# Metabolic Stability Study Using Cassette Analysis and Polarity Switching in an Ultra High Performance Liquid Chromatography (UHPLC)-Triple Quadrupole LC/MS System

## Application Note


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### Abstract

This application note demonstrates the performance of the Agilent 1290 Infinity LC System coupled to an Agilent 6460A Triple Quadrupole LC/MS for the evaluation of metabolic stability using the cassette analysis approach combined with fast polarity switching.

We present a UHPLC/MS/MS method that demonstrates:

- High throughput analysis (retention times less than 0.8 min at a mobile phase flow rate of 1.5 mL/min and binary pump pressure 1,100 bar)
- High speed MS/MS analysis with cycle time as low as 114 ms to monitor 6 transitions and switch polarity (polarity switching time 30 ms).
- Good precision with area RSD [%] or relative area RSD [%] less than 10%, enabled by collecting enough data points (at least 9 points) across the chromatographic peaks (as narrow as 0.37 sec at half height).
- Good accuracy over the expected concentration range using either external or internal standard calibration (within 90.7 – 107.1%)
- Comparable results (% parent drug remaining) when using polarity switching or non-switched analysis
- Excellent chromatographic resolution to avoid overestimation in quantitation due to in-source CID conversion of a metabolite into its parent drug


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## Introduction

*In vitro* ADME assays like metabolic stability play an important role in the early understanding of *in vivo* pharmacokinetic characteristics and help to discard non-drug-like compounds that would fail later stages of development. Thus, fast screening methods are needed to screen the large collection of new chemical entities that need characterization in the early stages of drug discovery. Typically, metabolic stability assessment is performed using the cassette approach and fast LC/MS/MS methods. In the cassette approach, a cocktail of substrates ionizing either in positive or in negative mode is created post-incubation in order to reduce the number of samples to analyze. Fast LC methods, which use high mobile phase flow rates and short sub-2 µm columns, allow reducing the analysis time and therefore increasing sample throughput [1, 2]. Detection is usually performed using a triple quadrupole mass spectrometer working in MRM scan mode.

Cassettes are typically designed so that substrates ionizing either in positive or negative mode are pooled together for LC/MS/MS analysis. The reason for that is because MS/MS analysis may show challenges with quantitative data quality if the MS detector is not able to acquire data fast enough in order to collect at least 9 to 10 data points across the extremely narrow peaks generated using fast chromatography. Nevertheless, pooling of substrates ionizing in positive and negative mode would present a pronounced flexibility advantage.

This work describes the advantage of combining not only cassette analysis and fast LC but also using fast polarity switching MS/MS analysis to increase throughput and flexibility in a metabolic stability assay, while maintaining good precision and accuracy. Moreover, the Agilent 1290 Infinity LC system flow range of up to 2 mL/min at 1,200 bar allowed excellent chromatographic resolution between critical peaks.

## Experimental

### *Reagents and supplies*

Buspirone hydrochloride and verapamil hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO USA). Dextromethorphan, diclofenac, and dextrorphan-$d_3$ (ISTD) were purchased from Cerilliant (Round Rock, TX USA). Diclofenac-$d_4$ (phenyl-$d_4$) was purchased from CDN Isotopes (Quebec, Canada). NADPH regeneration system, solution A (beta-nicotinamide adenine dinucleotide phosphate sodium salt (NADP), glucose-6-phosphate and $MgCl_2$ in water) and solution B (glucose-6-phosphate dehydrogenase in 5 mM sodium citrate) as well as 0.5 M potassium phosphate buffer, pH 7.4 were purchased from BD Biosciences (Woburn, MA USA). Male Sprague Dawley rat liver S9 homogenate was purchased from In vitro Technologies (Baltimore, MD). All other reagents and organic solvents were of analytical grade and from VWR (Darmstadt, Germany).

