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Introduction

LC-MS/MS is widely used to monitor immunosuppressive drug panels on a routine basis, for example for therapeutic drug monitoring of organ transplant patients.[1] Multiplexing is a popular approach to speeding the analysis time between individual samples. However, such techniques utilize a single electrospray probe and fast serial injections, typically requiring cumbersome wash cycles to ensure minimum carryover.[2] This poster reports a novel approach to multiplexing the immunosuppressant analyses using a single ion source equipped with two electrospray probes. Previous reports demonstrated linearity and use of two ESI probes in a single source for analysis of Vitamin D [3]. Here we demonstrate fast quantitation of Sirolimus, Tacrolimus, Everolimus and Cyclosporin A using Ascomycin and Cyclosporin D as internal standards.

Method

Sample and Preparation: The Tacrolimus, Sirolimus, Everolimus and Cyclosporin A standard stock solutions in liquid form were purchased from Cerilliant Inc (Round Rock, Texas) and stored at -4°C. Whole blood spiked samples were cleaned up by mixing one volume of serum with two volumes 0.1 M ZnSO₄ precipitation solution containing the internal standards: Cyclosporin D (1234/1217) and Ascomycin (809.6/756.6). Three level QCs were purchased from UTAK (Valencia, CA). The mixture was vortexed for one minute followed by centrifugation for 15 min. The supernatant was transferred to a clean vial for quantitation.

LC-MS/MS Conditions: The LC-MS/MS was performed using an IONICS 3Q 120 triple quadrupole mass spectrometer (Bolton, ON Canada) with a Shimadzu UFLC system. 20 µL of supernatant were loaded on a porous R1/20 pretreatment column (30x2.1mm) for on-line washing with water for 0.25 minutes at a liquid flow rate of 3 mL/min, then eluted by an Intakt Cadenza CD-C18HT analytical column (50x2.0mm, 3µm) at flow rate of 0.6 mL/min using Solvent A (water:methanol = 98:2, v/v, with 0.1% formic acid and 10mM ammonium acetate) and Solvent B (water:methanol = 2:98, v/v, with 0.1% formic acid and 10mM ammonium acetate)

Mass Spectrometry Conditions: Electrospray Voltage: 5000V, HSID: 150°C, Nebulizer Gas: 450, Drying Gas: 120, Heating Gas: 350, Source Temperature: 325°C.

The total LC cycle time for an injection is 3 min and the sample analysis time is 1.5 min with alternating injections from each LC. All the solvents used in this method are HPLC grade.

Figure 1: LC Cycle Time

Time (min)	Loading Pump		Eluting Pump	
	Solvent A (%)	Solvent B (%)	Solvent B (%)	Valve Position
0.01	100	0	100	Washing
0.25	100	0	100	Eluting
1.3	100	0	100	Washing
1.5	0	100	100	
2.0	0	100	100	
2.1	100	0	100	
3.0	100	0	100	

ESI/ESI LC-MS Configuration

Figure 2: The LC-MS/MS Setup Used During the Development of this Method.

