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Liquid chromatography

Increased robustness in LC-MS analysis of immunosuppressant drugs from human blood using FAIMS technology for clinical research

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Keywords

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Goal

LC-MS analysis of human whole blood presents various challenges, including matrix contamination of the mass-spectrometer source and API stack that can compromise data quality. Therefore, instrument maintenance must be performed to restore data quality, which interrupts data acquisition and reduces productivity. In this study, we demonstrate that incorporating the Thermo Scientific™ FAIMS Pro Duo interface enables users to run their LC-MS system, analyzing complex matrices continuously for 1,800 injections with minimum decrease in performance. We show this by performing analysis of the immunosuppressant drugs Sirolimus, Tacrolimus, Everolimus, and Cyclosporin A in human whole blood using high-flow LC-MS augmented with the FAIMS Pro Duo interface on a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer and comparing it to the performance without FAIMS technology.

Introduction

Analysis of immunosuppressant drugs (ISDs) after organ transplantation surgeries is critical because ISDs have narrow therapeutic ranges: low doses result in therapeutic inefficiency and can lead to transplant organ rejection and overdoses lead to toxicity. In addition, ISDs can cause various adverse effects such as cardiovascular disease or nephrotoxicity.[1] Monitoring levels of ISDs is usually done by immunoassay methods or liquid chromatography with different detectors. Immunoassay performance can suffer from cross-reactivity, inability to detect more than two drugs at once, and high cost.[2]

LC-MS is considered a superior technique because of its selectivity, sensitivity, and accurate multiplexed quantitation of ISDs in biological fluids. However, there are many challenges that laboratories face when using LC-MS. For instance, samples composed of biological fluids such as plasma, whole blood, and urine have very complex matrices. In addition, hundreds of samples are analyzed per day, which is significantly higher than a few dozen analyzed in general research labs. A combination of these factors often leads to interruption of the sample acquisition queue for basic system maintenance, decreasing throughput and productivity.

Incorporating the FAIMS Pro Duo interface improves instrument uptime and throughput by limiting matrix ions entering and contaminating the mass spectrometer. FAIMS technology operates by applying an asymmetric waveform, alternating high and low electric fields between an inner and outer electrode. Only ions with discreet ion mobilities that correlate with applied compensation voltages (CVs) are transmitted between the FAIMS electrodes into the mass spectrometer inlet for detection, and ions with non-optimized differential mobilities are neutralized. In addition, the cylindrical electrode geometry blocks neutrals and salts from direct line-of-sight further improving instrument robustness. Optimization of CVs for all analytes is done empirically and uses a quick and easy online LC process. If the FAIMS Pro Duo interface experiences contamination from the biological samples, its removal, cleaning, and reinstalling takes minutes and does not require breaking the instrument vacuum or recalibration.

The FAIMS Pro Duo interface has been used with various sample inlets such as low-flow LC[3] and the Advion TriVersa NanoMate™ direct nanospray infusion[4]. Introduction of orthogonal selectivity has increased signal-to-noise ratios and improved detection limits for the analysis of testosterone[5] and veterinary drugs.[6] The increased selectivity has also been demonstrated to improve reproducibility. To evaluate improved robustness, as well as analytical precision, we performed a large-scale study to mimic clinical laboratory routines by continuously injecting 360 samples per day for five days without instrument maintenance. To increase the complexity of the matrix, only a 5-fold solvent dilution was performed while precipitating proteins. No further processing that could add time and cost to the workflow was performed. Intra- and inter-day evaluation of raw peak areas and %RSD measurements were performed on spiked curves and replicate sample analysis. We have applied acceptance criteria similar to those used in clinical laboratories to assess relative performance between experiments performed using the standard workflow and experiments using the FAIMS Pro Duo interface.

