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Nam Nguyen, Ph.D.
Science Editor
Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA 02140

March 29, 2015

Re.: Our manuscript JoVE52424R3 entitled "Quantification of the Immunosuppressant Tacrolimus on Dried Blood Spots Using LC-MS/MS."

Dr. Nguyen:

Please find the revision of our above-mentioned manuscript attached.

Below you will find a list of changes and our response to the reviewers' comments.

Respectfully,

Uwe Christians, MD, PhD
Professor and Laboratory Director
Facharzt für Pharmakologie und Toxikologie
Facharzt für Klinische Pharmakologie
Diplomate, American Board of Clinical Pharmacology
Master in Research Quality Assurance (BARQA)

List of Changes

Editor's comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
DONE.

2. Please reduce the repetition between the Short and Long Abstract.
DONE.

3. How are the ions detected in step 3.6?
THIS INFORMATION WAS ADDED TO 3.5.

4. Please provide conditions for steps 2.6 and 2.7. (RPM, speed setting, etc.)
DONE.

5. Step 3.7 is an action step and not a note. Please revise.
THE ACTION INFORMATION IN 3.7. WAS MOVED TO 3.5. AS THIS IS MORE IN LINE WITH THE WORKFLOW.

6. Step 4.1 needs more detail or a reference.
THE COMPLETE SECTION 4 WAS REWRITTEN.

Reviewers' comments:

Reviewer #1:
Major Concerns:
N/A

Minor Concerns:
1. The authors must compare the merits of their methods with those reported in literature.
THIS IS DISCUSSED IN DETAIL IN THE PARAGRAPH STARTING ON LINE 583. IT IS IMPORTANT TO NOTE THAT NONE OF THE NUMEROUS TACROLIMUS DRIED BLOOD SPOT ASSAYS DESCRIBED IN THE LITERATURE WAS TRUELY NOVEL AS MOST OF THESE WERE VARIATIONS OF TACROLIMUS WHOLE BLOOD ASSAYS. AS OUTLINED IN SAID PARAGRAPH, THE PRESENT METHOD UNIQUELY COMBINES SAMPLE PROCESSING STRATEGIES RESULTING IN A RELIABLE ROUTINE ASSAY, WHICH HAS SUCCESSFULLY BEEN APPLIED TO THE ANALYSIS OF MORE 5000 DRIED BLOOD SPOT SAMPLES WITHOUT LOOSING A SINGLE SAMPLE.

2. Tables for intra-day and inter-day accuracy and precision and stability can be condensed by presenting mean values at different QC values.
WE HAVE DISCUSSED THIS SUGGESTION AMONG THE AUTHORS AND FELT THAT IT WILL BE IMPORTANT TO REPORT INDIVIDUAL RESULTS IN COMBINATION WITH THE APPROPRIATE DISTRIBUTION STATISTICS ANALYSIS. THE RATIONALE IS THAT THE GOAL OF THIS PUBLICATION AND ITS

SUPPORTING VIDEO COMPONENT IS TO ENABLE THE READER TO REPRODUCE OUR ASSAY. IN OUR OPINION THIS PUBLICATION PLATFORM IS A UNIQUE OPPORTUNITY TO GIVE THE INTERESTED READER A FEEL FOR WHAT THE EXPERIMENTS LOOK LIKE IN REAL LIFE AND WHAT DATA TO EXPECT, INCLUDING OUTLIERS AND FAILED ANALYSES. THIS IS IMPORTANT INFORMATION THAT TENDS TO GET LOST IN A DISTRIBUTION STATISTICAL ANALYSIS WITHOUT ACCESS TO THE RAWDATA. WE HOPE THAT THIS POSITION IS ACCEPTABLE.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
In their manuscript, Shokati and colleagues describe the development and validation of an LC-MS/MS method for the quantification of tacrolimus in dried blood spots. Overall, the article is well written and the experiments are clearly described. Although in general the experiments appear well-performed, there are several important issues that this reviewer would like to have addressed. An experiment to better address matrix effects is required and more extensive discussion of and comparison with existing literature is required.