### *Incubation of substrates with rat liver S9 fractions*

The incubation mixtures for phase I metabolism consisted of an amount of S9 preparation equivalent to 0.3 mg protein, 1 µM substrate (buspirone, verapamil, dextromethorphan or diclofenac from a 100 µM stock solution in 70% water / 30% acetonitrile), 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 3.3 mM magnesium chloride and 0.4 U/mL glucose-6-phosphate dehydrogenase in 0.1 M phosphate buffer (pH 7.4) made up to a total volume of 300 µL. Incubation was carried out at 37°C. A cocktail of the 4 substrates was created post-incubation to reduce analysis time. A 25 µL aliquot was taken at 0, 5, 10, 15, 25 and 35 min from each incubate and the reaction was stopped by adding 300 µL acetonitrile containing the internal standards (dextrorphan-$d_3$ and diclofenac-$d_4$) 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 v/v containing 0.1% formic acid for UHPLC/MS/MS analysis.

### *Equipment*

- Agilent 1290 Infinity LC System comprising 1290 Infinity Binary Pump with integrated degasser, 1290 High Performance Autosampler with Thermostat and 1290 Infinity Thermostatted Column Compartment
- Agilent 6460A Triple Quadrupole LC/MS System with Agilent Jet Stream technology
- Agilent MassHunter Workstation software for instrument control, data acquisition and data processing
- Agilent MassHunter Optimizer Software
- Agilent Rapid Resolution High Definition (RRHD) Zorbax SB-C18, 2.1 x 50 mm, 1.8 µm column

### *Agilent 1290 Infinity Methods*

The mobile phase consisted of:

Solvent A: water with 0.1% formic acid

Solvent B: acetonitrile with 0.1% formic acid

Injection volume: 1 µL

Needle wash: 20 sec in flushport with methanol/water 50/50 v/v (0.1% formic acid)

**UHPLC 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.

**UHPLC 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 6460A triple quadrupole conditions*

Scan type: MRM (MassHunter optimizer software allows to quickly determine the optimal fragmentor voltage, MRM transitions and collision energy for the selected transitions. Optimized parameters are shown in **Tables 1, 2 and 3**)

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: 4,000 V (+/-)

Polarity switching time: 30 ms

| Compound | ISTD | Precursor Ion | MS1 Res | Product Ion | MS2 Res | Dwell time [ms] | Frag V [V] | CE [V] | Polarity |
|---|---|---|---|---|---|---|---|---|---|
| Verapamil | | 455.3 | Unit | 165.1 | Unit | 5 | 185 | 26 | Positive |
| Buspirone | | 386 | Unit | 122.1 | Unit | 5 | 185 | 28 | Positive |
| Diclofenac -$d_4$ | X | 298 | Unit | 254 | Unit | 5 | 80 | 4 | Negative |
| Diclofenac | | 294 | Unit | 250 | Unit | 5 | 95 | 5 | Negative |
| Dextromethorphan | | 272.2 | Unit | 215.2 | Unit | 5 | 190 | 22 | Positive |
| Dextrorphan -$d_3$ | X | 261.2 | Unit | 157.1 | Unit | 5 | 125 | 40 | Positive |

*Table 1: MRM acquisition with positive/negative switching.*

| Compound | ISTD | Precursor Ion | MS1 Res | Product Ion | MS2 Res | Dwell time [ms] | Frag V [V] | CE [V] | Polarity |
|---|---|---|---|---|---|---|---|---|---|
| Verapamil | | 455.3 | Unit | 165.1 | Unit | 22 | 185 | 26 | Positive |
| Buspirone | | 386 | Unit | 122.1 | Unit | 22 | 185 | 28 | Positive |
| Diclofenac | | 296 | Unit | 250 | Unit | 22 | 80 | 8 | Positive |
| Dextromethorphan | | 272.2 | Unit | 215.2 | Unit | 22 | 190 | 22 | Positive |
| Dextrorphan -$d_3$ | X | 261.2 | Unit | 157.1 | Unit | 22 | 125 | 40 | Positive |