Experimental

Four types of samples were prepared for the study: whole blood samples, calibrators, controls, and blanks. Cyclosporin A, Everolimus, Tacrolimus, and Sirolimus were purchased from Cerilliant (Round Rock, Texas, P/N C-093, E-068, A-094, S-025) and diluted in LC-MS grade methanol to prepare stock solutions. Whole human donor blood was purchased from BioIVT and spiked with immunosuppressants from stock solutions, yielding final concentrations of 132.0 ng/mL for Sirolimus, 134.5 ng/mL for Everolimus, 115.5 ng/mL for Tacrolimus, and 2,205.0 ng/mL for Cyclosporin A. Calibrators (Iris Technologies International GmbH, Cursdorf, Germany, P/N 9933, levels 0–6 and P/N 9028, level 7) were prepared at six different levels plus a blank and were within the following ranges: Cyclosporin A (21.7–1,626 ng/mL), Everolimus (1.22–57.1 ng/mL), Tacrolimus (1.25–56.7 ng/mL), and Sirolimus (1.38–60.1 ng/mL). Vial contents were reconstituted in 2 mL of HPLC water and gently shaken on a roller mixer for 30 to 60 min. Controls III and IV (manufactured by RECIPE Chemicals + Instruments, GmbH) were prepared in a similar way to the calibrators and were used for system suitability checks.

To prepare samples for LC-MS analysis, 3 mL of 0.1 M zinc sulfate were added to a 2 mL aliquot of each sample, calibrator, and control to precipitate large proteins. Ascomycin (Cerilliant, Round Rock, Texas, P/N A-094) was selected as general internal standard (IS) for all immunosuppressants. 5 mL of stock solution of Ascomycin in methanol (LC-MS grade) was spiked into each sample, calibrator, and control, resulting in a final concentration of 23.1 ng/mL. All samples were equilibrated for 30 minutes at 4–15 °C and centrifuged at 3,000 rpm for 10 minutes to create a stable pellet. The supernatant was aliquoted to individual HPLC vials. LC-MS grade methanol/water (50:50) solution was used as blanks and added to separate vials. See Table 1 for the daily injection sequence used for the study.

Table 1. Recommended daily injection sequence, which was consistent between each workflow

Sample type	Number of injections	Injection volume
1. Blanks	2, as needed	15 µL
2. QC samples	1 each	
3. Calibrators: Level 0–Level 7	1–5 replicates per level	
4. Whole blood samples (up to 20)	1 each (20 per bracket)	
5. QC samples	1 each	
6. Repeat steps 4 and 5 until the total number of injections reaches 360	1 each (20 per bracket)	
7. Blank	1	

Chromatographic separation was performed on a Thermo Scientific™ Vanquish™ Horizon UHPLC system by injecting 15 µL of sample onto a Thermo Scientific™ Hypersil GOLD™ C8, 50 × 2.1 mm, 5 µm column (P/N 25205-052130). The LC flow rate was 0.8 mL/min and the UHPLC column temperature was 60 °C for the separation. Mobile phase A was 0.1% formic acid and 10 mM ammonium formate in water; mobile phase B was 0.1% formic acid and 10 mM ammonium formate in methanol. The gradient information is provided in Table 2.

Table 2. LC gradient parameters used for the entire study

No	Time (min)	%B	Curve
1	0.000	30.0	5
2	0.250	30.0	5
3	0.750	95.0	5
4	1.900	95.0	5
5	2.000	30.0	5
6	3.000	30.0	5

The Thermo Scientific™ OptaMax™ NG heated electrospray (H-ESI) ion source was aligned between M and L in vertical and 1.5 in horizontal positions. Optimized spray conditions are listed in Table 3. Note that the probe positioning and gas settings are the same for experiments with and without the FAIMS Pro Duo interface to minimize method optimization.

Table 3. H-ESI source conditions used for experiments performed with and without the FAIMS Pro Duo interface

Ion source type	Heated ESI
Positive ion spray voltage (V)	3,000
Sheath gas (Arb)	50
Aux gas (Arb)	25
Sweep gas (Arb)	2 (for runs without the FAIMS Pro Duo interface)
Ion transfer tube temperature (°C)	350
Vaporizer temperature (°C)	350
FAIMS mode	Standard Resolution (for study with the FAIMS Pro Duo interface) or Not Installed (for study without the FAIMS Pro Duo interface)
Total carrier gas flow	0.7 mL/min (for runs with the FAIMS Pro Duo interface)

The Orbitrap Exploris 240 mass spectrometer was operated in full-scan mode over the *m/z* range 700–1,400 at 60,000 resolution setting (Table 4). Optimization of CVs was performed for all immunosuppressants simultaneously by on-line injection of an ISD standard mixture and cycling through a range of CV values during compound elution (Figure 1). Data analysis was performed using Thermo Scientific™ TraceFinder™ 5.1.SP2 software. Quantitation was done using raw peak area response ratios of each ISD to Ascomycin.

Table 4. Orbitrap Exploris 240 MS instrument method parameters. Instrument settings were consistent across the two workflows.

