Variables that Impact Immunosuppressant LC-MS/MS Analysis

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1. Overview

Purpose

- To investigate LC-MS/MS variability with immunosuppressants

Methods

- UHPLC-MS/MS and HPLC-MS/MS for analysis of multi and single component solutions

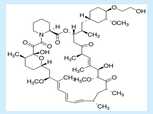
Results

- For concentrations below 5 µg/mL sample to sample concentration variability was observed for everolimus, tacrolimus, and sirolimus. This was linked to sample container/surface interactions and volume per container at low concentrations.

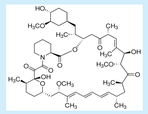
2. Introduction

Clinical analysis of immunosuppressants by LC-MS/MS can be challenging because patient samples are in whole blood and require extensive sample preparation. In addition, the large size of these molecules makes obtaining reasonable peak shape chromatographically challenging. LC-MS/MS immunosuppressant method development revealed extensive sample to sample variability for everolimus, sirolimus, and tacrolimus. Several parameters such as sample preparation, MS interference, and surface interaction between the compounds and the glass sample containers were investigated to determine the cause of the observed analytical variability.

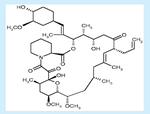
3. Methods



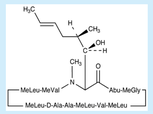
Everolimus



Sirolimus



Tacrolimus



Cyclosporin A

1) Multi-component spiking solutions containing sirolimus, everolimus, tacrolimus, and cyclosporin A were prepared in acetonitrile at 6 concentration levels ranging from 12.5-1500 ng/mL for tacrolimus, everolimus, and sirolimus, and 250-50,000 ng/mL for cyclosporin A.
   - LCMS system: Agilent 1290 UHPLC with 6460 tandem MS system
   - Column: Phenomenex Kinetex 1.7µm C8, 2.1x50mm column
   - Mobile Phase: 0.1% formic acid in H_2O:MeOH, gradient from 40:60 to 2:98 at 0.4 mL/min
   - MSMS Transitions:

Compound Name	Precursor Ion	Product Ion	Fragmentor	Collision Energy
Cyclosporin D	1239	1239	250	5
Cyclosporin A	1225	1225	250	5
Everolimus-D_4	984.6	393.2	280	58
Everolimus	980.6	389.2	350	62
Sirolimus	936.5	409.3	300	58
Tacrolimus	826.5	616.3	220	34
Ascomycin	814.5	604.3	220	34

2) Single component solutions of the above analytes were prepared in acetonitrile at 3 µg/mL for tacrolimus, everolimus, and sirolimus, and 15 µg/mL for cyclosporin A concentrations
   - LCMS system: Agilent 1100 HPLC with 6410 tandem MS system
   - Column: Waters XSelect CSH C18 3.5µm 2.1x10mm Guard Cartridge
   - Mobile Phase: 0.1% formic acid in H_2O: 0.1% formic acid in MeOH, gradient from 50:50 to 1:99 with a flow rate of 0.5 mL/min
   - MSMS Transitions:
     - Sirolimus: 936.5 > 409.3, Everolimus-D_4 (IS): 984.6 > 393.2
     - Everolimus: 980.6 > 389.2, Everolimus-D_4 (IS): 984.6 > 393.2
     - Tacrolimus: 826.5 > 616.3, Ascomycin (IS): 814.5 > 604.3
   - SIM Analysis
     - Cyclosporin A: 624.2, Cyclosporin D (IS): 631.2

4. Sample to Sample Variability in Analysis of Multi-Component Immunosuppressants in Solution

A series of immunosuppressant multi-component spiking solutions consisting of cyclosporin A, everolimus, sirolimus and tacrolimus were prepared in acetonitrile at six concentrations from certified single component 1 mg/mL stock solutions and filled to 0.2 mL, in amber ampoules. The concentration of each of the 6 calibrator levels was verified against an independently prepared check standard, testing five samples per level with duplicate injections of each sample. For cyclosporin A no sample to sample variability was observed and RSDs were below 2% across all sample analyzed for each of the 6 calibrator levels. For everolimus, sirolimus, and tacrolimus significant sample to sample inconsistency was observed at each concentration level. Analyzed concentration of the replicate injections of the same sample had RSDs ≤ 2%, but overall RSD's across the 5 samples analyzed per level were as high as 10%. The multi-component solution standards were remade and tested. When analyzed immediately after dispensing, no sample to sample variability was detected. However, after even a few days of storage in the freezer or sub freezer the sample to sample concentration variability was observed.

