An Improved Immunosuppressant Drug Research Method Based on a Novel SPLC-MS/MS System

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Overview

Purpose: Demonstrate robust and rugged method performance utilizing an automated two-channel sample preparation-liquid chromatography (SPLC) system that minimizes matrix interferences from whole blood when measuring immunosuppressant drugs (ISDs) for research purposes by tandem mass spectrometry (MS/MS) with electrospray ionization (ESI).

Methods: A 5 minute method involved automated clean up of whole blood preparations (cell rupture and protein precipitation by aqueous zinc sulfate and methanol) using TurboFlow technology followed by high-resolution liquid chromatography using a short Accucore C8, 2.6 µm HPLC column. Reversed-phase extraction, elution and final separations were done in a way that avoided the accumulation and co-elution of phospholipids, which would have suppressed ionization of ISDs in ESI sources. Quantitation of four ISDs was achieved by stable-isotope dilution using two internal standards (IS).

Results: Performance specifications were consistently reproduced within systems and across different laboratories as whole-blood levels were reliably measured: between 2.5 and 50 ng/mL for Everolimus, Sirolimus and Tacrolimus; and between 25 and 1,250 ng/mL for Cyclosporin A. A throughput of 21 samples per hour was achieved when multiplexing across both channels, which generated only 165 mL of solvent waste. No significant carryover between samples was detected.

Introduction

Immunosuppressant drugs (ISDs) are often analyzed in whole-blood using LC-MS with electrospray ionization, which is prone to interference by phospholipids. Although stable isotopes for each ISD are available to compensate, minimizing such interferences would improve data quality. The Thermo Scientific™ Prelude™ SPLC system—a novel dual-channel system that automates sample preparation and liquid chromatography (SPLC), was interfaced to the ESI of a tandem mass spectrometer (MS/MS) for the analysis of ISDs. The Prelude SPLC system incorporated Thermo Scientific™ TurboFlow™ technology and high-efficiency LC utilizing solid-core packing. Stable isotope derivatives D_{12}-Cyclosporin-A and Tacrolimus-$^{13}CD_2$ were used as internal standards in the whole-blood sample preparation procedure. The method was optimized to reliably minimize interferences from phospholipids to improve data quality. The method was also designed to minimize solvent waste.

FIGURE 1. Immunosuppressant Drugs Analysed

Cyclosporin A

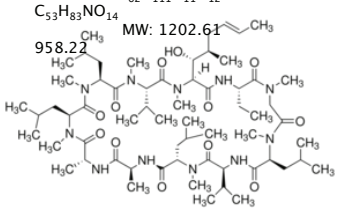
$C_{62}H_{111}N_{11}O_{12}$

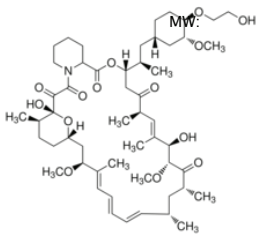
MW: 1202.61

Everolimus

$C_{53}H_{83}NO_{14}$

MW: 958.22



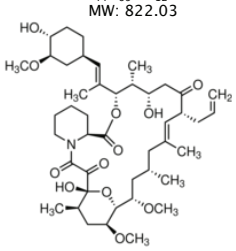


Tacrolimus (FK-506)

(Rapamycin)