Application mode	Small molecule
Pressure mode	Standard
Expected LC peak widths (s)	3
Default charge state	1
Advanced peak determination	False
Mild trapping	False
Start time (min)	0
End time (min)	3
Cycle time (s)	3
Orbitrap resolution	60,000
Scan range (*m/z*)	700–1,400
Microscans	1
Maximum injection time mode	Auto
AGC target	Standard
RF Lens (%)	60
Data type	Profile
Polarity	Positive
Source fragmentation	Disabled

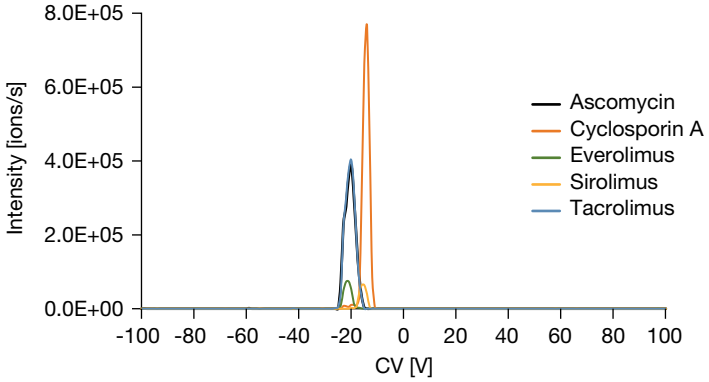

Figure 1. Optimization of CVs for immunosuppressants that identified final settings of -14 V for Cyclosporin A, -16 V for Sirolimus, and -21 V for Tacrolimus, Ascomycin, and Everolimus

Results and discussion

Workflow robustness with and without the FAIMS Pro Duo interface was evaluated by collecting data over five consecutive days of sample injections, yielding 360 injections per day and a total of 1,800 injections for each study. Sample dilution to perform protein crash was kept to a minimum to maximize the matrix complexity since the study was performed for only five days. Calibration of the mass spectrometer and cleaning of the ion transfer tube were performed only at the beginning of each study. The linear calibration curves for each immunosuppressant were generated daily using calibrators to evaluate the detection capabilities at the previously published levels for each ISD. Crashed whole blood samples were injected in brackets after the calibrators and were followed by QC samples to assess system suitability for robustness. Each bracket consisted of 20 samples, resulting in analysis of 280 QC samples per day. Refer to Table 1 for the daily injection sequence.

Initial comparative analysis was performed using the QC sample acquisition both per day and across the entire study. Average raw peak areas for Cyclosporin A in samples declined more than 20% after three days of injections using the standard workflow (Figure 2A) and does not meet our acceptance criteria. This occurs due to clogging of the ion transfer tube by various components in human whole blood and leads to the signal loss. However, peak areas remained steady across all five days of continuous analysis when using the FAIMS Pro Duo interface with only a 4% decrease on any day relative to the average peak area value from day 1 (Figure 2B). When utilizing the FAIMS Pro Duo interface, the peak area signal had slightly lower peak area (approximately 62%) than without FAIMS selectivity on day 1, which is expected due to decreased ion transmission through the electrode assembly.

A similar trend is observed for Tacrolimus, Sirolimus, and Everolimus using the two experimental methods (Figures 2C and 2D). The average peak area decreased more than 20% after three days of injections for all immunosuppressants using the standard workflow, requiring data acquisition to be interrupted for mass spectrometer maintenance. The experiments using FAIMS technology, however, maintained acceptable performance throughout the five-day study, increasing productivity. It should also be noted that the amount of transmission loss on FAIMS electrodes varies for each compound individually and is less for Tacrolimus than for Cyclosporin A, Sirolimus, and Everolimus.

