A New Validated Stability Indicating RP-HPLC Method For Estimation of Pimecrolimus In Bulk And Topical Formulations

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Abstract:A new, rapid, economical, and gradient reverse phase high-performance liquid chromatography (RP-HPLC) method has been developed and validated for the quantitative determination of Pimecrolimus in the bulk and pharmaceutical dosage form. The chromatographic separation was achieved with YMC Pack pro (C-18, 250×4.6 mm) and 5 µm particle size column. The optimized mobile phase consists of a phosphate buffer, pH adjusted to 2.5 with o-phosphoric acid and acetonitrile. The flow rate was 1 mL/min and eluents were detected at 210 nm using PDA detector. The retention time of Pimecrolimus was found to be 13 min. The percentage recoveries for molecules were found to be in the range of 99–101%. The calibration curve demonstrated good linearity in the range of 5–30 g/ml for Pimecrolimus. The degradation studies were conducted under a variety of stress conditions, including oxidation, thermal, photochemical, and ultraviolet radiation. The developed technique was approved in accordance with ICH recommendations. The validated method was suitable for the detection of Pimecrolimus in bulk drug substances and pharmaceutical dosage forms.

Keywords: Pimecrolimus; Reversed Phase High Performance Liquid Chromatography; Degradation studies; Acetonitrile

1. Introduction

A novel family of immunomodulating macrolactams called Pimecrolimus (SDZ ASM981) is an ascomycin derivative, specifically effective in the treatment of inflammatory skin diseases [1]. Pimecrolimus has earned a lot of interest because of its powerful anti-inflammatory and immunomodulatory properties, in addition to its minimal potential for systemic immunosuppression. The blocking of T cell activation is the mechanism of action of Pimecrolimus [2]. Like other ascomycins, Pimecrolimus is an immunophilin ligand that only interacts with the Immunophilin macrophilin-12 cytosolic receptor. The protein phosphatase calcineurin is efficiently inhibited by the Pimecrolimus-macrophilin complex by preventing calcineurin from dephosphorylating the transcription factor nuclear factor of activated T cells. As a result, signal transduction pathways in T cells are blocked and the production of inflammatory cytokines, especially those of the Th1-and Th2-type, is inhibited. Additionally, Pimecrolimus has been demonstrated to suppress mast cell release of cytokines and pro-inflammatory mediators. [3,4] It has been shown to be beneficial in treating a number of

inflammatory skin conditions, like vitiligo [5], seborrheic dermatitis [6], oral lichen planus [7], cutaneous lupus erythematosus [8], and psoriasis [9-16]. Pimecrolimus as cream and Tacrolimus as an ointment are USFDA-approved calcineurin inhibitors for the treatment of skin diseases [17].

Pimecrolimus (Figure. 1) is a white to off-white fine crystalline powder [17]. It is soluble in methanol and ethanol but insoluble in water. IUPAC name is *(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- ,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.* The molecule has a molecular weight of 810.47g/mol and the empirical formula $C_{43}H_{68}ClNO_{11}$.

