Orthogonal method development and validation of reverse phase ultra-performance liquid chromatographic-mass spectrometry (using PDA and QDa mass detector) for quantification of temsirolimus in temsirolimus pharmaceutical dosage forms

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Abstract

In this study a sensitive, specific and stability-indicating Ultra performance liquid chromatographic (Waters ACQUITY UPLC H-class PDA with integrated QDa mass detector) assay method is developed and validated for the quantification of Temsirolimus in Temsirolimus injection. UPLC separation is achieved with a YMC Pack Pro C18 RS (100 mm x 4.6mm id x 3µm) as stationary phase and 0.05% Trifluoro acetic acid (pH adjusted to 3.0 with ammonia solution):Methanol : Acetonitrile (25:60:15, v/v) as eluent, with a flow rate 0.7 ml/min. UV detection was performed at 280 nm. The retention time of Temsirolimus peak is about 20 minutes. The method has been fully validated and is linear. Results of analysis are validated statistically and by recovery studies. The standard and sample solutions are stable up to 24 hours at 5˚C. Temsirolimus was found to degrade in all stress conditions. Purity of Temsirolimus was found to be less than purity threshold and no additional mass interference was found at Temsirolimus peak in all controlled and stressed samples. No interference was found from corresponding stressed blank with Temsirolimus peak. This method offers advantages over using photodiode-array UV detection (LC-PDA) for the determination of UPLC peak purity, namely components with similar UV spectra can be distinguished, the molecular mass of the impurity can be determined and structural data can be obtained by using QDa mass detector. The result of studies showed that the proposed RP-UPLC method is found to be precise, linear, accurate, rugged, selective, specific, and robust and stability indicating. Hence this method can be used for the routine analysis in bulk drug and in its pharmaceutical dosage forms.

Keywords: Temsirolimus; UPLC-MS-QDa mass detector; Orthogonal Method Development; Validation.

Introduction

Renal cell carcinoma (RCC) is the most common malignancy of kidneys and accounts for 2-3% of all adult cancers.[(1)] Although surgical resection can be curative in localized disease, prognosis of advanced renal cell carcinoma is very poor with a 5-years survival rate of 5-10%. Immunotherapy with interferon-α (IFN-α) has produced modest survival rates in clinical trials[(2-7)] while high dose interleukin-2, though active in highly selected patients, is associated with severe toxicity.[(8,9)] Phase III studies, since 2007, have emphasized the importance of targeting angiogenesis through vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibition with sunitinib[(10)] and sorafenib[(11)] or direct VEGF inhibition with bevacizumab in combination with IFN-α.[(12,13)] These anti angiogenic agents have demonstrated improved overall survival (sunitinib)[(14)] or progression free survival (Sorafenib[(15)] and bevacizumab/IFN)[(16,17)] for patients with advanced RCC. The mammalian Target Of Rapamycin (mTOR), a member of the phosphatidylinositol 3 kinase family, is a multifunctional serine-threonine kinase that acts as central regulator of cell growth, proliferation, and apoptosis.[(18,19)] It modulates the expression and stability of hypoxia-inducible factor (HIF)-1α, which regulates expression of VEGF. Temsirolimus, also known as cci-779, is a potent and selective inhibitor of mTOR. It has been demonstrated its efficacy as a first line immunotherapy in poor prognosis metastatic RCC in comparison with IFN-α.[(20)] It is a derivative of sirolimus and is sold commercially as Torisel TM, 25 mg/ml concentrate and diluents for infusion solution in the treatment of advanced RCC. The recommended dose of Temsirolimus is 25mg infused over a 30 to 60-minute period once in a week. Temsirolimus (sirolimus -42-[2, 2-bis-(hydroxymethyl)]-propionate) is an ester analogue of rapamycin (Figure 1), a natural macrolide antibiotic with antifungal, antitumor, and immunosuppressive activities. Temsirolimus has demonstrated significant inhibition of tumour growth both in vitro and in vivo. It binds to the cytoplasmic protein FKBP, forming a complex that antagonizes the mTOR signalling pathway[(21)] which consequently inhibits many of the downstream process affected by mTOR kinase activity, including transcriptional and translational control of important cell cycle regulators, resulting in cell cycle arrest.[(22)] Temsirolimus is currently in phase III clinical development for the treatment of renal cancer.

There is no reported HPLC method for assay of Temsirolimus and no published RP-Ultra Performance Liquid Chromatography (UPLC)[(21-25)] method for assay of Temsirolimus in bulk and pharmaceutical dose forms. According to the international conference on Harmonization (ICH) guideline Q1(R2) entitled ‘stability testing of new drug substances and products,

stress testing of drug substances should be carried out to elucidate the inherent stability characteristics of the active substance.[26] Temsirolimus is stressed and degraded by treating with acid, base, peroxide, thermal, and photolytic stability studies. An ideal stability-indicating method shall quantify the drug and the presence of its potential degradation products and also resolve its degradation products.

Molecular formula of Temsirolimus is $C_{56}H_{87}NO_{16}$ and molar mass is 1030.28. Systematic (IUPAC) name is 1R, 2R, (4S)-4-{(2R)-2-(3S, 6R, 7E, 9R, 10R, 12R, 14S, 15E, 17E, 19E, 21S, 23S, 26R, 27R, 34aS)-9, 27-dihydroxy-10, 21-dimethoxy- 6, 8, 12, 14, 20, 26-hexa-methyl-1, 5, 11, 28, 29-pentaoxo-1, 4, 5, 6, 9, 10, 11, 12, 13, 14, 21, 22, 23, 24, 25, 26, 27, 28, 29, 31, 32, 33, 34, 34 a-tetracosa–hydro - 3H - 23, 27-epoxypyrido [2,1-c] **[1,4]** oxazacyclohentriacontin-3-yl] propyl}-2-methoxy cyclohexyl 3-hydroxy- 2-(hydroxymethyl)-2-methylpropanoate (Figure 1). Temsirolimus is a white to off-white non-hygroscopic powder, poorly soluble in water but freely soluble in ethanol. Temsirolimus exists in three isomeric forms A, B and C and they interconvert in solution. Isomer B is predominant (≥ 97 %) both in solution and solid states, whereas isomer A is only observed in solution state.

