HT QUANTIFICATION FOR A DRUG MIXTURE IN RAT PLASMA: A COMPARISON OF UPLC™/MS/MS AND HPLC/MS/MS

Waters

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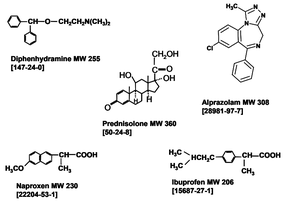
INTRODUCTION

- One major application of the tandem LC/MS analysis is quantification. An optimum quantification protocol requires a fast LC separation with sufficient MS analysis in order to obtain good linearity and low limit of detection.
- Major challenges in achieving an ultra-sensitive quantification protocol include matrix interference, limitations of LC/MS system such as column efficiency, system volume, system back pressure etc., as well as the data collection speed and the stability of the MS.
- Improvement in LC/MS/MS quantification relies on either to improve LC separation (column efficiency, peak capacity, system volume etc) via increasing the separation efficiency, improving column chemistry as well as reducing column dimension and/or particle size, or to improve the data acquisition rates in the mass spectrometer.
- This work demonstrates the advantages in quantification analysis by systematically improving both LC and MS.
- In LC, an analytical method was developed for a 5 drug mixture with the Ultra Performance LC™ (UPLC™). To put things in perspective, similar experiment protocol was also performed on the traditional High Performance LC (HPLC), and the comparative advantages of the UPLC™ is demonstrated.
- In MS, analytes eluting from LC were detected by Waters Micromass® Quattro Premier™ triple quadrupole mass spectrometer. The innovated T-Wave collision cell in the Quattro Premier allows an analytical dwell time as well as the inter-channel delay to be as low as 5 ms. In addition, the inter-scan delay is reduced down to 20 ms.
- The combination of the UPLC™ with the Quattro Premier enables the analytical quantification to be performed at higher speed with increased sensitivity, negligible carryover and solid robustness.

Overview

- LC-MS/MS for 5 drug mixture
  - Comparison of UPLC/MS/MS vs. HPLC/MS/MS
- Simultaneous Polarity switching
  - Analysis by both ESI+ and ESI- in a single injection
- Quantification performed in crashed Rat Plasma
  - Complete quantification limits defined for both UPLC/MS/MS and HPLC/MS/MS
- Complete method evaluation for UPLC/MS/MS
  - System robustness and carryover in crashed rat plasma

Model Analyte



Experimental Conditions

UPLC Conditions

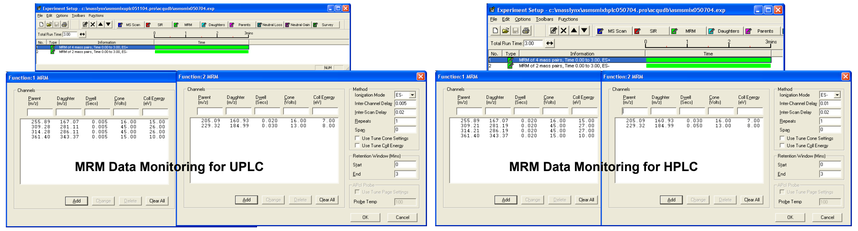
- Waters ACQUITY Ultra Performance LC ™ System (UPLC™)
- Waters ACQUITY Ultra Performance LC™ Column:
  - 2.1 x 50 mms, 1.7 µm, 40ºC
  - The Bridged Ethane-Silicon C_{18} particles ($BEHC_{18}$)
- Injection Volume: 5 µL
- Flow Rate: 0.6 ml/min
  - Back Pressure at about 11,000 psi
  - No splitting for MS detection
- Mobile Phase
  - 10 mM NH4OAc at pH 5.0 in 20% MeOH (A)
  - 10 mM NH4OAc at pH 5.0 in MeOH/Acetonitrile at 80/20 (B)
- Gradient:
  - 45%B to 50% in 0.4 minute
  - Hold at 95% B for another 0.1 minute
  - Total run time was 2 minute