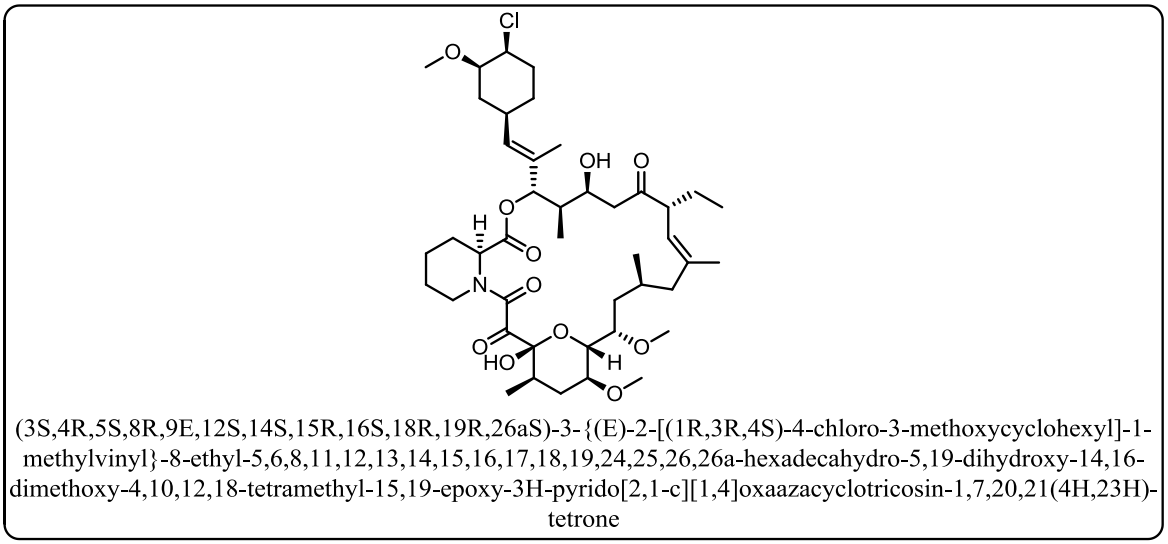

Figure 1. Structure of Pimecrolimus

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

A review of the literature revealed that only one High Performance Liquid Chromatography (HPLC) technique for Pimecrolimus quantification in "Pimecrolimus 1% w/w cream" has been proposed by *Sreedhar lade and Y. Rajendra Prasad* [17]. We have combined and modified method utilized by authors. The principal goal of the current work is to develop and validate a new Reversed Phase- High Performance Liquid Chromatography (RP-HPLC) method to estimate Pimecrolimusin bulk and pharmaceutical dosage forms. Pharmaceutical parameter analysis is a crucial and important step in the entire drug development process. Thus, quick and easy procedures for testing the quality of commercial formulations are required. In light of this, the authors have developed a new, easy, accurate, and efficient technique for determining Pimecrolimus in a pharmaceutical dosage form.

2. Materials and Methods

2.1. Chemicals and solvents.

Pimecrolimus working standard was obtained as a gift sample from Concord

Biotech,India. The commercial pharmaceutical preparation Elidel cream (Novartis India Ltd.) with a label claim of 1% w/w was procured from a local pharmacy. Benzyl alcohol, Acetonitrile and water are of HPLC grade.

2.2. Instrumentation.

The chromatographic separation was performed on Waters Alliance HPLC systems (Waters, USA) equipped with Photodiode array detector (PDA; Model-2998) detector. It has an in-built auto sampler and a column oven. The output signals were monitored and processed using Empower-3 software. A Sonicatorand pH meter of make Lab India was used to enhance the dissolution of the compounds. All weighing was done on Sartorious balance (model AE-160).

2.3. Preparation of Solution A (Buffer).

1.36g of Potassium Dihydrogen Ortho Phosphate weighed and transferred to 1 liter of water and the pH adjusted to 2.5 ± 0.5 with Ortho Phosphoric Acid.

2.4. Preparation of Solution B and Diluent.

Acetonitrile

2.5. Preparation of Pimecrolimus Standard Stock Solution.

25 mg of Pimecrolimus working standard weighed and transferredinto a 25 mL volumetric flask. 10ml of diluentadded, sonicated and, made up to the mark with diluent.

2.6. Preparation of Benzyl Alcohol Standard Stock Solution.

1000 mg of benzyl alcohol weighed and transferred into a 100 mL volumetric flask. 70ml of diluentwas added, sonicated and, made up to the mark with diluent.

2.7. Preparation of Standard Solution.

Pimecrolimus(10ml) and Benzyl alcohol (1ml) from each stock solution was taken and 50ml diluent added (200ppm each).

2.8. Preparation of sample solution.

The sample was taken from the tube (2 gm) and mixed uniformly. In a 100ml volumetric flask, about 70 ml of diluent was added, vortex to disperse the sample uniformly. It was sonicated for about 15 minutes, at room temperature with some intermediate shaking, and diluted up to the mark with diluent. Filter through 0.45µ Teflon membrane Filter (200 ppm).

2.9. Selection of wavelength .

The wavelength was selected on the basis of scanning of Pimecrolimus solution over the range of 200 to 400 nm.

2.10. Chromatographic conditions.

The chromatographic separation was accomplished using gradient elution at temperature of 60°C on an analytical column of dimensions YMC Pack pro (C-18, 250×4.6 mm, 5µm). 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.

2.11. Analytical Method Validation.

The following parameters: specificity, linearity, precision, accuracy, and forced degradation studies are used to validate the proposed RP-HPLC technique. The validation was done in accordance with the standards for validating analytical processes established by the International Conference on Harmonization (ICH) [19].

2.11.1. Specificity.

The method's specificity and selectivity were evaluated by injecting each of the Blank, Placebo solution, Sample solution, and Standard solution to verify the absence of interference with Pimecrolimus elution in standard samples or pharmaceutical formulations.