A simple analytical method is reported in this paper that can quantitatively estimate Temsirolimus in presence of its potential degradation products. This paper describes the development and validation of stability indicating RP-UPLC-MS method for the assay of Temsirolimus as a bulk drug and its pharmaceutical dosage forms. A technique is described where the purity of an UPLC peak can be determined by using liquid chromatography-electro-spray ionization QDa mass detector. Electro spray mass spectra acquired across an UPLC peak are summed and examined for co-eluting impurities. The mass spectrometer is set up to produce so cationised species and background noise is minimized so that minor co-eluting impurities can be observed down to a level of < 0.1% of the major component. This method offers advantages over using photodiode-array UV detection (LC-PDA) for the determination of UPLC peak purity, namely components with similar UV spectra can be distinguished. The molecular mass of the impurity can be determined and structural data can be obtained by using mass QDa detector (MS). The effectiveness of the technique is demonstrated with the drug of pharmaceutical interest, which has been on an UPLC system design to intentionally generate maximum number of impurities with main peak. With great detection power, great possibility – minimize the risk of unexpected co-elution or components and confirm trace components with the analytical confidence of mass detection to enhance the analytical value and productivity of each analysis.

Material and Methods

Reagents and chemicals: Water used to prepare the solution had been purified by a Milli-Q system (Millipore). Methanol (HPLC grade) and Acetonitrile (HPLC grade) were purchased from Rankem (Ranbaxy India). Trifluoroacetic acid (HPLC grade), Acetic acid (HPLC grade) and ammonia (HPLC grade) were purchased from spectrochem (Spectrochem Ltd). Temsirolimus was obtained from Hetero drugs Limited Hyderabad, India as gift samples.

Instrumentation: The Waters ACQUITYUPLC H-class system delivers the flexibility of quaternary solvent blending with the advanced performance of UPLC separations. The system is comprised of a quaternary solvent manager (QSM), a sample manager with flow-through needle (SM-FTN) design, Waters auto injector with 10µL standard injection, thermostat column compartment, ACQUITY UPLC-H-class Photo Diode Array Detector (PDA) and QDa Mass detector. Data acquisition was performed on Empower 3 software.

Chromatographic and Mass Conditions: The chromatographic separation was performed on The Waters ACQUITY UPLC H-class system consisting of a quaternary solvent manager (QSM), a sample manager with flow-through needle (SM-FTN) module PDA detector, Auto sampler and QDa mass detector. The HPLC method was developed and validated for the analysis of Temsirolimus. Analytical conditions used were, C18 column (YMC Pack Pro C18 RS, 100 mm X 4.6mm id. 3µm particle size), column oven temperature 35˚C with a flow rate of 0.7 ml/min, injection volume 10µL, the isocratic condition developed using 25:60:15(v/v) mobile phase consisting of 0.05% Trifluoroacetic acid in water with pH 3.0 (adjusted by using ammonia solution): Methanol: Acetonitrile. Detection carried out at 280 nm and runtime 30min. Mass detector: ACQUITY QDa (Extended performance), Ionization mode ESI + (Electro spray ionization), MS acquisition range 100-1500Da, Scan type TIC (Q1 MS), sampling rate 10pts/s, Capillary voltage 0.8kV, Cone voltage 15V, probe temperature 550°C and data centroid.

Column Selection: Several columns were investigated in order to obtain a single method for the separation and quantization of Temsirolimus. The columns investigated are inertsil ODS-3, inertsil ODS-3V, a waters symmetry C-8 and C-18. Each column had the dimensions of 150 mm X 4.6 mm, 3µm particle size. In each case, poor retention and peak tailing were observed for Temsirolimus peak. In order to avoid the use of ion-pairing reagent to improve retention time and peak shape, a YMC Pack Pro C18 RS (150 x 4.6mm, 3µm) column were investigated. The design of these columns makes them suitable for the analysis of Temsirolimus. The YMC Pack Pro C18 RS column produced the best peak shape and was, therefore, used

for the reminder of the method development process; all peaks of interest were eluted within 30 min.

Mobile phase Composition/Column Temperature: The composition of the mobile phase was examined next using an initial isocratic method. As a preliminary guide to the selection of the mobile phase, the standard solution of Temsirolimus was injected into chromatographic system and elution was studied using mobile phase comprising tertiary mixture of buffer, methanol and acetonitrile with varying ratios and finally optimized the mobile phase composition of buffer:methanol:acetonitrile (25:60:15) ratio at pH 3.0 It was found to give good peak shape and retention without sacrificing resolution. The critical pair under these conditions was Temsirolimus and Temsirolimus isomer-C. With these parameters, Temsirolimus isomer-C eluted close to the tail of the Temsirolimus peak. Further optimization was performed by varying column temperature. A decrease in retention time was observed with increasing temperature. However, no significant improvements in resolution were observed with changes in temperature. If the temperature is kept lower than 25˚C resulted in high back pressure while resolution of several other impurities was sacrificed at temperature above 40 ˚C. Finally a column temperature of 35˚C was chosen for further method development.

Effect of pH: The effect of pH on resolution of the critical pair was also examined. Separation of pharmaceutical compounds containing basic functionality can be challenging due to the complexity of interactions with the stationary phase.[27] In addition pH effects can add to this complexity, when impurities generated during processing possess a wide range of pK. Using the results from the initial development strategy was used in pH values 2.5, 3.0, 3.5 and 4.0 and the column temperature was maintained at 35˚C for all runs. The resolution was improved at pH buffer value 3.0.

Stability –Indicating Capability: One dimensional (1D) liquid chromatography (LC) has been widely adopted in the separation and identification of pharmaceutical compounds. However, this technique has proven to have limitations in selectivity and peak capacity when analyzing chemically complex samples. Therefore, the use of multidimensional LC-MS (QDa) can serve as an additional analytical tool providing enhanced peak capacity and selectivity. Thus superior assessment of peak purity and impurity identification can be achieved. Therefore, this method discusses the application LC-QDa as a tool for peak purity assessment and as a technique that can be used to identify unknown impurities. Hence 2D-HPLC/MS provides orthogonality as well as a means of performing mass spectral analysis on the main chromatographic peak. At least the key predictive samples should be screened by both PDA and QDa mass detector. In order to develop the stability-indicating power of the assay, stress-testing was carried out under extreme acidic, basic, oxidative, thermal and photolytic conditions. All degradation products were analyzed in the system with PDA detector and QDa mass detector to evaluate peak purity of Temsirolimus. The Temsirolimus was found to degrade under all stress conditions. Purity of Temsirolimus was found to be less than purity threshold and no additional mass at Temsirolimus peak. Only Temsirolimus mass number has been found in the form of ammonium, sodium and potassium adducts (1047, 1052 & 1068 Da). No interference was found from corresponding stressed blank, stressed placebo, known impurity and any potential degraded impurities with Temsirolimus peak. The purity data of Temsirolimus peak indicates that the peak is homogeneous and no co-eluting peaks indicating specificity of the method, hence this method was found stability indicating method.

