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BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF TACROLIMUS IN HUMAN BLOOD USING LC-MS/MS

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ABSTRACT

A simple, accurate and precise method was developed using high performance liquid chromatography with electron spray ionizationtandem mass spectrometry (HPLC-ESI-MS) to quantify the concentration of tacrolimus in human blood with K_2EDTA anticoagulant was developed and fully validated. Sirolimus was used as an internal standard (ISTD). The sample extraction procedure utilized solid phase extraction method. The chromatographic analysis was conducted on a Thermo scientific Hypurity (50 x 4.6 mm) 2.6µm within 2 min, using methanol with 10mM ammonium acetate (80:20%, v/v) was used as mobile phase at the flow rate of 0.6mL/min under an isocratic condition. The ionization was performed on electron spray ionization interference with positive mode by multiple reaction monitoring (MRM). The mass transitions were 821.60→768.60 m/z for tacrolimus and 931.70→864.70 m/z for ISTD. Method validated as per USFDA guidelines and calibration curve was found to be linear in the range of 0.200-100.176 ng/mL.The results were within the acceptance limits. The extraction efficiency

was 82.52% at the three quality control levels. The lower limit of detection (LLOQ) was found to be 0.200 ng/mL.Stability studies demonstrated that tacrolimus was stable in blood during Bench-Top (4hr 10min at room temperature), Auto-sampler (27hr 45 min at 4°C), Freeze-Thaw (5cycles) and Long term analyte stability in blood (37days at -20°C).

Keywords: Tacrolimus; Human Blood; Stability; Solid Phase Extraction; Validation

INTRODUCTION

Tacrlimus belongs to the immunosuppressive medication with highly albumin protein binding (>98%) and low bioavailability 20-25% **[1]**. Chemically it is (1R,9S,12S,13R,14S,17R,18E,21S,23S,24R, 25S,27R)-1,14-dihydroxy-12-[(1E)-1-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-23,25-dimethoxy-13,19,21,27-tetramethyl-17-(prop-2-en-1-yl)-11,28-dioxa-4-azatricyclo octacos-18-ene-2,3,10,16-tetrone **(Figure 1a)** and molecular formula $C_{44}H_{69}NO_{12}$ with compound weight 804.09 g/mol **[2]**.

The pharmacological act as macrolide calcineurin inhibitor. In T-cells, activation of the T-cell receptor normally increases intracellular calcium, which acts via calmodulin to activate calcineurin. Calcineurin then dephosphorylates the transcription factor nuclear factor of activated T-cells (NF-AT), which moves to the nucleus of the T-cell and increases the activity of genes coding for IL-2 and related cytokines. Tacrolimus prevents the dephosphorylation of NF-AT **[3]**.

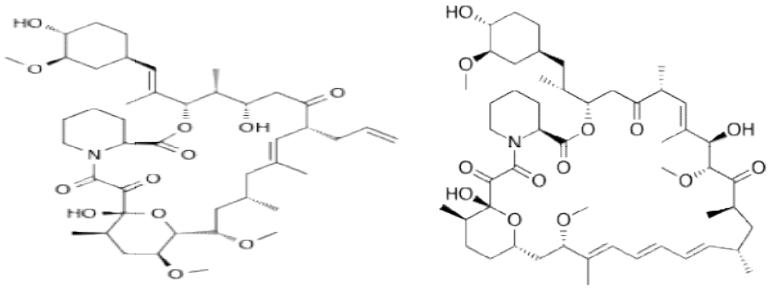

Figure 1: Chemical structure of (a) Tacrolimus (b) Sirolimus (ISTD)

Drug literature review reveals that few analytical quantification methods have been reported for the tacrolimus in bulk, formulations, and biological matrices. Which includes UV spectrophotometric **[4-6]**, high performance liquid chromatography **[7-8]** and ultra-high performance liquid chromatography tandem mass spectrometric detection (UPLC-MS/MS) **[9-14]**. The present work designed to develop a simple,rugged, economic and validated LC-MS/MS method for the determination of

tacrolimus in human blood (K_2EDTA-anticoagulant) with internal standard sirolimus **(Figure 1b)**.

