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Rapid and Sensitive Ultra Performance Liquid Chromatography Tandem Mass Spectrometry for Quantitation of Tacrolimus in Human Whole Blood

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Abstract
Therapeutic drug monitoring of immunosuppressive agents in organ transplanted Patient's is crucial to prevent intoxication or transplant rejection due to inadequate dosage. The commonly used immune assays have been gradually undergoing replacement by mass spectrometry since this physical method offers both a higher Sensitivity and specificity. A simple rapid, novel, sensitive Ultra performance liquid Chromatography (UPLC) coupled with electron spray mass spectrometry method was developed and validated for quantification of tacrolimus (I) in human plasma. The analyte and internal standard (Sacrolimus II) were extracted by liquid-liquid extaction with hexane and four levels of quality control samples of k2 EDTA human whole blood were used. The chromatographic separation was performed on reverse phase thermo hypurity advance column (46 x 50 mm, 5um) with mobile phase of 90% methanol and 10mM ammonium acetate buffer (90:10)low at a flow rate of 0.5ml/min and run time of 2.5 minute .The retention time of both analyte and ISD were 1.37mints.The deprotonate of analyte was quantitated in positive ionization by multiple reaction monitoring (MRM)with mass spectrometry. The mass transitions m/z 821-768.54 and m/z 931.6-864.8 were used to measure I&II in whole blood –I(Q1)and m/z 821.5-768.54 and m/z 931.5-884.6for I&II in whole blood –II(Q2) respectively. The method exhibited a linear response in the range of 0.495-99.430ng/ml for tacrolimus in human plasma with co-relation coefficient of greater than 0.998.The lower limit of quantification was 0.5ng/ml with C V % of 5%.The %accuracy for QC samples were 98.33%LQC, 93.03%, GMQC 93.60%MQC and 96.87%HQC.The %CV for QC samples were 5.19%LQC 931.78%GMQC, 3.94%MQC and 0.21%HQC. The % stability by freeze and thaw stability was 97.27%for LQC, 96.42%for HQC and by bench top stability was 100.33% & 98.20% for LQC and HQC respectively. The % accuracy and CV% for dilution integrity in the ratio of 1:5 was 159.248&4.02%. This method can be used for the quantification of tacrolimus in human whole blood in routine and bioequivalence studies.

Keywords: Tacrolimus, UPLC-MS/MS, Liquid phase extraction, Bioequivalence, sensitive

INTRODUCTION

Tacrolimus (also FK-506 or Fujimycin) is a 23-membered macrolide lactone discovered in 1984 from the fermentation broth of a Japanese soil sample that contained the bacteria *Streptomyces tsukubaensis*. **Tacrolimus** is the most frequently used immunosuppressive drugs in organ transplantation [1] mainly used after allogeneic organ transplant to reduce the activity of the patient's immune system and so lower the risk of organ rejection.). Initially, it was employed in the management of liver transplants; it is now routinely used in the management of kidney, heart, pancreas, small bowel, lung and bone marrow transplants [2, 3] FK-506 acts by binding to a cytoplasmic protein called immunophilin; the resultant complex then inhibits the function of an intracellular protein calcineurin, a Ca and calmodulin-dependent serine/threonine phosphatase. Furthermore, this interaction leads to the inhibition of T-lymphocyte signal transduction and decreases IL-2 transcription, which gives rise to immune suppression [2-4]. These immunosuppressive drugs have narrow therapeutic ranges. it is also used in a topical preparation in the treatment of severe atopic dermatitis (eczema) severe refractory uveitis after bone marrow transplants, and the skin condition vitiligo) FK-506 is a critical dose drug with a narrow therapeutic index; i.e., it exhibits the desired therapeutic effect with acceptable tolerability within a narrow range of blood concentration. As a result, at low blood levels there is a risk of rejection of the organ transplant, while elevated circulating concentration can lead to serious toxicity and long-term morbidity [5, 6] In addition, there is important variation for blood levels of these immunosuppressive drugs in different individuals, and ethnicities may also affect these parameters [7, 8]. Thus, the accurate determination of FK-506 is essential to correlate its blood concentration and clinical outcomes for therapeutic drug monitoring [9-11]. TDM has been used to monitor drug levels in routine patient care. The methodology of TDM must be precise and accurate for immunosuppressive drugs [12].

There are two main analytical methods for determination of immunosuppressive drugs in transplant patients: immunoassays (micro particle enzyme immunoassay, cloned enzyme donor immunoassay, etc.) and liquid chromatography-based methods (high-performance liquid chromatography (HPLC) with ultraviolet detection, LC-mass spectrometry (LC-MS), and LC-tandem mass spectrometry (LC-MS/MS)) [13]. Immunoassays are widely used for the routine determination of FK-506; however, they lack specificity due to endogenous compounds and cross reactivity of monoclonal antibodies with the metabolites of the drug [14, 15] On the other hand, LC–MS-MS based methods are highly selective because

they depend on the physicochemical properties of the drug for detection and quantitation [16]. Although there are several LC–MS-MS methods reported in literature to determine FK-506, either alone [17–24] or with other immunosuppressant drugs [25-41] in human whole blood.. An ultra-fast liquid chromatography–tandem mass spectrometry method has also been reported for the simultaneous determination of FK-506, cyclosporine A, sirolimus and everolimus in human whole blood [42]. The method was validated in the calibration range of 1.0–44 ng/mL for FK-506 using a sample volume of 100 µL for processing. Only few analytical methods have been reported for Quantification of tacrolimus and all the methods used HPLC as liquid chromatography. Very few analytical methods have been reported for Quantification of tacrolimus by UPLC –MASS spectrometry. A rapid and Sensitive UPLC–MS-MS Determination of Tacrolimus in Wister Rats and Human Blood [43].So the present work is to develop and validate UPLC coupled with MASS spectrometric method for Quantification of tacrolimus in human plasma.

Ultra-performance liquid chromatography (UPLC) can serve as a superior alternative to high-performance liquid chromatography (HPLC), especially in reducing the rapid UPLC–MS-MS method has been developed analysis time when large number of samples to be analyzed in a clinical setting. This technology is capable of achieving higher peak capacity, speed and sensitivity than conventional HPLC. In addition, solvent consumption can be considerably reduced compared to conventional 4.6 mm I .d. columns [44]. Thus, in the present work, an accurate, simple, sensitive and rapid UPLC coupled with electron spray mass spectrometry method was developed and validated for measurement of FK-50 in non biological solvents as well as human whole blood samples.

The method requires only a 0.300 mL human blood sample for extraction and demonstrates excellent performance in terms of ruggedness and chromatographic efficiency (2.5 min per sample). Interference due to matrix was ascertained by post column infusion technique. The method was also validated in human blood by using 6 healthy human subjects.

EXPERIMENTAL

Chemicals and materials.

Reference standards of sirolimus (99.6%) and tacrolimus (IS, 98.5%) were obtained from Toronto Research Chemicals (Toronto, Canada). HPLC grade acetonitrile and methanol were procured from Merck (Darmstadt, Germany). Acetic acid and zinc sulfate were purchased from Spectro chem. Pvt. Ltd. (Mumbai, India) and SD Fine Chem. Ltd (Mumbai, India) respectively. Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Bangalore, India).

Liquid chromatographic and mass spectrometric conditions.

