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# High-quality Chromatography for Bioanalysis of Small Molecule Pharmaceuticals

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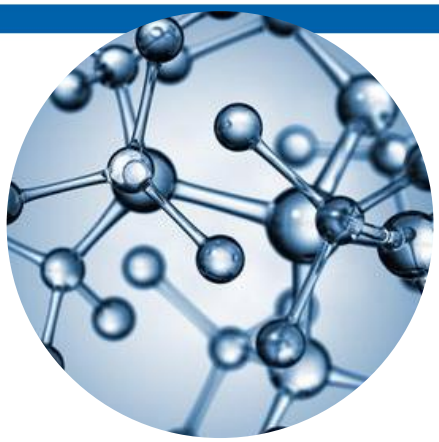

## Introduction

Robust LC-MS/MS methods are essential to support pharmaceutical drug development through all phases from discovery to clinical trials. The bioanalytical scientist is constantly challenged to achieve sufficient sensitivity and selectivity in method development, while maintaining robustness such that the final method can be used across large sample sets. At all concentration levels across the calibration range and from many different sources of matrix, the method must provide high reproducibility in order to produce consistent results.

An important aspect of a successful mass spectrometry (MS) experiment in achieving selectivity, reproducibility, and robustness is combining it with some form of high-quality, upfront sample separation. In most cases, this is achieved using liquid chromatography (LC). In this example, we used an ultra high efficiency core-shell Kinetex 1.7 µm C18 column to achieve the desired chromatographic separation.

In this technical note, key performance of the SCIEX® ExionLC™ 2.0 system was investigated, specifically linearity, precision, and carryover, for small molecule drug analysis in rat plasma. A SCIEX Triple Quad™ 5500+ system was used for detection.

## Sample Preparation

Calibration curves were prepared in precipitated rat plasma, and a bulk matrix sample was prepared using an Acetonitrile precipitation approach. Three parts of cold Acetonitrile were added to 1 part rat plasma. The solution was vortexed for 5 minutes, then centrifuged for 10 minutes at 10,000 x g. The supernatant was removed and added to an equivalent volume of water to make the final processed matrix solution. Standard solutions were prepared using a serial dilution approach using the prepared matrix solution. **Table 1** shows the standards that were prepared.

**Table 1.** Calibration Standard Concentrations.

| Standard | Concentration (ng/mL) |
|---|---|
| 1 | 0.007 |
| 2 | 0.021 |
| 3 | 0.067 |
| 4 | 0.211 |
| 5 | 0.662 |
| 6 | 2 |
| 7 | 6 |
| 8 | 20 |
| 9 | 64 |
| 10 | 202 |

## LC Conditions

**Column:** Kinetex™ 1.7 µm C18
**Dimensions:** 50 x 2.1 mm
**Part No.:** 00B-4475-AN
**Mobile Phase:** A: 0.01 % Formic Acid in Water
B: 0.01 % Formic Acid in Acetonitrile

**Gradient:**

| Time (min) | %B |
|---|---|
| 0 | 10 |
| 0.1 | 10 |
| 2.25 | 65 |
| 2.26 | 99 |
| 2.75 | 99 |
| 2.76 | 10 |
| 3.5 | 10 |

**Flow Rate:** 0.5 mL/min
**Injection Volume:** 5 µL
**Temperature:** 40 °C
**LC System:** SCIEX ExionLC 2.0
**Detection:** MS/MS
**Detector:** SCIEX Triple Quad 5500+

Note: The syringe speed was set to Normal and the speed factor to 1. The SCIEX ExionLC 2.0 system autosampler was used in the standard configuration consisting of a 250 µL syringe, 100 µL sample loop, 250 µL buffer tubing and 15 µL needle tubing. For all injections, the µL pick-up plus mode was selected in order to minimize the injection cycle time while optimizing sample consumption. An injection volume of 5 µL was used. The ExionLC 2.0 system autosampler was operated in advanced rinse mode using 2 mL 100 % Isopropanol with 0.1 % Formic Acid for wash solvent and a wash sequence that included 2 valve washes.

