Original Article

Forced Degradation Studies Of Pimecrolimus As Per ICH Guidelines

NEHA SHARMA*[1] AND MUKESH KUMAR GUPTA[2]

[1]Associate Professor, Department of Pharmacy, University of Kota, Kota, Rajasthan, India.
[2]Dean, Faculty of Pharmacy, Lords University, Chikani, Alwar, Rajasthan, India.

*Address for correspondence E-mail: sharmaneha2706@gmail.com
DOI: 10.47750/pnr.2022.13.S09.654

Abstract

Forced degradation studies are used to validate analytical procedures for the drug substance and degradation products. Forced degradation studies of Pimecrolimus drug substance and its cream were performed under the stressed alkaline, acidic, oxidative and thermal conditions according to ICH guidelines ICH Q1A(R2). A sensitive stability indicating RP-HPLC method was purposed and validated for the separation of Pimecrolimus and Desmethyl Pimecrolimus. The chromatographic separation was achieved with Phenomenax Luna, C18, 150 x 4.6mm and 3µm particle size column. The flow rate was 1.5 mL/min and eluents were detected at 210nm using PDA detector. For the examination of Pimecrolimus and its associated compounds, our newly designed HPLC approach proved extremely exact, specific, reliable, and accurate.

Keywords: Pimecrolimus, Reversed Phase High Performance Liquid Chromatography, ICH, Forced Degradation.

Introduction

Pimecrolimus (Figure1) is a white to off-white fine crystalline powder [17]. It is soluble in methanol and ethanol but insoluble in water. The molecule has a molecular weight of 810.47g/mol and the empirical formula $C_{43}H_{68}ClNO_{11}$. It is ascomycin derivative and member of a new class of immunomodulating macrolactams, that works particularly well to treat inflammatory skin conditions[1]. A lot of attention has been gathered for its strong anti- inflammatory and immunomodulatory properties. The mechanism of action involves the blocking of T cell activation[2]. It is an immunophilin ligand that only interacts with the Immunophilin macrophilin-12 cytosolic receptor, similar to other ascomycins. This complex effectively inhibits the protein phosphatase calcineurin by preventing it from dephosphorylating the transcription factor nuclear factor of activated T cells. Because of this, signal transduction pathways in T cells are stopped, which prevents the production of inflammatory cytokines, particularly those of the Th1 and Th2 type. It has been demonstrated that Pimecrolimus inhibits cytokine and pro-inflammatory mediator release from mast cells [3,4]. Inflammatory skin disorders such vitiligo[5], seborrheic dermatitis[6], oral lichen planus [7], cutaneous lupus erythematosus [8], and psoriasis[9-16] have all responded favorably to its use. The USFDA has authorized the calcineurin inhibitors pimecrolimus and tacrolimus for the treatment of skin

conditions[17].

For drug impurity control analysis, high-performance liquid chromatography (HPLC) technologies are highly suggested[27]. The International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines[28] state that HPLC methods are primarily used because they have higher resolution, capacity, sensitivity, and specificity than other traditional techniques like thin-layer chromatography and gas chromatography[29].

Each gram of Elidel Cream 1% w/w (Novartis India Ltd.), contains 10 mg of Pimecrolimus in a whitish cream base of benzyl alcohol, citric acid, mono- and di-glycerides, oleyl alcohol, cetyl alcohol, sodium cetostearylsulphate, propylene glycol, sodium hydroxide, stearyl alcohol, triglycerides, and water[18].

Forced degradation studies are both scientific and regulatory need throughout the drug development process. Therefore, we conducted stress experiments using a range of ICH-recommended test settings to examine the Pimecrolimus's intrinsic stability. The newly developed approach was also evaluated for its capacity to identify process impurities and Pimecrolimus degradation impurities. This is the first publication of its kind on Pimecrolimus's stability-indicating HPLC approach and the characterization of its related compounds using HPLC.

MATERIALS AND METHODS

Chemicals and solvents:

The working standard Pimecrolimus was received as a gift sample from Concord Biotech, India. The commercial pharmaceutical product Elidel cream (Novartis India Ltd.), with a label claim of 1% w/w, was purchased from a local pharmacy. Water, Acetonitrile, and Benzyl alcohol are all of HPLC quality.