Chromatographic analysis was conducted on a Waters Acquity U PLC system (Milford, MA) equipped with binary solvent manager, sample manager, and column manager was used for setting of reversed phased liquid chromatographic condition. The analysis OF TACROLIMUS and IS was performed on thermo hypurity advance column (46 x 50 mm, 5um) that was maintained at 45C in a column oven. The mobile phase consisted of 10 mM ammonium acetate, pH 5.00 adjusted with acetic acid and methanol (10:90 v/v), and was delivered at a flow rate of 0.50 mL/min. Ionization and detection of FK-506 and IS were conducted on a multiplier AP14000 mass spectrometer from Waters Micro Mass Technologies, equipped with a turbo ion spray interface and operated in positive ionization mode. Quantitation was performed using multiple reaction monitoring (MRM), product! Product ion transitions of m/z 821-768.54 and m/z 931.6-864.8 was used to quantify FK-50 and IS in whole blood –(Q1)and m/z 821.5-768.54 for tacrolimus and m/z 931.5-884.6 for IS was used in whole blood –II(Q2) respectively.

The source-dependent parameters maintained for FK-506 and IS were as follows: desolvation gas, 850 L/h; capillary voltage, 355kV; desolvation temperature, 400C; entrance potential, 1.0 V; and cone gas flow, 50 L/h. The optimum values for compound-dependent parameters like cone voltage and collision energy were 31 and 21 eV for FK-506 and 30 and 17 eV for IS respectively. Quadruples 1 and 3 were maintained at unit mass resolution and the dwell time was set at 0 .2sec for both drugs. Data collection, peak integration and calculations were performed using Mass Lynx software, version 4.1.

Standard stock solutions, calibration curve standards and quality control samples.

The stock solution of FK-506 (1,000 ug/mL) was prepared by dissolving the accurately weighted reference standard in methanol. Calibration curve standards were prepared by spiking 4.75ml of k2 EDTA human whole blood with 0.25ml of appropriated stock/intermediate solutions and quality control (QC) Samples were prepared by spiking 0.95 mL of k2 EDTA human whole blood with 0.05 mL of the appropriate stock/intermediate solutions. Calibration curve standards were made with k2 EDTA human whole blood at concentrations of 0.50, .0,2.5,5.0,15,30,70,100 ng/mL where as high, medium, geometric mean, low and low limit of quantification QC samples were prepared with k2 EDTA human whole blood at concentrations of 80.0, 50.0 and 8.65,1.5,0.5 ng/mL, respectively. A stock solution (1000 g/mL) of the IS was prepared by dissolving the accurately weighted reference standard of sirolimus in methanol. Its working solution (1000 ug/mL) was prepared by appropriate dilution of the stock solutions in 60%methanol. Standard stock solutions were stored at 2-8*c. whole blood blanks, calibration curve standards and QC sample were stored at -25*C in deep freezer until use.

Blood sample preparation.

Prior to analysis, all frozen subject samples, calibration curve standards and quality control samples (in K2EDTA) were thawed and allowed to equilibrate at room temperature before extraction and Vortex the thawed samples to ensure complete mixing of contents. To an aliquot of 0.300mL of whole blood sample in a relabeled

ria vial, 30ul of 60% methanol in water solution was added followed by vortex the samples to ensure complete mixing of contents. 0.300ML of zinc sulfate hepta hydrate in methanol solution was added to all the ria vials and vortex the samples to ensure complete mixing of contents. 2.500 mL of extraction mixture was taken, shake for 15 minutes and centrifuge for 10 minutes at 4000rpm at 20*c and the supernatant approximately 2.000mL was transferred in to another ria vial .evaporate this layer under a stream of nitrogen at 40*c . The residue was reconstituted with 0.300mL of mobile phase and vortex. The sample was loaded in to auto injector vials and injects 10 uL on to LC-MS/MS system.

METHOD VALIDATION PROCEDURE.

The validation protocol and the acceptance criterion were essentially based on the ICH guide lines. The method was validated for system suitability, selectivity, Specificity, ruggedness, linearity, accuracy, precision, dilution integrity, Sensitivity, matrix effect, stability.

System suitability was checked by injecting 6 successive injections of aqueous samples of tacrolimus (50ng/ml) and IS (sirolimus) (100ng/ml) during method validation. The precision (%CV) in the measurement of retention time it was in the range of 0.00-0.37 %, and 0.63 to 1.23 for area response of tacrolimus and IS. The system performance was also verified with one processed blank sample, one upper limit of quantitation and one LLOQ along with the IS at the beginning and end of each. The auto sampler carry over for the analyte was checked by injecting the following sequence of injections, Processed blank plasma, upper limit of quantitation (ULOQ) sample, processed blank plasma, LLOQ sample, and processed blank plasma.

The selectivity of the method was checked in six blank samples from the k2EDTA whole blood (without spiking tacrolimus) obtained from six different donors. Spiked Six samples at LLOQ concentrations of tacrolimus and IS (sirolimus) in K2EDTA whole blood of any one donor and mean peak response was calculated.

The specificity of the method was established in terms of interference at retention time. It was checked by injecting six replicates of whole blood bank with IS and six replicates at MQC concentration of Tacrolimus and calculate the % interference at retention time of IS in presence of tacrolimus and % interference at retention time of tacrolimus in presence of IS.

The sensitivity was measured in terms of % accuracy and precision, which was denoted by CV% and Sensitivity was determined by limit of quantification quality control sample(LOQ-QC).The % mean accuracy should be within 80-120% and CV% at mean concentration should not exceed 20%.

Two calibration curves were performed for linearity. A calibration curve consists of a blank sample, blank with IS and 6 non zero standards covering the expected range. The method linearity was evaluated by using least square weighted (1/*x2*) linear regression, and is the best fit for the concentration /response relationship.

The intra-batch accuracy and precision, was assessed by analyzing six replicates of QC samples (low, geometric mean, medium and high) from batch-I, II and III on the same day. The inter-batch accuracy and precision were assessed by analyzing three batches on three consecutive days in a similar manner. The intra and inter batch accuracy at each concentration level should be within 85-115%.The precision [coefficient of variation (CV)] at each concentration level from the nominal concentration should not be greater than 15%.

The extraction recovery and relative matrix effect were determined at three QC levels (LQC, MQC, and HQC) in six replicates. The Relative recovery or extraction recovery for FK-506 and IS was calculated by comparing the mean area response of extracted samples (spiked before extraction) to that of un extracted sample (spiked after extraction) at each QC level. The assessment of relative matrix effect was based on the calculation of precision (% CV) values for slopes of calibration lines prepared from these batches. For a method to be practically free from relative matrix effect the % CV should not exceed 3-4 %.

The stability results were evaluated by measuring the area responses ratio (FK-506/IS) of stability samples against freshly prepared comparison samples with identical concentration. Stock solutions of FK-506 and IS were checked for short-term stability at room temperature after 16hr The Bench top stability for thawed QC samples were processed at room temperature after 18 hr and The freeze-thaw stability (at -20°C) in spiked blood samples were determined at LQC and HQC levels in six replicates after freezing and thawing the QC samples . The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed QC samples was within+15.0%.

Dilution integrity was performed by using app. 1.6 times of CC8 concentration (159.248ng/mL) in six replicates. The precision and accuracy for dilution integrity standards at 1:5 dilutions were determined by analyzing the samples against freshly prepared calibration curve standards.

Run acceptance of dilution integrity, sensitivity was performed at LQC, GMQC, MQC, and HQC in six replicates .Run acceptance of freeze and thaw stability was determined in six replicates of freshly prepared three quality control samples (LQC, MQC, and HQC). The %accuracy for at least 67%of quality control samples should be within 15%.