$C_{44}H_{69}NO_{12}$

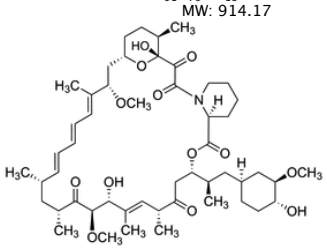
MW: 822.03



Sirolimus

$C_{51}H_{79}NO_{13}$

MW: 914.17



Methods

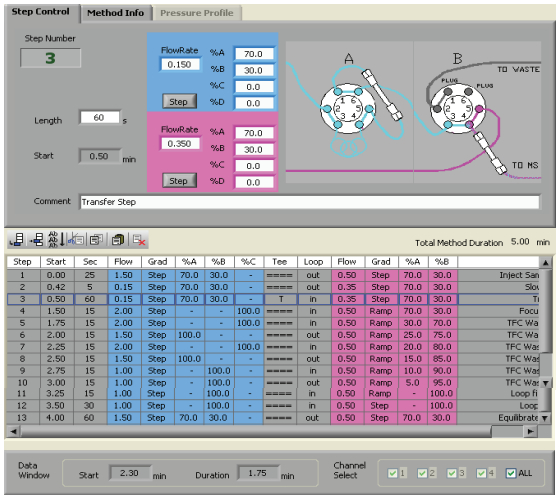
Off-Line Sample Preparation

ChromSystems 6PLUS1® ISD multilevel calibrator set and MassCheck® whole-blood controls as well as in-house test samples were mixed with aqueous zinc sulfate solution and then with methanol containing internal standards: Tacrolimus-$^{13}CD_2$ (Toronto Research Chemicals, Canada) and D_{12}-Cyclosporin A (Alsachim, France). After centrifugation, supernatants were harvested into glass autosampler vials.

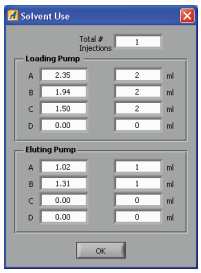
On-Line Sample Preparation & Liquid Chromatography (SPLC)

In each channel, 20 µL injections of supernatants were extracted with a Thermo Scientific™ TurboFlow™ Cyclone-P™ TurboFlow column (0.5 x 50mm) using a mobile phase mixture of 7:3 water:methanol containing 10 mM ammonium formate and 0.05% formic acid at 1.5 mL/min. A slow flow of methanol eluted extracted ISDs, which merged with a higher flow of a 7:3 water: methanol mixture, to transfer and focus the ISDs to an Accucore C8, 2.6 um, 3.0 x 30 mm HPLC column, which was maintained at 70 °C by the built-in heater. The ISDs were separated from matrix interferences and eluted to the heated electrospray ionization (HESI) source by a gradient of increasing methanol. Figure 2 shows this focus method.

FIGURE 2. Summary of SPLC Focus Method.



Solvents:
A: Water + 10mM NH_4OOCH + 0.05% HOOCH
B: Methanol + 10mM NH_4OOCH + 0.05% HOOCH
C: 45% Acetonitrile + 45% Isopropanol + 10% Acetone



Total solvent consumption is 3.37 mL A, 3.25 mL B, 1.5 mL C for each injection.

Mass Spectrometry

The Thermo Scientific™ TSQ Vantage™ triple-stage quadrupole system with heated electro-spray interface (HESI-II) was used to measure the transitions from ammonium-adduct precursor ions to product ions:

Everolimus: 975.7 > 908.4 Sirolimus: 931.6 > 864.6

Tacrolimus: 821.5 > 824.4 Tacrolimus IS: 824.4 > 771.0

Cyclosporin A: 1202.8 > 425.3 Cyclosporin A IS: 1214.9 > 437.2

During method development, the elution of phospholipids and dioctylphthalate were tracked by adding the following transitions:

Dioctylphthalate: 391 >149

Lyso-Phosphotidylcholine;16:0: 496 > 184

Lyso-Phosphotidylcholine;18:0: 524 > 184

Phosphotidylcholine;38:6: 806 > 184

Data Analysis

Thermo Scientific™ TraceFinder™ software with Aria MX was used for instrument control, data acquisition and data processing. The internal standards (IS) shown above were used for quantitation by stable-isotope dilution technique.

Results

Identifying the HPLC Column and Conditions that Minimize Interferences

Because ISDs are as hydrophobic as phospholipids and phthalates, all are extracted and transferred to the HPLC column during the TurboFlow process. Therefore, the HPLC conditions must be optimized to elute the ISDs to the detector in a reasonable timeframe while avoiding co-elution of interferences as well as buildup of interfering compounds in the HPLC column while processing many samples. Figure 3 shows buildup and co-elution from non-optimized conditions, which resulted in poor reproducibility (RSDs > 20%) of peak areas for internal standards in sample batches. Figure 4 shows results from optimized conditions, which resulted in improved IS peak area reproducibility (RSDs < 10%).