HPLC Conditions

- Waters Alliance HT solvent managing system
- Waters Xterra Column
  - 2.1 x 50 mm, 3.5 µL, 40ºC
  - Reverse phase C_{18} particles
- Injection Volume: 5 µL
- Flow Rate: 0.6 ml/min
  - Back Pressure at about 3000 psi
  - No splitting for MS detection
- Mobile Phase
  - 10 mM NH4OAc at pH 5.0 in 20% MeOH (A)
  - 10 mM NH4OAc at pH 5.0 in MeOH/Acetonitrile at 80/20 (B)
- Gradient:
  - 20% B hold for 0.2 minute then to 80% B in 1.8 minute
  - Hold at 95% B for another 0.2 minute
  - Total run time was 5 minute

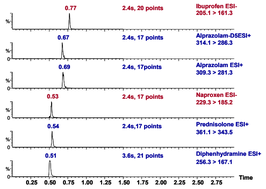
MS Conditions

- Waters Micromass Quattro Premier triple quadrupole MS
- Ionization Mode: ESI+ and ESI-
- Capillary Voltage: 0.5 kv
- Source Temperature: 130ºC
- Desolvation Temperature: 400ºC
- Desolvation Gas Flow: 800 L/Hr
- Cone Gas Flow: 50 L/Hr
- Collision Gas Flow: 0.23 L/Hr (Collision Cell Pressure at 3.2e-3)

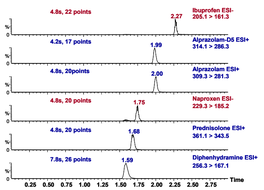


QUANTIFICATION RESULTS

UPLC/MS/MS
UPLC MRM Chromatograms



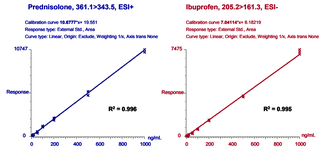
HPLC/MS/MS
HPLC MRM Chromatograms



Simultaneous Polarity Switching

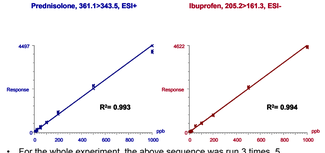
- For UPLC/MS/MS, the total cycle time was 110 ms
  - ESI+, dwell time was 5 ms each for all ions
  - ESI-, dwell time was 20 ms for ibuprofen, and 30 ms for naproxen
  - Inter-channel delay was 5 ms
  - Inter-scan delay was 20 ms
- Both LC/MS/MS detections were performed with simultaneous polarity switching. As a result, analytical results of both ESI+ and ESI- were obtained with a single injection
- For HPLC/MS/MS, the total cycle time was 220 ms
  - ESI+, dwell time was 30 ms for ibuprofen and 50 ms for naproxen
  - Inter-channel delay was 10 ms
  - Inter-scan delay was 20 ms
- The sole difference in the MS/MS detection for the UPLC vs. the HPLC is the cycle time
- The UPLC peaks are about half the width of the HPLC peaks.

UPLC Calibration Curves
3 Injections per Concentration



- Standards were spiked into crashed rat plasma
  - Rat plasma was crashed with acetonitrile at 2/1 ratio (2 AcN/1 rat plasma)
  - After vortex, centrifuged at 12000 rpm for 15 minutes in 4ºC
  - Supernatant was diluted with water in 1:1 ratio
  - Standard was spiked into this diluted supernatant and injected directly into LC/MS/MS system
- The injection sequence was the following
  - Rat plasma blank (3 injections)
  - Standard rat plasma solution low to high concentration (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100, 200, 500, 1000 ng/mL)
  - Rat plasma blank (3 injections)
  - Low QC (6 injections)
  - Rat plasma blank (3 injections)
  - Mid QC (6 injections)
  - Rat plasma (3 injections)
  - High QC (6 injections)
  - Rat plasma

