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Original Article

A QBD-ORIENTED RP-HPLC ANALYTICAL METHOD FOR CONCURRENT ESTIMATION OF LAMIVUDINE AND ZIDOVUDINE IN TABLET DOSAGE FORM

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ABSTRACT

Objective: This article aimed to develop a method for the simultaneous estimation of Lamivudine (3TC) and Zidovudine (AZT) by incorporating a quality-by-design (QbD) approach, which is used to develop the most accurate and precise analytical method for tablet formulation in comparison with traditional method development.

Methods: At the initial phase, researchers conducted reverse phase high performance liquid chromatography (RP-HPLC) trials as per the traditional method development protocol and the factors like mobile phase, flow rate and wavelength are taken into consideration for finalizing most suitable trial. Analytical quality by design (AQbD) approach is then implemented as per ICH Q14 guidelines for further steps in method development. The analytical method was systematically optimized by implementing QbD principles, utilizing a design of experiments (DoE) approach specifically through Box-Behnken design (BBD) by using Design Expert® 13 software. The most suitable optimized solution is used for validation. Method was validated in accordance with ICH Q2(R2) guidelines.

Results: Based on traditional method development, Methanol (MeOH): Buffer (Potassium dihydrogen phosphate buffer with sodium salt of pentane sulphonic acid) selected as a mobile phase in 40: 60 (v/v) ratio, the flow rate was selected as 1.5 ml/min and wavelength was selected at 268 nm. On the basis of these factors 17 RP-HPLC runs performed as per BBD protocol. Method was optimized by QbD and the outcomes were 50:50 (v/v) same mobile phase, 1.7 ml/min flow rate and 268 nm wavelength. Optimized parameters were validated. The accuracy for Lamivudine and Zidovudine was found to be 99.43% and 99.84% respectively. The linearity was calculated from range of 5-45 µg/ml for Lamivudine and 10-90 µg/ml for Zidovudine, correlation coefficient (R^2) was found to be 0.9998 and 0.9984 for Lamivudine and Zidovudine respectively. Recovery data lies in between 98-102%.

Conclusion: Through systematic risk assessment and Box-Behnken design, a stable method operable design region (MODR) was established. The QbD optimized method achieved a ~39.5% reduction in analysis time (from 5.953 to 3.603 min), which resulted in a ~31.5% net saving in mobile phase volume per injection (from 8.93 to 6.12 ml) despite a slight increase in flow rate. This proves the cost effectiveness of the developed method. The MODR ensures method reliability and consistency across different pharmaceutical scaffolds, providing a cost-effective, high-throughput tool for routine industrial quality control and laboratory testing.

Keywords: Lamivudine, Zidovudine, Quality by design, Box-behnken design, Anti-retroviral agents, HPLC, Simultaneous estimation, Method development, Validation, Design expert® 13, 3TC, AZT

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INTRODUCTION

When it comes to bulk medications and formulations, a pharmaceutical analysis is crucial to both quality assurance and quality control. As a consequence, analytical method development has become the basic activity of analysis. Analytical chemistry is separated into two predominant classes, a qualitative evaluation and quantitative evaluation [1].

Analytical method development and validation play important roles in the drug discovery, drug development and manufacturing of pharmaceuticals. It involves the detection of the purity and toxicity of a drug substance. A number of chromatographic parameters have been evaluated in order to optimize the methods like mobile phase, column, column temperature, wavelength and pH. High pressure liquid chromatography (HPLC) is the most widely applied analytical technique because of its high selectivity and high reliability, especially in pharmaceutical formulations [2, 3].

Regulatory authorities place utmost importance on analytical methods in manufacturing. Drug approval by regulatory authorities requires the applicant to prove control of the entire process of drug development by using validated analytical methods [4]. The main objective of the method validation process is to prove that an analytical method is acceptable for its intended purpose [5].

There are several internationally renowned organizations offering guidelines on method validation and related topics [6]. These guidelines for validation focus on selectivity, specificity, limit of detection, limit of quantitation, linearity, range accuracy, precision, recovery solution stability, ruggedness, and robustness of liquid chromatographic methods to support routine, in-process and stability analysis [7]. Analytical method validation has advanced significantly with the transformation of ICH Q2(R1) to Q2(R2) guidelines. The new guideline provides a more solid, adaptable, and scientifically sound framework for method development and control by supporting a lifecycle-based approach, incorporating risk management, and fostering alignment with analytical quality by design (AQbD) [8, 9]. Updated guidance for validating analytical methods is provided in Q2(R2), while Q14 introduces, for the first time, comprehensive guidance on the development of analytical methods [10, 11]. This research bridges the existing gap by implementing ICH Q14 and ICH Q2(R2) guidelines for quality by design (QbD) based method development and validation to get advantageous method in comparison with traditional method development approach. While methods for Lamivudine and Zidovudine already exists, they typically suffer from long run times, high solvent consumption, or a lack of robustness testing. This QbD based study addresses that method is not only faster but also statistically validated to be robust against environmental and instrumental variations, filling a critical gap in high throughput quality control for tablet dosage forms. The validated method was successfully applied to the commercially available pharmaceutical dosage form, yielding very good and reproducible results [12, 13]. The pharmaceutical industry is rapidly adopting the QbD principles for the fabrication of safe, effective and quality products. However, we are still on a journey and the process of gathering all experience and metrics required for connecting and demonstrating QbD benefits to all stakeholders [14]. The QbD is a systematic approach to development that begins with predefined objectives and emphasizes product,

process understanding and process control, based on sound science and quality risk management [15]. The QbD approach is a tool for developing economical and quality pharmaceutical products [16].

