The Investigation of Factors Contributing to Immunosuppressant Drugs Response Variability in LC-MS/MS Analysis

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Overview

Purpose: To investigate the factors that cause variation in response of immunosuppressant drugs including Cyclosporin A, Tacrolimus, Sirolimus, and Everolimus. The type of extraction vial and cross-talk between analytes were investigated in this study.

Methods: The sample preparation for the immunosuppressants was performed manually to allow for all the variables to be tested. Each compound was spiked into whole blood and allowed to equilibrate for several minutes. Samples where lysed and the protein precipitated prior to analyses. A new Thermo Scientific Transcend system was equipped with a Thermo Scientific TurboFlow Cyclone-P column (50 x 0.5 mm) and a Thermo Scientific Accucore PFP analytical column (50 x 2.1 mm, 2.6 μm) for online sample clean-up. The detector for the system was a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer with a HESI-II source.

Results: There are many factors that cause variation in response for the immunosuppressant drugs. Adsorption to plastic and cross-talk between analytes are two factors that have a significant impact on the results.

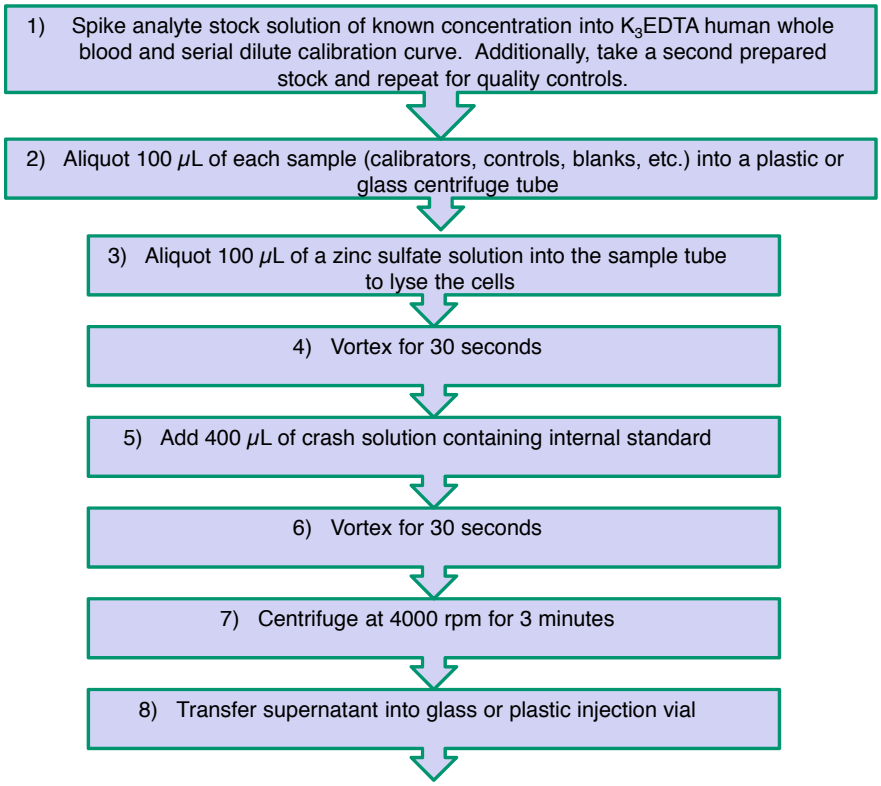
Introduction

The use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the analysis of immunosuppressant drugs (ISDs) is well established in the literature. The development of the reagent kits for ISDs (which includes Cyclosporin A, Sirolimus, Everolimus and Tacrolimus) has tremendously simplified sample preparation and quantitation for LC-MS/MS for routine analyses. Here we present data that is part of an ongoing investigation into the response variability of these compounds even when using existing reagent kits.

