Certified Spiking Solutions of Immunosuppressant Drugs

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Introduction

Immunosuppressant drugs such as Sirolimus, Tacrolimus, Cyclosporin and Mycophenolate mofetil have a narrow therapeutic index. In addition, they exhibit a wide range of inter-individual pharmacokinetic variability.[1]

Careful individual monitoring of drug levels in blood is required to optimize prevention of organ rejection while minimizing side effects.

Accurate quantitation of patient drug levels requires robust analytical methods and high quality reference standards. Due to the variability of traditional immunoassay methods, LCMSMS methods have been developed for patient monitoring of these critical therapeutic drugs. There is a need for high quality reference materials with certified values suitable for use in preparation of controls and as calibrators and tuning standards.

Sirolimus, Tacrolimus, and Cyclosporin are complex peptide macromolecules. The materials are toxic and unstable in solution presenting handling and stability challenges.

The goal was to develop high quality certified spiking solutions suitable for use in therapeutic drug monitoring assays and for use as calibration and tuning standards. These pre-made stock solutions would be highly accurate with certified values (not ranges), and would provide convenience of use in a shelf stable format.

1. Kahan, B.D. et al Clinical Therapeutics, 2002, 24, 330-350.

Design & Preparation of Immunosuppressant Certified Spiking Solutions

- Process Controls: Reference materials are prepared and certified to ISO Guide 34 and ISO/IEC 17025. Extensive and robust process controls ensure accuracy of weighing and dilution operations, homogeneity, and provide assurance against contamination as well as operator safety in handling toxic materials.
- Certification includes verification of solution concentration, ampoule-to-ampoule consistency in concentration and verification of solution purity by HPLC and LCMS.
- Spiking solutions are packaged under inert atmosphere in flame sealed ampoules.
  - Eliminates degradation caused by exposure to oxygen
  - Eliminates concentration changes due to evaporation of solvent
  - Single use format eliminates contamination concerns

Specific considerations for Immunosuppressants:

- Neat material characterization due to hygroscopicity.
- Stability and safe handling of both neat materials and solutions.

Certification of Immunosuppressant Neat Materials

Certified Values					
Compound	Chrom. Purity (%)	Residual Water (wt %)	Residual Solvent (wt %)	Residual Inorganic (wt %)	Purity Factor for Quantitative Use (%)
Sirolimus	98.755	0.106	0.009	BQL*	98.64
Tacrolimus	98.636	1.858	None detected	BQL*	96.80
Cyclosporin	99.786	0.408	0.040	BQL*	99.34
Mycophenolic acid	99.003	0.026	0.022	BQL*	98.96

*BQL: Below Quantitation Limit <0.2%

- Complete characterization of all neat materials is performed vs. reliance on vendor certified values.
- A mass balance equation is used to account for chromatographic and residual impurities and calculate the amount of material needed to accurately prepare a certified spiking solution at a specified concentration. Purity factor = (100-%water-%solvent-%inorganic)x(Chrom Purity)/100).
- Sirolimus, Tacrolimus and Cyclosporin are peptide macromolecules – likely to contain residual water. Absorption of moisture over time will impact subsequent weighing of the material and must be re-evaluated prior to use in quantitative applications.

Residual impurities such as water, residual solvent, and trace inorganic impurities must be accounted for when weighing neat reference materials for quantitative applications. Chromatographic purity, when used alone, may yield an inaccurate solution.

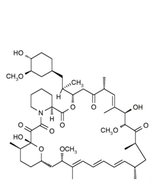
Evaluation of Solvents, Material Handling, Stability and Storage

- Solutions were prepared in methanol and/or acetonitrile.
- Samples were handled under inert atmosphere in a glove box to control exposure to humidity and oxygen; and for operator safety due to their toxic nature.
- Solutions were ampouled under inert atmosphere and stored at ambient, refrigerate (2 to 8 C), freezer (-10 to -25 C) and sub-freezer (-60 to -80 C) conditions.
- Purity was evaluated for each of the solutions for 4-8 weeks.

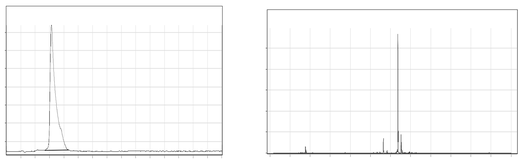
Initial observations

- Sirolimus and Tacrolimus are highly unstable in methanol.

