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STABILITY-INDICATING RP-HPLC CHROMATOGRAPHIC METHOD TO STUDY THE DEGRADATION BEHAVIOUR OF TACROLIMUS

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ABSTRACT

Tacrolimus (TAC) is (1R,9S,12S,13R,14S,17R,18E,21S,23S,24R,25S,27R)-1,14-dihydroxy-12-[(1E)-1-[(1R,3R,4R)-4-hydroxy-3-methoxycyclohexyl] prop-1-en-2-yl]-23,25-dimethoxy-13,19,21,27-tetramethyl-17-(prop-2-en-1-yl)-11,28-dioxa-4-azatricyclo[22.3.1.0,]octacos-18-ene-2,3,10,16-tetrone. (TAC), its available as a topical decongestant in formulations, it is also used to atopic dermatitis. In this study, degradation behavior of Tacrolimus was studied by subjecting the drug to various ICH stress conditions. Also a new, simple, sensitive and accurate stability-indicating method was established for quantitative determination of Tacrolimus in the presence of various and related compounds (Impurity I and II). An expectable separation was achieved with ODS C_{18} column with flow rate 1.0 ml/min. UV Detection wavelength was used for estimation of Tacrolimus over a concentration range of 10 – 300 µg/ml with mean recovery of 99.87 – 100.12%. Methods can analysis Tacrolimus related compound LOQ limit up to 0.031 µg/ml. Method can well resolve all degraded product as compare to Tacrolimus. Developed method can routinely use for the estimation of Tacrolimusrelated compounds from the dosage form and also for stability sample.

KEYWORDS: Related Compounds, Stability Indicating, HPLC.

INTRODUCTION

Stability testing and forced degradation studies play a very crucial role during drug development. Stability is fundamental to all product characteristics, and the term "stability indicating assay" has been used to describe "a procedure which affords specific determination of a drug substance in the presence of its degradation products". The prime goal of studying the stability of a drug is to determine the shelf-life of the drug. Identification of the degradation products, establishment of degradation pathways, determination of intrinsic stability of the drug molecules, and validation of the analytical procedure are some of the goals achieved by stress testing.

The various conditions specified for forced degradation studies include thermal, acidic, alkaline, and neutral hydrolysis conditions, and oxidative and light stress.

It is a white to almost white crystalline powder. It is soluble in methanol, ethanol and water. The mechanism of action of tacrolimus in atopic dermatitis is not known. While the following have been observed, the clinical significance of these observations in atopic dermatitis is not known. It has been demonstrated that tacrolimus inhibits T-lymphocyte activation by first binding to an intracellular protein, FKBP-12. A complex of tacrolimus-FKBP-12, calcium, calmodulin and calcineurin is then formed and the phosphatase activity of calcineurin is inhibited. This prevents the dephosphorylation and translocation of nuclear factor of activated T-cells (NF-AT), a nuclear component thought to initiate gene transcription for the formation of lymphokines. Tacrolimus also inhibits the transcription for genes which encode IL-3, IL-4, IL-5, GM-CSF, and TNF-, all of which are involved in the early stages of T-cell activation. Additionally, tacrolimus has been shown to inhibit the release of pre-formed mediators from skin mast cells and basophils, and to downregulate the expression of FceRI on Langerhans cells.[2,3]

Several methods have been described for determination of Tacrolimus in pharmaceutical preparations including HPTLC, HPLC and NMR had been used for the determination of Tacrolimus. The proposed method was validated as per ICH guidelines According to International Conference on Harmonization (ICH).[4,7]

In the present study, hydrolytic degradation, it was observed significant degradation behavior of TAC to form Impurity-I. While during Oxidative degradation potential degradation product has been observed as Impurity-II. The structure of possible related

compounds/degradants is identified/characterized by the various characterization techniques such as UV, IR, and NMR & Mass and chromatographically by HPLC spiking studies.

Also developed methods are precise, accurate, specific and sensitive stability indicating methods for estimation of TAC in presence of its degradation products.

