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COST EFFECTIVE, ROBUST PRECISE & STABILITY INDICATING ANALYTICAL METHOD VALIDATION OF RELATED SUBSTANCES FOR PIMECROLIMUS CREAM BYRP-HPLC

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Keywords:

Pimecrolimus, Desmethyl Pimecrolimus, High-Performance Liquid Chromatography, Validation, Related substances

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ABSTRACT: A new, precise, economical and gradient reverse-phase high-performance liquid chromatography (RP-HPLC) method has been validated for the relative substance determination in pimecrolimus cream. The chromatographic separation was achieved with phenomenax luna, C18, 150 x 4.6mm and 3 µm particle size column. The flow rate was 1.5 mL/min and eluents were detected at 210 nm using PDA detector. The retention time of pimecrolimus and desmethyl pimecrolimus was found to be 31.5 min and 27.5 min respectively. The calibration curves were linear for both pimecrolimus and desmethyl pimecrolimus. The LOQ was 0.50µg/ ml for pimecrolimus and 0.47µg/ml for desmethyl pimecrolimus. The approach has been validated in accordance with the international conference on harmonization's regulatory criteria. The evaluated parameters are precision, linearity, detection limit, quantification limit, specificity, accuracy and robustness. The technique may be applied to stability investigations as well as routine analysis to identify and quantify known and unidentified impurities pimecrolimus in the pharmaceutical dosage form.

INTRODUCTION: Pimecrolimus is an ascomycin derivative that belongs to a novel class of immunomodulating macrolactams and is especially effective in treating inflammatory skin conditions [1]. Pimecrolimus has a low risk of systemic immunosuppression and significant anti-inflammatory and immunomodulatory effects, which have received much attention. Pimecrolimus works by preventing T cell activation [2–5], which is how it works. Similar to other ascomycins, pimecrolimus is an immunophilin ligand that only engages the immunophilin macrophilin-12 cytosolic receptor.

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By preventing it from dephosphorylating activated T cells' transcription factor nuclear factor, the Pimecrolimus-macrophilia complex efficiently suppresses the protein phosphatase calcineurin. This causes T-cell signal transduction pathways to be closed off, which limits the generation of inflammatory cytokines, especially those of the Th1 and Th2 types. Pimecrolimus has been shown to prevent mast cell release of cytokines and pro-inflammatory mediators [6–8].

Its use has successfully treated inflammatory skin conditions like vitiligo, seborrheic dermatitis, oral lichen planus, cutaneous lupus erythematosus, and psoriasis [8–12]. The calcineurin inhibitors pimecrolimus and tacrolimus have been approved by the USFDA for the treatment of skin conditions [13–14]. Desmethyl pimecrolimus has a molecular weight of 796.44 g/mol and an empirical formula of $C_{42}H_{66}ClNO_{11}$, whereas pimecrolimus has a

molecular weight of 810.47 g/mol and $C_{43}H_{68}ClNO_{11}$ as its empirical formula [15-17]. The principal goal of the current work is to validate a new reversed phase-high performance liquid chromatography (RP-HPLC) method to estimate pimecrolimus and desmethyl pimecrolimus impurity in pharmaceutical dosage forms. Pharmaceutical parameter analysis is a crucial and important step in the entire drug development process. Thus, rapid and easy procedures for testing the quality of commercial formulations are required. In light of this, the authors have developed a new, accurate and efficient technique for determining pimecrolimus and desmethyl pimecrolimus impurity in a pharmaceutical dosage form. The suggested RP-HPLC technique is validated using the following factors: specificity, linearity, precision, accuracy, LOD and LOQ experiments. The validation was performed in compliance with the International Conference on Harmonization's (ICH) requirements for validating analytical procedures [18-20].

