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Future Journal of Pharmaceutical Sciences (2024) 10:142
https://doi.org/10.1186/s43094-024-00716-z

Future Journal of Pharmaceutical Sciences

RESEARCH Open Access

Simultaneous estimation of nebivolol hydrochloride and amlodipine besylate in human plasma employing an innovative HPLC chromatographic method

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Abstract

Background This study developed and validated a simple, robust, and cost-effective RP-HPLC bioanalytical method for the determination of nebivolol hydrochloride (NBH) and amlodipine besylate (AMB) in human plasma. Briefly, NBH and AMB were extracted from plasma through protein precipitation using 5% formic acid and acetonitrile. Chromatographic separation was achieved using an Inertsil ODS-3 V column (150 mm × 4.6 mm, 5 µm) with a mobile phase composed of acetonitrile and buffer (40:60, v/v). The analysis was conducted using UV detection at 215 nm.

Results The bioanalytical method demonstrated linearity for NBH (4.50–180.12 µg/mL) and AMB (3.50–140.06 µg/mL). It exhibited good selectivity and sensitivity, with LLOQ responses within ≤ 20% of the analyte signal. Accuracy and precision were within acceptable limits. The extraction recovery from human plasma showed a CV (%) of 1.15% for NBH and 1.35% for AMB, indicating consistent recovery rates. Stability studies on drug-spiked human plasma at LQC and HQC levels confirmed the stability of the drugs under various conditions.

Conclusion The present bioanalytical method successfully quantified NBH and AMB simultaneously in plasma samples. It demonstrated suitability, supported by high recovery rates and low relative standard deviations. With its proven linearity, accuracy, and precision, this technique is well suited for drug identification in plasma samples.

Keywords Nebivolol hydrochloride, Amlodipine besylate, Human plasma, HPLC, Method validation

Background

The modern lifestyle, coupled with societal and work-related stress, has led to a significant rise in blood pressure (BP) levels beyond the normal range. This increase has resulted in various harmful health consequences, including cerebral hemorrhage, hypertensive heart failure, progressive renal failure, accelerated atherosclerosis, and related complications in the aorta, coronary arteries, and cerebral arteries. As a result, hypertension remains a critical global clinical challenge, contributing to the unfortunate deaths of 7.6 million individuals annually worldwide [1]. In managing hypertension, a broad spectrum of pharmacological agents from various classes is utilized. Among these, β1 receptor blockers and calcium channel blockers have proven to be particularly effective interventions. These agents demonstrate great promise in managing hypertension by addressing both the direct effects of elevated blood pressure and the secondary complications associated with atherosclerosis. Nebivolol hydrochloride (NBH), a widely used β1 receptor blocker, is employed in the treatment of hypertension, either as a monotherapy or in combination with other medications [2]. Chemically, NBH is represented as (1R)-1-[(2R)-6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl-2-

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{[(2R)-2-[(2S)-6-fluoro-3,4-dihydro-2H-benzopyran-2-yl] hydroxyethyl]amino}ethan-1-ol hydrochloride (Fig. 1A). The pharmacological action of NBH involves its adrenergic agonist activity at the beta-3 adrenergic receptor, which leads to the activation of endothelial nitric oxide synthase. This activation results in vasodilation and a subsequent reduction in blood pressure [3]. Moreover, NBH effectively reduces peripheral vascular resistance while significantly enhancing stroke volume, thereby helping to maintain optimal cardiac output [4, 5].

Amlodipine besylate (AMB) is an oral dihydropyridine calcium channel blocker widely used for its therapeutic effects. Chemically, AMB is identified as 3-ethyl-5-methyl(±)-2-(2-aminoethoxymethyl)-4-(O-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridine dicarboxylate (Fig. 1). AMB exerts its pharmacological effects by inhibiting the transmembrane entry of calcium ions, which modulates the function of vascular smooth muscle cells [6]. This pharmacological action leads to vasodilation and a reduction in peripheral vascular resistance, ultimately lowering blood pressure. AMB has a longer half-life of 30 to 50 h compared to NBH, which allows for once-daily dosing and offers practical advantages for patient compliance [7].

AMB is officially recognized in the Indian Pharmacopoeia (IP), British Pharmacopoeia (BP), and European Pharmacopoeia (EP). In the Indian pharmaceutical market, AMB is available in tablet form under the brand names AMCARD and AMCHECK, with strengths of 2.5, 5, and 10 mg. These tablets are primarily prescribed for hypertension and to prevent angina (heart-related chest pain). By lowering blood pressure and reducing cardiac strain, they effectively mitigate the risk of heart attacks and strokes [8].

Combination dosage forms of NBH and AMB are available under various brand names, such as Amlopres-NB, Nebilong-AM, and Nebistar-SA tablets. These combination products are recommended when single-drug therapy is insufficient for controlling hypertension. By effectively reducing blood pressure, these tablets play a crucial role in minimizing the risk of future heart attacks and strokes [9].