MATERIALS AND METHOD

Chemicals and Reagents

The pure standard of tacrolimus (purity 99.91% by hplc) and sirolimus (purity 98.65% by hplc) as is basis were purchased from Vivan life sciences hydrabad, India. Emparta grade of ammonium acetate, LC-MS grade of methanol, deionized milli-Q-weter, Zinc sulphate and acetonitrile purchased from Merck Specialties Private Limited India, matrix: human blood.

Instrument and Equipment

Quantitative analysis was performed on an exion LC^{TM} chromatographic system (AB Sciex, USA). The detection of analyte and ISTD performed using ESI and triple quadrupole mass spectrometer API 6500. Data acquisition and processing were performed by using analyst software version 1.6.3 (AB Sciex) to control all parameters of LC and mass spectrometry.

Chromatographicand MRM Condition

The chromatography separation of analytewas achieved by using Thermo scientific Hypurity (50 x 4.6 mm) 2.6µmand the isocratic mobile phase consist of Methanol: 10mM Ammonium acetate (80:20%, v/v) was delivered with flow rate of 0.600mL/min. The column compartment (oven) and autosampler temperature were at 40^{o}C and 4^{o}C, respectively with an injection volume of 20 µL. the analysis run time was completed within 2min.The main working parameters of the mass spectrometer are given in **Table 1**.

Table 1: Multiple Reaction Monitoring (MRM) conditions

Parameters	Tacrolimus	Sirolimus
General Dependent		
Mass spectrometer	API 6500	
Tuning mode	Manual	
Ion source	Turbo Ion Spray (ESI)	
Ionization Mode	Positive Ionization	
Spray needle set point (X/Y)	5/5	
Compound Dependent		
Transition (m/z) Q1→Q3	821.60→768.60	931.70→864.70
Declustering Potential (V)	80	75
Entrance Potential (V)	10	10
Collision Energy (V)	26	31
Collision Cell Exit Potential (V)	24	26
Source Dependent		
Curtain Gas (psi)	25	
Ion Spray Voltage (V)	5000	
Temperature (^{o}C)	500	
Gas Source 1 (psi)	40	
Gas Source 2 (psi)	45	
Collision gas (psi)	6	
Dwell Time Per Transition (msec)	200	

Sample preparation

The sample preparation was performed by solid phase extration method. Exactly 0.500 ml of blood sample was aliquoted and transferred into a tarsons ria vial polypropylene tube and 0.050 ml of ISTD (750ng/ml) working concentration solution was added, except for standard blank, to which 0.050 ml of 60% methanol solution (v/v) was added and vortexed. To this 0.250 ml of 0.5M Zinc sulphate solution (w/v) was added, vortexed and to this 0.5mL of 100% acetonitrile added with proper vortex. The samples were vortexed 10 min in vibromax and followed by centrifuged at 4200rpm for 5min with 4°C. Sample clean-up was conducted by using the following Solid Phase Extraction procedure, the waters cartridges (Oasis HLB) were first conditioned with methanol 1 ml to activate the chemical bonds then equilibrated by using water 1 ml, in a positive pressure manifold SPE. Blood supernatant samples were loaded onto SPE cartridges. Then, the cartridges were washed with water 1 ml twice and followed by 5% methanol solution (v/v) 1 ml to wash the interferences and the analyte was successively eluted with 0.300 ml of 100% methanol with elution tarsons ria vial polypropylene tube contained 0.050 ml of 5mM Ammonium Acetate solution (w/v). Finally 20µL was injected into the LC-MS system.

METHOD VALIDATION

Method validation was done as per the criteria of industrial guidance for bioanalytical method validation of USFDA **[15]**.

System Suitability

System suitability was evaluated by analyzing6 repeated injections from same vial of standard aqueous mixture equivalent to an about middle concentration of the calibration curve of tacrolimus and working concentration of ISTD during the start of the method validation and at the start of the respective day. The area ratio and retention time (Analyte and ISTD) of system suitability has within the toleance limits of 5% CV.

Carryover Effect

Carryover effect was performed in order to remove the carryover from the previous injection to the next injection. Extracted blank, LLOQ and ULOQ samples were prepared from biological matrix of human blood as mentioned above extraction process. These samples were injected in the sequnce of mobile phase, extracted blank (without analyte and ISTD), extracted LLOQ, extracted ULOQ and above extracted blank urine samples during the start of the method

validation. The area of interfering peaks at the RT of analyte has ≤ 20% of area of extracted LLOQ and at the RT of ISTD have ≤ 5% of area of extracted LLOQ.

