21
7 OH Warfarin	323.1	177.0	20	15	21
Lipid *m/z* 682.6	682.6	682.6	60	4	21

RESULTS AND DISCUSSION



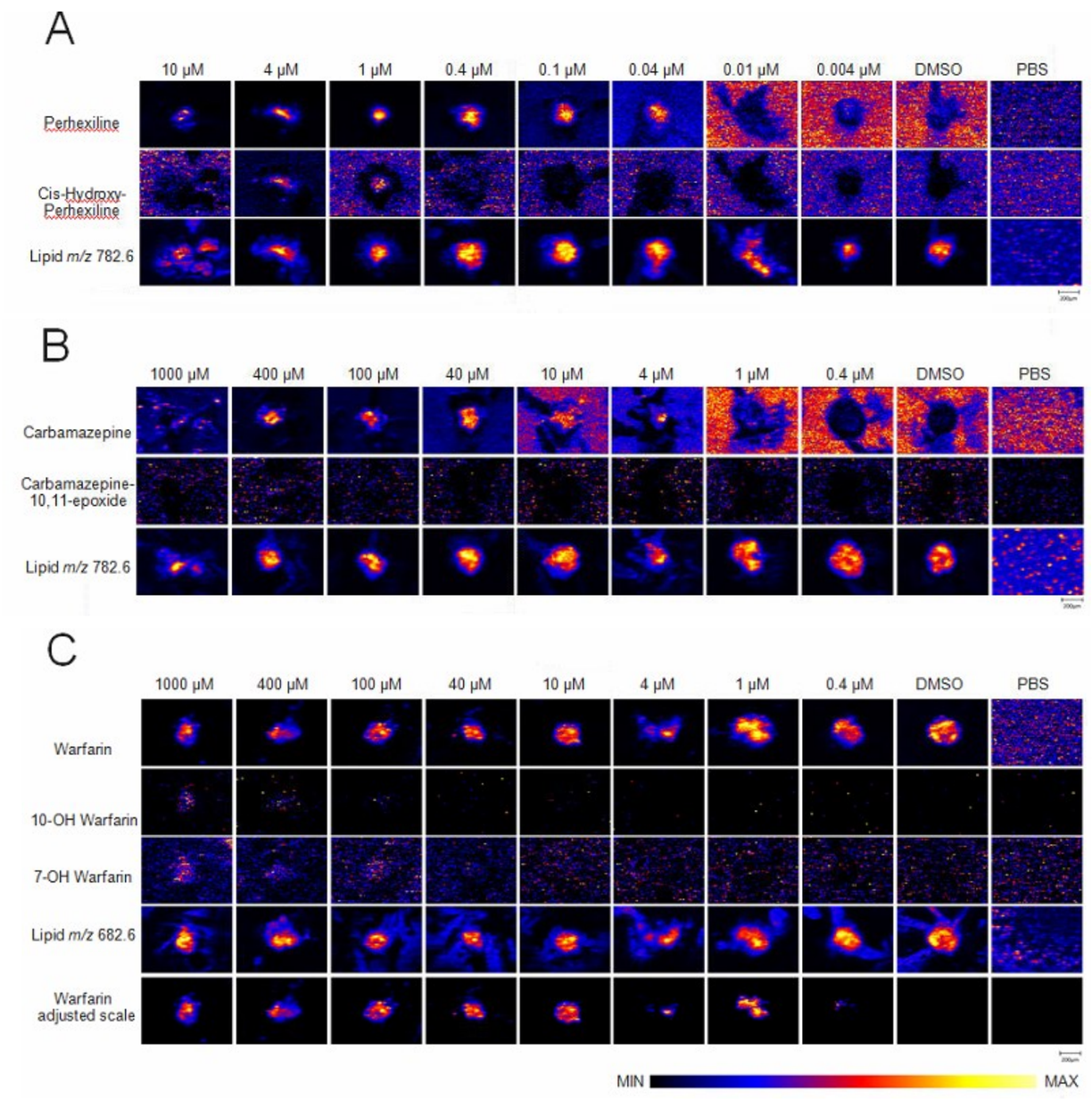
Dilution series

Using a dilution series of each standard: warfarin and carbamazepine were detected at the lowest dosing level in this study 0.4 µM. This is equivalent to a total amount present of 94.48 pg and 123 pg respectively (based on a 1 µL volume on target). Perhexiline, was detected down to 0.04 µM or 9.1 pg on target based on a 1 µL volume), ***Figure 1***.

