

Determination of Metabolic Stability using Polarity Switching and Cassette Analysis in an Ultra High Performance Liquid Chromatography (UHPLC)-Triple Quadrupole Mass Spectrometry System

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Introduction

Metabolic stability is one of the primary assays performed in the early stage of drug discovery to characterize new chemical entities and discard non-drug like compounds that would fail during later stages of development. Fast LC/MS/MS screening methods are required, which should be able to provide good chromatographic resolution and precise quantitation of large numbers of samples requiring analysis.

This work describes the advantage of combining high speed/high resolution UHPLC and high speed MS/MS analysis of pooled incubates to increase throughput in a metabolic stability assay, while maintaining good precision and accuracy.

Experimental

Sample preparation

The incubation mixtures consisted of an amount of rat liver S9 preparation equivalent to 0.3 mg protein, 1 μ M substrate (buspirone, verapamil, dextromethorphan or diclofenac), NADPH regeneration system and 0.1 M phosphate buffer (pH 7.4) up to a total volume of 300 μ L. Incubations were carried out separately at 37°C. A 25 μ L aliquot was taken at 0, 5, 10, 15, 25 and 35 minutes from each incubate and the reaction was stopped by adding 300 μ L acetonitrile containing the internal standards (dextropran-d3 and diclofenac-d4) followed by centrifugation for 10 min at 14,000 g. The supernatant was evaporated to dryness using a gentle stream of nitrogen and reconstituted with water/acetonitrile (80/20) containing 0.1 % formic acid for UHPLC/MS/MS analysis as described below.

Agilent 1290 Infinity UHPLC:

Column: Agilent Rapid Resolution High Definition (RRHD) Zorbax SB-C18, 2.1 x 50 mm, 1.8 μ m
Mobile phase: A= 0.1% formic acid in water, B= 0.1% formic acid in ACN; Injection volume: 1 μ L

Method 1:

Column temperature: 25°C or 40°C, Flow rate: 1.0 mL/min
Gradient: 25%B during 0.2 min, 80%B at 1 min, 80%B at 1.25 min, 25%B at 1.26 min, stop time at 1.8 min.

Method 2:

Column temperature: 60 °C, Flow rate: 1.5 mL/min
Gradient: 25%B during 0.2 min, 80%B at 0.73 min, 80%B at 1.00 min, 25%B at 1.01 min, stop time at 1.5 min.

Agilent 6460 Triple quadrupole MS with Agilent Jet Stream:

Scan type: MRM (Mass Hunter optimizer software used to optimize MRM transitions, fragmentor voltage and collision energy)

Polarity: positive/negative, positive only or negative only

Parameters: Drying gas temperature: 350°C, Drying gas flow: 10 L/min, Sheath gas temperature: 400°C, Sheath gas flow: 12 L/min, Nebulizer pressure: 35 psig, Nozzle voltage: 0 V (+) 1000 V (-), Capillary voltage: 4000 V (+/-)

Polarity switching time: 30 ms

MRM transitions: 6 (+/-) or 5 (non-switched analysis)

Dwell time: 5 ms (+/-) or 22 ms (non-switched analysis)

