STABILITY INDICATING BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF TACROLIMUS USING LCMS/MS

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ABSTRACT:

Tacrolimus in human whole blood was quantified utilizing a simple, efficient, and reproducible approach. With Phenomenex, Strata-X SPE cartridges, Tacrolimus and internal standard Tacrolimus 13CD4 were extracted from human whole blood using a solid phase extraction technique. Chromatographic separation was performed using an ACE CN (4.6 x 75mm) 1.5µm column as the stationary phase, while a mobile phase made up of acetonitrile and ammonium acetate (90:10 v/v) flowed at a rate of 0.60 mL/min followed by MRM. Electrospray ionization was used to monitor the eluents with Tacrolimus set at transition m/z 821.4→ 768.5 for Tacrolimus and m/z 826.43→ 773.27 for Tacrolimus (IS), respectively. Tacrolimus and its internal standard had a retention period of 3.25 minutes. In accordance with US-FDA guidelines, the technique was validated for precision, accuracy, specificity, recovery, matrix effect, linearity, and limit of detection. Tacrolimus quantification was carried out with a precise and accurate approach, as shown by the assay validation findings, which all fulfilled the necessary acceptance criteria. The range of the validated test was 0.204 to 60.080 ng/mL. No matrix impact, carryover, or interference were noticed. The calibration curve was confirmed to be linear, with a r^2 value of 0.99. Tacrolimus was found to have a LOQ signal-to-noise (S/N) ratio of 23.789. The devised technique demonstrated high analyte and IS recovery. When the concentration of Tacrolimus in the matrix was evaluated for stability, no discernible change was found. The bioanalytical method validated in this research is suitable and practicable for quantitating Tacrolimus in human whole blood samples obtained using LC-MS/MS.

KEYWORDS: Human whole blood, LC-MS/MS, Method development, Method validation, Tacrolimus.

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INTRODUCTION:

An immunosuppressive drug called Tacrolimus is used to prevent organ rejection after transplantation. It can be used as a therapy or preventative measure for some autoimmune disorders. It was initially discovered to be a 23-membered macrolide lactone in 1987. Chemically, Tacrolimus is an inhibitor of the macrolide calcineurin. It works by preventing the dephosphorylation of NF-AT, which in turn reduces the activation of T cells. The peak blood plasma concentrations (C_{max}) of oral tacrolimus are achieved one to three hours after ingestion, with a total bioavailability of 20 to 25% (although with variations from 5 to 67%) and slower absorption in the gastrointestinal system. Tacrolimus is mostly associated to erythrocytes in the blood; just 5% is detected in the plasma, and more than 98.8% of that is bound to plasma proteins. The structure of Tacrolimus is given in Figure.1.

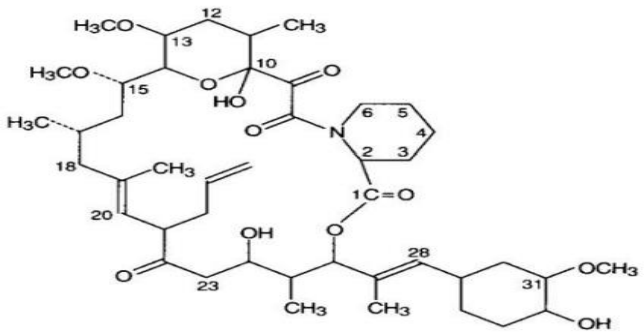

Figure.1 Structure of Tacrolimus

There aren't many methods that have been reported in the literature for determining Tacrolimus levels in bodily fluids. [1-10] Analytical techniques have been used to quantify Tacrolimus in multiple ways. They consist of immunoassay, Tandem liquid chromatography-mass spectrometry, and High-performance liquid chromatography. The gold standard approach is the LC-MS, which has significant costs and complete validation criteria. For this kind of assessment, a high level of technical proficiency and significant training is needed. The purpose of this work was to create a more rapid, sensitive, and accurate approach for Tacrolimus in human whole blood using LC-MS-MS. The method used 400 µl of human whole blood sample and highly repeatable solid phase extraction. The internal standard used was Tacrolimus 13CD4.The stability of Tacrolimus was evaluated in various laboratory settings using the validated approach.

