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Development and Validation of UPLC Method for Determination of Lamivudine Impurity Profile in Tablets

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Lamivudine and its related impurities method separation was performed on Acquity UPLC BEH Phenyl C18 column 2.1 mm × 100 mm, 1.7 µm. The mobile phase consists of 0.025 mol L^{-1} ammonium acetate buffer pH 3.8 ± 0.05 and with gradient elution. Ammonium acetate buffer and methanol were used as diluent with 95:5 v/v ratio. The flow rate was 0.5 mL min^{-1} and the detection wavelength was 277 nm. Lamivudine was completely separated from salicylic acid impurity and unknown impurities. The linear range of lamivudine and salicylic acid was 0.12-4.51 and 0.20-3.38 ppm, respectively. The recovery of salicylic acid was found to be in between 80 to 120 % with RSD of 3.48 %. Method was robust against small variations in wavelength, pH, flow rate and column oven temperature. The RP-UPLC method is an ultra-fast, specific, precise, reproducible, accurate, rugged and robust, which may be useful for the routine estimation of lamivudine related substances in several pharmaceutical dosage forms.

Keywords: Lamivudine, Salicylic acid, RP-UPLC, Impurities, Validation.

INTRODUCTION

Lamivudine is an analogue of cytidine. It inhibits both types I and II of HIV reverse transcriptase and also the reverse transcriptase of hepatitis B. It is phosphorylated to active metabolites that compete for incorporation into viral DNA. They inhibit the HIV reverse transcriptase enzyme competitively and act as a chain terminator of DNA synthesis. The lack of a 3′-OH group in the incorporated nucleoside analogue prevents the formation of the 5′-3′ phosphodiester linkage essential for DNA chain elongation from and therefore, the viral DNA growth is terminated [1].

Literature survey revealed that a few analytical methods has been established for the determination of lamivudine and its impurities by UPLC in human plasma and estimation of lamivudine in combined dosage forms [2,3]. To the best of our knowledge, there is no reported UPLC method for estimation of lamivudine related substances in pharmaceutical formulations. Thus, efforts were made to develop fast, selective and sensitive analytical method for the estimation of lamivudine related impurities in 300 mg tablet dosage form using ultra performance chromatographic method [4-6]. In the present work, a simple, reliable and reproducible UPLC method is author developed, which was duly validated by statistical parameters precision and accuracy.

EXPERIMENTAL

Lamivudine standard was a generous gift from a reputed pharmaceutical organization. Lamivir 300 mg tablets (Cipla, India) was purchased from local market. Gradient grade methanol was procured from Merck, India. Ammonium acetate and glacial acetic acid were purchased from Loba Chemie, Mumbai, India. Water used in the UPLC analysis was prepared by the water purifier Arium, 611UF, Sartorius, Germany. The mobile phase filtered through a 0.22µ membrane filter, Fisher brand and all the test solutions were filtered through a 0.45 µ millex PVDF filter, Millipore, India [7].

The method has been satisfactorily applied to the simultaneous estimation of related substances of lamivudine in bulk and pharmaceutical dosage forms 50, 100, 150 and 300 mg [8,9].

Chromatographic conditions: Lamivudine, salicylic acid and its unknown impurities were separated on a Aquity UPLC BEH phenyl 2.1 × 100 mm, 1.7 µm with gradient program using 0.025 Mm ammonium acetate as buffer (pH 3.80) and

methanol as mobile phase at a flow rate of 0.5 mL/min with column oven temperature at 40 ºC and the detection was carried out at 277 nm.

Preparation of solutions

Mobile phase A: Weighed and dissolved about 1.9 g of ammonium acetate in 900 mL of deionized water in 1000 mL beaker and adjusted the pH of the solution to 3.8 with dil. glacial acetic acid and diluted up to volume with deionized water to make up the volume 1000 mL and filtered the solution through 0.22 µm membrane filter (0.025 M ammonium acetate buffer).

Mobile phase B: Gradient grade methanol filtered through 0.22 µm membrane filter.

Preparation of diluent: Mixed mobile phase A and B in the ratio 95:5 % v/v and sonicated for 10 min. The mixture was filtered through 0.45 µ membrane filter and sonicated again before use.

Lamivudine standard stock solution (30 ppm): A standard stock solution was prepared by dissolving 50 mg of lamivudine with diluent in a 100 mL volumetric flask and sonicated for 30 min. Diluted 3 mL of the above solution further to 50 mL with diluent and mixed well.