In order for a method to be successful it is subjected to a number of bioanalytical validation requirements. These include, but are not limited to, the lower limit of quantitation (LLOQ) and low quality control need to be ±20% of the expected concentration; all remaining calibrators and controls need to be ±15% of the expected concentration; and carryover response cannot exceed 20% of the LLOQ response. These criteria were used to evaluate the effect of various method parameters on the validation of methods for analyzing immunosuppressant drugs in whole blood.

Methods

Sample Preparation Workflow for Immunosuppressant Drugs



LC System: A new Transcend™ system that maximizes efficiency and minimizes solvent consumption.

Mobile Phases:

1) A: aqueous phase, 10 mM ammonium formate, 0.05% formic acid in water
2) B: organic phase, 10 mM ammonium formate, 0.05% formic acid in methanol
3) C: column wash, 45% isopropanol, 45% acetonitrile and 10% acetone

Needle washes:

1) 60% water, 40% methanol, and 0.5% formic (aqueous)
2) 45% isopropanol, 45% acetonitrile, and 10% acetone (organic)

Columns:

TurboFlow™ Cyclone-P column (50 x 0.5 mm) and Accucore™ PFP analytical column (50 x 2.1 mm, 2.6 μm) encased in a 70 ºC column heater

MS System: TSQ Vantage™ triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI II) probe in positive ion mode.

Results

The analytical measurement range for cyclosporin A was 10 to 2000 ng/mL. The range for Tacrolimus, Everolimus, and Sirolimus was 1 to 50 ng/mL. All quantitation was performed using Thermo Scientific LCQUAN Software.

Extraction Vials

Several types of extraction vials were compared for the immunosuppressant drugs. Plastic (treated and untreated vials) and glass (silanized and nonsilanized) vials were evaluated. Different combinations of each type of vial were tested to compare the variability in response of each of the compounds. Cyclosporin A had no response variability regardless of vial type. Tacrolimus also had very little response variability due to vial type. However, Everolimus and Sirolimus had significant variability between vial types. Table 1 shows two consecutive calibration curves with the calculated concentration and percent difference for Everolimus assayed in glass where Everolimus was prepared in silanized glass, extracted in silanized glass, and injected in silanized glass. The percent difference is listed to show the difference of the determined value from the expected value. Table 2 shows the representative data from Everolimus assayed in plastic where Everolimus was prepared in plastic, extracted in plastic, and injected in plastic. The results for the all plastic preparation show more variability than the results for the all glass preparation. Additionally, the calibration curves for the glass and the plastic preparation are plotted in Figures 3 and 4 respectively. The coefficient of variability (r^2) is 0.9977 for the glass vials and 0.9487 for the plastic. The r^2 for the glass vials pass validation criterion but the plastic vials do not.

Table 1. Two consecutive calibration curves for Everolimus assayed in glass.

Conc. (ng/ml)	Calculated Conc. (ng.ml)	% diff
1	1.00	0.0
2	2.50	-25.0
5	5.34	-6.8
10	10.25	-2.5
25	23.38	6.5
50	50.04	-0.1
1	1.12	-12.0
2	1.78	11.0
5	5.08	-1.6
10	9.19	8.1
25	24.61	1.6
50	52.21	-4.4

Table 2. Two consecutive calibration curves for Everolimus assayed in plastic.

Conc. (ng/ml)	Calculated Conc. (ng.ml)	% diff
1	1.35	-35.0
2	1.55	22.5
5	5.74	-14.8
10	11.47	-14.7
25	24.47	2.1
50	51.93	-3.9
1	0.82	18.0
2	1.72	14.0
5	5.27	-5.4
10	8.40	16.0
25	26.23	-4.9
50	47.03	5.9

FIGURE 3. Everolimus calibration curve with no internal standard in glass (pass).

