# Separation and Quantitation of Multicomponent Solutions Using a Novel UHPLC-MSMS System

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## Overview

**Purpose:** To demonstrate the fast chromatographic separation of a panel of standard drug compounds in the presence of a biological sample matrix while maintaining sensitivity and robustness throughout sample analysis.

**Methods:** Mass spectrometry (MS) analysis was performed in the selected reaction monitoring (SRM) mode with generic mass spectrometer source conditions. Chromatographic separation was by ultra-high performance liquid chromatography (UHPLC).

**Results:** Sample analysis demonstrated chromatographic separation for a panel of 18 analytes utilizing a 2.5 min gradient. A working range of 100 pg/mL to 100 ng/mL for the analyte set was demonstrated with a linear signal response across the concentration range, and with a coefficient of variance of less than fifteen percent for all replicate injections.

## Introduction

Quantitative assays in bioanalytical laboratories typically require chromatographic methods that allow for fast gradient conditions and provide separation of target analytes as well as from isobaric interferences while at the same time delivering high levels of sensitivity. The implementation of a UHPLC system capable of operation at pressure limits of up to 1500 bar allows for the use of increased linear flow rates when coupled with a 1.5 micron particle size column producing an increase in peak capacity and improved chromatographic resolution. The combination of enhanced chromatographic performance coupled with a high performance mass spectrometer operating at fast analytical scan speeds allows for improved assay sensitivity and significantly reduced method run time.

## Methods

### Sample Preparation

Crashed plasma stock solutions were prepared using an Acetonitrile (ACN) crash at a ratio of 3:1, ACN to plasma. The resulting solution was centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed and added to an equivalent volume of water to make the final crashed plasma stock solution. Stock solutions of were prepare for a mix of 18 standard drug compounds at 1 mg/mL. Stock solutions were diluted in the crashed plasma solution to produce a concentration curve with a range of 100 pg/mL to 100 ng/mL. Isotopically labeled Paroxetine-D6 was added at each concentration level as an internal standard to produce a final internal standard concentration of 1 ng/mL. All reagents were obtained from Cerilliant Corporation, Round Rock, Texas, at 1 mg/mL in methanol.

### Liquid Chromatography

Chromatographic separation was achieved using a Thermo Scientific™ Vanquish™ UHPLC System. Samples were injected (5 µL) onto a 2.1 x 100 mm, 1.5 um Thermo Scientific™ Accucore™ Vanquish™ C18 UHPLC column. Column temperature control was maintained at 60°C for the duration and in “still air mode” for the length of the analytical run. Gradient elution was accomplished using water + 0.1% formic acid (FA) (A) and acetonitrile + 0.1% formic acid (FA) (B), with a generic 2.5 minute gradient at a flow rate of 850 uL/min (Table 1). Total run time including column equilibration was approximately 4 minutes. (Figure 1)

### Mass Spectrometry

Sample analysis was performed using a Thermo Scientific™ TSQ Quantiva™ triple-stage quadrupole mass spectrometer in positive ionization mode, with heated electrospray ionization, and acquired in selected reaction monitoring (SRM) mode. All compounds were optimized by direct infusion to determine optimal transition and collision energy settings. Generic source conditions suitable for a 850 µL/minute LC column flow rate were applied for all data collection (Table 2).

### Data Analysis

All data was acquired and processed using Xcalibur software. All chromatographic integration was accomplished using automated processing settings.

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**TABLE 1. LC gradient method used for sample analysis.**

| Time (min) | Flow rate (uL/min) | %A | %B |
|---|---|---|---|
| 0 | 850 | 97 | 3 |
| 0.05 | 850 | 97 | 3 |
| 2.2 | 850 | 30 | 70 |
| 2.3 | 850 | 5 | 95 |
| 3.0 | 850 | 5 | 95 |
| 3.1 | 850 | 97 | 3 |
| 4.0 | 850 | 97 | 3 |

