Original Article

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High-performance Liquid Chromatography Analysis of Nebivolol and Amlodipine and Some Related Compounds in Synthetic Mixture

Nebivolol ve Amlodipin ve Bazı İlgili Bileşiklerinin Sentetik Karışımlarda HPLC ile Analizi

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ABSTRACT

Objective: This study aimed to develop and validate a method using a high-performance liquid chromatography (HPLC) to perform a quantitative analysis of nebivolol (NEB) and amlodipine (AML) along with some related substances in the synthetic mixture.

Methods: The separation in the described chromatographic system was accomplished using a mobile phase consisting of a mixture of acetate buffer (pH: 4.5) and acetonitrile and a HPLC C18 column (150 mm x 4.6 mm, 2.6 µm) with gradient elution on a consistent flow rate of 1.3 mL/min. Photodiode array detection was carried out at a wavelength of 265 nm. According to The International Conference on Harmonisation guidelines, the drug was exposed to various stress conditions; including photolysis, oxidation, thermal degradation and hydrolysis under acidic, basic and neutral mediums.

Results: Ranges of detection and quantitation limits were determined to be 0.2-10.0 $\mu g.mL^{-1}$ and 0.25-10.0 $\mu g.mL^{-1}$ for NEB and AML, respectively. The relative standard deviation values within and between days precision were determined to be <2%. For all substances, the average recovery values were determined within the range of 98.00%-101.50%.

Conclusion: We conclude that this developed analytical procedure applies to the quality control of drug formulations.

Keywords: Nebivolol, amlodipine, high-performance liquid chromatography, validation, synthetic mixture

ÖZ

Amaç: Bu çalışma, nebivolol (NEB) ve amlodipinin (AML) kantitatif analizini yapmak için yüksek performanslı sıvı kromatografisi (HPLC) kullanarak bir yöntem geliştirmeyi ve validasyonunu amaçlamıştır

Yöntemler: Kromatografik sistemdeki ayırma, asetat tamponu (pH: 4,5) ve asetonitril karışımından oluşan bir mobil faz kullanılarak, 1,3 mL/dakika akış hızında, C18 kolonda (150 mm x 4,6 mm, 2,6 m) gerçekleştirildi. Diyot sıralı dedektör 265 nm dalga boyuna ayarlandı. Uluslararası Uyum Konferansı yönergelerine uygun olarak ilaç, çeşitli stres koşullarına maruz bırakılmıştır. Bunlar; fotoliz, oksidasyon, termal bozunma ve asidik, bazik ve nötr ortamlar altında hidrolizdir.

Bulgular: NBV ve AMV için kantitasyon limit aralıkları sırasıyla 0,2-10,0 g/mL^{-1} ve 0,25-10,0 g/mL^{-1} olarak belirlenmiştir. Gün içi ve günler arası kesinlik bakımından RSD değerlerinin %2'den düşük olduğu belirlenmiştir. Analizlenen tüm maddeler için ortalama geri kazanım değerlerinin %98,00-%101,50 aralığında olduğu belirlenmiştir.

Sonuç: Geliştirilen analitik sürecin ilaç formülasyonlarının kalite kontrolü için uygulanabilir olduğu sonucuna vardık.

Anahtar Sözcükler: Nebivolol, amlodipin, yüksek basınçlı sıvı kromatografisi, validasyon, sentetik karışım

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Introduction

Nebivolol hydrochloride's (NEB) chemical name is (1R)-1-[(2R)-6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl]-2-{[(2R)-2-[(2S)-6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl]-2-hydroxy ethyl]amino}ethan-1-ol hydrochloride. NEB hydrochloride is a third-generation antihypertensive drug, acting as a very specific β1-blocker. The mechanism of action of the drug substance occurs via its β1-receptor blocking activity and nitric oxide potentiation, resulting in its vasodilation effects (1-3).