## MRM Transitions and Parameters

| Analyte | Q1 (m/z) | Q2 (m/z) | DP (V) | CE (V) |
|---|---|---|---|---|
| Carbamazepine 1 | 237 | 194 | 60 | 30 |
| Carbamazepine 2 | 237 | 179 | 60 | 48 |
| Fluoxetine 1 | 310 | 44 | 50 | 52 |
| Fluoxetine 2 | 310 | 148 | 50 | 12 |
| Sulfamethoxazole 1 | 254 | 156 | 60 | 22 |
| Sulfamethoxazole 2 | 254 | 92 | 60 | 36 |
| Trimethoprim 1 | 291 | 230 | 100 | 31 |
| Trimethoprim 2 | 291 | 261 | 100 | 34 |



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## Results and Discussion

**Figure 1** shows the retention time reproducibility for fluoxetine across 50 injections, highlighting the stability of the gradient generated by the ExionLC™ 2.0 system and the reproducibility of the Kinetex™ C18 column. Retention time precision of each of the analytes across a range of retention times for these injections is less than 1 % RSD. Retention time variability %RSD for Trimethoprim, Sulfamethoxazole, Carbamazepine, and Fluoxetine was 0.40, 0.10, 0.06, and 0.07 respectively, giving a maximum retention time difference of less than a second for all compounds.

An example chromatogram from std 8 (20 ng/mL) is shown in **Figure 2** in which very good separation of the compounds was achieved. Note the excellent peak shape (symmetry) and narrow peak widths (efficiency) provided by the core-shell Kinetex C18 column.

The LLOQ of each analyte in matrix was determined based on current bioanalytical method guidance: the lowest standard concentration with a precision of <20 %, accuracy between 80 and 120 %, and a response (peak area) that is at least 5 times the response compared to the matrix blank.

Listed in **Table 2** are the LLOQs achieved for each analyte and the calibration curve range tested for each. Example calibration cruves are shown in **Figure 3**. Linear regression with 1/x weighting was used. The linear coefficient of determination ($r^2$) was higher than 0.99 for all compounds. The accuracy and precision of the calibration standards are shown in **Table 3**.

The concentration range tested in this work covers a typical linear dynamic range requirement when analyzing these small molecule pharmaceuticals in plasma. **Figure 4** shows extracted ion chromatograms for Sulfamethoxazole from extracted plasma standards and blanks. The blank injection after standard 10 (200 ng/mL) shows no carryover peak detected showing that the simple and quick autosampler wash sequence used provided acceptable carryover performance for the assay requirements.