Figure 1. Dilution series MRM results for carbamazepine, warfarin (1000-0.4 µM) and perhexiline (10-0.004 µM). The maximum of the intensity scale has been set to highlight the signal at lower concentrations.

Spheroid analysis

The dosed spheroids were imaged using the appropriate MRM settings listed in Table 1. All three drugs were successfully detected within/on the spheroids (***Figure 2***).



Perhexiline was detected down to the 0.04 µM dosing level, the cis-hydroxylperhexiline metabolite was detected down to the 1 µM dosing level. **(A)**

Carbamazepine was detected down to the 4 µM dosing level, its carbamzepine-10,11-epoxide metabolite was not detected. **(B)**

Warfarin signals appear in the lowest dosing level of 0.4 µM however, they are also observed within the DMSO control vehicle spheroid, suggesting the presence of an interfering compound. When the maximum signal detected in the DMSO spheroid is subtracted from the signal observed within the dosed spheroids, an unambiguous result is returned for the 1 µM dosing level (>2.4 times increase in signal compared to the DMSO). **(C)**

Figure 2. Spheroid imaging MRM results for Spheroids dosed with perhexiline (A) (10-0.004 µM +DMSO Control + PBS Blank) carbamazepine (B) and warfarin (C) (1000-0.4 µM + DMSO Control + PBS Blank). Each image is displayed on its own independent scale (0 to maximum signal) except the final row of C where the maximum signal from the DMSO control Vehicle has been set to the minimum value (115002 to maximum signal).

The compounds selected were chosen due to their known hepatotoxicity and so are known to cause metabolic changes within the HepaRG cells. At the highest dosing levels for perhexiline and carbamazepine it appears that the spheroids have lost some structural integrity (no longer appearing solid and round). This is likely related to the high level of compound exposure causing cell damage.

In particular, the perhexiline seems to show an interesting distribution: with a significantly higher signal response from the center of the spheroid (***Figure 3***). If a presumption is made that the compounds are being extracted from the complete depth of the sample –then the internal distribution of the drug can be inferred (based on microscopy images).

This will form part of future work comparing the results of sectioned vs. un-sectioned spheroids. As well as investigating the metabolomic changes of the spheroids using a full discovery platform to demonstrate that these drug distributions can be mapped to metabolic changes in the HepaRG cells.

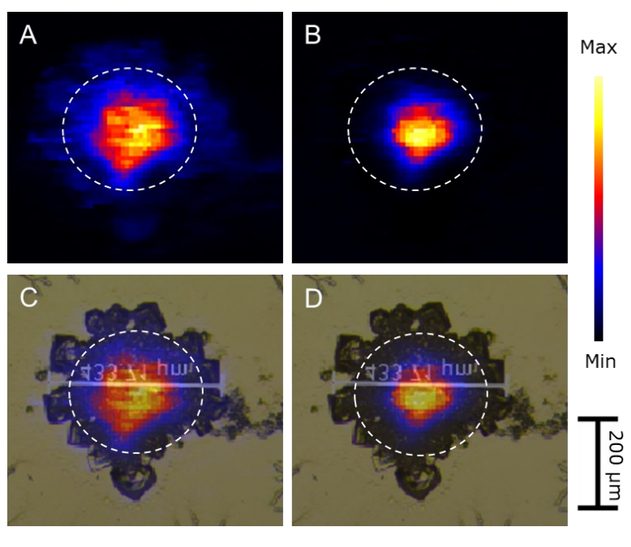


Figure 3. Expansion and over lay of the perhexiline 1 µM dosed spheroid. A) ion image of lipid m/z 782.6 MRM B) ion image of perhexiline MRM. C) overlay of A with spheroid D) overlay of B with spheroid.

CONCLUSION

- **The Targeted MS Imaging solution with DESI XS and Xevo TQ Absolute is a highly sensitive imaging tool able to image:**
  - **drug distribution within spheroids at typical dosing levels**
  - **drug metabolites within spheroids at typical dosing levels**
- **Relative concentration differences can be visualized using HDI software giving a clear picture of limits of detection and level of absorbance for the analytes of interest.**
- **The compounds chosen in this study are known to be hepatotoxic and there is clear evidence of spheroid deformation.**

References

1. W Ju *et al*, Austin J Anal Pharm Chem. 2014 ; 1(2)
2. G.F van Rooyen *et al,* Journal of Chromatography B, 769 (2002) 1–7
3. Mei Zhang *et al,* Journal of Chromatography B, 877 (2009) 3025–3030

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