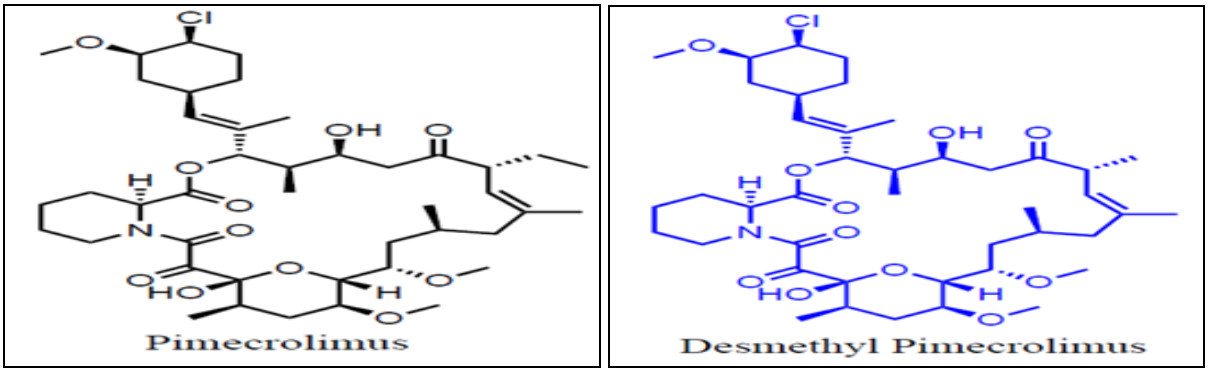

FIG. 1: STRUCTURE OF PIMECROLIMUS AND DESMETHYL PIMECROLIMUS

MATERIALS AND METHODS: Apparatus instruments, chemicals, and experiment reagents are listed in **Tables 1** and **2.**

TABLE 1: APPARATUS / INSTRUMENTS USED

Sl. no.	Material/Reference Standard
1.	Pimecrolimus
2.	Desmethyl pimecrolimus

TABLE 2: NAME OF CHEMICALS / REAGENTS

Sl. no.	Name of Chemicals /Reagents	Grade	CAS No.
1.	Water (HPLC Grade)	ACS	7732-18-5
2.	Acetonitrile	ACS	75-05-8
3.	Methyl Tertiary Butyl ether	ACS	1634-04-4
4.	Formic acid	ACS	64-18-6

HPLC Instrumentation and Conditions:

Chromatographic Conditions: Gradient elution was used to achieve the chromatographic separation using an analytical column with the dimensions Phenomenex Luna (C18, 150 x 4.6mm, 3) at a temperature of 60°C. Solutions A and B constitute together the mobile phase. The mobile phase was filtered through a 0.45 m Millipore Nylon 6 membrane filter. Before injection, the column was equilibrated with the mobile phase for at least 30 minutes.

Gradient Programming:

S. no.	Parameter	Chromatographic conditions
1.	Flow rate	1.5ml per minute.
2.	Column	Phenomenex Luna, C18, 150 x 4.6mm, 3µ
3.	Detector wavelength	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		
9.		**Time**	**Mobile Phase A**	**Mobile Phase B**
10.		0.0	70	30
11.		20.0	70	30
12.		35.0	38	62
13.		40.0	5	95
14.		48.0	5	95
15.		50.0	70	30
16.		60.0	70	30
17.		0.0	70	30

Sample and Standard Solution Preparation:

Preparation of Solution A: Water: Acetonitrile: Methyl Tertiary Butyl ether: Formic acid (650:240:70:0.2).

Preparation of Solution B: Water: Acetonitrile: Methyl Tertiary Butyl ether: Formic acid (200:660:70:0.2).

Preparation of Desmethyl Pimecrolimus Impurity Stock Solution: Desmethyl pimecrolimus impurity (1mg) weighed into a volumetric flask (10ml) then 5ml of acetonitrile added and sonicated to dissolve. Volume made up with acetonitrile.

Preparation of System Suitability Solution: Pimecrolimus working standard (30mg) was weighed and transferred to 50mL volumetric flask. To this 3ml of desmethyl pimecrolimus impurity stock solution in 35ml of acetonitrile and sonicated to dissolve, volume made up by Acetonitrile.

Preparation of Diluted Standard Solution: 30mg of pimecrolimus working standard with 30 ml of diluent transferred in a volumetric flask (50ml). The solution sonicated and volume made up with diluent. Further, 1ml of solution take into100 ml volumetric flask to make 6 ppm solution.

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

Selection of Wavelength: The wavelength was chosen by scanning a solution of pimecrolimus between 200 and 400 nm.