MATERIALS AND METHOD:

Chemicals:

All of the chemicals were of an analytical grade. The pure forms of Tacrolimus and Tacrolimus IS were purchased from Clearsynth. Pvt Ltd. in India. Acetonitrile, methyl t-butyl ether, and methanol of the LCMS grade were bought from JT. Baker in Mumbai, India. Ammonium Acetate, Formic acid and Zinc sulphate was purchased from Merck Limited, India. Human whole blood (K_2EDTA) was acquired from Symbiosis laboratories & research centre in Ahmedabad, India.

Instrument used:

The Liquid Chromatography -tandem mass spectroscopy(LC-MS/MS)WaterXevo TQD using Mass lynx 4.1software.

Instrumentation and chromatographic conditions:

Tacrolimus was quantitated using Liquid Chromatography-Tandem Mass Spectrometry. With ACE CN (4.6 x 75mm) 1.5µmas the stationary phase and acetonitrile: ammonium acetate in the ratio of 90:10 with a flow rate of 0.6 ml/min and injection volume of10µl, the chromatographic separation was accomplished. It is necessary to tune the mass spectrometer. The mass spectrometer was equipped with electron spray ionization (ESI), which was used to estimate the concentrations of Tacrolimus and Tacrolimus (IS) with the precursor to product ion transitions of m/z 821.4→ 768.5 and m/z 826.43→ 773.27, respectively.

The other parameters included the source temperature of 150 °C, a capillary voltage of 3.5 kV, a desolvation temperature of 550 °C, a collision gas flow of 3 to 4 10^{-3} mbar, and a probe flow rate of 600µL/min. The Phenomenex Strata-X 33, 30mg/ml SPE cartridge was used to extract the sample using the solid phase extraction method.

Preparation of Tacrolimus stock solution:

2.5 mg of Tacrolimus was precisely measured and transferred into a 50 mL volumetric flask. It was dissolved and methanol was added to the remaining amount to get an equal concentration of50µg/mL.

Preparation of Tacrolimus IS stock solution:

For the preparation of Tacrolimus IS stock solution, 2 mg of Tacrolimus 13CD4 was precisely weighed and transferred into a 20 mL volumetric flask. It was dissolved and methanol was added to the volume to make it equal to concentration of 100µg/mL.

Preparation of tuning solution:

Aqueous solution with analyte and internal standard was prepared with 50% acetonitrile and had a final concentration of 500 ng/ml.

Calibration Curves

The C_{max} value, which was chosen based on the dosage form and strength of the medication to be dosed for the pharmacokinetic research, is the major

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factor in the calibration range selection. The value of C_{max} dictates the concentration range, and the ULOQ and LLOQ should be 2 times of C_{max} and 5-7 half-lives of C_{max}, respectively.

Method validation:

The procedure of method validation is used to show that the quantitative measurements in the specified matrix are accurate and reproducible. Specificity/selectivity, sensitivity, matrix factor, accuracy, precision, recovery, stability, and dilution effects are among factors that are included in a validation. [11]

Calibration curve:

Data derived from recognized calibration curves were used to evaluate the verified procedure. The calibration curves maintained a high level of accuracy and precision. Using a linear regression model, the quality of fit for calibration standards was assessed. The back calculated values of each concentration point on an inter-assay basis were used to generate the standards, which make up the calibration curves.

Precision and accuracy:

On an intra- and inter-assay basis, the assay's precision and accuracy were evaluated. Matrix samples from pre-defined P&A extraction ran with varying Tacrolimus concentrations were analyzed. Six repetitions of each quality control from the low limit of quantification and high limit of quantification were included in each run along with a calibration curve (lower limit of quantification to upper limit of quantification).

Sensitivity- Limit of Quantification (LOQ):

On an intra- and inter-assay basis, the quantitative precision and accuracy (P&A) of the lower limit of quantification were assessed. Comparing the signal of each Lower Limit of Quantification quality control sample to its baseline noise allows investigators to experimentally estimate sensitivity by injecting six Lower Limit of Quantification quality control samples.

Recovery of Analyte:

By comparing the mean peak areas of six extracted low, medium, and high quality control samples to six post-extracted samples spiked with neat standards containing the same amounts of low, medium, and high Tacrolimus quality control samples, the recovery of Tacrolimus was assessed.