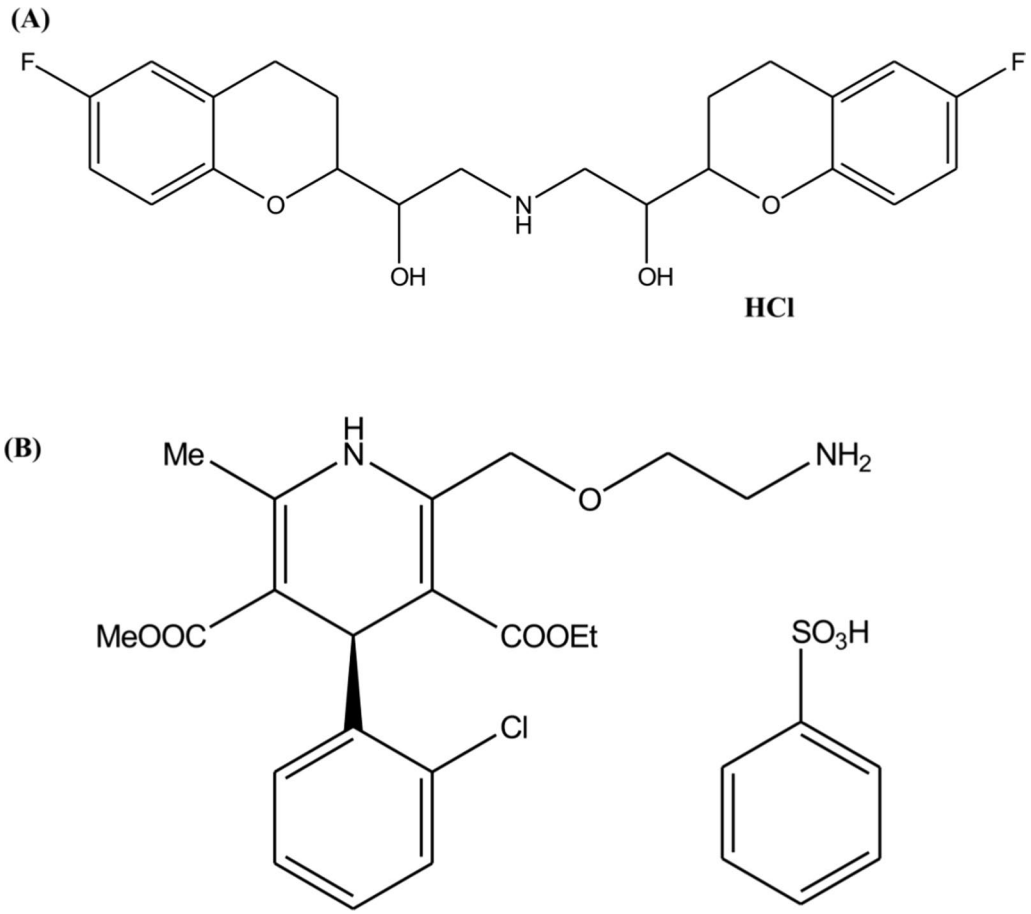

Fig. 1 Structures of NBH **A** and AMB **B**

A comprehensive literature review revealed the use of various analytical techniques for AMB, such as LC–MS [10–12] and HPLC [13, 14], and for NBH such as HPLC [4] and UV spectrophotometry [15]. In the case of NBH and AMB in combination, UV spectrophotometry [16, 17], spectrofluorimetry [18], HPLC [8, 17, 19–23], HPTLC [24, 25], and UPLC/MS [26] have been used as analytical methods.

Current research lacks a validated RP-HPLC method for the simultaneous estimation of NBH and AMB in human plasma with protein precipitation extraction. This approach is cost-effective, less time-consuming, and simpler than other extraction methods. Given the increasing use of this drug combination for hypertension treatment, there is a clear need for a more specific, accurate, and efficient method for determining NBH and AMB in human plasma. To address this gap, a new bioanalytical method is being developed and validated using HPLC with protein precipitation for NBH and AMB. This method is anticipated to be simple, cost-effective, and robust, offering a practical solution to the current research deficiency.

Methods

Materials

Nebivolol hydrochloride and amlodipine besylate were received as gift samples from Mylan Laboratory Pvt. Ltd., India. Analytical HPLC-grade methanol and formic acid were purchased from Merck Specialties Pvt. Ltd., Mumbai, India. Orthophosphoric acid was procured from Qualigens (Thermo Fisher Scientific). Acetonitrile and trifluoroacetic acid (TFA) were acquired from Merck. Ultra-pure HPLC-grade water was obtained from Siddhi Lab, India.

Instrumentation

The method development and validation process utilized an HPLC system comprising a 1260 Infinity II module, which included a G7111A pump, degasser, G7115A detector, and G7129A autoinjector. Analysis and separation were conducted using an Inertsil ODS-3 V column (150 mm × 4.6 mm, 5 µm). Sonication was performed with a Bio Technics India 12L300H ultrasonicator, and material weighing was carried out using an Aczet CY224 model.

Chromatographic conditions for HPLC

The analytical method was developed by preparing various solutions and optimizing the chromatographic conditions, including the composition of the mobile phase [27].

Preparation of standard stock solution (A and B)

A weighed amount of 10 mg NBH was carefully added to a 20 mL volumetric flask containing 10 mL of methanol (A). The mixture was sonicated for 10 min, and the volume was then adjusted with methanol to reach a final concentration of 500 µg/mL, resulting in a stock solution. Similarly, a separate 20 mL volumetric flask (B) was used to prepare a 500 µg/mL stock solution of AMB following the same procedure.

Working solution preparation

To prepare the working solution of NBH and AMB, 0.8 mL of each stock solution was pipetted from flasks A and B, respectively, into a 20 mL volumetric flask. The solution was then diluted with methanol to the 20 mL mark, yielding a final concentration of 20 µg/mL for both compounds.

Mobile phase preparation

The mobile phase composition was optimized by varying the ratios of acetonitrile, methanol, water, and buffer. Initially, a column with a 5 µm particle size was used for separation, and various modifiers such as chloroform, tetrahydrofuran (THF), ethanol, isopropyl alcohol (IPA), n-hexane, dichloromethane, and trifluoroacetic acid (TFA) were tested. The final mobile phase was established using a combination of acetonitrile and 0.1% TFA as the buffer, resulting in an acetonitrile–buffer ratio of 40:60 (v/v). The injection volume for all samples was set at 20 µL, and the mobile phase flow rate was maintained at 1 mL/min in isocratic mode.

Plasma sample preparation

The extraction and preparation process for plasma samples is outlined as follows [28].

Blank plasma preparation

To prepare the samples, 475 µL of human plasma was combined with 20 µL of 5% formic acid, and 1 mL of acetonitrile was added. The mixture was vortexed for 2 min and then centrifuged at 4000 rpm for 3 min. The resulting supernatant was filtered through a 0.22 µm filter, and 0.5 mL of the filtered supernatant was extracted for subsequent chromatographic analysis.

Plasma sample preparation with 5% spiking

For a 5% spiking of the sample in plasma, 475 µL of human plasma was spiked with 12.5 µL of NBH and 12.5 µL of AMB, both dissolved in methanol. The mixture was transferred to a Tarson tube and centrifuged at 4000 rpm for 10 min. The plasma was carefully

separated using disposable polypropylene tips and stored in a deep freezer at −20 ± 2 °C until further analysis.

5% spiked plasma sample extraction preparation

The protein precipitation method was used to extract the plasma samples. To prepare the samples, 500 µL of human plasma was mixed with 20 µL of 5% formic acid and the volume was adjusted to 1 mL with acetonitrile. The mixture was allowed to stand for 2 min and then thoroughly mixed. The samples were centrifuged at 4000 rpm for 3 min. The resulting supernatant, which contained the target drugs, was carefully separated. This supernatant was then dried and reconstituted with 1 mL of the mobile phase. The sample was filtered through a 0.22 µm filter, and 0.5 mL of the filtered supernatant was transferred for chromatographic analysis.

Quality control sample preparation

The quality control (QC) samples were prepared using procedures similar to those for the NBH and AMB stock solutions. To prepare the stocks, 200 mg of AMB and 250 mg of NBH were dissolved in 25 mL of methanol. From these solutions, 1 mL was pipetted and diluted with human plasma. Four concentrations of QC samples were prepared for NBH: 4.51, 13.52, 90.11, and 150.19 µg/mL, representing the LLOQ, LQC, middle-quality control (MQC), and HQC standards, respectively. Similarly, AMB QC samples were prepared at concentrations of 3.50, 10.51, 70.06, and 116.09 µg/mL for the LLOQ, LQC, MQC, and HQC standards, respectively [28].

