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Method Development and Validation for Clevudine by RP-HPLC Using Green Chemistry Approach

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ABSTRACT

Objectives: To establish and validate a straightforward, precise, accurate, and stability-indicating RP-HPLC procedure for the quantitative analysis of clevudine in pharmaceutical bulk material and oral tablet formulations, incorporating a green analytical chemistry framework to meet the growing demand for eco-compatible methods in hepatitis B antiviral drug analysis. **Methods:** Separation conditions were methodically refined through a three-factor, three-level Box-Behnken Design within a Response Surface Methodology framework, with isopropyl alcohol content, flow rate, and column temperature as input factors and retention time, peak asymmetry, and theoretical plate count as responses. The method was subsequently validated in accordance with ICH Q2(R2) guidelines. **Results:** Optimal separation was achieved with isopropyl alcohol and ammonium formate buffer (pH 3.5) at 35:65 v/v, a flow rate of 0.8 mL/min, column temperature of 35°C, and UV detection at 262 nm, selected at a composite desirability of 1.0. The validated method exhibited linearity across 20–140 µg/mL (r^2 = 0.9999), mean accuracy of 99.78%, repeatability %RSD of 0.08%, LOD of 0.189 µg/mL, and LOQ of 0.573 µg/mL. Forced degradation studies established its stability-indicating character. Assay of commercial tablets returned a mean label claim of 99.91% (%RSD = 0.39%). **Conclusion:** The validated green RP-HPLC method offers a reliable, environmentally responsible tool for routine quality control of clevudine, with significant potential for pharmacokinetic monitoring in high HBV-burden regions and future clinical translation into biological matrix analysis.

1. INTRODUCTION:

Hepatitis B virus (HBV) infection ranks among the most severe infectious disease threats to public health globally [1]. According to the World Health Organization (WHO), an estimated 296 million individuals are currently living with chronic HBV infection, with roughly 1.5 million new infections occurring annually [2]. The disease constitutes a major contributor to global morbidity and mortality, accounting for approximately 820,000 deaths each year, predominantly driven by end-stage liver complications including cirrhosis and hepatocellular carcinoma. Almost two-thirds of the total burden in the world is borne by Sub-Saharan Africa and East Asia, and in South-East Asian Region (including India), hepatitis B surface antigen (HBsAg) prevalence in the general population is between 2

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and 4 per cent [3]. Although the prevention vaccine is very effective, having been introduced into the world since the 1980s, the prevalence of the disease is still very high, because vaccination does not remove the pool of chronically infected people, but only prevents new infections [4]. In addition, less than 3% of those who are infected with HBV currently receive anti-HBV therapy, highlighting the large treatment gaps. The economic burden of liver disease due to HBV is huge, with direct health care costs including hospitalisation, antiviral therapy and liver transplantation, and indirect costs including loss of productivity and early death [5].

Clevudine (L-FMAU; 1-(2-deoxy-2-fluoro-β-L-arabinofuranosyl) thymine) is a pyrimidine nucleoside analogue with a pharmacologically unique and scientifically intriguing mechanism of action against hepatitis B virus [6]. The triphosphate metabolite of clevudine inhibits the protein priming and second-strand elongation of the HBV polymerase in a noncompetitive fashion, which is in contrast to the mechanism of action of all other nucleoside analogues [7]. Clevudine has been clinically approved in South Korea and Philippines, with a 48-week virological response rate of 81% [8]. It is also unique due to the very long intracellular half-life of its active triphosphate metabolite in hepatocytes that leads to a prolonged antiviral activity beyond the dosing window. Physicochemical characterization of clevudine shows it to be a white to off-white crystalline powder with a melting point of 184–185°C, with the highest solubility in alkaline media and in methanol; it has UV absorption maxima at 214 nm and 262 nm [9]. These clinically relevant properties, however, made resistance as high as 11% after 60 weeks of clevudine monotherapy and raised concerns about myopathy during prolonged use, making it imperative to precisely analytically quantify the properties for pharmacokinetic monitoring as well as pharmaceutical quality control [10].

RP-HPLC is the most common analytical method used in pharmaceutical quality control in complex drug matrices, which has the advantages of good selectivity, sensitivity and repeatability [11]. Antiviral nucleoside analogues are commonly analyzed by conventional RP-HPLC methods that involve a high percentage of acetonitrile, a solvent that can cause serious health risks, especially neurological issues, due to its neurotoxicity and high flammability and can produce toxic amounts of hydrogen cyanide when it is burned, which leads to a high proportion of laboratory waste and health risks for users [12]. Green analytical chemistry not only encourages the use of environmentally friendly solvents like isopropyl alcohol instead of the hazardous solvents, but also aims to reduce the volume of mobile phase and shorten the analysis time as well as decrease the amount of waste generated during the process [13]. In contrast, one factor at a time optimisation methods provide a non-statistic and non-systematic method for assessing method robustness and evaluating multiple chromatographic variables, and they do not have as much advantage as the method optimisation under Response Surface Methodology with Box-Behnken Design [14]. Evaluation frameworks for greenness, such as GAPI, AGREE and Analytical Eco-Scale, are objective and quantitative indicators of the environmental qualities of new methods and are recommended in many influential analytical chemistry journals [15].

This work describes the development and validation of a stability-indicating RP-HPLC procedure for the quantitative determination of clevudine in pharmaceutical bulk material and oral tablet formulations, embedded within a green analytical chemistry framework. The primary objectives encompass systematic chromatographic optimisation via Box–Behnken Design, comprehensive method validation per ICH Q2(R2) guidelines spanning specificity, linearity, accuracy, precision, LOD, LOQ, robustness, and forced degradation studies, along with environmental impact evaluation using the AGREE, GAPI, and Analytical Eco-Scale tools.