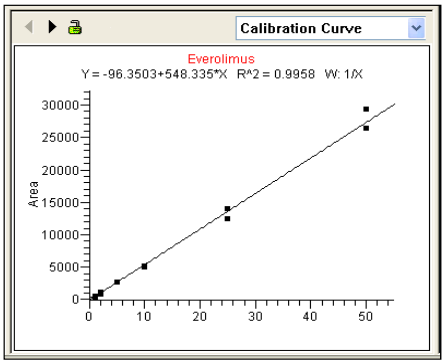
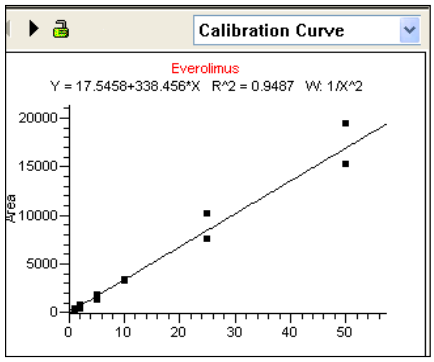


FIGURE 4. Everolimus calibration curve with no internal standard in plastic (fail).



Cross Talk

When Sirolimus is assayed alone, a signal is detected in the Everolimus transition. This can be seen in Table 5. Sirolimus expected concentrations are seen in the leftmost column with the respective responses shown in the middle. The right column shows the response generated in the Everolimus transition from Sirolimus. When Everolimus is assayed alone, signal is detected in the Sirolimus transition. This can be seen in Table 6. Everolimus expected concentrations are seen in the leftmost column with the respective responses shown in the middle. The right column shows the response generated in the Sirolimus transition from Everolimus.

Lastly, it is common practice in bioanalytical chemistry to use a labeled internal standard. For Tacrolimus, the internal standard Tacrolimus 13C-d2 was chosen initially. However, it was determined that there was cross-talk on the Tacrolimus analyte transition. Figure 7 (top) shows two consecutive Tacrolimus calibration curves, with the LLOQ's highlighted in red. Figure 7 (bottom) shows the response generated in the Tacrolimus transition when the Tacrolimus internal standard, Tacrolimus 13C-d2, is injected alone.

Individual Assay vs. Combined Assay

Immunosuppressant drugs are often assayed together in matrix. However, this can present some issues due to cross talk and other factors. Figure 8 shows the calibration curve and percent difference for Sirolimus prepared, extracted, and injected individually. Figure 9 shows the calibration curve and percent difference for Sirolimus prepared, extracted, and injected in the presence of the other immunosuppressant drugs.

Table 5. Two consecutive calibration curves for Sirolimus with Everolimus contribution.

Concentration (ng/ml)	Sirolimus Response	Everolimus Response
1	574	ND
2	1382	56
5	3211	ND
10	5791	49
25	15698	124
50	34372	391
1	722	ND
2	1026	ND
5	3187	41
10	6132	106
25	20624	325
50	43059	472

Table 6. Two consecutive calibration curves for Everolimus with Sirolimus contribution.

Concentration (ng/ml)	Everolimus Response	Sirolimus Response
1	390	ND
2	1130	ND
5	2698	54
10	5090	40
25	12541	63
50	26364	179
1	525	ND
2	909	ND
5	2756	ND
10	5136	ND
25	13989	36
50	29308	218

FIGURE 7. Tacrolimus 13Cd2 contribution to Tacrolimus (bottom) compared to Tacrolimus LLOQ (top)