Experimental

Apparatus: A Shimadzu HPLC, Model: LC-10ATvp (Shimadzu) with rheodyne injector, UV-Visible detector, Model: SPD-10 AVP (Shimadzu) and class VP software. HPLC Column, C_{18} (size-250 x 4.6 mm, I.D-5 µ) (Phenomenex). Nylon filters 0.45 µm. PH meter (Thermo Electro Corporation). Drug was weighed on balance, Model ALC 210.4 (Acculab). Sonicator used was Ultra Sonicator (Fast Clean Ultrasonic Cleaner).

Reagents and Materials

Tacrolimus (TAC) was kindly supplied as gift samples by Torrent Research Center, Ahmadabad, India. HPLC grade Acetonitrile and sodium acetate was purchased from S.D. Fine Chemicals Ltd. (Mumbai). The water for HPLC was prepared by triple glass distillation and filtered through a nylon 0.45 µm – 47 mm membrane filter (Gelman Laboratory, Mumbia, India). Sodium hydroxide, hydrochloric acid and 30% Hydrogen peroxide was purchased from Qualigens Fine Chemicals (Glaxo Ltd.). AR grade Acetonitrile was purchase form ACS chemicals (Ahmedabad). Impurity-I and Impurity-III is inhouse isolated by degradation process of TAC.

Chromatographic conditions

(a) HPLC method. – The mobile phase has been used for separation consisting of buffer (Acetate buffer in water, pH adjusted to 5.0 with tri ethyl amine)-Acetonitrile (30 : 70, v/v) using phenomenax C_{18} column with flow rate 1.0 ml/min. The elution was monitored by peak area at 240 nm, and the injection volume was 20 µL.

Stress studies

Acidic conditions: For acidic hydrolysis, Acid degradation study was performed by treating sample with 1 N hydrochloric acid, kept at about 100°C for 3 hours and analyzed as per method.

Alkaline conditions

Alkaline degradation studies were performed by keeping the drug content in 1 N NaOH at about 100°C for 3 hours and mixture was neutralized.

Oxidation

Oxidative degradation study was performed by treating sample with 30%w/v Hydrogen peroxide, kept at about 100°C for 3 hours and analyzed as per method. Prominent peak at around 8.541 min was observed.

Photo degradation studies: Photo degradation studies were carried out by exposing the drug powder drug in a photostability chamber for 10 days. The powder was spread as a thin layer in a petri plate. The samples of both solution and powder were kept in parallel in darkness for the same period.

Thermal stress studies: The bulk drug, in a thin layer in a petri plate, and drug solution (1 mg/mL) were exposed to thermal stress conditions in a hot air oven at 100ºC for 24 hours.

Preparation of standard stock solutions

TAC (100 mg) was weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml acetonitrile properly and diluted up to mark with acetonitrile to obtain final concentration of 1000 µg/ml. 10 µg/ml solution was prepared for related compound.

Analysis of TAC related compound in TAC formulation

Full content was transferred into a 10 ml volumetric flask containing 5 ml ACN, sonicated for 15 min and further diluted to 10 ml with ACN. The resulting solution was sonicated for 10 min and supernatant was filtered through whatman filter paper no.41. 20 µl of this solution was injected into HPLC column for two times and peak area was measured at 240 nm and average was considered for HPLC method.

The amount of TAC in sample solution was determined by fitting the responses into the regression equation of HPLC.

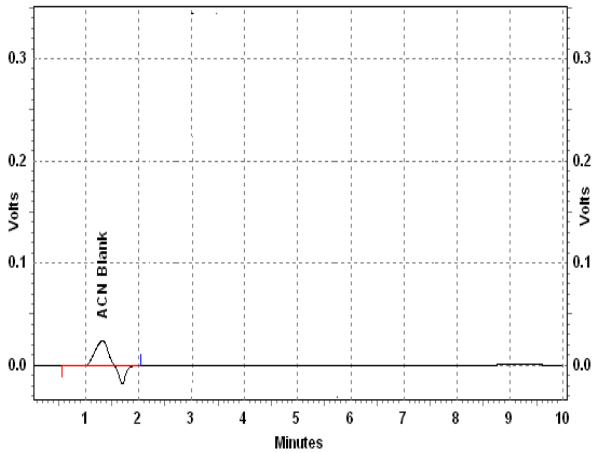

Figure 1: Blank solution by HPLC with UV detection.