Application of the method

The method was applied for a clinical study in 18 fasted healthy volunteers receiving a single oral dose of 3, 7, and 10 mg capsules. The study was performed as per the International Conference on Harmonization and USFDA guidelines [46]. An Independent Ethics Committee approved the study protocol and a written consent was provided by all the subjects before enrolment in the study. The pharmacokinetic parameters of tacrolimus have been determined following oral (PO) administration in healthy volunteers, and in kidney transplant, liver transplant, and heart transplant patients. Tacrolimus maximum blood concentrations (C max) and area under the curve (AUC) appeared to increase in a dose-proportional fashion in 18

fasted healthy volunteers. In 18 kidney transplant patients, tacrolimus trough concentrations from 3 to 30 ng/mL measured at 10-12 hours post-dose (C min) correlated well with the AUC (correlation coefficient 0.93). In 24 liver transplant patients over a concentration range of 10 to 60 ng/mL, the correlation coefficient was 0.94. In 25 heart transplant patients over a concentration range of 2 to 24 ng /mL, the correlation coefficient was 0.89 after an oral dose of 0.075 or 0.15 mg/kg/day at steady-state.

The effect of food on both rate and extent of absorption was determined in 15 healthy volunteers. The presence of food decreased the absorption rate. The effect was more pronounced with a high-fat meal (848 kcal, 46% fat).The mean AUC and C max were decreased 37% and 77%, respectively; T max was lengthened 5-fold. A high-carbohydrate meal (668 kcal, 85% carbohydrate) decreased mean AUC and mean C max by 28% and 65%, respectively. The time of the meal also affected the bioavailability of tacrolimus in 16 healthy volunteers. The mean C max was reduced 71%, and mean AUC was reduced 39% when administered immediately following the meal and the mean C max was reduced 63%, and mean AUC was reduced 39% with relative to the fasted condition when administered 1.5 hours following the meal. The mean AUC and C max were decreased by 27±18% and 50±19% in 11 liver transplanted patients compared to a fasted state when administered 15 minutes after a high fat (400 kcal, 34% fat) breakfast. Hence the rate and extent of tacrolimus absorption was greatest under fasted conditions.

RESULTS AND DISCUSSION

Method development

To achieve the desired sensitivity and selectivity, the extraction procedure, chromatography and mass detection parameters were suitably optimized. In the present work, electro spray ionization (ESI) source for MRM UPLC–MS-MS analyses was used to maximize sensitivity and obtain good linearity in the regression curves. As observed in several reports [45, 46, 47 and 48] tacrolimus has low affinity for protons and therefore the protonated precursor ions in the positive ionization mode have very low abundance and thus remain undetected. The Q1 scan for FK-506 and IS showed strong ability to bind with ammonium ions (from ammonium acetate used in the mobile phase) to give peaks at *m/z* 821.50 and 931.60 corresponding to ammonium ion adducts with much higher abundance. These adducts can be readily fragmented to give stable and consistent product ions. The most abundant product ions obtained from the ammonium ion adducts in the Q3 scan corresponded to *m/z* 768.54 (neutral loss of H2O, NH3 and CH3OH) and 864.6 (neutral loss of 2H2O and NH3) for FK-506 and IS respectively at collision energy of 21 eV (**Figure 1 and 2**). In addition to the quantification transition, a qualifying transition was also monitored for the identification of the analyte (*m/z* 821.5→ 768.54) and IS (*m/z* 931.6 → 864.6). A dwell time of 0.2 min for tacrolimus and IS was adequate to have sufficient no. of data points for quantification.

Several chromatographic methods have been reported for the analysis of FK-560 from whole blood using isocratic [49] as well as gradient conditions [50, 51, and 46]. Initially, during development, different analytical columns were tested to produce a short run time, good peak shapes and minimum matrix interference and solvent consumption. Because several columns of different dimensions and particle sizes have been used in reported methods to analyze FK-506 [18, 20, 22-33, 35, 43, 45], three different columns were tested in the present work. These include Genesis C18 column, Xterra ODS column and Thermo hypurity advance column. Furthermore, the mobile phase was optimized using a different compositions of Acetonitrile: water (10:90), 10mM ammonium format: methanol (10:90), 10mM ammonium acetate buffer: methanol (10: 90) low peak responses and higher retention time were observed on Genesis C18 column with mobile phase of Acetonitrile: water (10:90), the chromatography was poor on Xterra ODS column with mobile phase of 10mM ammonium format: methanol (10:90). Nevertheless, the best chromatographic conditions as functions of analyte peak intensity and symmetric peak shape and analysis run time was achieved with the Acquity UPLC Thermo hypurity advance(4.6 x 50mm,5um) column using 10 mM ammonium acetate as the mobile phase, pH 6.00, adjusted with glacial acetic acid and methanol(10: 90) by maintaining a flow rate of 0.5ml/min under isocratic conditions The auto sampler cooler temperature and injection volume were optimized as 10*c & 10 ul respectively. The total chromatographic run time was 2.5min with a retention time of 1.37 min for FK-506 and 1.38 for IS respectively. Sirolimus which is also an immunosuppressant drug was efficiently used as an internal standard in the present study. It had similar chromatographic elution pattern and no interference at retention times and did not affect the overall accuracy and precision of the method.

Sample preparation is a decisive step for precise and accurate quantitation by LC-MS/MS methods. However, the majority of the methods have used protein precipitation (PP) [35, 36, 38, and 41] a combination of PP and SPE [18 , 22,25,26,31,37and 39] or PP and LLE [20,24]for quantitative and Consistent recoveries of FK-506 from human blood. Because of FK-506 is Sequestered within the erythrocytes, it is essential to lyses the cells with a protein precipitant to free the analyte. In the present work, ZnSO4 was used as the protein precipitant, as reported previously (25, 26), followed by liquid phase extraction using hexane and tertiary butyl methyl ether, and there was no interferences at the retention time of analyte and IS.

Assay validation results

Carryover evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. Practically, there was no carry-over observed during auto sampler carryover experiment. No enhancement in the response was observed in blank after subsequent injection of highest calibration standard (aqueous and extracted) at the retention time of tacrolimus and IS respectively .The results of system suitability have been tabulated in tables **1.0 & 2.0** .The results were within the acceptance criteria

TABLE-1.0 SYSTEM SUITABILITY-1

Sl. No.	RT (in minutes)		Peak response (area)		
	Analyte	Internal standard	Analyte	Internal standard	Analyte area / ISTD area
1	1.37	1.38	75231	67462	1.12
2	1.37	1.38	77852	68978	1.13
3	1.37	1.38	77210	69315	1.11
4	1.37	1.38	78420	69480	1.13
5	1.37	1.38	77671	69603	1.12
6	1.37	1.38	77410	69450	1.11
Mean	1.37	1.38			1.12
SD (±)	0.0052	0.0000			0.0071
Cv%	0.37	0.00			0.63

TABLE-2.0 SYSTEM SUITABILITY-I1

Sl. No.	RT (in minutes)		Peak response (area)		
	Analyte	Internal standard	Analyte	Internal standard	Analyte area / ISTD area
1	1.36	1.36	83508	82561	1.01
2	1.36	1.36	86075	81969	1.05
3	1.36	1.36	84759	82935	1.02
4	1.36	1.36	85412	82901	1.03
5	1.36	1.36	84918	82711	1.03
6	1.36	1.36	85764	83559	1.03
Mean	1.36	1.36			1.03
SD (±)	0.0000	0.0000			0.0127
Cv%	0.00	0.00			1.23

TABLE-3.0 SELECTIVITY

(I) SELECTIVITY (LLOQ)

