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HPLC method development and validation for simultaneous determination of lamivudine and tenofovir in tablet dosage

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ABSTRACT

A simple, reverse-phase high performance liquid chromatographic method has been developed and validated for the simultaneous estimation of Lamivudine and Tenofovir disoproxil fumarate in pure and tablet dosage form. The chromatographic Separation was carried out using on Hypersil BDS C18 column (250 x4.6mm id, 5 μ particle size) in isocratic mode with flow rate of 1mLmin⁻¹ and the detection was carried out by photo diode array detector at 260nm. The mobile phase consists of 0.01N Sodium Dihydrogen Ortho Phosphate Buffer (pH 4.5 \pm 0.5) and acetonitrile in the ratio of 50:50 (v/v). The retention time for Lamivudine and Tenofovir was found to be 2.18mins and 3.64mins respectively. The linearity was obtained in the concentration range of 75- 450 μ g/mL for both the Lamivudine and Tenofovir Disoproxil Fumarate. The LOD was found 0.92 μ g/mL and 2.37 μ g/mL for Lamivudine and Tenofovir respectively, whereas LOQ was found to be 2.79 μ g/mL for Lamivudine and 7.17 μ g/mL for Tenofovir. The percentage recovery was found to be 99.84 – 100.54 for Lamivudine and 99.72 – 100.82 for Tenofovir respectively. The method was statistically validated as per ICH guidelines and %RSD was found to be less than 2 indicating high degree of accuracy and precision of the proposed HPLC method.

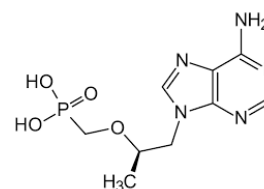
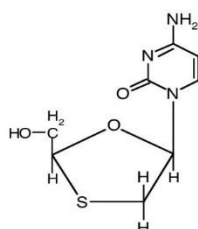
KEY WORDS: Lamivudine, Tenofovir, Method development, RP-HPLC, Validation.

INTRODUCTION

Lamivudine (LMV) (Fig.1) chemically it is (2R-cis)-4-amino-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-2(1H) pyrimidinone, is a synthetic nucleoside analogue with potent activity against human immune deficiency (HIV) and hepatitis B viruses (HBV) through inhibition

of reverse transcriptase activity. Tenofovir (TNF) (Fig.1) chemically, it is 9-[(R)-2-[[bis [(Isopropoxycarbonyl) oxy] methoxy] phosphinyl] methoxy] propyl] adenine fumarate (1:1) is an antiretroviral agent belonging to the class of nucleotide reverse transcriptase inhibitor. [1]

Fig.1: Structures of Lamivudine and Tenofovir



Both Lamivudine and Tenofovir active substances interfere with enzymes used by HIV and thereby blocking the reproduction of the virus. Antiviral medicines used for HIV infection are known as antiretrovirals. To prevent the virus from becoming resistant, Lamivudine and Tenofovir Disoproxil Fumarate tablets should always be given in combination with other antiretrovirals when treating HIV. Literature survey reveals different methods for their analysis in formulations [2-10]. Our present plan is to develop a new, simple, precise RP-HPLC method and validated the same as per ICH norms [11-12].

EXPERIMENTAL

MATERIALS AND METHODS

APPARATUS AND SOFTWARE

Chromatography measurements were achieved using waters alliance equipped with a waters 2695 separations module, and waters 2996 PDA detector. The system was controlled through a system controller. The data acquisition was performed by the empower-2 software. The mobile phase was degassed using power sonic sonicator (Hwashin technology, seoul, korea). Absorbance spectra were recorded by using an UV visible spectrophotometer (Model UV-1800, Japan) employing quartz cell of 1.00 cm of path length (Fig 2). The rest of the calculations for the analysis were performed by use of micro soft excel 2007 software (Microsoft, USA).

CHEMICALS AND REAGENTS

Working standard of LMV and TNF were donated by Pharma Analytical labs, Puducherry. Tablet dosage form was procured from local market with the brand name Lamivir HBV containing 300mg of Lamivudine by cipla and 300mg of Tenofovir with the brand name Viread by Gilead Sciences. HPLC Grade Water and Acetonitrile were of HPLC grade supplied by Merck, sodium dihydrogen ortho phosphate and ortho

phosphoric acid of AR Grade supplied by Rankem, were used during the study.