For the1 mg/mL certified single component stock solutions no sample to sample variability was detected for any of the 4 immunosuppressants, which have demonstrated stability of ~36 months at sub-freezer storage.

The 10% variability in the spiking solutions was a concern because this can contribute to variability in clinical end-use results.

Cyclosporin A 50,000 ng/mL

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Avg Conc (ng/mL)	50554	51128	50925	51145	52235
%RSD (per sample)	0.00	0.09	0.22	0.21	0.56
% RSD (overall)	1.18				

Cyclosporin A 250 ng/mL

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Avg Conc (ng/mL)	251.7	250.9	249.1	252.9	249.9
%RSD (per sample)	0.75	0.58	0.24	1.12	0.03
% RSD (overall)	0.75				

Everolimus 1,500 ng/mL

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Avg Conc (ng/mL)	1436	1272	1444	1300	1535
%RSD (per sample)	0.84	0.46	0.79	0.43	0.70
% RSD (overall)	7.38				

Everolimus 25 ng/mL

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Avg Conc (ng/mL)	22.29	21.93	20.55	21.68	20.62
%RSD (per sample)	2.42	0.01	1.20	0.10	0.93
% RSD (overall)	3.59				

Sirolimus 1,500 ng/mL

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Avg Conc (ng/mL)	1470	1299	1452	1323	1532
%RSD (per sample)	0.75	0.66	0.84	0.67	0.21
% RSD (overall)	6.66				

Sirolimus 25 ng/mL

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Avg Conc (ng/mL)	24.41	22.26	22.52	22.43	21.88
%RSD (per sample)	0.92	1.80	0.69	2.28	1.35
% RSD (overall)	4.25				

Tacrolimus 750 ng/mL

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Avg Conc (ng/mL)	716.1	711.0	683.8	678.9	653.3
%RSD (per sample)	0.12	0.47	1.67	0.28	0.16
% RSD (overall)	5.55				

Tacrolimus 125 ng/mL

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Avg Conc (ng/mL)	124.3	112.6	110.8	124.2	124.7
%RSD (per sample)	0.17	0.22	0.10	0.05	0.06
% RSD (overall)	3.50				

Investigation

- In order to investigate the high RSD's observed for Sirolimus, Tacrolimus, and Everolimus; sample handling and analytical methods were evaluated and tested to see what factor(s) were influencing variability.
- Parameters investigated included:
  - Transitions (SIM vs MRM)
  - Use of Internal Standard
  - Sonication of Samples Following Storage
  - Fill volume (0.2 mL vs. 1 mL)
  - How Internal Standard was Added (Manual vs Automated addition by using injector program)

SIM vs MRM

	Everolimus	Sirolimus	Tacrolimus
MRM %RSD	2.34	2.74	6.40
SIM %RSD	2.02	2.10	5.70

IS vs no IS

	Everolimus	Sirolimus	Tacrolimus
IS %RSD	5.17	4.65	2.78
no IS %RSD	4.69	4.38	2.44

Manual vs Automated IS addition

	Everolimus	Sirolimus	Tacrolimus
Manual IS %RSD	8.38	7.45	5.55
Auto IS %RSD	6.41	6.14	9.10

Sonication vs no Sonication

	Everolimus	Sirolimus	Tacrolimus
Sonication %RSD	9.30	9.44	2.52
no Sonication %RSD	7.54	7.10	2.18

Fill volume comparison

	Everolimus	Sirolimus	Tacrolimus	Cyclosporin A
Concentration (ng/mL)	25	25	12.5	250
0.2 mL Fill %RSD	5.70	7.10	3.80	0.60
1 mL Fill %RSD	3.10	3.40	3.80	0.70