Amlodipine besylate (AML) is dihydropyridine class calcium channel blocker used via oral route and has the chemical name as follows: 3-ethyl 5-methyl 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1, 4-dihydropyridine-3, 5-dicarboxylate; benzenesulfonate (4). AML is used for hypertension management as the first line therapy agent according to therapeutical guidelines based on its suitable adverse event profile and relatively long action. Its mechanism of action involves reducing the calcium ion influx. However, this effect occurs quite selectively on the smooth muscles rather than affecting the cardiac muscle cells. In addition, it does not affect calcium ion mobilisation across the cell membrane, resulting in a reduction in the peripheral vascular resistance and blood pressure (5,6). Concomitant use of NEB and AML for hypertension management is evaluated to be beneficial due to the involvement of mechanisms of action of these two molecules, yielding a synergetic effect in the living organism to lower the blood pressure. Furthermore, lower doses of both molecules are sufficient with this combination therapy.

A literature review was performed and discovered that most of the analytical techniques, employed for determining NEB and AML in the combination formula, were mainly based on spectrophotometry (7-15), high-performance liquid chromatography (HPLC) (16-19) and high-performance thin-layer chromatography (20-22). The quantification of related substances in the combination formula of NEB and AML are individually reported in literature. Based on literature review and current available information, no method which can analyse related substances of simultaneous NEB and AML in combined dosage forms was reported. Related compounds of NEB and AML should be determined in the combination formula of NEB and AML without a need for prior separation for practical reasons. Therefore, our study targeted the development and validation of the developed analytical method to allow quantification of related compounds of NEB and AML in fixed dose combination pharmaceutical dosage forms avoiding any a prior separation procedure.

Some of these related compounds were determined to be possible degradants of AML and NEB in the drug product. This fact, too, shows the need for a method to determine the related compounds of NEB and AML in combined dosage forms without prior separation and supports our aim to develop and validate an analytical method that meets the needs for the combination formula. Method validation for related compounds was accomplished according to the International Conference on Harmonisation (ICH) requirements, by carrying out stress tests for fixed dose combination tablets covering various conditions; photolysis, oxidation, hydrolysis (at different pH mediums) and thermal degradation (23). Results obtained out of the stability testing procedures were anticipated to provide important contributions to properly developed manufacturing processes; contributing to decision-making processes for selecting proper packaging and determining the storage conditions, shelf life of the product and expiration date.

Experimental

Reagents and Solutions

Working standards of AML and NEB and related compounds were obtained from Hetero Drug Ltd. (Telangana Limited, India). Laboratory-prepared tablets were made, containing 10 mg of NEB and 10 mg of AML, Starch maize 50.0 mg, Hydroxypropyl Methyl Cellulose 45.0 mg, Lactose Monohydrate 70.0 mg, Croscarmellose Sodium 15.0 mg, Microcrystalline Cellulose 38.0 mg, Silicon Dioxide 5.0 mg and Magnesium Stearate 2.2 mg per tablet. All used chemical substances and chemical reagents were procured as analytical reagent grade. Ultra pure water was obtained using an aquaMAX™ (Younglin Instrument, Korea) water purification system for HPLC.

Chromatographic Conditions and Instrumentation

Shimadzu HPLC, Binary Pump (Shimadzu, Kyoto, Japan) was used for the chromatographic separation. The HPLC chromatographic system was established using the following parts: SPD M20A photodiode array (PDA) detector, SIL 20AC Autosampler, LC 20AB pump and CTO-10As column oven. Data gathered and derived from chromatographic procedure was processed by the software of the same chromatographic system**.** Separation of substances in chromatographic system was performed on a Phenomenex Kinetex 150 mm x 4.6 mm, 2.6 µ C18 (4.0x100 mm, 3 µm) using a mobile phase consisting