Chromatographic conditions

In the HPLC analysis, specific chromatographic conditions were established. The analysis employed an Inertsil ODS-3 V column with dimensions of 150 mm × 4.6 mm and a particle size of 5 µm. The mobile phase was a mixture of acetonitrile and buffer in a 40:60% v/v ratio. A UV detector was used, set to a wavelength of 215 nm. The flow rate was maintained at 1.0 mL/min, and a 20 µL injection volume was utilized. The autosampler temperature was set appropriately for the analysis. To ensure the clarity of the mobile phase, it was filtered through 0.45 µm pore-size membranes and degassed. Throughout the study, the mobile phase was continuously degassed using a degasser connected to the column and was pumped in isocratic mode at a flow rate of 1 mL/min. The HPLC system received the 20 µL sample, which was then detected by the UV detector.

Method validation

Following the guidelines set by the USFDA and the EMEA, the developed and optimized method for the simultaneous determination of NBH and AMB underwent a comprehensive validation process. The validation assessed the method's linearity, selectivity, sensitivity, accuracy, precision, recovery, and stability [29–31].

Linearity

Calibration curves were constructed to evaluate the linearity of NBH and AMB. The calibration ranges for NBH and AMB were established as 4.50–180.12 µg/mL and 3.50–140.06 µg/mL, respectively. Drug standard solutions were spiked into human plasma to create calibration standards. The relationship between the analyte peak area and concentration was used to generate the calibration curves [32].

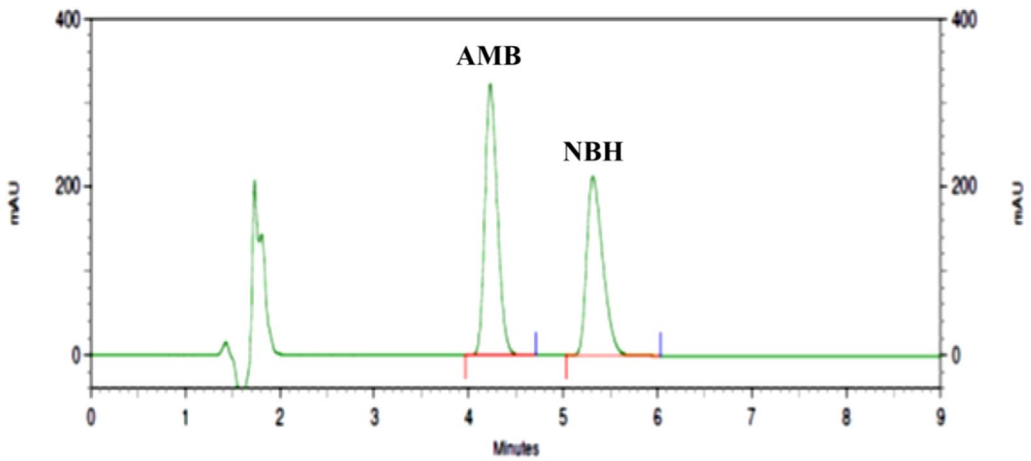

Fig. 2 HPLC chromatogram of NBH and AMB (100 PPM each)

Selectivity and sensitivity

The selectivity and sensitivity of the method were evaluated by analyzing six batches of human blank plasma, as well as hemolyzed and lipemic plasma samples. This assessment aimed to demonstrate the absence of chromatographic interference at the retention times of NBH and AMB. Acceptance criterion for selectivity was set at ≤ 20% of the response observed at the lower limit of quantification.

Extraction recovery

The extraction recovery of NBH and AMB was assessed as a percentage to evaluate the efficiency of the analytical procedure. This was done by comparing the response of QC samples with post-extraction spiked QC samples at the same concentration. The extraction recovery for NBH was calculated to be 97.69%, while for AMB it was 97.19%.

Accuracy and precision

Six replicates of QC samples were analyzed at four different concentration levels on three separate days to evaluate the intra-day and inter-day precision and accuracy of NBH and AMB in plasma samples. The assessment included QC samples of NBH with concentrations of 4.51 µg/mL (LLOQ), 13.52 µg/mL (LQC), 90.11 µg/mL (MQC), and 150.19 µg/mL (HQC). Similarly, QC samples of amlodipine besylate were prepared at concentrations of 3.50 µg/mL (LLOQ), 10.51 µg/mL (LQC), 70.06 µg/mL (MQC), and 116.09 µg/mL (HQC). For intra-day precision, the acceptable range was set at 85–115% of the nominal value for all concentrations, except for the LLOQ, which had a required range of 80–120%. The intra-day precision was evaluated based on the percentage relative standard deviation (% RSD), which should not exceed 15%. The LLOQ QC concentration was not to exceed a maximum of 20% RSD. For inter-day precision, the acceptable range was set at ≤ 20% of the nominal value for all concentrations except the LLOQ, where the range was ≤ 20%. The between-run precision (inter-day) criteria required that the RSD of QC samples at

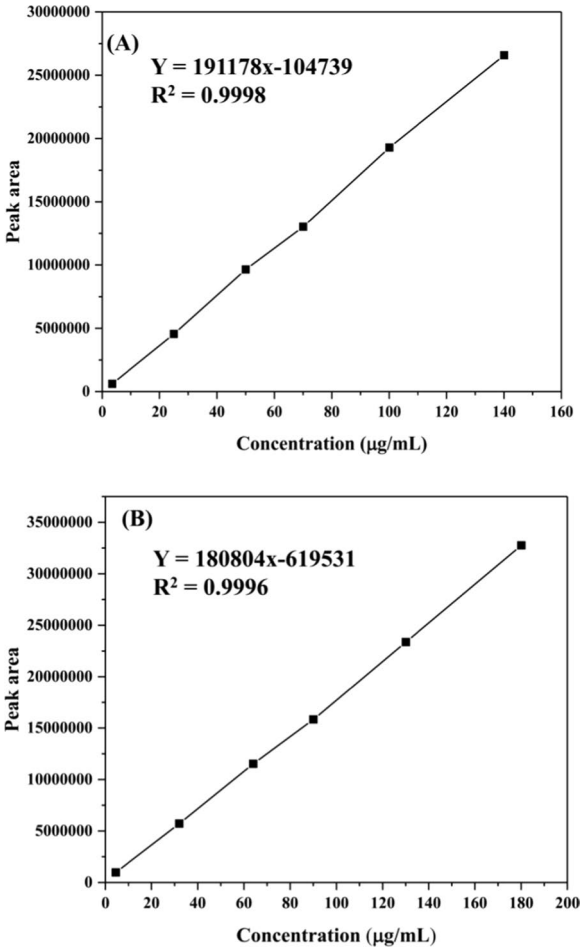

Fig. 3 Linearity of **A** AMB and **B** NBH

Table 1 The summary of the linearity study

Conc. (µg/mL) of AMB	Peak area	Conc (µg/mL) of NBH	Peak area
3.50	610,517	4.50	978,615
25.01	4,551,203	32.02	5,721,633
50.02	9,639,587	64.04	11,526,410
70.03	13,025,698	90.06	15,828,499
100.05	19,286,989	130.08	23,361,794
140.06	26,578,870	180.12	32,761,409
Regression equation	Y = 191,178 x-104739	Regression equation	Y = 180804x-61953
R^2	0.9998	R^2	0.9996
Intercept	− 104,739	**Intercept**	− 61,953
Slope	191,178	**Slope**	180,804