Salicylic acid standard stock solution (22.5 ppm): A standard stock solution was prepared by dissolving 37.5 mg of salicylic acid with diluent in a 100 mL volumetric flask and sonicated for 30 min and mixed well. Diluted 3 mL of the above solution further to 50 mL with diluent and mixed well.

Standard preparation: Standard solution was prepared by adding 5 mL of lamivudine standard stock solution (30 ppm) and 5 mL of salicylic acid standard stock solution into 50 mL volumetric flask and diluted with diluent.

Sample solution: Average weight of 20 tablets of lamivir (300 mg) was crushed into fine powder and taken the sample weight equivalent to 150 mg lamivudine and transferred into a 100 mL volumetric flask. To it 70 mL of diluent was added and sonicated for 5 min with intermittent shaking, further the volume made up with diluent and filtered the solution through 0.45 µm PVDF membrane syringe filter.

Placebo solution: Weighed and transferred 20 tablets of lamivir (300 mg) placebo powder into a 100 mL of volumetric flask which is equivalent to150 mg of lamivudine and added 70 mL of diluent sonicated for 5 min with intermittent shaking, further the volume made up with diluent and filtered the solution through 0.45 µm PVDF membrane syringe filter.

Forced degradation studies: Intentional degradation (n = 3) was attempted by using water, heat, light, acid, base, humidity and oxidizing agent. For acid degradation, the sample was treated with 1N HCl at 80 ºC for 4 h and then neutralized with 1N NaOH. For alkali degradation, the sample was treated with 0.1N NaOH at 80 ºC for 2 h and then neutralized with 0.1N HCl. For oxidative degradation, the sample was treated with 0.1% H_2O_2 on bench top for 2 h. For photolytic degradation, sample was exposed to ultraviolet (UV) (200 watt h/m^2) as per ICH Guidelines [10]. For thermal degradation, sample was exposed to temperatures at 105 ºC for 2 days. For hydrolytic degradation, the sample was treated with water by heating on water bath at 80 ºC for 24 h. For humidity degradation, exposed the sample at 85 % humidity (saturated solution of potassium nitrate) at 7days.

After completion of the degradation treatments, the samples were cooled to room temperature, diluted with the diluent, and injected for chromatographic analysis.

RESULTS AND DISCUSSION

Method development: A HPLC method was developed by using Phenyl 4.6 × 250 mm, 3.5 µm with isocratic mode. The mobile phase is 0.025 Mm ammonium acetate buffer (pH 3.80, mobile phase-A) and methanol (mobile phase-B) as mobile phase at a flow rate of 0.7 mL/min with column oven temperature at 40 ºC and the detection was carried out at 277 nm with a run time of 20 min. The separation was good between lamivudine and salicylic acid (Fig. 1).

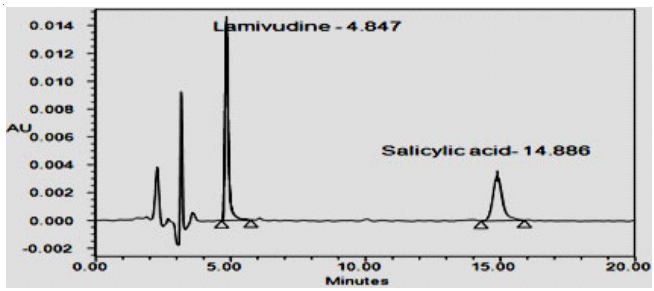

Fig. 1. HPLC chromatogram of standard

Later to decrease the run time a short UPLC method was developed and validated by converting the above HPLC method to UPLC method by using Aquity UPLC BEH phenyl 2.1 × 100 mm, 1.7 µm with gradient program shown in Table-1.

TABLE-1
GRADIENT PROGRAM

Time	Flow	A1 (%)	B1 (%)	Curve
Initial	0.5	98	2	Initial
2.2	0.5	98	2	6
3.5	0.5	85	15	6
4.0	0.5	98	2	6
5.0	0.5	98	2	6

Specificity: The specificity parameter was performed to check blank, placebo and impurity interferences with lamivudine, salicylic acid and all unknown impurities.