*Table 2: MRM acquisition in positive polarity.*

| Compound | ISTD | Precursor Ion | MS1 Res | Product Ion | MS2 Res | Dwell time [ms] | Frag V [V] | CE [V] | Polarity |
|---|---|---|---|---|---|---|---|---|---|
| Arbitrary transition 1 | | 455.3 | Unit | 165.1 | Unit | 22 | 185 | 26 | Negative |
| Arbitrary transition 2 | | 386 | Unit | 122.1 | Unit | 22 | 185 | 28 | Negative |
| Diclofenac | | 294 | Unit | 250 | Unit | 22 | 95 | 5 | Negative |
| Arbitrary transition 3 | | 272.2 | Unit | 215.2 | Unit | 22 | 190 | 22 | Negative |
| Diclofenac -$d_4$ | X | 298 | Unit | 254 | Unit | 22 | 80 | 4 | Negative |

*Table 3: MRM acquisition in negative polarity.*

## 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 1,100 bar enabled a run time of only 1.5 min and generated peak widths less than a second at half height (typically 0.4 to 1.0 sec). 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 [%] = area target / area internal standard < 10). **Fig. 1** shows in comparison the MRM chromatograms, data quality and analysis time achieved using flow rates of 1.5 and 1.0 mL/min.

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 monitor 6 transitions and switch polarity. This is enabled by 30 ms polarity switching time and a total cycle time of only 114 ms as shown in **Fig. 2** for the substrate diclofenac. The graphic shows that even for peaks exhibiting a width of 0.37 sec at half height, the mass spectrometer was able to collect a total of 9 points, ensuring good area and relative area precision of 4.8 and 7.9%, respectively.