**TABLE 2. Mass spectrometer settings used for sample analysis.**

| HESI Source Settings | Value |
|---|---|
| Spray Voltage (V) | 3000 |
| Vaporizer temperature ($^{O}$C) | 500 |
| Capillary Temperature ($^{O}$C) | 350 |
| Sheath Gas Pressure (Arb) | 55 |
| Aux Gas Pressure (Arb) | 25 |
| Ion Sweep Gas Pressure (Arb) | 1 |

**FIGURE 1. Graphical view of LC gradient and flow conditions. LC flow rate 850 uL/min.**

# Results

### Chromatographic Resutls

Chromatographic peak separation is an important aspect of any robust quantitative analysis. The capability to resolve multiple components in a complex mixture from one and other as well as from matrix interferences can dramatically improve assay performance and robustness. A mix of 18 standard compounds was injected onto a 2.1 x 100 mm, 1.5 µm Thermo Scientific™ Accucore™ Vanquish™ C18 UHPLC column and separation was achieved using a generic 2.5 minute gradient. Fast separation for all 18 compounds was achieved with all but two of the component sets in the mixture achieving baseline chromatographic separation. The generic gradient combined with the 1.5 µm particle size C18 column provided adequate separation for all components with a retention times ranging from 0.69 minutes to 1.98 minutes. The chromatographic peak with for the component mixture ranged between 2 - 6 seconds at the base and excellent peak shaped was observed for the majority of the components. (Figure 2)

**FIGURE 2. Representative chromatogram for the compound mixture. Chromatographic retention times ranged from 0.69 minutes to 1.98 minutes.**

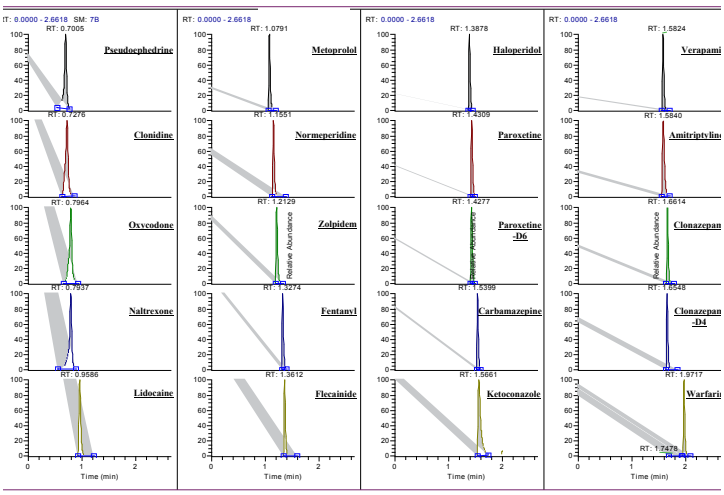
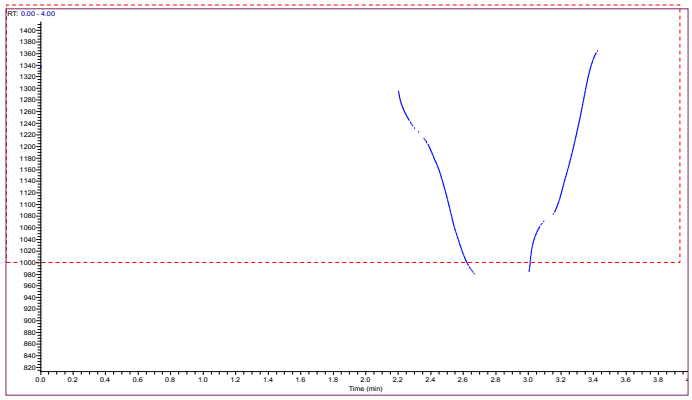
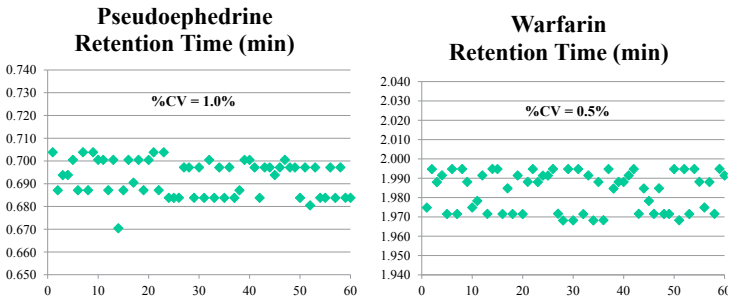