System suitability results (Table-2) was evaluated by % RSD, tailing factor and resolution between lamivudine and salicylic acid. The chromatograms for specificity parameter were shown from Figs. 2-12. In force degradation studies, all the generated impurities have not interfered with the lamivudine peak, salicylic acid peak and also with each other. The purity angle of lamivudine and salicylic acid is less than the purity threshold (Figs. 13 and 14). The results for forced degradation

TABLE-2
SYSTEM SUITABILITY

Component	Parameters	Results
Lamivudine	RSD (%)	0.5
	Tailing factor	1.2
Salicylic acid	RSD (%)	2.3
	Tailing factor	1.2
Resolution between lamivudine and salicylic acid		13.5

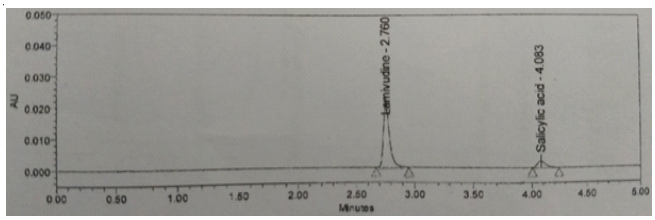

Fig. 2. Chromatogram of standard

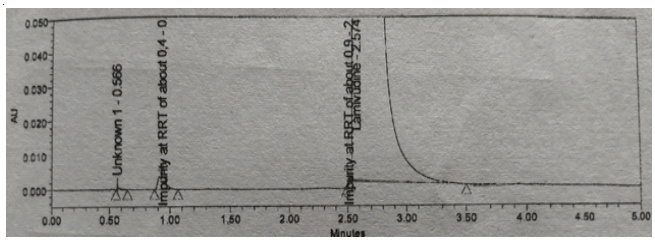

Fig. 3. chromatogram of sample

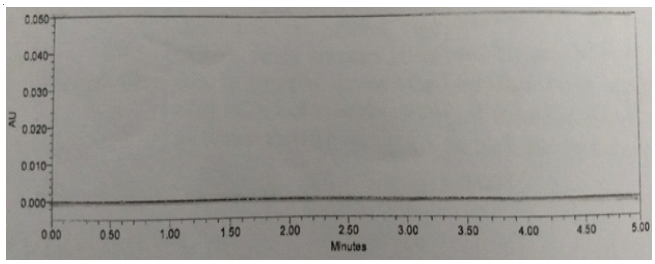

Fig. 4. Chromatogram of blank

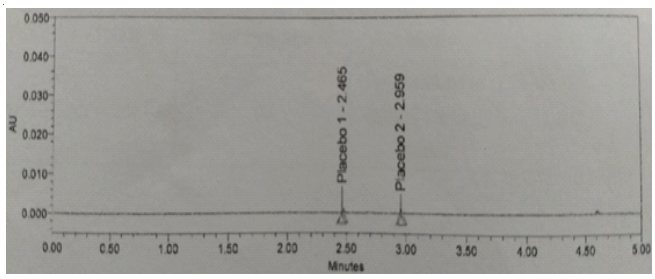

Fig. 5. Chromatogram of placebo

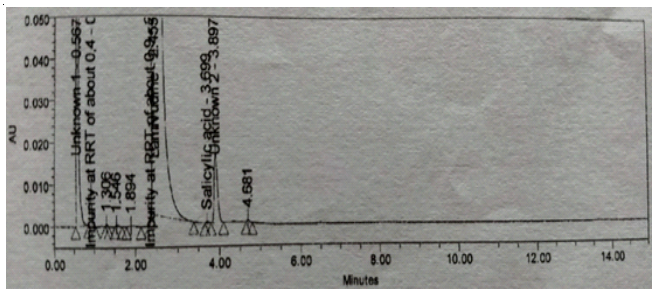

Fig. 6. Chromatogram of acid degradation sample

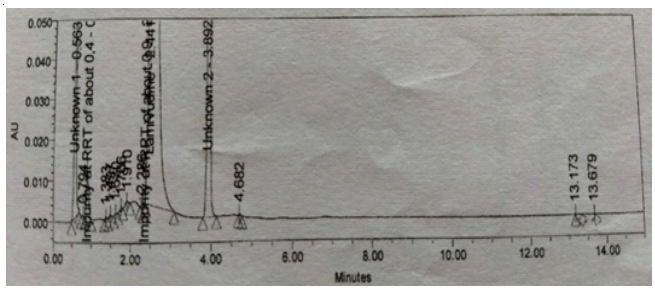

Fig. 7. Chromatogram of base degradation sample

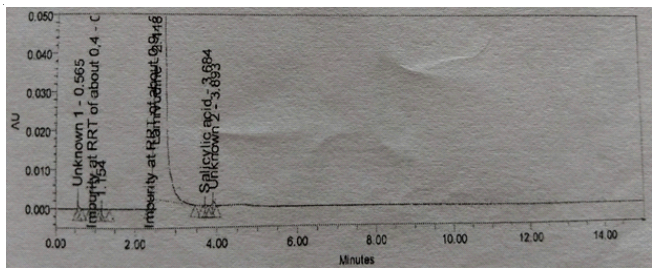