Flow rate and Detector Selection: Detection was performed at 280 nm for low noise level and baseline consistency purpose. The anticipated degradation products were expected to absorb at this wavelength and therefore can be detected. Mobile phase of buffer: methanol: acetonitrile (250:600:150% v/v) at a flow rate of 0.7 mL/min was found to be good for the separation of Temsirolimus.

Preparation of Diluent: Transfer 500 mL of acetonitrile and 500 mL HPLC grade water in 1L bottle. Add 0.5 mL of acetic acid and mixed well. Degas the solution by sonicating for 5 minutes.

Preparation of standard solution: Weigh accurately and transfer about 25mg of Temsirolimus standard into a 50 ml of volumetric flask and add about 20mL of diluents. Sonicate to dissolve the drug and dilute to volume with diluent and mix thoroughly. The diluent is used as a blank.

Preparation of sample solution: Dilute 1mL of sample solution into a 50 mL of volumetric flask with diluent and mix.

Results and Discussion

Method Development: An understanding on the nature of API (functionality, acidity or basicity), the synthetic process, related impurities, possible degradation pathways and their degradation products is needed for successful method development in reverse-phase UPLC. In addition, successful method development should results a robust, simple, and time efficient method that is capable of being utilized in manufacturing setting.

Analytical Method Validation: Validation of the optimized UPLC-MS method was done with respect to various parameters, as required under ICH guideline Q2 (R1).[27] This validation study covered selectivity, specificity, linearity, precision (system precision, method precision and intermediate precision), accuracy

as recovery, range, stability in analytical solution and robustness.

Selectivity: Blank, placebo, standard, sample, spiked sample solution and individual impurities solutions were injected into system. Results are given in Table 1. No interference was observed from blank and placebo at retention time of Temsirolimus peak. The % variation in assay of spiked sample from control was found to be 0.13. The purity of Temsirolimus peak was found to be less than purity threshold in PDA detector analysis and no additional mass was observed at Temsirolimus peak in QDa mass detector analysis. The purity data of Temsirolimus peak indicates that the peak is homogeneous; hence the analytical method is selective. The typical chromatogram and mass spectra are shown in Figure 2.

Specificity: Temsirolimus was stressed and degraded by treating with acid, base, peroxide, thermal, and photolytic stress for different lengths of time. Samples were analysed in the system with PDA detector and QDa mass detector to peak purity of Temsirolimus peak. All degradation chromatograms and mass spectra are shown in Figure 3 & 4.

Base stress: Temsirolimus was subjected to base stress by treating with 1.0 mL of 0.02N NaOH solution at room temperature for 30 minutes. The results are given in Table 2. Temsirolimus was found to degrade in base stress conditions. Purity of Temsirolimus was found to be less than purity threshold in PDA detector analysis and no additional mass was observed at Temsirolimus peak in QDa mass detector analysis.

Acid stress: Temsirolimus was subjected to acid stress by treating with 1.0 mL of 0.1N HCl for 2 hours at room temperature. Temsirolimus was found to degrade in acid stress conditions. Temsirolimus purity was found to be less than purity threshold in PDA detector analysis and no additional mass was observed at Temsirolimus peak in QDa mass detector analysis. The purity data of Temsirolimus peak indicates that the peak is homogeneous and the results are presented in Table 2.

Oxidative stress: Temsirolimus was subjected to oxidative stress by treating with 1mL of 1% w/v m-chloroperbenzoic acid for 30 minutes and the results are given in Table 2. Temsirolimus was found to degrade in oxidative stress conditions. Temsirolimus purity was found to be less than purity threshold in PDA detector analysis and no additional mass was observed at Temsirolimus peak in QDa mass detector analysis. The purity data of Temsirolimus peak indicates that the peak is homogeneous.

Thermal stress: Temsirolimus was subjected to thermal degradation by keeping at 70˚C for three days followed by analysis as per method. Temsirolimus was found to degrade in thermal stress conditions. Purity of Temsirolimus was found to be less than purity threshold in PDA detector analysis and no additional mass was observed at Temsirolimus peak in QDa mass detector analysis. and results are shown in Table 2.

Photolytic stress: Temsirolimus was exposed to white fluorescent light exposure of total 1.2 million lux hours and near UV Fluorescent light exposure of 200 WH/Sq.mtr. The results are presented in Table 2. Temsirolimus was found to degrade in all stress conditions. Purity of Temsirolimus was found to be less than purity threshold in PDA detector analysis and no additional mass was observed at Temsirolimus peak in QDa mass detector analysis. The purity data of Temsirolimus peak indicates that the peak is homogeneous. No interference was found from corresponding stressed blank, Placebo and potential degradation impurities with Temsirolimus peak. Temsirolimus peak spectrally pure, hence this analytical method stability indicating method.

Linearity: Linearity was established over a specified range of the 80% to the 120% of the test concentration. The respective concentrations were plotted against respective average area counts to draw linearity graph and correlation coefficient was calculated. The method is linear and the correlation coefficient is 0.9980. The calibration curve is shown in Figure 5.

Precision

System precision: System precision was performed by injecting six replicate injections of standard solution of Temsirolimus. % RSD of assay was found to be very less value (0.03%) suggesting the precision of the system. The results are given in the Table 3.

Method Precision: Method precision was performed by analysing six sample preparations as per method. The results are given in the Table 4. The % RSD of assay results was found to be 0.8 indicating the precision of the method and may be followed in the method development.

Intermediate precision: Intermediate precision was performed by analysing six sample preparations as per method by a different analyst, on a different day, on a different instrument, using a different column. The results are given in the Table 4. The % RSD of assay results was found to be 0.6 and overall % RSD of assay results for intermediate was found to be 0.7. The intermediate precision has RSD within acceptance criteria; therefore the method is rugged.