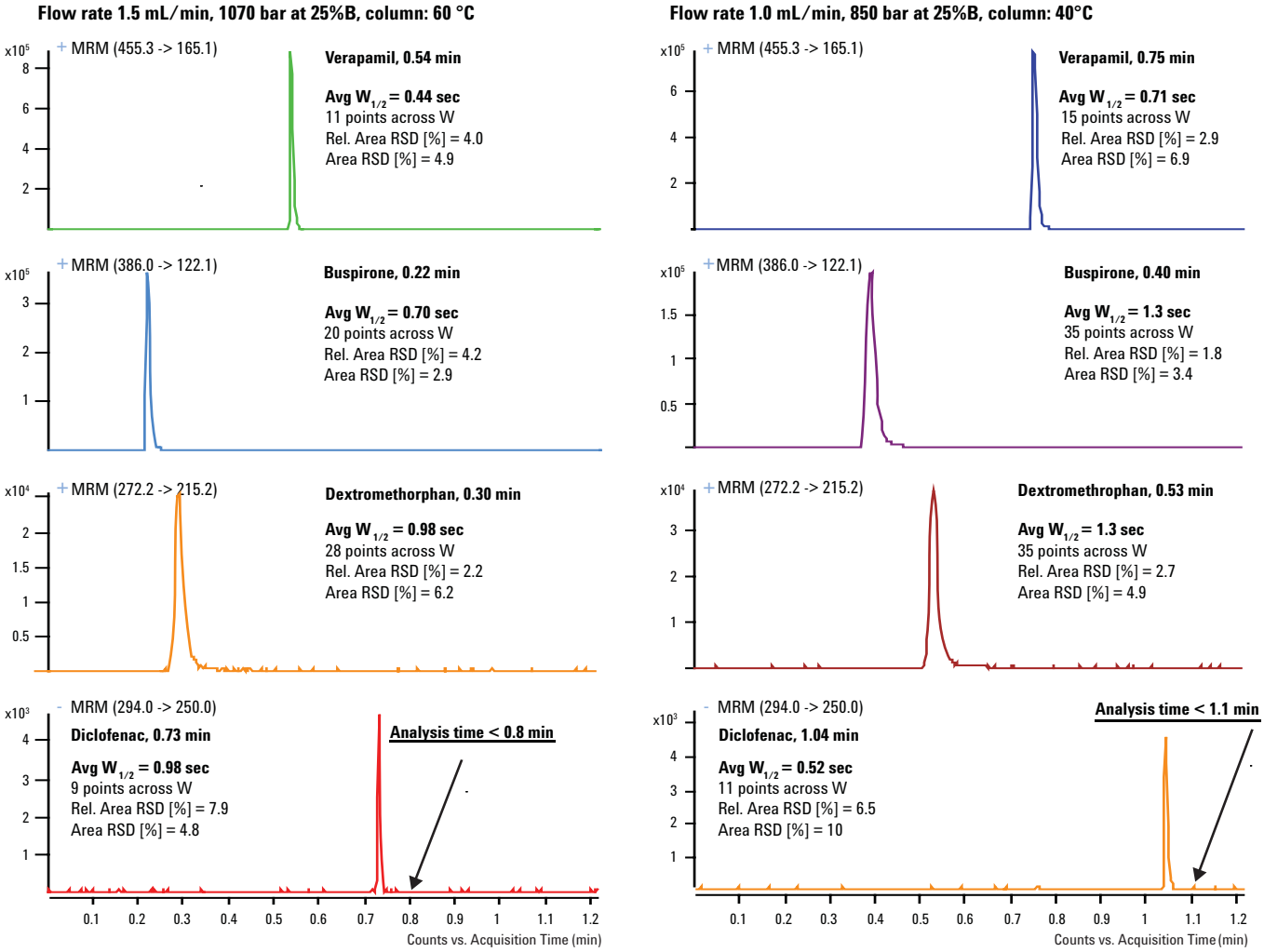



*Fig. 1: MRM chromatograms, data quality and analysis time achieved using flow rates of 1.5 and 1.0 mL/min.*

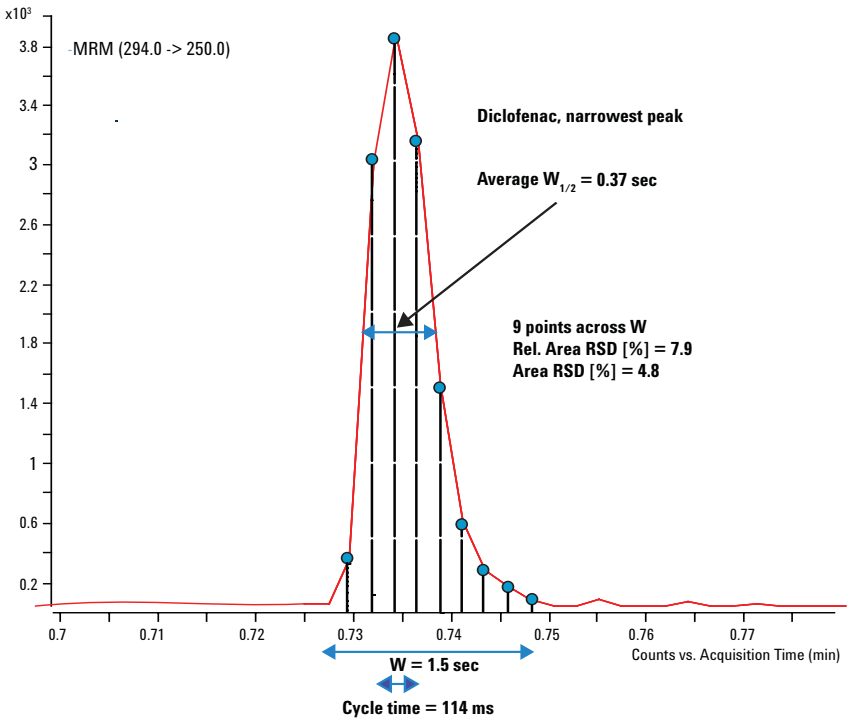



*Fig. 2: MRM chromatogram of diclofenac obtained using a flow rate of 1.5 mL/min. The graphic shows the peak width at half height (0.37 sec) and the number of data points collected across the peak (9 data points).*