RESULTS AND DISCUSSION: To create an accurate, selective, and precise stability-indicating RP-HPLC method for quantifying Pimecrolimus in stressed samples, various mobile phases with various compositions and flow rates were tested.

The chromatographic conditions were developed and modified after a number of compositions and combinations. An acceptable quantification of Pimecrolimus was observed at 1.5 mL/min with the gradient mobile phase, good peak symmetry, and a steady baseline. A clear baseline at 210 nm and one unique peak with a retention time (RT) of 31.5 min both were observed in the Pimecrolimus. Below is a description of each parameter's specific result.

Specificity: In order to confirm that there is no interference with pimecrolimus elution in standard samples or pharmaceutical formulations, the specificity and selectivity of the technique were assessed by injecting each of the system suitability solution, Standard solution, sample solution, individual impurity solution and spiked sample solution. Peak purity passes for pimecrolimus and desmethyl pimecrolimus impurity peaks, the result of specificity given in **Table 3** and **Fig. 2** to **Fig. 4.**

TABLE 3: PEAK PURITY OF STANDARD AND CONTROL SAMPLE

	Purity angle	**Purity Threshold**
Sample		
Standard	4.619	7.318
Control sample	0.326	1.102
Spiked sample		
Pimecrolimus	0.355	1.079
Desmethyl pimecrolimus	5.121	6.648