BUFFER PREPARATION

Accurately weighed 1.41 gm of sodium dihydrogen ortho phosphate in a 1000mL of volumetric flask, add about 900mL of HPLC grade Water and sonicate for few minute to degas and finally make up the volume with HPLC water and then the pH was adjusted to 4.0 with dil.ortho phosphoric acid.

STANDARD PREPARATION

Accurately Weighed and transferred 30mg of Lamivudine and Tenofovir working Standards into a 10mL⁻¹ clean dry volumetric flask separately, add 3/4th amount of diluent, sonicated for 30 minutes and make up to the final volume with diluents. From the above stock solution, 1 mL⁻¹ was pipette out in to a 10 mL⁻¹ volumetric flask and then make up to the final volume with diluents.

SAMPLE PREPARATION

Prepare standard stock solutions of both the drugs as given above. Locally available marketed formulations contain 300mg of Lamivudine and 300mg of Tenofovir Disoproxil Fumarate was taken. From the formulation prepare the sample stock solution of 1.5mgmL⁻¹. Further 0.5 mL was taken from the sample stock solution into a 10mL volumetric flask and made up with diluents.

CHROMATOGRAPHIC CONDITIONS

Chromatographic separations were achieved using C18 analytical Column, Hypersil BDS (250x4.6mm, 5μ). The mobile phase consists of Phosphate Buffer (pH4.0±0.5) and Acetonitrile taken in the ratio 50:50. The injection volume of sample was 10μL. The mobile phase was degassed by ultra sonication before pumping into HPLC system. The flow rate was set at 1.0mLmin⁻¹ and the wavelength 260nm was selected for detection and the corresponding UV-overlay spectrum was shown in the fig.2. The column temperature was maintained at 30°C.

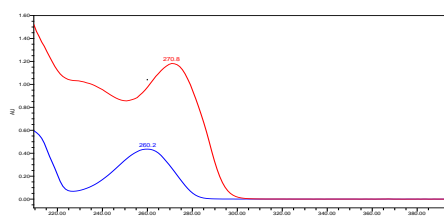


Fig.2: UV- Overlay Spectrum of LMV and TNF

METHOD DEVELOPMENT

Several trials were performed for the method development and the best peak with least fronting factor was found to be the seventh trail with RT for 2.18

Lamivudine and 3.63 for Tenofovir. Table.No:1 shows the best optimum separation condition and the corresponding chromatograms shown in Fig. 3.

Table 1: Optimum chromatographic separation conditions

Sl. No.	Chromatographic conditions	
1	Mode of separation	Isocratic elution
2	Mobile phase	Buffer (pH 4.0±0.5) and Acetonitrile (50:50)
3	Column	Hypersil BDS 250mm x 4.6 mm, 5 μ .
4	Flow rate	1.0 ml/ min
5	Detection wavelength	260 nm
6	Injection volume	10 μ l
7	Column oven temperature	30 °C
8	Run time	7 min