Isolation of TAC related compounds

Drug substance was kept under Alkaline medium for 24 hrs and impurity formation compound was filtered and isolated to check the retention time of degradation product formation. Formed product retention time is matching with the degradation product observed during alkali degradation. Further Impurity was purified by preparative TLC method. Alkali degradation impurity was detonated as Impurity – I.

Drug substance was kept under with 30% H2O2 in alkaline medium for 24 hrs and impurity formation compound was filtered and isolated to check the retention time of degradation product formation. Retention time of product formed is matching with the degradation product observed during peroxide degradation.

Further Impurity was purified by preparative TLC method. Alkali degradation impurity was detonated as Impurity –II.

Preparation of related compounds stock solutions
Separate stock solution of related compounds Impurity I and II of 10 µg/ml were prepared by dissolving 10 mg of each of related substance in 100 ml of acetonitrile. Further diluted 5 ml of resulted solution to 50 ml with Acetonitrile.

System Suitability Test
System suitability test of the chromatographic system was performed before each validation run using five replicate injections of a standard solution. Theoretical plates and tailing factor were determined. Refer Table 1,

Table 1: System suitability parameters of TAC.

Sr. No	System suitability parameters	Retention time (minutes)	RRT	Theoretical Plate	Tailing Factor
1	TAC	6.145	**1.0**	8962	1.05
2	Impurity I	4.025	**0.7**	7566	1.03
3	Impurity II	8.541	**1.4**	9536	1.07

Method validation
Calibration curve– From the stock solution of TAC (1000 µg/ml) and TAC related compound (10 µg/ml), appropriate aliquots selected to prepared final concentrations of 10 to 300 µg/ml of TAC and 0.1 to 3 µg/ml of TAC related compound solution. All these solutions were injected into HPLC column and the peak area of each solution was measured at selected wavelength. Figure shows the resolution of TAC and its related substance.

Accuracy (% Recovery)
To ensure the accuracy of method, recovery studies were performed by standard addition method at 80%, 100% and 120% levels of drug concentrations, to the pre-analyzed samples and they were re-analyzed.

Accuracy of the method for all the related substances was determined by analyzing TAC sample solutions spiked with all the related substances at three different concentration levels of 0.1 µg/ml, 1 µg/ml and 3 µg/ml and sample concentration of 1000 µg/ml each in triplicate.

Precision
Repeatability: Repeatability was performed by analyzing six separate TAC solutions of concentration 1000µg/mL that were prepared by spiking the related substances to give 1 µg/mL of each of Impurity I and II. The %R.S.D for each related substance was evaluated.

Intermediate Precision: The intermediate precision of the method for TAC and related substances was determined on three separate sample solutions prepared by spiking the related substances by two different analysts on two different days. The mean values of results for each day and for each analyst were compared.

Robustness: The robustness of the method was checked by repeatedly injecting (n = 5) standard solutions of 100 µg/ml in two C_{18} column one was made by phenomenex and one by hypersil for the HPLC method.

Limit of Detection and Limit of Quantification
The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were calculated using the following equations as per International Conference on Harmonization (ICH) guideline.[17]
LOD = 3.3 x (X/ S)
LOQ=10 x (X/S)
Where X = the standard deviation of the response and S = the standard deviation of y-intercept of regression lines.

RESULT AND DISCUSSION
HPLC Method: To optimize the HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for TAC was obtained with a mobile phase consisting of buffer (Acetate buffer in water, pH adjusted to 5.0 with Triethyl amine)-Acetonitrile (30 : 70, v/v). The elution was monitored by peak area at 240 nm, and the injection volume was 20 µL using phenomenax C_{18} column with flow rate 1.0 ml/min. Detection wavelength was 240 nm. A complete resolution of the peaks with clear baseline separation was obtained (Figure 1).

Two related substances were detected and well resolved by the method. The retention data for TAC and related substances is indicated in Table 1.

Validation of the Proposed Method
Linearity – Linear correlation was obtained between peak areas and concentrations of TAC in the range of 10 – 300 µg/ml and 0.1 to 3 µg/ml for Tacrolimus related compounds. The linearity of the calibration curves was validated by the high value of correlation coefficients of regression (Table 2).

Table 2: Summary of Validation parameters by HPLC with UV detection.