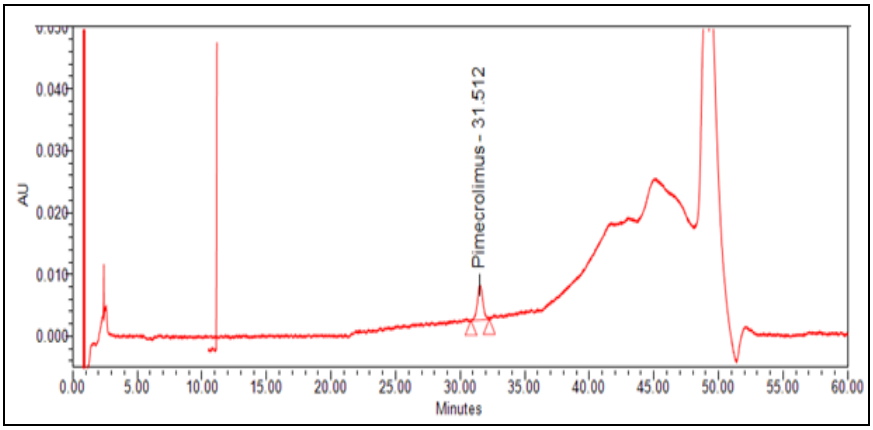

FIG. 2 STANDARD CHROMATOGRAM OF PIMECROLIMUS

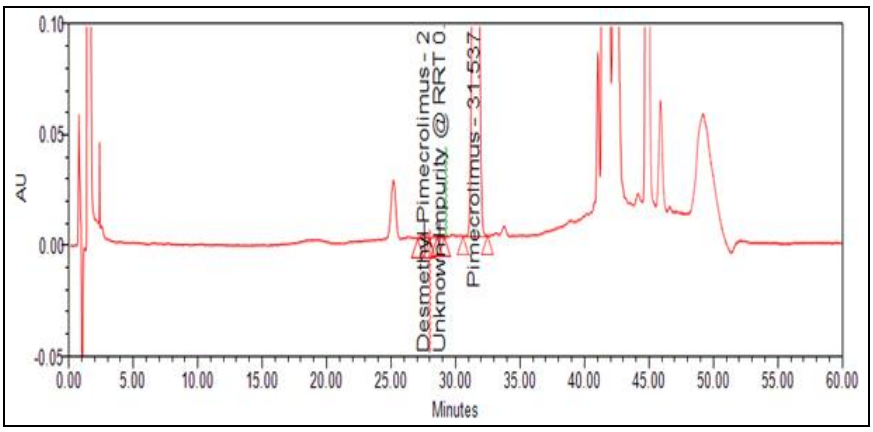

FIG. 3 CHROMATOGRAM OF SAMPLE

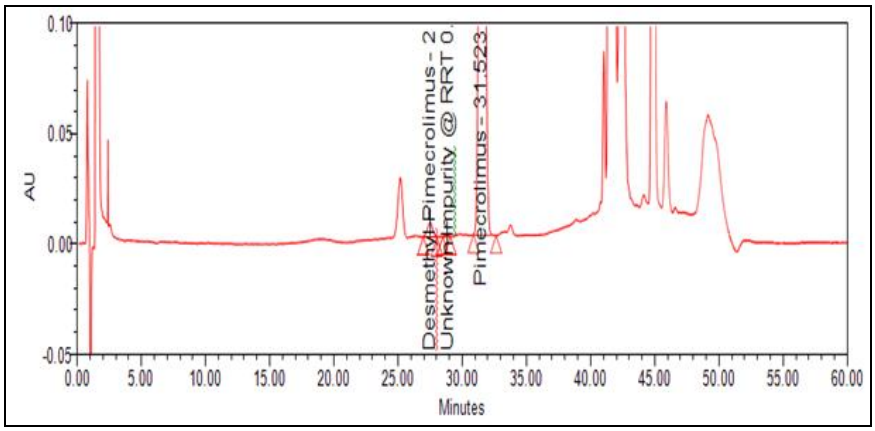

FIG. 4 CHROMATOGRAM OF SPIKED SAMPLE SOLUTION

Limit of Detection and Quantification: The limit of quantitation (LOQ) can be calculated using the formula: LOQ=10 standard deviation (SD)/S, where the standard deviation is response based on either the SD of the blank or the residual SD of the regression lines or the SD of y=intercepts of regression lines and S=Slop of the linear regression. The LOD and LOQ values of pimecrolimus and desmethyl pimecrolimus are given in **Table 4.**

TABLE 4: LIMIT OF DETECTION AND QUANTIFICATION FOR PIMECROLIMUS AND DESMETHYL PIMECROLIMUS

	Limit of Detection		Limit of Quantification	
	Pimecrolimus	**Desmethyl Pimecrolimus**	**Pimecrolimus**	**Desmethyl Pimecrolimus**
Conc. (µg/mL)	0.253	0.236	0.506	0.473
1	8913	7545	16503	16289
2	8099	5889	16156	15451
3	7092	7821	14692	14893

4	7491	5877	15993	15625
5	6991	6992	16742	16848
6	8603	7777	15102	16328
Mean	7865	6984	15865	15906
SD	799.870	902.070	804.400	710.530
%RSD	10.170	12.920	5.070	4.470

Linearity: The linearity of response for pimecrolimus and desmethyl pimecrolimus was determined in the concentration range of the limit of quantitation to 150% of the specification limit. A calibration curve was prepared each for Pimecrolimus and impurity 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. The acceptance criteria squared correlation coefficient was not less than 0.99. The observations and calibration curve was shown in **Table 5** and **Fig. 5** and **6.**

TABLE 5: LINEARITY STUDIES FOR PIMECROLIMUS AND DESMETHYL PIMECROLIMUS

Injection	Pimecrolimus		Desmethyl Pimecrolimus	
	Conc. (µg/ mL)	Response (Area)	Conc. (µg/ mL)	Response (Area)
LOQ	0.506	15865	0.473	15906
Lin-1	1.190	31242	1.113	29095
Lin-2	2.974	81969	2.782	77342
Lin-3	4.758	126750	4.451	116754
Lin-4	5.948	155114	5.563	139205
Lin-5	7.137	188890	6.676	174373
Lin-6	8.922	243839	8.345	209871
Slope	26760.67		24901.882	
Intercept	349.606		4336.566	
Correlation Coefficient	0.9993		0.9991	