Selectivity/ Specificity

The selectivity of the method was evaluated by analysing six different lots of human blood matrix and two lipemic lots. From each lot, blank and LLOQ were processed using the above extraction method. For specificity, interference from analyte was established by processed minimum of six individual matrix lot with MQC concentration level without ISTD and interference from ISTD was established by processed minimum of six individual matrix lot with working concentration of ISTD without analyte.

Sensitivity

Assessed the sensitivity in the terms of percentage accuracy and precision which was denoted by %CV. It was evaluated with the lower limit of quantification (LLOQ QC) 0.200ng/mL of quality control sample along with all precision and accuracy bathch. The tolerance limit of percentage accuracy within ±20 and %CV ≤20.

Calibration curve

Calibration curve was constructed by plotting the ratio of peak area of tacrolimus and sirolimusagainst the nominal concentration of calibrators. The calibration curve were fitted by weighting factor $1/X^2$ least square linear regression equation method (y=mX+c) which are distributed throughout the calibration curve range from 0.200-100.176 ng/mL of tacrolimus. The curve constructed by using balnk, zero and nine non-zero standards0.200, 0.400, 0.800, 1.599, 10.098, 25.244, 42.074, 70.123 and 100.176 ng/mL. The tolerance limit of calibration curve was a correlation coefficient (R^2) of 0.98 or greeter, and each back-calculated standard concentration have ±15% deviation from the nominal value with the exception of LLOQ, which was set at ±20%.

Precision and Accuracy

Precision and accuracy batch was calculated by analysing four batches. For P & A studies five concentration level of quality control samples were prepared as lower limit of quantification (LLOQ), lower quality qontrol (LQC), medium quality control (MQC), high quality control (HQC) and dilution integrated quality control (DIQC)equivalent to 0.200, 0.570, 35.643, 77.484 and 387.421 ng/mL respectively, with six replicates each. The intra-run and inter-run precision (% CV) for LOQ, MQC, HQC and DIQC should be ≤ 15% except for LLOQ, which was set at ≤20% andthe intra-run and inter-run accuracy for LQC, MQC, HQC and DIQC should be

within ±15% except for LLOQ, which was set at within ±20%.

Recovery

The percentage extraction efficency of tacrolimus from human blood was calculated by comparing the mean peak response of six extracted low, medium and high (0.570, 35.643, and 77.484ng/mL) respectively, quality control samples to the mean peak response of six post-extracted low, medium and high quality control samples with the same concentrations.

The percentage extraction effiency of ISTD from human urine was calculated by comparing the mean peak area of the prepared extracted ISTD to the mean peak area of post extracted ISTD at the concentration level intended for use. The % recovery of analyte and ISTD has to be less than 110%.

Matrix Factor

Matrix factor was evaluated at LQC and HQC level by using six screened different lots of human blood and one lipemic matrix. To determine the matrix factor two sets of blank matrices were processed from individual lots using the above extraction method. Post extraction samples were prepared by the standard of LQC and HQC containing internal standard were spiked into the extracted black matrices. In the same way, standard aqueous solution equal to LQC and HQC concentration containing internal standard was prepared using diluent and mobile phase as injected single batch. The acceptance criteria for IS normalised matrix effect was that the %CV should be less than 15%.

Dilution Integrity

Dilution integrity was evaluted to ensure that samples could be diluted with screened blank matrix of human blood without affecting the final concentration. Tacrolimus spiked human blood samples were prepared at concentrations of 387.421ng/mL, above the upper limit of the calibration range. These samples were further diluted with human pooled blood five times dilution in six replicates and analysed with all P&A batch. The six replicates have a precision of ≤ 15%CV and accuracy of 100 ± 15%.

Ruggedness

The ruggedness of the method was assessed by the deliberate changes in the experimental state with a precision and accuracy batch. The batch was supervised using a similar chemistry type of column to another column manufacture (Phenomenex Luna) and different analyst in the same laboratory.

Run Size Evaluation

Evaluate the run size during method validation, which should include the number

of samples to be analyzed under a run during actual study sample analysis. Establish run-size based on the chromatographic run time and analyte stability.

Reinjection Reproducibility

Reproducibility is the precision between two laboratories. It also represents the precision of the method under the same operating conditions over a short period. Re-injection reproducibility shall be evaluated by re-injecting anyone of the accepted P & A.