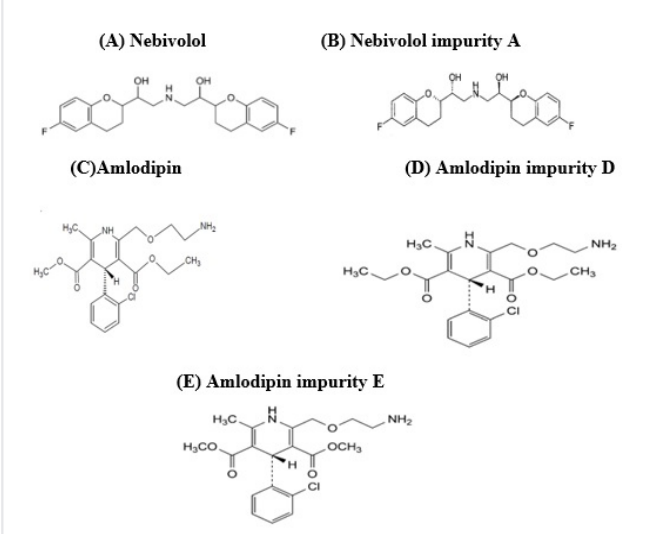

Figure 1. Chemical structures: NEB and AML and some related compounds

NEB: Nebivolol, AML: Amlodipine

of a mixture of acetate buffer (pH: 4.5) and acetonitrile with gradient elution on a consistent flow rate of 1.3 mL/min. Specific wavelength of 265 nm was selected for PDA detection. Temperature Column was kept at 35 °C.

Standard Solution Preparation

Stock solution of 500.0 μg/mL NEB and impurity of NEB A, AML and impurity of AML D and E were formed through dissolution of reference standard materials of the investigated pharmaceutical active ingredients and related compounds in 100 mL of acetonitrile: acetate buffer (pH: 4.5) (50:50, v/v) in a calibrated flask using an ultrasonic bath. Working standard solutions were formed through adequate dilution of the stock solution with the above mentioned solution mixture. Stock solutions were kept at 4 °C and were stable for a month.

Ammonium acetate of 1.5 g was accurately weighed and dissolved in 1,000 mL of water to get 20 mM of solution for the buffer solution preparation. The pH of the final solution was adjusted to 4.5 with acetic acid. It was then filtered with a 0.22 μ filter. The filtered solution was degassed and used as a buffer in the mobile phase.

General Procedures

Construction of the Calibration Curves

Accurately measured aliquots of NEB and AML standard solutions covering the working concentration ranges of 0.20-10.0 μg/mL and 0.25-10.00 μg/mL were prepared, respectively, and then transferred into a series of 10 mL volumetric flasks. Solutions were diluted to the mark with acetonitrile: acetate buffer (pH: 4.5) (50:50, v/v) and mixed well. Related compounds of NEB A, AML D and E stock solutions were formed through diluting 1 mg of related compounds in 1 mL of acetonitrile: acetate buffer (pH: 4.5) (50:50, v/v) and further diluted with the same solvent. Stock solutions were stable when kept at 4 °C in a refrigerator.

Sample Preparation

Fixed dose combination tablets with NEB and AML based on a ratio of 1:1 were prepared in the laboratory. An accurate amount of prepared tablets having equivalent milligrams of active ingredients of 10 mg of NEB and 10 mg of AML (in line with their ratio in the pharmaceutical formulation) was weighed and transferred into a 100 mL volumetric flask, and the volume was completed to final a volume of 100 mL with acetonitrile: acetate buffer (pH: 4.5) (50:50, v/v). Flasks were kept in an ultrasonic bath for 30 min to best dissolve the contents, and then the content was filtered by 0.45 μm membrane polytetrafluoroethylene. The above mentioned procedure was implemented to construct the calibration curves. The quantification of the nominal value of each compound within the content was calculated based on the established calibration curve or the corresponding regression equation.

Validation of Analytical Method

The analytical method development and validation were carried out in line with the ICH guidelines (23). Methods were validated based on the parameters covering quantification, accuracy, precision, specificity, linearity, range and detection limit.