Table 2 Selectivity for LLOQ NBH and AMB

Plasma LOT	Area of blank plasma at R.T. of NBH	Area of blank plasma at R.T. of AMB	Area of NBH	Area of AMB
LOT 1	0	0	1,064,156	615,320
LOT 2	0	0	946,517	646,829
LOT 3	0	0	986,416	628,513
LOT 4	0	0	1,041,497	617,630
LOT 5	0	0	1,029,416	619,527
LOT 6	0	0	986,075	639,856
Hemolyzed	0	0	996,843	626,106
Lipemic	0	0	1,011,261	631,613

Table 3 Sensitivity for LLOQ NBH and AMB

LLOQ	Area of NBH	Area of AMB
	1,021,647	641,507
	986,411	609,631
	999,873	629,504
	1,021,419	610,529
	1,016,522	608,150
	1,005,123	602,934
Mean	1,008,499	617,043
STDV	13,984.6332	15,028.52678
CV (%)	1.39	2.44

each concentration be within ± 2%, except for the LLOQ, which should not exceed 20% [33].

Recovery study and matrix effect

To evaluate the effectiveness of extracting the substances from plasma samples and assess the impact of the matrix, a recovery study was conducted. The substances were first added to blank plasma at standard concentrations (low, medium, and high levels) and analyzed. Solutions without the biological matrix were then prepared and spiked with the same standard concentrations. The ratio of the area of the NBH peak to the AMB peak in samples with and without the biological matrix was determined. The impact of the matrix on the quantification of NBH at the LLOQ was compared between the responses obtained with and without the matrix.

Stability studies

Stability studies of NBH and AMB in plasma samples were conducted at two concentration levels: LQC and HQC. The purpose was to determine the drug concentrations after each storage period and compare them to the initial baseline concentration. The following section details the investigations performed to assess the stability of the samples [34–37].

Bench-top stability

The bench-top stability of NBH and AMB was evaluated using low and high-concentration samples at a temperature of 25 °C. After keeping the samples at room temperature for 6 h, aliquots of the low and high-concentration plasma samples were processed and analyzed by HPLC to determine their respective drug concentrations.

Freeze–thaw stability

The freeze–thaw stability study of NBH and AMB was conducted on plasma samples with low and high concentrations over three cycles within three days. The frozen plasma samples were thawed for 2 h at ambient temperature and then refrozen for 24 h each cycle. The drug concentrations in the samples were analyzed at the end of the third cycle and compared with those at the initial (zero) cycle.

Autosampler stability

In the autosampler stability study, LQC and HQC samples obtained from the precipitate solution were reinjected 24 h after the initial injection. The percentage recovery was calculated by comparing the measured concentrations of the reinjected samples with those of freshly prepared samples.

Stability study of processed sample

The stability study of processed samples included three low-quality control (3LQC) and three high-quality control (3HQC) samples for NBH and AMB. These samples were subjected to a 6 h stability period and then analyzed by injecting them into the HPLC system.