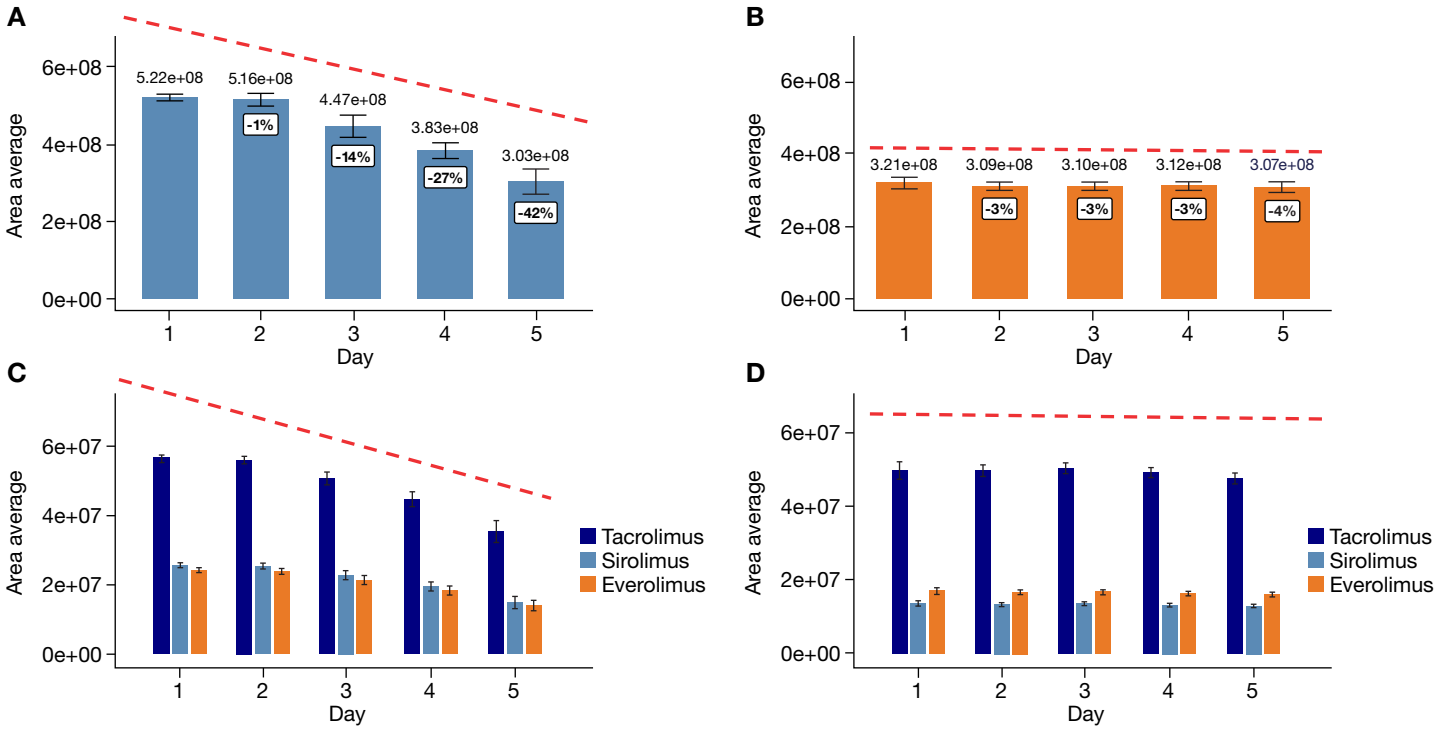

Figure 2. Evaluation of the average Cyclosporin A peak area in human whole blood across the five-day study acquired using (A) the standard workflow and (B) with the FAIMS Pro Duo interface. The dashed lines represent the trend in measured peak areas across the five days. The values listed on the daily response for (A) and (B) represent the difference in average peak areas per day relative to that measured for day 1. Similar measurements were presented for the remaining three ISDs (C) using the standard workflow and (D) data acquisition with the FAIMS Pro Duo interface.

The decline in measured peak area values using the standard workflow correlates with the fore-vacuum pressure drop (Figure 3) and is indicative of transfer tube clogging by the whole blood matrix components. While performing simple maintenance steps, such as replacing/cleaning the ion transfer tube on the Thermo Scientific mass spectrometer, is not an overly time-consuming process, it still interrupts data acquisition. The maintenance steps involve stopping data acquisition, removing the ion transfer tube to clean, replacing it, and then running QC samples to confirm system suitability. Incorporating the FAIMS Pro Duo interface helps to maintain stable performance over the course of the five-day experiment without the requirement for basic maintenance, increasing throughput and productivity.