Major Concerns:
Lines 141 & 515:The authors state that coagulation takes place in a dried blood spot. However, the reviewer is not aware of evidence that coagulation does take place (even when blood is directly applied from a fingerprick). Either the reviewers provide a reference demonstrating that coagulation does take place in filter paper, or they replace "coagulated" by "dried". Moreover, in the authors' experiments, EDTA blood was used, which does not coagulate.
ALTHOUGH TO THE BEST OF OUR KNOWLEDGE THIS STATEMENT IS CORRECT (BLOOD WITHOUT ANTICOAGULANT SUCH AS A CAPILLARY BLOOD DROP AFTER FINGER STICK WILL AT LEAST PARTIALLY COAGULATE), WE HAVE COMPLIED WITH THE REVIEWER'S REQUEST AND DELETED THE STATEMENTS TO THIS EFFECT FROM THE INTRODUCTION AND DISCUSSION SECTIONS.

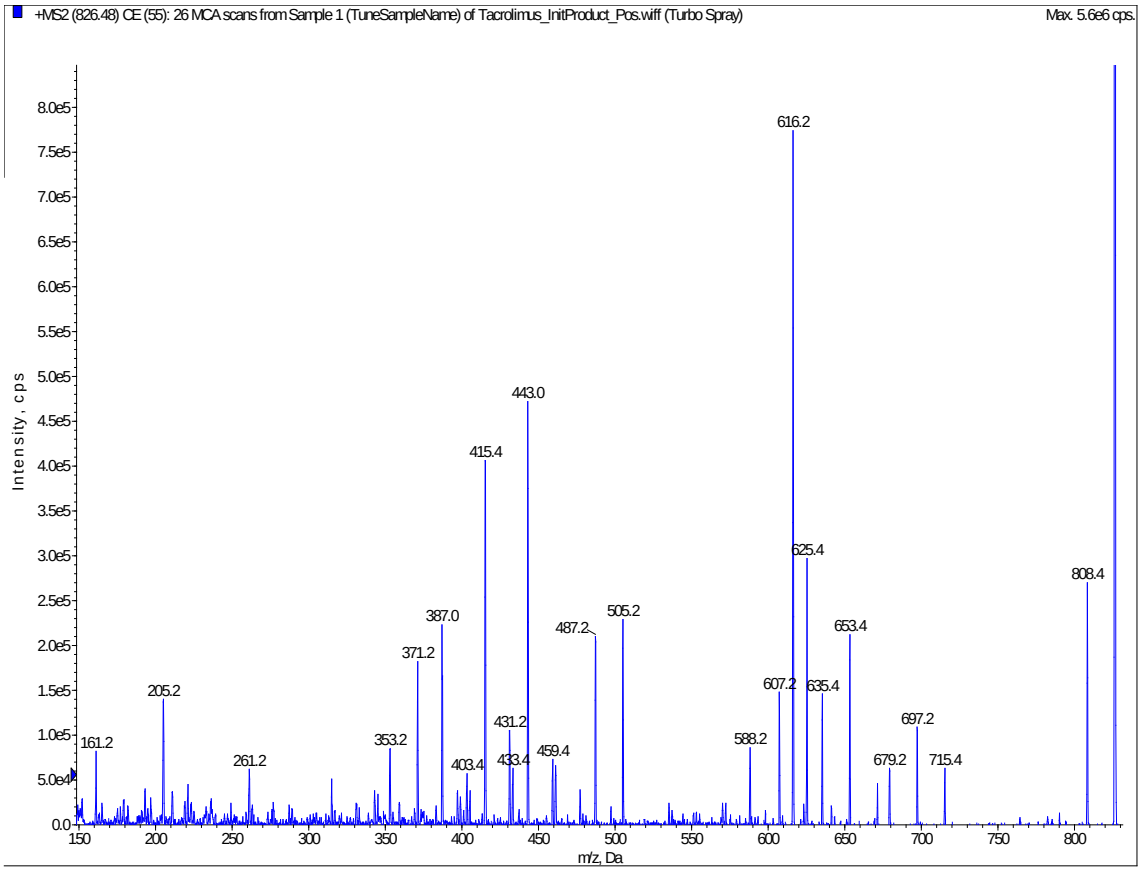
Line 181-183: The authors have in the end 10% methanol in blood. Organic solvent should not exceed 5%. The authors need to mention that this 10% is not optimal.
THIS STATEMENT WAS POTENTIALLY MISLEADING. AS COMMON, CALIBRATORS AND QUALITY CONTROL SAMPLES WERE PREPARED IN BULK AND AFTER SPIKING AND INCUBATION AT 37°C UNDER GENTLE SHAKING, SAMPLES WERE ALIQUOTED INTO 1.5 ML POLYPROPYLENE TUBES WITH CONICAL BOTTOMS AND SNAP-ON LIDS. THE PARAGRAPH IN THE TEXT HAS BEEN RE-WRITTEN.