**FIGURE 3. Chromatographic system back pressure profile. The red dotted line highlights the portion LC gradient above a 1000 bar system back pressure.**




Chromatographic separation was enhanced with the use of 1.5 µm solid core particle column technology. The decreased particle size diameter plus the core enhanced technology provides short diffusion paths that improve separation efficiency and allow for reduced LC method cycle time. Additionally, the 1500 bar pressure capability of the chromatographic system coupled with the decreased particle size column enabled an extended range of flow rate operation that further enabled fast chromatographic separation and while providing higher overall sample throughput. Using a flow rate of 850 µL/min the system pressure at the start of the gradient was approximately 1300 bar with a maximum of 1410 bar. (Figure 3) More than 85% of the gradient produced a system back pressure of greater than 1000 bar. The system was run routinely under these conditions for extended periods and for multiple runs with no system errors or adverse effects on chromatographic performance.

**FIGURE 4. Retention time reproducibility for Pseudoephedrine (Left) and Warfarin (Right). The retention time %CV for both compounds was observed to be 1% or less with a standard deviation of approximately 500 milliseconds across the analytical run.**




Retention time reproducibility is an important characteristic for both targeted and untargeted screening assays, providing an increased level of confidence and overall data quality. The improved solvent delivery and injection capabilities of the chromatographic system combined to provide consistently reproducibly peak retention times over the injection series and from run to run. The retention time %CV for the 18 component mixture was 5% or less for all components with the majority of components at 1% or less. (Table 3). Both early and late eluting components were observed to have a %CV of 1% or less and a standard deviation of approximately 500 milliseconds over the injection series. (Figure 4)

### Quantitative Results

Rugged quantitative analysis must be precise, robust, and reproducible across the working range of the assay. Good reproducibility at both high and low concentration range are essential to assay quality. To evaluate the performance and reproducibility of the generic LC-MS method, calibration curves were analyzed with replicates of n=6. Linearity and reproducibility were calculated across the working range of the curve and the percent difference was calculated and reported at both the upper and lower end of the concentration range. The percent difference or difference of the linear fit at both the 100 pg/mL and 100 ng/mL concentration levels was observed to be less than 15% for all components and less than 5% for the majority of the components. The %RSD for each of the replicate sets at both the 100 pg/mL and 100ng/mL concentration levels was observed to be less than 15% for all components and less than 5% for the majority of the components.

**TABLE 3. Percent difference in the linear fit of the calibration curve and %RSD of replicate injections at both 100 pg/mL and 100 ng/mL (Left). Average retention time and retention time %CV for the injection series (Right)**

