Ultrahigh pressure liquid chromatography for analysis of therapeutic drugs in whole blood

Repeatability performance of the ExionLC 2.0 system

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Tandem mass spectrometry (MS/MS) has become the analytical choice for the analysis of therapeutic drug compounds in clinical research samples. Many instances require monitoring these compounds in whole blood, which poses a challenge in managing matrix effects. Matrix effects can cause ionization suppression and/or enhancement, which can reduce the precision and accuracy of quantification. Many traditional methods overcome matrix effects by using time-consuming and expensive extraction methods.[1-4] More recently, simple and rapid methods[5] have been developed that can be used to analyze drug compounds in whole blood. These methods meet benchmarks for assay performance, while reducing cost and time investment.

To be successful, tandem mass spectrometry (MS/MS) experiments must be combined with a method that achieves high-quality upfront compound separation. This is typically achieved using liquid chromatography (LC) and the chromatographic performance is crucial to implement a rapid and robust method. In this technical note, the performance of a method for the analysis of therapeutic drugs in whole blood using the ExionLC 2.0 system for chromatographic separation, was investigated.

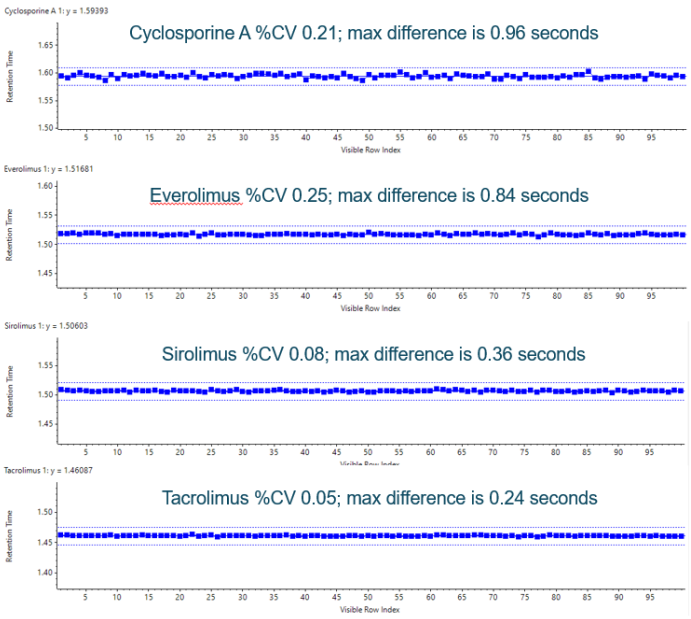

Figure 1. Retention time precision. Retention time variability of the ExionLC 2.0 system for the therapeutic drug compounds for 100 injections is shown. It exhibited good retention time stability well within a mean regression of ±1% deviation (dashed lines).

Key features of therapeutic drug quantification using the ExionLC 2.0 system

- Fast and robust assay for the detection of therapeutic drugs in whole blood
- Simplified sample preparation using protein precipitation
- Reproducible quantification using the ExionLC 2.0 system with the SCIEX Triple Quad 4500 system
  - High-pressure, dual, serial piston pump rated to 860 bar at flow rates of 0.001 to 2 mL/min for maximum flexibility
  - Precise and stable solvent flow delivering less than 0.5% RSD retention time variation (Figure 1)
- Excellent accuracy, precision and robustness achieved.
  - Accurate and precise quantification results with linear coefficient of determination performance (r^2) > 0.99 and

coefficient of variation <10% for all concentration levels studied

Methods

Material and solutions: Cyclosporine A, tacrolimus, ascomycin and sirolimus were purchased from MilliporeSigma. Everolimus was purchased from Fischer Scientific. Zinc sulfate heptahydrate was purchased from MilliporeSigma. LC-MS grade methanol was purchased from Avantar/VWR. Calibration was accomplished using a commercially available, multilevel, whole blood calibration kit.

