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Research Article

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Method Development and Validation for Simultaneous Estimation of Lamivudine and Zidovudine by RP-HPLC

Vatchavai Bhaskara Raju*, Bonthu Mohan Gandhi, Kamatham Sumanth, Kolli Srinivas, Boppana Sowmya

Sri Vasavi Institute of Pharmaceutical Sciences, Pedatadepalli, Tadepalligudem-534101, Andhra Pradesh

Abstract A new, accurate, rapid and effective isocratic reverse-phase high performance liquid chromatographic (RP-HPLC) method was developed, optimized and validated for the estimation of Lamivudine (LAM) and Zidovudine (ZID) in bulk and pharmaceutical dosage forms. The drugs were estimated using Shiseido C18 (250 mm x 4.6 mm, 5 µm particle size) column. A mobile phase composed of phosphate buffer (pH 3) and ACN in the ratio of 70:30, v/v at a flow rate of 1.0 ml/min was used for the separation. Detection was carried out at 228 nm. The linearity range used was 7.5-22.5µg/ml for LAM and 15-45µg/ml for ZID with retention times (Rt) of 2.512 min and 3.721 min for LAM and ZID respectively. The correlation coefficient values were found to be 0.999. Precession studies showed %RSD values less than 2% for both the drugs in all the selected concentrations. The percentage recoveries of LAM and ZID were found to be 99.06% and 99.88% respectively. The assay results of LAM and ZID were within the limits of 98-102%. The limit of detection (LOD) and limit of quantification (LOQ) were 0.1ng/ml and 0.1µg/ml for LAM and 0.1µg/ml and 0.5µg/ml for ZID respectively. The method was validated as per the International Conference on Harmonization (ICH) guidelines. The developed method was successfully used for the quantitative analysis of commercially available dosage form.

Keywords Lamivudine, Zidovudine, RP- HPLC, Shiseido C18 Column, Validation

Introduction

Lamivudine (Fig. 1) is chemically 4-amino-1-[(2R, 5S)-2-(hydroxymethyl)-1,3-oxathiolan-5yl]- 1,2dihydro pyrimidin-2-one. It is a potent nucleoside analog reverse transcriptase inhibitor (nRTI). This compound belongs to pyrimidine nucleosides and analogues. These are compounds comprising a pyrimidine base attached to sugar [1].

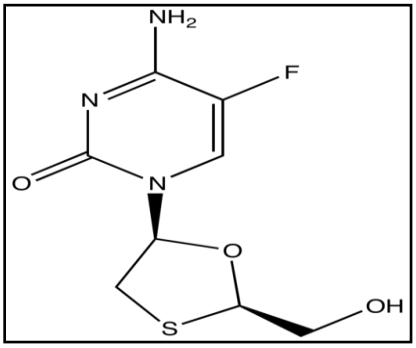

Figure 1: Chemical structure of Lamivudine

Zidovudine (Fig. 2) is 1-[(2R, 4S, 5S)-4-Azido-5-(hydroxymethyl) oxalon-2-yl]-5-methyl pyrimidine- 2, 4-dione. I t is a structural analog of thymidine, is a pro-drug that must be phosphorylated to its active 5'-triphosphate metabolite. It inhibits the activity of HIV-I reverse transcriptase (RT) via DNA chain termination after incorporation of the nucleotide analogue. It competes with the natural substrate dGTP and incorporates itself into viral DNA. It is also a weak inhibitor of cellular DNA polymer [2].