FIGURE 3. Non-Optimized HPLC Conditions

Elution from Accucore PFP, 2.6 µm, 3.0 x 50 mm column:

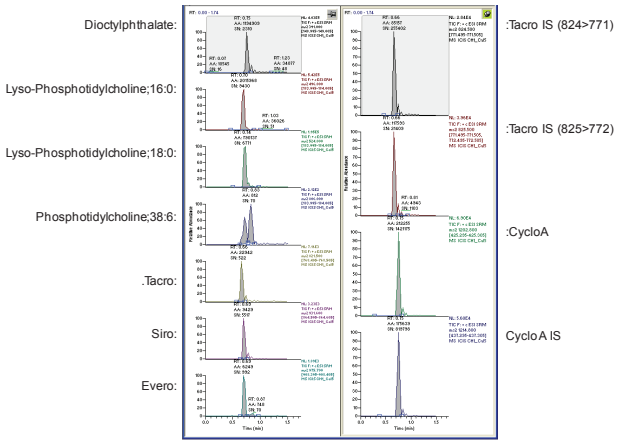
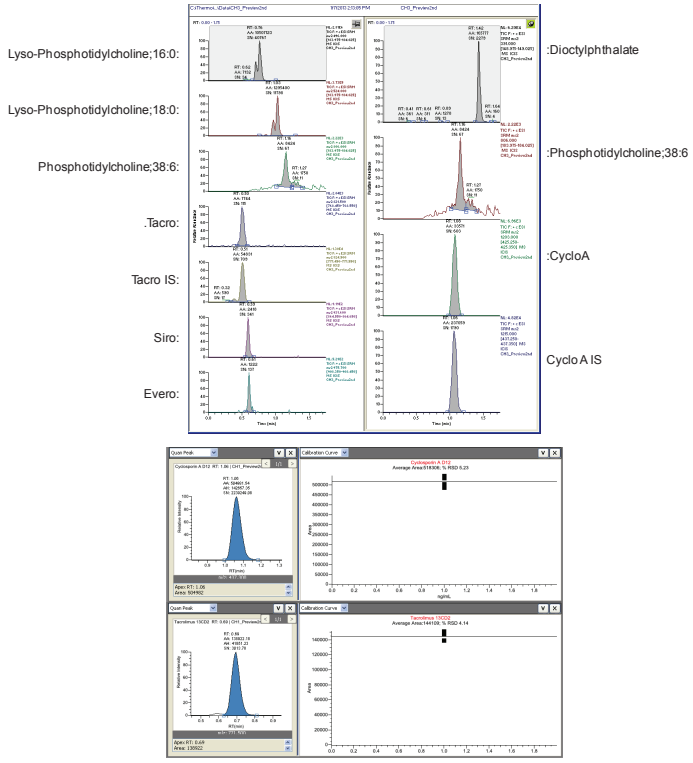


FIGURE 4. Optimized HPLC Conditions

Elution from Accucore C8, 2.6 µm, 3.0 x 30 mm column:



Achieving Required Linear Range with No Significant Carryover

As shown in Figures 5 and 6, the method consistently showed linear responses between 2.5 and 50 ng/mL for Everolimus, Sirolimus and Tacrolimus and between 25 and 1,250 ng/mL for Cyclosporin A. Weighting the data by 1/x minimized differences between expected and calculated concentrations in calibrators.

FIGURE 6. Everolimus Calibrators and QCs

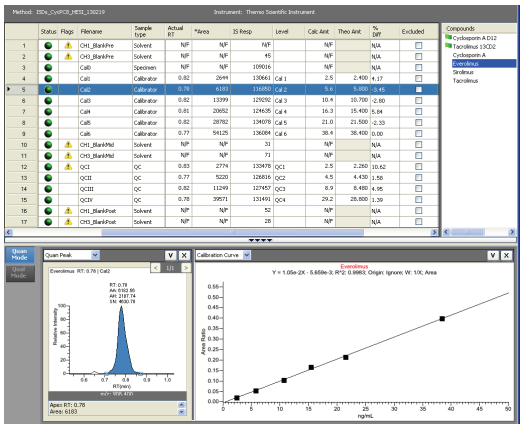


FIGURE 7. Cyclosporin A Calibrators and QCs

Reproducible QC Results were Reported Across 3 Different Test Sites.