| Compound | % Diff 100 pg/mL | %RSD 100 pg/mL | % Diff 100 ng/mL | %RSD 100 ng/mL | Average Retention Time (min) | Retention Time (min) %CV |
|---|---|---|---|---|---|---|
| Pseudoephedrine | 4.3% | 3.8% | 8.0% | 4.0% | 0.6930 | 1.0% |
| Clonidine | 8.4% | 6.1% | 1.6% | 1.3% | 0.7202 | 1.0% |
| Oxycodone | 2.3% | 7.6% | 3.6% | 2.7% | 0.7828 | 1.7% |
| Naltrexone | 4.3% | 7.3% | 1.5% | 3.5% | 0.7838 | 1.8% |
| Lidocaine | 2.6% | 3.1% | 9.9% | 4.6% | 0.9282 | 4.2% |
| Metoprolol | 13.0% | 10.6% | 7.9% | 9.7% | 1.0322 | 5.2% |
| Normeperidine | 4.7% | 3.3% | 1.9% | 1.5% | 1.1184 | 3.8% |
| Zolpidem | 0.4% | 5.3% | 3.4% | 9.0% | 1.1909 | 2.8% |
| Fentanyl | 2.7% | 8.8% | 12.6% | 6.4% | 1.3515 | 1.7% |
| Flecainide | 4.4% | 4.5% | 6.4% | 7.3% | 1.4013 | 2.3% |
| Haloperidol | 2.1% | 6.9% | 10.8% | 4.9% | 1.4330 | 2.5% |
| Paroxetine | 2.5% | 2.4% | 5.6% | 2.2% | 1.4761 | 2.5% |
| Carbamazepine | 1.6% | 6.3% | 0.8% | 4.9% | 1.5652 | 1.1% |
| Ketoconazole | 2.3% | 12.8% | 1.3% | 1.5% | 1.5885 | 1.3% |
| Verapamil | 2.0% | 13.0% | 9.1% | 3.2% | 1.6001 | 1.1% |
| Amitriptyline | 2.9% | 6.0% | 13.0% | 2.9% | 1.6016 | 1.0% |
| Clonazepam | 1.2% | 3.5% | 3.4% | 1.7% | 1.6718 | 0.7% |
| Warfarin | 2.2% | 4.6% | 2.8% | 2.3% | 1.9835 | 0.5% |

**FIGURE 5. Representative calibration curves for the component mixture. Oxycodone (Top Left), Carbamazepine (Top Right), Warfarin (Bottom Left), Clonazepam (Bottom Right). Linear regression was performed using 1/x2 weighting.**

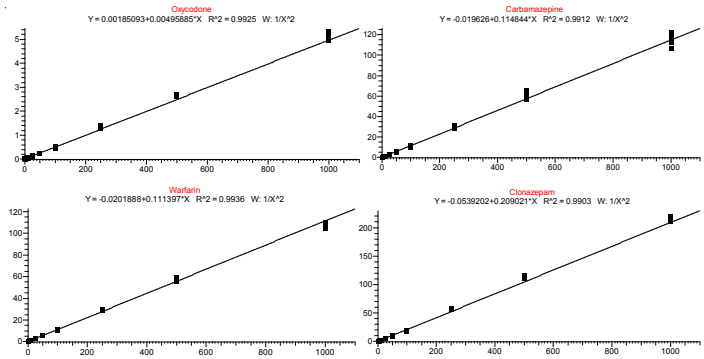
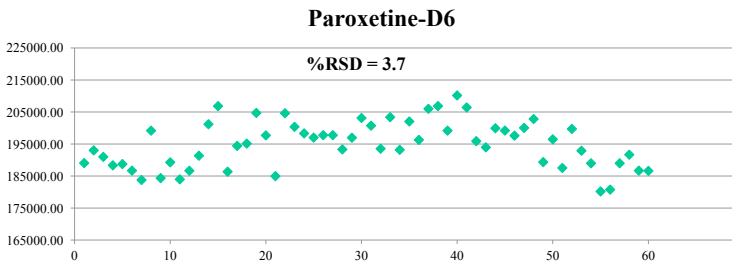



**FIGURE 6. Peak area reproducibility, 1ng/mL Paroxetine-D6 internal standards at across the injection sequence.**




A linear response was observed across the working range of the curve from 100 pg/mL 100 ng/mL for all 18 components in the sample mixture. Representative calibration curves for early, middle, and late eluting compounds demonstrate a linear regressions with R2 values of greater than 0.990. (Figure 5 ).

Injection reproducibility was evaluated using signal response from the Paroxetine–D6 internal standard. The peak area for the internal standard response was plotted for each injection in the run and the %CV determined to be 3.7%. (Figure 6)

## Conclusion

- Chromatographic separation for a panel of 18 analytes utilizing a 2.5 min generic gradient was demonstrated.
- Increased pressure capability and decrease column particle combine to increase separation efficiency and decrease overall analysis time.
- Improved chromatographic system technology and design provided highly reproducible retention times for all components.
- Rugged and reliable MS system provided reproducible and linear response across the working range of the assay.



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