Due to QbD, increased understanding and control beyond the traditional, International Council for Harmonisation (ICH) procedure of method validation, flexibility in analysis of active pharmaceutical ingredient (API), impurities in dosage forms, stability of samples [17]. A Box-Behnken design (BBD) was employed to implement the QbD approach for method optimization. The BBD is response surface methodology that is particularly useful for establishing cause-and-effect relationships between factors and responses in experiment [18]. The reverse phase high performance liquid chromatography (RP-HPLC) based method development and its validation was depended on earlier research [19]. However, no statistical methodology such as QbD was used in earlier research [20].

Lamivudine and Zidovudine are synthetic nucleoside analogues with activity against human immunodeficiency virus (HIV). Lamivudine was initially developed for the treatment of HIV infection. The chemical name of lamivudine is (2*R*, *cis*)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1H)-pyrimidin-2-one. Lamivudine the (−) enantiomer of 2′-deoxy-3′-thiacytidine, is a nucleoside analog in which the 3′ carbon of the ribose of zalcitabine has been replaced by sulfur. The (−) enantiomer of the racemic mixture shows much less cytotoxicity than the positive enantiomer. Although generally less potent than zidovudine in inhibiting HIV-1 and 2 replication *in vitro*, lamivudine has very low cellular cytotoxicity. It is rapidly absorbed with a bioavailability of approximately 80% [21].

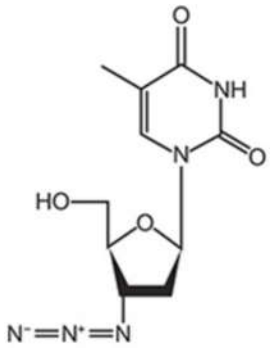

Fig. 1: Chemical structure of Zidovudine (AZT) [22]

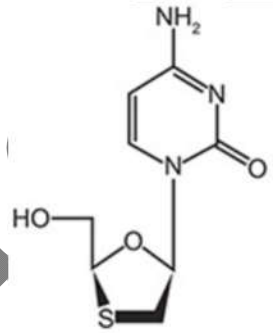

Fig. 2: Chemical structure of lamivudine (3TC) [23]

MATERIALS AND METHODS

Instrumentation

Chromatographic analysis was performed on a Shimadzu Ultra High-Performance Liquid Chromatograph (UHPLC) system equipped with an quaternary gradient, LC-20AD pump and a UV-Visible detector. Although a UHPLC system was utilized for its superior precision and pressure stability, the developed method maintains full compatibility with conventional HPLC platforms using an Inertsil ODS-3V C18 column (250 × 4.6 mm, 5 µm). A UHPLC system was utilized for the experimental phase of this study, all critical chromatographic parameters were optimized according to standard HPLC specifications. Consequently, the developed method is fully compatible with and transferable to conventional HPLC instrumentation without compromising analytical performance. Details about instrument is given in table 1.

Table 1: Details about high-performance liquid chromatograph (HPLC) instrument

Parts of instruments	Information	Model/description
System	Ultra high-performance liquid chromatograph	UHPLC
Model no.	LC 20 AD	LC 20 AD
Company	Shimadzu Japan	Shimadzu Corporation, Kyoto, Japan
Pump	Quaternary gradient	LC-20 AD
Column	C-18, 250 x 4.6 mm, 5 µm	Make: G. l Sciences Model: Inertsil ODS-3V
Detector	Dual wavelength UV-Vis	SPD-20 A

Column oven	Max. temp. 80 °C	CT0-10 AS VP
Autosampler	0.1 µl to 100 µl (20 µl injection volume is used in this study)	SIL-20AC HT
Software	Lab solution	Version DB 6.110

*UHPLC: Ultra high-performance liquid chromatograph; ODS: Octadecylsilane; SPD: Shimadzu photodiode detector or Spectro-photometric detector.

Chemicals and reagents

A gift sample of Lamivudine and Zidovudine APIs was provided by Lupin Ltd. Chhatrapati Sambhajinagar (Auranagabad). The same combination was available in tablet formulation under the brand name Duovir, which is manufactured by Cipla Ltd. and bought from the local market. Table 2 provides a list of chemicals.

Table 2: List of chemicals and reagents

S. No.	Chemical	Manufacturer	Grade
1	Methanol	Rankem labs	HPLC
2	Acetonitrile	Rankem labs	HPLC
3	Water	Molychem	HPLC
4	Ammo. dihydrogen ortho-phosphate	Ozone Int. India	AR
5	Potassium dihydrogen ortho-phosphate	Ozone Int. India	AR
6	Acetic acid	Ozone Int. India	AR
7	Ortho phosphoric acid	Ozone Int. India	AR
8	Monobasic potassium phosphate	Merck	AR
9	Sodium salt of pentane sulphonic acid	Merck	Pure

***AR: Analytical reagent (grade); HPLC: high-performance liquid chromatograph.**

Solution preparations

Preparation of standard solution

The standard solutions of Lamivudine and Zidovudine were prepared using 1000 µg/ml of Lamivudine and Zidovudine separately to get final concentration of 15 µg/ml of Lamivudine and 30 µg/ml of Zidovudine.

Preparation of tablet solution

About 20 tablets were weighed, powdered and then placed equivalent weight of powder in 100 ml volumetric flask and to that 50 ml of mobile phase was added, sonicated for 5 min, remaining volume was filled with the mobile phase and filtered through Nylon syringe filter of 0.22 µm. About 1.0 ml of filtered solution was pipetted out into a 10 ml volumetric flask and make up to the mark with diluent, mobile phase itself used as a diluent.

Label claim: 150 mg Lamivudine, 300 mg Zidovudine.

Buffer preparation

3.400 g of Potassium dihydrogen phosphate and 0.50 g of sodium salt of pentane sulphonic acid were dissolved and diluted to 500 ml with HPLC grade water. pH of this solution was adjusted to 3.0 with ortho phosphoric acid.