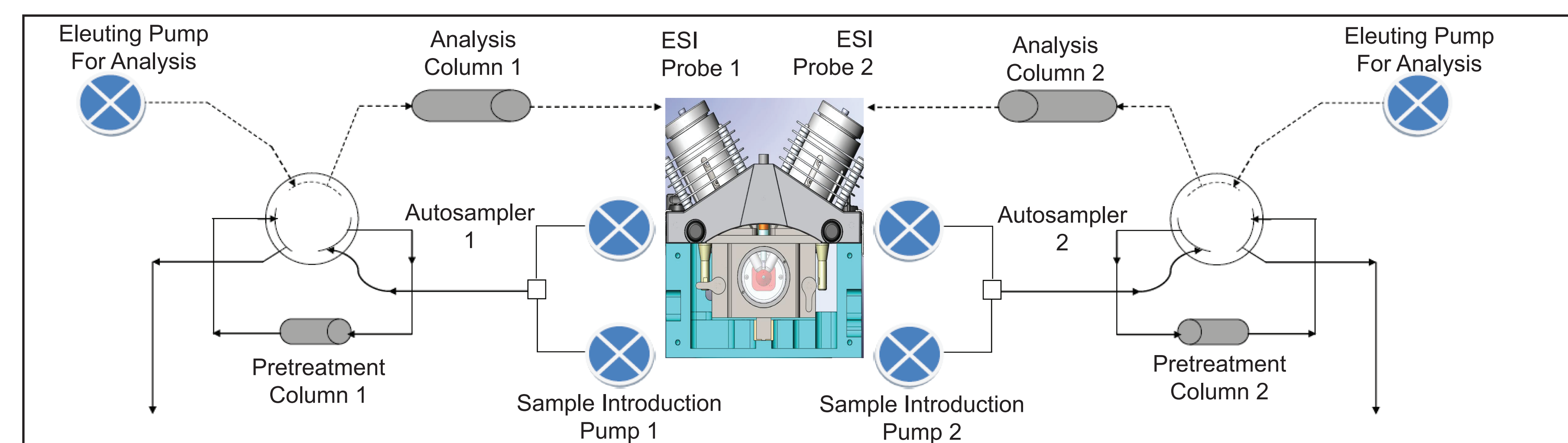
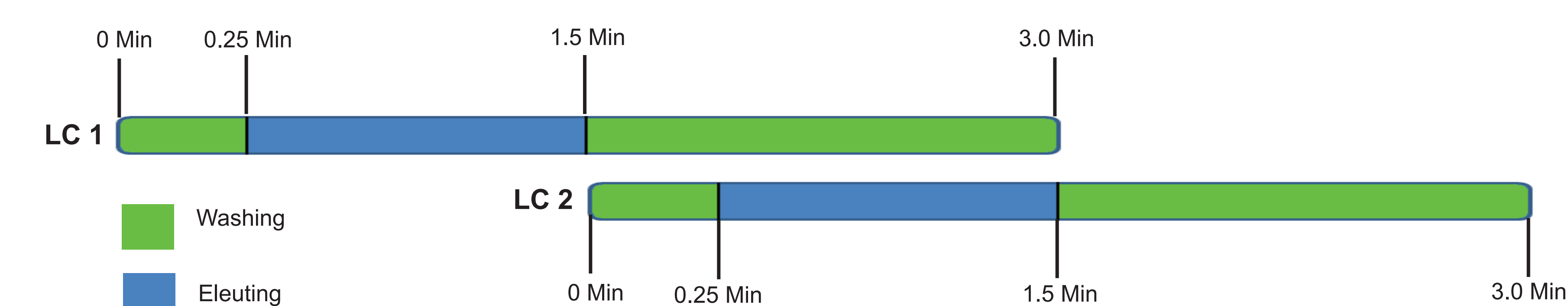


