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Liquid-liquid Extraction of Everolimus an Immunosuppressant from Human Whole Blood and its Sensitive Determination by UHPLC-MS/MS

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ABSTRACT

A sensitive and precise ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method has been developed and fully validated for therapeutic drug monitoring of everolimus in human whole blood. Sample preparation involved liquid-liquid extraction of everolimus and its deuterated internal standard (IS, everolimus-d4) from 100 µL blood sample using diethyl ether: ethyl acetate (30:70, *v/v*) solvent system under alkaline conditions. The chromatography was conducted on a COSMOSIL 2.5C18-MS-II (50 mm × 2.0 mm, 2.5 µm) analytical column. The analyte and IS were eluted within 2.5 min under isocratic conditions using 10mM ammonium acetate, pH 6.00 adjusted with formic acid and acetonitrile (20:80, *v/v*). Multiple reaction monitoring was used for the quantitation of everolimus (*m/z* 975.5 → 908.5) and everolimus-d4 (*m/z* 979.6 → 912.6) in the positive ionization mode. The method was shown to be linear over the entire concentration range from 0.10-50.0 ng/mL. The recovery ranged from 90.9-94.8 % for everolimus and 91.4-95.6 % for everolimus-d4. The selectivity of the method is demonstrated in six different sources of blank human blood. The method is free from matrix effect as apparent from the post-column analyte infusion experiment, absolute and relative matrix effect results. The stability of everolimus in whole blood was thoroughly established under different storage conditions.

Keyword: Everolimus; everolimus-d4; UHPLC-MS/MS; liquid-liquid extraction; sensitivity; matrix effect

INTRODUCTION

Everolimus a macrocyclic lactone is a derivative of sirolimus having 2-hydroxyethyl chain at position 40 of the macrolide ring. It has potent antiproliferative and immunosuppressive effects with greater stability and solubility as well as more favourable pharmacokinetics [1]. Nevertheless, the immunosuppressive action of everolimus is similar to its parent drug sirolimus. Everolimus is a selective mammalian target of rapamycin (mTOR) inhibitor that specifically targets the mTOR signal transduction complex (mTORC1). It has shorter half-life (18-35 h across different treatment groups) than sirolimus and is thus administered twice a day. It is mainly bound to red blood cells (>75 %) and therefore blood matrix is recommended for pharmacokinetic studies. The oral bioavailability of everolimus is approximately 16 % and the time to reach peak plasma concentration is 0.5-1.0 h [2, 3]. Like sirolimus, it is metabolized by the CYP-450 enzyme system and is also a substrate for P-glycoprotein. Further, everolimus exhibits substantial intra- and inter-subject variation in its pharmacokinetics. Everolimus has a narrow therapeutic index and a variable bioavailability. The projected therapeutic range is 3.0–8.0 µg/L. At levels greater than 3.0 µg/L, there has been less incidence of organ rejection, while at concentrations greater than 8.0 µg/L there are increased chances for toxicity [2-5]. Thus, sensitive and rugged methods are required for therapeutic drug monitoring (TDM) of everolimus.

Several methods are presented in the literature to estimate everolimus from different human specimens like mouse plasma [6], human peripheral blood mononuclear cells [7], dried blood spots (DBS) [8, 9] and human whole blood [10-33] samples. The common techniques used for analysis includes immunoassay [3, 23, 26], HPLC-UV [13, 23] and LC-MS/MS [8-12, 14-25, 27-32]. Due to cross reactions between the drug and their metabolites, immunoassay method are generally not preferred as they may cause overestimation of drug concentration with unacceptable bias. Thus, more selective LC-MS/MS methods are recommended

for TDM. Additionally, few other methods based on UPLC-MS/MS are presented for high-throughput applications [6, 7, 33]. Hsieh et al. [6] described a ultra-performance hydrophilic interaction liquid chromatography (UPHILIC) for the determination of everolimus in the range of 10-5000 ng/mL in mouse plasma samples within 1.0 min. The plasma samples were prepared by protein precipitation with acetonitrile. Similarly, everolimus quantification (linear range 1.25-12.5 ng/mL) in peripheral blood mononuclear cells was also carried out by a UPLC-MS/MS method [7]. In the third UPLC-MS/MS method, five immunosuppressants were determined by improved sample preparation procedure using $ZnSO_4$ (for haemolysis) and acetonitrile (for protein precipitation). The linear range established for everolimus was 1.0-28 ng/mL [33].

In the present work a sensitive, precise and a rugged UHPLC-MS/MS method has been developed using liquid-liquid extraction (LLE) of everolimus from whole blood samples. The method was thoroughly validated for selectivity, matrix effect, and stability. Under the optimized conditions, the sensitivity achieved was 0.10 ng/mL employing 100 µL human blood sample.