Sirolimus

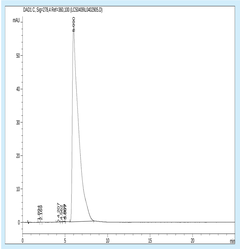


LCMS

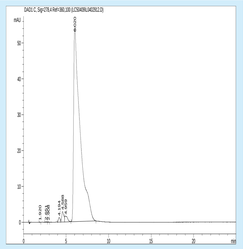


Analysis Method: HPLC
Column: Agilent Eclipse XDB-C18 4.6x150
Mobile Phase: 65:35
Acetonitrile: 0.1%TFA in water
Flow Rate: 1.8 ml/min
Wavelength: 278 nm
Column Temp: ambient

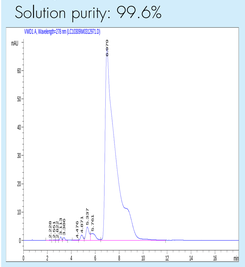
Stability of Sirolimus in Methanol



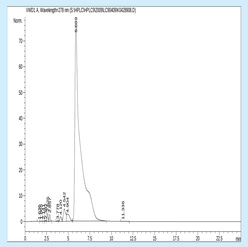
Freshly prepared solution
Time 0
Solution purity: 99.6%



Ambient; 4 hours
Solution purity: 96.4%



Freezer; 1 week
Solution purity: 95.0%

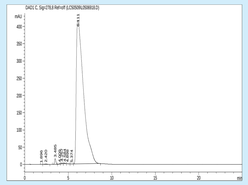


Freezer; 2 months
Solution purity: 93.5%

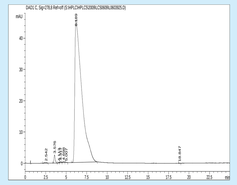
- Concentration: 1 mg/mL.
- Solution degradation:~3% after 4 hours at ambient temperatures.
- Shoulder peak observed along with early eluting impurities, indicative of isomerization and/or degradation in solution.
- ~1.5% degradation observed in the solution stored in freezer for 8 weeks.

Rapid degradation in methanol

Stability of Sirolimus in Acetonitrile



Time 0
Solution Purity: 98.8%



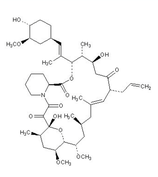
Freezer; 4 weeks
Solution Purity: 98.5%

Stability Assessment: Purity of Sirolimus in Acetonitrile			
Testing Interval	Refrigerator	Freezer	Sub-freezer
Time 0	98.9		
1 Week	98.3	99.1	99.1
2 Weeks	96.6	98.7	98.2
4 Weeks	95.0	98.5	98.8
4 Months	-	-	99.0

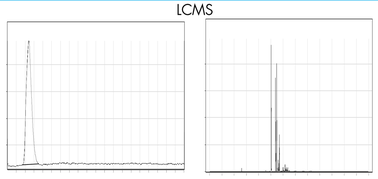
- Stable during routine analysis under ambient conditions (3 hours).
- Degrades to 96% within 2 weeks in the refrigerator.

Stable in freezer and sub-freezer

Tacrolimus



LCMS

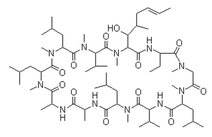


Analysis Method: HPLC
Column: Eclipse XDB-C18 150x4.6 mm
Mobile Phase: A: 88:12 Methanol/0.2% aq. TFA ; B: 0.1% TFA in water; C: Acetonitrile
Flow Rate: A:1.2; B: 1.8 mL/min
Wavelength: 254 nm

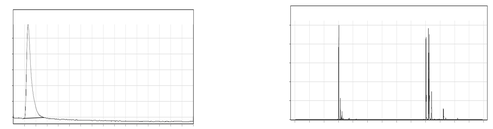
Stability Assessment: Purity of Tacrolimus in Acetonitrile						
Method (Mobile Phase)	100%A			65:35 C:B		
Testing Interval	Refrigerator	Freezer	Sub-freezer	Refrigerator	Freezer	Sub-freezer
Time 0	99.9			98.5		
1 Week	99.1	99.8	n/a	97.3	98.2	n/a
2 Weeks	99.0	99.2	98.8	97.9	98.0	97.7
4 Weeks	99.9	99.7	100.0	98.3	97.9	98.3

- Acetonitrile was selected because Tacrolimus demonstrated instability in methanol with degradation similar to Sirolimus.
- Concentration: 1 mg/mL.
- Evaluation by two HPLC methods illustrates the importance of method selection to ensure resolution of impurities.