Fig. 8. Chromatogram of water degradation sample

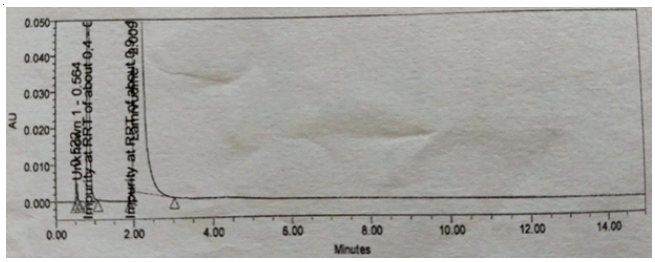

Fig. 9. Chromatogram of peroxide degradation sample

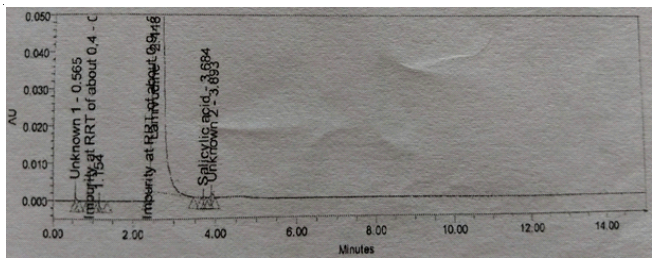

Fig. 10. Chromatogram of UV degradation sample

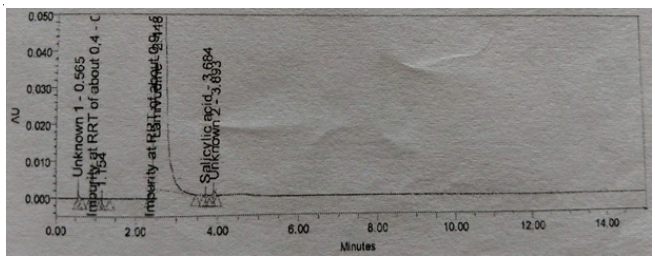

Fig. 11. Chromatogram of humidity degradation sample

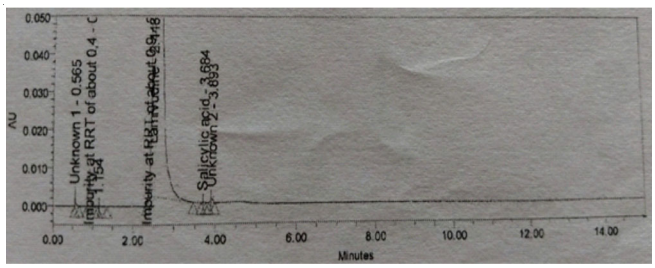

Fig. 12. Chromatogram of thermal degradation sample

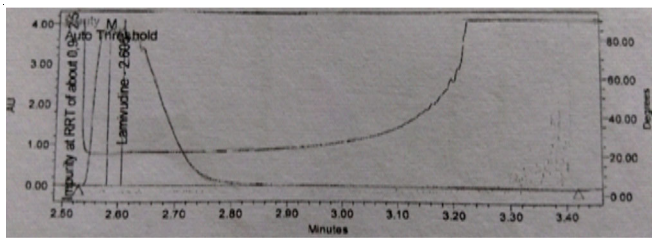

Fig. 13. Peak purity plot of lamivudine in spiked sample

TABLE-3
DEGRADATION STUDIES

Degradation condition	Salicylic acid		Lamivudine		Total impurities (% w/w)	Net degradation (%)	Mass balance (%)
	Purity angle	Purity threshold	Purity angle	Purity threshold			
Unstressed sample	ND	ND	0.716	28.564	0.07	NA	NA
1N HCl/80 °C For 4 h	0.837	21.154	0.816	32.332	5.67	5.60	98.5
0.1N NaOH/80 °C For 2 h	ND	ND	0.915	31.824	6.06	5.99	99.1
Water/80 °C For 24 h	12.653	36.476	1.348	35.878	2.27	2.20	99.5
UV light at 254 nm for 168 h	ND	ND	1.454	28.939	0.08	0.01	99.8
Humidity 90 % RH at 25 °C for 168 h	11.454	46.808	0.751	33.488	0.08	0.01	99.7
0.1 % H_2O_2 for 2 h on bench top	ND	ND	0.418	5.702	3.54	5.99	98.9
Thermal 105 °C for 48 h	ND	ND	0.290	4.573	0.09	0.09	99.9