EXPERIMENTAL

Chemicals and materials

Reference standards of everolimus (99.1 %) and everlimus-d4 (IS, 98.8 %) were obtained from Clearsynth Lab (Mumbai, India). HPLC grade acetonitrile and boric acid/potassium chloride/sodium hydroxide, pH 10.0 buffer was procured from Merck (Darmstadt, Germany). Formic acid and ammonium acetate was purchased from Spectrochem Pvt. Ltd. (Mumbai, India), and Sigma-Aldrich (St. Louis, MO, USA) respectively. Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Bangalore, India). Lichrosep Sequence SPE Cartridge (30 mg, 1 mL) was purchased from Merck. Blank human blood was obtained from in-house clinical department and was stored at –20 □C till use.

Liquid chromatographic and mass spectrometric conditions

A Waters Acquity UPLC system (MA, USA) consisting of binary solvent manager, sample manager and column manager was used for setting the reverse-phase liquid chromatographic conditions. The analysis of everolimus and IS was performed on a COSMOSIL 2.5C18-MS-II (50 mm × 2.0 mm, 2.5 µm) analytical column maintained at 40°C in a column oven. For isocratic elution a mobile phase consisting of 10mM ammonium acetate, pH 6.00 adjusted with formic acid and acetonitrile (20:80, *v/v*) was used. The flow rate of the mobile phase was kept at 0.400 mL/min and the sample manager temperature was maintained at 10 °C.

Ionization and detection of everolimus and IS was carried out on a triple quadrupole mass spectrometer from Waters – Micro Mass Technologies (MA, USA), equipped with turbo ion spray interface and operating in positive ionization mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor precursor → product ion transitions of *m/z* 975.5 → 908.5 for everolimus and *m/z* 979.6 → 912.6 for IS. A qualifying transition of *m/z* 975.5 → 926.6 and *m/z* 979.6 → 930.5 was also measured for the identification of analyte and IS respectively. For both the compounds, the optimized mass spectrometer parameters were as follows, capillary voltage 4.0 kV, desolvation temperature 400 °C, desolvation gas flow 800 L/h, cone gas flow 50 L/h, and source temperature 100 °C. The compound specific parameters like cone voltage and collision energy were set at 27 V and 19 eV for everolimus and 30 V and 17 eV for IS respectively. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 200 ms for both the drugs. Data collection, peak integration, and calculations were performed using Mass Lynx software version 4.1.

Standard stock, calibration standards and quality control samples

The standard stock solution of everolimus (1000 µg/mL) was prepared by dissolving requisite amount in methanol. Calibration standards (CSs) and quality control (QC) samples were prepared by spiking (2% of total blood volume) blank blood with stock solution. Calibration curve standards were made at 0.100, 0.200, 0.600, 2.00, 4.00, 10.0, 25.0, 40.0, and 50.0 ng/mL concentrations respectively, while quality control samples were prepared at three levels, viz. 45.0 ng/mL (HQC, high quality control), 25.0/3.00 ng/mL (MQC-1/2, middle quality control 1/2), and 0.300 ng/mL (LQC, low quality control). Stock solution (0.2 mg/mL) of the internal standard was prepared by dissolving 5.0 mg of in 25.0 mL of methanol. Its working solution (100 ng/mL) was prepared by appropriate dilution of the stock solution in methanol. Standard stock and working solutions used were stored at 5 °C, while CSs and QC samples in plasma were kept at -70 °C until use.

Sample extraction protocol

Prior to analysis, all frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 100 µL of spiked blood sample, 50 µL of internal standard was added and vortex-mixed for about 15 sec. Further, 100 µL, boric acid/potassium chloride/sodium hydroxide pH 10.0 buffer was added and vortex for 1 min. Extraction of analyte and IS was done with 2.5 mL of diethyl ether: ethyl acetate (30:70, *v/v*) on a rotary mixer (rotospin) for 10 min at 32 × g. Subsequently, centrifugation of the sample was done at 3204 × g for 5 min at 10 °C. The organic layer (2.0 mL) was separated and evaporated to dryness in a thermostatically controlled water-bath maintained at 40°C under a gentle stream of nitrogen. The dried samples were reconstituted in 100µL of mobile phase and 5µL was used for injection in the chromatographic system.

Method validation procedures

The validation protocol and the acceptance criterion were essentially based on the USFDA guidelines [34] and similar to our previous work [35].

System suitability was checked by injecting 6 successive injections of aqueous samples of everolimus (25 ng/mL) and IS (100 ng/mL) at the beginning of each batch. The precision (%CV) in the measurement of retention time it was in the range of 0.11-0.15 %, and 0.45 to 1.19 for area response of everolimus 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 batch. The S/N ratio was ≥ 40 for the analyte and IS. 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 different batches/lots of blank blood which included 5 normal and 1 lipemic blood lots. The method linearity was evaluated from four linearity curves using least square weighted ($1/x^2$) linear regression. Intra-batch accuracy and precision was assessed by analyzing six

replicates of LQC, MQC-1/2, and HQC samples from a single batch on the same day, while for inter-batch, five batches were analyzed on three consecutive days in a similar manner.