MATERIALS AND METHODS:

MATERIALS:

Clevudine working standard (purity 99.8%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Isopropyl alcohol (HPLC grade) and ammonium formate (analytical grade) the latter being a thermally labile ammonium salt widely selected for its compatibility with mass spectrometric ionisation and its characteristically low UV background absorbance were both purchased from Merck Life Sciences Pvt. Ltd. (Mumbai, India) (Stoll & Gilar, 2023). HPLC-grade methanol was procured from Fisher Scientific (Mumbai, India). Hydrochloric acid (35–38% w/v) and sodium hydroxide, both of analytical grade, were purchased from SD Fine Chemicals Ltd. (Mumbai, India) for deployment as acid- and base-catalysed hydrolytic stress reagents, respectively, as required under ICH Q1A(R2) forced degradation testing protocols (ICH, 2003). Hydrogen peroxide (analytical grade, 30% w/v), serving as the primary oxidative stress agent, was obtained from Loba Chemie Pvt. Ltd. (Mumbai, India). An aqueous ammonium formate buffer (pH 3.5) was constituted freshly in the laboratory using ultrapure water drawn from a Milli-Q purification system (Millipore, Bedford, MA, USA). The commercially available tablet formulation of clevudine (30 mg per tablet)

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was sourced from a licensed local retail pharmacy. All remaining reagents and solvents were of analytical reagent grade unless noted otherwise.

METHODS:

Determination of Absorption Maximum (λ_{max}) of Clevudine:

The wavelength of maximum UV absorbance (λmax) of clevudine was established using a Jasco V-730 double-beam UV-Visible spectrophotometer (Jasco India Pvt. Ltd., Mumbai, India), a general-purpose instrument with a 1 nm spectral bandwidth and an operational wavelength range of 190 to 1,100 nm. A stock solution (1000 µg/mL) was prepared by dissolving 10 mg of accurately weighed clevudine working standard in 10 mL of methanol, with ultrasonic agitation applied for 5 minutes to ensure complete dissolution. An aliquot of 1.0 mL from this stock was transferred into a 100 mL volumetric flask and made up to volume with ammonium formate buffer (pH 3.5), producing a 10 µg/mL working solution. The UV absorption spectrum was recorded from 200 to 400 nm at a scanning rate of 200 nm/min, using a 1 cm quartz cuvette and referencing against ammonium formate buffer (pH 3.5) as the spectral blank. Operating at λ_{max} optimises detector sensitivity and signal-to-noise ratio during chromatographic analysis (Dong, 2006); the experimentally derived value was therefore fixed as the detection wavelength for all subsequent chromatographic measurements. The procedure was performed in triplicate (n = 3) [16].

Solubility Study of Clevudine:

Clevudine was found to be soluble in 10 buffer systems and pharmaceutical solvents. Ten milligrams (10.0 mg) of clevudine was weighed out separately into clean, dry glass test tubes and 10 mL of each solvent was added to these. The solvents examined were distilled water, methanol, ethanol, isopropyl alcohol, acetonitrile, 0.1 N HCl, 0.1 N NaOH, phosphate buffer (pH 6.8), phosphate buffer (pH 7.4), and ammonium formate buffer (pH 3.5). The test tubes were then sealed, vortexed for 2 min and left undisturbed at 25 ± 2°C for 24 h. The suspensions obtained were filtered through a nylon membrane syringe filter of 0.45 µm pore size, suitably diluted with the corresponding solvent and the absorbance was recorded at the predetermined λmax using a UV-Visible double beam spectrophotometer (Jasco V-730, Jasco India Pvt. Ltd., Mumbai, India). The calibration curve was used to determine the solubility, which was expressed in mg/mL. Each experiment was run three times (n=3) [17][18].

Experimental Design:

Building on chromatographic parameters identified as most influential in preliminary trial experiments, a three-factor, three-level Box-Behnken Design (BBD) embedded within a Response Surface Methodology (RSM) framework was employed to optimise the RP-HPLC conditions for clevudine. Developed by Box and Behnken (1960), this design was selected for its statistical efficiency in estimating linear effects, pairwise variable interactions, and quadratic curvature without simultaneously driving all factors to their extreme settings an advantage over both classical one-factor-at-a-time approaches and full factorial designs in terms of experimental burden. The isopropyl alcohol proportion in the mobile phase (X_1), mobile phase flow rate (X_2), and column temperature (X_3) were identified as the three independent variables and assigned coded factor levels of −1 (low), 0 (centre), and +1 (high). The total experimental matrix comprised 17 runs 12 factorial points and 5 replicated centre points where the centre-point replicates provide an unbiased estimate of pure experimental error and allow direct evaluation of model reproducibility (Myers et al., 2016). Retention time (Y_1), peak asymmetry factor (Y_2), and theoretical plate count (Y_3) were designated as the chromatographic response variables. Each response was modelled by a full second-order polynomial equation that accounts for all linear, two-factor interaction, and quadratic terms, enabling construction of the response surface and systematic identification of optimal operating conditions. The experimental factor levels and optimisation objectives are summarised in Table 1 [18][19–21].

Table 1: Independent and Dependent Variables with Levels and Goals for Box-Behnken Design

Parameter	Units	Low	Middle	High
IPA Proportion	% v/v	32	35	38
Flow Rate	mL/min	0.6	0.8	1
Column Temperature	°C	27	30	33
Dependent Variables				**Goal**
Retention Time				Range (6-9)
Assymetry				Minimize (≤1.5)
Theoretical Plates				Maximize

Preparation of Standard Solutions and Execution of DoE Experimental Runs:

Clevudine was dissolved in a stock solution at a final concentration of 1000µg/mL by sonicating 10mg of accurately weighed clevudine in methanol with sonication for 5 min and then making up to 10 mL with methanol. An appropriate dilution of the stock solution with ammonium formate buffer (pH 3.5) was used to prepare a working standard solution with 100 µg/mL. Syringe filters (0.45 µm nylon) were used to filter all the solutions before they were injected. Sixteen experimental runs were designed using the BBD and were performed by systematically changing isopropyl alcohol

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proportion (X_1), flow rate (X_2) and column temperature (X_3) at their coded levels. For each run, the mobile phase was freshly prepared, the HPLC system was equilibrated for 30 min, and 20 µL of working standard solution was injected onto a Phenomenex Luna C18 column (250 × 4.6 mm, 5 µm) at a UV detection wavelength of 262 nm. The value of retention time (Y_1), asymmetry factor (Y_2) and theoretical plates (Y_3) were noted for each run. Table 2 shows the entire BBD layout. The experimental design layout of the Box-Behnken Design is shown in Table 2.Table 2 shows the Box-Behnken Design layout with 17 experimental runs [22].