Recovery of internal standard:

By comparing the mean peak areas of extracted quality control samples to the mean peak areas of Tacrolimus in six standard post-spiked samples with neat standards including samples with the same quantities of extracted tacrolimus, the recovery of tacrolimus internal standard was assessed.

Auto sampler Carry-Over Test (ASCOT):

Using the extracted LLOQ, Blank, and ULOQ, carryover was examined. The blank sample, which was injected in accordance with a ULOQ standard, was used to assess the outcomes of carryover.

Matrix Stability:

The stability of samples under anticipated conditions is tested to demonstrate matrix stability when handling, storing, processing, and analysing subject materials.

a) Freeze-Thaw Stability in Matrix:

The analyses' stability in the following of freeze-thaw cycles is referred to as freeze-thaw stability in the matrix. A sample was frozen for at least 12 hours before being thawed, at which point the freeze-thaw cycle is deemed finished. For this test, a single aliquot was taken from each of six different tubes of test samples at low and high concentrations (subjected to stress conditions) and compared to QCs (High and Low) and a freshly spiked calibration standard curve in the same run. Five freeze-thaw cycles were used to verify stability (in a freezer with a fixed point of -70°C&room temperature).

b) Bench Top Stability in Matrix:

The stability of the analyte in the matrix under circumstances that might arise during the analysis of the subject sample. For this test, a single aliquot from each of six different tubes containing low and high concentration samples that were stored in benchtop conditions was extracted and compared in one run to a newly spiked calibration standard curve and QCs (high and low). A 21.0-hour demonstration of stability was made at room temperature.

c) Processed Sample Stability (PS-RT and PS-Refg):

One aliquot from each of six different tubes, including both low and high concentrations, was submitted to stress conditions such room temperature and auto sampler settings in accordance with the analytical procedure. The samples were then

extracted and tested against a recently spiked calibration standard curve as well as QCs (high and low) in the same run. For 25 hours of storage at room temperature, stability was proven. Storage under auto sampler conditions for 58.0 hours was shown to be stable.

d) Solution Stability:

Under predefined storage circumstances, the stability of the analyte and internal standard were assessed separately and collectively in solution. On the day of the test, the master stock solution of Tacrolimus and its internal standard underwent short-term storage at ambient temperature for 6.0 hours and long-term storage in a refrigerator for 30 days. The test samples were assessed against newly made solutions of the same concentration after being put through the test procedures (reference samples).

Ruggedness:

The impact of bioanalytical method remains unchanged by small variation. Ruggedness of the method was evaluated by one precision and accuracy run using different column and different instrument.

RESULTS AND DISCUSSION:

METHOD DEVELOPMENT:

The MS tuning is the first phase of method development. The key job in mass spectrometry tuning is to acquire the appropriate fragmented ions and response for both TL and TL13CD4. This is done by adjusting the mass spectrometer parameters, fragmentation pattern, and mode of ionisation. Since it meets the sensitivity and selectivity required for analytical procedures, ESI-LC-MS/MS is a particularly effective technology for pharmacokinetic research. The assay development was done using the MRM method. The analyte response was maximised by optimising the MRM settings. Mass spectrum of Tacrolimus and Tacrolimus13CD4 IS given in Figure2 and 4. The mass spectrum of daughter ions of Tacrolimus and its internal standard are given in Figure.3 and 5.