Ion suppression/enhancement effects on the method sensitivity and selectivity was studied by the post column analyte infusion experiment. A standard solution containing everolimus (25 ng/mL) and IS (100 ng/mL) was infused post column via a 'T' connector into the mobile phase at 10µL/min employing an in-built infusion pump. Further, 5 µL aliquots of extracted control blood were then injected into the column and chromatogram was acquired for everolimus and IS.

The extraction recovery of everolimus and matrix effect were determined at four QC levels in six replicates as reported previously [36]. Relative recovery or extraction recovery was calculated by comparing the mean area response of extracted samples (spiked before extraction) to that of unextracted samples (spiked after extraction) at each QC level for the analyte and IS. Absolute matrix effect was computed by comparing the mean area response of unextracted samples (spiked after extraction) with mean area of neat standard solutions. The relative matrix effect was also checked in six different batches/lots of blood at LQC and HQC levels. The precision and accuracy in the measurement of concentration was estimated at both these levels.

The stability of everolimus and IS was examined in stock solutions and for the analyte in matrix by comparing the area response ratio (sirolimus/IS) of the stability samples with freshly prepared comparison samples. Bench top stability at room temperature, wet extract (autosampler) stability at 5 °C, freeze-thaw and long-term stability (at -20°C and -70°C) in spiked blood samples were determined at LQC and HQC levels in six replicates.

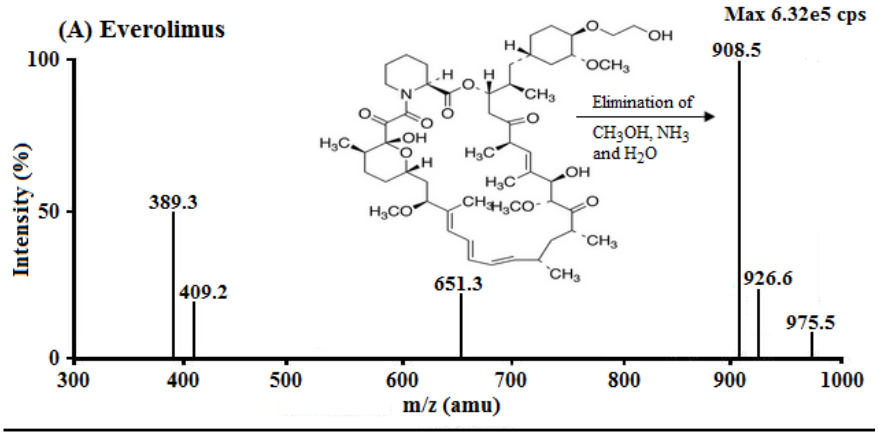
Dilution integrity was established from six replicates of standards prepared at 1/2 (45.0 ng/mL) and 1/10th (9.00 ng/mL) dilution, by spiking standard stock solution of everolimus (90.0 ng/mL) in the screened blank blood. The precision and accuracy were evaluated by comparing the results against freshly prepared calibration curve standards. Method ruggedness was ascertained by analyzing two precision and accuracy batches. The first batch was analyzed by different analysts while the second batch was studied on two different columns.

RESULTS AND DISCUSSION

Method development

As everolimus is predominantly present in red blood cells, its measurement in whole blood is required for TDM. The aim of this work was to develop a simple, sensitive and rugged protocol for everolimus for routine subject sample analysis. For selective extraction of immunosuppressants from whole blood sample preparation plays a crucial role. Several reports have highlighted the importance of protein precipitation with methanol/ $ZnSO_4$ followed by on-line SPE for rapid and reproducible results [12, 16, 21, 22, 27]. Few other methods have optimized sample preparation with methanol-acetonitrile [17], followed by on-line SPE [19]. In the present work an attempt was made to use liquid-liquid extraction of everolimus without the need for on-line SPE as it has very low water solubility and a high log P value of 5.01. Many trials were undertaken with different organic diluents like methyl *tert*-butyl ether, diethyl ether, dichloromethane, ethyl acetate and their combinations in different proportions (50:50, 60:40, 70:30 and 80:20, *v/v*) at different pH (8.0, 9.0 and 10.0). The best extraction solvent system for quantitative and precise recovery of everolimus and everolimus-d4 was diethyl ether-ethyl acetate (30:70, *v/v*) at pH 10±0.1.

To maximize the desired sensitivity and selectivity, electrospray ionization (+ESI) source was used to obtain good linearity in the regression curves. As reported by several authors [17, 21, 28, 33], protonated precursor ions $[M+H]^+$ were not observed in the Q1 full scan mass spectra as it has less affinity for protons in the positive ionization mode. In the negative ionization mode the deprotonated ions were observed but the intensity was too low to achieve the desired sensitivity of 0.10ng/mL. The formation of sodium $[M+Na]^+$ and ammonium ion $[M+NH_4]^+$ adducts were observed in the positive ionization mode for everolimus and its deuterated analog. However, higher sensitivity was found for $[M+NH_4]^+$ compared to $[M+Na]^+$ adduct as precursor ions using multiple reaction monitoring and hence was selected in the present work. The Q1 mass spectra for everolimus and IS showed peaks at *m/z* 975.5 and 979.6 respectively corresponding to the ammonium ion adducts. The most abundant product ions obtained in the Q3 scan were at *m/z* 908.5 and 912.6 for everolimus and IS respectively, which were due to neutral loss of H_2O, NH_3 and CH_3OH (**Figure 1**). To check the identity of the analyte and IS, a qualifying transition of *m/z* 975.5 → 926.6 and *m/z* 979.6 → 930.5 respectively was also monitored. A dwell time of 200 ms provided adequate number of data points for quantitation.