Table 2: Box-Behnken Design Layout for 17 Experimental Runs

Run	IPA Proportion (%v/v)	Flow Rate (mL/min)	Column Temperature (°C)
1	35	0.8	30
2	32	0.8	33
3	32	0.8	27
4	38	0.8	33
5	35	0.8	30
6	35	1.0	27
7	35	1.0	33
8	32	0.6	30
9	32	1.0	30
10	35	0.8	30
11	38	0.6	30
12	35	0.8	30
13	35	0.8	30
14	35	0.6	27
15	38	1.0	30
16	38	0.8	27
17	35	0.6	33

Preparation of Standard and Sample Solutions for Validation:
Stock solution of clevudine (1000 µg/mL) was prepared by dissolving 10 mg of clevudine, which was accurately weighed, with sonication for 5 min in methanol and then making up to 10 mL in the same solvent. For all of the validation studies, the diluent used was ammonium formate buffer (pH 3.5) and a working standard (100 µg/mL) was prepared by appropriate dilution of the stock solution. A blank ammonium formate buffer solution (pH 3.5) was injected to ensure that there were no interfering peaks in the reference solution at the clevudine retention time. All solutions were prepared fresh and filtered through a 0.45 µm nylon membrane syringe filter before injection and kept away from light during preparation and storage. The developed Optimized RP-HPLC Method has been validated as per ICH Q2(R2) Guidelines [23].

Validation of Optimized RP-HPLC Method as per ICH Q2(R2) Guidelines:
System Suitability:
Six successive injections of the clevudine working standard (100 µg/mL) were made onto a Phenomenex Luna C18 column (250 × 4.6 mm, 5 µm) to verify system suitability before formal validation. Peak area, retention time, tailing factor, and theoretical plate count were recorded at each injection. The system was deemed acceptable when the tailing factor did not exceed 2.0, theoretical plates numbered no fewer than 2000, and the %RSD of peak area across all six injections was no greater than 2.0% (n = 6) [24].

Specificity and Peak Purity:
Optimized chromatographic conditions were used to inject separately blank (ammonium formate buffer, pH 3.5), placebo and standard solutions of clevudine to confirm the absence of interfering peaks at the retention time of clevudine. The peak purity was assessed by comparing the UV spectra from the peak apex, upslope and downslope, and by a photodiode array (PDA) detector. The peak purity angle, < peak purity threshold, indicates a spectral homogeneity of the clevudine peak [25].

Forced Degradation Studies:
The developed method was shown to be capable of distinguishing between the degradations by carrying out the forced degradation studies. Clevudine solution (100 µg/mL) in 1 N HCl was incubated at 60ºC for 1 hour, neutralized with 1 N NaOH and diluted to volume with ammonium formate buffer (pH 3.5) for acid degradation. Clevudine solution (100 µg/mL) prepared in 1 N NaOH was heated at 60°C for 1 h and then neutralized with 1 N HCl and diluted as above for alkaline degradation. In oxidative degradation, clevudine solution (100 µg/mL) was stored at room temperature for 1 h in the dark in the presence of 3% v/v hydrogen peroxide. Clevudine solution (100 µg/mL) in ammonium formate buffer (pH 3.5) was heated in a dry heat oven at 105°C for 1 hour and then cooled before being injected. The clevudine solution (100 µg/mL) was subjected to an exposure of UV light of 254 nm and the dark control was kept wrapped in aluminium foil for a minimum period of 1.2×10^6 lux hours, as specified in ICH Q1B guidelines. Stressed solutions were all filtered through the nylon membrane 0.45 µm syringe filter before injection. Peak areas from the stressed samples were compared to the unstressed sample to determine the percentage degradation [26][27].

Linearity:
Linearity of the analytical response was assessed across seven concentration levels of clevudine (20-140 µg/mL), each prepared through stepwise dilution of the stock solution with ammonium formate buffer (pH 3.5). Every concentration level was injected in triplicate under the established chromatographic conditions, and the resulting mean peak areas were computed and recorded. A

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calibration graph was constructed by plotting average peak area against the corresponding analyte concentration, and the dataset was subjected to least-squares linear regression analysis. Linearity was considered satisfactory when the coefficient of determination (r^2) reached or exceeded 0.999 (n = 3) [28].

Limit of Detection and Limit of Quantification:
In compliance with ICH Q2(R2) guidelines, the LOD and LOQ were computed from calibration curve regression parameters using LOD = 3.3σ/S and LOQ = 10σ/S, where σ denotes the y-intercept standard deviation and S is the fitted slope. Experimental confirmation was obtained through signal-to-noise ratios of not less than 3 and 10 at the respective limits [29].

Accuracy:
Accuracy was assessed using the standard addition technique across three spiking levels 50%, 100%, and 150% of the nominal concentration (100 µg/mL) with each level prepared in triplicate. All solutions were run under the established chromatographic conditions, and percentage recovery was calculated from the ratio of the measured concentration to the nominal concentration. The criterion for acceptable accuracy was a mean recovery within 98.0–102.0% and a %RSD not exceeding 2.0% (n = 3) [30].

Precision:
Three levels of precision were tested. Repeatability was determined by the analysis of six independent standard solutions of clevudine (100 µg/mL) on the same day by the same analyst under the same conditions and calculating the %RSD of the peak areas (n=6). Intermediate precision was determined by performing the analysis three separate days using six separate preparations and two different analysts and instruments on each day. If the %RSD was less than or equal to 2.0% for both levels, the method was deemed to be precise [31].

Robustness:
The robustness of the optimized chromatographic conditions (proportion of IPA (±2% v/v), flow rate (±0.1 mL/min), column temperature (±5°C) and buffer pH (±0.2 units)) were evaluated by intentionally introducing small changes. In each different condition, clevudine standard solution (100 µg/mL) was injected thrice and the following were recorded: retention time, tailing factor, theoretical plates, and %RSD of peak area. Robustness was considered to be if the system suitability parameters were within the acceptance limit under all varied conditions (n=3) [32].

Solution Stability:
The stability of the clevudine standard and sample solutions (100 µg/mL) were tested at time intervals of 0, 6, 12, 24 and 48 h at bench-top conditions (25 ± 2°C) and refrigerated conditions (2–8°C). The peak area percentages were computed from the initial value at every time point. The percentage change in peak area within the study period of ±2.0% was considered to be solutions that were stable [33].

Filter Compatibility:
The compatibility of the filters was determined by comparing the peak areas of clevudine standard solution (100 µg/mL) using filtration through a 0.45-µm nylon membrane syringe filter after discarding the initial filtrate and using centrifugation at 3,000 rpm for 10 min. The percent difference in peak area between both preparations was determined and a difference of 2.0% or less was acceptable for filter compatibility [34,35].