2.11.2. System Suitability.

The system suitability test is a pre-use test to verify the compatibility and efficacy of a chromatographic system. Any chromatographic system's performance could fluctuate continually throughout routine operation, which could compromise the accuracy of the findings of analytical procedures. The system's suitability was tested by injecting five Pimecrolimus and Benzyl alcohol replicas.The process was repeated every day during the validation of the method [20]. Different parameters, including theoretical plates, tailing factor, and %RSD, were calculated. The acceptance criteria is that the % RSD of five replicate injections for Pimecrolimus and Benzyl alcohol should not be more than 2.0%. Theoretical plates in standard solution should not be less than 3000.

2.11.3. Linearity.

The linearity of response for Pimecrolimus and Benzyl alcohol was determined in the concentration range of the limit of quantitation to about 50%–150% of the specification limit. A calibration curve was prepared each for Pimecrolimus and Benzyl alcohol by plotting the concentration on the x-axis and the average peak area on the y-axis. A linear regression analysis was used to construct the regression equation. Acceptance criteria squared correlation coefficient was not less than 0.99. A volume of 10 µl of each sample was injected five times for each concentration level, and a calibration curve was constructed by plotting the peak area versus drug concentration. A linear relationship between peak area vs. concentration was observed in the range of study.

2.11.4. Precision.

A method's precision is a measure of its ability to produce repeatable results. For system precision, five replicate injections of the standard preparation of both Pimecrolimus and Benzyl Alcohol were used, whereas for method precision, six sample preparations for both.

2.11.5. Accuracy.

The methodological recovery was used to verify the measurement method's accuracy. The recovery of the procedure was indicated by the percentage difference between the sample's measured concentration and its theoretical concentration. The Placebo of Pimecrolimus Cream was spiked with Pimecrolimus and Benzyl Alcohol at three different levels: 80%, 100%, and 120% of the label claim in triplicate. The acceptance criteria for

accuracy should be in the range of 98.0% to 102.0% and the RSD should not be more than 2.0%.

3. 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 1.0 mL/min, reasonable estimate of Pimecrolimus with good peak symmetry and constant baseline was observered. The drug had one distinct peak with retention time (RT) of 13 min and a distinct baseline at 210 nm. The detailed result for every parameter is described below. Each injection had a volume of 10 µl. The optimized chromatographic conditions shown in Table 1.

Table 1. Optimized Chromatographic Conditions And System Suitability Parameters For Proposed HPLC Method For Pimecrolimus.

S.No.	**Parameter**	**Chromatographic conditions**		
1.	Flow rate	1ml per minute.		
2.	Column	YMC Pack Pro, C18, 250mm x 4.6mm, 5µm		
3.	Detector wave length	210 nm		
4.	Oven temperature	60°C		
5.	Injection volume	10 µL		
6.	Run time	30 min		
7.	Diluent	Water: Acetonitrile (50:50)		
8.	Mode of separation	Gradient		
		Time	Mobile Phase A	Mobile Phase B
		0.0	55	45
		5.0	55	45
		7.0	20	80
		16.0	20	80
		18.0	2	98
		23.0	2	98
		25.0	55	45
		30.0	55	45

3.1. Analytical Method Validation.

3.1.1. Specificity.

No interference observed from blank and placebo at the retention time of Pimecrolimus and Benzyl Alcohol peaks (**Figure.** 2-6).

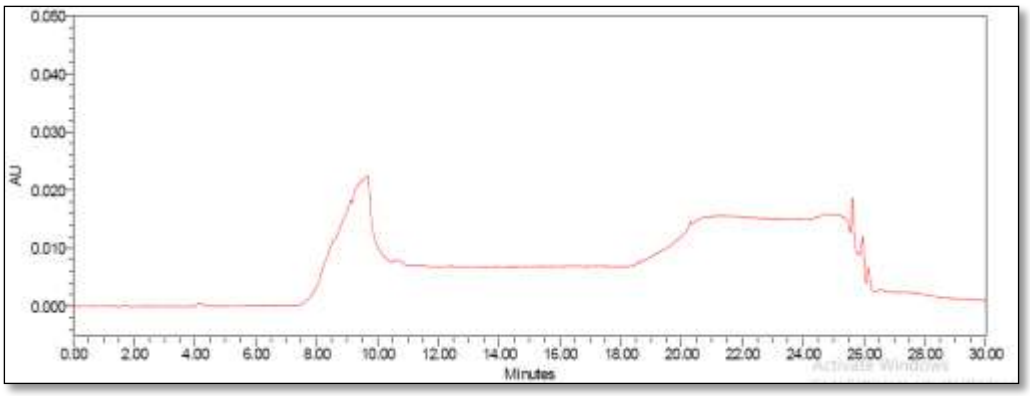

Figure 2. Chromatogram of Placebo.

