

THE BENEFITS OF EXTENDED DYNAMIC RANGE FOR METABOLITE ID USING UPLC/MS/MS

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INTRODUCTION

An important area in the drug discovery and development process is the identification of drug metabolites for both *in vitro* and *in vivo* samples. The challenge in these analyses lies in the inherent complexity of the biofluid sample to be analyzed (i.e., plasma, urine, bile, feces, microsomal and hepatocyte incubations), and the large number of endogenous interferences that are often present. These matrix interferences may slow down the metabolite identification process as additional experiments may be required to isolate the analytes of interest and eliminate false positives.

To eliminate this multi-step approach and obtain maximum characterization information in a single analysis, LC/MS has long been the ideal technique for the identification of structurally diverse metabolites in complex matrices at wide concentration ranges as it is extremely robust, rapid, and sensitive. Of the wide range of mass analyzers which may be utilized for metabolite identification, the Waters® Micromass® Q-ToF Premier™ orthogonal hybrid quadrupole time-of-flight mass spectrometer is particularly suited. The extra specificity, and hence, confidence in results afforded by the wide dynamic range and exact mass MS and MS/MS capabilities of the Q-ToF Premier can assist in streamlining the metabolite identification process. The Q-ToF Premier[1,2] when paired with the Waters® ACQUITY UPLC™ System simplifies the task of finding and identifying drug metabolites by producing superior chromatographic separations, increased sensitivity, and higher throughput. Moreover, exact mass can detect biological changes which may occur as a result of metabolism and thus can be used as the first point of reference to eliminate false positives. Further MS/MS exact mass experiments together with precursor and neutral loss acquisitions can decipher the structure of interest for the putative metabolite.

Past TOF technologies have suffered from a limited dynamic range of between 2.5 and 3 orders of magnitude. This had an effect not only on the quantitative capabilities of the instrument but also on the ability to mass measure putative metabolites at high concentrations with great accuracy. Due to the variety of metabolites and the range of concentrations which may be found in any sample, the task of carrying out exact mass measurements required care in data processing in order to avoid using signal that was in dead time. The Q-ToF Premier's programmable dynamic range (pDRE™) technology allows routine exact mass measurement and greatly simplifies the identification of drug metabolites in biological matrices.

EXPERIMENTAL

In this study we demonstrate the use of pDRE for highly concentrated metabolite samples. pDRE uses data from an attenuated ion beam for ion currents that would normally lead to detector dead time effects. This process extends the dynamic range of the instrument to greater than 4 orders, allowing mass accuracy and quantitative responses to be achieved with high concentration samples. All data shown were acquired using pDRE, except for the example in Figure 1, where we simply illustrate the effect of dead time.

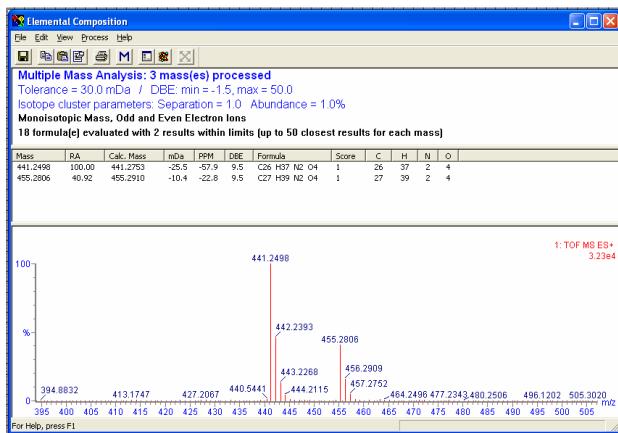


Figure 1. 'Dead time' for highly saturated signals for Verapamil and its dealkylated metabolite

Sample Preparation

In vitro metabolism: Verapamil was incubated using rat microsomes at a 10 μ M level. The incubation period was 60 minutes at 37 °C, in a solution of 50 mM potassium phosphate (adjusted to pH 7.4 with NADPH). The reaction was then terminated with 2 volumes of cold acetonitrile to 1 volume of sample. The samples were stored frozen at -80 °C prior to LC/MS analysis.