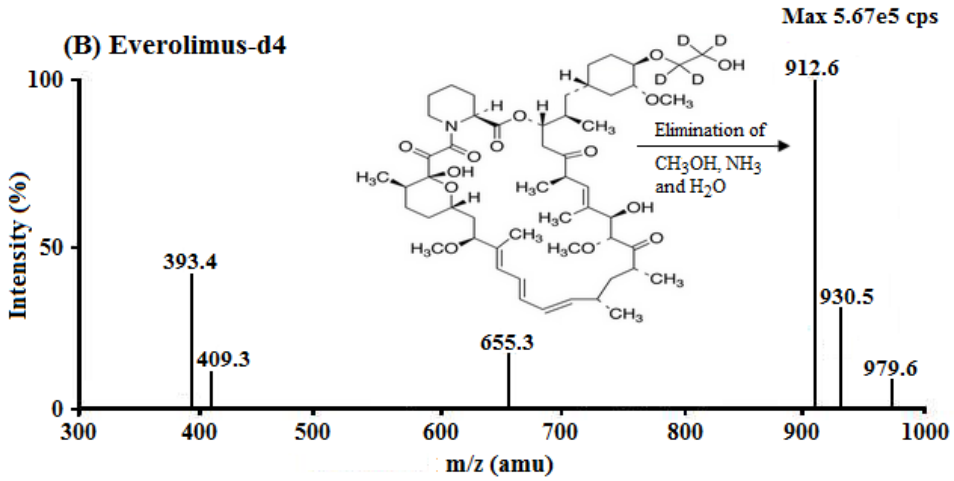
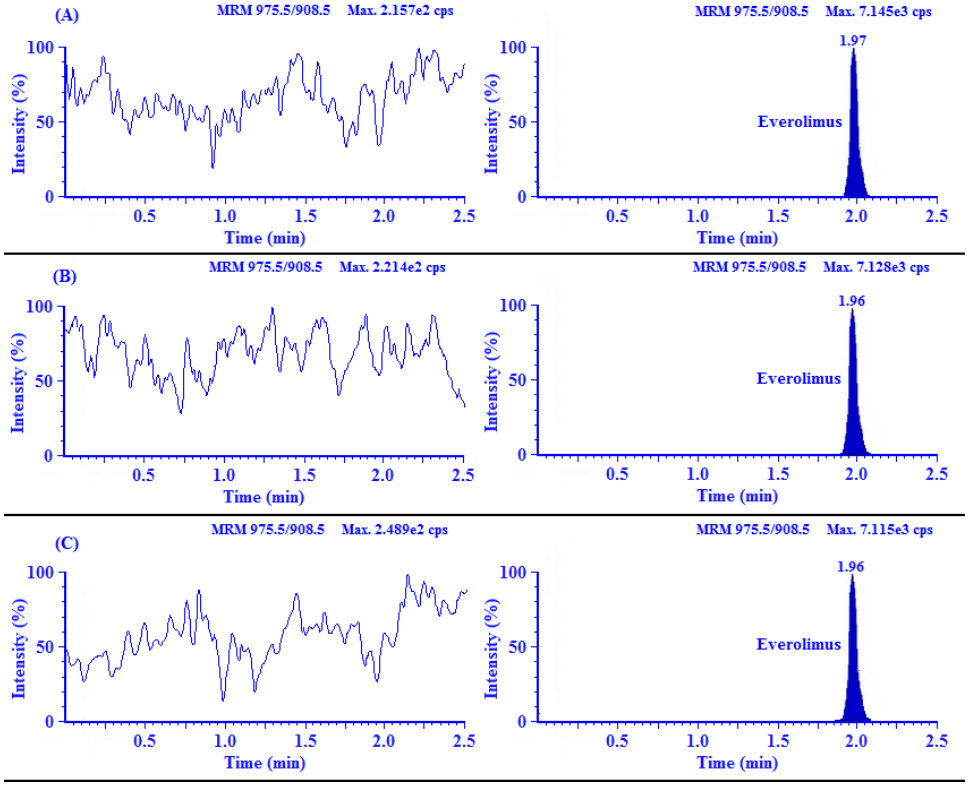


Figure 1 Product ion mass spectra of (A) everolimus (*m/z* 975.5 → 908.5, scan range 300-1000 amu) and (B) everolimus-d4 (IS, *m/z* 979.6 → 912.6, scan range 300-1000 amu) in the positive ionization mode.