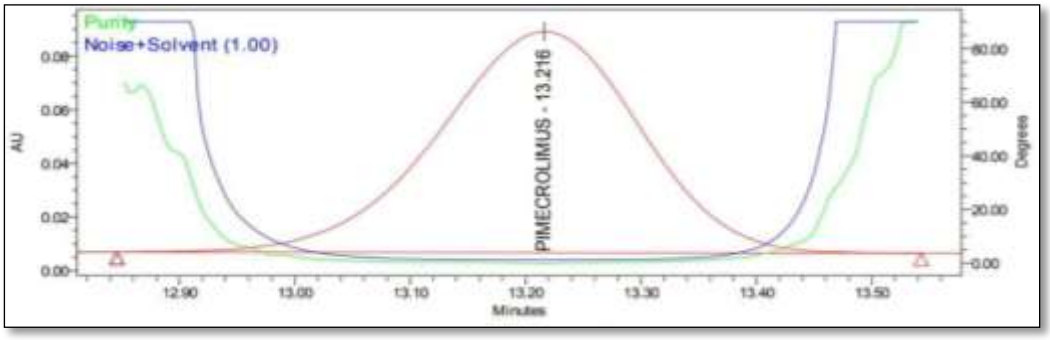

Figure 3. StandardChromatogram of Pimecrolimus.

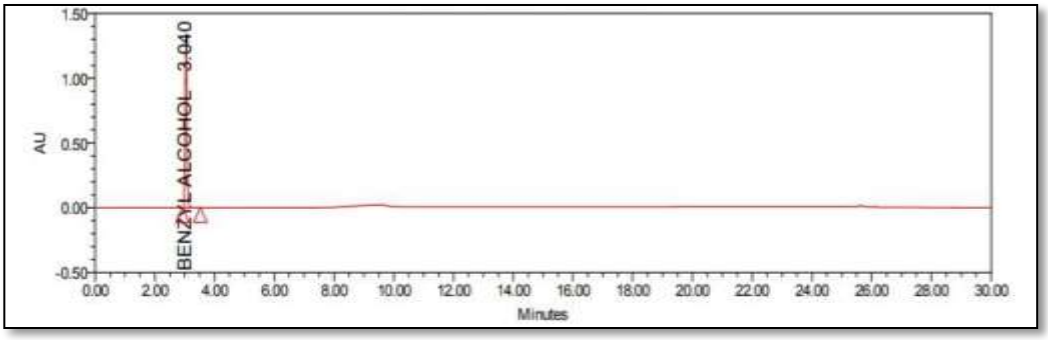

Figure 4. StandardChromatogram of Benzyl Alcohol.

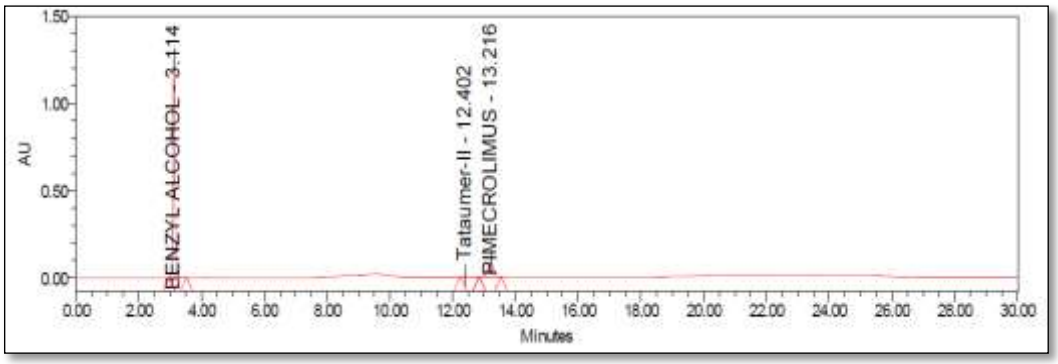

Figure 5. Chromatogram of Pimecrolimus& Benzyl alcohol mixture spiked.