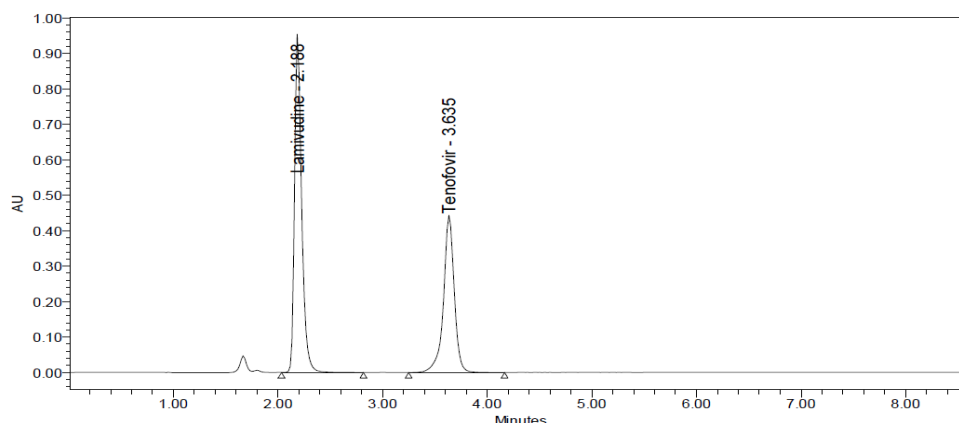


Fig. 3: Chromatogram corresponding to synthetic mixtures of LMV and TNF

METHOD VALIDATION

Validation of final assay condition was carried out by analyzing the synthetic mixtures of two analyzed drugs based on the principles of validation described in the ICH guidelines “Text on Validation of Analytical Procedures and Q2B”, Validation of Analytical Procedures: Methodology”. Key analytical parameters, including specificity, accuracy, precision, linearity, detection limit and quantitation limit were evaluated [12].

SYSTEM SUITABILITY

System suitability tests are an integral part of liquid chromatographic method. These tests are used to verify that the chromatographic system is adequate for the intended analysis. The system suitability test was performed for theoretical plates (not less than 2000) and tailing factor (less than 2), the results were within the acceptable limit are summarized in Table 2.

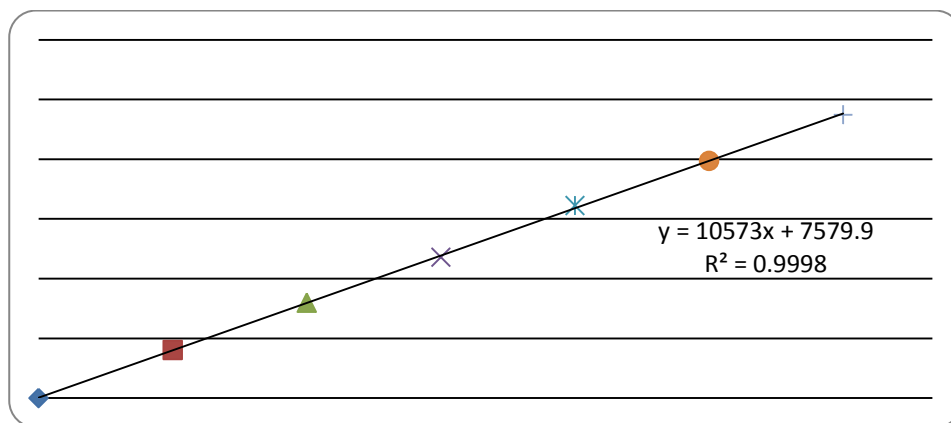
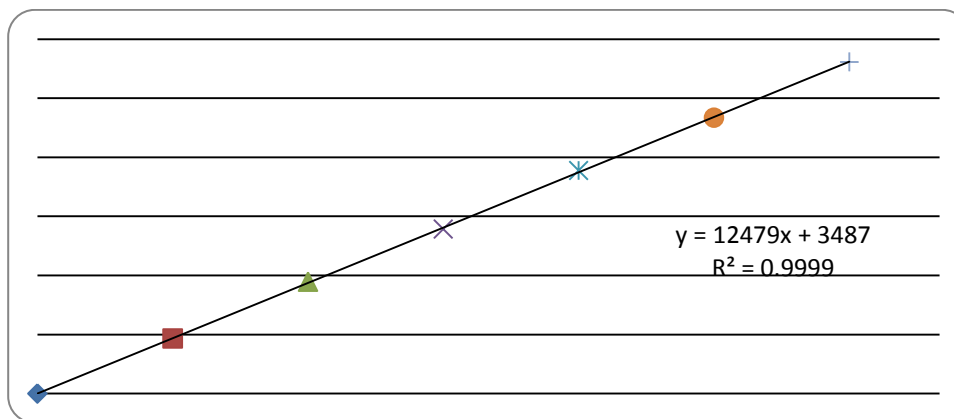
Table 2: System Suitability data of LMV and TNF

Sl. No	Parameters	Lamivudine	Tenofovir	Acceptable criteria
1	Tailing Factor	1.30	0.89	Less than 2
2	Theoretical plates	5200	6834	Not less than 2000
3	Retention Time	2.183	3.643	Less than 10
4	Average Area	4497652	3171539	-
% RSD		0.37	0.63	Less than 2 %

LINEARITY AND CALIBRATION CURVE

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Linearity experiments were performed for both the components and the response

were found to be linear in the range of 75-450 ppm for Both LMV and TNF. The regression equation of calibration curves were $y=10573x+7579.9$ for LMV and $y=12479x+3487$ for TNF respectively and the corresponding calibration graphs were shown in fig.4.