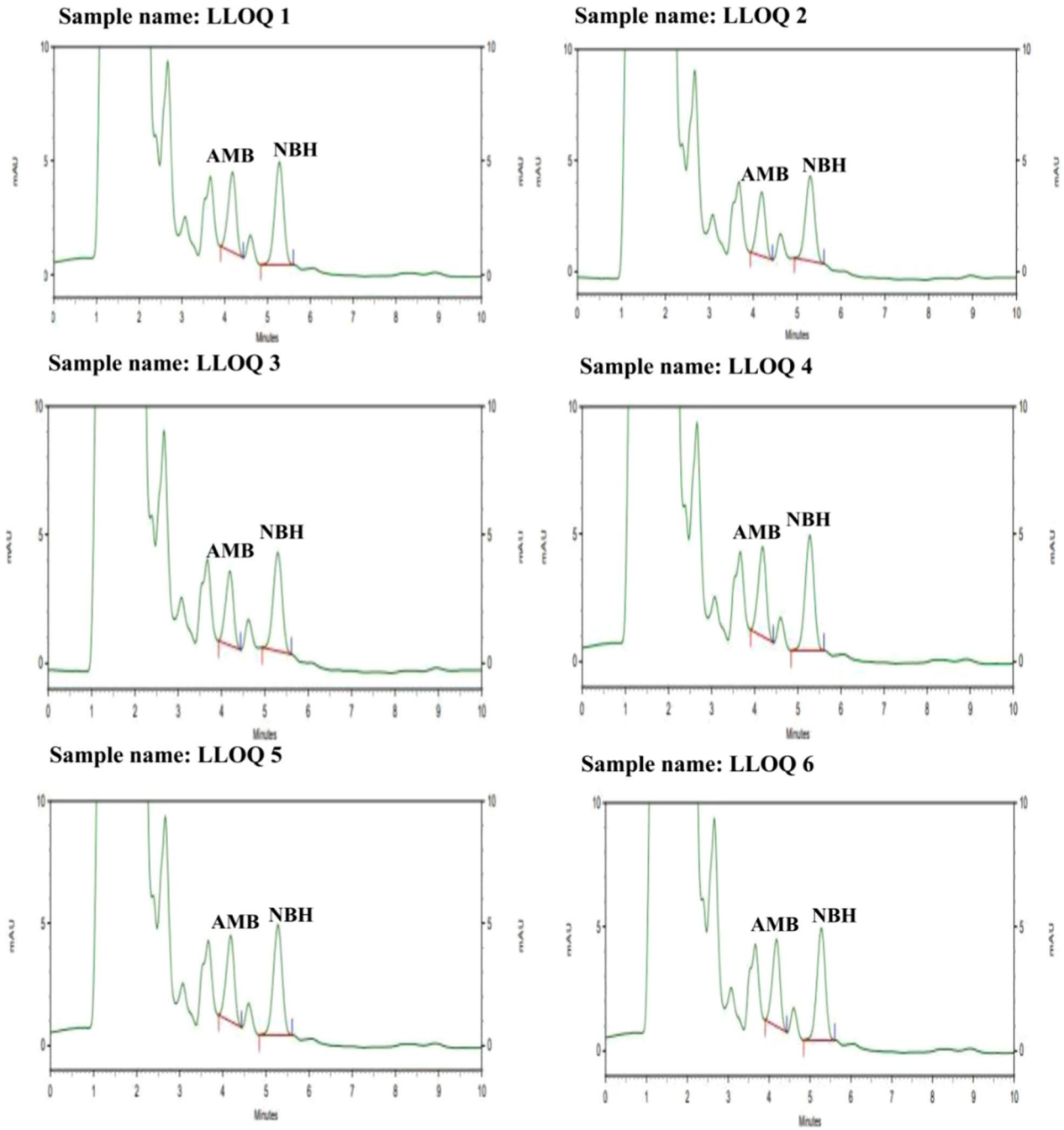

Fig. 4 Sensitivity chromatogram of NBH and AMB

Results

Development of HPLC method for NBH and AMB

The mobile phase, consisting of acetonitrile and buffer in a 40:60 ratio, combined with a column size of 150 mm × 4.6 mm, a particle size of 5 µm, and a flow rate of 1 mL/min, was optimal. Analytes were detected at a wavelength of 215 nm. The chromatograms showed distinct peaks for NBH with a retention time of 5.22 min and for AMB with a retention time of 4.18 min (Fig. 2).

Method validation for NBH and AMB in human plasma

Linearity

Calibration curves for NBH and AMB were constructed to assess linearity. The developed bioanalytical method demonstrated linearity across a concentration range of

Table 4 Recovery study of NBH and AMB in human plasma

QC	% Recovery	Mean recovery	% CV	Overall recovery	Overall % RSD
% Recovery of NBH at 3 levels					
LQC	98.02	97.85	0.18	97.69	1.15
	97.66				
	97.87				
MQC	99.44	97.32	1.89		
	96.38				
	96.14				
HQC	98.19	97.90	1.19		
	96.62				
	98.9				
% Recovery of AMB at 3 levels					
LQC	98.57	98.23	1.19	97.19	1.35
	99.19				
	96.93				
MQC	95.69	96.41	0.64		
	96.73				
	96.8				
HQC	95.28	96.92	1.62		
	98.40				
	97.08				

4.50–180.14 µg/mL for NBH and 3.50–140.06 µg/mL for AMB, with correlation coefficients (R^2) of 0.9996 and 0.9998, respectively (Table 1, Fig. 3).

Selectivity and sensitivity

Regarding selectivity, no interference was observed at the drug retention times, indicating that the method is selective. The selectivity of both drugs is shown in Table 2. The coefficient of variation (CV) for both drugs was calculated to be 2.44% and 1.39%, respectively, further confirming the method's sensitivity. The sensitivity of both drugs is detailed in Table 3, with a graphical representation provided in Fig. 4.

Extraction recovery

Table 4 presents the percentage recovery of NBH and AMB after extraction from human plasma. The overall coefficient of variation (% CV) for NBH and AMB was calculated to be 1.15% and 1.35%, respectively, indicating a high level of extraction recovery. Figure 5 depicts the retention times of NBH and AMB at different concentration levels. Figure 6 displays the chromatogram of both blank plasma and plasma samples containing NBH and AMB.

Accuracy and precision

The accuracy of NBH and AMB in sample solutions was evaluated at four levels: LLOQ, LQC, MQC, and HQC. The % RSD for both drugs was found to be less than 20% at LLOQ and less than 15% at LQC, MQC, and HQC, which is within the acceptable range. Detailed results of the accuracy study are provided in Tables 5 and 6.

The peak areas were recorded for four injections of sample solutions, and the % RSD was calculated for the QC samples at each level. The % RSD values for all concentrations were found to be below 20% at LLOQ and below 15% at LQC, MQC, and HQC, indicating a high level of precision within the acceptable range. Detailed results of the precision assessment are provided in Tables 7 and 8.

Recovery study and matrix effect

NBH was recovered from plasma and other organs using the standard addition method at three different concentration levels (10.50, 70.01, and 116.02 µg/mL) to cover the entire linearity range. The results for the quality control samples at low, medium, and high concentrations are presented in Table 4. The data show that the mean %