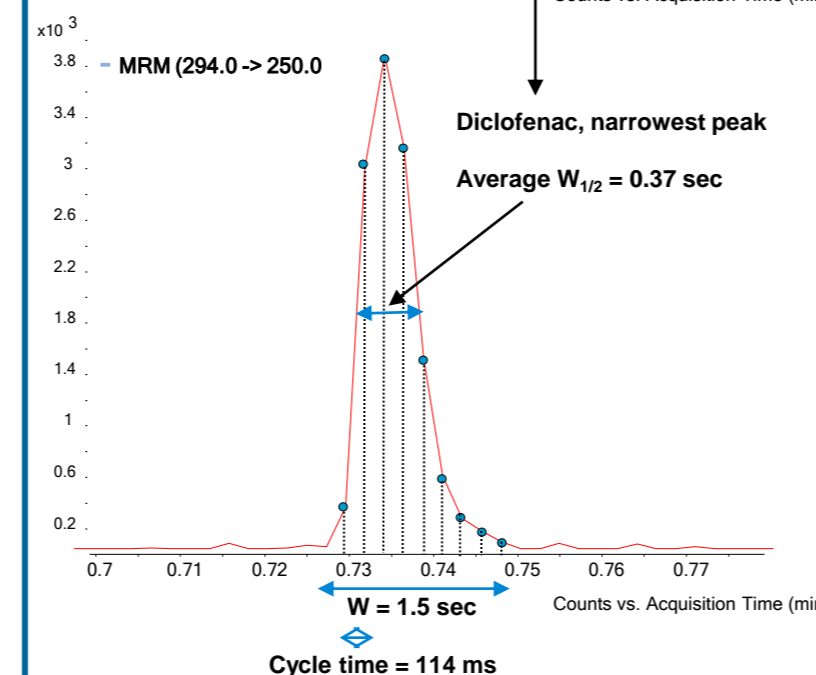
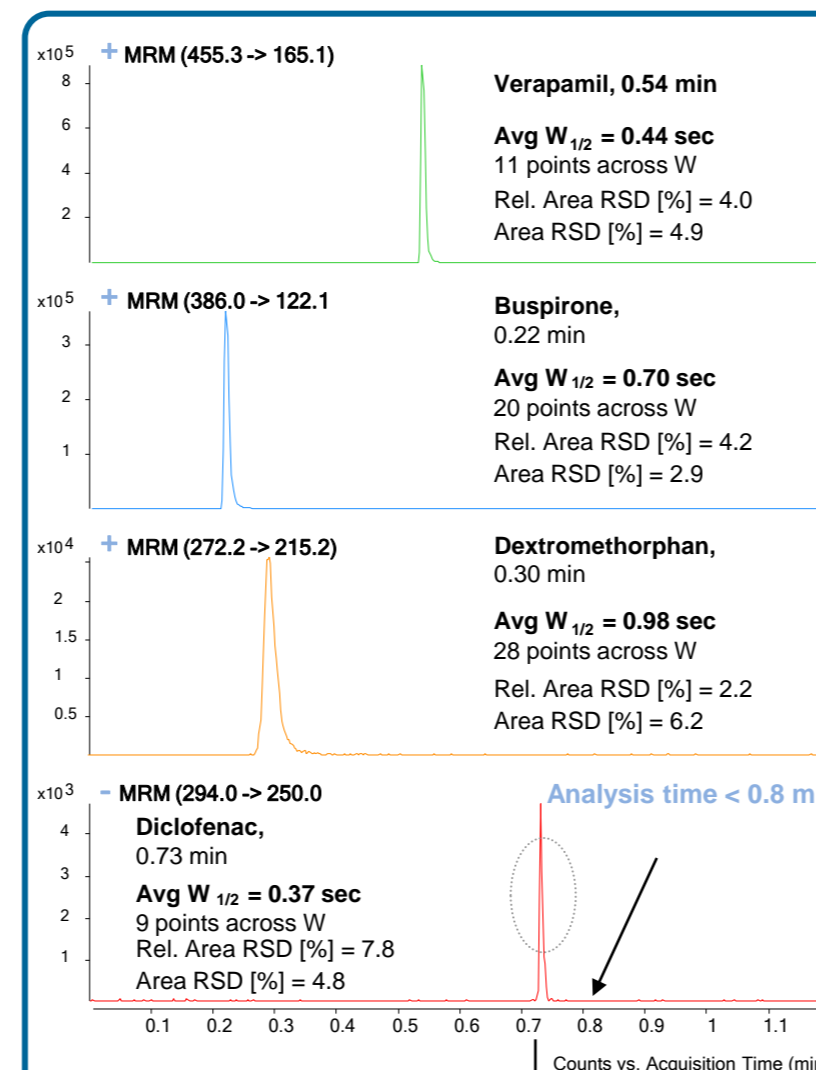
When using internal standard calibration, a conflict between $^{35}\text{Cl}_2$ -Diclofenac- d_4 (m/z 298) and $^{37}\text{Cl}_2$ -Diclofenac (m/z 298) was observed. The response of the transition 298 \rightarrow 254 arising from $^{37}\text{Cl}_2$ -Diclofenac contributes to the response of the internal standard $^{35}\text{Cl}_2$ -Diclofenac- d_4 . This led to a quadratic calibration curve due to artificially "increasing" internal standard. To solve this, either external calibration or a unique transition to diclofenac- d_4 like 298 \rightarrow 217 can be used.