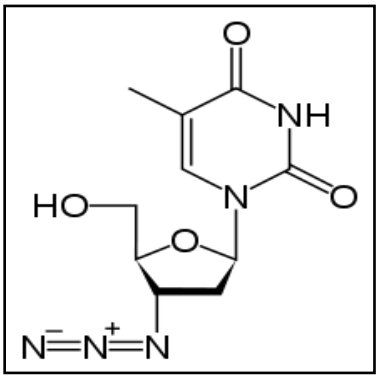

Figure 2: Chemical structure of Zidovudine

Lamivudine (LAM) and Zidovudine (ZID) in combination are available in tablet dosage forms in the ratio of 1:2. Few analytical like UV [3], Ion-exchange [4], counter current extraction [5], HPLC [6-14] methods are available for the simultaneous estimation of LAM and ZID in pharmaceutical dosage forms. We tried to develop a simple and alternative method for the estimation of LAM and ZID by RP-HPLC in pharmaceutical dosage forms. The developed method was optimized and validated as per the International Conference on Harmonization (ICH) guidelines [15].

Materials and Methods

Instrumentation

To develop a high pressure liquid chromatographic method for simultaneous estimation of LAM and ZID using Shimadzu HPLC system, Shiesiedo C18 column (250 mm x 4.6 mm, 5µ) was used. The instrument is equipped with SPD 20A UV, VIS dual absorbance detector. A 20 µL rheodyne injector port was used for injecting the samples. Data was analyzed by using lab solutions software.

Chemicals and Solvents

The working standard of LAM and ZID were procured from Yarrow chemicals, Mumbai. The market formulation Trizivir tablets (Lamivudine (150mg) and Zidovudine (300mg)) were procured from local market. Methanol and Acetonitrile of HPLC grade was supplied by Merck Limited, Mumbai. Water HPLC grade was supplied by Thermo fisher scientific India Pvt ltd, Mumbai.

Selection of detection wavelength for LAM and ZID:

10 µg/ml solutions of LAM and ZID were prepared using methanol as solvent. This solution was scanned in the UV region of 200 - 400 nm and the UV spectrum was recorded. From the spectra, detection wavelength of 228 was selected.

Optimized chromatographic conditions

The following chromatographic conditions were selected for the estimation of selected drugs in the bulk and marketed product.

Stationary phase	: Shiseido C_{18} (250 x 4.6 mm, 5µ)
Mobile Phase	: Acetonitrile: phosphate buffer (pH 3.0)
Mobile phase ratio	: 30:70
Flow rate	: 1.0 ml/min
Sample volume	: 20 µl

Detection : 228nm
Diluent : Acetonitrile: Buffer (30:70)

Preparation of standard and sample solutions

a. Standard stock solution of LAM and ZID

10 mg of LAM and ZID working standards were accurately weighed and transferred into two 10 ml volumetric flasks and dissolved in methanol and water (50:50) solution and made up to the volume with the same solvent to produce a 1mg/ml of LAM and ZID respectively. The stock solutions were stored in refrigerator at -20 ± 2^0 C until analysis. The stock solutions were diluted to suitable concentrations with acetonitrile and phosphate buffer (30:70, v/v) solution to obtain calibration curve (CC) standards and quality control (QC) samples.

b. Calibration curve standards and quality control samples

Working *solutions for calibration* and controls *were prepared* from the *stock solutions by an adequate dilution using* acetonitrile and buffer (30:70) solution. Calibration standards for control samples were prepared by diluting this stock solution to obtain the concentration levels of 7.5, 11.25, 15, 18.75, 22.5µg/ml for LAM and 15, 22.5, 30, 37.5, and 45µg/ml for ZID respectively. Quality control samples were prepared as bulk, at a concentration of 7.5µg/ml (LQC), 15µg/ml (MQC) and 22.5µg/ml (HQC) for LAM and 15 µg/ml (LQC), 30µg/ml (MQC) and 45 µg/ml for ZID respectively.

Validation of HPLC method

Specificity: Known concentrations of standard drug were prepared individually and injected into HPLC. A solution containing two drugs was prepared and injected into HPLC.

Acceptance criterion: There should not be any change in the retention times of the two drugs when injected individually and in combination. A blank chromatogram should also be recorded to check the interference of mobile phase.

Sensitivity

It is expressed as limit of detection and limit of quantitation.

- **Limit of Detection:** The lowest amount of analyte in sample that can be detected, but not necessarily quantified, with acceptable precision and accuracy was determined by comparision of S/N value of standard solution with that of blank.