(The presence of Sodium salt of pentane sulphonic acid was intentional in complete study and acts as an ion-pairing reagent to enhance the retention and peak symmetry of Lamivudine, which is highly polar. It also facilitated the optimization of the selectivity factor between the two analytes, it modifies the stationary phase hydrophobicity, the ion-pairing reagent allowed for the reduction of Zidovudine's retention time without compromising the resolution between the two peaks.)

Plan of method development

Schematic representation of QbD based method development plan is given in fig. no. 3. The foundational chromatographic conditions and material selections were adapted from the peer-reviewed literature [9, 15, 17, 18, 22, 24].

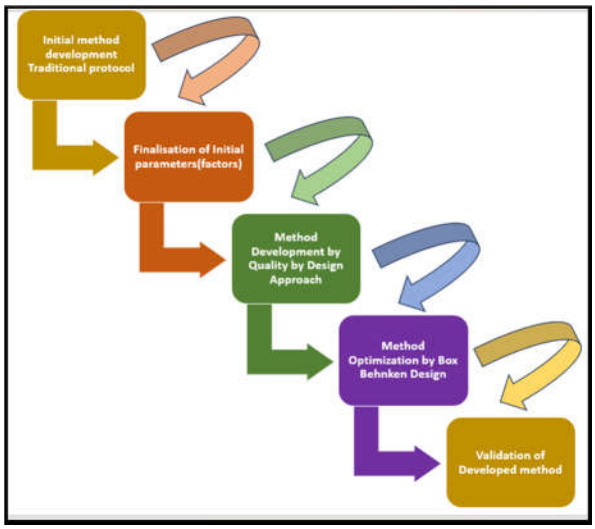

Fig. 3: Plan of QbD based method development

RESULTS AND DISCUSSION

Implementation of traditional method development approach

At the initial stage the appropriate method was finalised with the help of traditional method development approach. The mobile phase composition, flow rate and wavelength were evaluated as a critical method parameter. Details about factors and outcome for trials are given in table 3.

Table 3: Initial trials for finalizing critical method parameters/factors for QbD

Trials no and column	Mobile phase	Ratio (%)	Flow rate (ml/min)	Wavelength (nm)	Inference
1 (C18 Shimadzu)	ACN: Acetic Acid 0.1%	75:25	0.8	274	Peak resolution was not good.
2 (C18 Shimadzu)	MeOH: Tetrahydrofuran	75:25	1	274	No separation of peaks observed.
3 (C18 Agilent)	MeOH: Tetrahydrofuran	80:20	0.8	274	Single peak observed.
4 (C18 Agilent)	ACN: MeOH: Buffer	30:40:30	0.8	266	The resolution between two peaks was not satisfactory.
5 (C18 Agilent)	MeOH: Buffer	50:50	0.8	266	Peak shape found broad.
6 (C18 G l Schiences)	MeOH: Buffer	40:60	1.5	268	RT, NTP, asymmetry and resolution found satisfactory.

*The column used for the final optimized method was Inertsil ODS-3V (C18, 250 x 4.6 mm, 5 µm); RT: Retention time; NTP: Number of theoretical plates; OPA: Ortho phosphoric acid, n=6, data in above table contains total 6 experimental runs.

In this study 20 µl injection volume was used. The isosbestic point observed for Lamivudine and Zidovudine was at 268 nm which is shown in fig. no. 4. The individual lambda max was 274 nm for Lamivudine and 266 nm For Zidovudine. Initial trials were carried out by considering individual lambda max as well as isosbestic point of Lamivudine and Zidovudine. An isosbestic point is observed in overlaid spectra when a chromophoric precursor is converted to a product with a different spectrum, so that it is often assumed that an isosbestic point occurs only when the precursor is quantitatively converted to a single product [25].

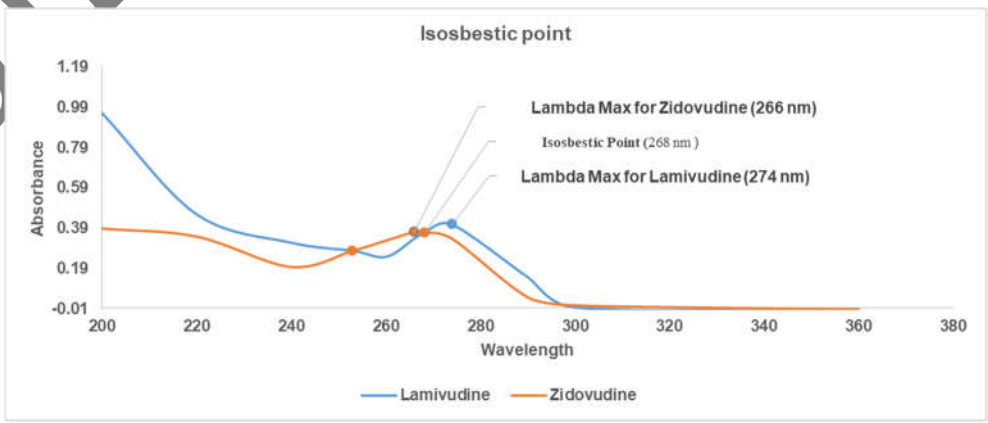

Fig. 4: Overlay of UV spectra for Lamivudine and Zidovudine showing isosbestic point at 268 nm

The sixth trial was found most suitable from traditional method development approach. Critical method parameters for trial 6 were mobile phase MeOH: Buffer v/v in 40:60 ratio, flow rate was 1.5 ml/min and wavelength were 268 nm. The Potassium dihydrogen phosphate buffer with sodium salt of pentane sulphonic acid as a ion pairing reagent, temperature and pH were maintained constant in 6th trial, in QbD based optimization process and in validation process. The pH was adjusted and maintained at 3 with the help of Ortho phosphoric acid (OPA). The column temperature also maintained constant at 25 °C. Trial no. 6 shown in fig. no. 5, this trial was used for further QbD approach. The Design of Experiment (DoE) software viz. Design Expert® 13 was utilized to execute the QbD protocol. The system suitability parameters for trial number 6 is given in table no. 4. Finalized parameters for QbD protocol is given in table 5.