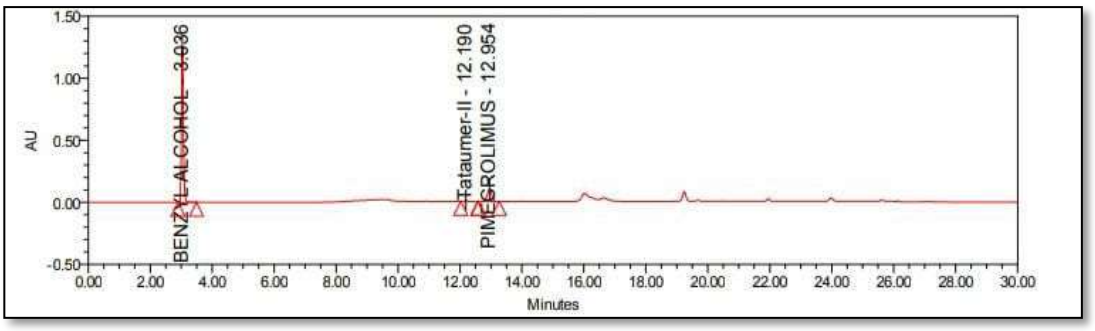

Figure 6. Chromatogram of sample.

3.1.2. System Suitability.

System suitability test combinations that have been carefully chosen can be used to test the operation parameters of the entire chromatographic system. These mixtures are used to set up common chromatographic parameters such as the number of theoretical plates, resolution, asymmetry, detection limit, and selectivity. Only if the responses fall within predetermined limits then system is appropriate.Pimecrolimusand Benzyl alcohol passes system suitability test.

3.1.3. Linearity.

The calibration curve was plotted by graphing the peak area *vs* concentration. Utilizing linear regression analysis, the calibration curve's linearity was assessed. The regression formula was y = 5400.3x - 39085. The co-relation coefficient was 0.9998 for Pimecrolimus and 0.9993 for Benzyl Alcohol, which satisfied the requirements for the validation of the analytical technique [19]. Thus, the method's linearity across the concentration range of 50%-150% was established.The observations and calibration curve was shown in **Table**-3 and **Figure**.7-8.

Table 3. Linearity studies for Pimecrolimus and Benzyl Alcohol.

% Concentration	Pimecrolimus		Benzyl alcohol	
	Concentration (µg per mL)	Response (Area)	Concentration (µg per mL)	Response (Area)
50%	99.1	494635	102.4	2572074
80%	160.4	835487	163.3	4198895
90%	179.2	923937	183.6	4718672
100%	197.9	1025652	203.8	5136722
110%	218.3	1136352	225.1	5700787
120%	239.5	1261150	243.4	6135373
150%	300.2	1580709	303.3	7707550