Sr. No	Parameters	TAC	Impurity I	Impurity II
1	Linearity range	1 - 300µg/ml	0.1 – 3 µg/ml	0.1 – 3 µg/ml
2	Correlation coefficient (r^2)	0.9999	0.9999	0.9999
3	Intercept	189567	93872	48392
4	Slope	65984	385	1288
5	Precision			
6	Intraday Average % RSD (n = 5)	0.34	0.37	0.27
	Inter day Average % RSD (n = 5)	1.06	0.87	1.19
	Reproducibility of measurements %RSD	0.33	0.57	0.44
7	% Recovery	99.61 – 102.24	99.87-100.13	99.67-100.02
8	Limit of detection (µg/ml)	0.0067	0.0015	0.0009
9	Limit of quantification (µg/ml)	0.022	0.0049	0.0029

TAC- Tacrolimus.
%RSD calculated from five replication of readings.

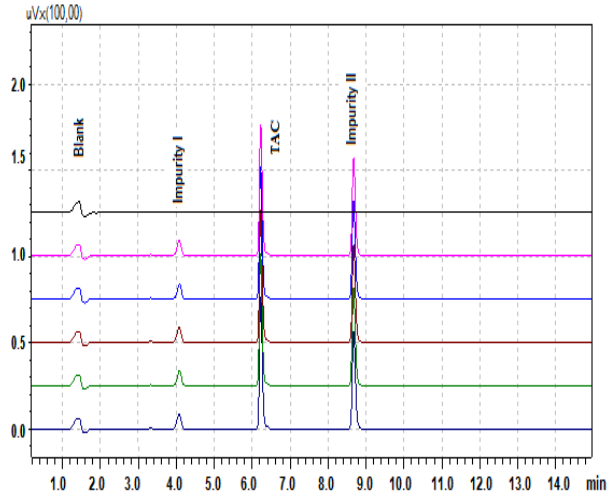

Figure 2: Linearity of Tacrolimus and Related substances.

Accuracy

The recovery experiments were carried out by the standard addition method. The recoveries obtained were ranged between 99.61 – 102.24%. The values of % assay range 99-102% indicated there is no any interference form excipient present in formulation. Figure shows that TAC can well separate form its all type of degradation products so developed HPLC method is specific and selective for TAC.

Precision was expressed in terms of % R.S.D. All values for precision were within recommended limits.

The % RSD values for precision and LOD and LOQ were reported in table 2.

Table 3: Accuracy data of TAC by HPLC with UV detection.

Initial conc. (µg/ml)(A)	Quantity of std. Added (µg/ml)(B)	Total Amount (A + B)	Peak Area	
			Total quantity Found*± S.D.	%Recovery ± S.D
100	150	250	251.32 ± 0.17	100.52 ± 0.68
100	300	400	399.89 ± 0.35	99.97 ± 0.23
100	450	550	550.54 ± 0.19	100.09 ± 0.17

***Average of five readings**

Table 4: Accuracy data of TAC related substance by HPLC with UV detection.

	Impurity I		Impurity II	
Amount Added	Total quantity Found*	%Recovery ± S.D	Total quantity Found*	%Recovery ± S.D
0.1	0.109	100.99±0.14	0.100	100.00± 0.31
1	1.02	102.09±0.64	1.02	102.06±0.08
3	3.02	100.66±0.23	3.01	100.33±0.28

***Average of five readings**

CONCLUSIONS

In this study, it was possible to develop a selective and validated stability indicating HPLC assay method for Tacrolimus on a C18 column, which could separate the drug and its degradation products formed under a variety of stress conditions. TAC was found to be sensitive to the hydrolytic and oxidative condition, whereas it was comparatively stable in thermal and photolytic condition. TLC used to isolated and separate TAC form its degradation products. Based on NMR and Mass data

three Impurities were isolated and characterized as Impurity-I (Tacrolimus Di ene) and Impurity II (Identified as oxidative impurity).

The results of the analysis of pharmaceutical dosage forms by the proposed methods are highly reproducible and reliable and are in good agreement with the label claim of the drug. The method can be used for relates substance analysis of TAC in pharmaceutical preparation and also it is hoped that this report on stability indicating method and degradation of TAC would be helpful for the multiple generic manufacturers of the drug around the globe by saving them for unnecessary repetition of the same studies.

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