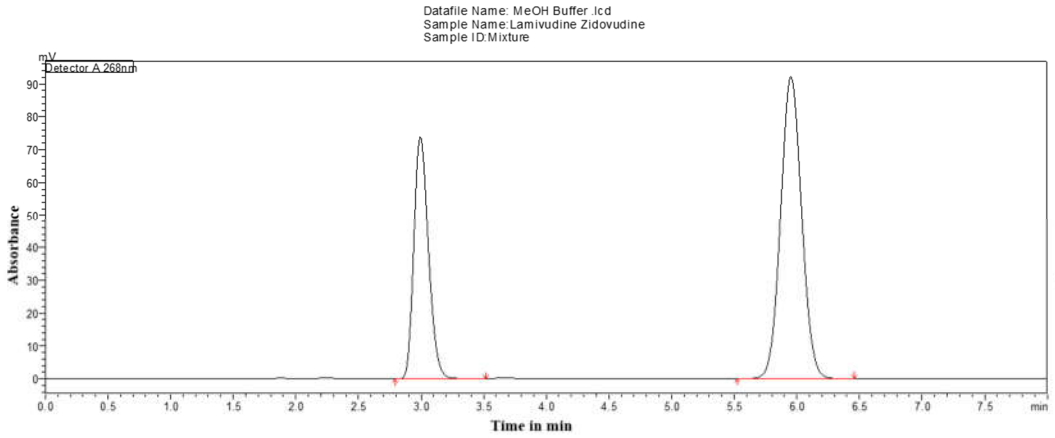

Fig. 5: Trial number 6

Table 4: System suitability parameters for trial number 6

Parameter	Results	
	Lamivudine	Zidovudine
Retention time (min)	2.994	5.953
Area	599491	1075968
Area in %	35.781	64.219
Asymmetry	1.236	1.050
Plates	2861	5696
Resolution	-	10.970

*The resolution value reported is for the separation between Lamivudine and Zidovudine peaks; high resolution in initial trials justified the QbD optimization, which ultimately achieved a more efficient resolution within the recommended range of 1.5–5 for the finally optimized method.

Table 5: Finalised parameters/factors for DoE specifically for BBD.

S. No.	Important parameters	Values
1	MeOH: Buffer	40:60 v/v
2	Flow rate	1.5 ml/min
3	Wavelength	268 nm

*Buffer: Potassium dihydrogen phosphate buffer with sodium salt of pentane sulphonic acid as a ion pairing reagent.

Implementation of QbD approach for method optimization

The DoE specifically BBD is implemented for method optimization. From traditionally developed method mobile phase, flow rate and wavelength considered as a factor for BBD. The BBD were suggested 17 runs protocol with 5 centre points. Evaluation of method optimization was based on six responses viz. retention time, area, asymmetry, number of theoretical plates, resolution and selectivity. Details of 17 run protocol is given in table no. 6. The variation in peak areas observed across the 17 trials in table no. 6 is a function of the shifting residence time within the detector flow cell, dictated by the varying flow rates and mobile phase velocities used to establish the design space. In accordance with the relationship area is inversely proportional to the flow rate, a decrease in retention time leads to a reduction in absolute peak area. However, system suitability was maintained in all runs with % RSD<2.0.

Table 6: Results of BBD

Run	Factor 1	Factor 2	Factor 3	1	2	Responses 3	4	5	6
	Mobile phase (MeOH %)	Flow rate (ml/min)	Wave-length (nm)	Retention time (min)	Area	Asymmetry	No. of plates (NTP)	Resolution	Selectiv-ity

1	40	1.7	264	4.1785	742.74	1.1340	4150.0	10.756	1.98
2	50	1.3	268	3.4180	928.50	1.2295	3526.0	5.502	1.46
3	30	1.5	272	5.7785	866.71	1.0795	5391.0	15.195	2.35
4	30	1.7	268	5.3370	798.90	1.0755	5155.0	14.83	2.34
5	40	1.5	268	4.4675	832.86	1.1420	4302.0	9.991	1.98
6	40	1.7	272	4.1830	786.34	1.1415	4179.5	10.784	1.98
7	40	1.5	268	4.4585	834.36	1.1415	4305.0	10.993	1.99
8	40	1.3	264	4.9985	890.18	1.1465	4738.5	11.505	1.98
9	40	1.3	272	5.0040	942.60	1.1470	4744.5	11.519	1.98
10	40	1.5	268	4.4520	836.80	1.1435	4315.0	10.997	1.98
11	50	1.7	268	2.9765	776.20	1.2015	3431.0	5.553	1.47
12	50	1.5	272	3.0590	822.68	1.2260	3224.0	5.247	1.45
13	40	1.5	268	4.4440	831.36	1.1435	4315.0	10.989	1.98
14	30	1.5	264	5.7955	813.38	1.0790	5386.5	15.204	2.35
15	50	1.5	264	3.0530	790.06	1.2255	3222.5	5.238	1.45
16	30	1.3	268	6.5520	982.03	1.0795	5880.0	15.883	2.35
17	40	1.5	268	4.4390	839.23	1.1335	4217.0	10.983	1.98

* All runs were performed in randomized order; BBD: Box Behnken design; Response values represent the mean of three injections.; Response values represent the mean of three injections; NTP = Number of theoretical plates.

ANOVA for quadratic model suggest that F-values for six responses shows the model is significant. On other side p-values are less than 0.0500 confirms the quadratic model is significant for the study.

However, to avoid statistical over interpretation, the lack of fit F-Value for six responses implies the lack of fit is not significant relative to the pure error. It indicates that the model successfully describes the relationship between Critical Method Parameters (CMPs) and Critical Quality Attributes (CQAs). Non-significant lack of fit is good and shows the model fits for our study. The summery of ANOVA for quadratic model is given in table 7.

