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 accordance with six concentrations from certified single component 1 mg/mL stock solutions and filled to 0.2 mL in amber vials. The concentration of each of the 6 calibration 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 variation was observed and RSDs were below 2% across all sample analyzed for each of the 6 calibration levels. For everolimus, sirolimus, and tacrolimus significant sample to sample variability was observed at each concentration level. Analyzed concentration of the replicate injections of the same sample had RSDs ≤ 2%, but overall RSDs across the 5 samples analyzed per level varied as high as 10%. The multi-component solution standards were reanalyzed and retested. When analyzed immediately after dispensing, no sample to sample variability was detected. However, over a few days of storage in the freezer or sub freeze the sample to sample concentration variability was observed. For the RSD of 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.

In order to investigate the high RSD’s observed for IS vs no IS Product Ion:

- Cyclosporin A
- Everolimus
- Sirolimus
- Tacrolimus

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 challenging. LC/MS/MS immunosuppressant method development revealed serious sample to sample variability for everolimus, sirolimus, and tacrolimus. Several parameters such as sample preparation, IS 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

1) Multi-component spiking solutions containing sirolimus, everolimus, tacrolimus, and cyclosporin A were prepared in accordance 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 C18 2.1x100mm column
- Mobile Phase: 0.1% formic acid in H2O:MeOH, gradient from 40:60 to 98:0 at 0.4 mL/min
- MSMS Transitions:
  - Cyclosporin A: 624.2 > 250
  - Everolimus: 980.6 > 389.2
  - Sirolimus: 936.5 > 409.3

- SIM vs MRM

- In order to investigate the high RSD’s observed for sirolimus, tacrolimus, and everolimus, sample handling and analytical methods were evaluated and found to be what behavior was influencing variability.

- Parameters investigated included:
  - Transition (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 project program)

- Manual vs Automated IS addition
- Sonication vs no Sonication

In order to investigate the high RSD’s observed for sirolimus, tacrolimus, and everolimus, sample handling and analytical methods were evaluated and found to be what behavior was influencing variability.

Investigation of mobile phase, column, gradient, autosampler vial self, and dispensing temperatures 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 amber vials

- 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 at 0.2 mL unit vials, with a larger glass surface to solution rate 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 interactions.

4. Conclusion

- Low concentration ng/mL to low µg/mL range solutions of sirolimus, tacrolimus, and everolimus intended for use as spiking solutions could contain high levels of pseudos for low RSD, which could contribute to variability in clinical end-use results.

- The variability was most pronounced for tacrolimus. A tacrolimus curve was prepared and dispensed 3 mL into a 2 mL amber vial and filled 5 mL into a 5 mL amber vial to investigate surface interactions.

- 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.

5. Single Component Solution to Sample Variability

In order to remove any compound to compound interaction variability, single component solution standards were prepared in accordance at 3,000 ng/mL for everolimus, sirolimus and tacrolimus, and 15,000 ng/mL cytochalasin 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 amber vial and 5 mL into a 5 mL amber vial 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 a good linear response of fill volume vs 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 R2 (0.99) for curves that had fill volumes varied by 10 - 20%, the R2 was not consistent. These results suggested fill volume to surface area impact on concentration variability.

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 could contain high levels of pseudos for low RSD, which could contribute to variability in clinical end-use results. The variability appears to be due to a combination of analyte interactions with glass and to work with sub-volume and fill accuracy.

- Cyclosporin A was hold 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 contains 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 play a significant role in the variability observed with low concentration immunosuppressant solutions. Consistent fill volume reduced variability.

- Higher fill volume could reduce variability, 5 mL fill volumes resulted in linear calibration curves for low concentration immunosuppressant solution standards.

- Higher concentration 100 ng/mL to 1 mg/mL single component solution standards are consistent and stable and do not exhibit sample to sample variability.

Variables that impact ICAMS/MS results

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