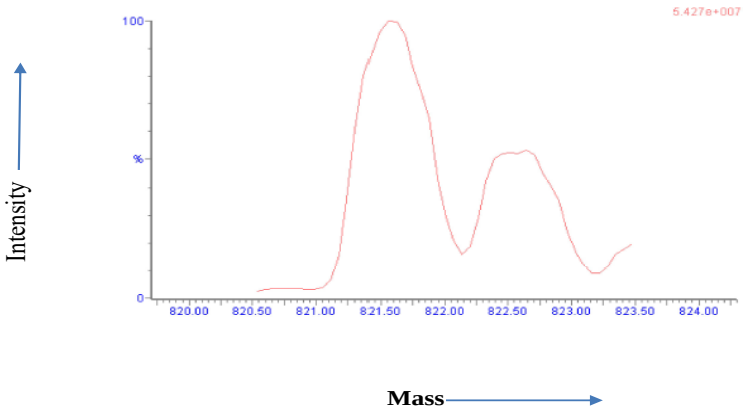

Figure.2 Mass spectrum of Tacrolimus

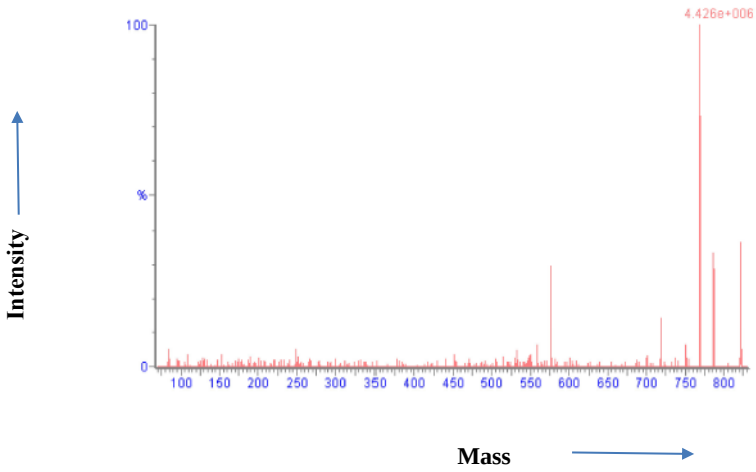

Figure.3Mass spectrum Tacrolimus daughter ions

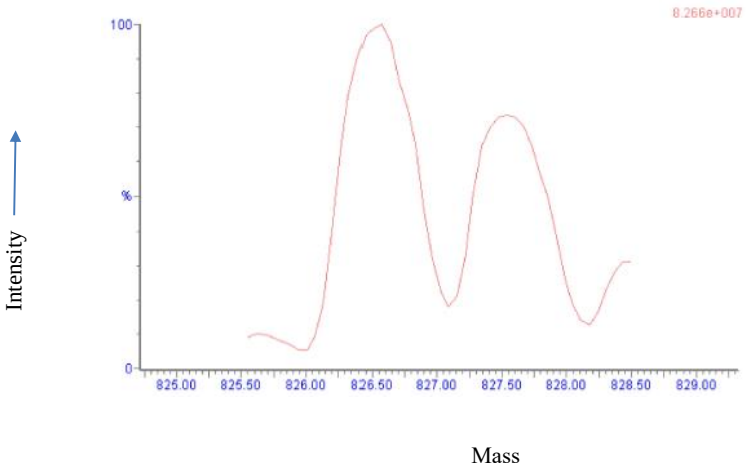

Figure.4 Mass spectrum of Tacrolimus13CD4

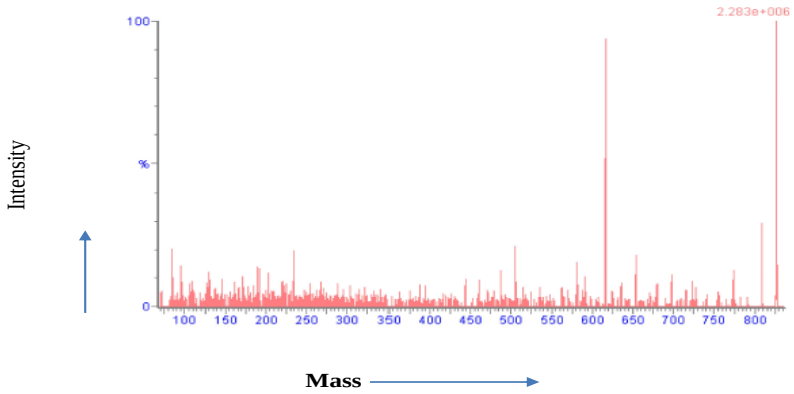

Figure.5 Mass spectrum of Tacrolimus13CD4 daughter ions

By running multiple trials, the instrumental parameters for mass spectroscopy were optimised. The mass spectrum of Tacrolimus collision-associated dissociation (CAD) reveals the creation of distinctive product ions at m/z 786.50, 768.50, 576.34, and 84.11. The main product ion for Tacrolimus was at m/z 768.50. Characteristic product ions at m/z 773.27, 616.37, 111.42, and 84.11 of the compound Tacrolimus 13CD4 were formed. The predominant product ion at 773.27 m/z. Figures 6 and 7 show the chromatograms of the blank human whole blood sample and the internal standard-spiked sample. Figures 8 and 9 show the chromatograms for the LLOQ level and ULOQ level, respectively.