As shown in Table 1, very similar results were reported from three different research test sites: Johns Hopkins University, Boston Children's Hospital and The Cleveland Clinic.

TABLE 1. Commercial Quality Control (QC) Reproducibility Results

	CyclosporinA			*Everolimus*		
Level	**Expected**	**Average**	**RSD%**	**Expected**	**Average**	**RSD%**
I	53	53	4.6	2.3	2.3	11.7
II	276	260	3.5	4.4	4.4	11.0
III	514	515	2.1	8.5	8.8	8.4
IV	1111	1172	6.4	28.8	28.6	6.1

	Sirolimus			*Tacrolimus*		
Level	**Expected**	**Average**	**RSD%**	**Expected**	**Average**	**RSD%**
I	2.9	2.9	8.5	2.6	2.8	5.3
II	10.1	10.0	4.6	7.3	7.1	6.1
III	20.4	20.6	5.2	16.7	16.4	4.1
IV	38.5	38.6	6.2	34.2	33.8	4.1

n=15 from 3 systems within 30 days

Matching Results from Legacy Method

As shown in Table 2, the Prelude method produced results that agreed with those produced by a legacy TurboFlow method for ISDs. Furthermore, the Prelude results were reproduced remarkably well from sample preparations that were almost 1 month old.

TABLE 2. Everolimus Calibrators and QCs

				Repeated						Repeated
		Ran on	1/9/2013	1/29/2013				Ran on	1/9/2013	1/29/2013
Test		Legacy	Prelude	Prelude		Test		Legacy	Prelude	Prelude
Sample	ISD	Method	Method	Method		Sample	ISD	Method	Method	Method
8KLE	Cyclosporin A:	86	105	103		120726-001	Everolimus:	3.5	3.0	4.5
8KBG	Cyclosporin A:	186	201	203		120726-002	Everolimus:	2.0	1.7	1.8
8KOU	Cyclosporin A:	84	99	93		120726-003	Everolimus:	2.0	1.8	2.0
8L20	Cyclosporin A:	80	81	75		120904-001	Everolimus:	4.0	4.3	3.9
8LB5	Cyclosporin A:	88	94	94		121227-001	Everolimus:	4.6	3.9	4.4
8JDF	Cyclosporin A:	168	176	176		121227-002	Everolimus:	2.3	2.2	2.5
8I6C	Cyclosporin A:	53	58	58		121227-003	Everolimus:	2.3	2.3	2.1
8KJNK	Sirolimus:	3.6	2.2	1.8		8LO5	Tacrolimus:	7.3	7.6	7.6
8KN6	Sirolimus:	3.0	1.2	2.0		8M3Y	Tacrolimus:	2.6	3.2	2.9
8L5K	Sirolimus:	8.4	9.5	7.3		8M4D	Tacrolimus:	12.5	11.1	12.5
8JB0	Sirolimus:	3.3	3.5	2.8		8M8F	Tacrolimus:	2.3	2.8	2.8
8GOC	Sirolimus:	14.4	12.5	10.9		8MI1	Tacrolimus:	16.2	15.0	17.9
8I27	Sirolimus:	3.2	2.5	1.9		8MDV	Tacrolimus:	8.9	8.8	9.6
86HF	Sirolimus:	5.7	5.5	4.2		8LRH	Tacrolimus:	20.0	17.7	19.0

Conclusion

Improved reliability and economy was achieved for ISD analysis for research purposes by using a novel SPLC-MS/MS system and method.

- Ion suppression of ISDs by co-eluting phospholipids was largely avoided by using the short Accucore C8 HPLC column.
- Using 1/x weighting, correlation coefficients (r2) > 0.995 were typical for:
  - Cyclosporin A, from 25 to 1250 ng/mL,
  - Everolimus, Sirolimus & Tacrolimus, from 2.5 to 50 ng/mL.
- Carryover, measured by peak areas corresponding to the ISDs from blank injections following the highest calibrators, was typically less than 0.1%.
- Reproducible ISD QC results were obtained from three research test sites evaluating this method with the PreludeSPLC-TSQ Vantage system.
- A reduction in solvent waste of about 40% was achieved, comparable to legacy TurboFlow methods for ISDs.

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