### *Evaluation of linearity, precision and accuracy using fast polarity switching*

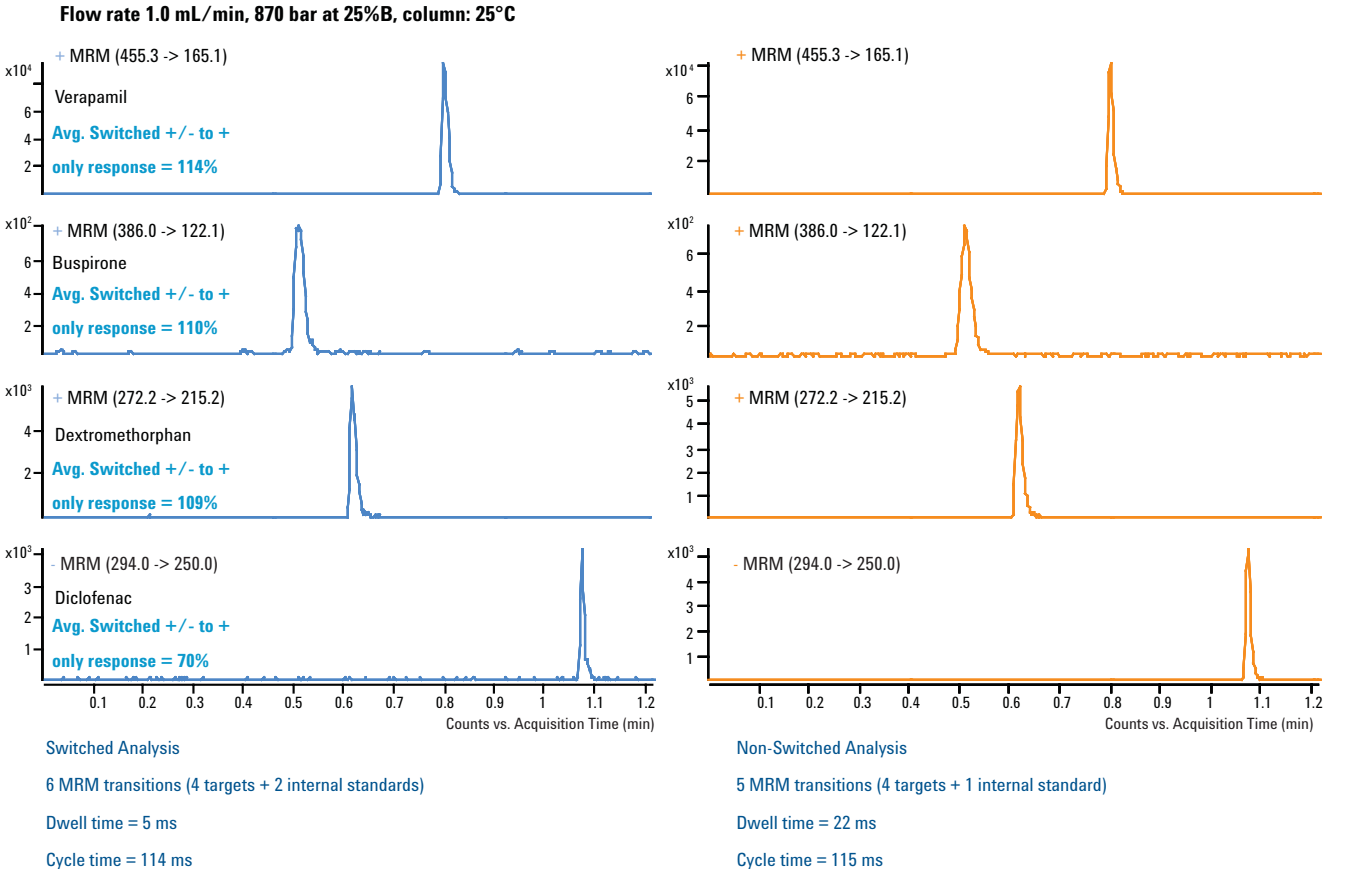
Linearity, precision and accuracy were tested using standards at a flow rate of 1.0 mL/min and column temperature 25°C (UHPLC method 1). The % of remaining parent was simulated over the expected range. The results obtained from the linearity test using either external or internal calibration demonstrate that the cassette analysis with polarity switching approach can provide linear results over the desired range. Moreover, good precision and accuracy values were achieved at all levels as shown in **Table 4**. When using internal standard calibration, a conflict between $^{35}Cl_2$-Diclofenac-$d_4$ (*m/z* 298) and $^{37}Cl_2$-Diclofenac (*m/z* 298) was observed. The response of the transition 298 → 254 arising from $^{37}Cl_2$-Diclofenac contributes to the response of the internal standard $^{35}Cl_2$-Diclofenac-$d_4$. This led to a quadratic curve due to artificially "increasing" internal standard. To solve this, either external calibration or a unique transition to diclofenac-$d_4$ like 298 → 217 (CE =17) can be used.

