Original Article

Simultaneous estimation of amlodipine besylate and nebivolol hydrochloride in tablet dosage forms by reverse phase-high-performance liquid chromatographic using ultraviolet detection

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Abstract

Background: The present study aimed to develop and validate the simultaneous estimation of amlodipine and nebivolol in tablet dosage forms. **Materials and Methods:** An isocratic reversed phase high-performance liquid chromatographic (HPLC) method with ultraviolet detection at 268 nm has been developed for the determination of amlodipine besylate (ADB) and nebivolol hydrochloride in dosage formulation. **Results:** Good chromatographic separation was achieved by using a stainless steel analytical column, the Lichrospher ODS RP-18 column (250 × 4 mm), particle size 5 µm. The system was operated at ambient temperature (25 ± 2°C) using a mobile phase consisting of acetonitrile (ACN) and a phosphate buffer (pH 3.0), mixed in a ratio of 40 : 60 at a flow rate of 0.8 ml/minute. The slope, intercept, and correlation coefficient were found to be 8818.2, - 18159, and 0.9993 for amlodipine and 9048.7, 108595, and 0.9998 for nebivolol, respectively. The proposed method was validated for its specificity, linearity, accuracy, and precision. **Conclusion:** The method was found to be suitable for the quality control of amlodipine besylate and nebivolol hydrochloride simultaneously in a bulk drug as well as in a formulation.

Key words: Amlodipine besylate, isocratic separation, method validation, nebivolol hydrochloride, RP-HPLC.

INTRODUCTION

Amlodipine Besylate (ADB), (RS)-3-ethyl-5-methyl-2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate benzenesulfonate (Merck Index, 1996) is a dihydropyridine analog, a long-acting Calcium Channel Blocker (Anti-Hypertensive), and inhibits the influx of extracellular calcium across the myocardial and vascular smooth muscle cell membranes. Amlodipine is a peripheral arterial vasodilator that acts directly on the vascular smooth muscle to cause a reduction in peripheral vascular resistance and in blood pressure. Amlodipine is official in the Indian Pharmacopoeia, British Pharmacopoeia, and European Pharmacopoeia.[1-5]

Nebivolol Hydrochloride α, α' [Iminobis (methylene) bis [6-fluoro-3,4-dihydro-2H-1-benzopyran-2-methanol] (Merck Index, 1996) is a β_1-Blocker (Anti-Hypertensive), reduces peripheral vascular resistance, and significantly increases stroke volume, with preservation of cardiac output.[1,2]

Various methods have been developed for the estimation of Amlodipine Besylate and Nebivolol Hydrochloride, in single and combined dosage forms, such as, UV spectrophotometry, HPLC, HPTLC alone, and only the spectrophotometric

method, in combination with both these. The titrimetric method is also available for the estimation of amlodipine. Here, ADB was directly titrated with a Bromate-Bromide mixture using methyl orange as an indicator.[6-16] However, to the best of our knowledge no HPLC-UV method has been reported for the simultaneous estimation of these drugs in any pharmacopoeia or in the available literature. Hence, the aim of our presented study is to develop and validate the simultaneous estimation of amlodipine and nebivolol in tablet dosage forms.

MATERIALS AND METHODS

High performance liquid chromatography was equipped with a Photodiode Array detector model Waters 2998, Controller (Waters 600) model code 6CE, and Pump (Waters Delta 600) model code 60F, of the Waters Corporation Limited, with Empower 2 software. The Lichrospher ODS RP-18 column (250 × 4mm), particle size 5 µm was used for the separation. All the reagents and chemicals were of HPLC grade and were purchased from Spectrochem Pvt. Ltd. Amlodipine Besylate and Nebivolol Hydrochloride reference standards with certificate of analysis were kindly gifted by Glenmark Pharmaceuticals Ltd., Goa. The marketed tablet formulations Nodon AM and Amlopress-NB were purchased from the local market.

Optimization of the chromatographic conditions

Several modifications in the mobile phase were made by changing proportions of acetonitrile, methanol, and water. Various modifiers were used such as chloroform, Tetrahydrofuran (THF), ethanol, Isopropyl alcohol (IPA), n-Hexane, and dichloromethane, with a 10 µ particle size column, used for separation initially. However, the best resolution of 1.72 was observed by using an Acetonitrile with a Potassium Hydrogen Orthophosphate Buffer (pH 3.0) in the ratio of 40 : 60, with THF and ethanol (1% in mobile phase). After switching to a 5 µ particle size column, with the same mobile phase composition, and without any modifier, a resolution of 4.78 was observed, much above the desirable limit of 2.0. The Retention Time of 7.47 and 10.25 of AMB and NBH was observed and this condition was then selected for our study.