Instrumentation:

The chromatographic separation was carried out using Waters Alliance HPLC equipment (Waters, USA) coupled with a Photodiode array detector (PDA; Model-2998) detector. It includes a column oven and an automatic sampler built in. Empower-3 software was used to monitor and process the output signals. To speed up the dissolving of the chemicals, a sonicator and pH meter made by Lab India were employed. On a Sartorious balance, all weighing was performed (model AE-160).

Preparation of Solution A:

Water: Acetonitrile: Methyl Ter Butyl ether: Formic acid (650:240:70:0.2)

Preparation of Solution B:

Water: Acetonitrile: Methyl Ter Butyl ether: Formic acid (200:660:70:0.2)

Preparation of Desmethyl Pimecrolimus Impurity Stock Solution:

1 mg of Desmethyl Pimecrolimus impurity weighed into a 10 mL volumetric flask then 5ml of acetonitrile added and sonicated to dissolve. Volume made up with acetonitrile.

Preparation of System suitability solution:

30 mg of Pimecrolimus working standard weighed and transferred to 50 mL volumetric flask. To this 3 ml of Desmethyl Pimecrolimus impurity Stock solution in 35ml of Acetonitrile, and sonicated to dissolve, volume made up by Acetonitrile.

Preparation of Diluted Standard solution:

30 mg of Pimecrolimus working standard with 30ml of diluent transferred in a volumetric flask (50ml). The solution sonicated and volume made up with diluent. Further,
1ml of solution taken to 100 ml volumetric flask to make 6ppm solution.

Preparation of Sample solution:

Sample (3gm) with 30 ml of diluent taken in 50 mL glass stopped test tube. The solution sonicated for about 15 minutes at room temperature with intermediate shaking and filtered through 0.45µ Teflon membrane filter.

Selection of wavelength:

The wavelength was chosen by scanning a solution of Pimecrolimus between 200 and 400 nm.

Chromatographic conditions:

The chromatographic separation was accomplished using gradient elution at temperature of
60°C on an analytical column of dimensions Phenomenax Luna (C18, 150 x 4.6mm, 3µ). The mobile phase is composed of solution A and B. The mobile phase was filtered through
0.45µm Milipore Nylon 6 membrane filter. The column was equilibrated with mobile phase prior to injection for at least 30 min.

Analytical Method Validation:

The published RP-HPLC technique was evaluated on parameters like stability in analytical solution, filter equivalency and forced degradation. The analyzed parameters were

performed in compliance with the International Conference on Harmonization's requirements for analytical procedures (ICH)[19].

Stability in Analytical solution:

The standard solution, sample and spiked sample solution were stored at room temperature and tested for stability after 72hours. The cumulative RSD should not be more than 10% for all the known impurities.

Filter Equivalency:

The sample of Pimecrolimus Cream spiked with known impurity was prepared after centrifugation and filtered through different membrane filters like Nylon 0.45µ and Teflon
0.45µ filters discarding first few mL of the filtrate.

Forced Degradation Studies:

The forced degradation study is regarded as a crucial analytical aspect of the small molecule drug development approach. Using HPLC,or a single analytical method that is capable of separating the degradant peaks from the drug substance/product peak demonstrates the specificity of a stability-indicating analytical method[21]. The acceptance criteria is that the Pimecrolimus peak and known impurity peak should be homogeneous and there should be no co-eluting peaks. Peak purity for analyte peak and known impurity peaks should pass.

Preparation of Sample solution A:

A sample solution was prepared by taking 3g of sample in a 50 mL volumetric flask and adding about 30 ml of diluent to disperse the sample uniformly. It was sonicated for about 15 minutes at room temperature with some intermediate shaking. All solutions were filtered through 0.45µ Teflon membrane filter.

Acid and Base Degradation:

With respect to acid degradation, sample solution A was allowed to equilibrate to room temperature, then 5 mL of 5N HCl and 5 mL of 5N NaOH were added immediately (for a 0hr sample). For collection of samples after 24 hours, sample solution A with 5 mL of 5N HCl was kept for 24 hours at room temperature, then 5N NaOH was added. About base degradation, sample solution A was equilibrated at room temperature, then 5 mL of 2N NaOH was added immediately preceded by 5 mL of 2N HCl to neutralize the solution. This was diluted up to the mark and kept for 15 minutes (0 hour sample). Sample solution A kept for 24 hour at room temperature and added 5 mL of 2 N HCl to neutralized the solution and diluted up to the mark with diluent and allowed sample solution to room temperature for 15 minutes (24 hour sample)[23,24].