**Fig.4: Linearity graph of LMV****Fig.4: Linearity graph of TNF**

SPECIFICITY

Specificity is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. In this study, the chromatograms

of standard and sample are identical with nearly same retention time. No interference due to placebo, mobile phase and sample at the retention time of analyte which shows that the method was specific [Fig.5].

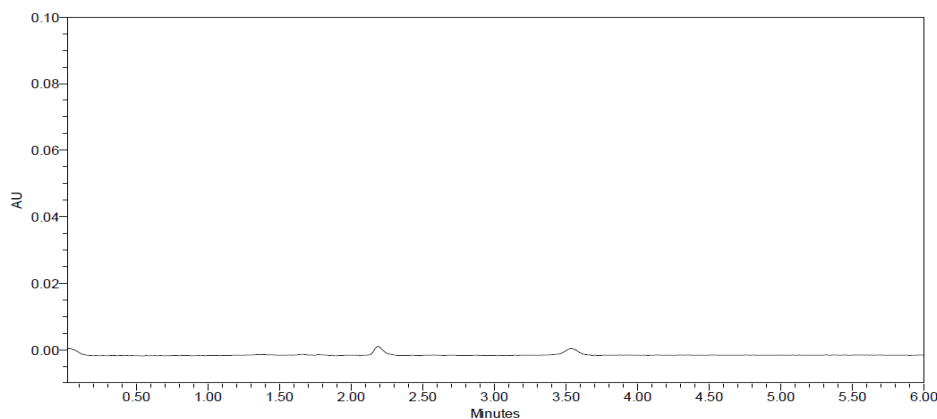


Fig.5: Chromatogram showing specificity-placebo

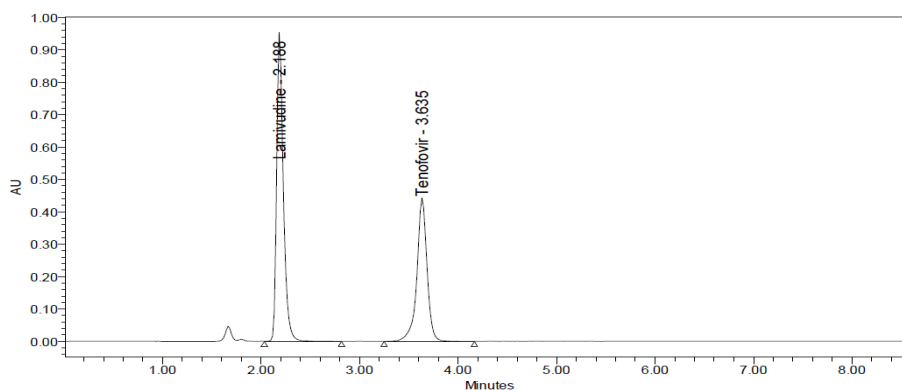


Fig.5: Chromatogram showing specificity-standard

PRECISION

Precision is the measure of the degree of repeatability of an analytical method under normal operation, and is normally expressed as the percent coefficient of variation (%CV). Precision may be performed at two

different levels: intra-day and inter-day precisions. Precision data representing the % CV values for both intra-day and inter-days were less than 2.0%, this indicates that the proposed method is precise [Table.3].

Table 3: Inter-day and Intra-day Precision data of LMV and TNF

S.No	Parameters	Inter day		Intraday	
		LMV	TNF	LMV	TNF
1	Retention time	2.188	3.607	2.183	3.585
2	Avg. peak area	4480415	3151162	4490319	3163663
3	SD	13158	11343.9	4984.2	17908.5
4	%RSD	0.3	0.4	0.11	0.56

ACCURACY

The accuracy of the method was determined by recovery experiment. Accuracy was determined by performing the recovery experiment at 50%, 100% and 150% of the target analyte concentration in the commercial

formulation. The % recovery of analytes at each concentration ($n = 3$) and mean % recovery ($n = 9$) for each analyte were determined. In this respect, the mean recovery ($n = 3$) at each concentration should be within the acceptance criteria of bias, $\pm 2\%$ [Table 4].