  * S/N Ratio should be 3: 1

- **Limit of Quantification:** The lowest amount of analyte in sample that can be quantified, with acceptable precision and accuracy was determined by comparision of S/N value of standard solution with that of blank.

  * S/N Ratio should be 10: 1

Linearity

Linearity and range of the methods were analyzed by preparing calibration curves using different concentrations of the standard solution. The calibration curve was plotted using concentration and peak area of the standard solutions. Linearity was established over the range of (7.5µg/ml to 22.5 µg/ml for LAM and 15µg/ml to 45µg/ml for ZID) using the weighted least square regression analysis.

Precision

The precision of the method was determined by analyzing three concentration levels from calibration curve standards with three replicates viz., [Quality Control samples at Low (LQC), Middle (MQC) and High (HQC) limits of quantifications].

- *Intra-run precision*

Intra-run precision was determined by calculating the percentage coefficient of variation (%CV) of the results obtained in the same run.

- *Intra-day precision*

Intra-day precision was determined by calculating the percentage coefficient of variation (%CV) of the results obtained in the same day.

- *Inter-day precision*

Inter-day precision was determined by calculating the percentage coefficient of variation (%CV) of the results obtained over at least two days.

Accuracy/Recovery

Absolute recovery of an analytical method is the measured response obtained from a certain amount of analyte added to sample and expressed as a percentage of the response obtained for the true concentration of the pure authentic standard which has not been subjected to the extraction procedure. The accuracy of the method was evaluated by determination of recovery of LAM and ZID at three levels of concentrations with three replicates. The sample solutions corresponding to LQC, MQC and HQC were analysed by adding known amount of standard drug.

Ruggedness

Ruggedness of the method was studied by changing the experimental conditions such as operators, instruments, source of reagents, solvents and column of similar type.

Robustness

Robustness of the method was studied by injecting the standard solutions with slight variations in the optimized conditions, namely, change in organic phase proportion, varying pH range ±1 and ± 0.1 ml of the flow rate.

Results and Discussion

Optimization of Chromatographic Conditions

Optimization of the chromatographic conditions are intended to take into account the various goals of method development and to weigh each goal (resolution, runtime, sensitivity, peak symmetry, etc) accurately, according to the requirement of HPLC methods being used for the estimation of drugs in marketed formulations. Reverse phase HPLC method was chosen for LAM and ZID.

The standard solutions of LAM and ZID were scanned from 200–400 nm and the UV spectra obtained were recorded. From the UV spectra, the detection wavelength selected was 228 nm.