Sample preparation: To perform the protein precipitation, 0.2 mL whole blood was placed in a 1.5 mL polypropylene microcentrifuge tube. Then, 400 µL of an 8:2, methanol with 2% zinc sulfate/water solution, spiked with internal standard, was added to the whole blood sample. This mixture was vortexed vigorously for 10-20 seconds and then centrifuged at 14,000 rpm for 10 minutes at room temperature. The supernatant was transferred to an autosampler vial and injected directly into the LC-MS/MS with a 20 µL injection volume.

Chromatography: LC separations were performed using the ExionLC 2.0 system. A Phenomenex Luna 5 µm Phenyl-Hexyl, 50 x 2.1 mm column (Phenomenex P/N 00B-4257-B0) with a Phenomenex Phenyl SecurityGuard ULTRA guard cartridge (Phenomenex P/N AJO-8774) was used. The SecurityGuard ULTRA guard cartridge holder (Phenomenex P/N AJO-9000) was required for pre-column plumbing of the guard cartridge. A simple gradient of water and methanol, both containing 2mM ammonium formate and 0.1 % formic acid, was used with a flow rate of 0.7 mL/min. The column oven temperature was set to 60°C. The analytical run, including equilibration, was 4.0 minutes. The low syringe speed was used with a speed factor of 0.5. The standard configuration of the ExionLC 2.0 system autosampler was used, which includes a 250 µL syringe, 100 µL sample loop, 250 µL buffer tubing and 15 µL needle tubing. The Microliter Pickup Plus mode was used for all injections to minimize the cycle time, while optimizing sample consumption. The ExionLC 2.0 system autosampler was operated in the advanced rinse mode using 2 mL of a 100% isopropanol with 0.1% formic acid wash solvent. The wash sequence included 1 valve wash.

Mass spectrometry conditions: Mass spectrometry was performed using a SCIEX Triple Quad 4500 system. The ionization source was operated using electrospray ionization (ESI) in positive mode. Two MRM transitions were monitored for each analyte and internal standard. The MRM conditions are detailed in Table 1.

Table 1. Optimized MS parameters for MRM transitions of the compounds used in this study.

Analyte	Q1	Q2
Cyclosporine A 1	*1219.9*	*1203.0*
Cyclosporine A 2	*1219.9*	*452.1*
Ascomycin 1	*809.6*	*756.7*
Ascomycin 2	*809.6*	*564.5*
Everolimus 1	*975.8*	*908.6*
Everolimus 2	*975.8*	*926.6*
Sirolimus 1	*931.6*	*864.6*
Sirolimus 2	*931.6*	*882.8*
Tacrolimus 1	*821.7*	*786.4*
Tacrolimus 2	*821.7*	*768.5*
d12-Cyclosporine A	*1232.0*	*1215.0*

Data acquisition was performed using Analyst software 1.7.1 with components for the ExionLC 2.0 system. Alternatively, data acquisition on the ExionLC 2.0 system could be performed using that the ExionLC 2.0 system is also fully supported for instruments in which data acquisition is performed using SCIEX OS software 2.1.5.

Data processing: Data processing of LC-MS/MS data was performed using SCIEX OS software 2.0.1, using the Analytics module to generate all calibration curves, and the precision and accuracy statistics tables.

Chromatography results

Figure 2 shows representative extracted ion chromatograms (XICs) for the MRMs of each compound obtained from protein precipitated whole blood calibrators at various concentrations. All compounds were effectively separated using a 4-minute LC run on the ExionLC 2.0 system. Peak symmetry was assessed using the asymmetry factor, which is defined as the distance from the center line of the peak to the back slope, divided by the distance from the center of the peak to the front slope. An asymmetry factor of 1.0 indicates symmetric peaks. This calculation was performed at 10% of the maximum peak for each compound. The average asymmetry factor across the concentration range was 1.2 for cyclosporine A, 1.0 for everolimus, 1.0 for sirolimus and 0.7 for tacrolimus, indicating that the peaks for each of these compounds were symmetric. The example chromatograms shown in Figure 2 highlight the