Peroxide Degradation:

Hydrogen peroxide is commonly employed in forced degradation experiments to oxidize drug substances. Sample solution A was allowed to equilibrate to room temperature, then 5mL of 50% H_2O_2 was added immediately and diluted up to the mark placed at room temperature for 15 minutes. In order to analyze degradation after 24 hours, sample solution A with 5mL of 50% H_2O_2 kept for 24 hours[24].

Thermal Degradation:

The sample was exposed to 60°C for 72 hours before being analyzed[24, 25].

Humidity Degradation:

The sample was exposed for 72 hours at 25°C/92%RH humidity and then analyzed

[24].

Photolytic Degradation:

The photo stability testing of pharmacological compounds must be assessed to show that light exposure does not cause an undesirable alteration. The principal degradants of a pharmacological substance are produced by photo stability tests when exposed to UV or fluorescent light [25, 26]. The sample kept

for 1.2 million lux hours and analyzed.

RESULTS AND DISCUSSION

Different mobile phases with different compositions and flow rates were tried to develop an accurate, selective, and precise stability indicating RP-HPLC method for estimating Pimecrolimus in stressed samples. After a number of compositions and combinations, the chromatographic conditions were devised and adjusted. With the gradient mobile phase and at a flow rate of 1.5 mL/min, reasonable estimate of Pimecrolimus with good peak symmetry and constant baseline was observered. The drug had distinct peak with retention time (RT) of 31.5 min and a clear baseline at 210 nm. The detailed result for every parameter is described below. Each injection had a volume of 50µl. The optimized chromatographic conditions shown in Table 1.

Analytical Method Validation

Stability of Analytical solution:

The standard solution is stable for 102 hours, sample solution is stable for 93 hours and spike sample is stable for 89 hours at room temperature (Table 2).

Filter Equivalency:

The overall % RSD is within limits. Therefore, Teflon 0.45µ and Nylon 0.45µ filters are suitable for filtration of samples.

Forced Degradation Studies:

The degradation products produced by forced degradation studies are prospective degradation products that might or might not arise under appropriate storage circumstances. The chromatograms from samples subjected to acidic, alkaline, oxidative, thermal, humidity, and photo degradation showed clearly separated peaks of Pimecrolimus in each degradation sample, demonstrating the homogeneity of the peak and the specificity of the method by the absence of co-eluting peaks (Table 4 and Figure.2-8).

The present study represents the first report that deals with the development of a stability-indicating HPLC method for determination of Pimecrolimus and related substances in "Pimecrolimus 1% w/w cream". This study is a typical example of development of a HPLC method following the recommendations of ICH guidelines. The sample preparation is simple, the analysis time is short and the elution is by gradient method. The Standard solution is stable for 102 hours and from the filter equivalency studies shown teflon and nylon filter is also suitable for sample preparation. It is revelaed from the forced degradation studies that peaks of Pimecrolimus and desmethyl Pimecrolimus is homogenous and there are no co- eluting peaks. From the economical point of view, the method involved the native UV- absorbing property of Pimecrolimus, rather than expensive analytical reagents. Statistical analysis for the results proved that the method is suitable for the determination of Pimecrolimus in bulk and Pharmaceutical dosage forms without any interference from the degradation products, and recommended for routine use in quality control laboratories.

Acknowledgms

Neha Sharma is thankful to Department of Pharmacy, University of Kota, Kota, Rajasthan, India.