Table 7: Summery of ANOVA study for quadratic model

Response number	Response	Source	Summary of ANOVA for quadratic model					
			Sum of squares	df	Mean square	F-value	p-value	Remark
1	Retention time	Model	16.60	5	3.32	37164.81	<0.0001	Significant
		Lack of fit	0.0005	7	0.0001	0.5185	0.7895	Not significant
2	Area	Model	63145.65	7	9020.81	258.08	<0.0001	Significant
		Lack of fit	275.21	5	55.04	5.59	0.0601	Not significant
3	Asymmetry	Model	0.0412	4	0.0103	524.64	<0.0001	Significant
		Lack of fit	0.0002	8	0.0000	1.19	0.4641	Not significant
4	NTP	Model	9.559	4	2.390	847.09	<0.0001	Significant
		Lack of fit	26909.23	8	3363.65	1.94	0.2733	Not significant
5	Resolution	Model	198.77	5	39.75	511.62	<0.0001	Significant
		Lack of fit	0.0554	7	0.0079	0.0396	0.9997	Not significant
6	Selectivity	Model	1.61	6	0.2677	29173.26	<0.0001	Significant
		Lack of fit	0.0001	6	0.0000	2.03	0.2575	Not significant

*ANOVA: analysis of variance; df: degrees of freedom; F-value: The signal-to-noise test; p-value: The probability test; NTP: Number of theoretical plates; n=17, above data is based on 17 experimental runs.

The predicted R^2 of six responses were in reasonable agreement with the adjusted R^2 of respective responses; i. e. the difference was less than 0.2. adequate precision measures the signal to noise ratio. A ratio greater than 4 was desirable. The study shows adequate signal to noise ratio, it suggests that, this quadratic model is suitable for further study. Details about study of fit statistics for quadratic model is given in table 8.

Table 8: Study of fit statistics for quadratic model

Parameters	Responses					
	Retention time	Area	Asymmetry	No. of plates	Resolution	Selectivity
Std. Dev.	0.0095	5.91	0.0044	53.11	0.2788	0.0030
Mean	4.51	842.05	1.15	4381.32	10.66	1.94
C. V. %	0.2098	0.7021	0.3869	1.21	2.62	0.1558
R^2	0.9999	0.9950	0.9943	0.9965	0.9957	0.9999
Adjusted R^2	0.9999	0.9912	0.9924	0.9953	0.9938	0.9999
Predicted R^2	0.9998	0.9688	0.9823	0.9869	0.9929	0.9997
Adeq. precision	634.5750	58.9605	64.3985	93.1921	65.0277	461.8041

*CV: coefficient of variation; Adeq. precision: signal-to-noise ratio; values>4 indicate adequate model discrimination; n=17, above data is based on 17 experimental runs.

The quadratic model was found suitable by ANOVA and fit statistic study, which was used for method optimisation. The six responses of BBD were theoretically in considerable limits but for finding out most suitable solution, this study focuses on retention time, selectivity and resolution. The minimum values 2.9765, 5.238, and 1.45 were set for retention time, resolution and selectivity respectively. On the basis of given input Design Expert® 13 suggested 94 solutions out of which the most suitable solution was finalised, which closely fulfils the set inputs.

The second order polynomial regression equations were presented here. After performing the ANOVA to ensure the statistical significance of the quadratic model, the mathematical relationship between the independent factors and the responses was established. The final equations in terms of actual factors for retention time, area, symmetry, number of theoretical plates, resolution and selectivity were presented below. (A represents flow rate, B represents wavelength and C represent mobile phase concentration of MeOH)

$$\text{Retantion time} = 26.49910 - 16.71413A - 0.261435C + 0.096687AC + 3.59523A^2 - 0.000257C$$

$$\text{Area} = -93859.72658 - 2568.88688A + 720.15003B - 15.26641C + 3.85362AC + 671.76219A^2 - 1.33295B^2 + 0.096141C$$

$$\text{Asymmetry} = 0.852708 + 0.088750A + 0.005168C - 0.003AC + 0.000081C^2$$

$$\text{Theoretical plates} = 25090.10243 - 17733.95833A - 223.23750C + 78.75000AC + 4450.17361A^2$$

$$\text{Resolution} = 47.38232 - 28.75664A - 0.196934C + 0.138000AC + 7.22763A^2 - 0.006309C^2$$

$$\text{Selectivity} = -8.47442 - 0.091678A + 0.082720B + 0.016826C + 0.002338AC - 0.000154B^2 - 0.000809C^2$$

The regression equations were generated in terms of actual factors to allow for the practical prediction of responses based on the original units of each chromatographic parameter. It should be noted that while these actual factor coefficients are essential for laboratory implementation and method transfer, they are scaled to accommodate the specific units of each variable.

As illustrated in fig. 6, the optimized chromatographic conditions for achieving the desired retention time, resolution, and selectivity were determined to be a mobile phase ratio of 50:50 (v/v), a flow rate of 1.69831 ml/min and a detection wavelength of 268 nm.

Fig. 7 shows that the large size of yellow region indicates method is robust. Small fluctuations in flow rate or wavelength will not cause method to fail. It also represents that mobile phase was constant for this plot. The boundaries of yellow region show curvature which represents interaction between flow rate and wavelength. Fig. 6 and 7 supports the most suitable solution given by BBD.