Results and Discussion

Speed and data quality using fast polarity switching

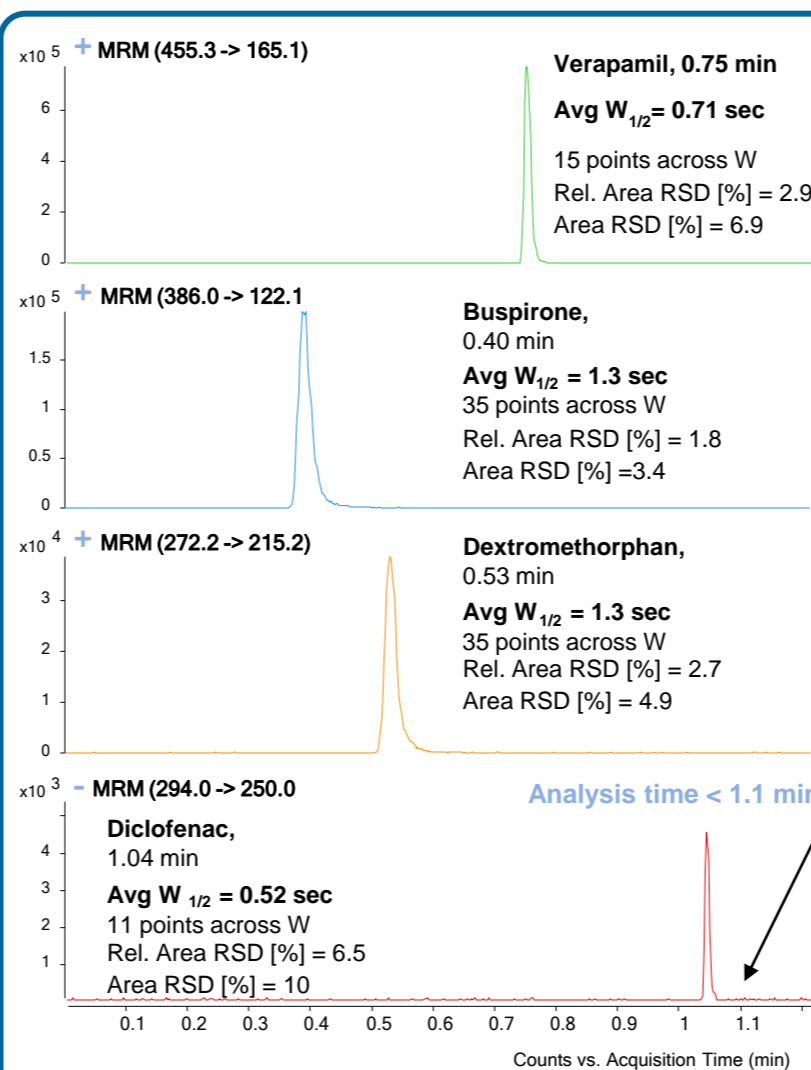
Operating the UHPLC system at high flow rates (1.0 mL/min or 1.5 mL/min) and pressures up to 1100 bar enabled a run time of only 1.5 minutes and generated peak widths less than a second at half height (typically 0.4 to 1.0 second). Sufficient data points across the peak (> 9 points) could be collected due to low MS cycle times, ensuring high precision quantitation (Area RSD [%] and Relative Area RSD [%] < 10)