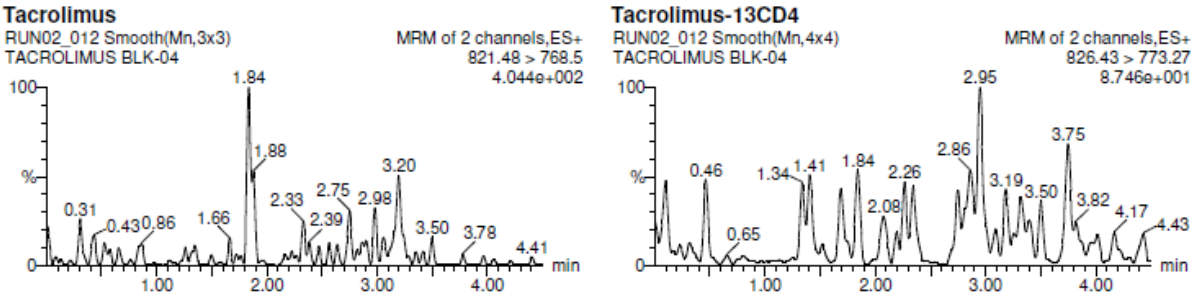

Figure.6. Representative chromatogram of blank human whole blood

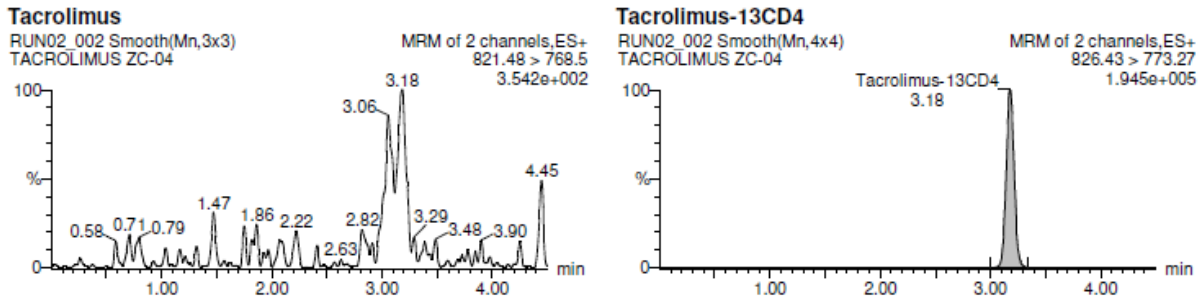

Figure.7. Representative chromatogram of blank human whole blood spiked with internal standard