LC Conditions

LC System:	ACQUITY UPLC System			
Column:	ACQUITY UPLC BEH C ₁₈ Column, 1.7 μ m, 2.1 mm x 100 mm			
Mobile Phase A:	Water + 0.1% formic acid			
Mobile Phase B:	Acetonitrile + 0.1% formic acid			
Gradient:				
Time (min.)	Flow (mL/min.)	%A	%B	Curve
Initial	0.5	100	0	1
7.00	0.5	20	80	6
7.10	0.5	20	80	6
8.00	0.5	100	0	6

MS Conditions

MS System:	Q-ToF Premier Mass Spectrometer			
Ionization Mode:	Electrospray, positive ion			
Capillary Voltage:	3 kV			
Cone Voltage	45 V			
Source Temp:	120 °C			
Desolvation Temp:	320 °C			
Acquisition Range:	70–900 amu			
Acquisition Mode:	Centroid with pDRE enabled			
Lock Mass:	Leucine enkephalin, $m/z=556.2771$			

RESULTS

Verapamil Metabolism

Orally administered verapamil undergoes extensive metabolism in the liver by O-demethylation (25%) and N-dealkylation (40%), and is subject to pre-systemic hepatic metabolism with elimination of up to 80% of the dose. The metabolism is mediated by hepatic cytochrome P450, and animal studies have implied

that the mono-oxygenase is the specific isoenzyme of the P450 family. This is confirmed by the results of the *in vitro* experiments carried out in this work. As a result of this, and to the initial 10 μ M Verapamil concentration in the microsomal incubation, the dealkylated metabolites were detected at high concentrations.

Figure 2 shows the extracted mass chromatogram ($m/z=441$) for the N-dealkylated metabolites of Verapamil. It can be observed that the peak height for the main N-dealkylated metabolite, at retention time of 3.84 minutes, was 4.49×10^4 in centroid mode. Before the introduction of pDRE, this signal intensity in centroid mode would have lead to detector saturation. Selection of spectra from the apex of the chromatographic peak would have given data that exhibited dead time effects, resulting in a mass error from the peak being shifted to lower mass, as illustrated in Figure 2. To avoid this, spectra from the falling side of the peak would need to be selected to obtain the best possible data with exact mass.

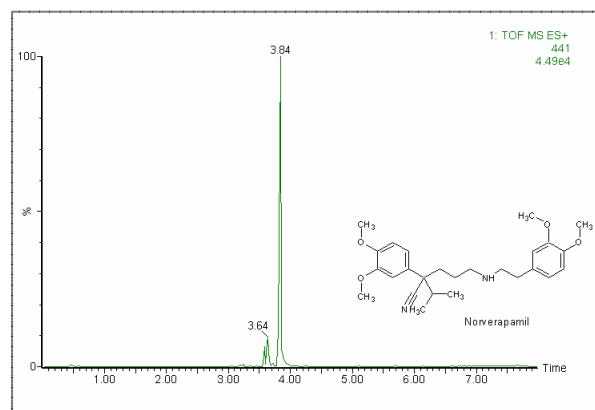


Figure 2. The extracted ion chromatogram for all dealkylated metabolites of Verapamil at $m/z=441$

When pDRE is applied, no pre-selection of the spectra is required and combining across the entire chromatographic peak produces excellent mass accuracy for both the parent compound (-0.8 ppm) and the coeluting Norverapamil metabolite (-2.3 ppm), as seen in Figure 3. The total combined trace intensity was 6.17×10^4 . This ion current without pDRE would have led

to detector saturation, resulting in a larger mass measurement error.