HPLC Calibration Curves
3 Injections per Concentration



- For the whole experiment, the above sequence was run 3 times. 5 calibration curves were obtained in the end
  - 3 calibration curves from ESI+ analysis
  - 2 calibration curves from ESI- analysis
- This experiment protocol resulted in a 13 point calibration
  - Each concentration point consists of 3 injections
  - Alprazolam was based on ***internal calibration*** (Alprazolam-D5 as the internal standard)
  - All other 4 compounds were based on ***external calibration***
- Three levels of QC results were obtained
  - Low QC at 2 ng/mL, med QC at 20 ng/mL, high QC at 200 ng/mL
  - Each QC level consists of 18 injections
- The calibration curves for both UPLC/MS/MS and HPLC/MS/MS were constructed in the similar manner
- Showing here are examples of calibration curves, one for ESI+, one for ESI-
- The complete quantification results are listed in tables below

UPLC Quantification Limits

		Alprazolam	Diphenhydramine	Prednisolone	Ibuprofen	Naproxen
	Linear Range	*0.1 – 1000* ng/mL	0.1 – 200 ng/mL	2 – 1000 ng/mL	0.1 – 1000 ng/mL	1 – 1000 ng/mL
	R2	*0.998*	0.998	0.996	0.995	0.999
	Slope	*0.109*	5249.22	10.68	7.04	2.21
	LOD	*0.0136*	0.025	1.642	0.0847	0.541
	LOQ	*0.0828*	0.150	9.852	0.508	3.246
Low QC 2 ng/mL	RSD%	*3.42*	8.67	------	7.92	7.02
	Bias%	*-0.5*	-4.71	------	0.315	2.735
Med QC 20 ng/mL	RSD%	*1.39*	5.63	10.21	6.30	6.95
	Bias%	*2.55*	-8.0	18.8	-1.05	4.10
High QC 200 ng/mL	RSD%	*1.20*	5.44	5.92	3.74	2.99
	Bias%	*-1.09*	-1.55	10.1	8.75	0.245

HPLC Quantification Limits

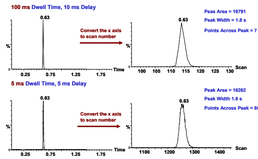
		Alprazolam	Diphenhydramine	Prednisolone	Ibuprofen	Naproxen
	Linear Range	*0.2 – 1000* ng/mL	0.1 – 200 ng/mL	5 – 1000 ng/mL	1 – 1000 ng/mL	5 – 1000 ng/mL
	R2	*0.998*	0.996	0.993	0.994	0.991
	Slope	*0.0090*	4903.31	4.48	4.62	0.939
	LOD	*0.0806*	0.116	2.102	0.852	4.22
	LOQ	*0.483*	0.696	12.61	5.112	25.32
Low QC 2 ng/mL	RSD%	*4.77*	9.10	------	------	------
	Bias%	*-9.5*	-21.5	------	------	------
Med QC 20 ng/mL	RSD%	*2.54*	6.01	21.13	12.11	15.36
	Bias%	*-5.35*	-15.9	-8.30	-14.7	-3.15
High QC 200 ng/mL	RSD%	*2.66*	3.23	5.98	6.23	9.95
	Bias%	*-2.68*	1.61	0.35	-7.70	-14.1

THE COMPARATIVE ADVANTAGES OF UPLC™/MS/MS

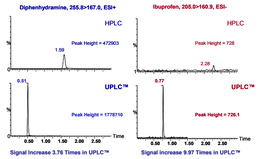
Higher Speed

- Faster separation was obtained by the ACQUITY UPLC™
  - High flow rate since the UPLC™ system can withstand up to 15,000 psi backpressure
  - Smaller system volume (150 µL)
  - LC column with smaller particle size (1.7 µm) plus innovated chemistry (the bridged ethane hybrid particle – $BEHC_{18}$)
- As a result, UPLC™ peaks are narrow (usually about half the peak width of the HPLC separation under similar protocols), typically about 2 second wide.
- The narrow UPLC™ peak demands shorter cycle time in MS/MS detection
  - Proper integration for quantification purpose requires minimum of 15 data point across the LC peak.
- The cycle time of an MS detection is the summation of the dwell times for all ions of interest plus the inter-channel delays as well as the inter-scan delays
  - The shorter the cycle time, the more challenging to the MS in terms of stability, and sensitivity
- Demonstrating here on the left is the effect of the MS dwell time. The UPLC™ peak was 1.8s wide, if 100ms dwell time were used for the detection, only 7 data points were obtained across the peak, insufficient for quantification. However, if 5 ms dwell time was used, 60 data points were collected across the peak, far more sufficient for quantification.
- The signal intensities were unaffected by the change of the dwell time.