Several analytical columns have been tried for rapid chromatographic analysis of different immunosuppressants. Recently, Tszyrsznic et al. [33] developed a rapid and robust ultra performance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) method for the quantification of four immunosuppressants in whole blood including everolimus on Waters UPLC BEH C18 column within 3.0 min. In the present work, two analytical columns namely BEH C18 (50 mm × 2.1mm, 1.7µm) and COSMOSIL 2.5C18-MS-II (50 mm × 2.0 mm, 2.5 µm) were tried to achieve symmetric peaks shapes, adequate retention and short run time under isocratic conditions. Several aqueous buffer-organic diluent combinations were tried using acetonitrile and methanol. Different buffer solutions having pH ranging from 4.0-9.0 were tested. The best elution conditions were optimized on COSMOSIL 2.5C18-MS-II column which provided better separation and shorter elution time compared to BEH C18 column. By maintaining a flow rate of 0.400 mL/min, everolimus and IS was eluted at 1.97 and 1.96 min respectively. The choice of internal standard is critical in mass spectrometric analyses; use of general internal standards can cause errors in measurements due to ionization process different from that of the analyte [28]. Thus, deuterated internal standard was used which shares close chemical properties with the parent compound and had similar retention time. This can adequately compensate for any possible matrix effect and also for true recovery. The chromatograms in **Figure 2** of six extracted blank blood samples and everolimus at 0.10 ng/mL concentrations, display the selectivity of the method. There were no peaks corresponding to the endogenous components at the retention time of everolimus. A summary of salient features of liquid chromatographic methods with mass spectrometry detection for determination of everolimus in human whole blood is presented in **Table 1**.



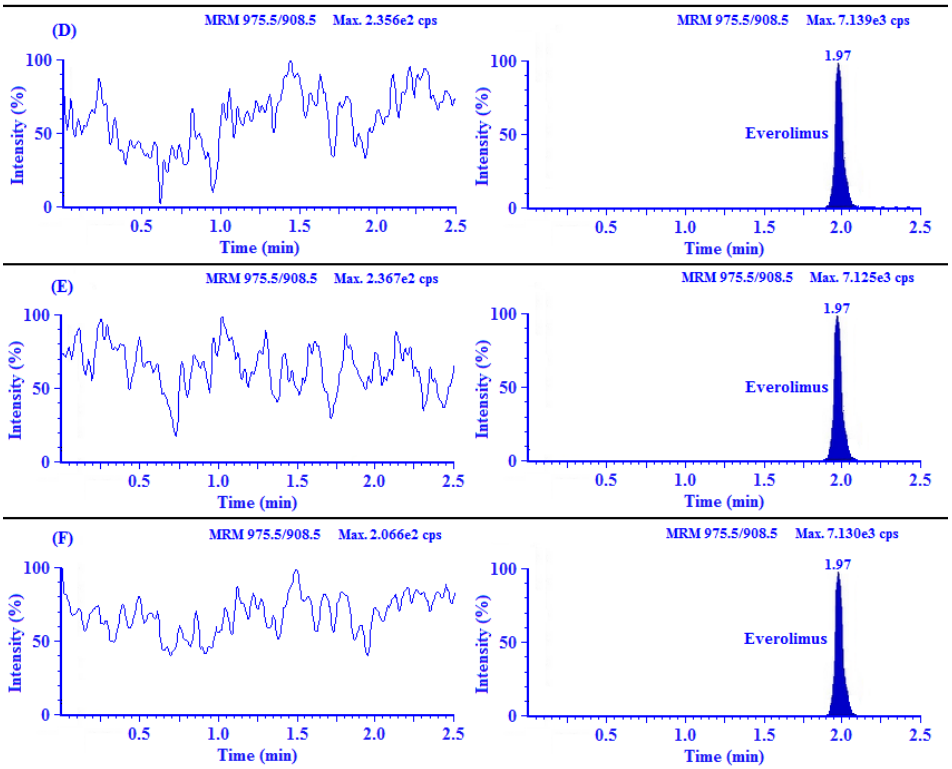


Figure 2 Chromatograms showing selectivity of the method with six extracted blank blood samples, (A-E) K_2EDTA and (F) lipemic and everolimus at lower limit of quantitation (0.10 ng/mL).

Table 1 Comparison of salient features of the present method with selected liquid chromatography-mass spectrometric procedures for everolimus in human whole blood

Extraction procedure	Sample volume (µL)	Linear range (ng/mL)	Retention time (min); run time (min)	Ref.
PP with methanol/$ZnSO_4$ followed by on-line SPE	100	1.0-50	2.03; 2.50	12
PP with methanol/$ZnSO_4$ followed by on-line SPE	250	2.5-30	7.00; 12.00	16
PP with methanol-acetonitrile	200	2.5-50	1.90; 2.60	17
PP with methanol-acetonitrile followed by on-line SPE	200	2.2-43.7	2.39; 6.00	19
PP with methanol/$ZnSO_4$ followed by on-line SPE	100	1.2-48	0.50; 1.00	21
PP with methanol/$ZnSO_4$ followed by on-line SPE	50	2.3-24.6	2.67; 3.5	22
PP with methanol/$ZnSO_4$ followed by on-line SPE	100	1.2-44.9	2.05; 2.80	27
PP with $ZnSO_4$ followed by on-line SPE	50	0.5-40.8	2.10; 2.40	28
PP with methanol/$ZnSO_4$	100	1.0-28	1.95; 2.50	33
LLE with diethyl ether-ethyl acetate	100	0.10-50	1.97; 2.50	PW