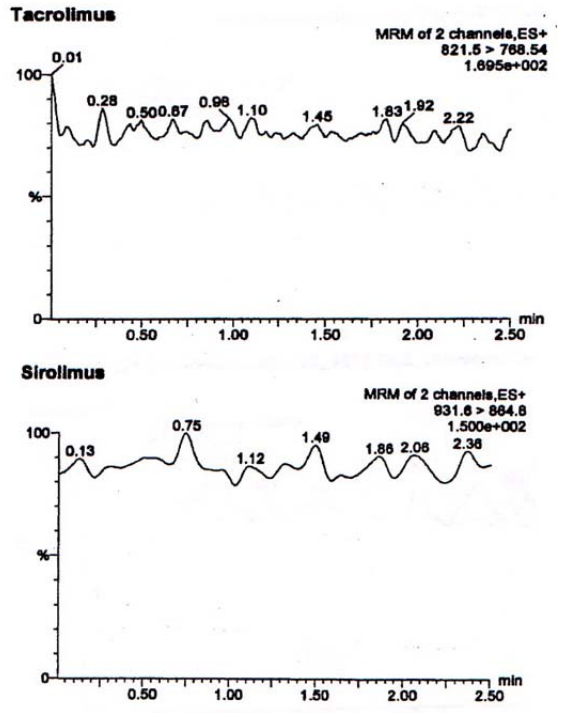
QC Level	Tacrolimus Peak area	ISTD Peak area
SP LLOQ-1	479	42310
SP LLOQ-2	435	41465
SP LLOQ-3	484	43337
SP LLOQ-4	533	44015
SP LLOQ-5	471	43664
SP LLOQ-6	503	43366
Mean	**484**	**43026**

(II) SELECTIVITY (WHOLE BLOOD BLANKS)

ID	Tacrolimus Peak area	ISTD Peak area	% interference at RT of Tacrolimus	% interference at RT of ISTD
VLL-BA/Whole Blood-2017/B	0	0	0	0
VLL-BA/Whole Blood-2018/B	0	0	0	0
VLL-BA/Whole Blood-2019/B	0	0	0	0
VLL-BA/Whole Blood-2020/B	0	0	0	0
VLL-BA/Whole Blood-2021	0	0	0	0
VLL-BA/Whole Blood-2022	0	0	0	0

There was no significant interference found at retention time of Tacrolimus and IS (Sirolimus) in whole bl ood blanks. The results for selectivity of the method for both tacrolimus and IS at LLOQ and in whole blood blanks have been tabulated in table **3.0.** The representative chromatograms of blank K2EDTA human whole blood samples shown in Figure 1

(A) Selectivity (whole blood 1)



(B) Selectivity (whole blood 2)

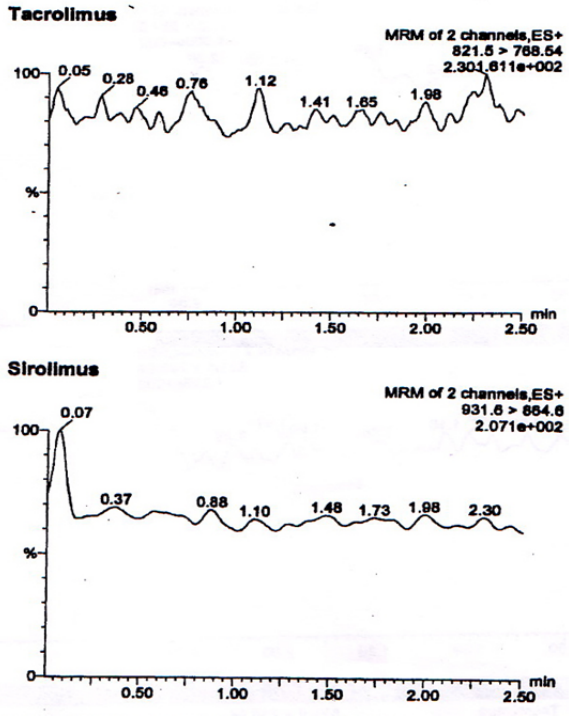


Figure 1 Chromatograms for Selectivity of tacrolimus and IS in Human Whole Blood

(A) Whole Blood-I (B) Whole Blood –II

There was no interfering peaks were found at respective retention time of tacrolimus due to IS (sirolimus) and respective retention time of IS (sirolimus) due to tacrolimus. The results of specificity have been tabulated in table **4.0**

TABLE 4.0 SPECIFICITY
(I) Specificity (MQC)

Q C Level	Tacrolimus Peak area	ISTD Peak area	% interference at RT of ISTD in presence of tacrolimus
SP MQC-1	46596	741	1.72
SP MQC-2	40424	626	1.45
SP MQC-3	40161	673	1.56
SP MQC-4	40409	723	1.68
SP MQC-5	40036	686	1.59
SP MQC-6	41147	672	1.56

(II) Specificity (IS)

ID	Tacrolimus Peak area	ISTD Peak area	% interference at RT of Tacrolimus in presence of ISTD
Whole Blood blank + ISTD -1	86	29647	17.77
Whole Blood blank + ISTD -2	61	29857	12.60
Whole Blood blank + ISTD -3	37	29881	7.64
Whole Blood blank + ISTD -4	57	29702	11.78
Whole Blood blank + ISTD -5	78	30347	16.12
Whole Blood blank + ISTD -6	42	30073	8.68

The lower limit of quantization (LOQ-QC) for sensitivity was found to be 0.500ng/mL. The % accuracy and the precision (CV %) for LOQ-QC were 108.40% and 5.00. The results are tabulated in Table **5.**

Table 5.0 PRECISION AND ACCURACY FOR LOQ-QC (SENSITIVITY)

Q C Level	LOQ-QC (ng/mL)	% Accuracy
LOQ-QC -1	0.551	110.21
LOQ-QC -2	0.543	108.66
LOQ-QC -3	0.570	114.01
LOQ-QC -4	0.499	99.81
LOQ-QC -5	0.521	104.12
LOQ-QC -6	0.565	112.96
Mean	**0.542**	
SD (±)	**0.0271**	
Cv%	**5.00**	
% Accuracy	**108.40**	
Actual Conc. (ng/mL)	**0.500**	

The calibration curves were linear over the concentration range of 0.495–99.430ng/mL. A straight-line fit was made through the data points by least square regression analysis and a constant proportionality was observed. The values of CC-I for slope, intercept and correlation coefficient (*r*2) observed were 0.0222991, 0.010946 and 0.999488 and the values of CC-II for slope, intercept and correlation coefficient were 0.00113101, 0.0205826 and 0.999136 respectively. The observed accuracy and precision (%CV) for the calibration curve standards were ranged from 98.18 to 104.65% and 2.51 to 7.8 % respectively. The representative graphs of linearity for calibration curves-I and II are shown in Figure 2.