Line 203: There is an issue with references, that became apparent here. Refs 44,45 should be 43,44. Also ref 45 (line 208) is not correct; also other references are not correct (e.g. there is no ref 49 in the ref list, while it is referred to on line 585)
WAS CORRECTED.

Line 217-219: The amount of IS is rather high. Calculations by this reviewer indicate that it likely corresponds to the concentration of the highest calibration standard. The authors

should motivate the choice for this high concentration.
AN INTERNAL STANDARD CONCENTRATION IN THE UPPER CONCENTRATION RANGE OF THE CALIBRATION CURVE WAS INTENTIONALLY CHOSEN TO MINIMIZE VARIABILITY OF THE INTERNAL STANDARD PEAK CAUSED BY RANDOM BASELINE SHIFTS AND NOISE. PLEASE NOTE THAT ESPECAILLY ERRORS AT THE PEAK BASE (EG BY THE INTEGRATION SOFTWARE INCLUDING A BASELINE SHIFTS IN THE INTERNAL STANDARD'S PEAK AREA) HAS A MARKED EFFECT ON THE AREA UNDER THE PEAK. THIS IS AN AUTOMATED HIGH-THROUGHPUT ASSAY USED FOR FDA STUDIES AND MANUAL RE-INTEGRATION IS NOT ALLOWED. THEREFORE IT WAS IMPERATIVE TO ENSURE THAT INTEGRATION BY THE SOFTWARE WAS EASILY REPRODUCIBLE AND THE INFLUENCE OF INTEGRATION ERRORS WAS MINIMIZED.

Line 249-251: are the mass transitions correct? A typical transition for tacrolimus in literature appears to be 821-768. The authors have to elaborate on this. Also the choice for a sodium adduct should be elaborated upon.
THE ION TRANSITIONS ARE CORRECT. FRAGMENTATION PATTERNS DEPEND ON MANY FACTORS INCLUDING, BUT NOT LIMITED TO INSTRUMENT DESIGN, COLLISION GAS AND COLLISION VOLTAGES. FOR THE REVIEWER'S INFORMATION, WE PROVIDE A REPRESENTATIVE MS/MS SPECTRUM RECORDED ON ONE OF THE INSTRUMENTS RUNNING THE PRESENT ASSAY:



THE LC-MS/MS INSTRUMENTS IN OUR LABORATORY ENVIRONMENT HAVE ALWAYS PRODUCED SODIUM ADDUCTS EVEN IF OTHER IONS WERE ADDED EG TO FACILITATE THE FORMATION OF AMMONIUM ADDUCTS. IN OUR

LABORATORY SODIUM ADDUCTS HAVE ALWAYS GIVEN THE BEST RESULTS. WE ACKNOWLEDGE THAT THIS MAY BE DIFFERENT IN OTHER LABORATORIES. NEVERTHELESS, BASED ON THE LITERATURE, THE RELIANCE ON SODIUM ADDUCTS FOR THE QUANTIFICATION OF TACROLIMUS AND OTHER IMMUNOSUPPRESSANTS IS NOT UNCOMMON.

Line 262: quadratic fit is sometimes used for very wide calibration ranges. However, the calibration range here is not wide (only 1-50 ng/ml). The authors should explain their choice for this model. Did they calculate residuals and used the lowest sum of residuals?
IN OUR EXPERIENCE A QUADRATIC FIT IN COMBINATION WITH AN MS/MS DETECTOR WILL ALWAYS GIVE MORE ACCURATE RESULTS AT THE LOWER AND UPPER LIMITS OF QUANTIFICATION THAN LINEAR FITS. THIS WAS ALSO THE CASE WITH PRESENT ASSAY. BEFORE THE DECISION FOR UTILIZING A QUADRATIC FIT MADE, SEVERAL WEIGHTING AND CURVE FITTING ALGORITHMS WERE TESTED AND THE QUADRATIC FIT, AS USUAL, GAVE THE BEST PRECISION AND ACCURACY AT THE ENDS OF THE CALIBRATION CURVE.