| | Buspirone | | Verapamil | | Dextromethorphan | | Diclofenac | |
|---|---|---|---|---|---|---|---|---|
| | external calibration | isotope dilution | external calibration | isotope dilution | external calibration | isotope dilution | external calibration | isotope dilution |
| Type | Linear | Linear | Linear | Linear | Linear | Linear | Linear | Quadratic |
| $r^2$ | 0.9989 | 0.9994 | 0.9965 | 0.9989 | 0.9978 | 0.9980 | 0.9990 | 0.9991 |
| Accuracy range [%] | 97.4 – 102.0 | 94.4 – 107.1 | 90.7 – 105.1 | 96.2 – 103.2 | 97.6 – 102.4 | 95.5 – 106.1 | 96.2 – 103.1 | 99,9 – 100.4 |
| Area RSD [%] | 2.4 – 6.1 | | 2.3 – 6.9 | | 3.7 – 5.1 | | 1.7 – 8.0 | |
| Rel. area RSD range [%] | n.a | 1.7 – 4.9 | n.a. | 1.4 – 6.0 | n.a. | 2.6 – 5.9 | n.a. | 0.9 – 4.1 |

*Table 4: Calibration curve type, Correlation coefficient ($r^2$), accuracy range [%], area RSD [%] and relative area RSD range [%] obtained using cassette analysis and fast polarity switching. References: n.a.: not applicable*

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

Average area responses obtained using switched analysis (fast polarity switching) or non-switched analysis (positive only or negative only analysis) were similar. Area RSD and relative area RSD values were less than 10.4% using polarity switching and less than 8.6% using non-switched analysis. **Fig. 3** shows in comparison the MRM chromatograms, average area response and relative area RSD [%] obtained for the pooled incubates analyzed using a flow rate of 1.0 mL/min and column temperature 25°C (UHPLC method 1).




| | Buspirone | | Verapamil | | Dextromethorphan | | Diclofenac | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Rel. Area RSD [%] | | Rel. Area RSD [%] | | Rel. Area RSD [%] | | Rel. Area RSD [%] | | Area RSD [%] | |
| Time [min] | pos./neg. | pos. only | pos./neg. | pos. only | pos./neg. | pos. only | pos./neg. | neg. only | pos./neg. | neg. only |
| 0 | 2.7 | 0.5 | 8.7 | 1.0 | 3.0 | 2.7 | 3.2 | 1.5 | 5.3 | 3.6 |
| 5 | 5.2 | 3.3 | 8.1 | 2.8 | 5.7 | 2.9 | 6.4 | 1.4 | 3.9 | 7.0 |
| 10 | 7.2 | 4.9 | 6.5 | 5.5 | 5.0 | 3.7 | 8.1 | 2.5 | 2.4 | 4.1 |
| 15 | 4.0 | 3.9 | 4.8 | 2.0 | 4.1 | 2.4 | 8.9 | 3.0 | 10.2 | 5.2 |
| 25 | 3.2 | 4.3 | 5.6 | 1.7 | 10.4 | 1.7 | 6.8 | 2.4 | 4.3 | 4.8 |
| 35 | 9.2 | 3.8 | 5.4 | 5.8 | 8.9 | 8.6 | 6.3 | 2.9 | 6.9 | 8.6 |