Accuracy (as recovery): The accuracy was performed by spiking Temsirolimus standard with placebo for Temsirolimus injection concentrate 25 mg/ml at 80%, 100% and 120% of test concentration. The solution was prepared in triplicate at each level. The results are given in Table 6. The average recovery was found within 98.0% to 102.0% at each level, hence method is accurate.

Analytical Solution stability: At room temperature and at temperature 2-8˚C, stability of

standard and sample preparations was performed, the results are given the Table 5 & 6. Cumulative % RSD of Temsirolimus area is 0.81 at 24 hours for Standard solution and cumulative % RSD of Temsirolimus area is 0.38 at 24 hours for Sample solution. Cumulative % RSD of Temsirolimus area is 0.19 at 24 hours for Standard solution and Cumulative % RSD of Temsirolimus area is 0.51 at 24 hours for Sample solution. The standard solution is stable up to 24 hours at 25˚C. Sample solution is stable up to 24 hours at 25˚C. The standard solution is stable up to 24 hours at 2-8˚C.Sample solution is stable up to 24 hours at 2-8˚C. These results exhibits the stability of the solutions prepared for analytical procedures.

Robustness: The robustness of the HPLC method for the determination of assay in Temsirolimus was established by varying analytical conditions one at a time from test method. System suitability parameters were monitored. The results are given in the Table 7. The system suitability parameters complied in every condition. The method was found to be robust. The summary of this drug validation is presented in the Table 8.

Conclusion

This is a simple analytical method for quantitative estimate of Temsirolimus in Temsirolimus drug substance and Temsirolimus injections 25mg/mL. ACQUITY QDa is as intuitive as an optical detector, with the robustness to handle all the analyses. Working in harmony with chromatography, it is pre-optimized to work with samples or without the sample-specific or user adjustments typical of traditional mass spectrometers. One dimensional (1D) liquid chromatography (LC) has been widely adopted in the separation and identification of pharmaceutical compounds. However, this technique has proven to have limitations in selectivity and peak capacity when analyzing chemically complex samples. Therefore, the use of multidimensional LC-MS (QDa) can serve as an additional analytical tool providing enhanced peak capacity and selectivity and thus superior assessment of peak purity and impurity identification. Minimize the risk of unexpected co-elutions and/or components with ACQUITY QDa Detector and confirm trace components with certainty because of the analytical confidence that mass detection brings. In Order to develop the stability-indicating power of the assay, stress-testing was carried out under extreme acidic, basic, oxidative, thermal and photolytic conditions. The method is selective, precise, accurate and stability-indicating and was successfully applied to analysis of commercially available Temsirolimus drug substance and Temsirolimus injection 25mg/mL. The mass spectral information combines seamlessly into the same workflow, routinely giving you more complete characterization of your separation. Process, interpret, visualize, and compare the most complex data, and turn it into meaningful information quickly and simply. This is a simple, cost effective, time saving and very effective means of enhancing the chromatographic detection of the compound.

Table 1: Results of standard, spiked, sample, placebo and blank temsirolimus.

Injection	RT (min.)	Purity angle	Purity threshold	Peak purity by LC-MS	% Assay Variation
Temsirolimus standard	21.801	0.062	1.588	No additional mass at Temsirolimus peak (Temsirolimus mass in form of ammonium, sodium and potassium adduct :1047.63, 1052.54 & 1068.52)	N/A
Temsirolimus Isomer-C	28.953	0.048	1.623	No additional mass at Temsirolimus peak (Temsirolimus mass form of ammonium, sodium and potassium adduct : 1047.43, 1052.384 & 1068.46)	N/A
Sample (Control)	21.813	0.056	1.445	No additional mass at Temsirolimus peak (Temsirolimus mass in form of ammonium, sodium and potassium adduct :1047.32, 1052.61 & 1068.54)	N/A
Spiked Sample	21.534	0.059	1.372	No additional mass at Temsirolimus peak (Temsirolimus mass in form of ammonium, sodium and potassium adduct : 1047.51, 1052.57 & 1068.45)	0.13

Table 2: Degradation studies of temsirolimus

S. No	Condition	Assay (%w/w)	% Degradation	Purity angle of Temsirolimus	Purity threshold	Peak purity by LC-MS
1	Control sample	100.6	--	0.04	90.00	No additional mass at Temsirolimus peak (spectrally pure)
2	Base stress	94.7	5.9	0.09	90.00	No additional mass at Temsirolimus peak (spectrally pure)
3	Acid stress	84.0	16.6	0.06	90.00	No additional mass at Temsirolimus peak (spectrally pure)
4	Oxidation stress	93.5	7.1	0.23	90.00	No additional mass at Temsirolimus peak (spectrally pure)
5	Thermal stress	66.3	34.3	0.06	90.00	No additional mass at Temsirolimus peak (spectrally pure)
6	Photolytic	63.7	36.9	0.106	1.785	No additional mass at Temsirolimus

	stress					peak (spectrally pure)

Table 3: System precision, method precision and intermediate precision of temsirolimus

Injection	System Precision Peak area of Temsirolimus	Method Precision Assay of Temsirolimus(% w/w)	Intermediate precision Assay of Temsirolimus (%w/w)
1	6801.65723	99.4	100.3
2	6804.63867	100.9	100.3
3	6803.26416	100.0	99.7
4	6806.23438	98.4	100.7
5	6801.29688	99.8	99.4
6	6803.05127	100.0	99.2
Mean	6803.35710	99.8	99.9
% RSD	0.03	0.8	0.6
Overall Mean		99.8	
Overall % RSD		0.7	

The RSD value indicates an acceptable level of precision of the analytical system.