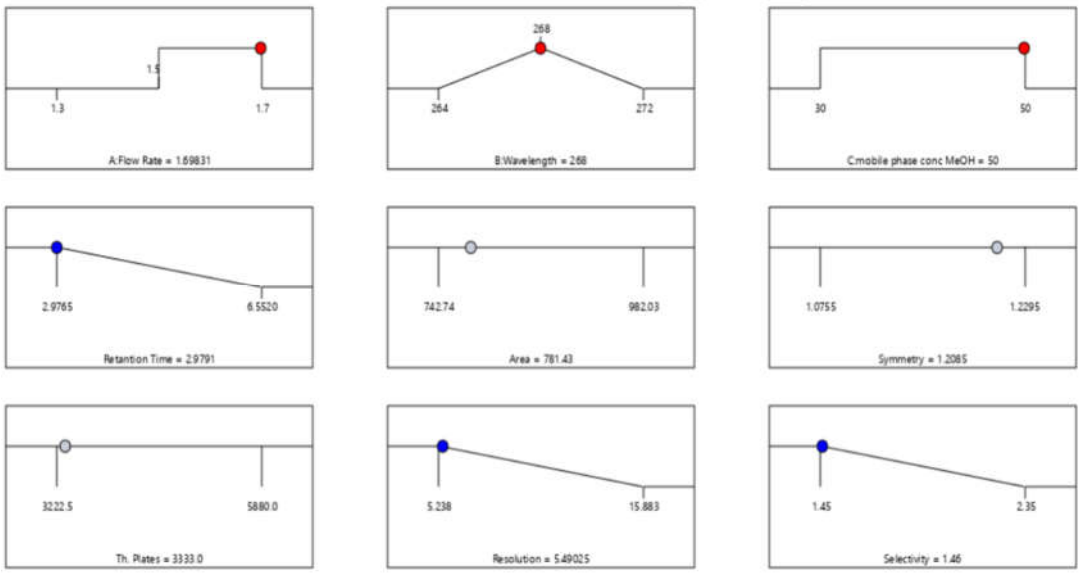

Fig. 6: Most appropriate solution by BBD, *The fig. shows minimum values i. e. 2.9765, 5.238, and 1.45 for retention time, resolution and selectivity respectively gives mobile phase concentration at 50:50 ratio, flow rate at 1.69831 ml/min and wavelength at 268 nm as a most suitable solution

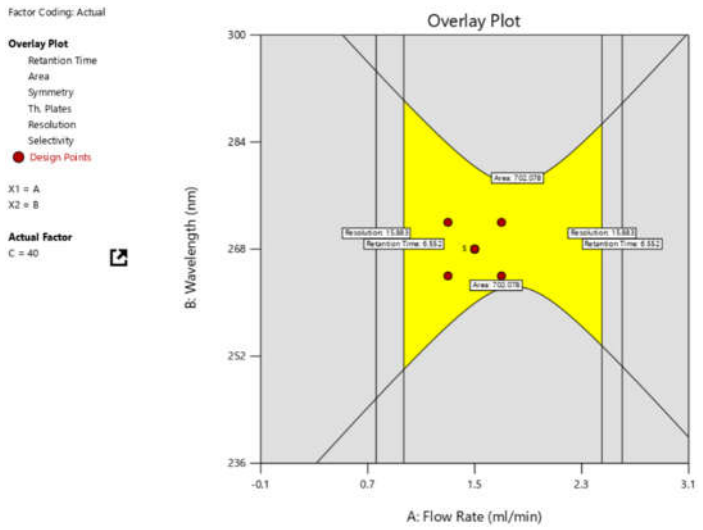

Fig. 7: Contour plot for most suitable solution by BBD, *Contour plot showing the effect of mobile phase composition and flow rate on the desirability function (set to minimize retention time while maintaining resolution>2.0). The highlighted area represents the design space

The optimised parameters by BBD given in table 9, which was based on 17 runs. The values given by BBD (i. e. Design Expert® 13) were practically achievable but for rounding to a standard value like 1.7 ml/min enhances method transferability and ruggedness, ensuring that the method remains valid and reproducible across different laboratories and equipment that's why for practical purpose values were rounded off. This rounding off will not affect any significant deviation because as per fig. 7 small fluctuations in flow rate or wavelength will not cause method to fail.

Table 9: Optimised parameters by BBD

S. No.	Important parameters	Traditional method values	Value by design expert® 13 software	Final values for experimentation
1	MeOH: Buffer	40:60	50:50	50:50
2	Flow rate	1.5 ml/min	1.69831 ml/min	1.7 ml/min
3	Wavelength	268	268	268

* Buffer: Potassium dihydrogen phosphate buffer with sodium salt of pentane sulphonic acid as a ion pairing reagent.

In comparison with traditional method development values and BBD optimised values the results for retention time, resolution and selectivity were improved. A comparative evaluation reveals that the QbD-optimized parameters significantly outperform literature-based conventional methods, offering a more robust and sustainable analytical profile. Under the initial traditional conditions, the retention time for Lamivudine and Zidovudine were 2.994 min and 5.953 min, respectively. Following by QbD based optimization values were successfully reduced to 2.420 min and 3.603 min. This represents a ~39.5% decreased analysis time while maintaining robust peak resolution and selectivity of the method.

The cost-effectiveness of the QbD optimized method is demonstrated through a significant decline in solvent consumption. Under initial traditional conditions the Flow rate was 1.5 ml/min and retention time was 5.953 min, the solvent requirement was 8.93 ml per injection/run. The optimized method the Flow rate was 1.7 ml/min and retention time was 3.603 min reduced this requirement to 6.12 ml. Despite the slight increase in flow rate, the 39.5% decrease in analysis time leads to a 31.5% net saving in mobile phase volume.

These results suggest that the BBD optimised parameters were advantageous over traditionally developed method. The QbD based method was finalised and the validation protocol was implemented for further study.

Method validation

Accuracy and recovery

Accuracy is the true value or closeness to the true value, the assay value for Lamivudine and Zidovudine was 99.43% and 99.84% respectively. Which was calculated on the basis of area. Details about accuracy given in table 10.