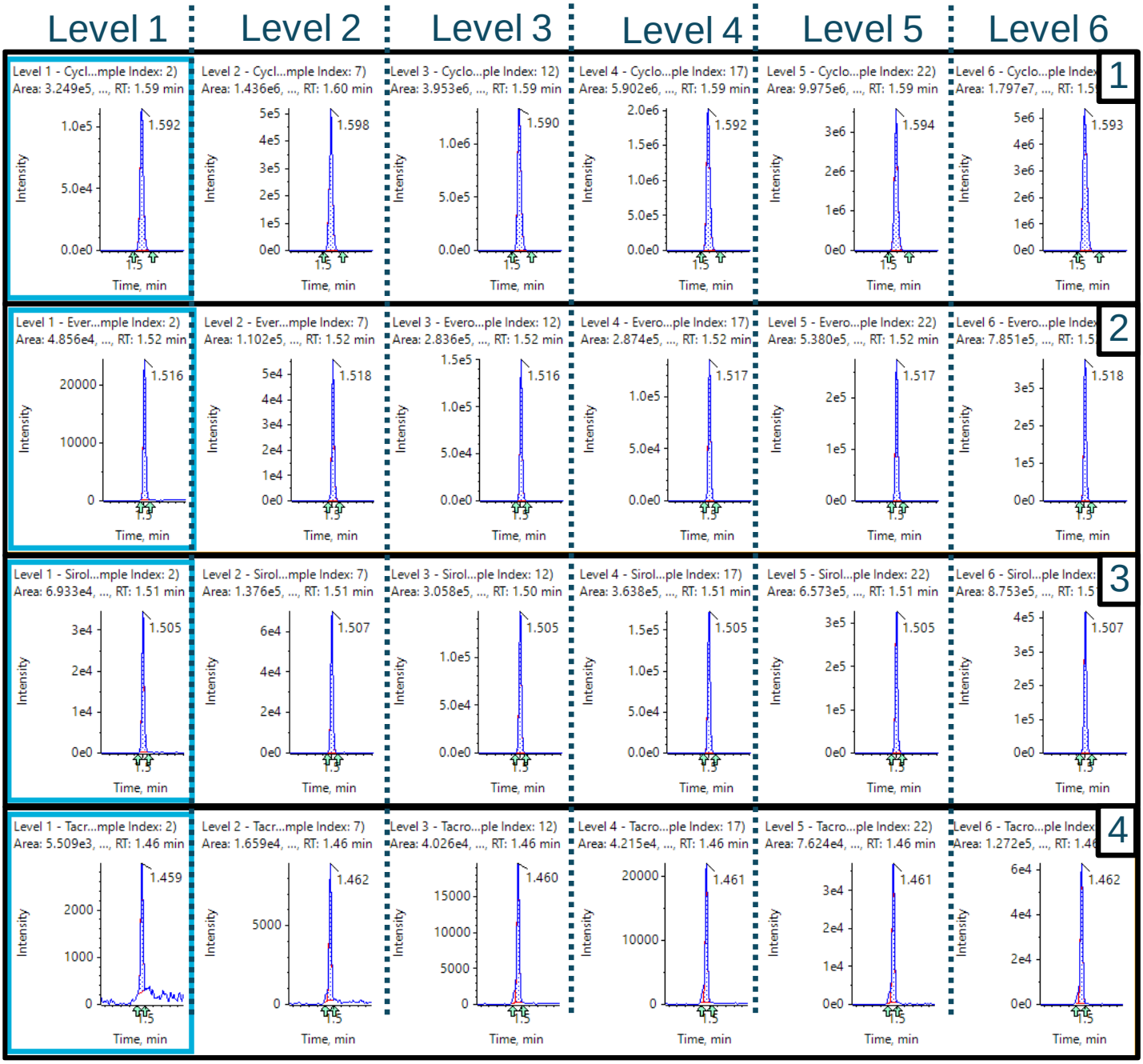

Figure 2. Extracted ion chromatograms (XICs) for drug compounds in whole blood extract at 6 calibrator levels. XICs are shown for cyclosporine A (1), everolimus (2), sirolimus (3), tacrolimus (4). Concentration levels correspond to 2, 6, 12, 18, 25 and 45 ng/mL for everolimus, sirolimus and tacrolimus. Concentration levels correspond to 23, 128, 294, 471, 745 and 1890 ng/mL for cyclosporine A.