The Effect of MS Dwell Time
Alprazolam in Rat Plasma, 10 ng/mL, 5 µl injection0



Increased Sensitivity
10 ng/mL Standard Injection

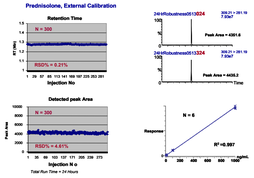


- Showing on the right is the comparison of the detected signals from UPLC/MS and HPLC/MS/MS
  - With the same column loading (10 ng/mL at 5 µL), a significant signal increase was obtained in UPLC™/MS/MS vs. the HPLC/MS/MS
- The chromatograms with blue labels were the LC results for diphenhydramine
  - ESI+ detection
  - Compared with HPLC, the signal increased 3.76 times was observed in the UPLC™
- The chromatograms with red labels were the LC results for Ibuprofen
  - ESI- detection
  - Compared with HPLC, the signal increased of 9.97 times was observed in the UPLC™
- Possible contributing factors for the signal increase in UPLC™ include
  - Narrower LC peak width due to the reduced system volume of the ACQUITY UPLC as well as the smaller particle size in the column (1.7 um for UPLC vs. 3.5 um for HPLC)
  - Shorter retention resulting in less sample dilution and dispersion during the chromatographic elution. Samples entering MS detector were more concentrated in UPLC compared with HPLC
- The scale of signal increase was compound dependent

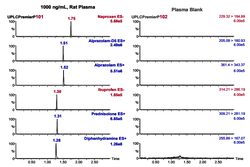
System Robustness

- The system robustness experiment was performed over the period of 24 hours with the following injection sequence
  - Rat plasma blank
  - Standard rat plasma solutions with concentrations low to high (1.0, 10.0, 100, and 1000 ng/mL)
  - Rat plasma blank
  - QC samples at 200 ng/mL was injected after the above sequence was run 3 times. 300 injections were made
  - Rat plasma blank
  - Standard rat plasma solution with concentrations low to high (1.0, 10.0, 100, and 1000 ng/mL)
  - Rat plasma blank
  - This sequence was also run 3 times after the 300 QC injection was completed
- The calibration curve shown on the figure consists 6 points at each concentration level
  - 3 points before the 300 QC injections
  - 3 points after the 300 QC injections
  - The signals were indistinguishable before and after the QC injection indicating the stability of the system
- The two chromatograms shown in the figure were the very first QC injection on the top and the very last QC injection on bottom
  - Consistent peak shape and peak area indicating the stability of the system

200 ng/mL Standard Spiked in Rat Plasma



Negligible Carryover



- The sequence of injections were as the following
  - Plasma blank
  - Standard rat plasma solution injections with concentration from low to high (the concentration sequence was 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100, 200, 500, and 1000 ng/mL)
  - Plasma blank
- Showing here in the figure are from two consecutive injections
  - The standard solution at the highest concentration (1000 ng/mL)
  - The rat plasma blank injected immediately after the injection of the highest standard solution
- No detectable carryover from rat plasma was observed for all analytes of interest

CONCLUSIONS

- Quantification methods for a 5 drug mixture were developed for HPLC/MS/MS and UPLC™/MS/MS
  - For HPLC/MS/MS, 3.5 orders of magnitude of linear range can be obtained
  - For UPLC™/MS/MS, 4 orders of magnitude of linear range can be obtained
- The improvement of speed and sensitivity of UPLC/MS/MS vs. HPLC/MS/MS was demonstrated
  - For the same column loading, the signal increased up to 10 times in UPLC vs. in HPLC
  - The scale of sensitivity improvement was compound dependent
- The system robustness for UPLC/MS/MS was demonstrated
  - For 300 injections of QC sample in cashed rat plasma over 24 hours, the retention time RSD% was 0.21%, the detected peak area RSD% was 4.61% for a externally calibrated compound
- The carryover for UPLC/MS/MS was studied
  - For the 5 compounds that were tested in this study, the carryover was negligible

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