One of the prime advantages that the authors put forward is lack of matrix effects. However, they have only examined matrix effects in a very rough way. It is essential that the authors perform the classical 'Matuszewski experiment', in which 3 series of sampled are compared: neat standards, extracts of DBS spiked prior to extraction and extracts of blank DBS spiked post extraction. This is an essential comment.
WE RESPECT THE REVIEWER'S CONFIDENCE IN THE MATUSZEWSKI EXPERIMENT. NEVERTHELESS, A POST-INFUSION EXPERIMENT CANNOT BE CONSIDERED "ROUGH" AND IS A VALID AND ACCEPTED APPROACH. PLEASE NOTE THAT THE MATUSZEWSKI EXPERIMENT IS ONLY A MOMENTARY SNAP-SHOT AS IT IS 'BLIND' TO POTENTIAL MATRIX EEFECTS THAT ARE DANGEROUSLY CLOSE TO THE ANALYTE PEAK, BUT DO NOT HAVE AN EFFECT YET, ESPECIALLY WHEN A RATHER NEW HPLC COLUMN IS USED AS USUALLY THE CASE DURING PRE-STUDY ASSAY VALIDATION. AS DURING ROUTINE MEASUREMENT SEPARATION POWER OF A COLUMN DECREASES, THERE IS THE RISK THAT AT SOME POINT A PREVISOULY UNNOTICED MATRIX EFFECT CLOSE TO THE ANALYTE PEAKS BECOMES A PROBLEM. IN CONTRAST TO THE MATUSZEWSKI EXPERIMENT, A POST-INFUSION EXPERIMENT WILL SHOW MATRIX EFFECTS OVER THE COMPLETE ANALYSIS PERIOD AND WILL GIVE INFORMATION ON HOW WELL SEPARATED FROM POTENTIAL MATRIX EFFECTS THE PEAKS OF INTEREST ARE. AS THE REVIEWER MAY ALREADY HAVE GUESSED BASED ON THE FACT THAT WE STUDIED EXTRACTION RECOVERY USING A MATUSZEWSKI EXPERIMENT, WE ALSO DETERMINED THE MATRIX EFFECTS DURING THIS EXPERIMENT. THESE WERE WITHIN ACCEPTABLE LIMITS (WITHIN 89.5-105.3% OF THE DETECTOR RESPONSE OF CORRESPONDING DRUG AMOUNTS IN NEAT STANDARD SOLUTIONS). AS IN OUR EXPERIENCE THE POST-INFUSION EXPERIMENT TO ASSESS THE PRESENCE AND RISK OF MATRIX EFFECTS IS OF MORE RELEVANCE FOR THE ROBUSTNESS OF AN ASSAY INTENDED FOR HEAVY ROUTINE USE, WE DECIDED TO FOCUS ON THOSE RESULTS. WE HOPE THAT THIS WILL BE ACCEPTABLE.

Another essential comment is that the authors did not evaluate accuracy & precision at LLOQ. Strictly taken, they cannot consider 1 ng/ml as the LLOQ, as they state themselves on line 271-272 that for LLOQ bias and imprecision should be less than

20%. However, no QC at LLOQ level was included, hence no true statement can be made that 1 ng/ml is a true LLOQ. This is a major shortcoming. However, looking at the available data for the 2 ng/ml level, this reviewer is convinced that the methodology will likely be OK for 1 ng/ml as well. Still, this shortcoming has to be clearly mentioned in the text (cfr on line 566 is it clearly mentioned that the LLOQ is 1 ng/ml).
ACCURACY AND PRECSION AT THE LLOQ WAS EXTENSIVELY TESTED. BASED ON CURRENT FDA GUIDANCE, THE LLOQ CAN BE DETERMINED BY BACK-EXPTRAPOLATION OF THE CALIBRATORS AND THERE IS NO REQUIREMENT FOR A QC AT THE LLOQ. IN FACT, FDA GUIDANCE RECOMMENDS THE LOWEST QC TO BE 3X THE LLOQ. PLEASE NOTE THAT WE COULD NOT REPORT ALL RESULTS IN DETAIL TO KEEP THE MANUSCRIPT WITHIN REASONABLE SPACE LIMITS. THE ACCURACY AT THE LLOQ WAS 101.3% AND THE PRECISON WAS 6.3 (MEASURED OVER 20 DAYS, N=2/ DAY, N=40 TOTAL). FOR THE REVIEWER'S REFERENCE WE HAVE INCLUDED THE RELEVANT RESULT TABLE IN THE APPENDIX TO THIS RESPONSE (COPIED FROM THE VALIDATION REPORT AS SUBMITTED TO THE FDA).