*Fig. 3: MRM chromatograms showing average area response obtained using fast polarity switching (on the left) in comparison to non-switched analysis (on the right). The table at the bottom shows the precision obtained using fast polarity switching or non-switched analysis for all incubation times.*

### *Switched versus non-switched analysis – Metabolic stability (% parent drug remaining)*

The % parent drug remaining was determined by comparing the average relative area of the parent compound measured in the 5-, 10-, 15-, 25- and 35-min samples to that in the 0-min sample and was calculated as follows:

% parent remaining = (avg. rel. area at $t_x$ / avg. rel. area at $t_0$) x 100

**Fig. 4** shows the correlation of results obtained for verapamil using fast polarity switching and non-switched analysis. The results of the switched and non-switched analysis were comparable for all substrates with $r^2 > 0.9848$.

| % Parent remaining (Switched vs. non-switched analysis | Buspirone | Verapamil | Dextromethorphan | Diclofenac |
|---|---|---|---|---|
| Linear Equation | y = 0.9911 x + 0.2671 | y = 0.9593 x + 0.2026 | y = 0.9430 x - 1.88 | y = 1.1473 x - 15.16 |
| $r^2$ | 0.9999 | 0.9914 | 0.9848 | 0.9946 |

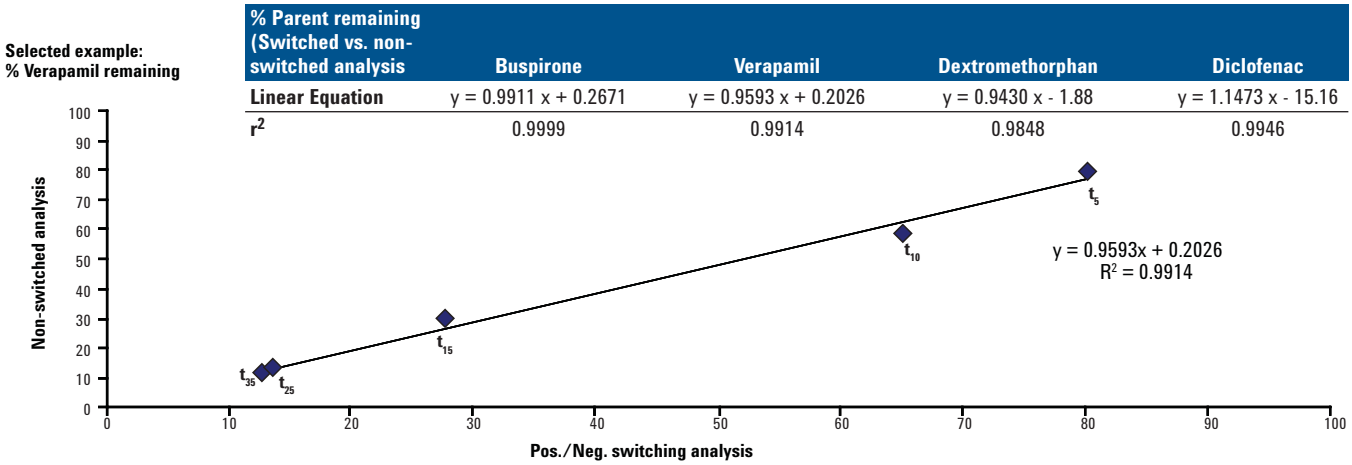



*Fig. 4: Comparison of % parent drug remaining from fast polarity switching with non-switched analysis using UHPLC method 1 (flow rate = 1.0 mL/min, column temperature = 25°C).*

