QUANTITATIVE BIOANALYSIS BY LC/MS/MS: A COMPARISON OF UPLC™ AND HPLC

Waters

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INTRODUCTION

The use of LC/MS/MS in drug discovery has increased in popularity due to instrumentation advances that have lead to mass spectrometers becoming smaller in size, less expensive, and easier to operate. These advances have resulted in the increased application of tandem mass spectrometers as universal detectors for LC analysis.

The most common mode of ionization MS/MS systems is electrospray (ESI). Although the vast majority of compounds can be detected by the positive ESI, many compounds prefer negative ESI. In order to simultaneously detect the widest range of compounds in a mixture, it is desirable to switch polarity during an analysis.

The recent advancement of Ultra Performance LC™ (UPLC™), which results in much faster analysis times and narrower peak widths (less than 2 seconds for high throughput analyses), places a significant challenge upon the data acquisition requirements of the MS systems. A tandem quadrupole system must be able to collect data rapidly enough to obtain sufficient points across the compound peaks for reproducible quantification.

This paper demonstrates the performance of the Waters Quattro Micro tandem quadrupole mass spectrometer with a Waters ACQUITY UPLC™ System for quantitative analysis. A drug mixture was prepared to demonstrate the improvements in quantitation attainable by combining UPLC with a tandem quadrupole mass spectrometer using electrospray ionization in both the ESI+ and polarity switching modes. The results obtained from the UPLC analysis were compared with that of HPLC analysis carried out under similar conditions.

LC/MS/MS CONDITIONS

LC Conditions

Instrument: ACQUITY UPLC™ System (UPLC™)
Waters Alliance® HT System (HPLC)
Column: ACQUITY UPLC™ BEH C18 Column, 2.1 x 50 mm, 1.7µm (UPLC)
XTerra® MS C18 Column, 2.1 x 50 mm, 3.5 µm (HPLC)
Flow Rate: 0.6 ml/minute (UPLC and HPLC)
Mobile Phase: 10 mM NH4OAc
A: 10% ACN, pH 5.0
B: 80/20 ACN/MeOH
Injection Vol.: 5 µl

Gradient (UPLC):

Time (min)	%A	Curve	Flow
0.0	90	1	0.60 ml/min
0.4	10	6	0.60 ml/min
0.5	0	1	0.60 ml/min
2.0	90	1	0.60 ml/min

Gradient (HPLC):

Time min)	%A	Curve	Flow
0.0	80	1	0.60 ml/min
1.5	0	6	0.60 ml/min
3.5	80	1	0.60 ml/min

LC/MS/MS CONDITIONS

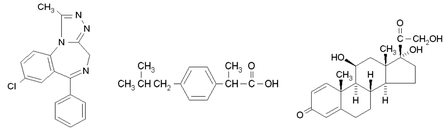
MS Conditions

Instrument: Quattro Micro™ API MS
Capillary Voltage: 0.5 kV
Source Temp: 130°C
Desolvation Temp: 350°C
Desolvation Gas: 800 L/hr.
Cone Gas Flow: 50 L/hr.

	ESI+ Only	**Polarity Switching**
Interscan Delay:	10ms (UPLC), 20ms (HPLC)	100ms (UPLC and HPLC)
Interchan. Delay:	10ms (UPLC), 20ms (HPLC)	20ms (UPLC and HPLC)
Dwell Time:	35ms (UPLC), 50ms (HPLC)	10ms (UPLC), 50ms (HPLC, ESI+)

EXPERIMENTAL

The results included in this paper were obtained using neat standards prepared fresh from 1mg/ml methanol stock solutions obtained from Cerilliant®. Calibration standards were prepared by appropriate dilution of the stock solutions in 90% water/10% acetonitrile to give concentrations ranging from 0.05 ng/ml to 1000 ng/ml.



Alprazolam **Ibuprofen** **Prednisolone**

Optimization of MS conditions and MRM transitions was carried out by infusing 10ng/ml of standard solutions at 5ul/min into mobile phase at 0.6ml/min and the results are shown in the following table.

Summary of Optimization Results for Standards

Compound	ESI+	ESI-	Ionization Mode	MRM Transition
Alprazolam	+++	-	ESI+	309.00 > 280.84
Prednisolone	+++	+	ESI+	360.95 > 343.07
Ibuprofen	-	+++	ESI-	205.02 > 160.87

Improved Sensitivity

The improvement in chromatographic separation using UPLC is demonstrated in Figure 1. A reduced analysis time was achieved with narrower peak widths and improved signal response. The average peak height increased by 3.7-fold and signal to noise increased by nearly 5-fold relative to the results obtained for HPLC. Figure 2 demonstrates the linearity obtained with the UPLC™/Quattro Micro™ system. A correlation coefficient greater than 0.994 over 4 orders of magnitude for Alprazolam was obtained.

SINGLE MODE IONIZATION: ESI+

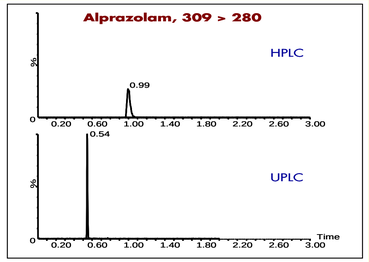


Figure 1. LC-MS/MS MRM chromatograms of Alprazolam: comparison of HPLC vs UPLC™.