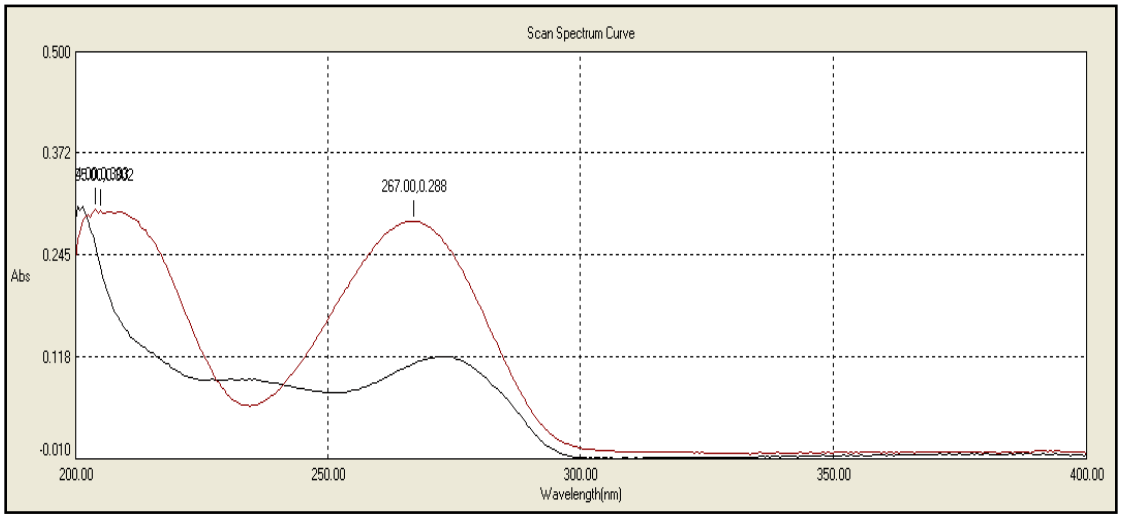

Figure 3: UV overlain spectrum of LAM & ZID

The wavelength selected gave good peak response and the typical chromatogram of standard solution of LAM and ZID are shown in Figure optimized.

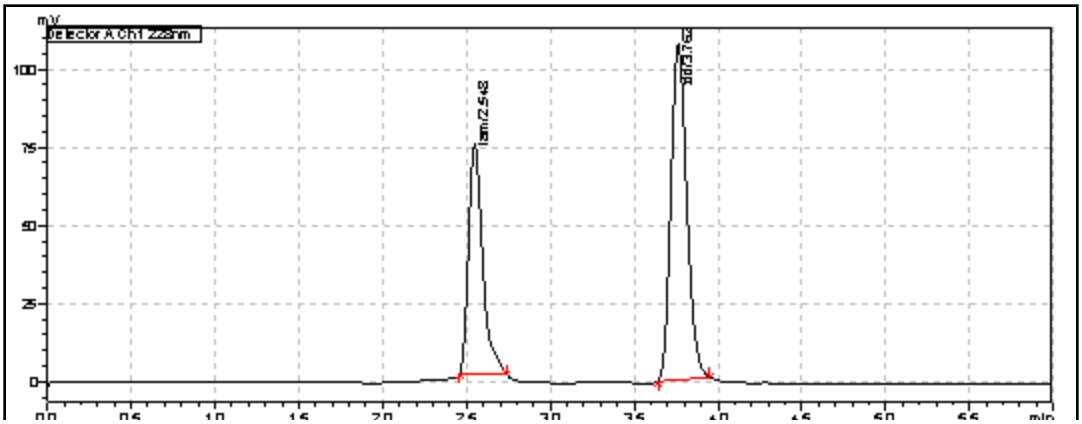

Figure 4: Chromatogram showing resolved peaks of LAM and ZID

Validation of HPLC method

Estimation of the drugs in marketed formulations was carried out using optimized chromatographic conditions. The validation parameters such as accuracy, precision (repeatability and reproducibility), linearity and range, sensitivity (limit of detection and limit of quantitation), robustness/ruggedness, stability, selectivity/specificity and system suitability were evaluated.

Assay

20µL of each standard and sample solution were injected into the chromatographic system and measured the areas of LAM and ZID peaks.

The assay results, expressed as % of the label claim, are in **table 1** .This indicates that the amount of each drug in the product meets the requirements.

Table 1: Assay of LAM and ZID

Drug	Label recovered (mg)	Amount found (mg)	Assay (%)
LAM	150.00	148.68	99.12
ZID	300.00	302.04	100.68

System Suitability

System Performance parameters of developed HPLC method were determined by injecting standard solutions. Parameters such as number of theoretical plates (N), tailing factor, resolution(R), retention time (RT) were determined. The results are shown in **table 2**, it indicates good performance.

Table 2: System suitability parameters of LAM and ZID

Drug	Theoretical plates	Tailing factor	Retention time	Resolution
LAM	3931	1.21	2.512	7.11
ZID	6636	1.201	3.721	

Specificity

Solutions of individual standard and combined samples were prepared as per the test procedure and injected into the HPLC system. There should not be any change in the retention times of the two drugs when injected individually and in combination. The chromatogram of blank can be seen in **fig.5**. The system suitability parameters are given in **table 3**.