Table 10: Area of lamivudine and zidovudine for accuracy

Injection number	Area of lamivudine	Area of zidovudine
1	591290	992839
2	594154	992879
3	592733	992899
Average	592725	992872

Assay calculation

% Assay = Avg. area of sample solution × conc. of standered × purity × 100 ÷ Area of standered solution × conc. of sample × 100

Lamivudine % assay = 592725 × 15 × 100 × 100 ÷ 596143 × 15 × 100 = 99.43%

Zidovudine % assay = 992872 × 30 × 100 × 100 ÷ 994483 × 30 × 100 = 99.84%

Recovery values are given in table 11 for Lamivudine and in table 12 for Zidovudine.

Table 11: Details about recovery for lamivudine

% Accuracy	Theoretical conc. (t)	Slope (y)	Spiked area (c)	Observed conc. in µg/ml (x)	% Recovery
75%	22.5 µg/ml	37648	836191	22.21	98.71%
100%	30.0 µg/ml	37648	1108162	29.43	98.10%
125%	37.5 µg/ml	37648	1390154	36.92	98.45%

Table 12: Details about recovery for Zidovudine

% Accuracy	Theoretical conc. (t)	Slope (y)	Spiked area (c)	Observed conc. in µg/ml (x)	% Recovery
75%	45.0 µg/ml	30699	1365485	44.48	98.84%
100%	60.0 µg/ml	30699	1850614	60.28	100.46%
125%	75.0 µg/ml	30699	2280021	74.27	99.03%

Range linearity

Linearity study was based on the concentration Vs absorbance area. Different concentrations were used for this study i. e. 5,10,15,20,30,45 and 10,20,30,45,60,90 for Lamivudine and Zidovudine respectively. The y-intercept for Lamivudine-29061 was evaluated against the response at the target concentration (100%), where it accounts for less than 2% of the total peak area, thus having a negligible impact on accuracy within the working range. To further validate the regression model, we performed a residual analysis for the intercept, confirming that it is not statistically different from zero at the 95% confidence level. Fig. 8 and fig. 9 represents the range linearity for Lamivudine and Zidovudine respectively.

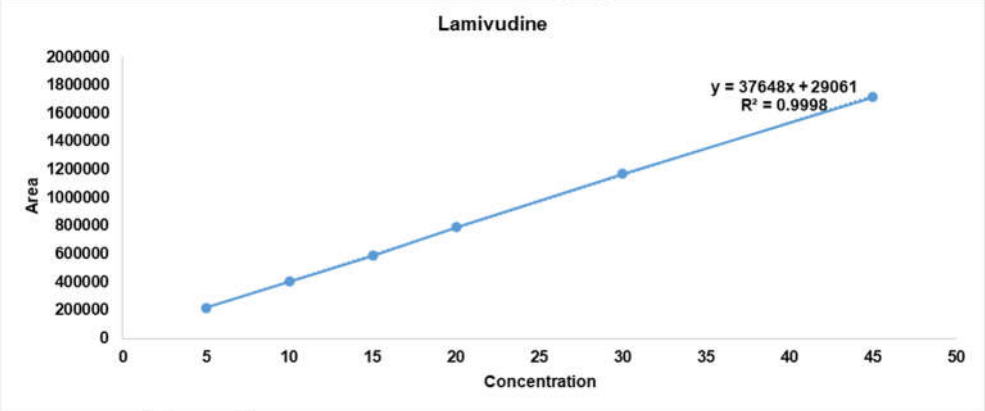

Fig. 8: Linearity details about lamivudine

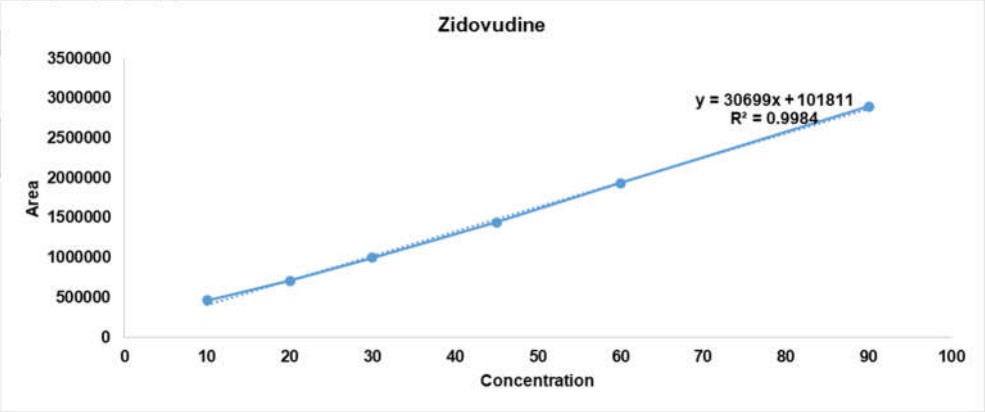

Fig. 9: Linearity details about zidovudine

The robustness of the method was intrinsically established through the 17 run of BBD. This QbD approach systematically evaluates the interaction between critical factors like flow rate, mobile phase ratio and wavelength. The resulting design space (shown in fig. 7) identifies a robust operating region where the method maintains baseline resolution and optimal system suitability.

All details about validation parameters i. e. intraday and interday precision, limit of detection (LOD), limit of quantification (LOQ), percent recovery, repeatability, linearity, regression equation, correlation coefficient and slope were given in table 13.

Table 13: Different validation parameters with results.