REFERENCES

1. Garside R, Stein K, Castelnuovo E. The effectiveness and cost-effectiveness of Pimecrolimus and tacrolimus for atopic eczema: a systematic review and economic evaluation. In: NIHOUR Health Technology Assessment programme: Executive Summaries. Southampton (UK): NIHOUR Journals Library; 2003-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK62257/
2 Ahn D, Robinson CA. Pimecrolimus. In: StatPearls. Treasure Island (FL): StatPearls Publishing; July 12, 2022.
3. Gupta A, Chow M. Pimecrolimus: A Review. J. Eur. Acad. Dermatol. Venereol.2003; 17 (5): 493–503.
4. Lipozencić J. Pimecrolimus --a safe and effective local immunomodulator in the treatment of inflammatory skin diseases. Acta.Dermato.Venerol.Croat.2005, 13(1), 63-69.
5. Seirafi H, Farnaghi F, Firooz A, Vasheghani-Farahani A, Alirezai NS, Dowlati Y, Pimecrolimus cream in repigmentation of vitiligo.Dermatology.2007; 214(3):253-259.
6. Kim G K, Rosso JD. Topical Pimecrolimus 1% Cream in the Treatment of Seborrheic Dermatitis.J. Clin. Aesthet. Dermatol., 2013; 6(2): 29-35.
7. Esquivel-Pedraza L, Fernandez-Cuevas L, Ortiz-Pedroza G, Reyes-Gutierrez E, Orozco- Topete R. Treatment of Oral Lichen Planus with Topical Pimecrolimus 1% Cream. Br. J. Dermatol. 2004; 150 (4): 771–773.
8. Kreuter A, Gambichler T, Breuckmann F, Pawlak FM, Stücke M, Bader A, Altmeyer P, Freitag M. Pimecrolimus 1% Cream for Cutaneous Lupus Erythematosus. J. Am. Acad. Dermatol. 2004; 51 (3): 407-410.
9. Esfandiarpour I, Ekhlasi A, Farajzadeh S, Shamsadini S. The efficacy of Pimecrolimus 1% cream plus narrow-band ultraviolet B in the treatment of vitiligo: a double-blind, placebo- controlled clinical trial. J. Dermatolog. Treat.2009; 20(1):14-18.
10. Yin ZQ, Xu J, Zhou B, Wu D, Xu Y, Zhang J, Luo, D. Topical Pimecrolimus Inhibits High-Dose UVB Irradiation-Induced Epidermal Langerhans Cell Migration, via Regulation of TNF-a and E-Cadherin. Drug Des.Devel.Ther.2014;1817.
11. Zhao Z, Gao XH, Li W, Wang H, Liang Y, Tang J, et al. Experts' Consensus on the Use of Pimecrolimus in Atopic Dermatitis in China: A TCS-Sparing Practical Approach. DermatolTher (Heidelb)2022; 12 (4): 933–947.
12. Alsmeirat O, Lakhani S, Egaimi M, Idris O, Elkhalifa M. The Efficacy and Safety of Pimecrolimus in Patients With Facial Seborrheic Dermatitis: A Systematic Review of Randomized Controlled Trials. Cureus2022.
13. Luger T, Paller AS, Irvine AD, Sidbury R, Eichenfield LF, Werfel T, Bieber T. Topical Therapy of Atopic Dermatitis with a Focus on Pimecrolimus. J. Eur. Acad. Dermatol. Venereol.2021; 35 (7): 1505–1518.
14. Madsen S, Price KN, Shi V Y, Lio PA. Pearls in Mitigating Application Pain of Topical Nonsteroidal Agents. Dermatology2020; 236 (5): 477–480.
15. Shin N, Jung N, Lee SE, Kong D, Kim NG et al. Pimecrolimus Interferes the Therapeutic Efficacy of Human Mesenchymal Stem Cells in Atopic Dermatitis by Regulating NFAT-COX2 Signaling. stem. Cell Res. Thers.2021, 12 (1).
16. Mrowietz U, Wustlich S, Hoexter G, Graeber M, Bräutigam M, Luger T. An experimental ointment formulation of Pimecrolimus is effective in psoriasis without occlusion. Acta.Derm.Venereol.2003; 83(5): 351-353.
17. Lade S, Rajendra Prasad Y. A new validated stability indicating rp-hplc method for estimation of Pimecrolimus in bulk and topical formulations. Int. J. Adv. Pharm. Biotechnol.2015; 1 (1): 33- 44.
18. Ayer J, Young HS. Pimecrolimus for psoriasis. Expert Opin.Pharmacother.2013, 14(6), 767-774.
19. Validation of analytical procedures: text and methodology. Proceedings of the International Conference on Harmonization. IFPMA, Geneva 1996.
20 Syed HK, Liew KB, Loh GOK, Peh KK. Stability indicating HPLC–UV method for detection of curcumin in Curcuma longa extract and emulsion formulation. Food Chem., 2015;170: 321-326.
21. Blessy M, Patel RD, Prajapati PN, Agrawal Y. Development of forced degradation and stability indicating studies of drugs-A review. J. Pharm. Anal., 2014;4(3): 159-165.
22. Singh S, Bakshi M. Guidance on conduct of stress tests to determine inherent stability of drugs, Pharm. Technol.2000; 24:1-14.
23. Alsante KM, Ando A, Brown R, Ensing J, Hatajik TD, Kong W, Tsuda Y. The role of degradant profiling in active pharmaceutical ingredients and drug products, Adv. Drug Deliv. Rev. 2007; 59 (1): 29-37.
24. Alsante K, Ahuja S. Handbook of Isolation and Characterization of Impurities in Pharmaceutical, Academics Press, New York, Solving impurity/degradation problems: case studies, 2003; pp. 380.
25. Photo stability Testing of New Drug Substances and Product, International Conference on Harmonization, 1996.(available at: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidanc es/ucm073373.pdf)
26. Shi Q, Li J, Ding F. Development and Validation of method for the determination of related substances of Tacrolimus in Tacrolimus Capsules and Degradation Studies. Int. J. Chem. Tech. Res.2012; 4(4):1543-1552.