**Figure 1.** Retention Time Variability of the ExionLC 2.0 System for Fluoxetine for over 50 Injections.

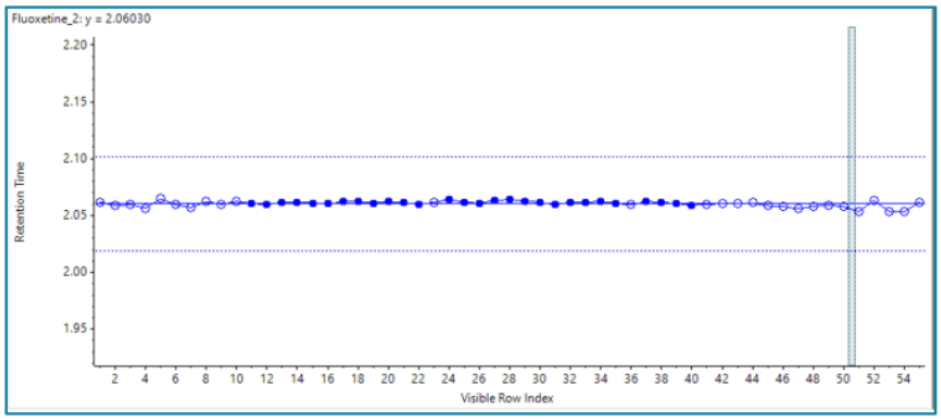


**Figure 2.** Extracted Ion Chromatogram of Select Compounds at a Concentration Level of 20 ng/mL.

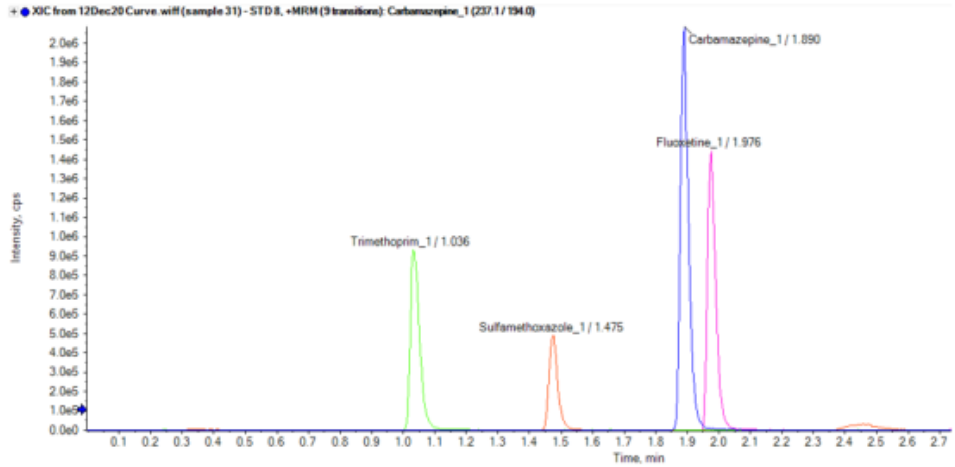




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**Table 2.** Calibration Range for Compounds in this Pharma Small Molecule Assay.

| Analyte | LLOQ (ng/mL) | Calibration Curve Range (ng/mL) | Retention Time (min) |
|---|---|---|---|
| Sulfamethoxazole | 0.067 | 0.067 – 200 | 1.48 |
| Fluoxetine | 0.067 | 0.067 – 200 | 1.98 |
| Trimethoprim | 0.067 | 0.067 – 64 | 1.04 |
| Carbamazepine | 0.021 | 0.021 - 67 | 1.90 |

**Figure 3.** Example Calibration Curves.

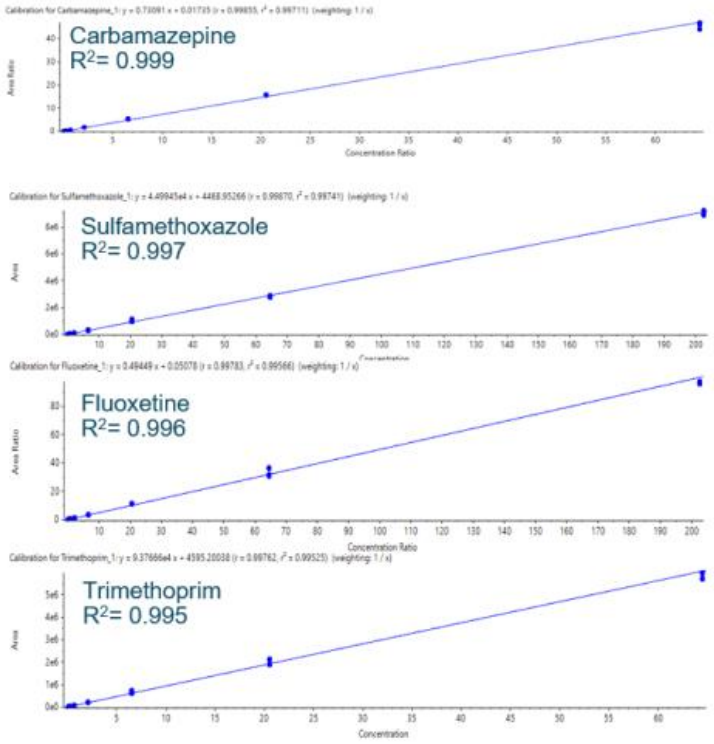


**Table 3.** Accuracy and Precision Statistics of Calibration Standards.

| | Carbamazepine | | Fluoxetine | | Sulfamethoxazole | | Trimethoprim | |
|---|---|---|---|---|---|---|---|---|
| Calibration Standard (ng/mL) | Accuracy (%) | CV (%) | Accuracy (%) | CV (%) | Accuracy (%) | CV (%) | Accuracy (%) | CV (%) |
| 0.021 | 80.00 | 0.40 | - | - | - | - | - | - |
| 0.067 | 86.33 | 1.16 | 82.67 | 4.33 | 95.97 | 3.40 | 98.52 | 11.93 |
| 0.211 | 99.75 | 6.11 | 84.72 | 3.69 | 90.53 | 1.02 | 98.22 | 10.41 |
| 0.662 | 110.37 | 1.23 | 105.33 | 5.53 | 110.09 | 2.36 | 114.07 | 3.77 |
| 2.08 | 111.0 | 2.32 | 107.71 | 3.05 | 120.89 | 0.31 | 115.94 | 1.39 |
| 6.54 | 111.12 | 1.10 | 110.37 | 9.73 | 111.83 | 11.70 | 109.74 | 9.38 |
| 20.53 | 104.81 | 0.69 | 111.88 | 1.79 | 110.22 | 9.78 | 104.17 | 7.69 |
| 64.50 | 96.89 | 3.19 | 103.35 | 8.20 | 98.38 | 1.95 | 96.52 | 3.00 |
| 202.64 | - | - | 96.15 | 1.06 | 98.88 | 2.04 | - | - |