Stability experiments

The aim of determining the stability of tacrolimus in human blood performed viz. bench-top stability, freeze-thaw stability, auto-injector stability, wet extract stability, Long-term analyte stability in blood, stock and working solution stability studies were carried out by using six replicates of the lower and higher quality control samples.

The stability was calculated by comparing the found concentration to the nominal concentration values against the freshly prepared calibration standard and bracketed run acceptance quality control (LQC, MQC and HQC) samples.

Stock and working solution stability

To assess the standard stock solution stability of analyte and ISTD, stability samples were prepared and maintained at 2-8^{o}C for 15 days. The percentage bias calculated mean peak area of of the stability standard stock solution of analyte and ISTD against the comparable freshly prepared standard stock solution of analyte and ISTD, then injected six replicates of fresh and stability samples at LLOQ and ULOQ level.

Bench-top stability

To determine the stability of analyte in human blood on the based-top condition, six replicates of stability quality control (LQC and HQC) samples were set separately at ambient temperature up to 4hr 10min then extracted and qualified.

Freeze-Thaw stability

Freeze Thaw stability of analyte was evaluated by six replicates of stability quality control (LQC and HQC) samples were frozen at -20 degree in the deep freezer. The frozen urine samples containing the analyte thawed at room temperature for a minimum 1 hour followed by refrozen for minimum 12 hours. The stability quality control samples were exposed to 5FT cycles before being extracted and analysed.

Auto-sampler stability

To determine the stability of processed sample in autosampler condition, six replicates of stability quality control (LQC and HQC) samples were processed and left in the autosampler rack up to 27hours 45 minutes at 4^{o}C then injected and quantified.

Wet Extract stability

To determine the stability of wet extract, six replicates of stability quality control (LQC and HQC) samples were processed and stored at 2-8^{o}C refrigerator condition for 17 hours as wet extract form prior to loading into LC autosampler.

Long-term analyte stability in Blood

To determine the long-term stability of analyte in urine, six replicates of 3 set stability quality control (LQC, HQC and DIQC) samples were stored at -20 ^{o}C in the deep freezer for 37 days. after completion of stability duration extracted and analyzed.

All stability experiments were stable if assay values were within the adequate tolerance of ±15% of accuracy and ≤15%CV of precision.

RESULTS AND DISCUSSION

The ionization techniques of positive and negative MRM mode was tried using harvard syringe pump was carried out to obtain Q1 and Q3 ion mass spectra of analyte and ISTD with electron spray ionization probe source and the signal intensity was good and higher in the positive mode of ionization tuning. For tacrolimus and sirolimus, the highly sensitive transitions were detected from precursor ion m/z 821.60 to product ion m/z 768.60 and precursor ion m/z 931.70to product ion m/z 864.70, respectively.

System Suitability

The system suitability %CVof the retention time was found to be 0.59-1.28% forfor tacrolimusand 0.66-1.92% for sirolimus. The %CV of the peak area ratio was found to be 2.01 to 3.69%. Prior to suitability few equilibration injections were given, and the results were found to be within the acceptance.

Carryover Effect

The results indicated that no carryover was observed throughout this chromatographic method for both tacrolimus and sirolimus. It does not affect the precision and accuracy of the individual run.

Selectivity/ Specficity

Selectivity of the technique was verified on ten blank human blood samples obtained from different volunteers. The chromatographic method determined analyte of interest in the analysed matrices without interference from endogenous components. This matrices lots were further selected for preparation of calibration curve and quality control samples. The % accuracy of individual lot's LLOQ samples were within the acceptable range of ±20%. The selectivity and specificity experiments ensured null interferenceat the retention time of analyte and ISTD. Results are given in **Table 2**.

Linearity

The linearity of the method was demonstrated peak area ratio of analyte to ISTD was linear with reliable reproducibility over the concentration range of 0.200 to 100.176ng/mL **Figure 2**. At nine non-zero calibrator levels. The correlation coefficient R^2 for the calibration curve ranged from 0.9998 - 1.0000 for torsemide.Results are given in **Table 3**.