PP: protein precipitation; LLE: liquid-liquid extraction; SPE: solid phase extraction

To ascertain the interference of co-eluting matrix components, post-column infusion method was employed. The results shown in Figure 3 shows major ion suppression in the time frame of 1.25 to 1.70 min, however, it did not interference in the quantitation of everolimus which eluted at 1.97 min. A similar observation was also reported by Buckwald and co-workers [28].

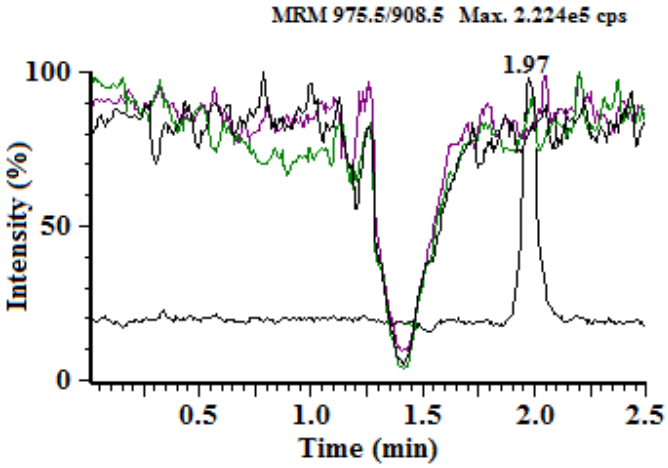


Figure 3 Chromatograms of three blank blood samples during post-column infusion of everolimus (3.00 ng/mL).

Assay validation results

Autosampler carryover study was performed for each analytical run to ensure that it does not affect the accuracy and the precision of the proposed method. There was negligible carryover in any of the runs with no enhancement in the response for analyte or IS in double blank after subsequent injection of highest calibration standard at the retention time of everolimus and IS respectively as shown in Figure 4.

The calibration curves were linear over the concentration range of 0.10–50.0ng/mL. A straight-line fit was made through the data points by least square regression analysis and a constant proportionality was observed. The mean and standard deviation value for slope, intercept and correlation coefficient (r^2) is shown in Table 2. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 89.3 to 99.8 % and 0.75 to 6.26 % respectively. The signal to noise ratio (S/N) at the LLOQ concentration (0.10ng/mL) was ≥ 40.