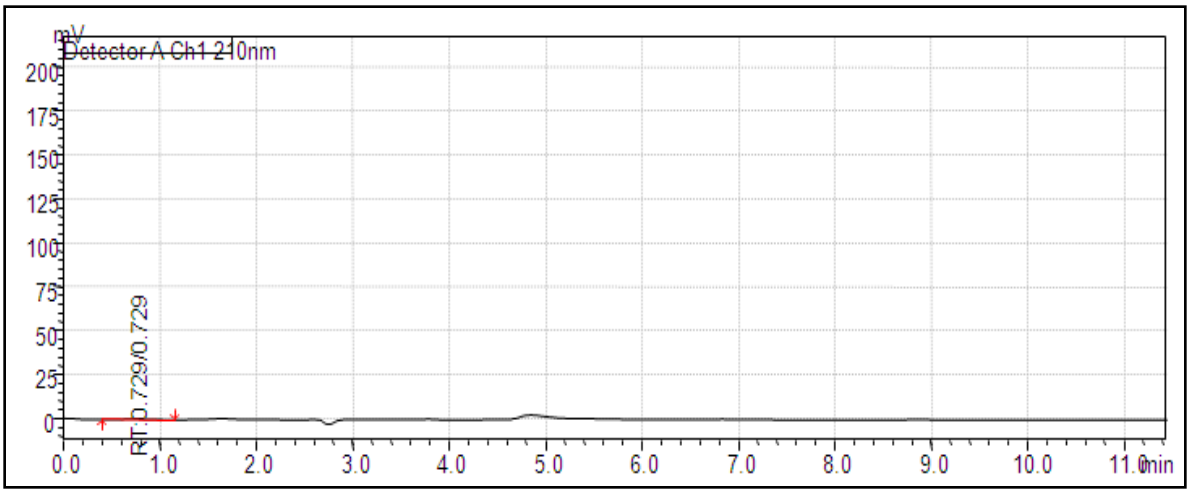

Figure 5: Chromatogram of blank

Table 3: Interference of blank and placebo

Name	Interference	Retention time (minutes)
Blank	Nil	Nil
Placebo	Nil	Nil

Sensitivity

The limit of detection for LAM and ZID were found to be 0.1ng/ml and 0.1µg/ml, limit of quantitation for LAM and ZID were found to be 0.1 µg/ml and 0.5µg/ml respectively.

Table 4: LOD & LOQ of LAM and ZID

Drug	LOD	LOQ
LAM	0.1	0.1
ZID	0.5	0.5

Linearity

The linearity was plotted within the range of 7.5, 11.25, 15, 18.75 and 22.5 µg/ml for LAM and 15, 22.5, 30, 37.5 and 45µg/ml for ZID. The results are given in **table 5**., and graphs were shown in **fig. 6 & 7** with correlation coefficient (r^2) greater than 0.99.