Specificity

Stress degradation investigation was carried out in line with the ICH guidelines Q1A (R2) (23) to show the stability-defining characteristic and specificity of the developed analytical method. Solutions from tablet preparation were investigated under various forced degradation conditions described as follows: under alkaline conditions (1 N sodium hydroxide (NaOH) at ambient temperature in 24 hours), acidic conditions (1N hydrogen chloride (HCl) at ambient temperature in 24 hours), neutral conditions (water at 70 °C in 1 hour) and oxidative conditions (3.0% v/v H_2O_2 at ambient temperature in 3 hours). Samples from alkaline and acidic degradation stress conditions were neutralised using adequate amount of 1 N HCl and 1N NaOH, respectively, and completed to the end volume with the diluent. Thermal stress conditions were created by keeping the investigational medicinal product in heat controlled oven at 80 °C in a week. The pharmaceutical preparation was exposed to ultraviolet lamp for 72 hours to check the photostability. Upon completion of pre-defined time, solutions resulting from stress condition testing were diluted with the methanol: acetate buffer (pH: 4.5) (50:50, v/v), and samples for degradation testing were subjected to analysis by the developed HPLC method as mentioned in the chromatographic condition section. A PDA detector was used to define peak purity concerning peaks resulting from all samples from stress condition testing.

Specificity is the ability of the method to measure the analyte response in the presence of all related substances (NEB A, AML D and E). For specificity determination, all related substances were prepared individually and injected into HPLC to confirm retention times. Later on, solutions of blank, sample and spiked sample (sample spiked with all related substances) were prepared and injected into HPLC to confirm any co-elution with analyte peaks from respective blank and any degradation peaks. From the injections of spiked sample, the known related substance peaks were confirmed to be well separated from each other and without collation, showing that the method is selective and specific. The stability indicating nature of the method was further evaluated by performing forced degradation studies. Stress testing was carried out to identify the likelihood degradation products or to elucidate the inherent stability characteristics of drug substance. In this study, drug was subjected to oxidation, hydrolytic, photolytic, thermal and humidity stress conditions, and the summary of results obtained from forced degradation experiment results is presented in Table 4. Results showed that peaks were found stable in all forced degradation conditions, without interference for related compound peaks from other peaks.

Linearity

The linearity of the method was established for drugs and their related compounds. Drugs solution and their related compounds were formed at five various concentrations within the range of 0.20 and 15.00 μg/mL of concentration of analyte. The regression line was constructed between the peak area and

corresponding analyte concentration based on method of least squares analysis. The slope and Y-intercept values of regression line were calculated.

Limit of Quantitation (LOQ) and Limit of Detection (LOD)

LOQ and LOD were calculated as the 10 and 3.3 times of the standard deviation of the peak area divided by the slope of the linearity calibration curve, respectively.

Precision

Method precision and accuracy were determined as within and between the days of precision. The within and between the day precision values were investigated at three different concentrations (n=5) of the analyte during five consecutive days. The relative standard deviation (RSD) was provided as the precision value.

Accuracy

Recoveries by spiking method were used for method accuracy calculation. Standard solutions with known amount (low, medium and high concentrations) were spiked with sample solutions of known amount (0.2 µg/mL). The regression equation of the calibration curve was used to estimate the spiked amount.

Robustness

Investigation of the robustness of the method was performed through deliberate modification of key method conditions like organic phase composition, flow rate of the mobile phase and key method parameters, such as selected wavelength for detection. Investigation of drug solution stability in mobile phase was performed by keeping the drug solution in ambient temperature for one day, 24h.

Results

Chromatographic Conditions

Separation in proposed chromatography system was performed on C18 column (150 mm x 4.6 mm, 2.6 µm) using a gradient elution system. For this procedure, the mobile phase consisting of a mixture of acetate buffer (pH: 4.5) and acetonitrile was formed, and the flow rate was determined as 1.3 mL/min. The wavelength of 265 nm was selected for PDA detection. The column temperature was kept at 35 °C during the procedure. Table 1 presents the gradient elution programme. Figure 2 presents the typical chromatograms.