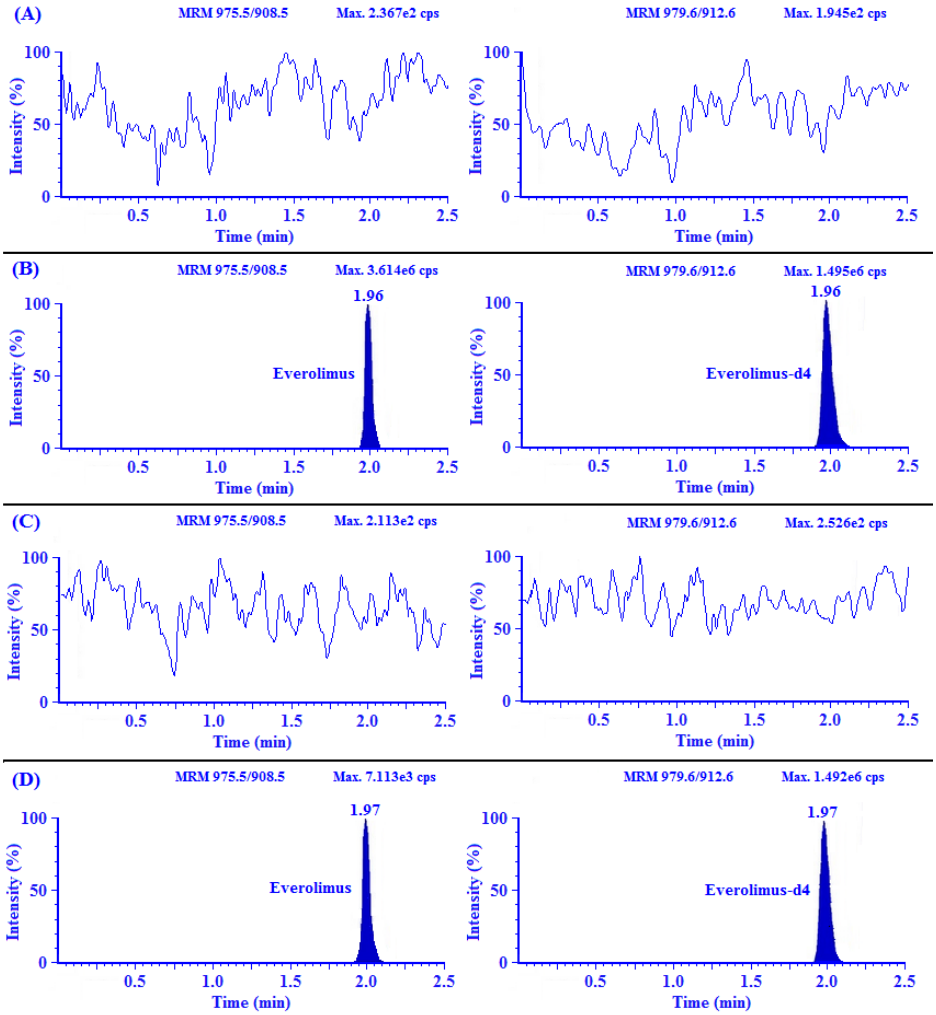


Figure 4 MRM chromatograms for carry over test of everolimus (*m/z* 975.5 → 908.5) and everolimus-d4 (IS, *m/z* 979.6 → 912.6) in (A) double blank blood (without analyte and IS), (B) everolimus (at ULOQ, 50.0 ng/mL) and IS (C) double blank blood (without analyte and IS) and (D) everolimus (at LLOQ, 0.10 ng/mL) and IS.

Table 2 Summary of back calculated concentrations for calibration curve standards of everolimus in human whole

ID No.	CS-1	CS -2	CS -3	CS -4	CS -5	CS -6	CS -7	CS -8	CS -9	Regression Parameters		
	Nominal concentration (ng/mL)											
	50.0	40.0	25.0	10.0	4.00	2.00	0.600	0.200	0.100	Slope	Intercept	r^2
1	49.2	39.0	23.8	9.72	3.59	2.16	0.602	0.194	0.089	0.000823	0.010814	0.9993
2	46.8	38.6	23.5	8.95	3.65	1.86	0.576	0.189	0.087	0.000920	-0.000855	0.9999
3	50.2	39.8	23.8	8.86	3.55	2.01	0.582	0.187	0.093	0.000874	0.007498	0.9988
4	48.1	37.9	23.5	9.22	3.51	1.97	0.575	0.194	0.095	0.000867	0.006661	0.9999
Mean	48.6	38.8	23.7	9.19	3.57	1.99	0.584	0.191	0.091	0.000871	0.006029	0.9995
S.D.	1.46	0.76	0.18	0.38	0.06	0.16	0.013	0.004	0.004	0.000040	0.004927	0.0006
CV (%)	3.01	1.97	0.75	4.18	1.74	6.26	2.23	2.09	4.40			
Nominal (%)	97.1	97.1	94.7	91.8	89.3	99.8	97.3	95.5	91.0			

The intra-batch precision (% CV) ranged from 1.27 to 8.25 % and the accuracy was within 95.78 to 102.8 %. For the inter-batch experiments, the precision varied from 3.37 to 6.06 % and the accuracy was within 97-102 % (**Table 3**).

Table 3 Intra-batch and inter-batch precision and accuracy for everolimus in human whole blood

QC level	Intra-batch (n = 6; single batch)			Inter-batch (n = 30; 6 from each batch)		
Nominal concentration (ng/mL)	Mean conc. found (ng/mL)	CV (%)	Accuracy (%)	Mean conc. found for 5 batches (ng/mL)	CV (%)	Accuracy (%)
HQC (45.0)	43.11	1.27	95.78	44.07	4.48	97.93
MQC-1 (25.0)	25.07	1.36	100.3	24.56	5.26	98.24
MQC-2 (3.00)	3.084	3.79	102.8	3.042	5.42	101.4
LQC (0.300)	0.297	3.70	99.00	0.296	3.37	98.67
LLOQ (0.100)	0.097	8.25	97.00	0.099	6.06	99.00

CV: coefficient of variation

The extraction recovery and absolute matrix effect results for everolimus and IS at different QC levels are presented in **Table 4**. The extraction recovery across QC levels for sirolimus and IS ranged from 90.9-94.8 % and 91.4-95.6 % respectively. The absolute matrix effect for everolimus varied from 98.6-102.3 %.

Table 4 Matrix effect and recovery of everolimus and IS from human whole blood (n = 6)

	Mean area response of everolimus			Absolute matrix effect† [B/A × 100]	Relative recovery† [C/B × 100]
QC level	In mobile phase, A (CV, %)	In post-extraction spiked sample, B (CV, %)	In pre- extraction spiked sample, C (CV, %)		
HQC	1870470 (4.1)	1867020 (1.5)	1771250 (2.4)	99.8 (97.3)	94.8 (92.3)
MQC-	1059995 (4.8)	1070970 (4.4)	982480 (2.2)	101.0 (98.6)	91.7 (93.1)
MQC-	121734 (0.9)	120136 (5.1)	109286 (2.7)	98.6 (97.5)	90.9 (91.4)
LQC	12194 (0.7)	12477 (3.2)	11751 (2.2)	102.3 (99.8)	94.2 (95.6)

CV: coefficient of variation; †values in parenthèses are for everolimus- d4

A more specific method to evaluate matrix effect, expressed as relative matrix in different blood lots/batches showed % CV values of 4.73 and 6.14 at LQC and HQC levels respectively as shown in **Table 5**.