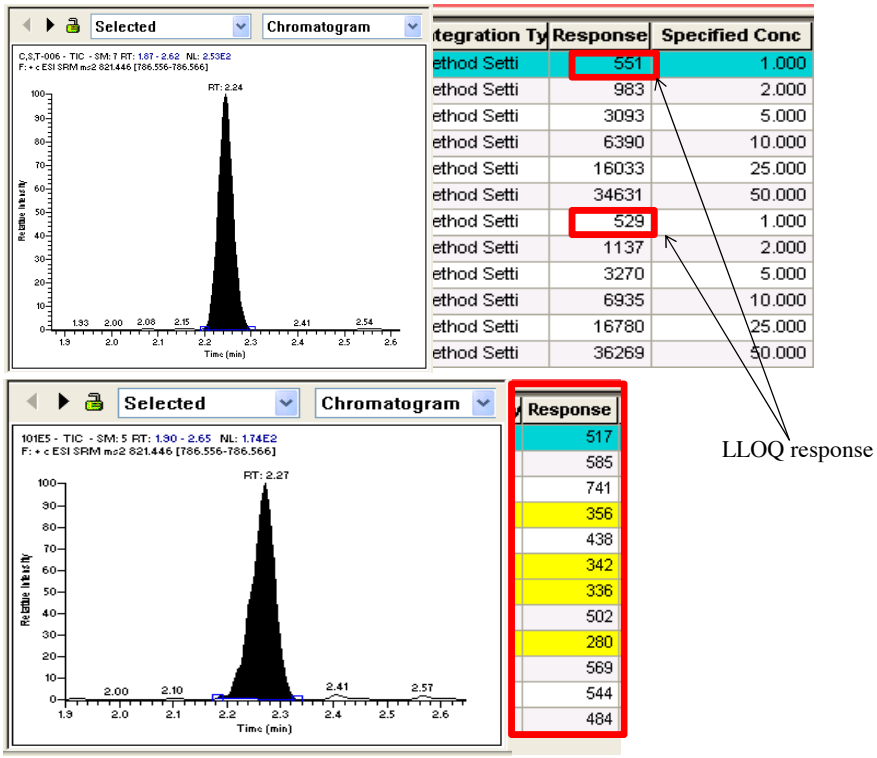


FIGURE 8. Sirolimus calibration curve and percent difference (individual prep).

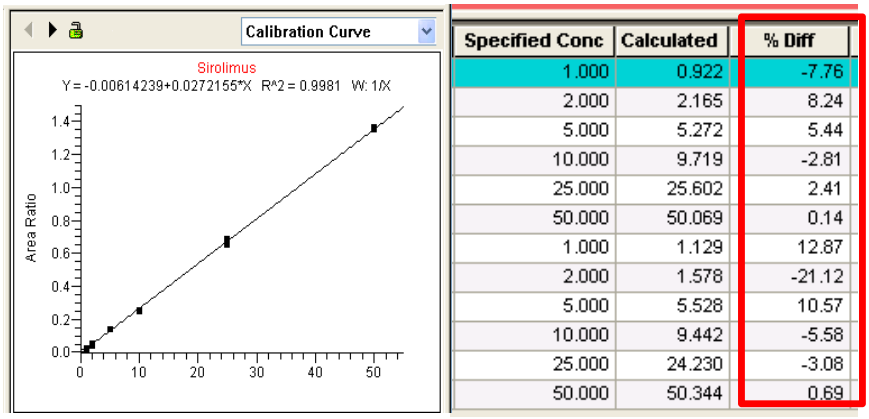


FIGURE 9. Sirolimus calibration curve and percent difference (combined prep).