Five replicates of freshly prepared substances were injected to evaluate the method adequacy for resolution between targeted peaks with high level of repeatability. The chromatogram was analysed based on the following factors: resolution (R), theoretical plates (N), tailing factor, retention time (tR) and symmetry factor (α). The system suitability test results proved that the developed method in this present article comply with the acceptable limits described by the requirements (Table 2).

Method Validation

ICH guidelines were the basis of method validation and optimisation (23). As described in the guideline, parameters consisting precision, accuracy, robustness, specificity, linearity, LOD and LOQ were investigated.

Linearity

The linearity of the method was tested both for the individual drug substances and their related compounds in the combination formula. Five different concentrations of drug solutions and impurity solutions were prepared. The concentration range of the analyte varied from 0.20 to 15.00 µg/mL. The calibration

Table 1. Gradient elution programme

Time	Acetate buffer (pH: 4.5)	Acetonitrile
0	80	20
5	80	20
40	35	65
45	80	20
50	80	20

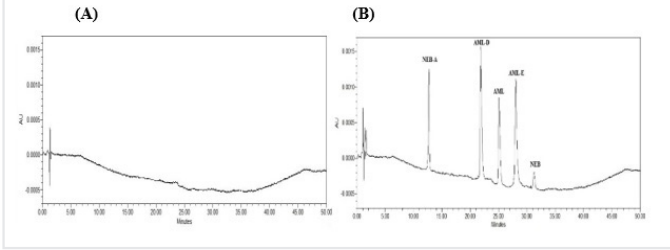

Figure 2. Representative chromatograms of (A) blank, (B) AML 0.2 µg/mL, NEB 0.5 µg/mL and related compounds spiked with 0.5 µg/mL

NEB: Nebivolol, AML: Amlodipine

Table 2. System suitability parameters

Name	(tR) ± SD (min)	Symmetry factor	N ± SD	RSD %	R ± SD	RSD %
NEB	31.094±0.001	1.09	33260±345	1.04	4.93±0.05	1.04
NEB Imp A	12.686±0.001	0.96	23544±158	0.59	5.76±0.03	0.59
AML	25.104±0.002	1.39	36758±419	1.20	6.31±0.08	1.20
AML Imp D	21.881±0.002	1.42	30772±320	1.04	22.19±0.23	1.04
AML Imp E	28.060±0.001	1.47	41048±570	1.34	5.50±0.07	1.34

N: Theoretical plates, R: Resolution, tR: Retention time and RSD: Relative standard deviation obtained from five replicate injections area

curve was plotted by drawing the corresponding impurity peak area against the concentration on the chart. The calculated coefficients of correlation, slopes of the curves, and y-intercepts of the calibration plots were presented. Calibration curves concerning the related substances were determined to be linear within the ranges involved in the study. Correlation coefficients were found to be >0.9990 for all molecules of interest (Table 3).

The LOQ and LOD were measured following a recommended formula (according to ICH Q2 (R1) (23) shown as

LOD = 3.3 SD / slope LOQ = 10 SD/slope (Eq. 1)

where SD is the standard deviation of the intercept and standard deviation of the peak area. The LOD and LOQ values presented in Table 3 prove that the proposed methods are adequately sensitive for determination of these drugs.

Precision

The precision trials were performed by a sequence of analyses of AML and NEB and related compounds for five consecutive days (each n=5). The RSD values were found in the range from 0.59% to 1.34% for intraday precision and from 1.11% to 1.61% for the interday precision. All values were found to be <2%, proving that the method was adequately precise. Results are presented in Table 3.