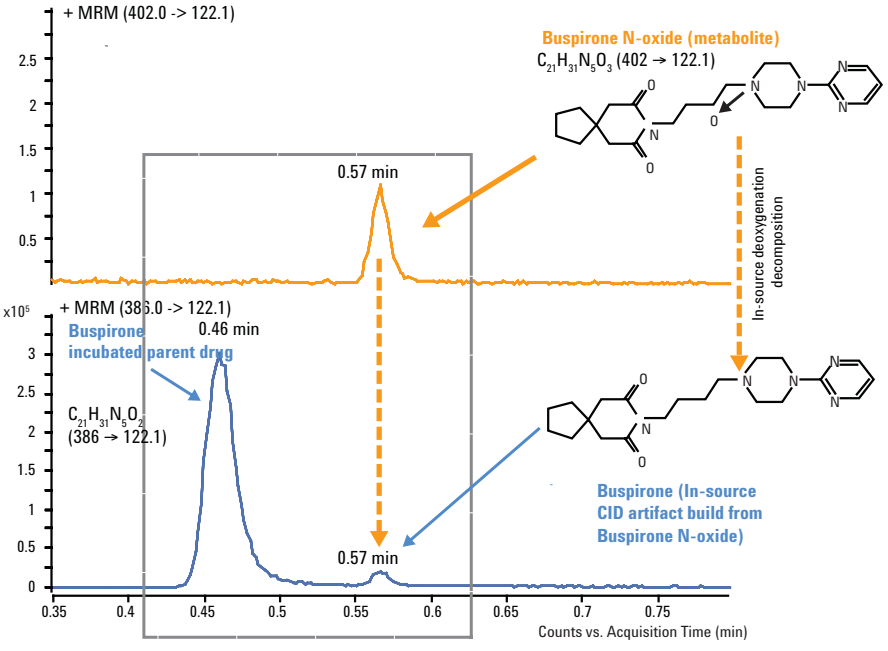



*Fig. 5: Excellent chromatographic resolution achieved between the incubated parent drug buspirone and one of its metabolites (buspirone N-oxide) which builds buspirone by in-source deoxygenation.*

### *Chromatographic resolution – Advantage for cassette analysis*

Excellent resolution between critical peaks was demonstrated. During the determination of metabolic stability, it is important to achieve high chromatographic resolution to ensure that the metabolites produced by the hepatic S9 fraction are resolved from the parent compounds. Coelution may result in overestimation of the amount of parent compound present if the metabolite is thermally unstable and can convert into the parent compound by in-source CID; **Fig. 5** shows as example the conversion of buspirone N-oxide to buspirone (parent drug) by deoxygenation. Excellent resolution between buspirone and buspirone N-oxide allowed to distinguish between the parent drug buspirone (originally present in the incubation sample, retention time = 0.46 min) and the artifactually build buspirone (build by in-source CID from buspirone N-oxide, retention time = 0.56 min).

## Conclusions

The combination of cassette analysis and fast polarity switching in the Agilent UHPLC/MS/MS system provided:

- Flexibility for cassette design: Compounds ionizing in positive and negative mode can be pooled together.
- High throughput analysis (retention times less than 0.8 min at a mobile phase flow rate of 1.5 mL/min and binary pump pressure of 1,100 bar)
- High speed MS/MS analysis with cycle time as low as 114 ms to monitor 6 transitions and switch polarity (polarity switching time 30 ms).
- Good precision with area RSD [%] and relative area RSD [%] less than 10%, enabled by collecting enough data points (at least 9 points) across the chromatographic peaks (as narrow as 0.37 sec at half height).
- Good accuracy over the expected concentration range using either external or internal standard calibration (accuracy within 90.7 - 107.1%)
- Comparable results (% parent drug remaining) when using polarity switching or non-switched analysis
- Excellent chromatographic resolution to avoid overestimation in quantitation due to in-source CID conversion of a metabolite into its parent drug

## References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