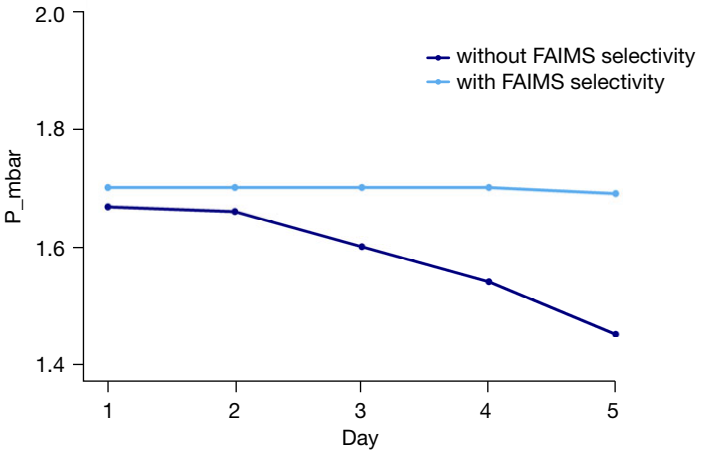

Figure 3. Fore-vacuum pressure change across all days of immunosuppressants analysis without FAIMS Pro Duo interface and with FAIMS Pro Duo interface

Relative standard deviation (%RSD) values of raw peak areas for immunosuppressants across all five days of robustness studies were compared between experimental methods (Table 5). Variability in peak areas was > 20% across all five days of injections without the FAIMS Pro Duo interface and does not pass the acceptance criteria we set for our study. Good precision of 4–5% was observed for all analytes when using the FAIMS Pro Duo interface.

Table 5. Measured %RSD of immunosuppressants analyzed with and without the FAIMS Pro Duo interface across the entire five days of sample analysis. Calculations were performed using raw ISD peak area values.

Compound	%RSD, without FAIMS selectivity	%RSD, with FAIMS selectivity
Cyclosporin A	20.9	4.3
Ascomycin	17.9	3.8
Everolimus	20.7	4.6
Sirolimus	20.6	4.3
Tacrolimus	17.6	3.9

Matrix effects can be addressed in the standard workflow by incorporating internal standards and performing normalization. Since the ion flux for both the target ISDs as well as Ascomycin are equally affected by the clogged ion transfer tube, normalization can mitigate matrix effects to maintain reliable area ratios. For each raw file used to originally plot Figure 2, the ISD area value was normalized against the area value for Ascomycin and replotted in Figure 4. The trends for each ISD show significantly reduced intra-day variance for results measured using the standard workflow. In addition, the inter-day analysis showed greater measurement consistency for the entire study, with overall variance of less than 20%. The results measured using the FAIMS Pro Duo interface remained consistent for the intra- and inter-day analysis for each of the ISDs. It should be noted that Ascomycin had a different retention time than the other ISDs, and the optimized FAIMS CV voltage overlapped only with Everolimus. The experimental differences of co-eluting matrix can contribute to slight perturbations in relative measurements, but the overall reproducibility was well within the stated acceptance limits.

Performing the comparative analysis on the calibration curves evaluated the matrix effect as a function of the spiking levels for each workflow. Calibration curves were linear and met experimental requirements, with the peak areas normalized to the internal standard Ascomycin and correlation coefficients (R^2) > 0.99 was measured for each ISD across each of the five days. The comparative response for Cyclosporin A is shown for day 1 and day 5, acquired with the standard workflow (Figures 5A and 5B) and using the FAIMS Pro Duo interface (Figures 5C and 5D). As shown for the standard sample analysis, the raw area values measured for ISDs decrease each successive day as was measured on the calibration curves. The area ratio of Cyclosporin A to Ascomycin at the most abundant spiking level on day 1 is approximately 5.8:1 as compared to an area ratio of approximately 5.2:1. Conversely, the area ratio for Cyclosporin A to Ascomycin remains approximately 4.2:1 for the same spiking level. Despite the decrease, all ISDs remained linear with a near-zero intercept and (R^2) > 0.99.