S. No.	Parameters		HPLC data			
			Lamivudine		Zidovudine	
1	System suitability		RT	Area	RT	Area
			2.420	616514	3.603	989209
		NTP	2568		4244	
		Asymmetry	1.281		1.160	
		Resolution	-------		5.739	
2	Linearity range		5 – 45 µg/ml		10 – 90 µg/ml	
3	Regression equation		y = 37648 x+29061		y = 30699 x+101811	
4	Correlation coefficient		R^2 = 0.9998		R^2 = 0.9984	
5	Slope		37648		30699	
6	Specificity		Specific		Specific	
7	Accuracy (% Assay)		99.43%		99.84%	
8	Recovery	50%	98.71%		98.84%	
	(98.0-102.0%)	100%	98.10%		100.46%	
		150%	98.45%		99.03%	
9	Intermediate precision (≤ 2%)	Level/Set	RT	Area	RT	Area
	Intra-day Precision %RSD	LQC	0.104%	0.099%	0.268%	0.071%
		MQC	0.168%	0.037%	0.330%	0.074%
		HQC	0.105%	0.040%	0.140%	0.038%
11	Inter-day Precision %RSD	LQC	0.127%	0.068%	0.197%	0.071%
		MQC	0.277%	0.490%	0.621%	0.063%
		HQC	0.087%	0.042%	0.147%	0.044%
12	Method precision-%RSD		0.129%	0.101%	0.188%	0.056%
13	Limit of detection (For assay)		0.76 µg/ml		4.28 µg/ml	
14	Limit of quantitation		2.31 µg/ml		12.9 µg/ml	

*NTP: Number of theoretical plates; LQC: Low quality control; MQC: Medium quality control; HQC: High quality control; RT: Retention time

The analytical method was developed by QbD approach to ensure robustness, reliability, and method efficiency. At the initial stage of method development, the isosbestic point was determined to be 268 nm and the individual lambda max was 274 nm for Lamivudine and 266 nm For Zidovudine, these wavelengths were used in initial traditional trials. Preliminary trials were conducted based on literature data, wherein various solvent systems were evaluated. Among them, a mixture of Methanol: Buffer v/v (40:60) (Buffer: Potassium dihydrogen phosphate buffer with sodium salt of pentane sulphonic acid as a ion pairing reagent) was found to be the most suitable solvent system, The flow rate and detection wavelength were found to be 1.5 ml/min and 268 nm, respectively, as confirmed through initial experiments. These experiments conducted using a traditional trial-and-error approach, helped to identify three CMPs significantly influencing the method's performance.

To further optimize and control these critical parameters, the QbD framework was implemented using Design Expert® version 13 software. A BBD was employed to construct the design of experiments (DoE), comprising 17 experimental runs. This statistical approach enabled the systematic evaluation of parameter interactions and their impact on method performance. As a result of the optimization process, the final method conditions were established as mobile phase composition, Methanol: Buffer v/v (50:50) (Buffer: Potassium dihydrogen phosphate buffer with sodium salt of pentane sulphonic acid as a ion pairing reagent), Flow rate-1.7 ml/min and Wavelength 268 nm. Under these optimized conditions, the method demonstrated robust performance and was subjected to a comprehensive method validation protocol. The validation included the assessment of parameters such as accuracy, precision, linearity, system suitability, LOD and LOQ.

The accuracy for Lamivudine and Zidovudine was found to be 99.43% and 99.84% respectively. The linearity was calculated from range of 5-45 µg/ml for Lamivudine and 10-90 µg/ml for Zidovudine, correlation coefficient (R^2) was found to be 0.9998 and 0.9984 for Lamivudine and Zidovudine respectively. Acceptable limit for precision study was less than 2% as per guidelines and all values were lying in specified limit which shows that the method was found precise. Recovery data lies in between 98-102%.

Specified parameters were found to be within acceptable limits, with most values aligning closely with theoretical expectations, confirming the method's validity and reliability. The successful mapping of the design space for the Lamivudine and Zidovudine combination aligns with our previous application of AQbD principles to Azilsartan Medoxomil and Cilnidipine [24]. This consistency across different pharmaceutical scaffolds reinforces the reliability of using systematic risk assessment to define robust method operable design regions (MODR).

CONCLUSION

This research demonstrates the successful development of a RP-HPLC method for the simultaneous estimation of Lamivudine and Zidovudine using an QbD approach for tablet formulation. This QbD approach was implemented on the basis of ICH Q14 guidelines for method development. By utilizing a systematic risk assessment and a BBD, a robust MODR was defined. The optimization process not only improved resolution and selectivity but also achieved a ~31.5% decline in solvent usage by ~39.5% less analysis time. The saving of mobile phase volume emphasizes the role of QbD in developing green analytical methods by minimizing solvent waste. This demonstrates that the proposed method was both high-performing and environmentally sustainable.

Validation as per ICH Q2R2 guidelines confirmed that the method is accurate up to 99.43% and 99.84% for Lamivudine and Zidovudine respectively as well as method was precise, and sensitive across the linear range, with recovery values between 98–102%. The consistency observed across different pharmaceutical scaffolds reinforces the reliability of the MODR established here. Ultimately, this method fulfils the existing gap in research by providing accurate, precise, robust, a cost-effective and time-efficient tool for routine analysis in pharmaceutical quality control laboratories and in industries.

ABBREVIATIONS

RP-HPLC: Reversed phase high performance liquid chromatography; DAD: Diode array detector; LOD: Limit of detection; LOQ: Limit of quantitation; RSD: Relative standard deviation; RS: Reference standard; UV: Ultra Violet; AR: Analytical reagent; RS: Reference standard; mg: Mili gs; µg**:** Micro Grams; mcg: Micro Grams; ml: Millilitres; min: Minutes; 3TC: Lamivudine; AZT: Zidovudine; MeOH: Methanol; OPA: Ortho phosphoric acid; MODR: Method operable design regions.

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AI USE STATEMENT

No artificial intelligence (AI) or AI-assisted tools were used in the writing, data analysis, or creation of fig. for this manuscript.

AUTHORS CONTRIBUTIONS

MBG (Mangesh B. Gadekar) was focused on the conceptualization and development of the methodology, including the Quality by Design (QbD) based optimization and validation studies. MBG also prepared the original draft of the manuscript. PBS (Prashant B. Shamkuwar) provided overall supervision of the research project and was involved in the critical review and editing of the final manuscript.

CONFLICTS OF INTERESTS

The authors declare that they have no conflict of interest for this research work.

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