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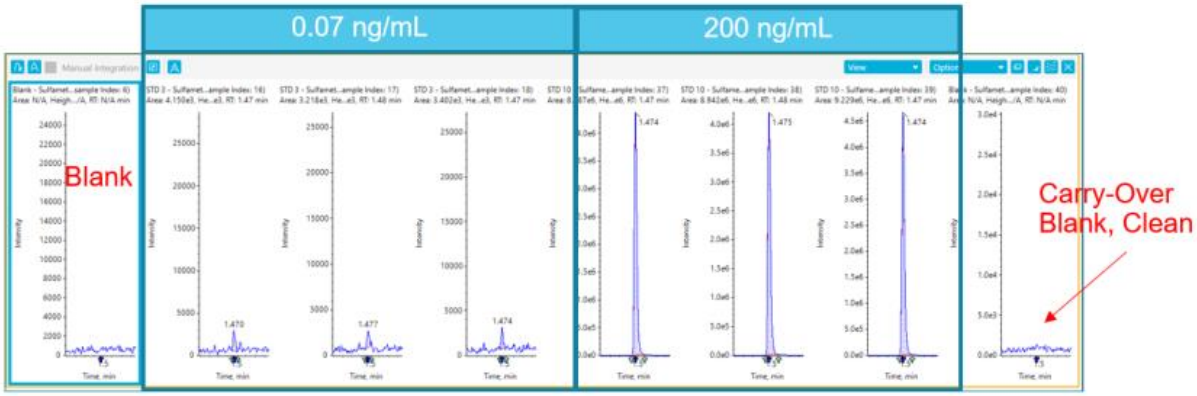
**Figure 4.** Extracted Ion Chromatograms for the LLOQ (3 Replicates), Highest Concentration Measured (3 Replicates), and Carry Over Blank using Sulfamethoxazole as an Example.




## Conclusions

The ExionLC™ 2.0 system, when coupled with a highly reproducible, highly efficient core-shell column (Kinetex™ 1.7 µm C18), has been shown to be a robust UHPLC system that is suitable for the bioanalysis of pharma small molecules in plasma matrix. Precise and stable solvent flow delivering less than 1 % RSD retention time variation. Accurate and precise quantification results with linear coefficient of determination performance ($r^2 > 0.99$) and precision <15% coefficient of variation for the concentration range tested, typical for this small molecule pharmaceutical assay.

## Kinetex™ Ordering Information

| 1.7 µm Minibore Columns (mm) | | | | | SecurityGuard™ ULTRA Cartridges‡ |
|---|---|---|---|---|---|
| | 30 x 2.1 | 50 x 2.1 | 100 x 2.1 | 150 x 2.1 | 3/pk |
| EVO C18 | — | 00B-4726-AN | 00D-4726-AN | 00F-4726-AN | AJ0-9298 |
| Biphenyl | 00A-4628-AN | 00B-4628-AN | 00D-4628-AN | 00F-4628-AN | AJ0-9209 |
| XB-C18 | 00A-4498-AN | 00B-4498-AN | 00D-4498-AN | 00F-4498-AN | AJ0-8782 |
| C18 | 00A-4475-AN | 00B-4475-AN | 00D-4475-AN | 00F-4475-AN | AJ0-8782 |
| C8 | 00A-4499-AN | 00B-4499-AN | 00D-4499-AN | 00F-4499-AN | AJ0-8784 |
| HILIC | 00A-4474-AN | 00B-4474-AN | 00D-4474-AN | — | AJ0-8786 |
| Phenyl-Hexyl | — | 00B-4500-AN | 00D-4500-AN | 00F-4500-AN | AJ0-8788 |
| F5 | — | 00B-4722-AN | 00D-4722-AN | 00F-4722-AN | AJ0-9322 |

for 2.1 mm ID

‡SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000



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