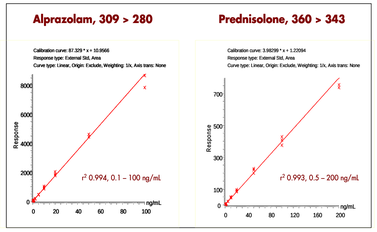


Figure 2. UPLC™/MS/MS Calibration curves for Alprazolam and Prednisolone. Both demonstrated good linearity and dynamic range.

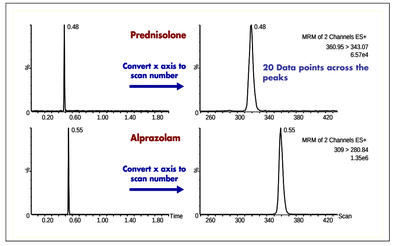


Figure 3. UPLC™/MS/MS MRM Chromatograms for Prednisolone and Alprazolam, with x-axis scaled as time (left) and as scan number (right). The peak width for each analyte was 1.8 second, the scan number obtained across each peak was 20.

POLARITY SWITCHING ON-THE-FLY: ESI+/ESI-

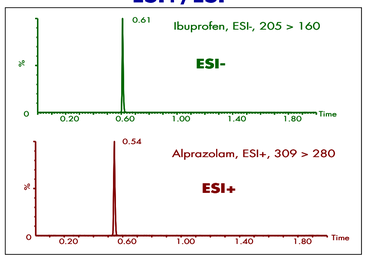


Figure 4. UPLC™/MS/MS MRM Chromatograms for Ibuprofen (ESI-) and Alprazolam (ESI+) obtained during a single injection with polarity switching on-the-fly.

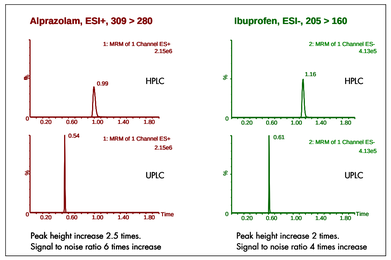


Figure 5. LC/MS/MS MRM chromatograms of Alprazolam and Ibuprofen: comparison of HPLC vs UPLC™.

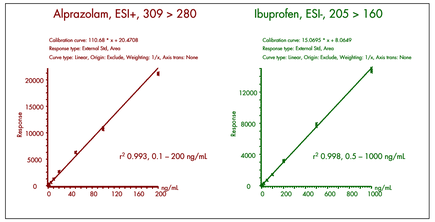


Figure 6. UPLC™/MS/MS Calibration curves for Alprazolam (ESI+) and Ibuprofen (ESI-) run with polarity switching. Both demonstrated good linearity and dynamic range.

Accurate Quantification

It is well accepted that for reproducible, reliable quantification the LC peak should be defined by no less than 10-12 data points. UPLC™ poses a challenge to any MS system due to the extremely narrow peak widths obtained. However, as demonstrated in Figure 3, acceptable LC-MS/MS quantification results were obtained for test compounds with the ACQUITY UPLC™/Quattro Micro™ system; approximately 20 data points (transitions) were collected across each peak, more than sufficient to achieve accurate, reproducible quantification of the sample.

Fast Polarity Switching On The Fly

Polarity switching requires a specific period of time to allow the electronics to stabilize after the switching. As a result, the detection cycle time for the mass spectrometer will be increased. Therefore, to perform polarity switching on-the-fly involves a balance between sensitivity and the number of data points that can be obtained across the LC peak. The delay time used for the polarity switching for the Quattro Micro™ is 100ms. Figure 4 demonstrates the MRM chromatograms obtained from an ACQUITY UPLC™/Quattro Micro™ system with polarity switching. The peak width obtained was 1.8 seconds, and the scan number collected across each peak was 10.

Figure 5 displays the comparative MRM chromatograms obtained with polarity switching on-the-fly. The alprazolam was analyzed by ESI+ (left) and the ibuprofen was analyzed by ESI- (right). The top chromatograms are from the HPLC analysis and the bottom chromatograms are from the UPLC™ analysis. As demonstrated here, the UPLC™ offered reduced LC run time with narrower peaks, plus increased signals. As a result, compared with the HPLC, the UPLC™ peak areas increased more than 2 fold, and signal to noise ratio increased more than 4 fold.

Figure 6 shows the calibration curves for the alprazolam obtained by ESI+ and ibuprofen obtained by ESI- by running the analysis with polarity switching on-the-fly. Both calibration curves demonstrated excellent linearity with correlation coefficients greater than 0.993 over more than 4 orders of magnitude.

MS dwell time was optimized for both UPLC™ and HPLC to give approximately 20 data points across peaks. It is well accepted that for Quadrupole (not T-Wave™) instruments the sensitivity is reduced as the dwell time is shortened. Therefore, as we have only 10ms dwell for UPLC™ and 30ms for HPLC, we are underestimating the actual increase in sensitivity for UPLC™. But in this experiment we are aiming to maximize the response for both modes and even under these unfavorable conditions UPLC™ is significantly more sensitive.

CONCLUSIONS

- The Waters Quattro Micro™ MS was able to provide accurate and precise quantitation when coupled with the Waters ACQUITY UPLC™.
- Significant improvements in sensitivity and throughput were achieved.
- Sufficient data points across the peaks were easily obtained for accurate peak integration and processing.
- The Waters Quattro Micro™ MS provides the speed and sensitivity required for polarity switching whether combined with the ACQUITY UPLC™ or traditional HPLC systems.

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