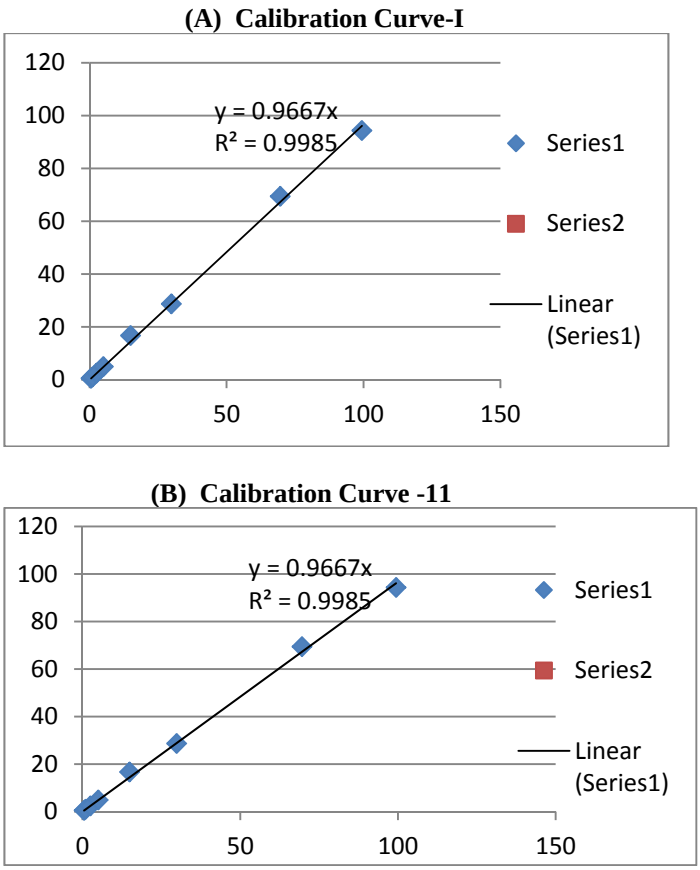


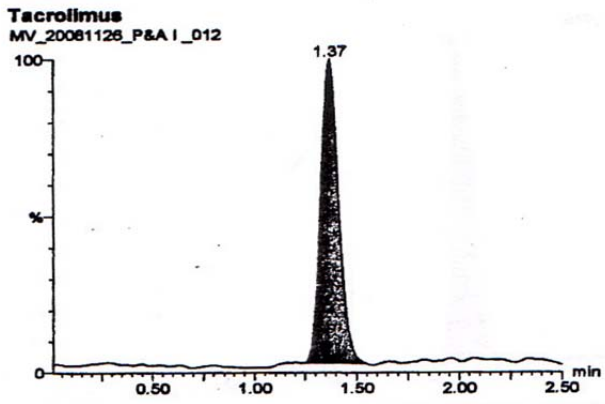
Figure 2 Graphs of Calibration Curves for Linearity (A) Calibration Curve –I (B) Calibration Curve –II

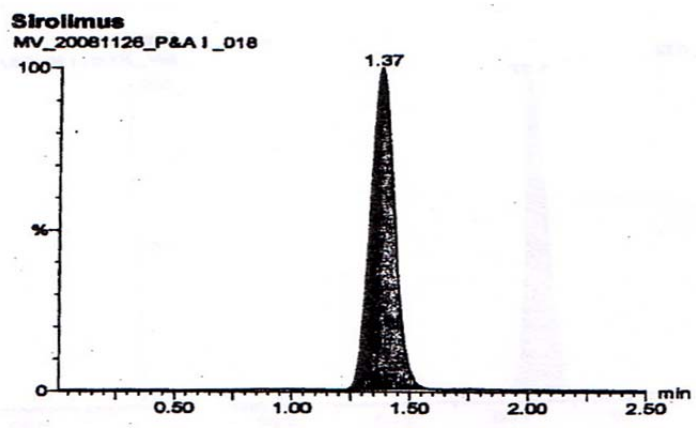
The precision (c v%) observed for the inter-batch QC samples were 5.19%, 1.78%, 3.94% and 4.21% for LQC, GMQC, MQC and HQC respectively .The c v% for the intra-batch QC samples ranged from 2.30% to 5.01%,1.35% to 2.05%,0.82% to 2.49%,and 0.85% to 2,91% for LQC,GMQC,MQC and HQC respectively. The % accuracy observed for inter-batch QC samples was 98.33%, 93.03%, 93.60% and 96.87 % for LQC, GMQC, MQC and HQC respectively. The % accuracy for intra-batch QC samples was ranged from 95.87% to 102.87%, 92.17 % to 93.85%, 90.24% to 97.88% and 93.19% to 101.74% for LQC, GMQC, MQC and HQC respectively. The results for precision and accuracy are tabulated in (Table **6**).The representative chromatograms of LQC, GMQC, MQC, and HQC shown in figures 3.

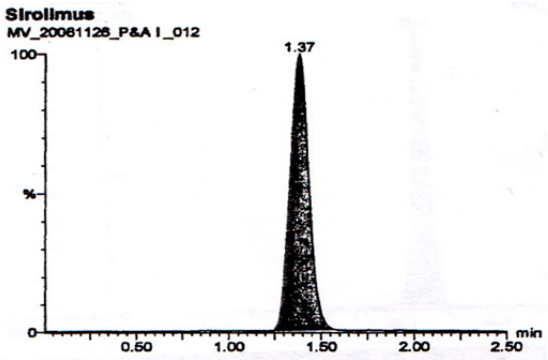
Table- 6 Inter batch and Intra batch Precision and Accuracy for Tacrolimus in Human Whole Blood

QC level Nominal Concentration ng/mL	Inter batch(n=18;6 from each batch)				Intra batch(n=18;6 from each batch)			
	Mean Conc.found ng/L	SD	CV(%)	Accuracy(%)	mean Conc found for batches ng/mL	SD	CV (%)	Accuracy(%)
LQC(1.5)	1.475	0.0765	5.19	98 33	1.475	0.0597	4 4.093	98.35
GMQC(8.607)	8.007	0.1428	1.78	93.03	8.007	0.1356	1.696	93.03
MQC(49.765)	46.579	1.8344	3.94	93.60	46.579	0.8344	1.813	93.59
HQ (79.624)	77.134	3.2465	4.21	96.87	77.133	1.2235	1.553	96.87