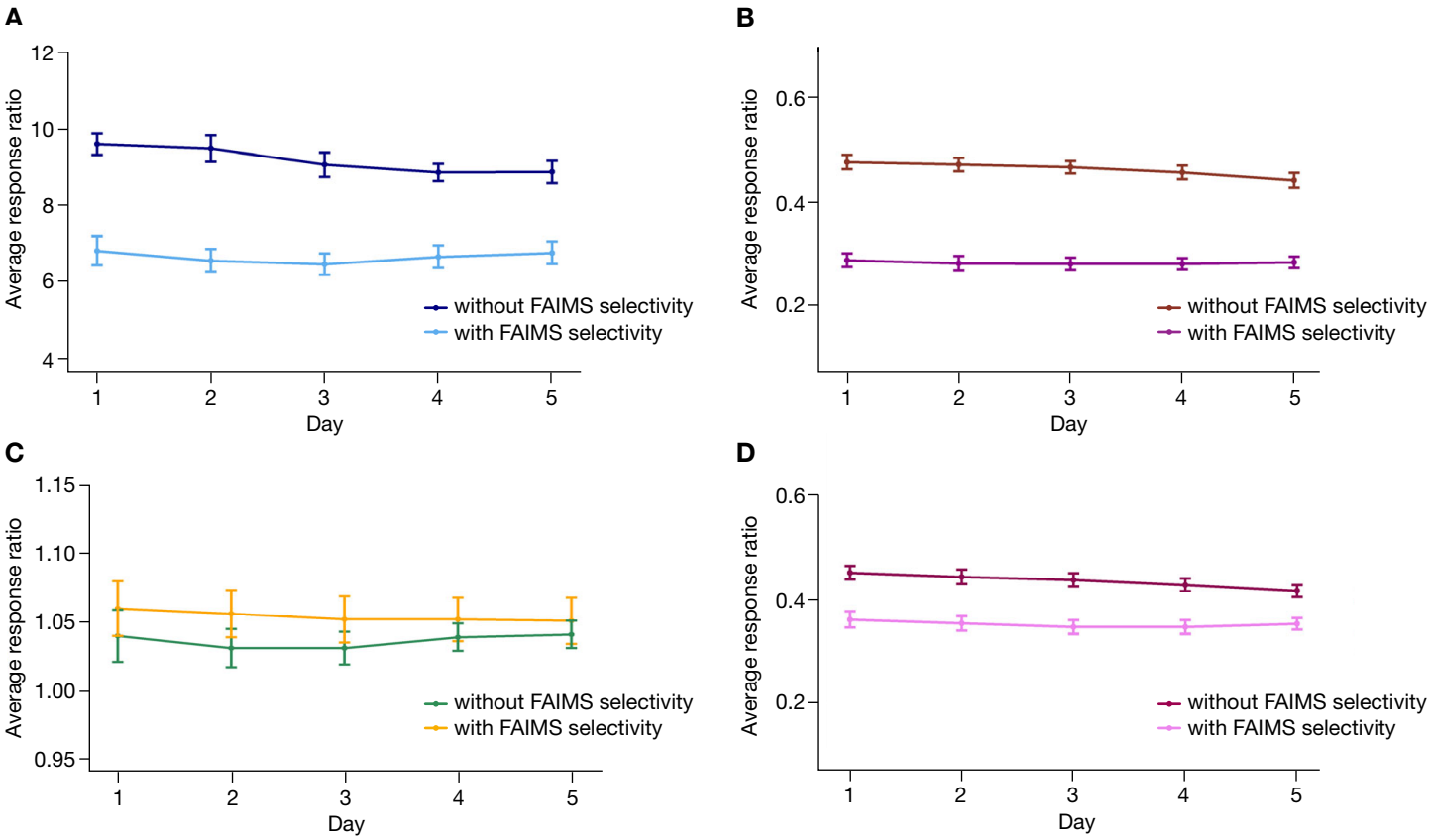

Figure 4. Reproducibility analysis for normalized ISD area values for the duration of the study using the standard method (no FAIMS) and experiments using the FAIMS Pro Duo interface. Measured ISD area values are normalized against the Ascomycin for (A) Cyclosporin A, (B) Sirolimus, (C) Tacrolimus, and (D) Everolimus.