Application to Tablet Formulation:
The method was validated and used for the quantitative estimation of clevudine in commercially available tablet formulation. A total of 20 tablets were weighed, averaged and crushed into fine powder. Tablet powder equivalent to 10 mg of clevudine was dissolved in methanol after sonication for 15 min, filtered through the nylon membrane (0.45 µm) of a syringe filter, and diluted with ammonium formate buffer (pH 3.5) to make a working solution at 100 µg/mL. The solution was injected triple blind under validated chromatographic conditions and the percentage claim of the label was measured by using the calibration curve equation obtained in the linearity study (n=3) [34,35].

RESULTS AND DISCUSSION:

RESULTS:

Scanning absorbance maxima determination
UV scanning of clevudine was carried out in the range 200-400 nm with two absorption maxima found at 214 and 262 nm (Figure 1), which corresponds to the chromophoric properties of pyrimidine nucleobase. Based on the above considerations, the wavelength of 262 nm was chosen for further chromatographic investigations with the least interference from mobile phase components and optimum selectivity.

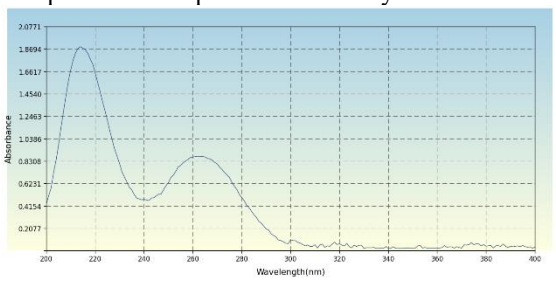

Figure 1: Scanning absorbance maxima of clevudine (214 and 262 nm)

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Solubility Study:

Clevudine's solubility in 10 solvents and buffer systems is shown in Table 3. Clevudine exhibited highest solubility in 0.1 N sodium hydroxide (68.40 ± 0.42 mg/mL), followed by methanol (45.20 ± 0.31 mg/mL) and ethanol (38.60 ± 0.27 mg/mL). Sparingly soluble behavior was seen in distilled water, isopropyl alcohol, and ammonium formate buffer pH 3.5, which was used to select methanol as the stock solution solvent and ammonium formate buffer as the diluent.

Table 3: Solubility Study of Clevudine

Sr. No.	Solvent	Solubility (mg/mL)	Inference
1	Distilled Water	12.40 ± 0.18	Sparingly Soluble
2	Methanol	45.20 ± 0.31	Soluble
3	Ethanol	38.60 ± 0.27	Soluble
4	Isopropyl Alcohol	28.50 ± 0.22	Sparingly Soluble
5	Acetonitrile	18.30 ± 0.19	Sparingly Soluble
6	0.1 N Hydrochloric Acid (pH 1.2)	14.80 ± 0.16	Sparingly Soluble
7	0.1 N Sodium Hydroxide	68.40 ± 0.42	Soluble
8	Phosphate Buffer pH 6.8	11.20 ± 0.14	Sparingly Soluble
9	Phosphate Buffer pH 7.4	13.50 ± 0.17	Sparingly Soluble
10	Ammonium Formate Buffer pH 3.5	22.60 ± 0.21	Sparingly Soluble

All values are expressed as mean ± SD

Results as per QbD Runs:

A summary of the responses observed for all 17 BBD experimental runs is summarized in Table 4. The retention time ranged from 6.30 to 7.59 min, the asymmetry factor ranged from 1.15 to 1.47 and the theoretical plates ranged from 5348 to 6712, which is significant variation between the experimental conditions and indicates that chromatographic responses are very sensitive to changes in the proportion of IPA, flow rate, and the column temperature.

Table 4: Observed Responses for Box-Behnken Design Experimental Runs

Run	Peak Area (mAU)	Retention Time (min)	Asymmetry Factor	Theoretical Plates (N)
1	74850	6.97	1.24	6150
2	64320	6.97	1.36	5862
3	61480	7.23	1.42	5524
4	82640	7.25	1.18	6624
5	74820	7.18	1.25	6460
6	71240	7.51	1.33	5948
7	72480	6.67	1.21	6184
8	60240	7.16	1.47	5348
9	63180	6.30	1.35	5748
10	74864	7.10	1.26	6260
11	79240	7.58	1.31	6248
12	74836	7.23	1.24	6020
13	74848	7.28	1.25	6670
14	68420	7.29	1.43	5412
15	84360	7.25	1.15	6712
16	80120	7.59	1.29	6124
17	74840	7.25	1.38	5924

Optimization of Method:

Retention Time:

The fit summary showed that the linear model was the most appropriate model for the retention time, as the p value of the sequential model was 0.0018 (Table 5). ANOVA was performed and the overall model was found to be statistically significant with an F-value of 8.90 ($p = 0.0018$), adjusted R^2 of 0.5969 and predicted R^2 of 0.3505. All three independent variables (IPA proportion (A), flow rate (B) and column temperature (C) were found to have statistically significant influence on retention time, with IPA proportion (A) being the most significant factor ($F = 12.49$, $p = 0.0037$), followed by flow rate (B) ($F = 7.43$, $p = 0.0173$) and column temperature (C) ($F = 6.77$, $p = 0.0219$). The lack of fit was not significant ($p = 0.1195$) and indicating a good fit of the model. The final polynomial equation for the coded factors of retention time was: Retention Time $= 7.1653 + 0.2513A - 0.1938B - 0.1850C$ By having a positive coefficient for A, it was determined that the reduction in IPA proportion shortened the retention time, and negative coefficients for B and C were determined, meaning that the increase in flow rate and column temperature decreased retention time. The contour plots (Figures 2A1-A3) and three-dimensional response surface plots (Figures 3A1-A3) showed a smooth surface for the decreasing retention time with increasing proportion of IPA and flow rate; the steepest gradient was along the IPA proportion axis indicating that this is the major parameter that affects the retention time.