Cross-talk between tacrolimus and its internal standard needs to be tested: is the signal for tacrolimus blank when only IS is present (and vice versa)?
THIS WAS TESTED (EG BY INCLUSION OF ZERO SAMPLES IN THE VALIDATION AND RUN BEFORE EACH OF OUR CALIBRATION CURVES) AND NONE WAS FOUND (NONE= <15% OF THE TACROLIMUS SIGNAL AT THE LLOQ). THIS WAS CRITICAL FOR OUR DECISION TO USE THE INTERNAL STANDARD AT A CONCENTRATION AT THE HIGHER END OF THE CALIBRATION CURVE.

Overall, there should be more extensive referral and comparison with existing methods. What is precisely the added-value of the presented method? As stated above, the authors suggest that their clean-up is better than that of other published methods, although they have failed to examine matrix effects in detail. Again, this is an experiment that needs to be performed.
THE COMPARISON WITH OTHER PUBLISHED ASSAYS IS DISCUSSED IN THE PARAGRAPH STARTING ON LINE 583. PLEASE NOTE THAT DUE TO THE REALTIVELY LARGE NUMBER OF PUBLISHED TACROLIMUS DRIED BLOOD SPOT LC-MS/MS ASSAYS, WE COULD NOT POINT OUT DIFFERENCES TO EACH INDIVIDUAL ASSAY AND RATHER WERE LIMITED TO DISCUSSING THE OTHER PUBLISHED ASSAYS IN AGGREGATE. IN TERMS OF OUR PRESUMED FAILURE TO APPROPRIATELY EVALUATE MATRIX EFFECT, WE WOULD LIKE TO REFER TO THE RELEVANT DISCUSSIONS ABOVE AND EMPHASIZE THAT WE USED BOTH POST-COLUMN INFUSION AND MATUSZEWSKI APPROACHES AND THAT BOTH CONISTENTLY INDICATED THE LACK OF RELEVANT MATRIX EFFECTS. DUE TO ITS GREATER RELEVANCE, WE DECIDED TO RELY HERE MAINLY ON THE POST-INFUSION EXPERIMENTS AS DESCRIBED BY MÜLLER ET AL. [REFERENCE 45].

Figure 3A: in fact, when this is a blank that is handled like any other sample, the signal of IS should be here. This reviewer understands that inclusion of the IS would not make it clear anymore to see the (low) background signal. Still, it has to be mentioned that this sample also lacks IS.
PLEASE NOTE THAT APPLICABLE FDA GUIDANCE DISTINGUISHES BETWEEN 'BLANK' AND 'ZERO' SAMPLES. A BLANK SAMPLE IS A TRUE BLANK WITHOUT ANALYTE AND INTERNAL STANDARD, WHILE A ZERO SAMPLE DOES NOT

CONTAIN ANALYTE, BUT IS SPIKED WITH THE INTERNAL STANDARD. THUS, THE LEGEND TO FIGURE 3A IS CORRECT AND A BLANK IS SHOWN. AS CORRECTYLY NOTED BY THE REVIEWER, WE DECIDED TO SHOW A BLANK INSTEAD OF A ZERO SAMPLE TO SHOW THAT THERE WAS NEITHER MATRIX INTERFERENCE WITH THE ANALYTE NOR WITH THE INTERNAL STANDARD PEAK.