Flow rate 1.5 mL/min, 1070 bar at 25%B, 60°C



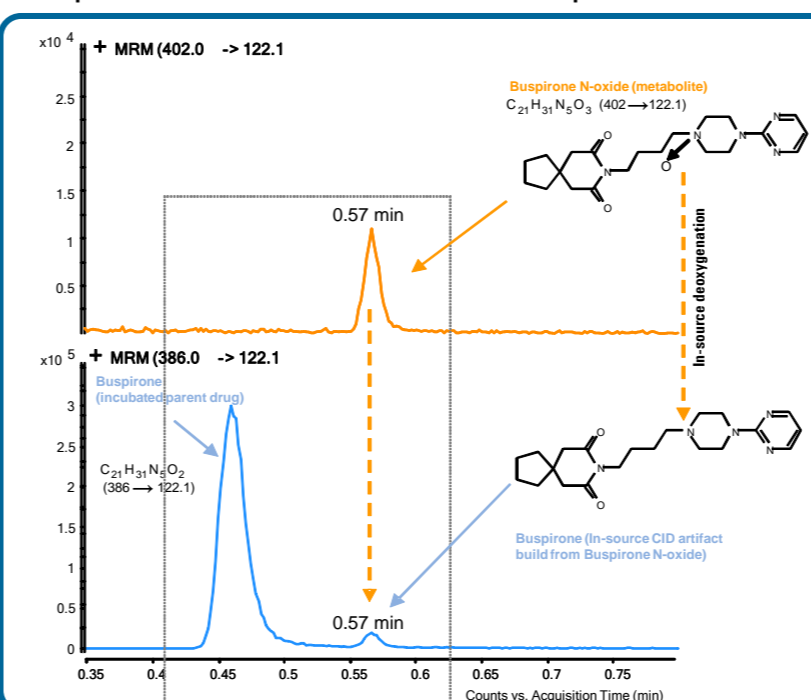
Good quantitation requires 9-10 data points across a peak. As peaks get narrower, the MS detector must be able to acquire faster. MS cycle times are reduced. During each cycle, the MS system must analyze 6 MRM transitions and switch polarity. This was enabled by 5 ms MRM dwell times and 30 ms polarity switching time.

Flow rate 1.0 mL/min, 850 bar at 25%B, 40°C



Chromatographic resolution - Pooled incubates

Excellent resolution between critical peaks was demonstrated. Coelution of metabolites and parent compounds may result in overestimation of the amount of parent compound present if the metabolite is thermally labile and can convert into the parent compound in the source of the mass spectrometer.