Table 4: Recovery studies of temsirolimus

Level	Sample	Amount added (mg)	Amount recovered (mg)	% Recovery	Average at each level
80%	1	18.94	18.90	99.8	99.6
	2	18.93	18.86	99.6	
	3	18.95	18.83	99.4	
100%	1	23.58	23.55	99.9	99.6
	2	23.71	23.60	99.5	
	3	23.46	23.35	99.5	
150%	1	28.59	28.42	99.4	99.5
	2	28.56	28.46	99.6	
	3	28.49	28.33	99.4	

Table 5: Stability of standard solution and sample solution at 25˚c

Stability of standard solution stored at (25˚C)			Stability of sample solution stored at (25˚C)		
Time point in hours	Area of Temsirolimus	Cumulative % of RSD	time point in hours	Area of Temsirolimus	Cumulative % of RSD
Initial	7126477	--	Initial	7576577	--
5	7104284	0.36	5	7566051	0.17
10	7089364	0.43	10	7557438	0.22
12	7076124	0.50	12	7551958	0.23
14	7061474	0.62	14	7544314	o.27
19	7047693	0.73	19	7539530	0.31
24	7035224	0.81	24	7531922	0.38

Table 6: Stability of standard solution and sample solution at 2-8˚c

Stability of standard solution stored at (2-8˚C)			Stability of sample solution stored at (2-8˚C)		
Time point in hours	Area of Temsirolimus	Cumulative % of RSD	time point in hours	Area of Temsirolimus	Cumulative % of RSD
Initial	7261672	--	Initial	7576577	--
5	7270847	0.15	5	7566051	0.28
10	7271529	0.12	10	7557438	0.55
12	7270352	0.11	12	7551958	0.59
14	7266692	0.15	14	7544314	o.57
19	7264387	0.16	19	7539530	0.54
24	7260901	0.19	24	7531922	0.51

Table 7: System suitability parameters of temsirolimus

Varying analytical conditions	System suitability parameter	Acceptance Criteria	Results	Complies/ Doesn't Comply
Change in column (AD/LC/128/1O)	USP tailing factor	Should be not more than 2.0	1.150	Complies
	% RSD of standard injections	Should be not more than 2.0	0.03	Complies
Chang in column (AD/LC/141/1O)	USP tailing factor	Should be not more than 2.0	1.147	Complies
	% RSD of standard injections	Should be not more than 2.0	0.200	Complies
Change in flow rate to 1.6 ml/min instead of	USP tailing factor	Should be not more than 2.0	1.142	Complies
	% RSD of standard injections	Should be not more than 2.0	0.100	Complies

1.8ml/min				
Change in flow rate to 2.0 ml/min instead of 1.8ml/min	USP tailing factor	Should be not more than 2.0	1.150	Complies
	% RSD of standard injections	Should be not more than 2.0	0.100	Complies
Change in detector wavelength to 277 nm instead of 280nm	USP tailing factor	Should be not more than 2.0	1.147	Complies
	% RSD of standard injections	Should be not more than 2.0	0.100	Complies
Change in detector wavelength to 283 nm instead of 280nm	USP tailing factor	Should be not more than 2.0	1.146	Complies
	% RSD of standard injections	Should be not more than 2.0	0.03	Complies
Change in column temperature to 33˚C instead of 35˚C	USP tailing factor	Should be not more than 2.0	1.163	Complies
	% RSD of standard injections	Should be not more than 2.0	0.04	Complies
Change in column temperature to 37˚C instead of 35˚C	USP tailing factor	Should be not more than 2.0	1.131	Complies
	% RSD of standard injections	Should be not more than 2.0	0.100	Complies
Change in Buffer: methanol: Acetonitrile (253 :598:149 instead of 250:600:150)	USP tailing factor	Should be not more than 2.0	1.151	Complies
	% RSD of standard injections	Should be not more than 2.0	0.03	Complies
Change in Buffer: methanol: Acetonitrile (247 :602:151 instead of 250:600:150)	USP tailing factor	Should be not more than 2.0	1.151	Complies
	% RSD of standard injections	Should be not more than 2.0	0.100	Complies

Table 8: Validation summary of temsirolimus

Validation parameters	Acceptance criteria	Results
Selectivity	There should not be any interference from diluent and placebo at the retention time of Temsirolimus peak and assay of spiked sample should not vary from that un-spiked sample by more than 2.0% Purity of Temsirolimus peak in standard, control and spiked sample should be than purity threshold.	No interference was observed from diluent and placebo at the retention time of Temsirolimus peak Purity of Temsirolimus in standard, control and spiked sample should be than purity threshold
Specificity	Purity angle of the Temsirolimus peak should be less than purity threshold.	Purity angle of Temsirolimus was found to be less than purity threshold in all the stress condition.
Linearity	Correlation coefficient of Temsirolimus should not be less than 0.999	Correlation Coefficient of Temsirolimus was found to be 0.9991
Precision		
System Precision	%RSD of six replicate standard injections should not be greater than 2.0	% RSD of six replicate injections of Standard solution was found to be 0.03
Method precision	%RSD of assay results from six sample preparation should not be be more than 2.0	% RSD of six results of assay results from six sample preparation was found to be 0.8
Intermediate precision	%RSD of assay results from twelve sample preparations should not be more than 2.0	%RSD of twelve results of assay from twelve sample preparation was found to be 0.7
Accuracy (as recovery)	Recovery of Temsirolimus at each level should be between 98.0% to 102.0%.	The recovery of Temsirolimus was found between 98.0% to 102.0% for al levels.
Range	From linearity accuracy and precision studies range of method shall be concluded.	The range of method was found to be from 80% to 120% of test concentration.
Stability of analytical Solution a) At Room temp. b) At 2-8˚C	Cumulative % RSD of area counts of Temsirolimus peak up to specified time point should not be more than 2.0. Cumulative %RSD of area counts of Temsirolimus peak up to specified time point should not be more than 2.0.	The standard solution is stable up to 24 hours with cumulative RSD of 0.85% and sample solution is stable up to 24 hours with cumulative RSD of o.51% The standard solution is stable up to 24hours with cumulative RSD of 0.24% and sample solution is stable up to 24hours with cumulative RSD OF 0.51%

Robustness	System suitability parameters should pass under each robustness condition.	System suitability parameters comply under every Robustness parameter.