Asymmetry Factor:

The quadratic model was selected as the best fit for asymmetry factor based on the fit summary (Table 5) which gave a sequential p-value of 8.00×10^{-6}. The results of ANOVA showed that the model was highly significant with F value of 169.66, adjusted R^2 value of 0.9896, and predicted R^2 value of 0.9575, which means the model has a good predictive ability. Among linear terms, IPA proportion (A) was the most dominant factor ($F = 649.24$, $p = 3.66 \times 10^{-8}$), followed by flow rate (B) ($F = 437.50$, $p = 1.44 \times 10^{-7}$) and column temperature (C) ($F = 167.19$, $p = 3.85 \times 10^{-6}$). Among interaction terms, BC was significant ($F = 14.17$, $p = 0.0070$) and AC was significant ($p = 0.0311$). Among quadratic terms, B^2 ($F = 114.59$), C^2 ($F = 81.89$), and A^2 ($F = 26.90$) were all statistically significant. The lack of fit was not significant ($p = 0.3329$) which indicates the excellent model adequacy. The coded factors final polynomial asymmetry equation was: Asymmetry $= 1.248 - 0.0838A - 0.0688B - 0.0425C - 0.0100AB - 0.0125AC - 0.0175BC + 0.0235A^2 + 0.0485B^2 + 0.0410C^2$ All the linear terms had negative

coefficients, which indicated that all the parameters – IPA proportion, flow rate and column temperature – were independent contributors to the decrease in the asymmetry factor, with the highest contribution from IPA proportion. When the quadratic terms of B^2 and C2 were positive, there were curvature effects at either end. The contour plot and three dimensional response surface plots (Figure 2B1-B3 and 3B1-B3) showed a curved bowl shape with the lowest level of asymmetry at higher proportion of IPA and moderate flow rate and temperature, which agrees with the quadratic behaviour of the model.

Theoretical Plates:

The linear model was best suited to the fit summary of theoretical plates, having a sequential p-value of 0.0008 (Table 5). The model was overall significant according to the ANOVA test (F value: 10.62 and p value: 0.0008), with a satisfactory linear fit (adjusted R^2 = 0.6434 and predicted R^2 = 0.5868). All three variables were seen to significantly affect the column efficiency with IPA proportion (A) being the most significant (F = 21.15, p = 0.0005), followed by flow rate (B) (F = 5.60, p = 0.0342) and then column temperature (C) (F = 5.11, p = 0.0415). There was no significant lack of fit (p = 0.5912), thus establishing the model's adequacy. The polynomial equation for theoretical plates of coded factors was: Theoretical Plates = 6071.65 + 403.25A + 207.50B + 198.25C The positive values for all three parameters showed that the number of theoretical plates increased as the proportion of IPA increased, the flow rate increased and the column temperature increased, with the largest effect being for the IPA proportion (coefficient = 403.25). Planar surfaces of the contour plots and the 3-D response surface plots (Figures 2 C1-C3 and 3 C1-C3) showed a generally increasing surface as both the proportion of organic modifier (IPA) and column temperature increased, and the maximum theoretical plate counts were found at the highest extremes of the IPA proportion, which corroborated that column efficiency is mainly determined by optimizing the organic modifier.

Table 5. Fit summary of Box-Behnken Design for optimization of RP-HPLC method for clevudine estimation.

Response	Model	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	Remarks
Retention Time (Y_1)	Linear	0.0018	0.1195	0.5969	0.3505	Suggested
Asymmetry Factor (Y_2)	Quadratic	8.00×10^{-6}	0.3329	0.9896	0.9575	Suggested
Theoretical Plates (Y_3)	Linear	0.0008	0.5912	0.6434	0.5868	Suggested

Table 6. ANOVA summary of selected models for all responses from Box-Behnken Design optimization of RP-HPLC method for clevudine.

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
Retention Time (Y_1)						
Model	1.0791	3	0.3597	8.90	0.0018	Significant
A-IPA Proportion	0.5050	1	0.5050	12.49	0.0037	
B-Flow Rate	0.3003	1	0.3003	7.43	0.0173	
C-Column Temperature	0.2738	1	0.2738	6.77	0.0219	
Residual	0.5255	13	0.0404	—	—	
Lack of Fit	0.4664	9	0.0518	3.51	0.1195	Not Significant
Pure Error	0.0591	4	0.0148	—	—	
Asymmetry Factor (Y_2)						
Model	0.1320	9	0.0147	169.66	2.36×10^{-7}	Significant
A-IPA Proportion	0.0561	1	0.0561	649.24	3.66×10^{-8}	
B-Flow Rate	0.0378	1	0.0378	437.50	1.44×10^{-7}	
C-Column Temperature	0.0145	1	0.0145	167.19	3.85×10^{-6}	
AB	0.0004	1	0.0004	4.63	0.0685	
AC	0.0006	1	0.0006	7.23	0.0311	
BC	0.0013	1	0.0013	14.17	0.0070	
A^2	0.0023	1	0.0023	26.90	0.0013	
B^2	0.0099	1	0.0099	114.59	1.36×10^{-5}	
C^2	0.0071	1	0.0071	81.89	4.12×10^{-5}	
Residual	0.0006	7	0.0001	—	—	
Lack of Fit	0.0003	3	0.0001	1.55	0.3329	Not Significant
Pure Error	0.0003	4	0.0001	—	—	
Theoretical Plates (Y_3)						
Model	1959759.00	3	653253.00	10.62	0.0008	Significant
A-IPA Proportion	1300884.50	1	1300884.50	21.15	0.0005	
B-Flow Rate	344450.00	1	344450.00	5.60	0.0342	
C-Column Temperature	314424.50	1	314424.50	5.11	0.0415	
Residual	799482.88	13	61498.68	—	—	
Lack of Fit	535202.88	9	59466.99	0.90	0.5912	Not Significant
Pure Error	264280.00	4	66070.00	—	—	

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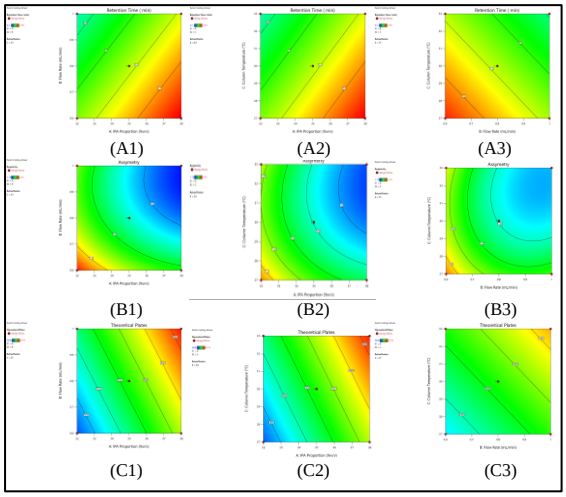

Figure 2. Two-dimensional contour plots depicting the effect of independent variables on chromatographic responses of clevudine optimized by Box-Behnken Design. (A1–A3) and (B1–B3) Combined effects of IPA proportion (X_1) and flow rate (X_2), IPA proportion (X_1) and column temperature (X_3), and flow rate (X_2) and column temperature (X_3) on retention time (Y_1) and asymmetry factor (Y_2), respectively; (C1–C3) corresponding effects on theoretical plates (Y_3). All plots generated at the fixed center-point level of the third variable.