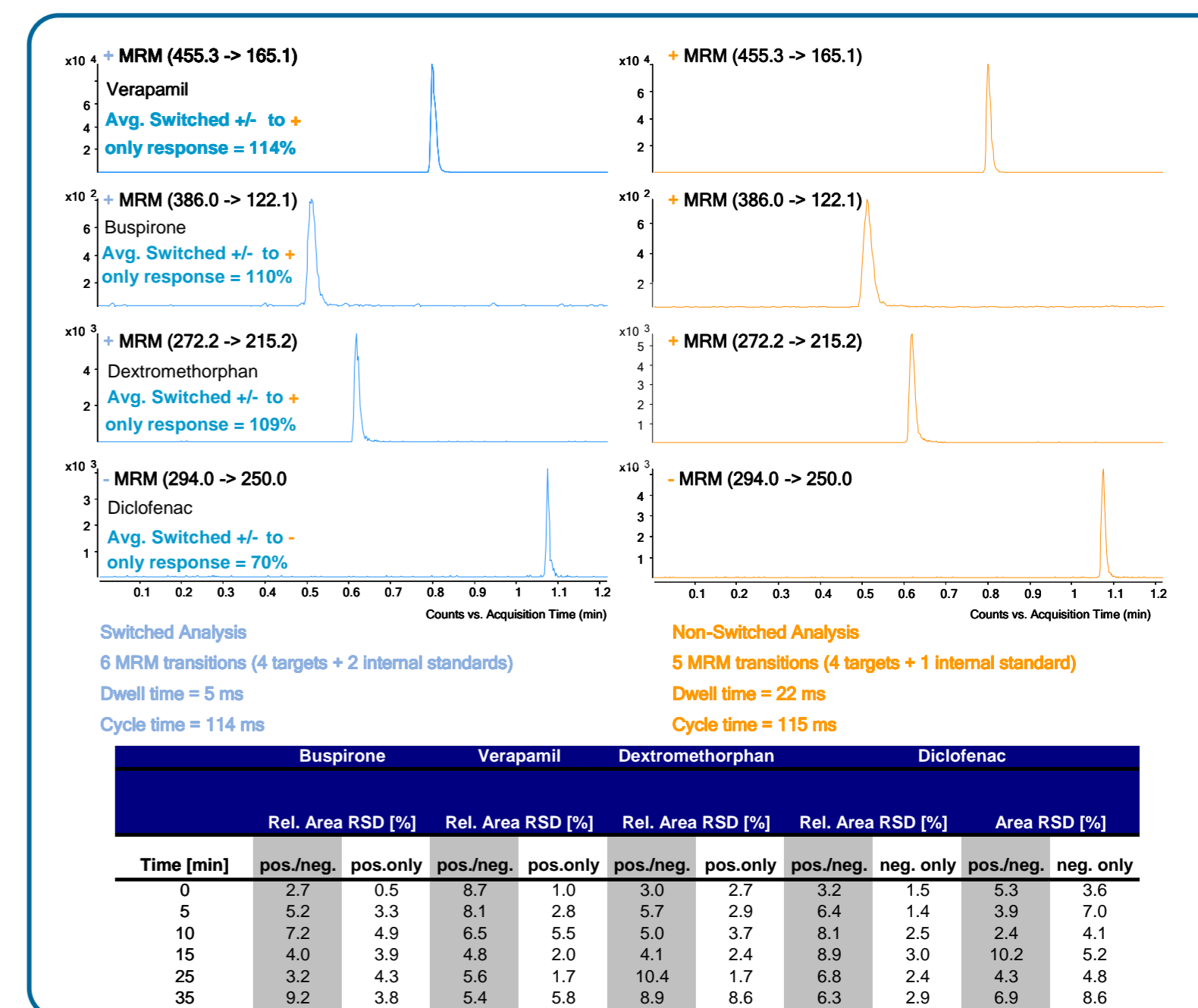


Results and Discussion

Switched versus non-switched analysis – Area response and precision for pooled incubates

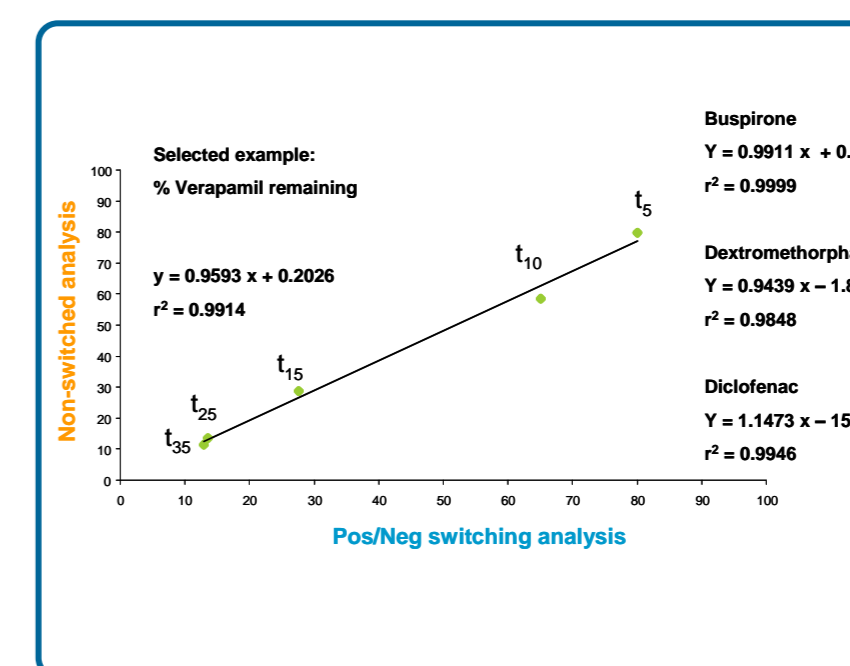
Average area responses obtained using polarity switching or non-switched analysis were similar. Area RSD and relative area RSD values were less than 10% using polarity switching and less than 9% using non-switched analysis.

Flow rate 1.0 mL/min, 870 bar at 25%B, 25°C



Switched vs. non-switched analysis – Metabolic stability

The results of the switched and non-switched analysis were comparable with $r^2 > 0.9848$



Conclusions

The high data acquisition rate provided by the Agilent 6460 triple quadrupole mass spectrometer (polarity switching time 30 ms, dwell time 5 ms) makes it perfectly compatible with the Agilent 1290 Infinity UHPLC System for excellent quantitation of LC peaks with sub-second peakwidths at half height.