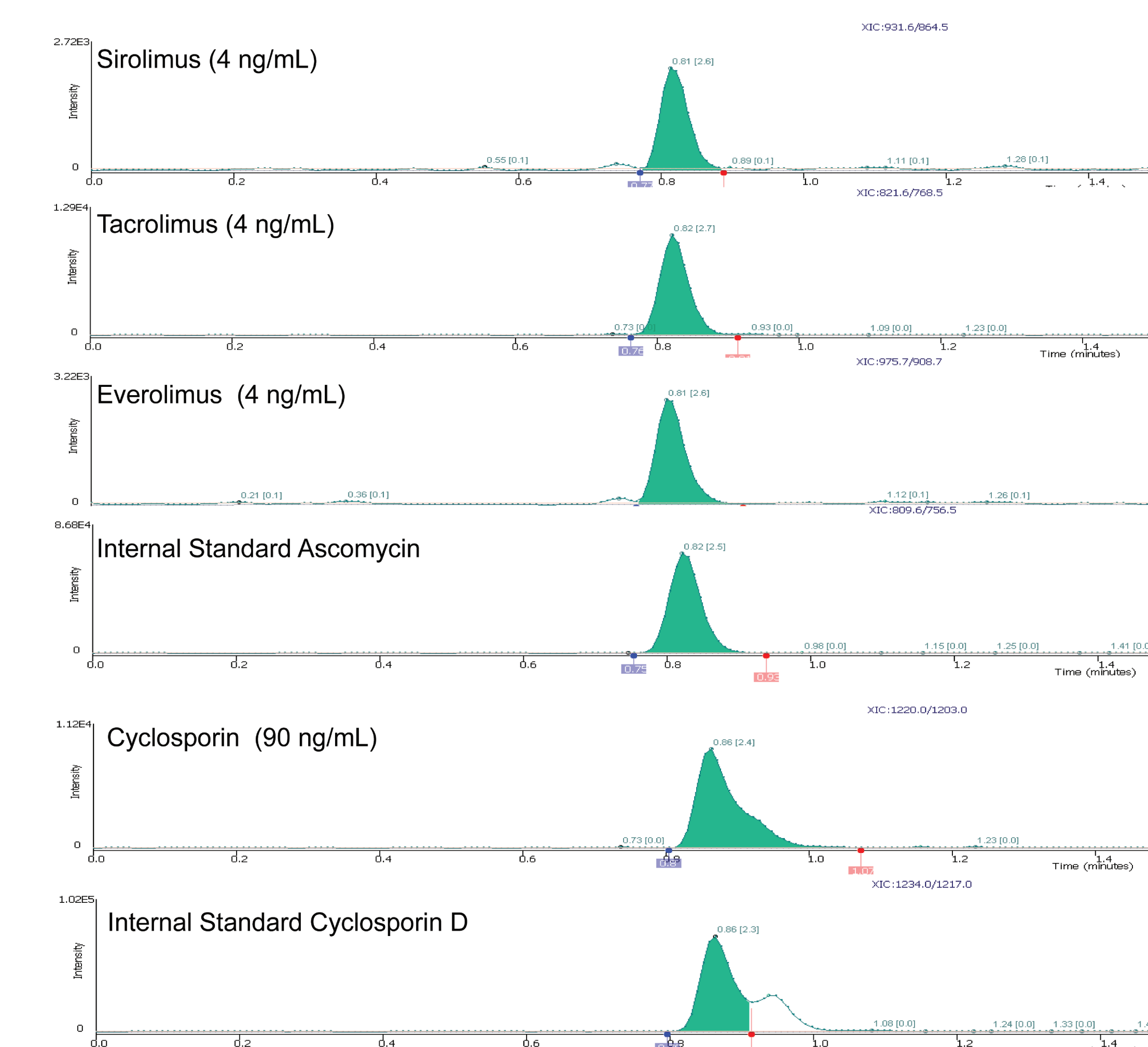
Figure 3: Injection Sequence



Results

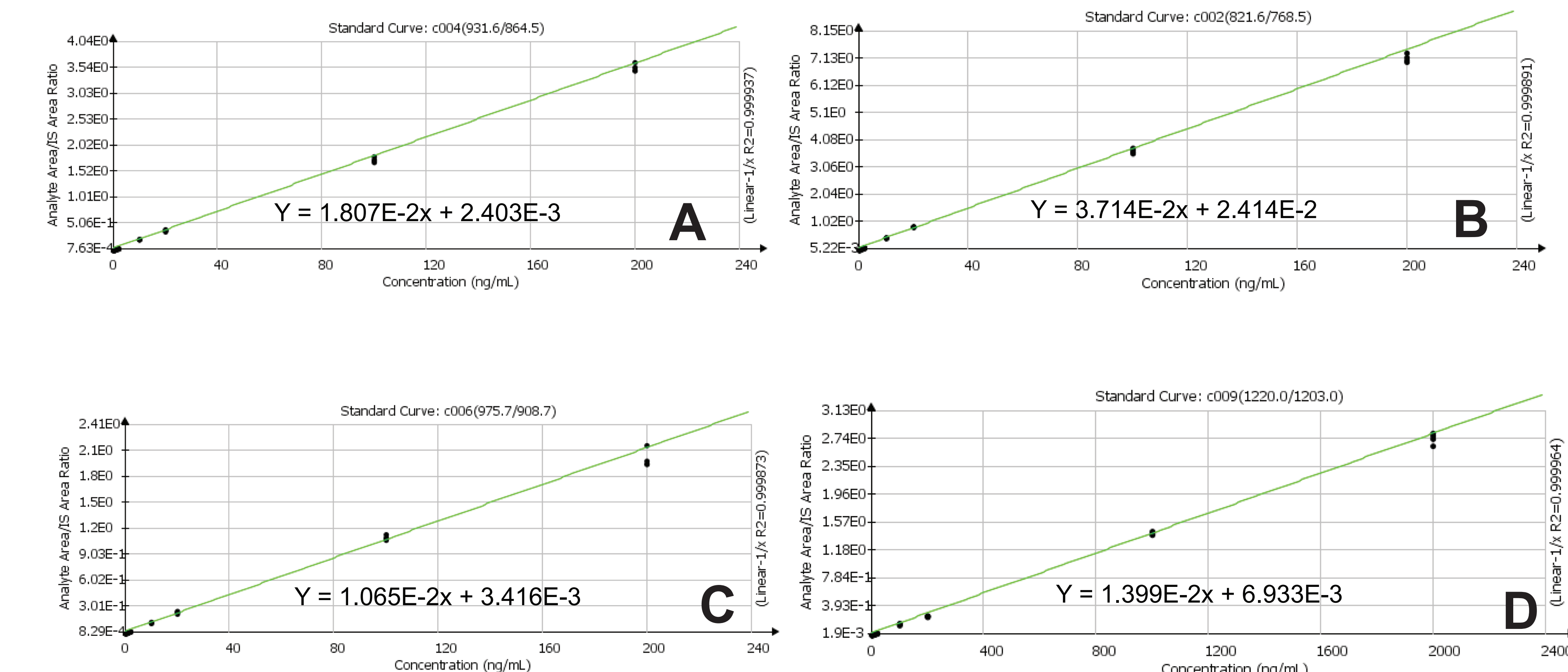
EIC Chromatograms

Figure 4: EIC Chromatograms of Compounds for QC Level I in Whole Blood



Calibration Curves

Figure 5A: Calibration Curve for Sirolimus (0.2-200ng/mL) 5B: Tacrolimus (0.2-200ng/mL) 5C: Everolimus (0.2-200ng/mL) 5D: Cyclosporin (2-2000ng/mL)



Results: This method covers a concentration range of three orders of magnitude from 0.2 to 200 ng/mL for Tacrolimus, Sirolimus, Everolimus and 2 to 2000 ng/mL for Cyclosporin A, while maintaining good linearity ($R^2 = 0.999$) with $1/x$ weighting. The intraday and interday variability for three levels QCs were all <7% and <11%, respectively. No interference or cross contamination was observed.

Figure 6: Intra and (Inter)-day Variability (%RSD)

Name	MRM	QC 1	QC 2	QC 3
Tacrolimus	821.6/768.5	3.5 (9.4)	1.4 (2.0)	2.1 (0.8)
Sirolimus	931.6/864.5	5.6 (4.9)	5.2 (2.1)	3.0 (2.0)
Everolimus	976.7/908.7	6.6 (10.1)	3.1 (2.1)	3.5 (0.9)
Cyclosporin A	1220.0/1203	1.9 (7.5)	3.6 (1.9)	3.5 (1.0)

Conclusion

A sensitive, reliable and accurate LC-MS/MS method was developed and validated for quantification of Tacrolimus, Sirolimus, Everolimus and Cyclosporin A in whole blood. The use of an ESI/ESI novel dual source allows a sample analysis time of only 1.5 minutes, which doubles the throughput. This LC-MS/MS method requires simple sample preparation and is well-suited for routine therapeutic drug monitoring of immunosuppressive drugs.

Reference: [1] Christoph Seger¹, et al. A rapid HPLC-MS/MS method for the simultaneous quantification of cyclosporine A, tacrolimus, sirolimus and everolimus in human blood samples. Nature Protocols, Vol 4, 526-534 (2009)
[2] Vogeser M, Spöhrer U. Pitfall in the high-throughput quantification of whole blood cyclosporin A using liquid chromatography-tandem mass spectrometry. Clin Chem Lab Med. 43(4):400-402 (2005).
[3] Sha Joshua Ye, et al, Method Development Time Reduction In Clinical Applications By Multiplexing Two HPLC Analyses Using a Dual Coaxial Flow Ion Source , 2011 ASMS meeting poster