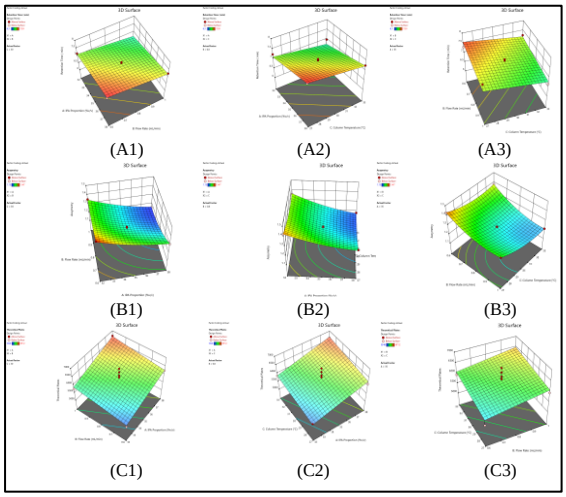

Figure 3. Three-dimensional response surface plots depicting the effect of independent variables on chromatographic responses of clevudine optimized by Box-Behnken Design. (A1–A3) and (B1–B3) Combined effects of IPA proportion (X_1) and flow rate (X_2), IPA proportion (X_1) and column temperature (X_3), and flow rate (X_2) and column temperature (X_3) on retention time (Y_1) and asymmetry factor (Y_2), respectively; (C1–C3) corresponding effects on theoretical plates (Y_3). Surface gradient and curvature depict the magnitude and nature of factor influence at the fixed center-point level of the third variable.

Validation of statistical model:

The BBD model was checked by running an experimental test at the predicted optimum conditions (Table 7) with a desirability value of 1.0. The experimental values of retention time (7.25 min), asymmetry factor (1.15) and theoretical plates (6712) were in good agreement with the predicted values (6.933 min, 1.155 and 6712.002) and the percentage relative errors were 4.57%, 0.43% and 0.00% respectively, indicating the excellent predictive accuracy of the developed model.

Table 7: Validation of Optimized Chromatographic Conditions Predicted by Box-Behnken Design

Run	Response	Predicted value	Experimental value	% Relative error	Desirability
15	Retention Time	6.933	7.25	4.57	1
	Asymmetry	1.155	1.15	0.43	
	Theoretical Plates	6712.002	6712	0.00	

Validation of Developed Methods:

System Suitability:

System suitability data for six consecutive injections of the clevudine standard (100 µg/mL) are summarised in Table 8. Mean retention time, peak area, tailing factor, and theoretical plate count were 7.25 min, 84269 mAU, 1.155, and 6703, with corresponding %RSD values of 0.14%, 0.08%, 0.50%, and 0.09%. All parameters remained within the prescribed acceptance limits, confirming reproducible and stable chromatographic performance under the optimised analytical conditions.

Table 8: System Suitability Parameters of Optimized RP-HPLC Method for Clevudine

Sr. No.	Injection	Retention Time (min)	Peak Area (mAU)	Tailing Factor	Theoretical Plates (N)
1	Injection 1	7.24	84218	1.16	6698
2	Injection 2	7.26	84356	1.15	6712
3	Injection 3	7.25	84284	1.16	6705
4	Injection 4	7.23	84196	1.15	6694
5	Injection 5	7.26	84312	1.16	6708
6	Injection 6	7.24	84248	1.15	6700
	Mean	**7.25**	**84269**	**1.155**	**6703**
	%RSD	**0.14**	**0.08**	**0.50**	**0.09**

Specificity and Peak Purity:

Specificity data are presented in Table 9. Chromatographic analysis of the blank and placebo solutions revealed no interfering signals at the retention time of clevudine, establishing the selectivity of the analytical procedure. Peak spectral homogeneity was further evaluated using a photodiode array (PDA) detector; the measured purity angle of 0.142 was markedly lower than the purity threshold of 0.318, demonstrating the absence of co-eluting species across the entire clevudine peak.

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Table 9: Specificity and Peak Purity Results for Clevudine

Sr. No.	Solution Injected	Retention Time (min)	Peak Observed at RT of Clevudine	Peak Purity Angle	Peak Purity Threshold
1	Blank (Ammonium Formate Buffer pH 3.5)	--	Absent	--	--
2	Placebo Solution	--	Absent	--	--
3	Standard Solution of Clevudine	7.25	Present	0.142	0.318

Forced Degradation Studies:

The results of forced degradation studies are shown in Table 10. The degradation was the greatest in alkaline (8.14%), acid (6.58%), oxidative (5.82%), photolytic (4.38%) and thermal (2.76%) stress. The retention time of clevudine was also found to be same in all the situations (7.25 min) and the degradation products were well separated, which demonstrated that the developed RP-HPLC method possesses the characteristic of being a stability indicating method.

Table 10: Results of Forced Degradation Studies of Clevudine

Sr. No.	Stress Condition	Stress Applied	Retention Time (min)	% Assay	% Degradation
1	Unstressed Control	--	7.25	100.00	--
2	Acid Degradation	1N HCl, 60°C, 1 hr	7.25	93.42	6.58
3	Alkaline Degradation	1N NaOH, 60°C, 1 hr	7.25	91.86	8.14
4	Oxidative Degradation	3% H_2O_2, RT, 1 hr	7.25	94.18	5.82
5	Thermal Degradation	105°C, 1 hr	7.25	97.24	2.76
6	Photolytic Degradation	UV 254 nm, 1.2 × 10^6 lux hr	7.25	95.62	4.38

Linearity:

The linearity data of clevudine in the concentration range of 20–140 µg/mL are shown in Table 11 and Figure 4. The plot of concentration versus mean peak area was linear with a regression equation of y = 842.24x + 131.83 and correlation coefficient (r^2) of 0.9999 which implies that there was excellent linearity and proportional response of the detector over the entire analytical range examined.