symmetric peak widths achieved using the ExionLC 2.0 system and selected chromatographic phase.

Figure 1 shows the retention time reproducibility across 100 injections for the compounds studied. The gradient generated by the ExionLC 2.0 system was highly stable and resulted in precise retention times for injections of each analyte with RSD values less than 0.5%. Retention times for each analyte varied by less than 1 second, with RSD values for cyclosporine A, everolimus, sirolimus and tacrolimus were 0.21%, 0.25%, 0.08% and 0.05%, respectively.

Quantification results

Calibration curves were generated using a commercially available whole blood calibration kit, using 6 concentration levels. These calibration curves were analyzed using linear

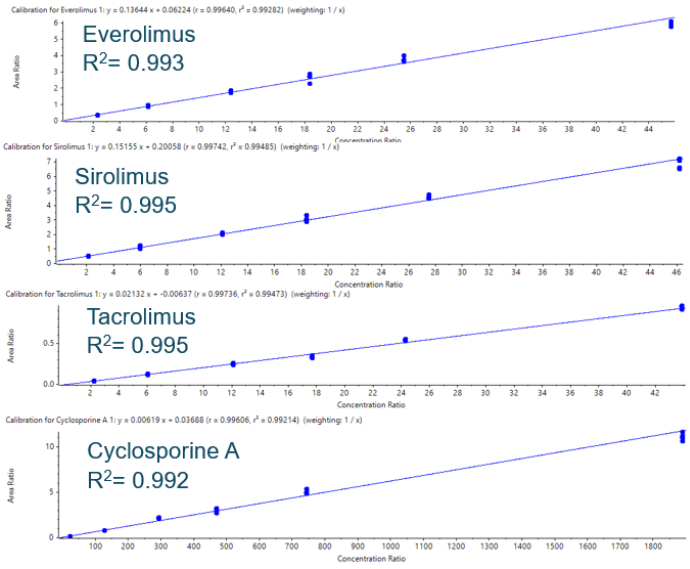

Figure 3. Calibration curves. Good linearity was observed for the compounds analyzed in whole blood matrix. Results were linear between 2 ng/mL and 45 ng/mL for everolimus, sirolimus and tacrolimus. Results were linear between 23 ng/mL and 1890 ng/mL for cyclosporine A.

regression with 1/x weighting. Examples are shown in Figure 3. The linear coefficient of determination (r^2) was typically higher than 0.99.

The peak area reproducibility was computed for 100 replicate 20 µL injections of the Level 4 calibrator. Typical variance results for this study are shown in Figure 4 using the internal standard, ascomycin. The peak area CV for ascomycin was 4.4 %, indicating high reproducibility over 100 injections.

Precision and accuracy values are shown in Table 2. Accuracy values ranged from 85 to 114 %, with precision values of less than 7 %.

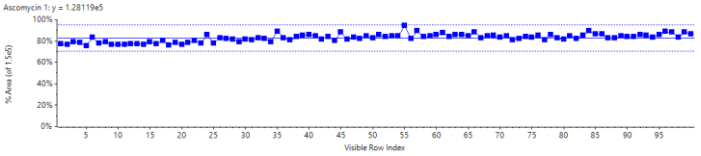

Figure 4 Reproducibility of data. Metric plot of internal standard area reproducibility over 100 injections. Mean regression within ±15% deviation (dashed lines).