27. Jenke DR. Chromatographic method validation: are view of common practices and procedures II. J. Liq. Chromatogr. 1996;19:737-757.
28. Maheswaran R. FDA perspectives:scientific considerations of forced degradation studiesinANDAsubmissions, Pharm.Technol. 2012; 36(5):73-80.
29. FDA Guidance for Industry, Analytical Procedures and Methods Validation: Chemistry, Manufacturing ,and Controls Documentation, Draft Guidance, Food and DrugAdministration. Availablefrom: ⟨http://www.fda.gov/downloads/Drugs/…/Guidances/ucm122858. pdf⟩, 2000.

Table 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS AND SYSTEM SUITABILITY PARAMETERS FOR PROPOSED HPLC METHOD FOR PIMECROLIMUS.

S.No.	Parameter	Chromatographic conditions		
1.	Flow rate	1.5ml per minute.		
2.	Column	Phenomenax Luna, C18, 150 x 4.6mm, 3µ		
3.	Detector wave length	210 nm		
4.	Oven temperature	60°C		
5.	Injection volume	50 µL		
6.	Run time	60 min		
7.	Diluent	Acetonitrile		
8.	Mode of separation	Gradient		
		Time	Mobile Phase A	Mobile Phase B
		0.0	70	30
		20.0	70	30
		35.0	38	62
		40.0	5	95
		48.0	5	95
		50.0	70	30
		60.0	70	30
		0.0	70	30

Table 2: STABILITY OF ANALYTICAL SOLUTION

Standard Solution		Control sample preparation					Spike sample preparation					
Time (Hours)	Area Pimecro li mus	Time (Hours)	Area Desmethyl Pimecrolim us	Area Unk. Impurity @ RRT 0.91	Time (Hours)	Area Pimecrolim us	Time (Hours)	Area Desmethyl Pimecrolim us	Time (Hours)	Area Unk. Impurit y @	Time (Hours)	Area Pimecrolim us
Initial	157437	Initial	3700	9670	Initial	14134911	initial	177341	initial	10520	initial	14461992
38	157011	30	3480	9219	30	14430017	23 HRS	176605	27 HRS	11054	27 HRS	14641565
68	158628	60	3703	9800	60	14813640	53 HRS	181151	38 HRS	10111	38 HRS	14820453
87	155204	78	3640	10698	78	15036441	71 HRS	182788	55 HRS	9871	55 HRS	14920636
94	159580	86	3619	10154	86	15117228	79 HRS	180790	72 HRS	11349	72 HRS	15028873
102	151119	93	3442	8334	93	15224356	86 HRS	181762	89 HRS	11813	89 HRS	15115406
%RSD	1.93	----	3.09	8.41	----	2.89	-----	1.39	----	6.95	------	1.65