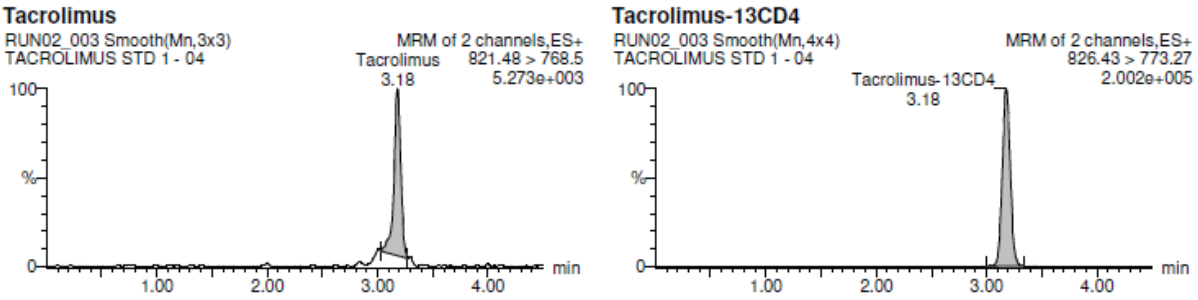

Figure.8. Representative chromatogram of LLOQ level

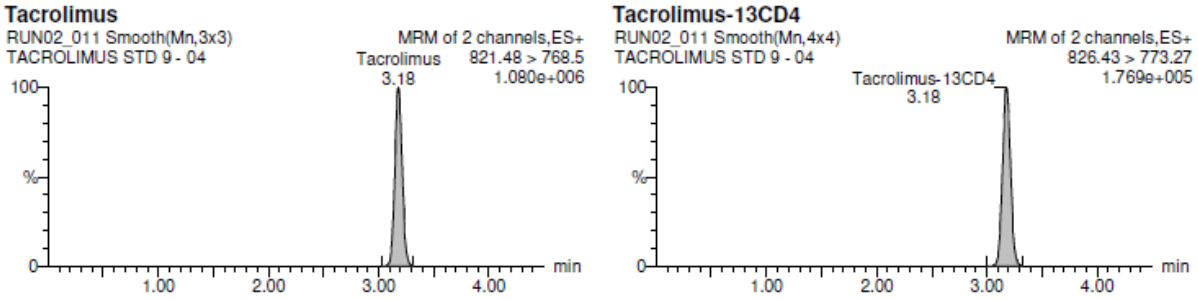

Figure.9: Representative chromatogram of ULOQ level

Calibration curve:

The calibration curve was determined to be linear, with a slope of 0.0925887, an intercept of 0.00158222, and a determination coefficient (r^2) of 0.998081. These findings show that this approach is reliable and reproducible within the analytical range of 0.204 to 60.080 ng/mL. The calibration curve's back calculated concentration is shown in Table.1. Figure.10 depicts the calibration curve.

Table.1.Back Calculated Concentration of Calibration Curve

Concentration of CC	Mean	S.D	%CV	% Nominal	% Deviation
STD 1 0.204 ng/ml	0.2014	0.00559	2.78	98.73	-1.27
STD 2 0.407 ng/ml	0.4166	0.02254	3.19	102.36	2.36
STD 3 3.018 ng/ml	3.0331	0.09683	3.19	100.50	0.50
STD 4 6.036 ng/ml	6.0158	0.23149	3.85	99.67	-0.33

STD 5 12.072 ng/ml	12.3540	0.42172	3.41	102.34	2.34
STD 6 24.144 ng/ml	23.6642	1.14312	4.83	98.01	-1.99
STD 7 36.144 ng/ml	36.2943	1.38400	3.81	100.42	0.42
STD 8 48.064 ng/ml	48.4216	1.56336	3.23	100.74	0.74
STD 9 60.080 ng/ml	58.4316	1.97443	3.38	97.26	-2.74

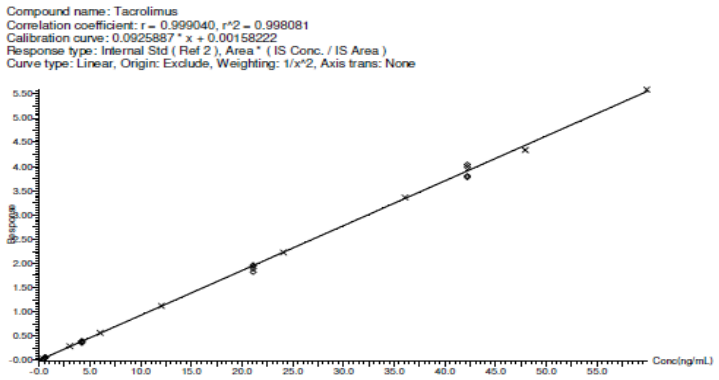

Figure.10 Calibration curve for Tacrolimus

Precision and accuracy:

Three Precision and Accuracy (P&A) batches made up of intraday and Interday batches were used to test the method's reproducibility. All of the results are confirmed to be within the parameters, proving the precision and accuracy of the approach. Table.2 lists the values for the intra- and inter-assay precision and accuracy of QC samples.

Table.2 Intra- and Inter- Assay Precision and Accuracy of QC samples

Type of assay	**Parameters**	**LLOQQC 0.206 ng/mL**	**LQC 0.603 ng/mL**	**MQC 21.091 ng/mL**	**HQC 42.181 ng/Ml**
Intra-assay P&A (n=6)	Intra run mean	0.2150	0.6357	22.0765	43.7838
	Intra run SD	0.01094	0.02437	0.97273	1.51636
	Intra run CV%	5.09	3.83	4.41	3.46
	Intra run % Nominal	104.37	105.42	104.67	103.80
	Intra run % deviation	4.37	5.42	4.67	3.80
Inter-assay P&A (n=18)	Inter run mean	0.2143	0.6313	21.7439	43.5355
	Inter run SD	0.00862	0.02361	0.78249	1.30231
	Inter run CV%	4.02	3.74	3.60	2.99
	Inter run % Nominal	104.03	104.69	103.1	103.21

Inter run % deviation	4.03	4.69	3.10	3.21

Sensitivity- limit of quantification (LOQ):

By injecting six LLOQ QC samples and comparing the signal of each sample to its baseline noise, the sensitivity was experimentally measured. The value of the LOQ signal-to-noise (S/N) for six Tacrolimus injections was reported. The mean was 23.789 which is in the acceptance criteria of mean S/N ≥ 5.