Table 11: Linearity Data for Clevudine by RP-HPLC

Sr. No.	Concentration (µg/mL)	Mean Peak Area (mAU)
1	20	16842
2	40	33648
3	60	50524
4	80	67318
5	100	84269
6	120	101124
7	140	117986
Slope		**842.24**
Intercept		**131.83**
Correlation Coefficient (r^2)		**0.9999**

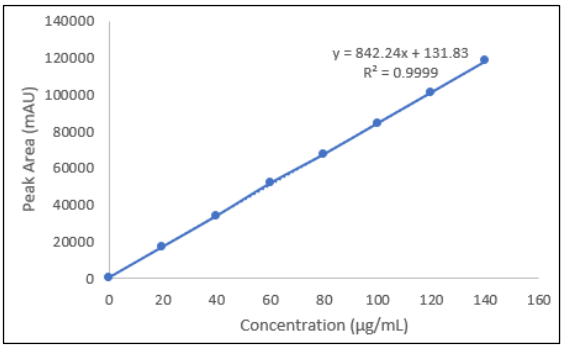

Figure 4: Linearity graph

Limit of Detection and Limit of Quantification:

Sensitivity parameters (LOD and LOQ) are reported in Table 12, derived in accordance with ICH Q2(R2) recommendations. Values of 0.189 µg/mL and 0.573 µg/mL were obtained for the LOD and LOQ, respectively, consistent with the theoretical estimates and corroborated experimentally through signal-to-noise measurements of 3.2 and 10.4 at the respective limits. These findings attest to the high analytical sensitivity of the method, enabling reliable detection and quantification of clevudine at trace concentration levels.

Table 12: LOD and LOQ for Clevudine by RP-HPLC

Sr. No.	Parameter	Value
1	Slope of Calibration Curve (S)	842.48
2	Standard Deviation of Y-intercept (σ)	48.26
3	LOD (3.3 × σ/S)	0.189 µg/mL
4	LOQ (10 × σ/S)	0.573 µg/mL
5	Signal to Noise Ratio at LOD	3.2
6	Signal to Noise Ratio at LOQ	10.4

Accuracy:

The results for accuracy, obtained by the standard addition method, are summarized in Table 13 and presented at three concentration levels. Mean percentage recoveries of 99.36%, 99.82%, and

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100.16% were obtained at 50%, 100%, and 150% levels respectively, with %RSD values not exceeding 0.44% at any level. The average percent recovery of 99.78% showed that there was no significant systematic error in the developed method.

Table 13: Accuracy Data for Clevudine by RP-HPLC (n=3)

Sr. No.	Level	Amount Added (µg/mL)	Amount Found (µg/mL)	% Recovery	%RSD
1	50%	50	49.68	99.36	0.42
2	100 %	100	99.82	99.82	0.38
3	150 %	150	150.24	100.16	0.44
Mean % Recovery				**99.78**	

Precision:

The repeatability and intermediate precision data are shown on Tables 14 & 15. The %RSD values of repeatability were 0.08% for the peak area and 0.07% for percentage assay using six replicate injections. The intermediate precision for three days and two analysts produced an overall %RSD of 0.11 which is satisfactory and shows that variations between day-to-day, analyst-to-analyst and instrument-to-instrument did not affect method performance, since all values are below the acceptance limit of 2.0%. The repeatability data for clevudine using RP-HPLC are given in table 14.

Table 14: Repeatability Data for Clevudine by RP-HPLC (n=6)

Sr. No.	Concentration (µg/mL)	Peak Area (mAU)	% Assay
1	100	84218	99.94
2	100	84356	100.10
3	100	84284	100.02
4	100	84196	99.91
5	100	84312	100.05
6	100	84248	99.97
Mean		**84269**	**100.00**
%RSD		**0.08**	**0.07**

Table 15: Intermediate Precision Data for Clevudine by RP-HPLC

Sr. No.	Day	Analyst	Mean Peak Area (mAU)	%RSD	% Assay
1	Day 1	Analyst 1	84269	0.08	100.00
2	Day 2	Analyst 1	84182	0.12	99.90
3	Day 3	Analyst 2	84318	0.14	100.06
Overall %RSD				**0.11**	

Robustness:

Robustness data which were obtained under intentionally changed chromatographic conditions are shown in Table 16. Changes in buffer pH, column temp, flow rate, and IPA proportion resulted in relatively small changes in the retention time, tailing factor, theoretical plates, and peak area %RSD, with all %RSDs being below 0.18%. All the parameters of the system indicated system suitability and were identical in all the varied conditions, which showed the robustness and reliability of the developed method.

Table 16: Robustness Data for Clevudine by RP-HPLC

Sr. No.	Parameter Varied	Condition	Retention Time (min)	Tailing Factor	Theoretical Plates (N)	%RSD of Peak Area
1	Optimized Method	--	7.25	1.15	6712	0.08
2	IPA Proportion	+2% (37%)	7.18	1.16	6698	0.14
3	IPA Proportion	-2% (33%)	7.34	1.18	6642	0.18
4	Flow Rate	+0.1 (0.9 mL/min)	7.18	1.16	6684	0.12
5	Flow Rate	-0.1 (0.7 mL/min)	7.36	1.17	6658	0.16
6	Column Temperature	+5°C (40°C)	7.18	1.14	6728	0.11
7	Column Temperature	-5°C (30°C)	7.34	1.17	6648	0.15
8	Buffer pH	+0.2 (pH 3.7)	7.22	1.16	6692	0.13
9	Buffer pH	-0.2 (pH 3.3)	7.28	1.17	6674	0.17

Solution Stability:

Table 17 shows solution stability data of clevudine standard solution and sample solution in bench-top and refrigeration. The maximum percentage change in peak area under bench-top conditions was 0.51% at 48 h, but was only 0.13% at 48 h under refrigeration (2–8°C). The results indicated good stability of the solution within the two limits of the ±2.0% acceptance limit, for both storage conditions, up to 48 h.

