SIMULTANEOUS QUALITATIVE AND QUANTITATIVE DATA GENERATION FROM DRIED BLOOD SPOTS

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INTRODUCTION

The simultaneous generation of qualitative and quantitative metabolism data (the 'qual/quan' approach) continues to increase in popularity, with the aim of maximising the amount of information available from early metabolic screens and *in vivo* studies.[1,2] Whilst *in vitro* and plasma samples have been shown to be amenable to qual/quan analysis, the increasing use of DBS for bioanalysis[3] necessitates an analysis of whether the resulting reduction in sample quantity compromises typical qual/quan methodologies.

EXPERIMENTAL

Quantitative data on the clearance of verapamil were obtained via MRM analysis on a triple-quadrupole (API5000) and accurate XIC extraction on a HRMS instrument (LTQ Orbitrap). On the triple quadrupole, predicted MRM transitions for known metabolites of verapamil were included in the MS method. On the HRMS instrument, full scan high-resolution MS data ± data-dependent MS^2 spectra were acquired using a range of MS resolution and IT fill time settings.

Verapamil was incubated (5 and 50µM) in rat liver microsomes and aliquots (500µl) of the incubation were precipitated with acetonitrile (2500µl) at various time points (0, 5, 10, 20, 30, 45 and 60 mins). The supernatant was evaporated to dryness and the samples were reconstituted in 25% acetonitrile (50µl). An aliquot (20µl) of the resulting solution was spiked into fresh whole rat blood (180µl) and the blood samples were then "spotted out" (20µl) on DMPK-B DBS cards (GE healthcare) and allowed to dry for at least 2 hours. Blank blood was also "spotted out" as a control.

A 3mm punch was taken from each DBS sample and extracted in methanol (300µl; containing imipramine as IS) by vortex mixing for 10 mins. After mixing the methanol was transferred to clean wells and evaporated to dryness. For triple quadrupole analysis each well was reconstituted in 25% acetonitrile (1000µl) prior to injection (15µl) onto a UPLC-MS system. The 50µM incubation samples were further diluted 1 in 10 before analysis. For HRMS analysis each well was reconstituted in 10% acetonitrile (100µl) prior to injection (15µl) onto a HPLC-MS system.

RESULTS

Quantitative analysis of verapamil

LC-MS analysis on both triple quadrupole and high-resolution instruments yielded very similar intrinsic clearance data (Figure 1), with $T_{1/2}$ values of 12.1 and 11.7 mins respectively. Varying the MS resolution and IT fill time had no significant impact on the clearance data.

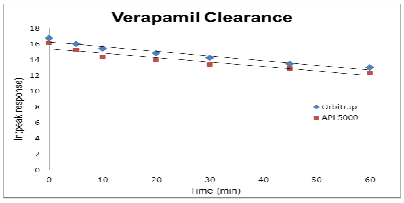

Figure 1: Comparison of verapamil clearance data

Qualitative analysis of verapamil metabolites

Initial analysis on an API5000 using a generic LC-MS/MS method (UPLC; 3min cycle ballistic gradient) showed significant co-elution of isobaric metabolites. Adequate resolution was achieved by extending the cycle-time to approximately 6 minutes. Subsequent analysis with the extended gradient using predicted MRM transitions showed evidence for products of demethylation, di-demethylation, mono-oxidation and cleavage via N-dealkylation in addition to unchanged verapamil (Figure 2). Although product ion spectra to enable structural elucidation of the metabolites could not be generated using the triple quadrupole instrument, due to insufficient scan speeds, knowledge of the parent compound fragmentation did allow some information about the sites of metabolism to be derived from the MRM data (Figure 2).