Cyclosporin



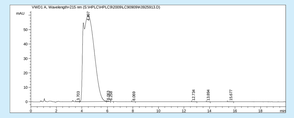
LCMS



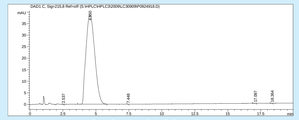
Stability Assessment: Purity of Cyclosporin				
Testing Interval	Acetonitrile (500 µg/ml)		Methanol (500 µg/ml)	
Time 0	99.6		99.9	
Storage	Refrigerator	Freezer	Refrigerator	Freezer
2 Weeks	99.6	99.7	99.8	99.7
3 Weeks	99.8	99.8	99.9	99.9
4 Weeks	99.9	99.9	99.9	99.9

Analysis Method: HPLC
Column: Synergi Polar RP 4.6x250 mm
Mobile Phase: 65:35
Acetonitrile: Water
Flow Rate: 2.0 ml/min
Wavelength: 215 nm
Column Temp: ambient

- Cyclosporin demonstrated stability in both methanol and acetonitrile.
- Concentration was evaluated at 500 and 100 µg/mL and determined not to be a factor in stability.
- Possible isomerization (a shoulder peak) was noted when analyzed on VWD detector.
- This was not observed using a PDA detector due to lower detector sensitivity. Isomerization of cyclosporin in solution is known in the literature.

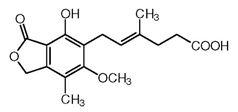


Cyclosporin, VWD detector

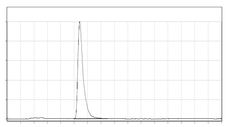


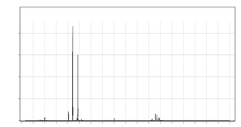
Cyclosporin, PDA detector

Mycophenolic Acid



LCMS





Stability Assessment: Purity of Mycophenolic Acid in Acetonitrile			
Testing Interval	Ambient	Refrigerator	Freezer
1 Week	98.7	99.0	99.0
2 Weeks	96.9	98.9	99.0
8 Weeks	98.9	98.9	98.9

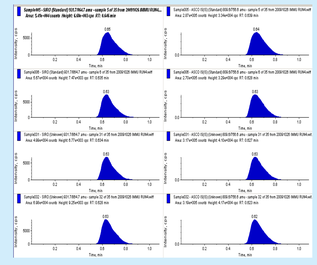
Analysis Method: HPLC
Column: Zorbax Eclipse SB-C8 4.6x250 mm
Mobile Phase: Gradient
Acetonitrile: 0.1% aq. phosphoric acid
Flow Rate: 1.2 ml/min
Wavelength: 215 nm

- Acetonitrile chosen due to observations documented with Sirolimus and Tacrolimus.
- Concentration: 1 mg/mL.
- Mycophenolic Acid demonstrated stability under various storage conditions in acetonitrile although degradation was observed at 40°C.

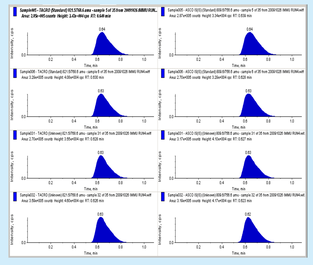
Evaluation in a 3rd Party Clinical Laboratory

- Sirolimus and Tacrolimus ampouled spiking solutions were evaluated by an independent third party clinical laboratory and used in preparation of quality control standards in comparison to standards prepared using the laboratory's house made stock solutions.
- Concentration range: 2 to 50 ng/mL in bovine blood.
- Top two rows: Using laboratory's in-house stocks; Bottom two rows: Using Cerilliant Certified Spiking Solutions™.

Sirolimus 40 ng/mL



Tacrolimus 40 ng/mL



CONCLUSIONS

- Accuracy and stability of reference standards is critical in the quantitation of drugs with a narrow therapeutic index.
- LCMSMS methods have been developed to achieve higher accuracy and greater specificity in patient monitoring of immunosuppressants drugs.
- Typical in-house preparation of standards for LCMSMS methods begins with preparation of stock/spiking solutions from neat materials.
- Certified Spiking Solutions™ of critical immunosuppressant drugs Sirolimus, Tacrolimus, Cyclosporin and Mycophenolic Acid were successfully prepared in an ampouled single use format.
- Solvent selection and storage were determined to be critical to short and long term stability.
- Ampouled format protects against degradation and changes in concentration.
- Use of pre-made solutions eliminates handling of toxic neat materials in the laboratory.

Cerilliant®
Analytical Reference Standards

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