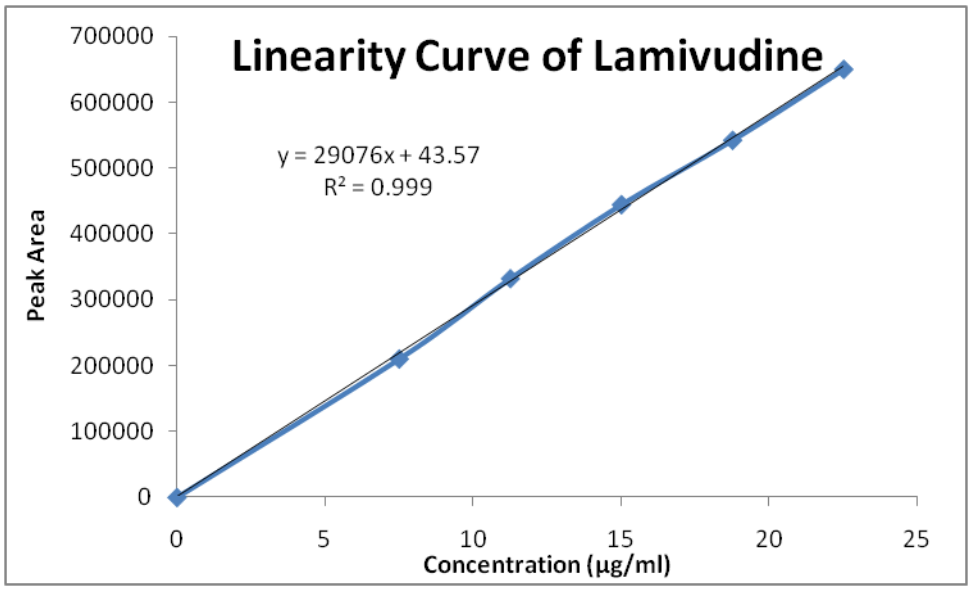

Figure 6: Linearity curve of LAM

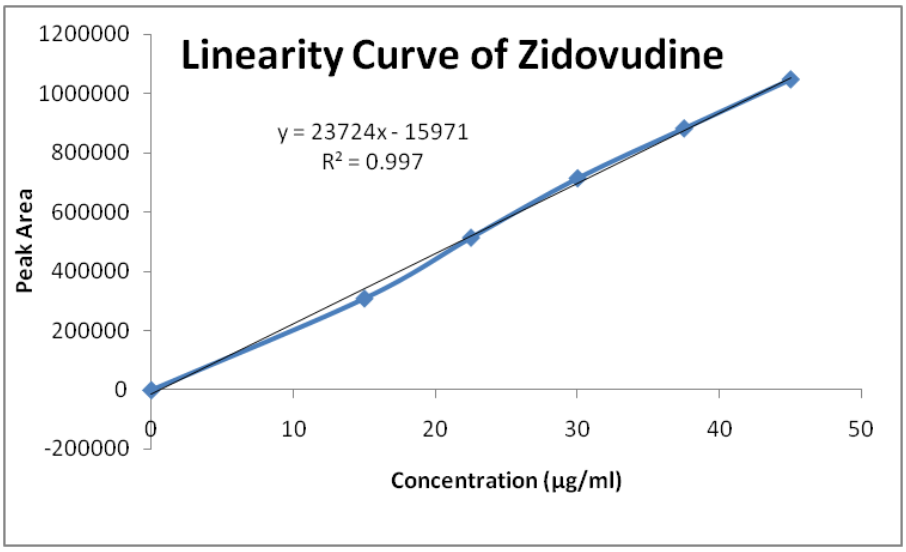

Figure 7: Linearity curve of ZID

Table 5: Linearity of LAM and ZID by RP-HPLC

S. No.	Conc. Taken in µg/ml (LAM)	Conc. Taken in µg/ml (ZID)	Peak area of LAM	Peak area of ZID
1	7.5	15	210433	308564
2	11.25	22.5	332506	513371
3	15	30	444911	713421
4	18.75	37.5	542646	881242
5	22.5	45	650446	1046235

Precision

The precision of the assay was measured by the percent coefficient of variation over the concentration range of low, middle and high quality control samples of LAM and ZID during the course of validation. The results for system precision and method (Inter day) precision were shown in **table 6 and 8**.

Table 6: System Precision Data

Lamivudine			
	LQC	**MQC**	**HQC**
	7.5 µg/ml	15 µg/ml	22.5 µg/ml
Mean	215346.667	435593.7	644391
S.D (+/-)	1411.3973	2427.092	4881.585
C.V. (%)	0.65	0.55	0.75
Zidovudine			
	15 µg/ml	30µg/ml	45 µg/ml
Mean	349419.333	70963.3	1053079
S.D (+/-)	4077.07914	6085.915	15035.52
C.V. (%)	1.16	0.85	1.42