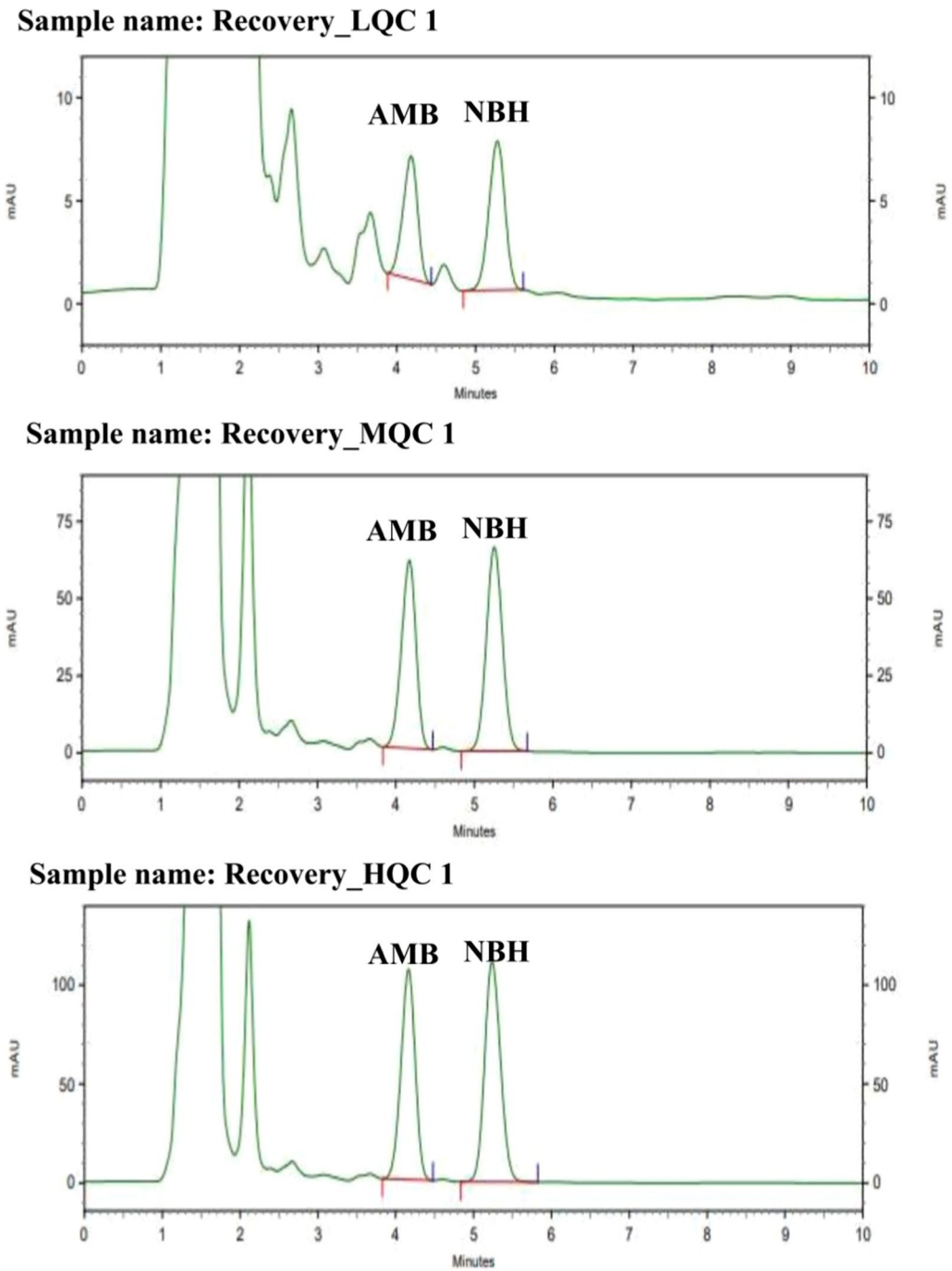

Fig. 5 Recovery chromatogram of NBH and AMB at 3 levels

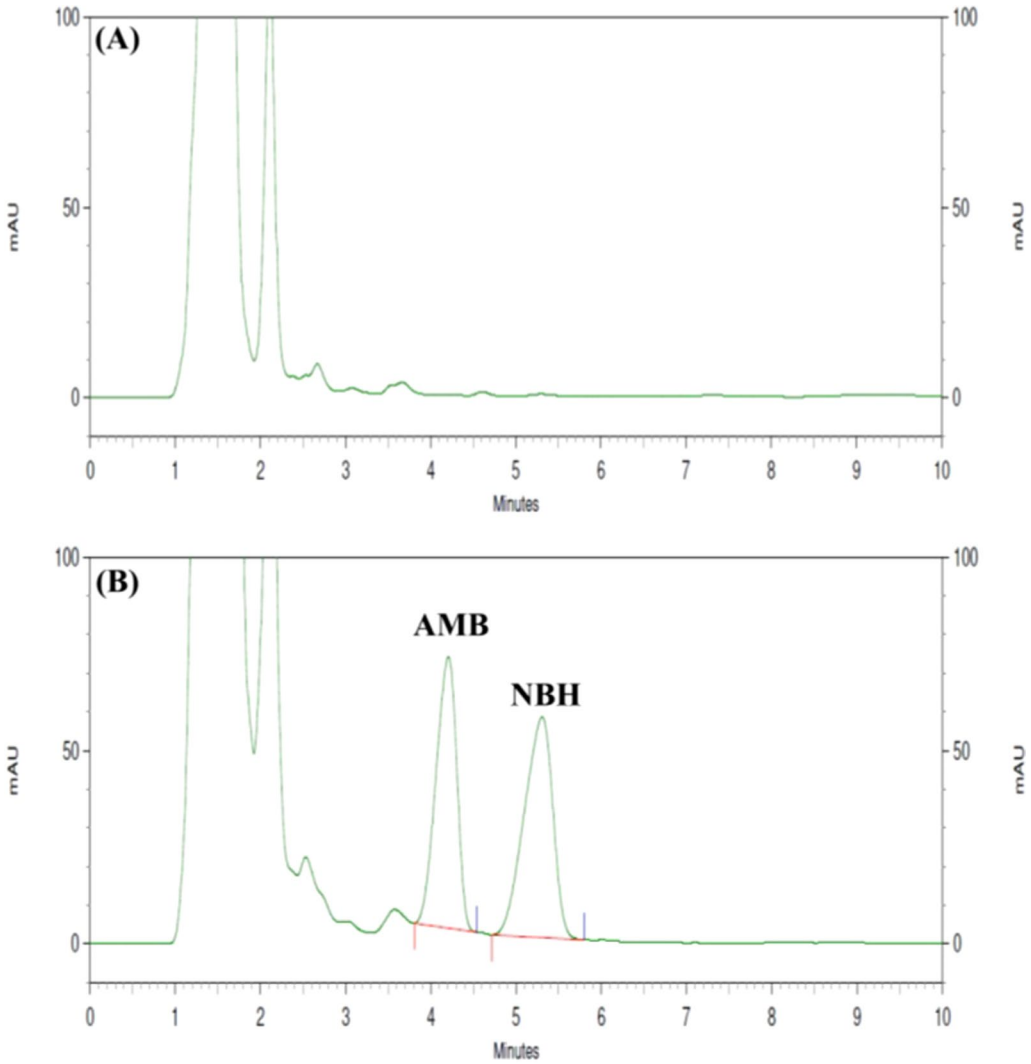

Fig. 6 Chromatogram of blank **A** as well as NBH and AMB **B** in the plasma sample

recovery of NBH from the matrix ranged from 97.32 to 97.90%. Additionally, the results confirmed that there was no matrix interference with NBH in the plasma sample.

Stability studies

In this study, the stability of NBH and AMB in human plasma was evaluated under various conditions, including bench-top stability (25 °C for 6 h), freeze–thaw stability (3 cycles), auto-sampler stability (24 h), and stability of process samples (6 h). The freshly prepared drug areas were compared to the resulting peak areas to assess stability. The results indicated that both drugs remained stable in human plasma. Additionally, the stability of plasma samples containing NBH and AMB at both low and high levels was not affected by freezing and thawing. Table 9 presents the mean % recovery for all drugs under the studied conditions, confirming their stability. The chromatogram of the stability study is depicted in Fig. 7.