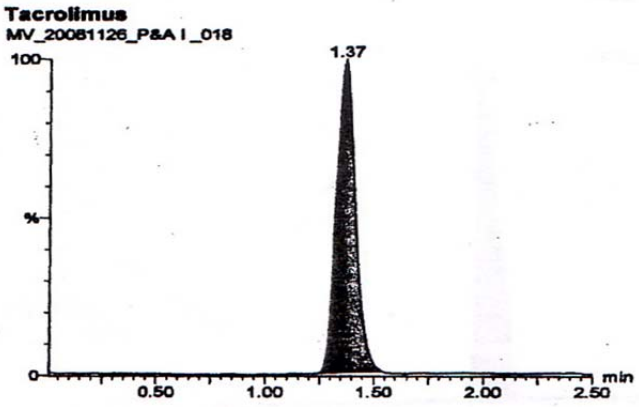
(A) LQC



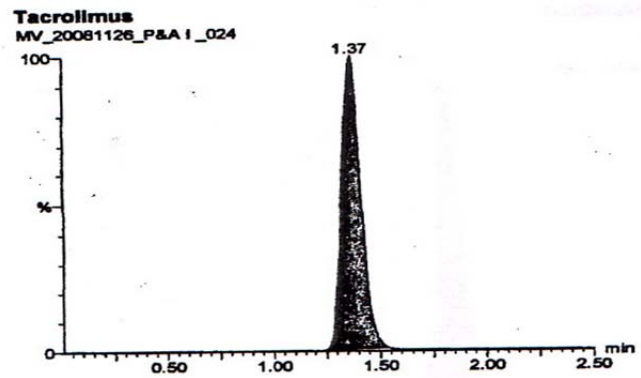


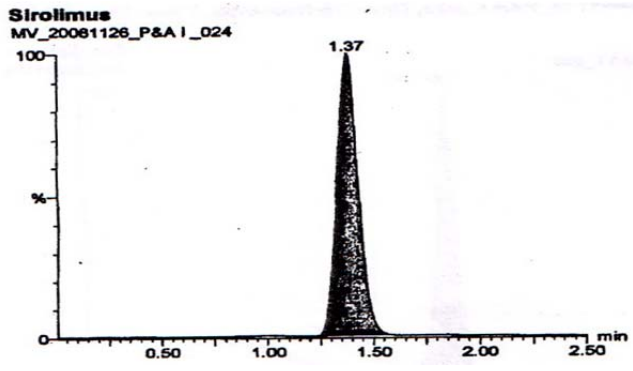


(B) GMQC



(C) MQC





(D) HQC

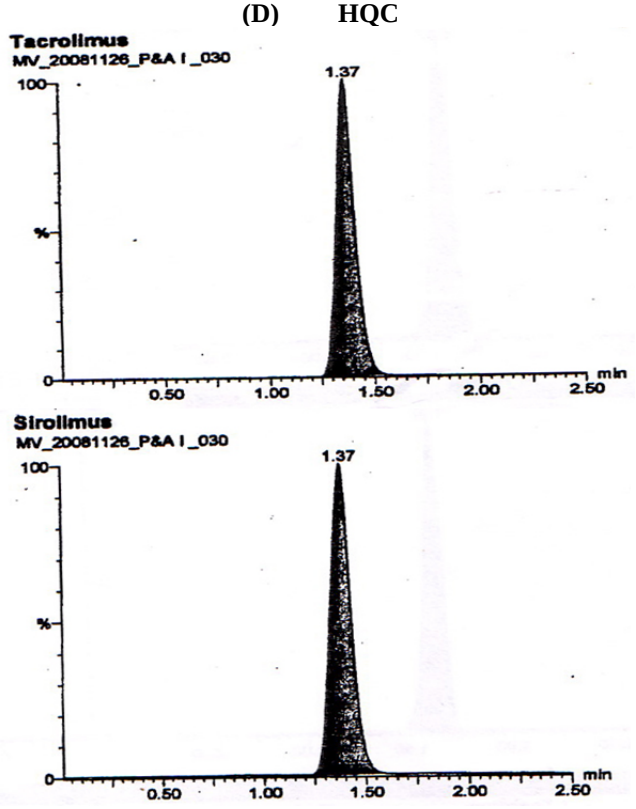


Figure 3 UPLC-MS/MS chromatograms of Tacrolimus and IS for Precision and Accuracy (A) LQC

(B) GMQC (C) MQC and (D) HQC.

The mean percentage recovery of the Tacrolimus and Sirolimus (IS) were 62.78% and 60.96% respectively. The recovery results are tabulated in table 7.

The % stability of Freeze and thaw stability for LQC and HQC samples after third cycle were found to be 97.27% and 96.42 % respectively. The %stability of Bench top stability after 18.00 hours was 100.33 % and 98.20% for LQC and HQC respectively. The run acceptance of Freeze and thaw stability for LQC, MQC and HQC samples were found to be 95.47%, 92.14% and 95.46 % respectively (Table-9.0).The % stability of stock solution for short term at LQC and HQC level was found to be 113.12&and 105.80% (Table-10).The results for the % stability of freeze and thaw stability for calibration curve standards were tabulated in table-11**.**The drug was found to be very stable up to 26 hours of in injector stability, up to 21 hrs dry extraction stability and up to 21 hours wet extraction stability.

The %accuracy and c v% of run acceptance of dilution integrity and sensitivity for LQC, GMQC, MQC and HQC were found to be 101.93 and 3.09, 107.52 and 2.29, 94.42 and 1.07, 94.89 and 0.87 respectively (Table-12). The % accuracy and CV% of dilution integrity at CC 8 level (1.6 times) for 1:5 dilutions were found to be 108.55% and 4.02% respectively. (Table- 13) Method ruggedness was evaluated along with accuracy and precision batches. The precision and accuracy values for column and analyst variation were within the acceptance criteria.

Table 7.0 Recoveries of Tacrolimus from matrix samples.

QC level N=6	Uextracted Standard peak area		Extracted matrix standard peak area		%Recovery	Mean %Recovery
	Mean area response	CV (%)	Mean area response	CV (%)		
LQC	96872	0.88	598 45	1.17	65.02	62.78
MQC	96872	1.07	598 45	0.67	61.78	
HQC	156101	1.27	960 58	1.47	61.54	

Table 8.0 Recoveries of Sirolimus (IS) from matrix samples.

QC level N=6	Un extracted Standard peak area		Extracted matrix standard peak area		%Recovery	Mean% Recovery
	Mean area response	CV (%)	Mean area response	CV (%)		
LQC	79382	0.44	475 44	0.813	59.89	60.96
MQC	80751	0.43	476 67	0.811	60.15	
HQC	78425	0.45	490 32	0.784	62.86	

Table 9.0 Stability of Tacrolimus under Different Conditions (n=6) in Human Blood.

Storage conditions	Nominal conc.ng/mL	Mean conc.ng/mL	CV%	% Stability
Freeze & thaw stability; 3 cycles, -20°C				
LQC	1.5	1.459	1.49	97.0
HQC	79.624	76.770	0.70	96.42
Bench top stability at room temperature; 18 h				
LQC	1.5	1.505	4.45	100.33
HQC	79.624	78.193	2.71	98.20
Run acceptance of freeze and Thaw stability				
LQC	1.5	1.432	2.53	95.47
MQC	50.050	46.116	0.37	92.14
HQC	80.080	76.443	1.32	95.46

Table-10 Stock Solution Stability for Analyte (Tacrolimus) (Short Term)

Storage conditions SHORT TERM STOCK SOLUTION STABILITY;16.0HR	Nominal Conc.ng/mL	Conc. after X hr ng/mL	Mean response area after X hr	CV %	% stability
LQC	1.5	1.49	2575	2.28	113.12
HQC	79.968	79.544	120381	3.14	105.80

Table-11 Freshly Spiked Calibration Curve Standards for Calculating the % Stability of Freeze and Thaw Stability.

CC Level	Sp. Cone (ng/mL)	CC set	% Accuracy
CC1	0.500	0.486	97.27
CC2	1.000	1.043	104.31
CC3	2.500	2.528	101.11
CC4	5.000	5.116	102.33
CC5	15.000	15.972	106.48
CC6	29.994	29.169	97.25
CC7	69.986	68.245	97.51
CC8	99.980	93.718	93.74

	CC set
Intercept	-0.0000870414
Slope	0.0264788
r	0.998806

Table-12 Quality Control Samples for Run Acceptance of Dilution Integrity and Sensitivity

Run acceptance Q C samples (N=6)	Nominal conc. ng/mL	Mean conc ng/mL	% Accuracy	%C V
LQC	1.5	1.529	101.93	3.09
GMQC	8.607	9.254	107.52	2.29
MQC	49.765	46.988	94.42	1.07
HQC	79.624	75.556	94.89	1.07

CONCLUSION

The assay described in this work is highly specific, sensitive and reproducible for the quantitative analysis of tacrolimus in non biological solvents as well as in human whole blood samples. The calibration curves were highly linear over a wide range of concentration. The developed UPLC-MS/MS method for the quantitation of tacrolimus in k2EDTA a whole blood was fully validated as per ICH guidelines. The specificity of the LC-MS/MS method makes the determination of these compounds in the presence of other endogenous and exogenous whole blood components possible .the chromatograms of tacrolimus and IS in human whole blood showed no significant interfering peaks.