Sensitivity/ Precision and Accuracy

The intra-run and inter-run precision for each concentration level within the range of 5.44 to 7.17%CV and 3.56to 4.24%CV, respectively and the intra and inter run accuracy for each concentration level was within the range of 94.16% - 100.32% and 95.32% - 100.56% respectively. The lowest concentration with %CV less than 20% was taken as LLOQ and was found to be 0.194ng/mL.

Recovery

The relative recovery for LQC, MQC and HQC of tacrolimus were found to be found 85.46%, 80.28% and 81.82% respectively. The percentage mean global recovery of analyte was found to be 82.52% with adequate precision of 3.22% CV and the ISTDpercentage mean recovery was found to be 81.23%. the result data shows that the solid phase extraction procedure efficiently extracts tacrolimus as well as sirolimus from human blood. The results were summarized in **Table 4**.

Matrix effect

The post-extraction spiked method indicated that no significant effect of matrix ion was observed at the retention time of analyte and ISTD for QC levels (LQC and HQC). The %CV was found to be IS normalised matrix factor 7.22 and 8.00, correspondingly. The result of matrix effect as within the acceptable limit.

Dilution integrity

Dilution integrity of tacrolimus was performed up to five fold. The percentage nominal values was found within the acceptance limit of ±15% and the diluted samples mean precision was 2.79 to 4.85 % and accuracy was 97.13 to 103.68%.

Ruggedness

The present method was shown good ruggedness when it was performed by using different analyst and column of different manufacture. The accuracy and precision result wasacceptable range of 94.34 - 98.85 % and 4.27-8.24 % CV respectively.

Stability

The stabilityof tacrolimuswas assessed under different environment expected to be encountered during the analytical process and sample storage. The analyte passed all the

stability parameter tests viz. stock solution stability (15 days at 2-8 ^{o}C), Auto-sampler (27h 45min at 4^{o}C), Bench-top (4h 10min), wet extract (17h at room temperature), Freeze-Thaw (5 cycles) and deep freezer stability (37 days at -20 ^{o}C). There was no significant decrease of the analyte concentration was observed. The summary of the stability parameters statistical data for torsemide presented in the **Table 5**.

Table 2: Selectivity-Interference from endogenous compound for Analyte and ISTD

Sl. no	Biological Matrix ID	Area response					
		Analyte Area Blank)	Analyte Area (LLOQ)	%Inteference for Analyte	ISTD Area (Blank)	ISTD Area (LLOQ)	%Inteference for ISTD
1	Tacr-Blood Lot-1	0	2652	0.00	0	289963	0.00
2	Tacr-Blood Lot-2	0	2220	0.00	0	269853	0.00
3	Tacr-Blood Lot-3	0	2492	0.00	0	278567	0.00
4	Tacr-Blood Lot-4	0	2301	0.00	0	289766	0.00
5	Tacr-Blood Lot-5	0	2295	0.00	0	286532	0.00
6	Tacr-Blood Lot-6	0	2396	0.00	0	275243	0.00
7	Tacr-Lipemic Lot-1	0	2124	0.00	0	267542	0.00
8	Tacr-Lipemic Lot-2	0	2286	0.00	0	275243	0.00