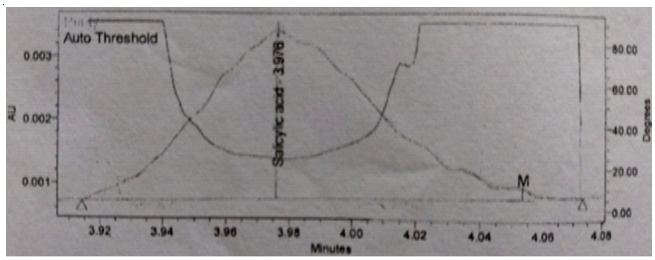

Fig. 14. Peak purity plot of salicylic acid in spiked sample

studies are given in Table-3. Blank, placebo and impurities have not shown any interference with lamivudine, salicylic acid and all the unknown impurities. Hence, the above method is specific.

Limit of detection (LOD) and limit of quantification (LOQ): The LOD and LOQ are expressed as a known concentration of lamivudine and salicylic acid at a specified signal to noise ratio, usually for LOQ 10:1, for LOD 3:1 can be quantitated or detected under the stated UPLC method. The LOQ S/N for lamivudine was 10.5 at concentration 0.1203 ppm, LOD S/N for lamivudine was 3.5 at concentration 0.038 ppm and LOQ S/N for salicylic acid was 10.8 at concentration 0.208 ppm, LOD S/N for salicylic acid was 3.1 at concentration 0.067 ppm, respectively.

Linearity and range: Linearity was conducted by preparing the six levels of linearity solutions for lamivudine and salicylic acid (n = 3) from 0.1203, 1.5037, 2.2555, 3.0073, 3.7591, 4.5110 ppm levels for lamivudine and 0.208, 1.129, 1.693, 2.258, 2.822 and 3.387ppm levels for salicylic acid, respectively.

The plot was linear over the concentration range for lamivudine and salicylic acid 0.1203-4.5110 ppm and 0.208-3.387 ppm, respectively (Figs. 15 and 16). The regression

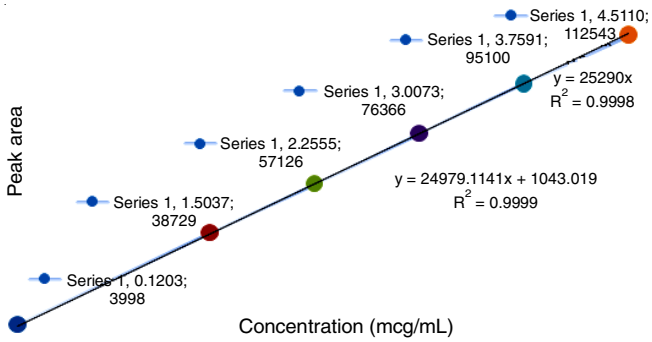

Fig. 15. Linearity graph for lamivudine

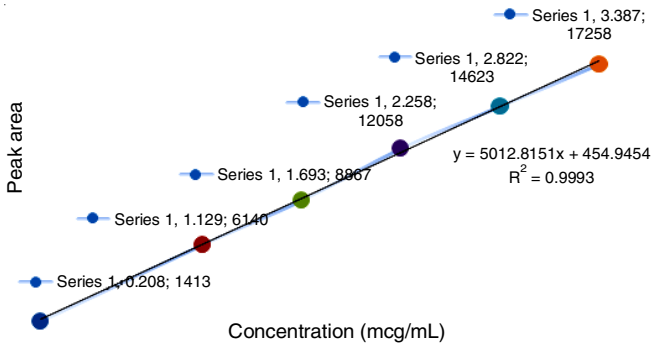

Fig. 16. Linearity graph for salicylic acid

equation was calculated by the least-square method for Lamivudine, y = 24,979.11x + 1043.01; correlation coefficient 0.9999 and for salicylic acid, y = 5,012.82x + 454.95; correlation coefficient 0.9996. The linearity data is given in Table-4.