Despite the fact that there are several methods for the quantitation of tacrolimus in whole blood, the present method offers significant advantage over those previously reported, in terms of sensitivity, reproducibility and overall analysis time. The chromatographic run time of 2.5 min per sample make it an attractive procedure in high-throughput bio analysis of tacrolimus. The specificity of the LC-MS/MS method makes the determination of these compounds in the presence of other possible endogenous and exogenous whole blood components .The chromatograms of tacrolimus and IS in human whole blood showed no significant interfering peaks. This analytical method demonstrates the robustness and reproducibility of the developed method as well as its high sensitivity. The detection limit of this method is as low as 0.495ng/ml and as high as 99.430ng/ml in human whole blood allowing the quantification of tacrolimus at low and high concentrations in body fluids.

This method can be efficiently used for quantification of tacrolimus in human whole blood in routine and bioequivalence studies

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REFERENCES

1. Kino, T., Hatanaka, H., Hasimoto, M., Nishiyama, M., Goto, T.,Okuhara, M., et al.; FK-506, novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation and physico-chemical and biological characteristics; The Journal of Antibiotics, (1987); 40:1249–125.
2. Jusko, W.J., Thomson, A.W., Fung, J., McMaster, P., Wong, S.H.,Zylber-Katz, E., et al.; Consensus document: Therapeutic monitoring of tacrolimus (FK-506); Therapeutic Drug Monitoring, (1995);17: 606–614.
3. Barun, K.D., Jimenez, E., De, S., Sawyer, J.C., McMillin, G.A.;Analytical performance characteristics of the Abbott Architecti2000 Tacrolimus assay; Comparisons with liquid chromatography tandem mass spectrometry (LC-MS/MS) and Abbott IMx methods;Clinica Chimica Acta, (2009); 410: 25–30.
4. Liu, J., Farmer, J., Lane, W., Friedman, J., Weissman, I., Schreiber, S.;Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes; Cell, (1991); 66: 807–815.

5. Oellerich, M., Armstrong, V.W.; The role of therapeutic drug monitoring in individualizing immunosuppressive drug therapy: recent developments; Therapeutic Drug Monitoring, (2006); 28: 720–725.
6. Shaw, L.M., Holt, D.W., Keown, P., Venkataramanan, R., Yatscoff,R.W.; Current opinions on therapeutic drug monitoring of immune suppressive drugs; Clinical Therapeutics, (1999); 21: 1632.
7. Skoog DA, Holler FJ, Niemen DA. Principles of Instrumental Analysis, 5thedition, Thomson Brooks/Cole Publication, 2004, 529-532.
8. James J Pitt, Clin biochem rev, vol 30, February 2009, 19-34
9. Capron, A., Lerut, J., Verbaandert, C., Mathys, J., Ciccarelli, O.Vanbinst, R., et al.; Validation of a liquid chromatography-mass spectrometric assay for tacrolimus in liver biopsies after hepatic transplantation: Correlation with histopathologic staging of rejection; Therapeutic Drug Monitoring, (2006); 28: 720–725.
10. Wallemacq, P.E.; Therapeutic monitoring of immune suppressant drugs. Where are we? Clinical Chemical Laboratory Medicine,(2004); 42: 1204–1211.
11. Venkataramanan, R., Swaminathan, A., Prasad, T., Jain, A.,Zuckerman, S., Warty, V., et al.; Clinical pharmacokinetics of tacrolimus; Clinical Pharmacokinetics, (1995); 29: 404–430.
12. Yu-Luan Chen . *et al*, Simultaneous determination of three isomeric metabolites of tacrolimus (FK506) in human whole blood and plasma using high performance liquid chromatography–tandem mass spectrometry . Journal of Chromatography B Volume 830, Issue 2, 18 January 2006, Pages 330-341
13. Snyder LR, Kirkland JJ, Practical H.P.L.C. method development**,** 2nd Edition, John Wiley and Sons Publication, 2000, 89-97.
14. Westley, I.S., Taylor, P.J., Salm, P., Morris, R.G.; Cloned enzyme donor immunoassay tacrolimus assay compared with high performance liquid chromatography-tandem mass spectrometry and microparticle enzyme immunoassay in liver and renal transplant ecipients; Therapeutic Drug Monitoring, (2007); 29:584-591.
15. Moscato, D., Nonnato, A., Adamo, R., Vancheri, M., Caropreso, A.,Therapeutic monitoring of tacrolimus: Aberrant results by an immunoassay with automated pretreatment; Clinica Chimica Acta,(2010); 411: 77–80.
16. Taylor, P.J.; High-performance liquid chromatography-mass spectrometry in the clinical laboratory; Therapeutic Drug Monitoring, (2005); 27: 689–693.
17. Lensmeyer, G.L., Poquette, M.A.; Therapeutic monitoring of tacrolimus concentrations in blood: Semi-automated extraction and liquid chromatography-electrospray ionization mass spectrometry;Therapeutic Drug Monitoring, (2001); 23: 239–249.
18. Hoogtanders, K., Van der Heijden, J., Christiaans, M., Edelbroek, P.,van Hooff, J.P., Stolk, L.M.L.; Therapeutic drug monitoring of tacrolimus with the dried blood spot method; Journal of Pharmaceutical and Biomedical Analysis, (2007); 44: 658–664.
19. Li, J.L., Wang, X.D., Wang, C.X., Fu, Q., Liu, L.S., Huang, M., et al.;Rapid and simultaneous determination of tacrolimus (FK506) and diltiazem in human whole blood by liquid chromatography tandem mass spectrometry: Application to a clinical drug–drug interaction Study; Journal of Chromatography B, (2008); 867: 111–118.
20. Capron, A., Musuamba, F., Latinne, D., Mourad, M., Lerut, J.,Haufroid, V., et al.; Validation of a liquid chromatography mass spectrometric assay for tacrolimus in peripheral blood Mononuclear cells; Therapeutic Drug Monitoring, (2009); 31:178–186.
21. Patil, R.S., Patil, S.R., Makone, S.S., Chaitanya, K.A., Thirugnanam, P.E.;Simple, rapid and sensitive method for determination of tacrolimusin human blood by using liquid chromatography/tandem mass spectrometry; spectrometry; Current Trends in Biotechnology and Pharmacy,(2010); 4: 684–690.
22. Napoli, K.L., Hammett-Stabler, C., Taylor, P.J., Lowe, W., Franklin,M.E., Morris, M.R., et al.; Multi-center evalaution of a commercialkit for tacrolimus determination by LC/MS/MS; Clinical Biochemistry, (2010); 43: 910–920.
23. Taillon, M.P.; Furtado, M., Garofolo, F.; Challenges of developing a bio analytical method for a macrodile immunosuppressant compound by LC-MS/MS; Bio analysis, (2011); 3: 1201–1215.
24. Dubbelboer, I.R., Pohanka, A., Said, R., Rosenborg, S., Beck, O.Quantification of tacrolimus and three demethylated metabolites inhuman whole blood using LC-ESI-MS/MS; Therapeutic Drug Monitoring, (2012); 34: 134–142.
25. Taylor, P.J., Salm, P., Lynch, S.V., Pillans, P.I.; Simultaneous quantification of tacrolimus and sirolimus in human blood by high performance liquid chromatography-tandem mass spectrometry; Therapeutic Drug Monitoring, (2000); 22: 608–612.
26. Christians, U., Jacobsen, W., Serkowa, N., Benet, L.Z., Vidal, C.,Sewing, K.F., et al.; Automated fast and sensitive quantification ofdrugs in blood by liquid chromatography-mass spectrometry withon-line extraction: Immuno suppressants; Journal of Chromatography B, (2000); 748: 41–53.
27. Volosov, A., Napoli, K.L., Soldin, S.J.; Simultaneous simple and fast quantification of three major immune suppressants by liquid chromatography-tandem mass-spectrometry; Clinical Biochemistry, (2001); 34: 285–290.
28. Hatsis, P., Volmer, D.A.; Evaluation of a cyano stationary phase for the determination of tacrolimus, sirolimus and cyclosporine A in whole blood by high-performance liquid chromatography-tandem mass spectrometry; Journal of Chromatography B, (2004); 809:287–294.
29. Ceglarek, U., Lembcke, J., Fiedler, G.M., Werner, M., Witzigmann, H.,Hauss, J.P., et al.; Rapid simultaneous quantification of immune suppressants in transplant patients by turbulent flow chromatography combined with tandem mass spectrometry; Clinica Chimica Acta, (2004); 346: 181–190.
30 .Koal, T., Deters, M., Casetta, B., Kaever, V.; Simultaneous determinationof four immunosuppressant by means of high speed and robust on-line solid phase extraction-high performance liquid chromatography-tandem mass spectrometry; Journal of Chromatography B, (2004); 805: 215–222.
31. Wang, S., Magill, J.E., Vicents, F.B.; A fast and simple high performance liquid chromatography/mass spectrometry method for simultaneous measurement of whole blood tacrolimus and sirolimus; Archives of Pathological and Laboratory Medicine, (2005);129: 661–665.
32. Poquette, M.A., Lensmeyer, G.L., Doran, T.C.; Effective use of liquid chromatography-mass spectrometry (LC/MS) in the routine clinical laboratory for monitoring sirolimus, tacrolimus and cyclosporine; Therapeutic Drug Monitoring, (2005); 27: 144–150.
33. Ceglarek, U., Casetta, B., Lembcke, J., Baumann, S., Fiedler, G.M.,Thiery, J.; Inclusion of MPA and in a rapid multi-drug LC-tandem mass spectrometric method for simultaneous determination o fimmuno suppressants; Clinica Chimica Acta, (2006); 373:168–171.
34. Bogusz, M.J., Enazi, E.A., Hassan, H., Abdel-Jawaad, J., Ruwaily, J.A.,Tufail, M.A.; Simultaneous LC-MS-MS determination of cyclosporineA, tacrolimus and sirolimus in whole blood as well as mycophenolic acid in plasma using common pretreatment procedure; Journal of Chromatography B, (2007); 850: 471–480
35. Salm, P., Taylor, P.J., Rooney, F.; A high-performance liquid chromatography-mass spectrometry method using a novel atmospheric pressure chemical ionization approach for the rapid simultaneous measurement of tacrolimus and cyclosporine in whole blood; Therapeutic Drug Monitoring, (2008); 30: 292–300
36. Ansermot, N., Fathi, M., Veuthey, J.L., Desmeules, J., Rudaz, S.,Hochstrasser, D.; Simultaneous quantification of cyclosporine,t acrolimus, sirolimus and everolimus in whole blood by liquid chromatography-electrospray mass spectrometry; Clinical Biochemistry, (2008); 41: 728–735.
37. Koster, R.A., Dijkers, E.C.F., Uges, D.R.A.; Robust, high-throughputLC-MS/MS method for therapeutic drug monitoring of cyclosporine,tacrolimus, everolimus and sirolimus in whole blood;Therapeutic Drug Monitoring, (2009); 31: 116–125.
38. Sallustio, B.C., Noll, B.D., Morris, R.G.; Comparison of blood sirolimus,tacrolimus and everolimus concentrations measured byLC-MS/MS, HPLC-UV and immunoassay methods; ClinicalBiochemistry, (2011); 44: 231–236.
39. Laverdie` re, I., Caron, P., Couture, F., Le´ vesque, E., Guillemette, C.; Aliquid chromatography–tandem mass spectrometry (LC-MS/MS)method for monitoring drug exposure in hematopoietic stem cell transplant recipients; Journal of Chromatography B, (2012); 885-886: 131–137.
40. He tu, P.O., Robitaille, R., Vinet, B.; Successful and cost-efficient replacement of immunoassays by tandem mass spectrometry for the quantification of immune suppressants in the clinical laboratory;Journal of Chromatography B, (2012); 883– 884: 95–101
41. Buchwald, A., Winkler, K., Epting, T.; Validation of an LC-MS/MS method to determine five immune suppressants with deuterated