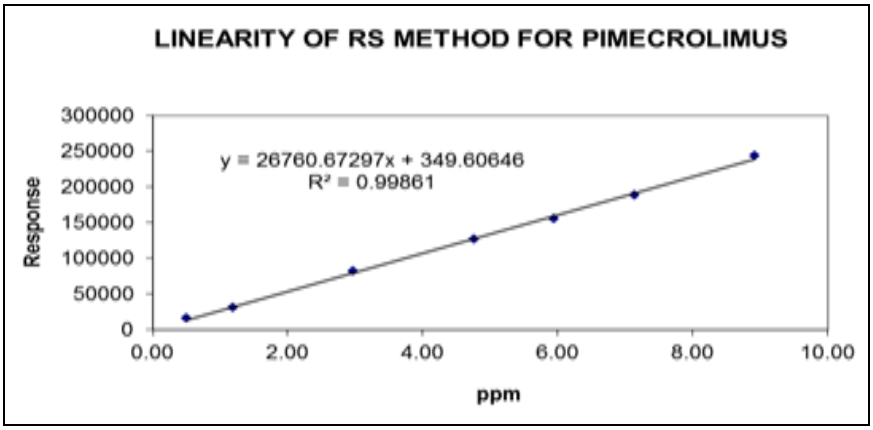

FIG. 5: CALIBRATION CURVE OFPIMECROLIMUS.

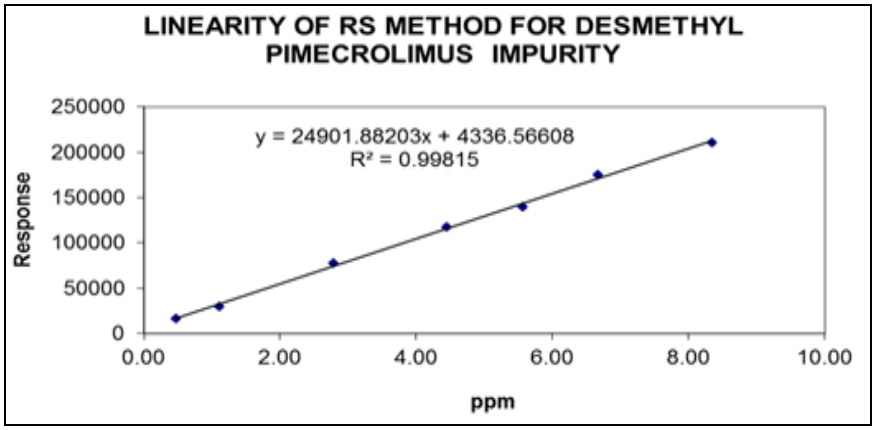

FIG. 6: CALIBRATION CURVE OFDESMETHYL PIMECROLIMUS

Precision: A method's precision is a measure of its ability to produce repeatable results. Six replicate injections of the standard preparation of desmethyl pimecrolimus were used for system precision, whereas, for method precision, six sample solution of Pimecrolimus cream and one sample spiked with known desmethyl pimecrolimus at specification limit was prepared. The precision of the method was determined by repeatability and intermediate precision. Repeatability was examined by performing six determinations of the same concentration of desmethyl pimecrolimus on the same day under the same experimental conditions. The result of repeatability and intermediate precision is given in **Table 6.**

TABLE 6: SYSTEM AND METHOD PRECISION STUDIES FOR DESMETHYL PIMECROLIMUS

Injection	System Precision	Method Precision			Intermediate Precision		
	Area	% Desmethyl Pimecrolimus	% Unknown Impurity@ RRT 0.91	% Total	% Desmethyl Pimecrolimus	% Unknown Impurity@ RRT 0.91	% Total
1	157437	0.249	BLQ	0.249	0.256	BLQ	0.256
2	160368	0.241	BLQ	0.241	0.227	BLQ	0.227
3	157153	0.232	BLQ	0.232	0.206	BLQ	0.206
4	151558	0.248	BLQ	0.248	0.224	BLQ	0.224
5	155314	0.250	BLQ	0.250	0.230	BLQ	0.230
6	156078	0.253	BLQ	0.253	0.250	BLQ	0.250
Mean (n=6)	156318	0.246	NA	0.246	0.232	NA	0.232
SD	2900.87	0.008	NA	0.008	0.018	NA	0.018
%RSD	1.86	3.252	NA	3.252	7.869	NA	7.869

Accuracy: The placebo of pimecrolimus cream was spiked with pimecrolimus and desmethyl pimecrolimus at three different levels: LOQ, 50%, 100% and 150% of the label claim in triplicate. The acceptance criteria for accuracy should be in the range of 90.0% to 110.0% for 50%, 100% and 150% levels. In the case of, LOQ mean recovery should be in the range of 85% to 115%. The recovery of desmethyl pimecrolimus is given in **Table 7.**