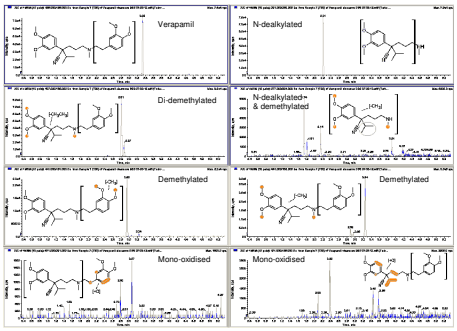

Figure 2: Predicted MRM chromatograms for verapamil metabolites, showing partial structural information

Qualitative analysis on the LTQ Orbitrap using the 6 minute LC system and extraction of accurate mass chromatograms resulted in the detection of the same metabolites observed using the predicted MRM approach (Figure 3). In this example, a resolution setting of 7500 was sufficient to generate adequate metabolism information and quantitative data.

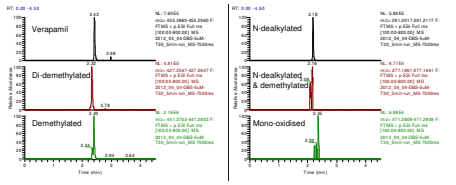

Figure 3: Example XICs derived from HRMS analysis

In addition, product ion spectra could be acquired on the LTQ Orbitrap in parallel with the full scan data using data-dependent scanning, enabling partial structural elucidation of the metabolites (Figure 4).

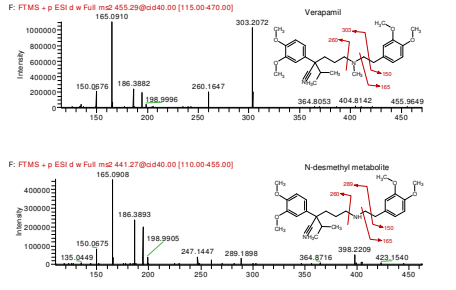

Figure 4: Example MSMS spectra for verapamil and its N-desmethyl metabolite using HRMS analysis and data-dependent acquisition

Furthermore, an investigation of UV data acquisition showed that, with suitable LC conditions and at selected wavelengths, useful metabolite abundance data could be generated in spite of the interfering components from the DBS paper (Figure 5).

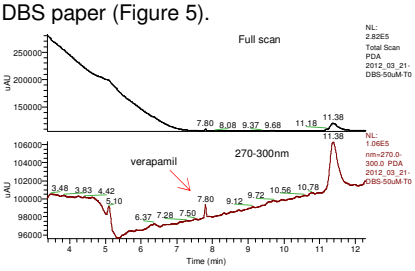

Figure 5: UV data from the t0 incubation (top: full scan; bottom: 270-300nm) to illustrate the detection of drug-related material

DISCUSSION

The quantitative analysis showed that comparable metabolic clearance information for verapamil could be obtained from DBS samples using both traditional MRM and high-resolution MS approaches. As expected, there were no issues with sensitivity or matrix effects when DBS samples were analysed using an API5000, whilst the LLOQ was significantly higher using the HRMS approach. Both approaches were able to provide additional information on the metabolites of verapamil. The full scan HRMS data could be interrogated to reveal the presence of metabolites and information on their structure could be obtained from MS^2 data acquired via data-dependent acquisition. In addition, UV data could be used to supplement the MS data, although the high background from matrix components eluted from the DBS paper meant that low wavelength UV acquisition was significantly compromised. Although useful information on metabolites was obtained on an API5000 via the prediction of metabolite MRM transitions, this approach is only likely to be successful for simple transformations, where the fragmentation is not significantly altered from the parent compound. In addition, useful full scan data could not be acquired on the API5000 as the scan speed and resolution requirements could not be reconciled with the cycle time.

CONCLUSION

This work demonstrates the feasibility of using qual/quan approaches on DBS samples to provide simultaneous information on clearance and metabolic pathways. Valuable information could be generated using MRM analysis on a triple quadrupole or by acquiring full scan data on a high-resolution instrument. As always, the choice of approach will depend on the desired balance between speed, sensitivity and the completeness of the derived metabolism information.

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3. Clark G and Haynes JJ. Utilization of DBS within drug discovery: a simple 2D-LC–MS/MS system to minimize blood- and paper-based matrix effects from FTA elute™ DBS. *Bioanalysis*, **3**, 1253-1270 (2011).

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