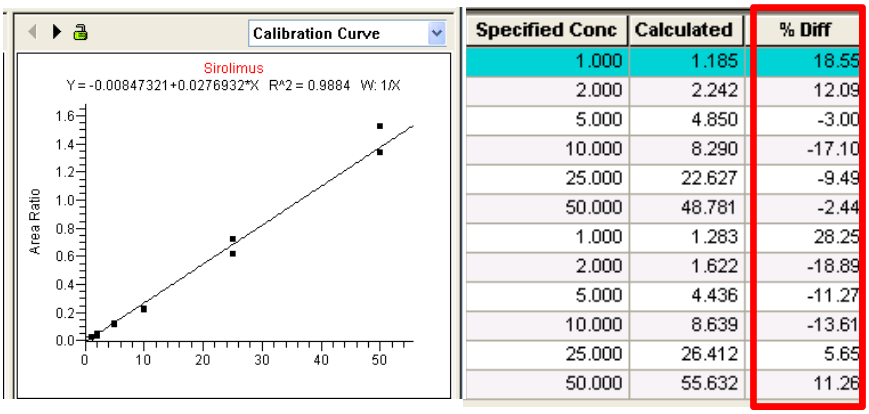
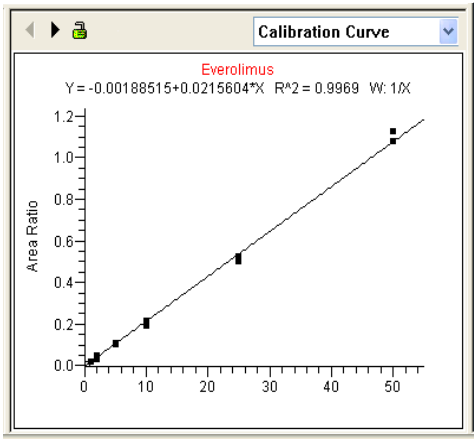


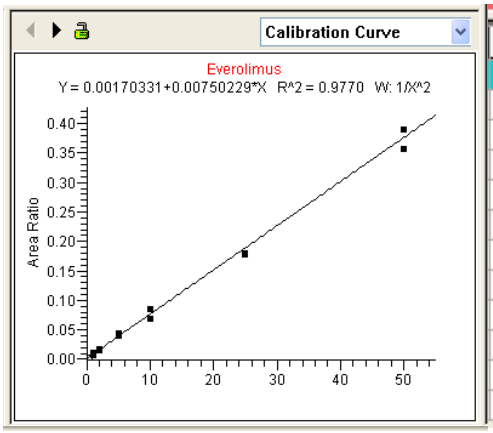
Figure 10 shows the calibration curve and percent difference for Everolimus prepared, extracted, and injected individually. Figure 11 shows the calibration curve and percent difference for Everolimus prepared, extracted, and injected in the presence of the other immunosuppressant drugs. For Sirolimus and Everolimus, less variability is observed when assayed alone then when assayed in combination with the other immunosuppressant drugs. The effect can be seen from the r^2 values, the calibration curve plots, or the percent differences.

FIGURE 10. Everolimus calibration curve and percent difference (individual prep).



Specified Conc	Calculated	% Diff
1.000	0.925	-7.51
2.000	2.434	21.68
5.000	5.279	5.57
10.000	10.197	1.97
25.000	23.351	-6.60
50.000	50.070	0.14
1.000	1.048	4.84
2.000	1.708	-14.61
5.000	5.014	0.27
10.000	9.139	-8.61
25.000	24.588	-1.65
50.000	52.248	4.50

FIGURE 11. Everolimus calibration curve and percent difference (combined prep).



Specified Conc	Calculated	% Diff
1.000	0.793	-20.65
2.000	1.797	-10.15
5.000	5.789	15.77
10.000	11.086	10.86
25.000	23.881	-4.48
50.000	51.658	3.32
1.000	1.209	20.88
2.000	2.052	2.62
5.000	5.212	4.23
10.000	8.918	-10.82
25.000	23.457	-6.17
50.000	47.294	-5.41

Conclusion

- Everolimus and Sirolimus can be validated when analyzed separately but not analyzed successfully together because there is cross-talk. This is most likely due to Sirolimus impurities in the Everolimus since Sirolimus is used as the starting material for Everolimus. Either compound can be analyzed with Cyclosporin A.
- Tacrolimus 13C-d2 contributes to the Tacrolimus transition; therefore, it is a poor choice of internal standard. Here again it is most likely due to impurities during synthesis.
- The material of the assay tube (i.e. plastic, glass, etc.) plays a role in the success or failure of the assay for Everolimus and Sirolimus. Both of these analytes prefer silanized glass for preparation and injection suggesting absorption issues with plastic containers.

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