Discussion

The development and validation of analytical techniques are crucial to the pharmaceutical product development process. This technical brief highlights the significance of these processes, particularly for pharmaceutical dosage forms [35]. Although the benefits of well-established

Table 5 Summary of accuracy study for QC samples of NBH

Set	QC	Actual concentration (µg/mL)	Recovered concentration (µg/mL)	% accuracy
SET 1	LLOQ 1	4.51	4.85	107.54
	LQC 1	13.52	13.17	97.41
	MQC 1	90.11	85.15	94.50
	HQC 1	150.19	149.37	99.45
SET 2	LLOQ 2	4.51	4.62	102.44
	LQC 2	13.52	13.46	99.56
	MQC 2	90.11	87.56	97.17
	HQC 2	150.19	147.08	97.93
SET 3	LLOQ 3	4.51	4.42	98.00
	LQC 3	13.52	13.58	100.44
	MQC 3	90.11	86.98	96.53
	HQC 3	150.19	152.21	101.34
SET 4	LLOQ 4	4.51	4.68	103.77
	LQC 4	13.52	13.90	102.81
	MQC 4	90.11	85.03	94.36
	HQC 4	150.19	146.83	97.76
SET 5	LLOQ 5	4.51	4.38	97.12
	LQC 5	13.52	13.30	98.37
	MQC 5	90.11	90.11	100.00
	HQC 5	150.19	145.92	97.16
SET 6	LLOQ 6	4.51	4.48	99.33
	LQC 6	13.52	13.40	99.11
	MQC 6	90.11	85.03	94.36
	HQC 6	150.19	145.76	97.05

Table 6 Summary of accuracy study for QC samples of AMB

Set	QC	Actual concentration (µg/mL)	Recovered concentration (µg/mL)	% accuracy
SET 1	LLOQ 1	3.50	3.89	111.14
	LQC 1	10.51	10.46	99.52
	MQC 1	70.06	72.11	102.93
	HQC 1	116.09	115.29	99.31
SET 2	LLOQ 2	3.50	3.81	108.86
	LQC 2	10.51	10.56	100.48
	MQC 2	70.06	74.21	105.92
	HQC 2	116.09	121.68	104.82
SET 3	LLOQ 3	3.50	3.71	106.00
	LQC 3	10.51	10.34	98.38
	MQC 3	70.06	69.54	99.26
	HQC 3	116.09	110.85	95.49
SET 4	LLOQ 4	3.50	3.76	107.43
	LQC 4	10.51	10.60	100.86
	MQC 4	70.06	68.51	97.79
	HQC 4	116.09	115.62	99.60
SET 5	LLOQ 5	3.50	3.98	113.71
	LQC 5	10.51	10.64	101.24
	MQC 5	70.06	74.05	105.70
	HQC 5	116.09	113.90	98.11
SET 6	LLOQ 6	3.50	3.88	110.86
	LQC 6	10.51	10.40	98.95
	MQC 6	70.06	68.50	97.77
	HQC 6	116.09	115.62	99.60

analytical techniques are widely recognized, their potential to significantly enhance the overall efficiency of a development program in terms of time and cost is often underestimated. The validation process involves evaluating the performance of a measurement technique and confirming its accuracy in assessing a specific parameter [36, 38]. A rapid and sensitive RP-HPLC bioanalytical method was developed for the simultaneous determination of NBH and AMB concentrations in human plasma samples. This method utilized optimized analytical parameters to effectively separate the two drugs. The optimized method resulted in peaks with minimal tailing, confirming its suitability. The overlay of NBH and AMB peaks is shown in Fig. 2. Linearity, defined as the ability of an analytical technique to produce results that are directly proportional to the analyte concentration within a specified range, was evaluated in this study. The calibration curves demonstrated a linear relationship between peak area and concentration across a wide range. The results of the linearity study are summarized in Table 1, with the corresponding chromatogram illustrating the linearity presented in Fig. 3. The method's selectivity and sensitivity were assessed by analyzing blank plasma, hemolyzed samples, and lipemic samples to ensure no chromatographic interference at the retention times of NBH and AMB.

Additionally, no significant changes were observed in the peak area of both drugs upon repeated injections at the same concentration, indicating the method's consistency and minimal variability. The LLOQ values demonstrated that the method is capable of detecting low concentrations of both drugs. Recovery studies are crucial for the utilization and validation of analytical methods, as they evaluate the performance of the procedure. These studies are a standard approach for assessing the effectiveness of an analytical method [13, 39, 40]. The

Table 7 Precision for QC samples of NBH

Level	QC	Recovered concentration (µg/mL)	Avg. recovered concentration (µg/mL)	% CV	% RSD
LLOQ	LLOQ 1	4.85	4.57	3.91	4.04
	LLOQ 2	4.62			
	LLOQ 3	4.42			
	LLOQ 4	4.68			
	LLOQ 5	4.38			
	LLOQ 6	4.48			
LQC	LQC 1	13.17	13.47	1.88	1.36
	LQC 2	13.46			
	LQC 3	13.58			
	LQC 4	13.90			
	LQC 5	13.30			
	LQC 6	13.40			
MQC	MQC 1	85.15	86.64	2.33	1.18
	MQC 2	87.56			
	MQC 3	86.98			
	MQC 4	85.03			
	MQC 5	90.11			
	MQC 6	85.03			
HQC	HQC 1	149.37	147.86	1.69	1.82
	HQC 2	147.08			
	HQC 3	152.21			
	HQC 4	146.83			
	HQC 5	145.92			
	HQC 6	145.76			

Table 8 Precision for QC samples of AMB

Level	QC	Recovered concentration (µg/mL)	Avg. recovered concentration (µg/mL)	% CV	% RSD
LLOQ	LLOQ 1	3.89	3.84	2.55	2.12
	LLOQ 2	3.81			
	LLOQ 3	3.71			
	LLOQ 4	3.76			
	LLOQ 5	3.98			
	LLOQ 6	3.88			
LQC	LQC 1	10.46	10.50	1.13	0.96
	LQC 2	10.56			
	LQC 3	10.34			
	LQC 4	10.60			
	LQC 5	10.64			
	LQC 6	10.40			
MQC	MQC 1	72.11	71.15	3.73	2.90
	MQC 2	74.21			
	MQC 3	69.54			
	MQC 4	68.51			
	MQC 5	74.05			
	MQC 6	68.50			
HQC	HQC 1	115.29	115.49	3.06	3.86
	HQC 2	121.68			
	HQC 3	110.85			
	HQC 4	115.62			
	HQC 5	113.90			
	HQC 6	115.62			

study confirmed the suitability and applicability of the established method for analyzing various samples. Precision and accuracy were evaluated using the proportion of error method. Accuracy refers to how closely the test results from an analytical method align with the actual values. The method's accuracy was assessed at four levels by performing six repeated measurements at each level, covering the entire range of the method. To evaluate the accuracy, three standard concentrations of NBH and AMB were used at four different levels, as specified by the guidelines. The findings demonstrated that the developed method exhibited excellent accuracy, with the % RSD for both drugs falling within an acceptable range. Detailed results from the accuracy study are provided in Tables 5 and 6. Precision measures the consistency of test results when the method is applied repeatedly to different samples. It is determined by injecting a series of standards or analyzing multiple samples from various aliquots of a homogeneous batch [29]. In the present study, the precision of the established method was assessed by evaluating the variation in results from multiple sample solution injections. Bioanalytical method validation (BMV) is used to quantitatively analyze pharmaceuticals and their metabolites in biological fluids. It plays a critical role in assessing bioavailability, bioequivalence, pharmacokinetics, and toxicokinetics [30]. During the development of any bioanalytical method, it is essential to verify the method's stability under various conditions such as time, temperature, and pH, and to ensure consistency between initial and final results. Regulatory guidelines emphasize the importance of evaluating analyte stability in prepared sample extracts. Analyte stability is a key criterion in validation guidelines provided by EMEA and USFDA, particularly in the bioanalytical field [30]. Assessing analyte stability is crucial to ensure the quality and reliability of results.