Specificity

Forced degradation under various experimental conditions was investigated using a starting concentration of 1 mg/mL of the sample. A PDA detector was used to ensure the homogeneity of drug peaks. After subjecting the drug to acid and base hydrolysis and oxidative degradation, the degradation products of NEB, Imp A and AML Imp D were observed. The thermal and water hydrolysis and the photolytic stress study showed that AML was degraded into AML Imp D (Figure 3). These results confirmed that the drug product maintained its stability well when exposed to forced degradation tests (Table 4).

Accuracy

The standard addition technique was used to prove the accuracy of the method. Certain amount (0.2 µg/mL) of pure sample solution were added to three concentration level of the drug standard solutions and related compounds (low, medium and high concentration) and analysed. Percentage recoveries for the drug and related compounds were within the range of 98.00%-101.50%. Results of recovery study were presented in Table 5.

Robustness

The method was evaluated to be robust based on the findings after making intentional changes in the process; including flow rate of the mobile phase (±0.1 mL/min), mobile phase pH

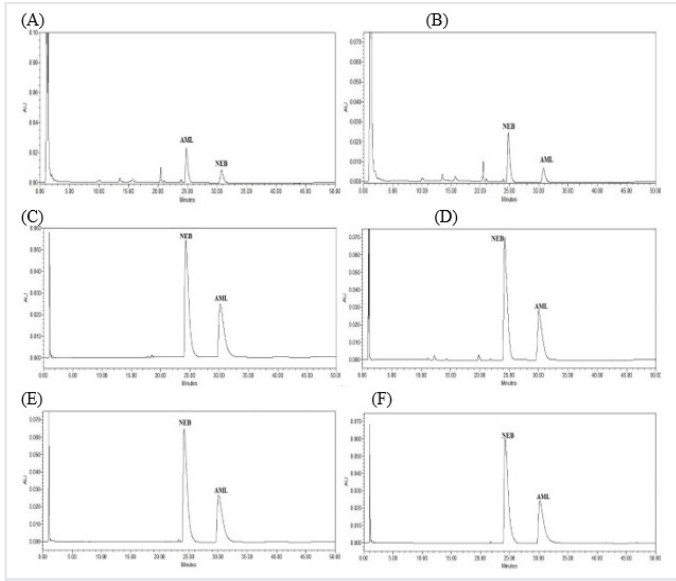

Figure 3. Chromatograms of AML and NEB at synthetic mixture (A) acid-degraded drug, (B) base-degraded drug, (C) water hydrolysis-degraded drug, (D) oxidation-degraded drug, (E) thermal-degraded drug and (F) daylight-degraded drug

NEB: Nebivolol, AML: Amlodipine

Table 3. Regression, precision, limit of detection (LOD) and limit of quantification (LOQ) data

Parameter	NEB	NEB Imp A	AML	AML Imp D	AML Imp E
Slope	1368748	1775624	2450505	3116410	1933305
Intercept	176	-182	-371	-358	-561
Linearity range (µg/mL)	0.20-10.00	0.20-10.00	0.25-10.00	0.30-10.00	0.40-15.00
LOD	0.05	0.05	0.06	0.04	0.03
LOQ	0.17	0.17	0.20	0.13	0.10
Intraday precision (RSD)[a]	1.04	0.59	1.20	1.04	1.34
Interday precision (RSD)[b]	1.13	1.36	1.14	1.61	1.11
[a]n=5					
[b]Results of five different days					

RSD: Relative standard deviation, NEB: Nebivolol, AML: Amlodipine

(4.5±0.1) and column oven temperature (35±2 °C). Some minor variations were observed in the proposed method; however, variables did not significantly affect the outcome. We concluded that the obtained data proved the robustness of the proposed method in this article.

The chemical stability of the stock solutions of the studied compounds has been tested to determine the outcomes of storage for 48 hours at room temperature (25 °C). All compounds were found to be stable in the mobile phase for 48 hours at room temperature, and were also stable in the refrigerator (4 °C). No unexpected peaks were detected in the chromatograms during the stability studies.