Recovery:

Comparing the mean peak areas of six extracted low, medium, and high quality control samples to six post-extracted samples spiked with neat standards containing the same quantities of low, medium, and high Tacrolimus quality control samples allowed researchers to assess the recovery of Tacrolimus. The devised technique exhibits satisfactory analyte and IS recovery as all values were determined to be within the acceptability criteria (% CV 15.0). Tables 3 and 4 show the recovery of Tacrolimus and Tacrolimus IS.

Table 3. Recovery for Tacrolimus

Sample level	Recovery	Mean	SD	%CV
HQC	63.35	64.1300	1.07791	1.68
MQC	65.36			
LQC	63.68			

Table 4. Recovery for TacrolimusC13D4 (IS)

Sample level	Recovery	Mean	SD	%CV
HQC	61.79	62.6600	1.21643	1.94
MQC	64.05			
LQC	62.14			

Auto sampler carry-over test (ascot)

When the auto sampler carryover test was run, there was no discernible carryover.

Stability studies:

When tested for freeze-thaw stability over 5 cycles, bench top stability for 25.0 hrs, dry extract stability for 24.0 hrs, processed sample stability of Tacrolimus for 25.0 hrs at room temperature and 58.0 hrs at auto sampler conditions, and long term stability for 55 days at -70 ℃ and -20℃ temperature, there was no discernible change in concentration of Tacrolimus in matrix.The stability of the Tacrolimus master stock solution was examined over a 30-day period in a refrigerator and for six hours in an RT environment. The stock solutions were determined to be stable, and the values were within accepted limits.The stability study's findings are shown in Table.5

Table.5 Results of stability tests of Tacrolimus QC samples under different conditions

	QC concentration	Mean	SD	%Nominal	%Deviation
Freeze & thaw stability	0.603ng/mL	0.5790	0.03536	96.02	-3.98
	42.181ng/mL	42.0125	0.36133	99.60	-0.40
Bench Top Stability	0.603ng/mL	0.6165	0.04031	102.24	2.24
	42.181ng/mL	41.8750	0.55579	99.27	-0.73

Processed sample Stability (R	0.603ng/mL	0.5815	0.00636	96.43	-3.57
	42.181ng/mL	43.6030	0.05091	103.37	3.37
Processed sample Stability (Auto sampler)	0.603ng/ml	0.5605	0.00919	92.95	-7.05
	42.181ng/mL	42.9175	0.91853	101.75	1.75
Long term Stability (-20°C)	LT-LOW	0.6095	0.03606	101.08	1.08
	LT-HIGH	44.6280	1.79605	105.80	5.80
Long term Stability (-70°C)	LT-LOW	0.5590	0.01980	92.70	-7.30
	LT-HIGH	44.7785	0.07283	106.16	6.16

Ruggedness:

Various lots/batch of analytical columns and equipment were used to attain ruggedness, and it was discovered that changes in the use of different columns and different instruments had no effect on it.

CONCLUSION

The development of a bioanalytical technique for the quantification of Tacrolimus in human whole blood is the primary focus of the current study. According to USFDA guidelines, the developed procedure was validated, and the outcomes were confirmed to be within acceptable bounds. The suggested technique is acceptable and applicable for normal laboratory analysis, according to the results of the system suitability and applicability of the method in the estimation of the drug in human whole blood. According to the results of the validation parameters, the suggested method is linear, exact, accurate, precise, robust, specific, and stable. The results of the proposed research thus support the suggested method's suitability as a methodology for Tacrolimus estimation in human whole blood. Pharmacokinetic studies involving BA/BE can benefit from the technique.

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CONFLICTS OF INTEREST: There was no conflict of interest.

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