TABLE 7: ACCURACY STUDIES FOR DESMETHYL PIMECROLIMUS

Sr. no.	mcg added	mcg found	% Recovery
LOQ-1	0.02255	0.02335	103.5
LOQ-2	0.02255	0.02327	103.2
LOQ-3	0.02255	0.02354	104.4
		Mean	103.7
		SD	0.620
		% RSD	0.600
50% -1	0.1409	0.1450	102.9
50% -2	0.1409	0.1456	103.3
50% -3	0.1409	0.1459	103.5
100% -1	0.2819	0.2945	104.5
100% -2	0.2819	0.2954	104.8
100% -3	0.2819	0.2981	105.8
150% -1	0.4228	0.4447	105.2
150% -2	0.4228	0.4483	106.0
150% -3	0.4228	0.4445	105.1
		Mean	104.5
		SD	1.170
		% RSD	1.120

Robustness: The HPLC method for the determination of related substances of pimecrolimus in pimecrolimus cream is robust for small changes in Column temperature, Flow rate and wavelength. The system suitability solution, standard solution, placebo, sample solution and sample spiked with known impurity were injected under different chromatographic conditions. The result of the different condition is mentioned in **Table 8** and the system suitability of different variable condition are mentioned in **Table 9.**

TABLE 8: ROBUSTNESS STUDIES FOR DESMETHYL PIMECROLIMUS

Sr. no.	Parameters	Variations	RRT Desmethyl Pimecrolimus
1	Control-1	-	0.87
2	Flow rate	-0.1ml/min	0.88
		+0.1ml/min	0.86
3	Column temperature	+5°C	0.87
		-5°C	0.88
4	Control-2	-	0.87
5	Wavelength	-5 nm	0.87
		+5 nm	0.87

TABLE 9: SYSTEM SUITABILITY FOR PIMECROLIMUS AND DESMETHYL PIMECROLIMUS

Sr. no.	Experiment	Resolution b/n Pimecrolimus & Desmethyl Pimecrolimus	USP Tailing	USP Theoretical Plates	% RSD
1	Control-1	3.2	1.1	10688	1.75
2	Flow - 0.1mL/min	3.3	1.1	12353	NA
3	Flow +0.1mL/min	3.2	1.0	9871	NA
4	Temp +5°C	3.2	1.0	9419	NA
5	Temp -5°C	3.4	1.0	12630	NA
6	Wavelength -5nm	5.2	1.1	28938	NA
7	Wavelength +5nm	5.4	1.1	29153	NA

System Suitability: The system suitability test is a pre-use test to verify the compatibility and efficacy of a chromatographic system. The system's suitability was tested by injecting the system suitability solution. The process was repeated every day during the validation of the method. The resolution between the pimecrolimus peak and the desmethyl pimecrolimus peak should not be less than 3. Tailing factor for the Pimecrolimus peak in the system suitability solution should not be more than 2 the and theoretical plate should not be less than 2000. The RSD of six replicate injections for a standard should be less than 5%. The system suitability of different validation parameters is given in **Table 10.**

TABLE 10: SYSTEM SUITABILITY FOR PIMECROLIMUS AND DESMETHYL PIMECROLIMUS

Sr. no.	Parameter	Resolution	USP Tailing	USP Theoretical Plates	% RSD
1	Specificity	5.0	1.1	29185	2.840
2	Linearity	3.1	1.0	10058	3.80
3	Accuracy	4.6	1.0	21319	1.856
4	Method precision	4.8	1.0	21319	2.560
5	Intermediate precision	4.7	1.0	20912	3.625
6	LOD and LOQ	4.7	1.0	20811	3.069

CONCLUSION: A novel gradient RP-HPLC approach has been developed to study related substances in pimecrolimus. The linearity, LOD and LOQ of pimecrolimus and desmethylpimecrolimus were determined. The technique has been proven to be precise. It can simultaneously identify and quantify impurities in pimecrolimus that are known and unknown. It is simple, sensitive, linear and exact. The technique may be applied to the routine and stability examination of dosage forms and pharmaceutical substances.

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