Minor Concerns:
Line 96-98 about physicochemical characteristics of tacrolimus is no essential information; this can be removed
WE RESPECTFULLY DISAGREE WITH THE REVIEWER. THE PHYSICOCHEMICAL PROPERTIES PROVIDE THE BASIS FOR THE EXTRACTION PROCEDURE AND HPLC CONDITIONS AND WE WOULD PREFER TO KEEP THIS INFORMATION IN THE MANSUCRIPT.

line 127: 50 ul is NOT typically collected from a fingertip. The reviewer refers to line 133, where it is stated that DBS are typically 20 ul. In this reviewer's experience, 20-30 ul is indeed typically collected.
THIS STATEMENT WAS MISLEADING. WHAT THIS STATEMENT WAS SUPPOSED TO REFER TO WAS THAT FILLING THE CIRCLE ON THE WHATMAN 903 PAPER IS EQUIVALENT TO 50 µL OF BLOOD. WE HAVE DELETED THIS STATEMENT FROM THE INTRODUCTION AND HAVE ADDED A CORRECTED STATEMENT STARTING ON LINE 537 OF THE REVISED MANUSCRIPT. WE HAVE ALSO ADDED REFERENCE [46].

Line 223: m n should be min
CORRECTED

Line 416: insert "calibrators" after the brackets
DONE.

Line 588: replace "have" by "has"

APPENDIX

Table 8.1-2: Intra- and Inter-day Accuracy and Precision of Calibrators (CCs)

Intra-Day Accuracy and Precision DBS

Day 1 - 20 CCs

Validation Day	1.0	2.5	5.0	10.0	25.0	50.0	R^2
Day 1	101.0	104.0	91.5	93.6	111.0	102.0	0.9982
	107.0	107.0	91.9	99.0	105.0	105.0	
	86.9	95.1	102.0	88.4	96.1	99.2	
	89.3	95.8	106.0	88.1	100.0	95.2	
	108.0	110.0	103.0	95.4	105.0	97.2	
	121.0	100.0	95.4	102.0	108.0	95.0	
Intra-day Accuracy	102.2	102.0	98.3	94.4	104.2	98.9	
Intra-Day Precision	12.7	6.1	6.2	5.6	5.4	4.0	
Day 2	94.4	101.0	95.5	94.4	102.0	102.0	0.9994
	98.8	106.0	110.0	95.7	101.0	98.0	
Intra-day Accuracy	96.6	103.5	102.8	95.1	101.5	100.0	
Intra-Day Precision	3.1	3.5	10.3	0.9	0.7	2.8	
Day 3	97.9	101.0	96.2	102.0	106.0	106.0	0.9990
	109.0	98.8	94.8	98.4	97.6	93.4	
Intra-day Accuracy	103.5	99.9	95.5	100.2	101.8	99.7	
Intra-Day Precision	7.8	1.6	1.0	2.5	5.9	8.9	
Day 4	104.0	102.0	96.1	94.4	108.0	96.6	0.9989
	106.0	93.8	103.0	94.0	100.0	102.0	
Intra-day Accuracy	105.0	97.9	99.6	94.2	104.0	99.3	
Intra-Day Precision	1.4	5.8	4.9	0.3	5.7	3.8	
Day 5	97.4	101.0	94.1	95.5	104.0	97.2	0.9994
	108.0	104.0	97.6	95.6	104.0	101.0	
Intra-day Accuracy	102.7	102.5	95.9	95.6	104.0	99.1	
Intra-Day Precision	7.5	2.1	2.5	0.1	0.0	2.7	
Day 6	113.0	93.6	97.8	101.0	107.0	90.0	0.9967
	113.0	94.9	87.2	90.3	105.0	107.0	
Intra-day Accuracy	113.0	94.3	92.5	95.7	106.0	98.5	
Intra-Day Precision	0.0	0.9	7.5	7.6	1.4	12.0	
Day 7	101.0	95.4	102.0	97.6	102.0	103.0	0.9996
	100.0	103.0	97.1	105.0	96.8	97.5	
Intra-day Accuracy	100.5	99.2	99.6	101.3	99.4	100.3	