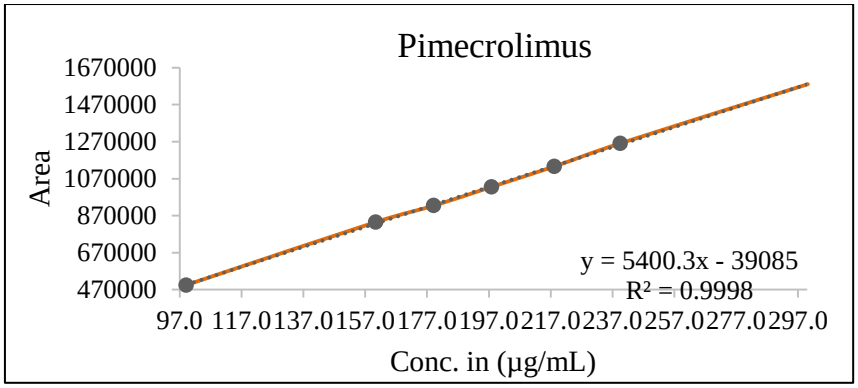

Figure 7. Calibration curve ofPimecrolimus**.**

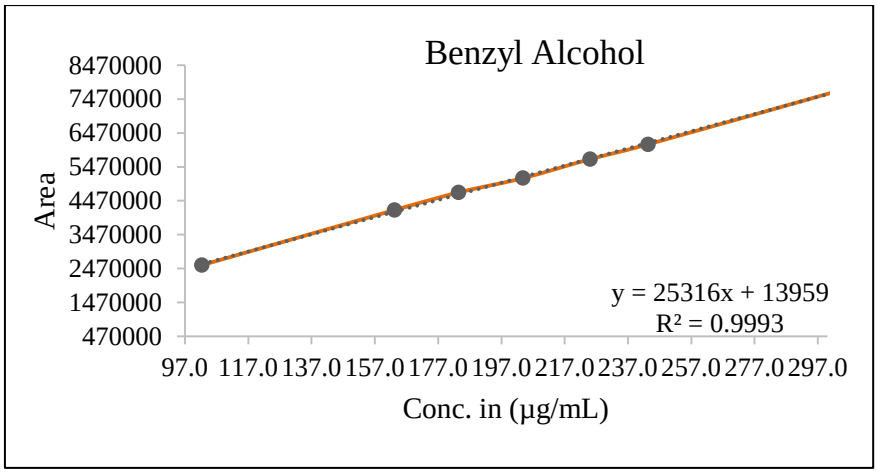

Figure 8. Calibration curve of Benzyl alcohol

3.1.4.Precision.

The precision of method was determined by repeatability and intermediate precision. Repeatability was examined by performing six determinations of the same concentration of Pimecrolimus and Benzyl alcohol on the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis intra-da and inter-day for Pimecrolimus (**Table** 4).The overall mean of intermediate precision is 99.71.The % RSD of system precision is 0.657% for Pimecrolimusand 0.711% for Benzyl Alcoholwhereas %RSD of method precision is 0.641% for Pimecrolimusand 0.505% for Benzyl Alcohol.Therefore, the HPLC method for the determination of Assay of Pimecrolimus and Benzyl Alcohol Preservative content inPimecrolimusCream is precise (**Table** 4).

Table 4. System and Method Precision studies for Pimecrolimus and Benzyl alcohol

Injection	Benzyl Alcohol		Pimecrolimus			
	System Precision	Method Precision	System Precision	Method Precision	Intermediate Precision	
					Interday	Intraday
	Area	% Assay	Area	% Assay	% Assay	% Assay
1	5067422	99.2	1016346	100.1	100.1	100.9

2	5112395	98.1	1026290	99.6	98.6	99.7
3	5147757	98.6	1031246	98.7	99.7	100.2
4	5094435	97.8	1024464	99.6	99.6	99.1
5	5058018	98.2	1035098	98.4	99.4	100.3
6	5056018	98.1	1032098	99.5	98.5	100.4
Mean (n=6)	5089341	98.33	1027590	99.32	99.32	100.10
SD	36171	0.497	6752	0.637	0.727	
%RSD	0.711	0.505	0.657	0.641	0.729	

Note:SD referred to Standard Deviation; RSD referred to Relative Standard Deviation.

3.1.5.Accuracy.

The results were analyzed and they were found to be within the limits.The mean recovery is 98.93 % and RSD is 1.883% for Pimecrolimus and Mean recovery is 100.5 % and RSD is 0.600% for Benzyl Alcohol (**Table** 5).

Table 5. Accuracy studies for Pimecrolimusand Benzyl alcohol

% Concentration	Benzyl alcohol			Pimecrolimus		
	Amount added (mg)	Amount recovered (mg)	% Recovery	Amount added (mg)	Amount recovered (mg)	% Recovery
80% -1	16.37	16.34	99.8	15.86	15.35	96.8
80% -2	16.37	16.43	100.4	15.86	15.45	97.4
80% -3	16.37	16.31	99.6	15.86	15.34	96.7
100% -1	20.46	20.14	98.4	19.82	19.35	97.6
100% -2	20.46	20.57	100.5	19.82	19.59	98.8
100% -3	20.46	20.91	102.2	19.82	19.96	100.7
120% -1	24.55	24.83	101.1	23.79	24.11	101.3
120% -2	24.55	24.86	101.3	23.79	23.87	100.3
120% -3	24.55	24.80	101.0	23.79	23.99	100.8
Mean			100.5	Mean		98.933
SD			0.603	SD		1.863
% RSD			0.600	% RSD		1.883

Note: Abbreviations same as Table 4.

4. Conclusions

The present study represents the first report that deals with the development of a stability-indicating HPLC method for determination of Pimecrolimus in "Pimecrolimus 1% w/w cream". This study is a typical example of development of a stability indicating assay, following the recommendations of ICH guidelines. The sample preparation is simple, the

analysis time is short and the elution is by gradient method. The proposed method showed acceptable accuracy, precision, selectivity, and wide linear concentration range. 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.

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Conflicts of Interest

The authors declare no conflict of interest.

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