Table 17: Solution Stability Data for Clevudine Standard and Sample Solutions

Time Point	Mean Peak Area (mAU)	% Change from Initial
Initial (0 hr)	84269	--
6 hrs	84186	0.10
12 hrs	84124	0.17
24 hrs	84018	0.30
48 hrs	83842	0.51
Initial (0 hr)	84269	--

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6 hrs	84248	0.02
12 hrs	84224	0.05
24 hrs	84198	0.08
48 hrs	84162	0.13
		≤2.0%

Filter Compatibility:

The results of the tests for filter compatibility are summarized in Table 18. The difference in the peak areas obtained from clevudine solutions by filtration using a nylon membrane, which has a pore size of 0.45 µm, and by centrifugation was not more than 0.10%, which is within the acceptance limit of 2.0%. The results showed that no adsorption or leaching occurred for clevudine during sample preparation with nylon membrane syringe filter.

Table 18: Filter Compatibility Data for Clevudine

Sr. No.	Method of Preparation	Mean Peak Area (mAU)	% Difference
1	Centrifugation (Reference)	84269	--
2	Filtration through 0.45 µm Nylon Membrane Filter	84186	0.10

Application:

The results obtained in the quantitative estimation of clevudine from the commercial formulation of tablet are presented in Table 19. The validated method was found to be suitable for routine quality control analysis of clevudine in pharmaceutical formulations as the percentage label claim values of 99.47%, 100.40% and 99.87% obtained for three replicate preparations were within acceptance limit of 98.0–102.0% with %RSD value of 0.39%.

Table 19: Application of Validated RP-HPLC Method for Estimation of Clevudine in Tablet Formulation

Sr. No.	Label Claim (mg/tablet)	Amount Found (mg/tablet)	% Label Claim	%RSD
1	30	29.84	99.47	0.38
2	30	30.12	100.40	0.42
3	30	29.96	99.87	0.36
	Mean	**29.97**	**99.91**	**0.39**

DISCUSSION:

The developed RP-HPLC method for clevudine showed excellent system suitability parameters, such as retention time (7.25 min), tailing factor (1.155), theoretical plates (6703) (Table 8), which fulfilled all the ICH recommended system suitability criteria. The organic modifier used in the mobile phase was isopropyl alcohol, which is also used in conventional HPLC methods, but is not typically used for pharmaceutical analysis; this is a conscious green chemistry approach that aligns with increased consideration given to environmentally friendly mobile phase solvent selection in pharmaceutical analysis [36]. Three critical variables, namely the proportion of IPA, flow rate, and column temperature were optimized simultaneously with the help of RSM to get statistically meaningful polynomial models for all three responses (Tables 5 and 6), especially the polynomial model for asymmetry factor showed high predictive accuracy (adjusted R2 = 0.9896). This DoE-based method is similar to recently published antiviral drug description analyses using BBD for chromatographic optimization [37], and is an added benefit to the traditional one factor at a time methods by systematically describing the interactions between variables. The method was fully validated according to ICH Q2(R2) guidelines and it showed linearity with $R^2 = 0.9999$ (Table 11), mean accuracy of 99.78% (Table 13), LOD of 0.189 µg/mL and LOQ of 0.573 µg/mL (Table 12) that are comparable to or better than the recently reported HPLC method for structurally related nucleoside analogues [18]. The stability-indicating ability of the method was verified by performing forced degradation studies, in which the highest amount of degradation was found under alkaline stress with 8.14% degradation and all degradation peaks were completely separated from the main peak (Table 10), satisfying the ICH Q1B photostability requirements [38].

The robustness test verified the reliability of the method when deliberately varying the proportion of IPA, flow rate, column temperature and buffer pH, with all the %RSD values < 0.18% (Table 16) similar to the results obtained for the green HPLC methods developed for antiviral agents [39]. Solution stability data (Table 17) showed that both the standard and sample solutions were stable for up to 48 h when stored at bench-top and refrigerated temperatures, respectively, with the maximum peak area difference of 0.51% and 0.13% being well within the acceptance limit of ±2.0%. Table 18 showed that the 0.45 µm nylon membrane filter was compatible with the filter and that the peak area difference was 0.10% which eliminated a potential source of analytical error. The validated method has been successfully used to a commercial clevudine tablet formulation, with a mean value of 99.91 % and %RSD of 0.39 (Table 19), a study that indicates that the method is directly applicable to routine pharmaceutical quality control. The use of IPA as mobile phase organic modifier provides a significant improvement in reducing laboratory waste and occupational health and safety risks compared to acetonitrile based methods, as is becoming more widely appreciated and measurable by greenness assessment tools like AGREE [40]. and GAPI that measure adherence to the twelve principles of green analytical chemistry. The present method addresses a significant analytical void, represents the first fully validated, stability-indicating, green chemistry compliant, DoE optimized RP-HPLC method for clevudine that is directly relevant for pharmaceutical

quality control in areas where the prevalence of HBV is high [41].

CONCLUSION:

The quantitative estimation of clevudine from its bulk drug and tablet dosage form by simple, sensitive, precise, accurate and stability indicating RP-HPLC method has been successfully developed and validated using green chemistry approach. Optimization of chromatographic conditions was done in a systematic way using Box-Behnken Design under Response Surface Methodology which resulted in a robust and statistically validated method with high predictive accuracy. The use of isopropyl alcohol as the organic modifier, instead of the traditional hazardous solvents used in the analysis, was shown to be a viable and environmentally friendly analytical approach. The method was validated with a commercial tablet formulation containing the same drug substance and satisfactory validation parameters were obtained, and this method was used successfully to analyze the sample, which was suitable for routine pharmaceutical quality control. The validated green method has a great clinical value for precise pharmacokinetic monitoring and quality surveillance of clevudine in high HBV-burden areas, and it should be further expanded to be applied in biological matrices and in vivo pharmacokinetic investigations for supporting the application in vivo.

ABBREVIATIONS:

RP-HPLC: Reverse-Phase High-Performance Liquid Chromatography; ICH: International Council for Harmonisation; BBD: Box-Behnken Design; RSM: Response Surface Methodology; IPA: Isopropyl Alcohol; UV: Ultraviolet; LOD: Limit of Detection; LOQ: Limit of Quantification; %RSD: Percentage Relative Standard Deviation; λmax: Wavelength of Maximum Absorbance; API: Active Pharmaceutical Ingredient; PDA: Photodiode Array; DoE: Design of Experiments; ANOVA: Analysis of Variance; HBV: Hepatitis B Virus; AGREE: Analytical GREEnness Metric; GAPI: Green Analytical Procedure Index; QbD: Quality by Design; RT: Retention Time; r^2: Correlation Coefficient.

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