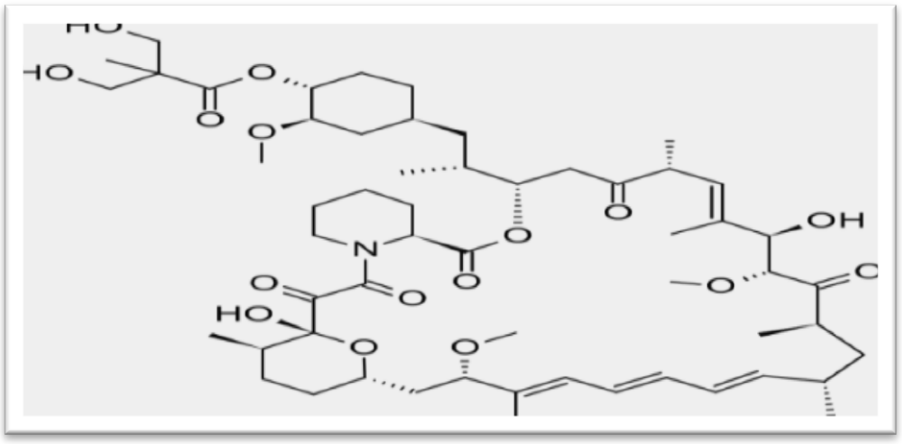

Fig. 1: Temsirolimus molecular structure

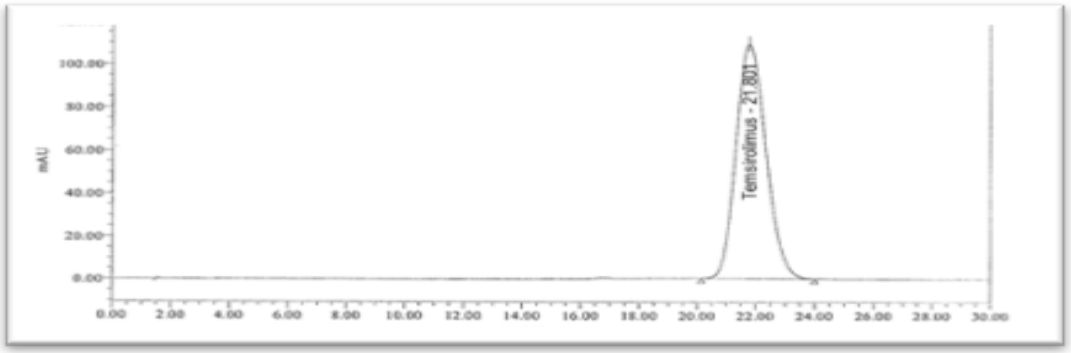

Fig. 2a: Temsirolimus Standard Solution chromatogram

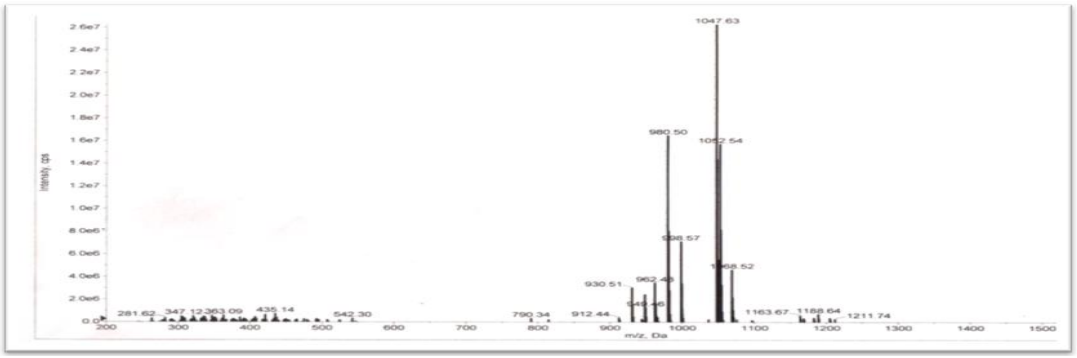

Fig. 2b: Temsirolimus Standard Solution QDa mass spectrum

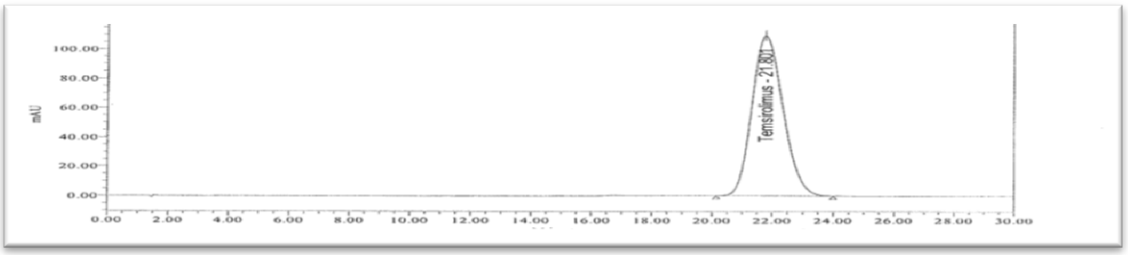

Fig. 3a: Chromatogram for Control sample

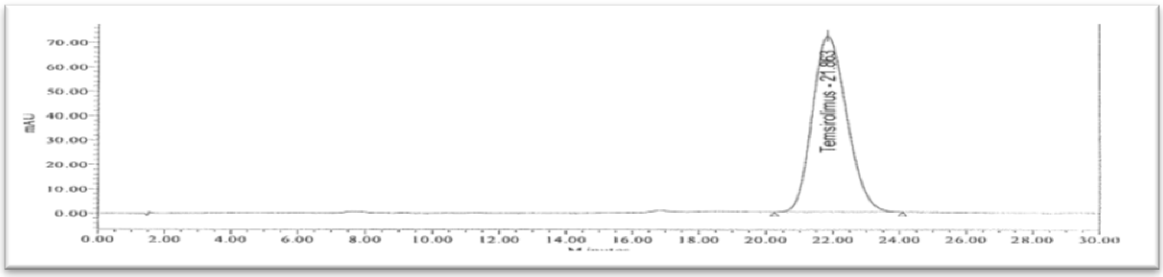

Fig. 3b: Chromatogram for Thermal stress sample

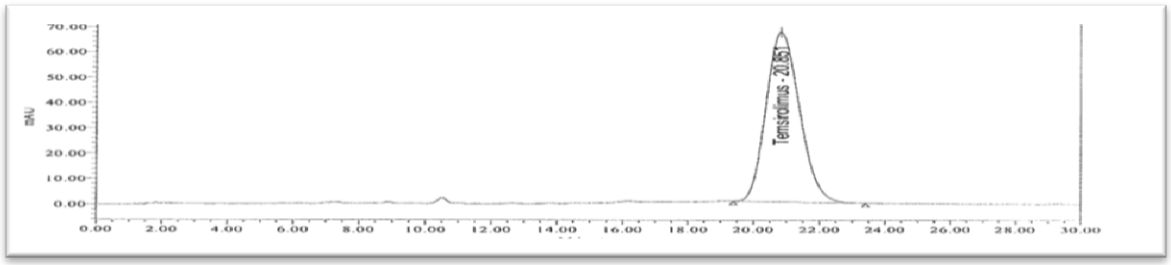