Table 4: Showing accuracy data of LMV and TNF
Accuracy for LMV

Sl.No	Conc.	Peak	Area	Amount added (mg)	Amount found (mg)	% recovery	Mean Recovery (%)	SD	%RSD
1	50%	2263376		2.5	2.51	100.54	100.3	0.2	0.2
2	100%	4494729		5	4.99	99.84		0.6	0.6
3	150%	6843189		7.5	7.54	100.54		0.37	0.36

Accuracy for TNF

Sl.No.	Conc.	Peak	Area	Amount added (mg)	Amount found (mg)	% recovery	Mean Recovery (%)	SD	%RSD
1	50%	1600504		2.5	2.52	100.82	100.19	0.2	0.2
2	100%	3167098		5	4.98	99.7		0.09	0.09
3	150%	4764721		7.5	7.5	100.06		0.05	0.05

LIMIT OF DETECTION

Limit of Detection (LOD) is the lowest concentration of an analyte in a sample that can be detected but not quantified. LOD is expressed as a concentration at a specified signal to noise ratio. The LOD will not only depend on the procedure of analysis but also on the type of instrument. In chromatography, detection limit is the injected amount that results in a peak with a height at least twice or thrice as high as baseline noise level. The LOD for LMV and TNF standard solutions were found to be $0.92\mu\text{g.mL}^{-1}$ and $2.37\mu\text{g.mL}^{-1}$ respectively.

LIMIT OF QUANTIFICATION

Limit of Quantification (LOQ) is defined as lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy and reliability by a given method under stated experimental conditions. LOQ is expressed as a concentration at a specified signal to noise ratio. In chromatography, limit of quantification is the injected amount that results in a peak with a height, ten times as high as base line noise level. The LOQ for Moxifloxacin and Bromofenac

standard solutions were found to be $2.79\mu\text{g.mL}^{-1}$ and $7.17\mu\text{g.mL}^{-1}$ respectively.

ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. In the present work Robustness of the method was determined by making slight changes in the composition of mobile phase $\pm 2\%$, flow rate by $\pm 0.2\text{ mL}^{-1}$ and temperature by $\pm 2^\circ\text{C}$. It was observed that there were no marked changes in the retention time and area of the chromatograms and the % RSD was less than 2% , which demonstrated that the RP-HPLC method developed was robust.

RESULTS AND DISCUSSIONS

The last step of the present study was to check method's validation for specificity, linearity, accuracy, intra/inter-day precision, and robustness. All the validation parameters were found to be well within the acceptance criteria, Table 5. The system suitability parameters also

reveals that the values within the specified limit for the proposed method. Theoretical plates and tailing factor for LMV and TNF were found to be NLT 2000 and NMT 2.0 respectively. The developed HPLC method was specific in relation to the placebo. All placebo chromatograms showed no interference peaks. An excellent linearity was established at six levels in the range of 75-450 $\mu\text{g mL}^{-1}$ for both LMV & TNF with R^2 of more than 0.999 for both the analytes. The LOD and LOQ were estimated as 0.92 and 2.79 $\mu\text{g mL}^{-1}$ for LMV, 2.37 and 7.17 $\mu\text{g mL}^{-1}$ for TNF respectively. Accuracy ($n = 9$), assessed by spike recovery, were found to be 100.03 and 100.19 % for LMV and TNF respectively, which were within acceptable ranges of $100 \pm 2\%$. The intra and inter-assay precision ($n = 6$) was confirmed

since, the %C.V. were well within the acceptable limit of ≤ 2 and ≤ 3 , respectively. Robustness study reveals that small changes did not alter the retention times, retention factor and resolution more than 2% and therefore it would be concluded that the method conditions are robust. From the above results, it was concluded that, the method was accurate, reproducible, repeatable, linear, precise and selective, proving reliability of method. The run time is relatively short i.e., 4.0 min which enables rapid quantitation of many samples in routine quality control analysis. These results show the method could find practical application as a quality control tool for estimation of the LMV and TNF in quality control laboratories.