Intra-Day Precision	0.7	5.4	3.5	5.2	3.7	3.9	
Day 8	106.0	102.0	103.0	N/D	99.5	105.0	0.9994
	87.9	105.0	95.8	102.0	98.1	95.8	
Intra-day Accuracy	97.0	103.5	99.4	102.0	98.8	100.4	
Intra-Day Precision	12.8	2.1	5.1		1.0	6.5	
Day 9	113.0	96.8	104.0	101.0	106.0	103.0	0.9992
	97.6	94.3	93.9	96.0	98.6	95.8	
Intra-day Accuracy	105.3	95.6	99.0	98.5	102.3	99.4	
Intra-Day Precision	10.9	1.8	7.1	3.5	5.2	5.1	
Day 10	108.0	103.0	94.1	92.0	108.0	98.3	0.9987
	101.0	103.0	94.4	95.2	104.0	98.4	
Intra-day Accuracy	104.5	103.0	94.3	93.6	106.0	98.4	
Intra-Day Precision	4.9	0.0	0.2	2.3	2.8	0.1	
Day 11	93.5	108.0	97.6	90.8	100.0	98.8	0.9993
	94.6	109.0	106.0	97.8	104.0	100.0	
Intra-day Accuracy	94.1	108.5	101.8	94.3	102.0	99.4	
Intra-Day Precision	0.8	0.7	5.9	4.9	2.8	0.8	
Day 12	108.0	101.0	Failed	106.0	103.0	100.0	0.9990
	99.8	91.4	91.2	105.0	93.3	101.0	
Intra-day Accuracy	103.9	96.2	91.2	105.5	98.2	100.5	
Intra-Day Precision	5.8	6.8		0.7	6.9	0.7	
Day 13	103.0	90.3	97.0	101.0	101.0	105.0	0.9987
	109.0	Failed	104.0	91.8	105.0	93.3	
Intra-day Accuracy	106.0	90.3	100.5	96.4	103.0	99.2	
Intra-Day Precision	4.2		4.9	6.5	2.8	8.3	
Day 14	99.1	108.0	102.0	93.8	104.0	94.7	0.9990
	98.2	99.0	101.0	93.8	103.0	104.0	
Intra-day Accuracy	98.7	103.5	101.5	93.8	103.5	99.4	
Intra-Day Precision	0.6	6.4	0.7	0.0	0.7	6.6	
Day 15	96.5	99.3	114.0	94.0	97.8	97.5	0.9992
	93.8	103.0	98.7	101.0	100.0	103.0	
Intra-day Accuracy	95.2	101.2	106.4	97.5	98.9	100.3	
Intra-Day Precision	1.9	2.6	10.8	4.9	1.6	3.9	
Day 16	100.0	108.0	103.0	99.1	98.9	106.0	0.9991
	102.0	92.1	92.2	103.0	101.0	94.2	
Intra-day Accuracy	101.0	100.1	97.6	101.1	100.0	100.1	

Intra-Day Precision	1.4	11.2	7.6	2.8	1.5	8.3	
Day 17	96.0	105.0	100.0	96.0	102.0	97.7	0.9996
	107.0	99.6	94.4	97.9	103.0	101.0	
Intra-day Accuracy	101.5	102.3	97.2	97.0	102.5	99.4	
Intra-Day Precision	7.8	3.8	4.0	1.3	0.7	2.3	
Day 18	99.7	104.0	98.1	94.7	106.0	101.0	0.9994
	107.0	96.1	95.4	100.0	100.0	97.4	
Intra-day Accuracy	103.4	100.1	96.8	97.4	103.0	99.2	
Intra-Day Precision	5.2	5.6	1.9	3.7	4.2	2.5	
Day 19	97.4	99.0	92.3	93.2	108.0	96.4	0.9985
	111.0	110.0	94.1	94.2	104.0	101.0	
Intra-day Accuracy	104.2	104.5	93.2	93.7	106.0	98.7	
Intra-Day Precision	9.6	7.8	1.3	0.7	2.8	3.3	
Day 20	93.7	104.0	97.9	94.8	101.0	97.5	0.9994
	98.4	107.0	107.0	94.5	103.0	102.0	
Intra-day Accuracy	96.1	105.5	102.5	94.7	102.0	99.8	
Intra-Day Precision	3.3	2.1	6.4	0.2	1.4	3.2	
Inter-Day Accuracy and Precision DBS							
Inter-day Accuracy	**101.3**	**101.0**	**98.4**	**96.7**	**102.5**	**99.4**	AVERAGE
Inter Day Precision	**6.7**	**5.3**	**5.5**	**4.4**	**3.7**	**3.9**	**0.9990**

- *N/D means no peaks were detected. This may be most likely due to wrong sample preparation or failure during sample injection.*
- *Those samples labeled as failed did show very high levels of tacrolimus which may be due to contamination with higher stock solution during sample preparation or a pipetting error of the internal standard solution. Those values were not taken into consideration for statistical calculation.*
- *Those values labeled as red did not pass the acceptance criteria. However, those values were included in the distribution statistical calculation.*