Analysis of Synthetic Mixture

The proposed method was implemented for the quantitation of NEB and AML and their related compounds using the synthetic mixture sample. The obtained results are in line with the labelled content for NEB and AML combination formula. Furthermore, the related chemical related compounds were found at levels below the acceptable limit.

Conclusion

In this article, validated HPLC method was presented to determine and quantify NEB, AML, and some related compounds in a synthetic mixture. This method was evaluated for linearity,

Table 4. Forced degradation results

Stress condition	Degradation (%)	Number of impurities	Retention time (min)	Peak purity (Amlodipin)	Peak purity (Nebivolol)
Acidic/1N HCl/RT/24 h	15.5	7	10.038;12.663; 13.537;15.719; 20.489;21.029; 23.927	0.99965	0.99975
Alkaline/1N NaOH/RT/24 h	16.4	7	10.072;12.695; 13.539;15.715; 20.489;21.047; 23.928	0.99981	0.99971
Oxidation/3% H202/3 h	6.5	9	11.158;11.737; 12.244;14.297; 16.541;16.983; 19.282;19.763; 21.822	0.99977	0.99967
Neutral/H2O/70°C/1 h	0.4	6	8.016;17.808; 18.474;19.182; 20.065;21.819	0.99972	0.99962
Photolytic/UV-lamp/72 h	1.3	10	8.731;10.323; 15.963;16415; 16.885;17.651; 18.475;19.190; 20.070;21.737	0.99989	0.99985
Thermal/80°C/1 week	0.9	9	17.419;17.881; 18.225;19.165; 19.554;20.023; 21.206;21.860	0.99990	0.99983

Table 5. Results of recoveries data of AML, NEB and related compounds

Analyte	Amount of drug taken[a] (µg/mL)	Amount of drug spiked[b] (µg/mL)	Total amount found (µg/mL) (Mean ± SD[c])	Recovery (%)	RSD[d]
		0.36	0.53±0.0058	99.6	1.09
Nebivolol A		4.30	4.48±0.0140	99.5	0.31
		6.55	6.72±0.0210	99.5	0.31
		0.21	0.41±0.0053	100.0	1.29
Amlodipine D		4.80	5.13±0.0300	100.6	0.59
		7.30	7.50±0.0430	98.0	0.57
		0.70	0.89±0.0079	98.8	0.89
Amlodipine E	0.2	4.80	4.97±0.0210	99.5	0.42
		7.30	7.46±0.0320	99.5	0.42
		0.70	0.50±0.0053	99.0	1.06
Amlodipine		4.80	5.04±0.0280	101.5	0.55
		7.30	7.56±0.0420	101.5	0.56
		0.65	0.85±0.0077	100.5	0.91
Nebivolol		1.80	2.01±0.0048	101.1	0.24
		2.80	3.02±0.0073	101.1	0.25

[a]synthetic mixture AML/NEB 10:10 mg, [b]Standard solution, [c]Standard deviation, [d]Five independent analyses, NEB: Nebivolol, AML: Amlodipine

precision, accuracy, LOD, LOQ, selectivity, robustness and solution stability. The selectivity of the method is evaluated as per ICH guidelines by carrying out forced degradation tests of NEB and AML combination tablets. Outcomes of these tests demonstrated that the method was stable based on the findings of NEB and AML-related substances. Thereby, we concluded that the developed and validated method proposed in this current article could be widely used in the routine practice to simultaneously determine NEB and AML and their related compounds in the synthetic mixture.

Peer-review: Externally peer review.

Authorship Contributions

Concept: C.Ö., K.A., A.Ö., Design: C.Ö., K.A., A.Ö., Data Collection or Processing: C.Ö., K.A., Ç.A., A.Ö., Analysis or Interpretation: C.Ö., K.A., Ç.A., Literature Search: C.Ö., K.A., Ç.A., A.Ö., Writing: C.Ö., K.A.

Conflict of Interest: No conflict of interest was declared by the authors.

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