Fig. 3c: Chromatogram for Photo stress sample

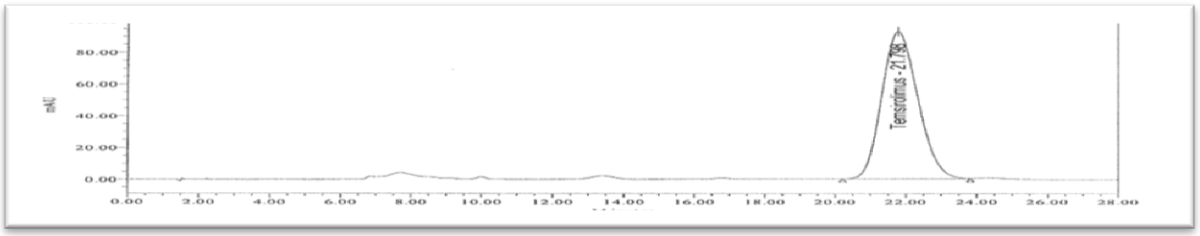

Fig. 3d: Chromatogram for Acid stress sample

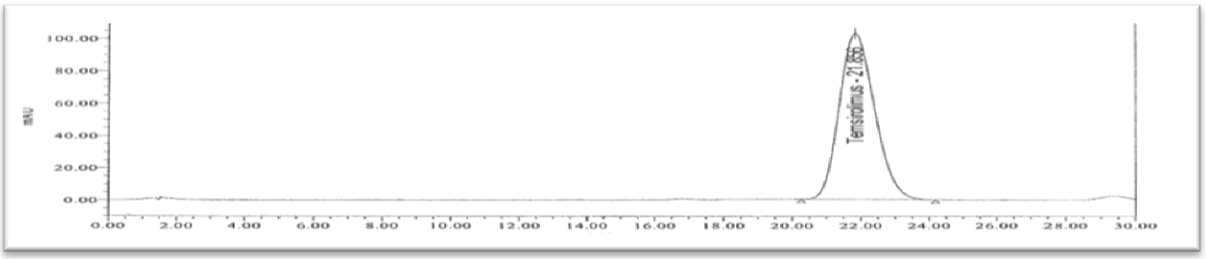

Fig. 3e: Chromatogram for Base stress sample

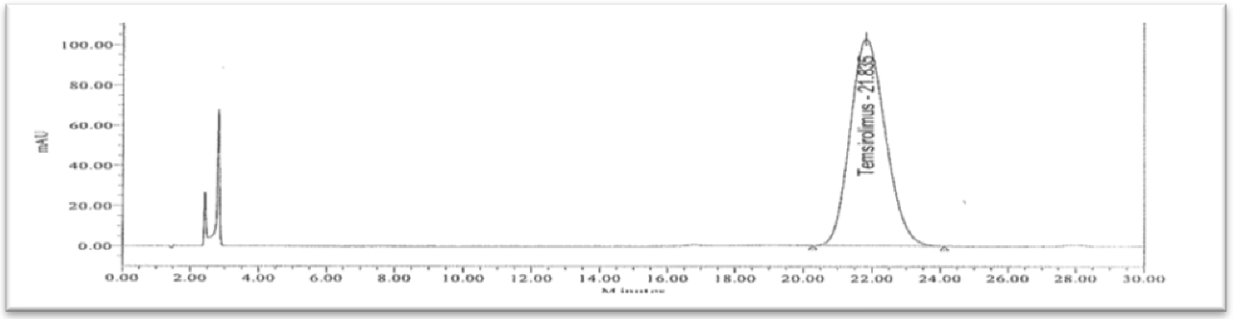

Fig. 3f: Chromatogram for Oxidative sample

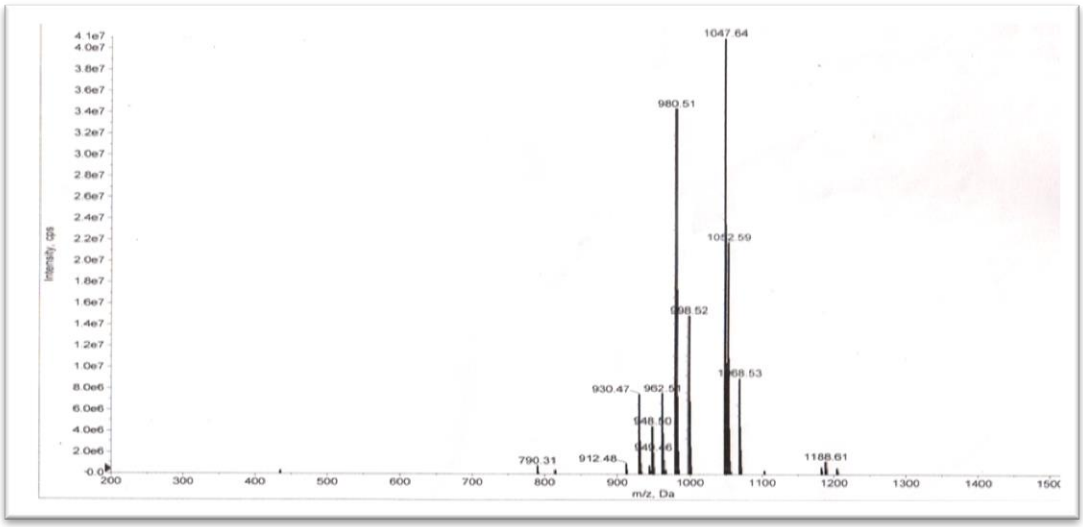

Fig. 4a: QDa mass spectrum for Control sample

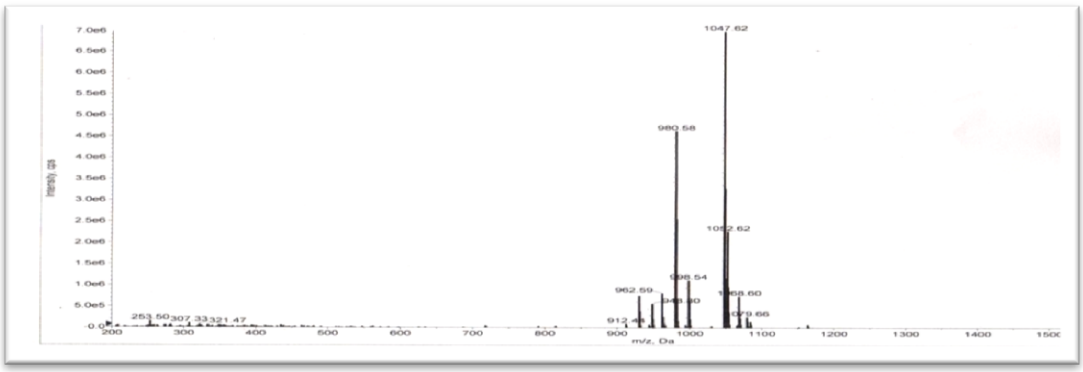

Fig. 4b: QDa mass spectrum for thermal stress sample