Conclusion

The present research work reported the development and evaluation of a method for the simultaneous and accurate estimation of NBH and AMB in human plasma. The results demonstrate that the developed approach is

Table 9 Summary of stability studies (% recoveries)

Nominal concentration (NBH)	Bench-top stability	Auto-sampler stability	Freeze–thaw stability	Process sample stability	Stock solution stability
LQC (13.52)	99.14	101.0	98.25	100.1	95.19
HQC (150.19)	99.57	99.27	95.74	97.32	98.57
Nominal concentration (AMB)	**Bench-top stability**	**Auto-sampler stability**	**Freeze–thaw stability**	**Process sample stability**	**Stock solution stability**
LQC (10.51)	99.46	98.8	96.38	100.4	96.42
HQC (116.09)	99.87	95.77	96.71	99.10	97.15

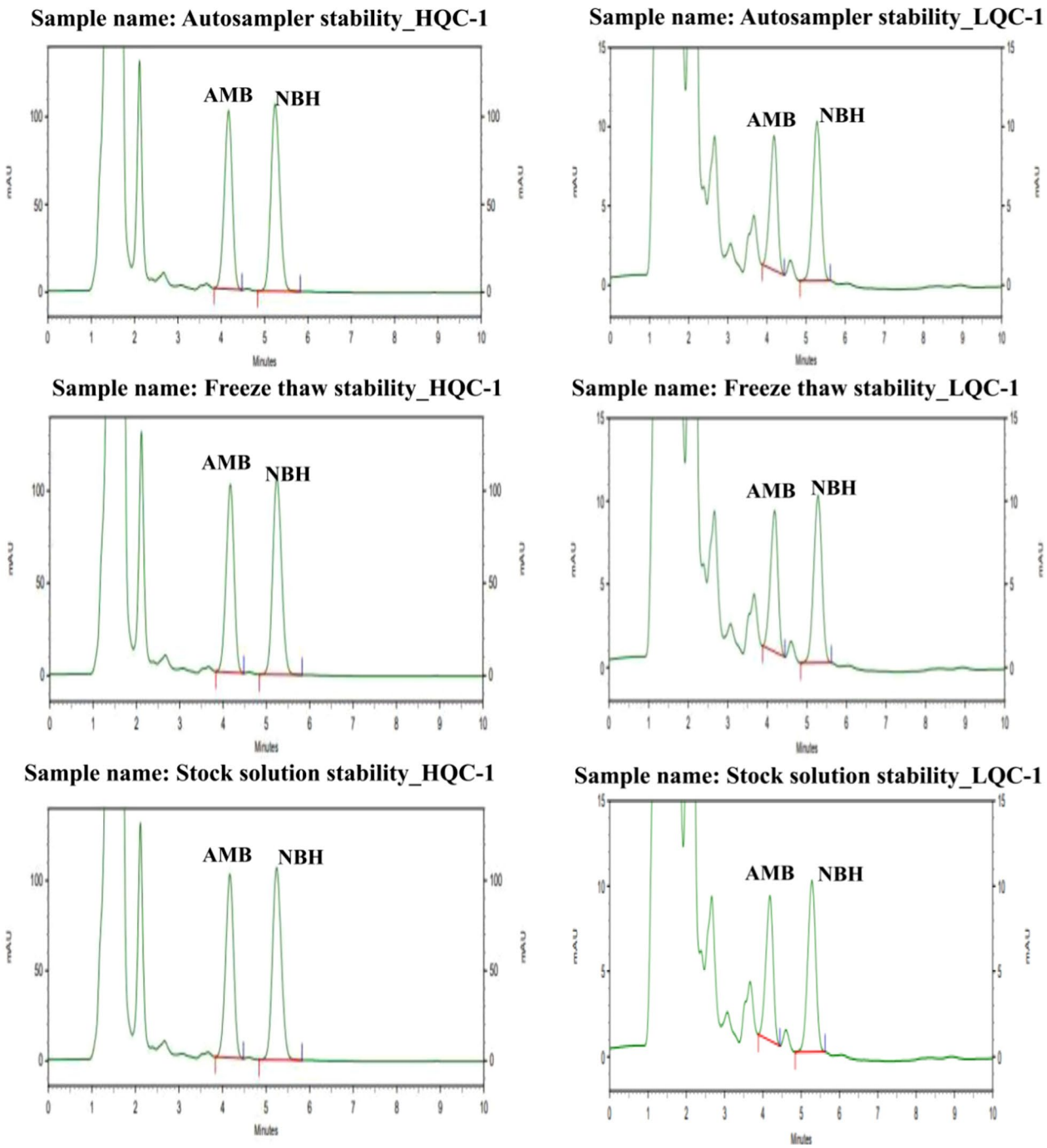

Fig. 7 Chromatogram of stability studies of NBH and AMB

characterized by its rapidity, simplicity, selectivity, and sensitivity. Additionally, the method exhibited high precision and accuracy, meeting the standards set by the US FDA. The stability study confirmed that the drugs remained stable under various conditions. Therefore, the proposed approach shows promise for routine analysis of NBH and AMB in human plasma.

Abbreviations

AMB	Amlodipine besylate
BP	Blood pressure
CV	Coefficient of variance
EMEA	European medicines agency
EP	European pharmacopoeia
HQC	Higher-quality control
IP	Indian pharmacopoeia
IPA	Isopropyl alcohol
LLOQ	Lower limit of quantification

LQC	Lower-quality control
MQC	Middle-quality control
NBH	Nebivolol hydrochloride
QC	Quality control
RP-HPLC	Reverse-phase high-performance liquid chromatography
RSD	Relative standard deviation
RT	Retention time
STDV	Standard deviation
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
USFDA	US Food and Drug Administration

Acknowledgements
The authors are thankful to Dr. S.J. Surana, Principal R. C. Patel Institute of Pharmaceutical Education and Research, for providing the necessary facilities. Studies involving plants must include a statement specifying the local, national, or international guidelines and legislation and the required or appropriate permissions and/or licenses for the study—NA.

Author contributions
Kaveri T. Vaditake performed the experimental work, generated the data, and drafted the manuscript. Dr. Atul A. Shirkhedkar has contributed in designing of the experiments as well as edited the drafted manuscript.

Funding
This research work has not received any funding support.

Availability of data and materials
The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
The authors declare no conflict of interest.

Competing interests
The authors declare that they have no competing interests.

Received: 31 March 2024 Accepted: 1 October 2024
Published online: 08 October 2024

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