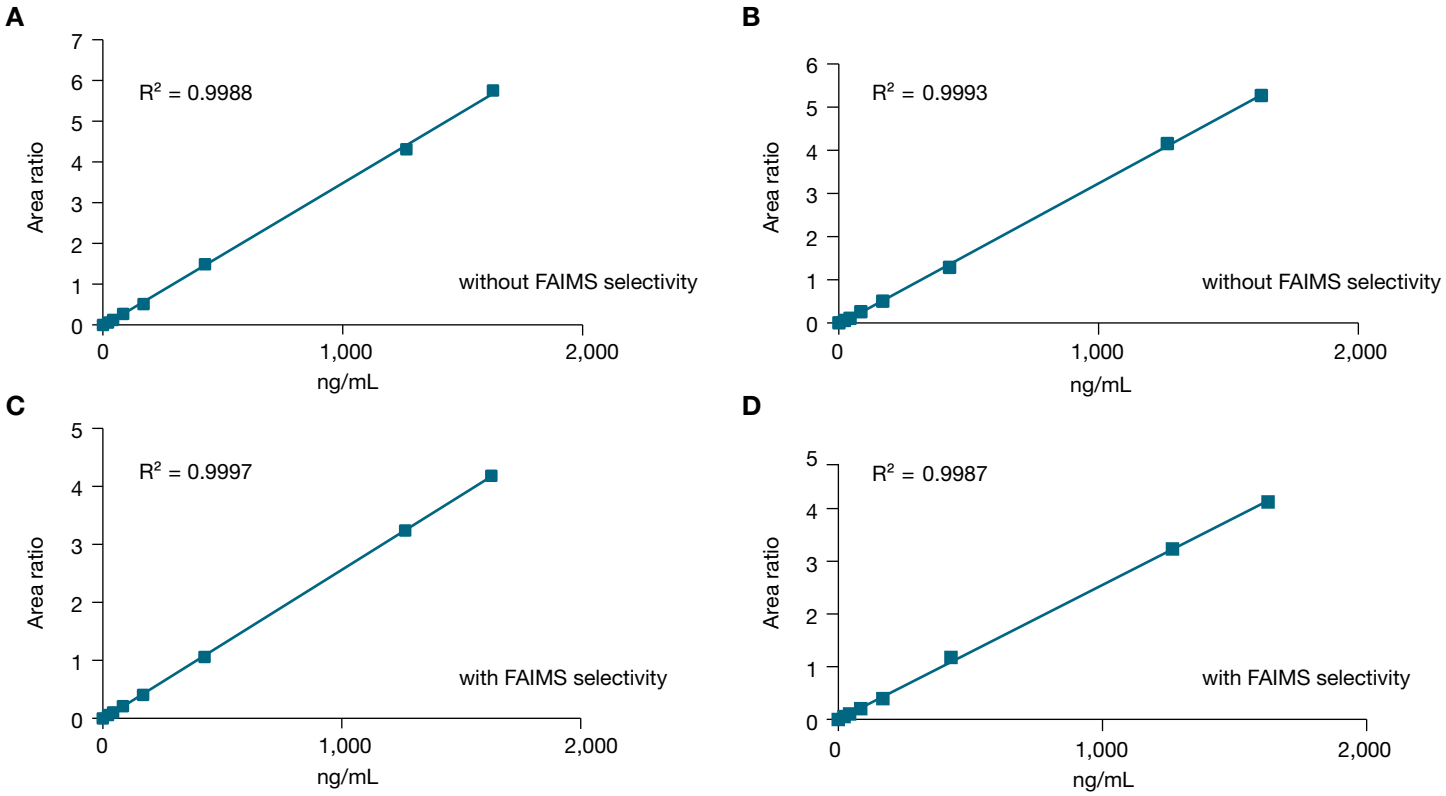

Figure 5. Comparative quantitation curves for Cyclosporin A in crashed whole human blood. Area under the curve ratios were determined as compared to the internal standard Ascomyocin. Quantitation curves 5A and 5B were acquired without FAIMS selectivity on days 1 and 5, respectively, and curves 5C and 5D were acquired with FAIMS selectivity on days 1 and 5.

Conclusion

We demonstrated that incorporating the FAIMS Pro Duo interface, that is able to support high-flow chromatographic separations, increases throughput and efficiency for LC-MS analysis of small molecules in complex biological matrices. The comparative results showed reproducible and robust immunosuppressant drugs quantitation in crashed human whole blood across 360 injections per day for five days. While the study was conducted for only five days, the matrix was substantially more complex than typical sample preparation procedures by purposely avoiding incorporation of a post-column divert valve, trap/elute SPE columns, or other off-line sample clean-up methods that can be introduced into the workflow and increase method development time and money. The number of injections were maximized in a 24-hour period to stress the ion transfer tube of the Orbitrap Exploris 240 mass spectrometer that once cleaned, regained the original performance. Incorporation of the FAIMS Pro Duo interface enabled accurate and precise quantitation over the same spiking levels, as measured for the standard workflow, despite the internal standard having a different optimized CV setting or retention time. In addition, the FAIMS Pro Duo interface operation was straightforward, with simultaneous CV optimization performed online, minimizing the amount of time needed for method development. Overall, the FAIMS Pro Duo interface upgraded to support high-flow chromatography possesses significant benefits for busy laboratories to perform routine analysis of samples without any interruptions.

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