Table 3: FILTER EQUIVALENCY STUDIES

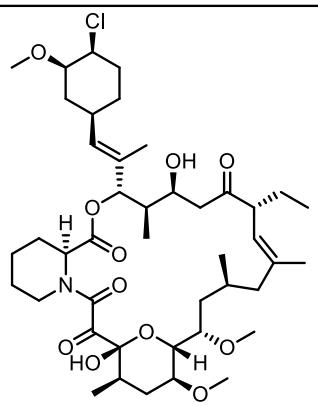
Sr. No.	Sample	% DesmethylPimecrolimus	Unk Impurity @ RRT 0.91	% Total Impurity
1	Centrifuge-1	1.184	BLQ	1.184
2	Centrifuge-2	1.197	BLQ	1.197
3	Centrifuge-3	1.211	BLQ	1.211
4	Nylon -1	1.142	BLQ	1.142
5	Nylon -2	1.154	BLQ	1.154
6	Nylon -3	1.146	BLQ	1.146
Mean		**1.172**	**NA**	**1.172**
SD		**0.029**	**NA**	**0.029**
%RSD		**2.474**	**NA**	**2.474**
7	Teflon -1	1.181	BLQ	1.181
8	Teflon -2	1.152	BLQ	1.152
9	Teflon -3	1.181	BLQ	1.181
Mean		**1.184**	**NA**	**1.184**
SD		**0.020**	**NA**	**0.020**
%RSD		**1.689**	**NA**	**1.689**

Note: Abbreviations: NA: Not Applicable, BLQ: Below Limit of Quantification

Table 4: FORCED DEGRADATION STUDIES FOR PIMECROLIMUS

Sr. No.	**Experiment**	**Degradation Condition**	**Purity Angle Pimecr-olimus**	**Purity Threshold Pimecrolimus**	**% Desmethyl Pimecrolimus**	**% Unk Impurity @ RRT 0.91**	**% Total Impurity**
1	Control	--	0.326	1.102	0.305	0.078	0.461
2	Acid Degradation	5N HCl – RT/0 hr	0.254	1.146	0.310	ND	0.310
		5N HCl – RT/24 hrs	1.023	2.973	ND	0.367	0.734
3	Base Degradation	2N NaOH– RT/0 hr	0.345	5.293	ND	1.546	3.092
		2N NaOH– RT/24 hr	0.521	2.054	0.262	ND	0.262
4	Peroxide Degradation	50% H_2O_2 – RT/24 hr	0.263	1.365	0.124	ND	0.124
5	Thermal Degradation	105°C –72 hours	0.403	1.073	0.215	0.279	0.773
6	Humidity Degradation	25°C/92%RH – 72 hours	0.358	1.091	0.200	0.098	0.396
7	Photolytic Degradation	1.2 million lux hours	0.518	2.183	1.617	0.887	3.391

Note: Abbreviations ND: Not detected.



(3S,4R,5S,8R,9E,12S,14S,15R,16S,18R,19R,26aS)-3-{(E)-2-[(1R,3R,4S)-4-chloro-3-methoxycyclohexyl]-1-methylvinyl}-8-ethyl-5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-5,19-dihydroxy-14,16-dimethoxy-4,10,12,18-tetramethyl-15,19-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclotricosin-1,7,20,21(4H,23H)-tetrone