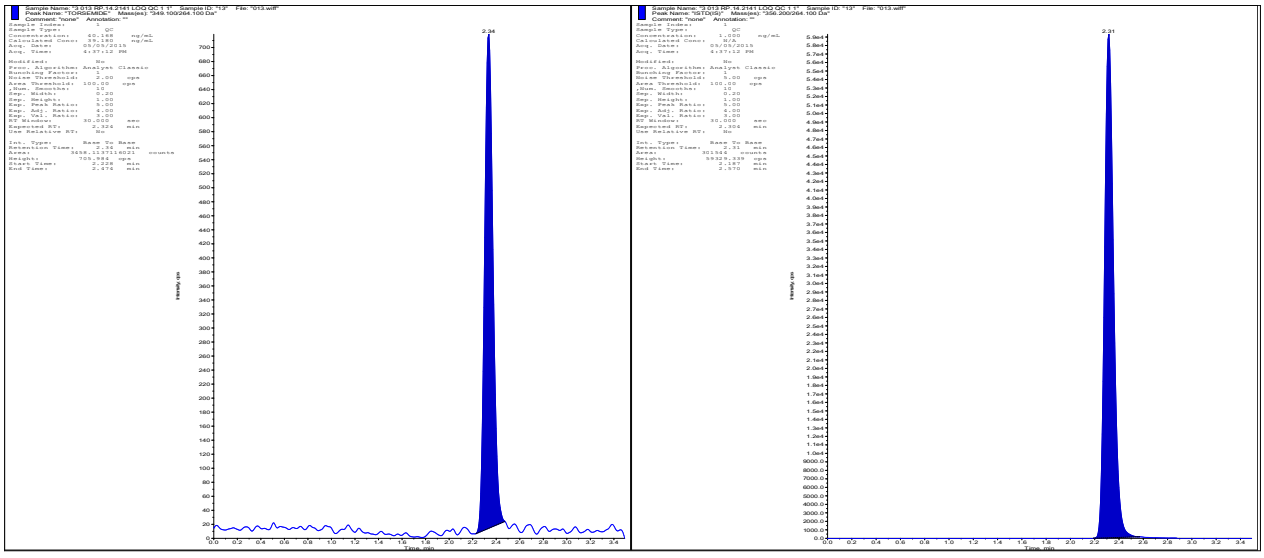

Figure 2: Mass Chromatograms of Tacrolimus(Left) and Sirolimus(Right)

Table 3: Linearity

Nominal Concentration (ng/mL)	STD-1 0.200	STD-2 0.400	STD-3 0.800	STD-4 1.599	STD-5 10.098	STD-6 25.244	STD-7 42.074	STD-8 70.123	STD-9 100.176
N*	4	4	4	4	4	4	4	4	4
Mean±SD	0.189	0.410	0.803	1.540	9.875	25.410	42.503	69.663	100.279
±SD	0.01	0.02	0.03	0.07	0.11	0.31	0.56	0.31	0.21
%CV	6.48	5.66	3.34	4.30	1.07	1.23	1.32	0.44	0.21
% Accuracy	94.27	102.41	100.35	96.31	97.79	100.66	101.02	99.34	100.10

***Number of each concentration injections**

Table 4: Recovery

QC ID	LQC		MQC		HQC		ISTD	
	Post Extracted Area	Extracted Area	Post Extracted Area	Extracted Area	Post Extracted area	Extracted Area	Post Extracted Area	Extracted Area
N*	6	6	6	6	6	6	6	6
Mean±SD	8940	7640	456840	366754	916705	750038	344963	280224
±SD	514	270	25127	20009	10937	48266	10887	16342
%CV	5.75	3.53	5.50	5.46	1.19	6.44	3.16	5.83
% Recovery	85.46		80.28		81.82		81.23	
%Global CV	82.52						-	
%Global recovery	3.22						-	

***Number of injections**

Table 5: Stability of Tacrolimus

Stability Experiment	QC ID	Nominal concentration (ng/mL)	Concentration found (ng/mL) (mean ± SD)*	Precision (% CV)	Accuracy (%)
Bench top Stability	LQC	0.570	0.555	5.08	97.36
	HQC	77.484	75.278	5.10	97.15
Auto sampler Stability	LQC	0.570	0.554	4.54	97.14
	HQC	77.484	76.124	4.84	98.25
Wet extract Stability	LQC	0.570	0.546	8.99	95.74
	HQC	77.484	76.880	5.30	99.22
Freeze thaw Stability	LQC	0.570	0.539	4.18	94.61
	HQC	77.484	74.688	6.17	96.39
Reinjection Reproducibility	LQC	0.570	0.557	6.00	97.69
	HQC	0.570	76.028	7.97	98.12
Long term urine stability	LQC	77.484	0.549	6.22	96.40
	HQC	0.570	79.512	4.54	102.62
	DIQC	387.421	404.091	2.79	104.30

***Number of each concentration injections-6**

CONCLUSION

Ahighly sensitive, selective, specific, accurate and precise LC-MS/MS method for the quantification of tacrolimus in human blood was developed.The extraction procedure of analyte in biological matrix simple with reproducible recovery and less matrix effect. Proposed chromatographic method was rapid, allowing for sample preparation procedure and analysis of a large number of sample in a short period of time and comprehensive method validation was carried out. All results were within the range of acceptable limits as specified in USFDA guidelines (2018). Hence, the developed method can be applied to PK and TDM studies in humans with desired precision and accuracy.

AUTHORS CONTRIBUTIONS

All author have contributed equally.

CONFLICT OF INTERESTS

Declared none.

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