System comparison

A back-to-back comparison performance study was performed on the ExionLC AC system by repeating the same analyse that was performed using the ExionLC 2.0 system, using the same column, solvents and mobile phase. Tables 2 and 3 highlights that very similar results were obtained on the two systems, in terms of accuracy and precision. The high degree of match between the results generated on the ExionLC 2.0 system and ExionLC 2.0 system confirms that the ExionLC 2.0 provides the high-quality LC performance expected for an analytical assay. This also confirms that analyzing whole blood that is equally obtainable on the both the established ExionLC AC system and the newer ExionLC 2.0 system. Under identical conditions, the results show better performance on the ExionLC 2.0 system for the more analytically challenging compounds, everolimus and sirolimus. Everolimus average %CV across the concentrations analyzed was 3.6% using the ExionLC 2.0 system compared to 6.6% using the ExionLC AC system. Sirolimus average %CV across the concentrations analyzed was 4.1% using the ExionLC 2.0 system compared to 7.3% using the ExionLC AC system.

Table 2. Accuracy and precision statistics of calibration standards using the ExionLC 2.0 system (n=5).

Calibration standard (ng/mL)	Everolimus		Sirolimus		Tacrolimus		Calibration standard (ng/mL)	Cyclosporine A	
	%Acc	%CV	%Acc	%CV	%Acc	%CV		%Acc	%CV
2	*93.75*	*3.34*	*97.27*	*5.13*	*100.34*	*5.55*	*23*	*93.03*	*5.05*
6	*99.85*	*5.71*	*96.31*	*3.18*	*95.76*	*3.45*	*128*	*89.72*	*4.73*
12	*99.78*	*3.46*	*101.59*	*3.63*	*111.59*	*3.19*	*294*	*113.93*	*2.07*
18	*107.49*	*2.99*	*102.04*	*5.65*	*85.04*	*4.41*	*471*	*100.01*	*5.86*
25	*105.98*	*3.98*	*105.04*	*2.29*	*108.96*	*0.66*	*745*	*107.76*	*2.08*
45	*94.65*	*2.51*	*96.12*	*4.83*	*98.45*	*2.28*	*1890*	*95.55*	*6.69*

Table 3. Accuracy and precision statistics of calibration standards using the ExionLC AC system (n=5).

Calibration standard (ng/mL)	Everolimus		Sirolimus		Tacrolimus		Calibration standard (ng/mL)	Cyclosporine A	
	%Acc	%CV	%Acc	%CV	%Acc	%CV		%Acc	%CV
2	*101.73*	*7.64*	*98.93*	*11.56*	*101.61*	*5.99*	*23*	*82.15*	*13.75*
6	*100.37*	*6.04*	*101.77*	*8.16*	*98.64*	*3.67*	*128*	*99.77*	*6.99*
12	*101.69*	*2.60*	*105.32*	*4.90*	*104.96*	*3.98*	*294*	*112.06*	*15.19*
18	*95.82*	*8.90*	*94.39*	*9.71*	*90.25*	*7.22*	*471*	*100.51*	*13.05*
25	*98.42*	*5.82*	*97.35*	*5.60*	*104.21*	*3.22*	*745*	*111.97*	*12.71*
45	*101.97*	*6.25*	*102.24*	*4.43*	*100.34*	*3.15*	*1890*	*93.52*	*14.26*

Conclusions

The ExionLC 2.0 system has been shown to be a robust UHPLC system that is suitable for the analysis of therapeutic drug compounds from whole blood matrix. Reproducible results and linearity, with quantitative accuracy and precision for calculated concentrations, were achieved with a fast four-minute LC run time using the ExionLC 2.0 system coupled to the SCIEX Triple Quad 4500 system.

- Precise and stable solvent flow delivering less than 0.5% RSD retention time variation
- Accurate and precise quantification results with linear coefficient of determination performance ($r^2 > 0.99$) and precision <10% CV for the concentration range tested

References

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