Table 5: VALIDATION PARAMETERS FOR LMV AND TNF

Sl. No	Requirement		Results for LMV	Results for TNF	Acceptance criteria
1	Linearity	Correlation coefficient	0.999	0.999	NLT-0.999
2	Specificity	Inference	Specific	specific	No interference
3	Accuracy & % Recovery	50 %	100.54%	100.82%	98-102%
		100 %	99.84%	99.7%	
		150 %	100.54%	100.06%	
4	LOD	$\mu\text{g. mL}^{-1}$	0.92	2.37	-
5	LOQ	$\mu\text{g. mL}^{-1}$	2.79	7.17	-
6	Method precision	Intraday % RSD	0.11	0.56	NMT-2%
		Inter day	0.3	0.4	
7	Robustness	Mobile Phase	52:48	1.1	NMT-2%
			48:52	1.8	
		Temperature	(29°C)	1.2	
			(31°C)	1.3	
		Flow rate	0.8 mL min ⁻¹	0.8	
			1.2 mL min ⁻¹	1.7	2.0

APPLICATION OF THE METHOD

Commercial products Lamivir HBV (300mg) (LMV) and Viread (300mg) (TNF) with the brand name were

assayed by the proposed HPLC method. 5 tablets were weighed and calculate the average weight of each tablet then the weight equivalent to 5 tablets was transferred

into a 250 ml volumetric flask, 200ml of diluent added and sonicated for 30 min, further the volume made up with diluent and filtered. From the filtered solution 0.5ml was pipetted out into a 10 ml volumetric flask and made upto 10ml with mobile phase. The solution was injected through 10 μ l loop system and chromatograms were obtained at flow rate of 1.0 mLmin⁻¹. The concentrations of both solutions were measured at

260nm and representative chromatograms are presented in (Fig.6). The percentage of drug content was calculated for MOX and BRO, which showed a Good agreement between the assay results and the label claim of the product. The %C.V. for both formulations was < 2, indicating the precision of the analytical methodology.

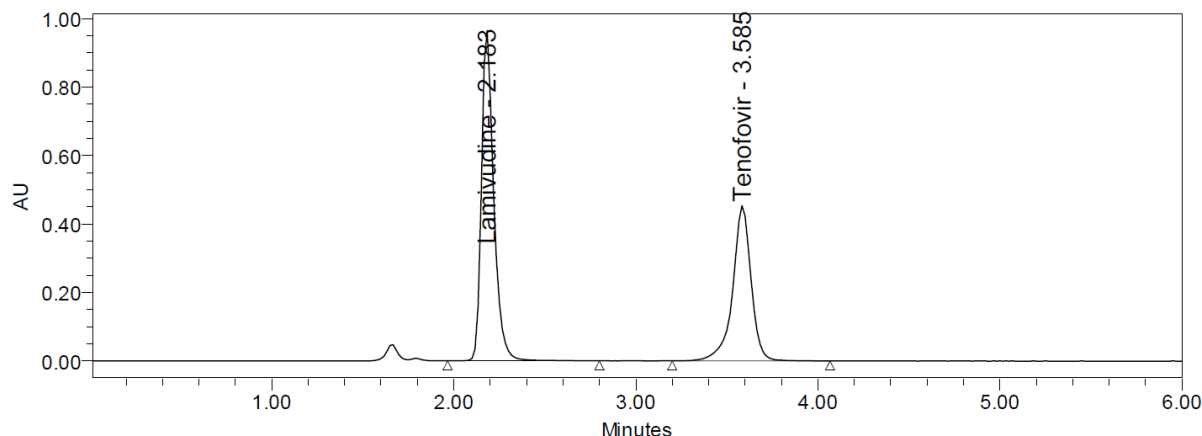


Fig.6: Assay of Lamivudine and Tenofovir

CONCLUSION

The evaluation of obtained values suggests that the proposed method is simple, fast and cost effective for the simultaneous estimation of LMV and TNF tablet dosage form. The mobile phase is simple to prepare and economical. The proposed method was validated for the various experimental parameters according to ICH guidelines. Influence of the mobile phase, column oven temperature and flow rate was evaluated for the

proposed method. The LMV and TNF analytes were well resolved and separated within 4 min. Hence this method can be applied for the simultaneous quantification of both LMV and TNF in tablet dosage form in routine analysis. This presentation in conjugation with the articles written on the subject hopefully provides a reasonably clear depiction of the current state of the art in development and validation of RP-HPLC method for the estimation of LMV and TNF.

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