internal standards including MPA; BMC Clinical Pharmacology,(2012); 12: 1–11.
42. Meinitzer, A., Gartner, G., Pilz, S., Stettin, M.; Ultra fast liquid chromatography-tandem mass spectrometry routine method for simultaenous determination of cyclosporin A, tacrolimus, sirolimus ,and everolimus in whole blood using deuterated internal standards for cyclosporin A and everolimus; Therapeutic Drug Monitoring,(2010); 32: 61–66.
43. Vivek Upadhyay, Vikas Trivedi, Gaurang Shah, Manish Yadav and Pranav S. Shrivastav Rapid and Sensitive UPLC–MS-MS Determination of Tacrolimus in Wistar Rats and Human Blood Journal , M. Fathi, J. Veutheyof Chromatographic Science(2012);1–9.
44. Chesnut, S.M., Salisbury, J.J.; The role of UHPLC in pharmaceutical development; Journal of Separation Science, (2007); 30:1183–1190.
45. R.A. Koster, E.C. Dijkers, D.R.Uges, Robust, high-throughput LC-MS/MS method for therapeutic drug monitoring of cyclosporine, tacrolimus, everolimus, and sirolimus in whole blood, Ther. Drug Monit. 31 (2009) 116-125.
46. M. Karapirli, M. Kizilgun, O. Yesilyurt, H. Gul, Z.I. Kunak, E.O. Akgul, E. Macit, T. Cayci, Y.G. Kurt, I. Aydin, H. Yaren, M. Seyrek, E. Cakir, H. Yaman, Simultaneous determination of cyclosporine A, tacrolimus, sirolimus, and everolimus in whole-blood samples by LC-MS/MS, Scientific World Journal (2012), doi:10.1100/2012/571201
47. R.A. Koster, J.C. Alffenaar, B. Greijdanus, D.R.A. Uges, Fast LC-MS/MS analysis of tacrolimus, sirolimus, everolimus and cyclosporine in dried blood spots and the influence of the hematocrit and immunosuppressant concentration on recovery, Talanta 115 (2013) 47–54.
48. B.K. Matuszewski, M.L. Constanzer, and C.M. Chavez-Eng. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal. Chem. 75: 3019-3030 (2003)
49. M.A. Campaneroa, E. Cardenas, B. Sadaba, E. Garcia-Quetglas, M.J. Munoz-Juarez, I.G. Aldea, D. Pazo, J.R. Azanza, J. Honorato, Therapeutic drug monitoring for sirolimus in whole blood of organ transplants by high-performance liquid chromatography with ultraviolet detection, J. Chromatogr. A, 1031 (2004) 265–273.
50. M.J. Bogusz, E. Al Enazi, H. Hassan, J. Abdel-Jawaad, J. Al Ruwaily, M. Al Tufail, Simultaneous LC–MS–MS determination of cyclosporine A, tacrolimus, and sirolimus in whole blood as well as mycophenolic acid in plasma using common pretreatment procedure, J. Chromatogr. B 850 (2007) 471–480.
51. N. Ansermot, J. Desmeules, S. Rudaz, D. Hochstrasser, Simultaneous quantification of cyclosporine, tacrolimus, sirolimus and everolimus in whole blood by liquid chromatography–electrospray mass spectrometry, Clin. Biochem. 41 (2008) 728–735.