Table 5 Relative matrix effect for everolimus in six different lots of human whole blood

Blood lots	Observed concentration (ng/mL)					
	LQC (0.300 ng/mL)			HQC (45.0 ng/mL)		
1. K_2EDTA	0.289	0.280	0.306	40.35	46.85	43.87
2. K_2EDTA	0.323	0.278	0.309	45.91	42.89	43.94
3. K_2EDTA	0.311	0.276	0.316	47.35	43.73	41.47
4. K_2EDTA	0.294	0.292	0.296	40.37	39.90	38.84
5. K_2EDTA	0.298	0.291	0.297	45.16	42.12	43.67
6. Lipemic	0.273	0.306	0.285	43.62	40.99	47.86
Mean		0.296			43.27	
SD		0.014			2.659	
CV (%)		4.73			6.14	
Accuracy (%)		98.67			96.16	

The stability results for everolimus in human whole blood under different storage conditions are shown in **Table 6**. Stock solutions of analyte and IS kept for short-term stability remained unchanged up to 11 h, while the long term stability of stock solutions under refrigerated temperature below 8°C was established for a minimum of 45 days. Everolimus samples in control human blood for bench top stability were stable for at least 8h at 25°C and for minimum of five freeze and thaw cycles at –20 °C and –70 °C. Spiked blood samples stored at these two temperatures for long term stability were found stable for a minimum period of 93 days. Autosampler stability (wet extract) of the spiked QC samples maintained at 5 °C was determined up to 42 h without significant drug loss.

Table 6 Stability of everolimus under different conditions in human whole blood (n = 6)

Storage condition	Nominal conc. (ng/mL)	Mean, stability samples ± SD	Change (%) *
Bench top stability at room temperature; 8 h			
HQC	45.00	44.40 ± 3.21	-1.33
LQC	0.300	0.294 ± 0.014	-2.00
Wet extract stability at 5 °C; 42 h			
HQC	45.00	41.57 ± 1.39	-7.62
LQC	0.300	0.309 ± 0.037	3.00
Freeze & thaw stability; 5 cycles, -20°C			
HQC	45.00	45.20 ± 3.27	0.44
LQC	0.300	0.296 ± 0.017	-1.33

Freeze & thaw stability; 5 Cycles, -70°C			
HQC	45.00	46.42 ± 1.83	3.16
LQC	0.300	0.292 ± 0.007	-2.67
Long term matrix stability in matrix; 93 days, -20°C			
HQC	45.00	43.23 ± 1.40	-3.93
LQC	0.300	0.284 ± 0.005	-5.33
Long term matrix stability in matrix; 93 days, -70°C			
HQC	45.00	45.51 ± 2.47	1.13
LQC	0.300	0.312 ± 0.014	4.00

$$*\text{Change } (\%) = \frac{\text{Mean stability samples} - \text{Mean comparison samples}}{\text{Mean comparison samples}} \times 100$$

The dilution test was performed to authenticate the method reliability for analyzing analyte concentration above the ULOQ concentration which may be encountered during subject sample analysis. The precision for dilution integrity of 1/2 and 1/10th dilution were 2.86 and 1.64 %, while the accuracy results were 97.62 and 96.22 % respectively, which is within the acceptance criteria for precision (15 % CV) and 85 to 115 % for accuracy (**Table 7**). Method ruggedness was evaluated using re-injection of analyzed samples on two different columns of the same make and also with different analysts. The precision (% CV) and accuracy values for two different columns ranged from 4.12 to 6.43 % and 94.3 to 98.1 % respectively at all four quality control levels. For the experiment with different analysts, the results for precision and accuracy were within 3.45-5.78 % and 97.5-101.3 % respectively.

Table 7 Dilution integrity experiment for everolimus at two dilutions

	Observed concentration (ng/mL)	
	Dilution Factor: 2	Dilution Factor: 10
Standard solution prepared in screened blank blood (90.0 ng/mL)	43.85 45.64 42.43 43.62 45.18 42.90	8.72 8.85 8.53 8.79 8.57 8.52
Mean	43.93	8.66
Standard deviation	1.25	0.14
Coefficient of variation (%)	2.86	1.64
Accuracy (%)	97.62	96.22

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CONCLUSIONS

A highly sensitive method has been proposed for therapeutic drug monitoring of everolimus in human whole blood. The method presents a new liquid-liquid extraction procedure for quantitative and precise recovery of everolimus from blood samples. With a turnaround time of 2.5 min, the method can be readily applied to a clinical setting where large numbers of samples are to be analyzed. Moreover, the sensitivity of 0.10 ng/mL achieved is the highest compared to all other methods developed for everolimus in human specimens. Further, the method has shown to be practically free from matrix interference and the stability of everolimus is extensively proved under different storage conditions.

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