Fig. 1: **Structure of Pimecrolimus.**

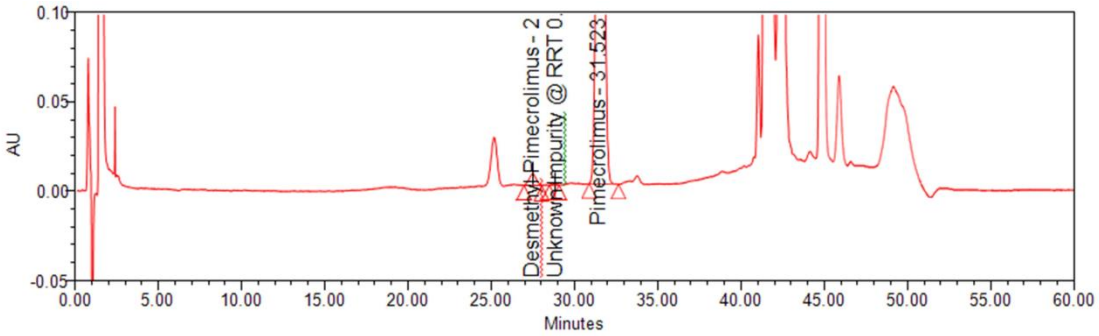


Fig. 2: **Chromatogram of Control Sample Solution.**

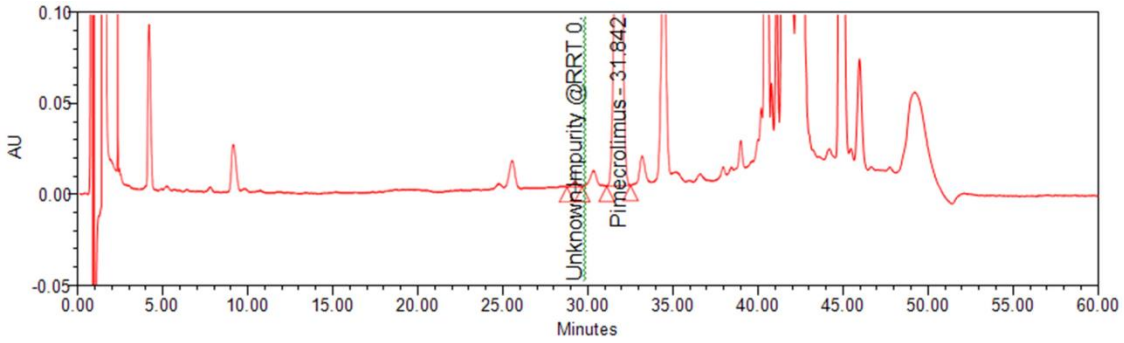


Fig. 3: **Chromatogram of Acid degradation (24 hours) study.**

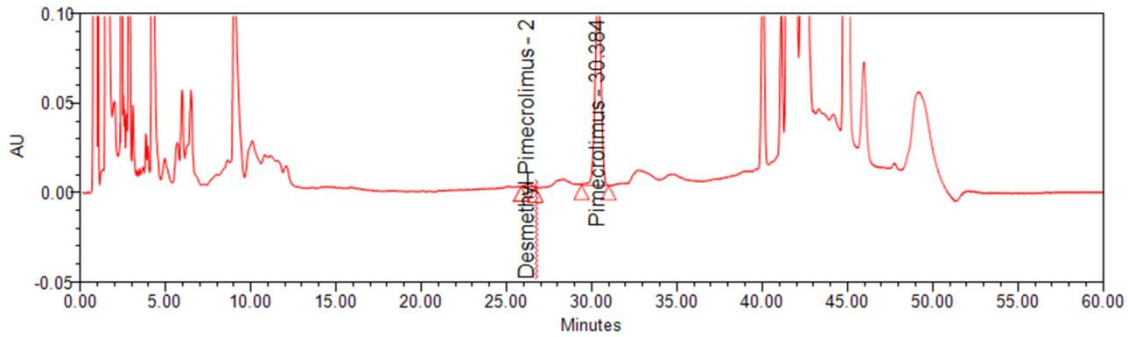


Fig. 4: **Base degradation (24 hours) – Sample.**

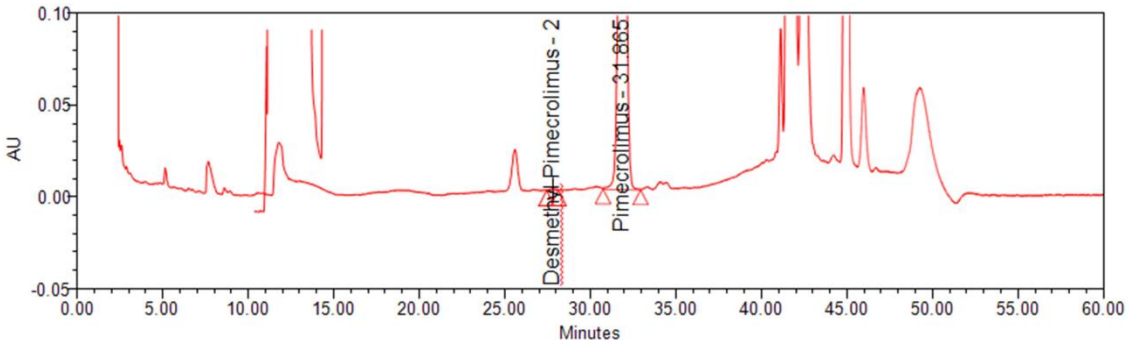


Fig. 5: **Chromatogram of Peroxide degradation (24 hours).**

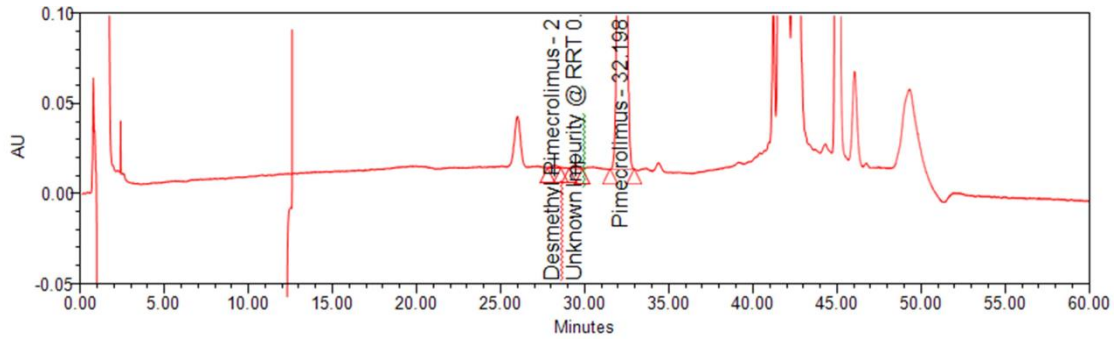


Fig. 6: **Chromatogram of Thermal degradation (105°C/72 hours).**