- Investigation of mobile phase, column, gradient, autosampler vial vendor, and dispensing temperature effects were also evaluated to see if modifying these parameters could mitigate the observed variability. No impact to the variability was observed.
- Cyclosporin A was consistent regardless of parameter modification.
- The check standard was observed to be consistent for all components and was filled to 1 mL in 2 mL ampoules.
- The largest contributor to high sample to sample variability for sirolimus, everolimus and tacrolimus appeared to be related to fill volume. The multi-component solutions were stored in 0.2 mL unit volumes, with a larger glass surface to solution ratio than a typical 1 mL fill volume. Non-specific binding to glass could be a contributor to the high %RSD's observed.
- Another contributor could be compound to compound interactions.

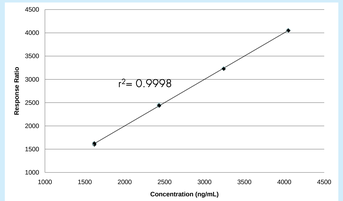
5. Single Component Solution Sample to Sample Variability

In order to remove any compound to compound interaction variability, single component solution standards were prepared in acetonitrile at 3,000 ng/mL for everolimus, sirolimus and tacrolimus, and 15,000 ng/mL cyclosporin A. A four point calibration curve was prepared for each of the 4 single component solutions. Triplicate injections were made from each calibration curve point. In addition, curve fill volumes were tested at both 1 mL into a 2 mL ampoule and 5 mLs into a 5 mL ampoule to see if there is an effect on solution fill volume.

Investigation of linearity of each compound across the 4 calibration points:

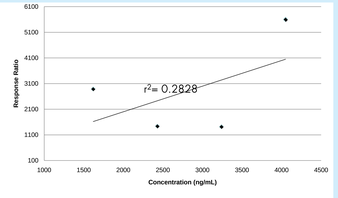
- Cyclosporin A had good linearity irrespective of fill volume or fill volume accuracy.
- Some of the 1 mL fill volume curves were dispensed by pipette into ampoules at different fill volumes. For curves that had equivalent fill volumes, the $r^2 \geq 0.99$; for curves that had their fill volumes varied by 10 - 20%, the r^2 was ≤0.98. These results suggested a fill volume to surface area impact on concentration variability.
- The variability was most pronounced for tacrolimus. A tacrolimus curve was prepared and dispensed 1 mL into a 2 mL ampoule and 5 mL into a 5 mL ampoule to investigate surface interactions.

Tacrolimus
Consistent 5 mL fill



Surface area to volume ratio volume was 5.4:1cm²:mL

Tacrolimus
Variable1mL fill



Surface area to volume ratio volume was 5.4:1cm²:mL

A correlation was observed between fill volume and linearity and also high RSD's at low concentrations. Higher concentration standards do not exhibit this sensitivity.

6. Conclusions and Discussion

- Low concentration (ng/mL to low µg/mL range) solutions of sirolimus, tacrolimus, and everolimus intended for use as spiking solutions can exhibit higher sample to sample RSD's which could contribute to variability in clinical test results. The variability appears to be due to a combination of analyte interactions with glass and/or with each other, and fill volume.
- Cyclosporin A was 10 fold higher in concentration than the other analytes and was stable and consistent at all concentrations tested. Cyclosporin A was not influenced by any of the parameters evaluated including other immunosuppressants and container fill volume.
- The analytical variability observed with Sirolimus, Tacrolimus, and Everolimus does not appear to be a result of the analytical method or sample processing.
- The single component investigation indicated that both surface area and solution volume may play a significant role in the variability observed with low concentration immunosuppressant solutions. Consistent fill volume reduced variability.
- Higher fill volumes can reduce variability; 5 mL fill volumes resulted in linear calibration curves for low concentration immunosuppressant solution standards.
- Higher concentration 100 µg/mL and 1 mg/mL single component solution standards are consistent and stable and do not exhibit sample to sample variability.

Surface interactions can impact LC-MS/MS results

Especially for low volume, low concentration immunosuppressants solutions

Special consideration should be applied during preparation & storage of spiking solutions used in the preparation of matrix calibrators