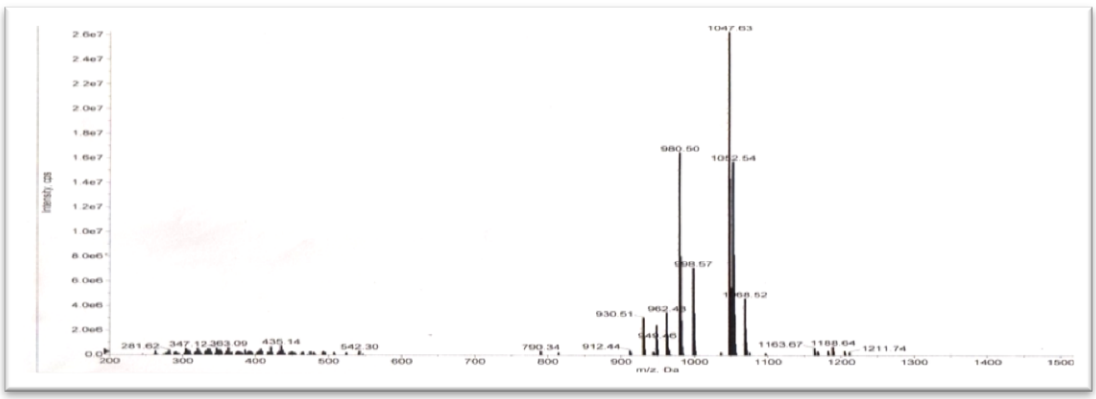

Fig. 4c: QDa mass spectrum for photo stress sample

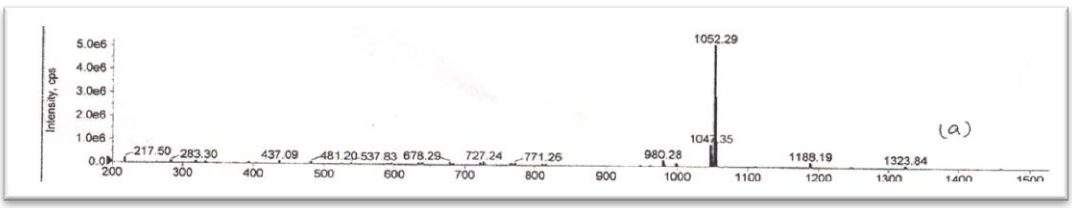

Fig. 4d: QDa mass spectrum for acid stress sample

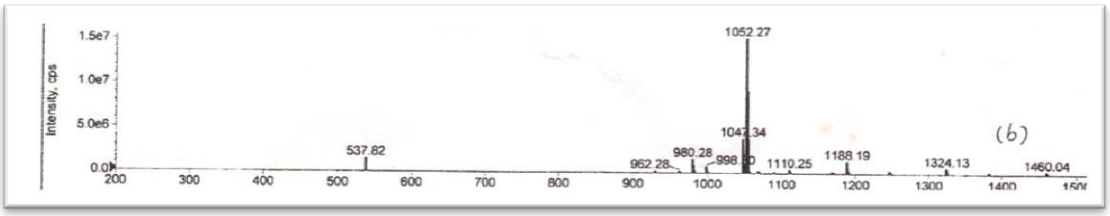

Fig. 4e: QDa mass spectrum for base stress sample

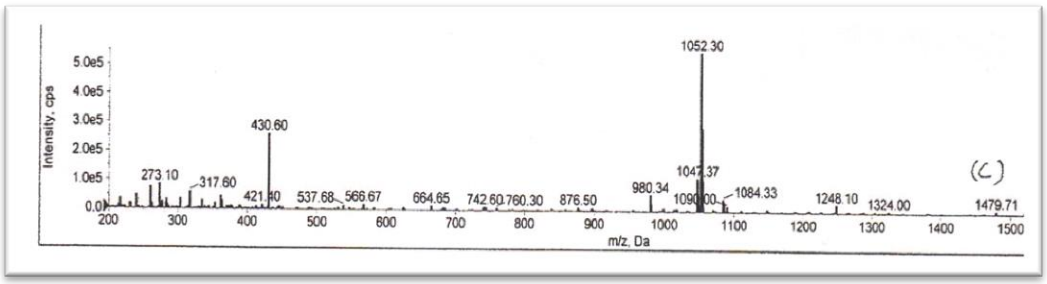

Fig. 4f: QDa mass spectrum for oxidative stress sample

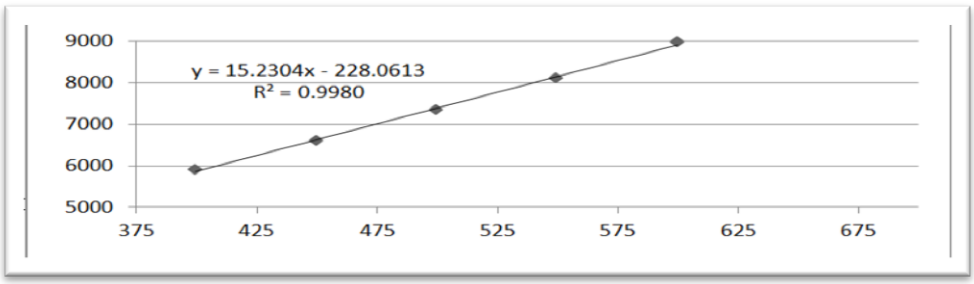

Fig. 5: Calibration Curve of Temsirolimus

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