Precision: Method precision was evaluated by injecting spiked known impurity salicylic acid solution for 6 times (n =

TABLE-4
LINEARITY OF LAMIVUDINE AND SALICYLIC ACID

Linearity levels	Lamivudine		Salicylic acid	
	Concentration (ppm)	Area response	Concentration (ppm)	Area response
LOQ	0.1203	3998	0.208	1413
50 %	1.5037	38729	1.129	6140
75 %	2.2555	57126	1.693	8867
100 %	3.0073	76366	2.258	12058
125 %	3.7591	95100	2.822	14623
150 %	4.5110	113545	3.387	17258
Correlation coefficient (R)	0.9999		0.9996	
Y-intercept at 100 % Level	1.40		3.80	
Slope	24979.11		5012.82	
Residual sum of squares	182671.34		125450.10	

TABLE-7
ROBUSTNESS STUDIES

Parameter	Variation	Salicylic acid			Total impurities (% w/w)	Impurity G and H	
		RT	RRT	% w/w		RT	RRT
Original conditions	None	3.91	1.52	0.15	0.33	0.91	0.35
Wavelength (nm)	275	3.91	1.53	0.13	0.32	0.91	0.35
	279	3.91	1.55	0.13	0.31	0.91	0.36
pH	3.6	4.03	1.91	0.15	0.35	0.83	0.39
	4.0	3.54	1.28	0.14	0.29	0.94	0.34
Flow rate (mL/min)	0.490	3.870	1.54	0.16	0.35	0.90	0.36
	0.510	3.73	1.56	0.12	0.31	0.87	0.36
Column oven temperature (°C)	38	3.91	1.55	0.16	0.35	0.90	0.36
	42	3.67	1.56	0.12	0.31	0.87	0.37

3). The results of precision and intermediate precision are shown in Table-5. % RSD values for both precision study and intermediate precision study are 2.9 and 0.0 for salicylic acid. The data demonstrated that the values are met the acceptance criteria.

TABLE-5
METHOD PRECISION/INTERMEDIATE PRECISION OF SPIKED KNOWN IMPURITY SALICYLIC ACID (% w/w)

Spiked sample	Method precision	Intermediate precision
1	0.14	0.14
2	0.14	0.14
3	0.15	0.14
4	0.14	0.14
5	0.14	0.14
6	0.14	0.14
Mean	0.14	0.14
RSD (%)	2.9	0.0
Overall mean (n = 12)	0.14	
Overall RSD (%) (n = 12)	2.05	

Accuracy: The accuracy was evaluated by measurement (n = 3) applying the method to the sample spiking with known amounts of salicylic acid corresponding to LOQ, 50, 100 and 150 % of specification. The recovery data of salicylic acid obtained from a study of formulation from LOQ level to 150 %. The test sample were prepared at each % level and tested against standard according to the description of the method. The total average recovery is 97.8 % with 3.48 % RSD (Table-6). This quantitative recovery of the impurity indicates that there was no interference from excipients present in the formulation and the method is accurate.

TABLE-6
ACCURACY OF SALICYLIC ACID

Level	Mean recovery (%)	Overall mean recovery (%)	Overall RSD (%)
LOQ	97.5	97.8	3.48
50 %	102.2		
100 %	93.9		
150 %	97.4		

Solution stability: The standard solution is found to be stable up to 40 h at laboratory temperature. Sample solution were found to be stable up to 16 h at laboratory temperature with the difference in % individual known impurity should not more than ± 0.04 absolute and total impurities should not more than ± 0.1 absolute from initial value.

Mobile phase stability: The mobile phase was found to be stable for 6 days with the appearance still remaining clear and no signs of turbidity present.

Robustness: Robustness of the method was assessed by varying the instrumental conditions such as flow rate (± 0.01 mL), column temperature (± 2 ºC), mobile phase pH (± 2 units) and wavelength (± 2 nm). The deliberate changes in the method have no significant changes in retention time, relative retention time and no distorted chromatography was observed for lamivudine and salicylic acid. This indicates that the method was robust. Results for robustness studies are given in Table-7.

Conclusion

A validated RP-UPLC method was specific and reproducible method for analyzing the related substances in 300 mg lamivudine tablets. The degrading impurities generated from force degradation studies are well separated and showed the satisfactory data as per ICH guidelines requirements. Hence, the proposed method can be used for routine and stability analysis.

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