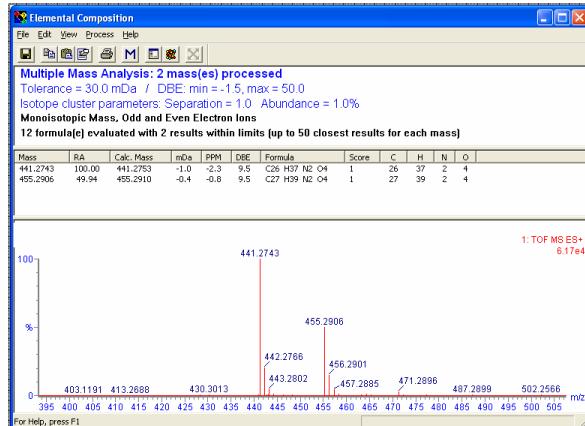


Figure 3. pDRE and exact mass for highly saturated signals for Verapamil and its dealkylated metabolite

In order to further illustrate the dynamic range enhancement, the extracted ion chromatogram for another metabolite ($m/z=277.2923$) is shown in Figure 4. The peak at a retention time of 3.07 minutes corresponded to the N-dealkylated cleavage metabolite. The peak height for this specific metabolite was 1.71 e^4 .

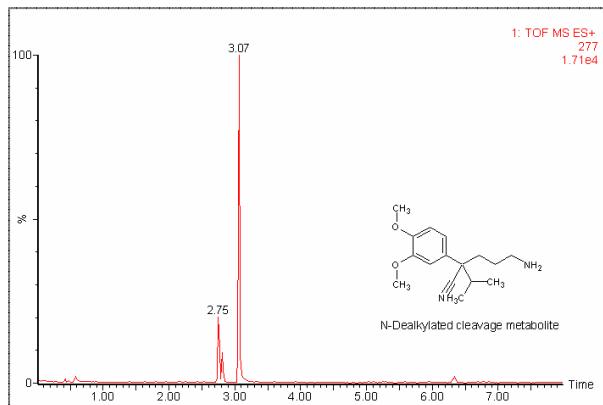


Figure 4. The extracted ion chromatogram for the dealkylated cleavage metabolite of Verapamil at $m/z=277$

The exact mass measurement for this metabolite was 2.5 ppm (Figure 5). From these results, it can be deduced that pDRE works across a wide mass range, enabling ease of use and good quantitative properties.

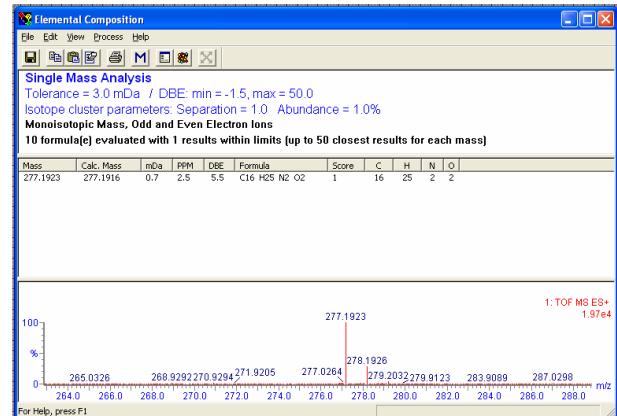


Figure 5. pDRE and exact mass for highly saturated signals for the dealkylated cleavage metabolite of Verapamil

CONCLUSIONS

The use of the Q-ToF Premier and pDRE in UPLC/MS/MS analyses provides routine exact mass measurement without the need to select spectra that are not saturated or diluting the sample of interest. This new technological advancement is of great importance when looking at unknowns and when good exact mass measurement is required to provide unequivocal results. Employing simple exact mass measurement with the resulting compound elemental composition allows accelerated data processing, reduces false positives, and prevents mis-assignment of metabolite structures. Furthermore, in this work we have focused on a specific application of qualitative detection and identification of metabolites. However, it should be known that pDRE also extends the quantitative properties of the instrument in both full scan MS and MS/MS with exact mass and high resolution. Further work will be published showing 4 orders of magnitude for quantitative bioanalysis.

REFERENCES

1. *Rapid Communications in Mass Spectrometry*, Volume 19, Issue 6, Date: 30 March 2005, Pages: 843-848.
2. *Rapid Communications in Mass Spectrometry*, Volume 18, Issue 19, Date: 15 October 2004, Pages: 2331-2337.

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