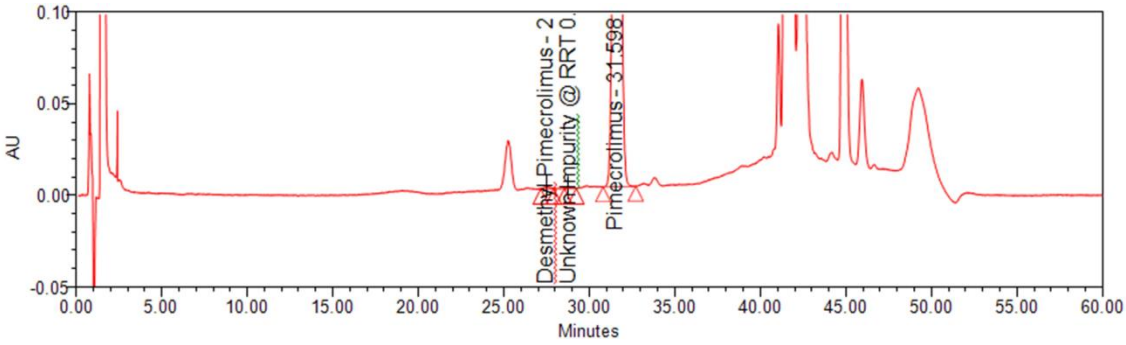


Fig. 7: Chromatogram of Humidity degradation.

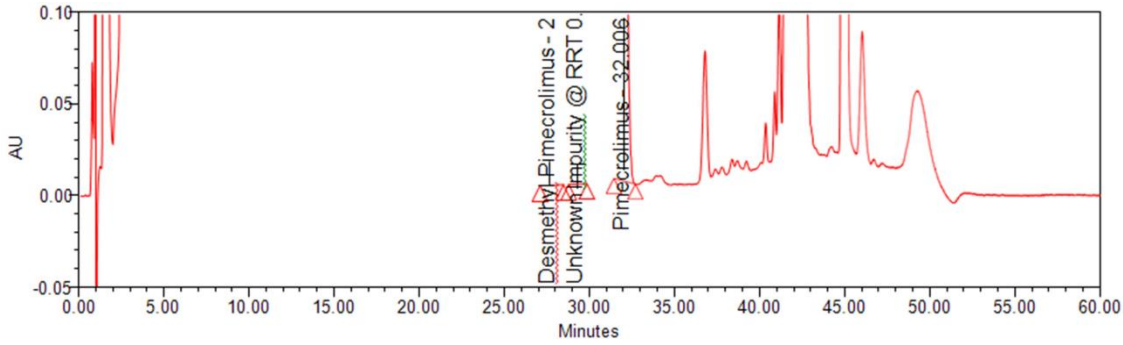


Fig. 8: Chromatogram of Photolytic degradation.

Tables and figure titles and legend:

Table 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS AND SYSTEM SUITABILITY PARAMETERS FOR PROPOSED HPLC METHOD FOR PIMECROLIMUS.

Table 2: STABILITY OF ANALYTICAL SOLUTION

Table 3: FILTER EQUIVALENCY STUDIES

Table 4: FORCED DEGRADATION STUDIES FOR PIMECROLIMUS