Table 7: Method Precision (inter-day) data

Lamivudine			
	LQC	**MQC**	**HQC**
	7.5 µg/ml	15 µg/ml	22.5 µg/ml
Mean	224169.3	452344.7	629465
S.D (+/-)	1067.439	153.6273	7075.641
C.V. (%)	0.47	0.03	1.12
Zidovudine			
	15 µg/ml	30µg/ml	45 µg/ml
Mean	224169.3	452344.7	629465
S.D (+/-)	1067.439	153.6273	7075.641
C.V. (%)	0.47	0.03	1.12

Table 8: Method Precision (intra-day) data

	Lamivudine		
	LQC	**MQC**	**HQC**
	7.5 µg/ml	15 µg/ml	22.5 µg/ml
Mean	237241.667	452207	629043
S.D (+/-)	1943.82107	489.7846	8546.859
C.V. (%)	0.81	0.10	1.35
	Zidovudine		
	15 µg/ml	30µg/ml	45 µg/ml
Mean	349419.3	709637.3	1053079
S.D (+/-)	4077.079	6085.915	15035.52
C.V. (%)	1.16	0.85	1.42

Accuracy (Recovery study)

Analyte recovery is a comparison of the analytical response from an amount of analyte added to quality control samples at three concentration levels. The detailed results are presented in **table 9.** The results indicate that the recovery of LAM and ZID were consistent at all levels.

Table 9: Accuracy and % recovery of each analyte

Accuracy Level %	Mean recovery of LAM (%)	Mean recovery of ZID (%)
50	99.85	99.88
100	99.06	99.85
150	95.40	98.78

Ruggedness and robustness

The ruggedness and robustness of the methods were studied by changing the experimental conditions. No significant changes in the chromatographic parameters were observed when changing the experimental conditions (mobile phase ratio and flow rate). The %CV was found to be 0.27 for change in mobile phase ratio and 0.39 for change in flow rate. The results were shown in **table 10**.

Table 10: Robustness parameters of LAM and ZID

S. No.	Parameter	LAM			ZID		
		RT	**Area**	**Tailing Factor**	**RT**	**Area**	**Tailing Factor**
1.	Initial Sample	2.341	428411	1.213	3.418	792362	1.121
2.	Flow (+0.2ml/min)	2.121	492765	1.209	3.208	792131	1.108
3.	Flow (-0.2ml/min)	2.820	417065	1.228	3.621	792101	1.112
4.	Temp Change 10 % more	2.118	417064	1.238	3.201	753224	1.123
5.	Temp Change 10 % less	2.913	419804	1.288	3.718	753114	1.189

Conclusion

The developed RP-HPLC method allows rapid and precise determinations of LAM and ZID with an economical mobile phase and a sensitive RP-HPLC method using phosphate buffer pH (3.0±0.1) and acetonitrile (70:30, v/v) as an ideal mobile phase, since it gives a good resolution and peak shapes with perfect optimization. The flow rate at 1ml/min was optimized. The linearity and correlation coefficient (R^2) of LAM and ZID was found to be 7.5-22.5µgl/ml and 15-45µg/ml respectively and R^2 value is 0.997and 0.997 respectively. The limit of detection for LAM and ZID was found to be 0.1ng/ml & 0.1µg/ml and the limit of quantification was found to be 0.1 & 0.5µg/ml. The percentage recoveries of LAM and ZID were found to be 99.06% for LAM & 99.88% for ZID. The summary of validation parameters are tabulated in **table 11**. The isocratic elution technique developed for the determination of LAM and ZID ideally suited for rapid and routine analysis. This method shows good reproducibility of the results.

Hence, the developed method can be used for the simultaneous estimation of the selected drugs in bulk and pharmaceutical dosage forms.

Table 11: Summary of system suitability and validation parameters of LAM and ZID

Parameter	Results	
	LAM	**ZID**
Linearity range (µg/mL)	7.5-22.5	15-45
Correlation coefficient	0.997	0.997
Theoretical plates (N)	3931	6636
Tailing factor	1.213	1.128
LOD (µg/mL)	0.1	0.1
LOQ (µg/mL)	0.5	0.5

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