Preparation of Potassium Hydrogen Orthophosphate Buffer (pH 3.0)

Potassium hydrogen orthophosphate of 6.8 gm was accurately weighed and dissolved in 1000 ml of water to get 50 mM of solution. The pH of the final solution was adjusted to 3.0 with the help of Orthophosphoric 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.

Preparation of mobile phase

Acetonitrile (ACN) and the Orthophosphate Buffer (pH 3.0) were mixed in a ratio of 40 : 60, and then filtered with a 0.45 µ filter. The filtered solution was degassed and used as the mobile phase.

Preparation of standard stock solution

Ten milligrams each of pure AMB and NBH were weighed accurately and separately dissolved in the mobile phase in a 10 ml volumetric flask and diluted up to the mark with the mobile phase, to get a 1 mg/ml solution.

Preparation of the calibration curve

Suitable aliquots of standard stock solution (1 mg/ml) of both the drugs, that is, AMB and NBH (0.3, 0.4, 0.5, 0.6, and 0.7 ml) were taken in a 10 ml volumetric flask and diluted up to the mark, to get 30, 40, 50, 60, and 70 µg/ml solution of the mixture of drugs. The prepared solutions were filtered with a 0.45 µ syringe filter and 20 µl was injected in each dilution. The same procedure was repeated six times. Calibration curve of both the drugs are presented in Figure 1 and 2 for AMB and NBH respectively. Figure 3 shows overlay spectra of the calibration curves of these drugs.

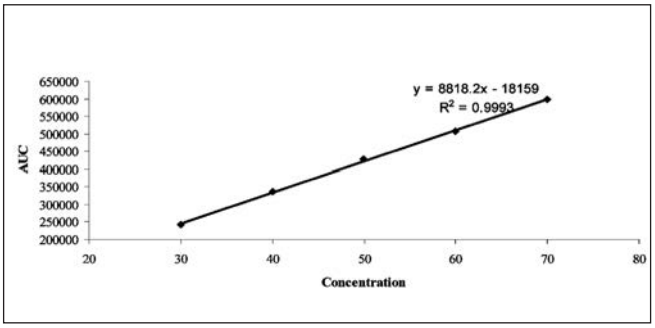

Figure 1: Calibration curve of Amlodipine Besylate

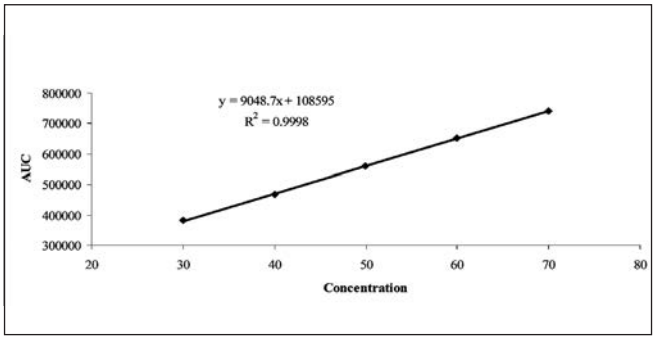

Figure 2: Calibration curve of Nebivolol Hydrochloride

Validation

Specificity

Generally used excipients like lactose, talc, starch, and magnesium stearate in a proportion of approximately 72 mg, 22.4 mg, 24 mg, and 1.6 mg, respectively, were transferred to a 10 ml volumetric flask and 5 ml of mobile phase was added to it and mixed, It was then diluted up to the mark after ultra-sonication for 10 minutes. This filtrate, of 0.1 ml, was diluted to 10 ml with the mobile phase. Filtrate of 0.1 ml was mixed with 0.1 ml of standard stock solution in a 10 ml volumetric flask and diluted up to the mark to produce 50 µg/ml. One dilution of 50 µg/ml standard solution was also prepared. All the solutions were injected in the order of the excipients mixture, 50 µg/ml of the standard solution, and then the excipient-drug mixture. An overlay of excipients, the pure drug and a mixture of the drug and excipients, showed no peak of the excipients at the RT of the drugs. The excipient peak showed a resolution of more than 2.0 with the drug peak, hence, the method was specific. Overlay spectra of both drugs with exipients is shown in Figure 4.

Linearity

Linearity was accessed by visualizing the calibration graph and plot of the residuals. The points distributed equally above and below the trend line showed linearity.

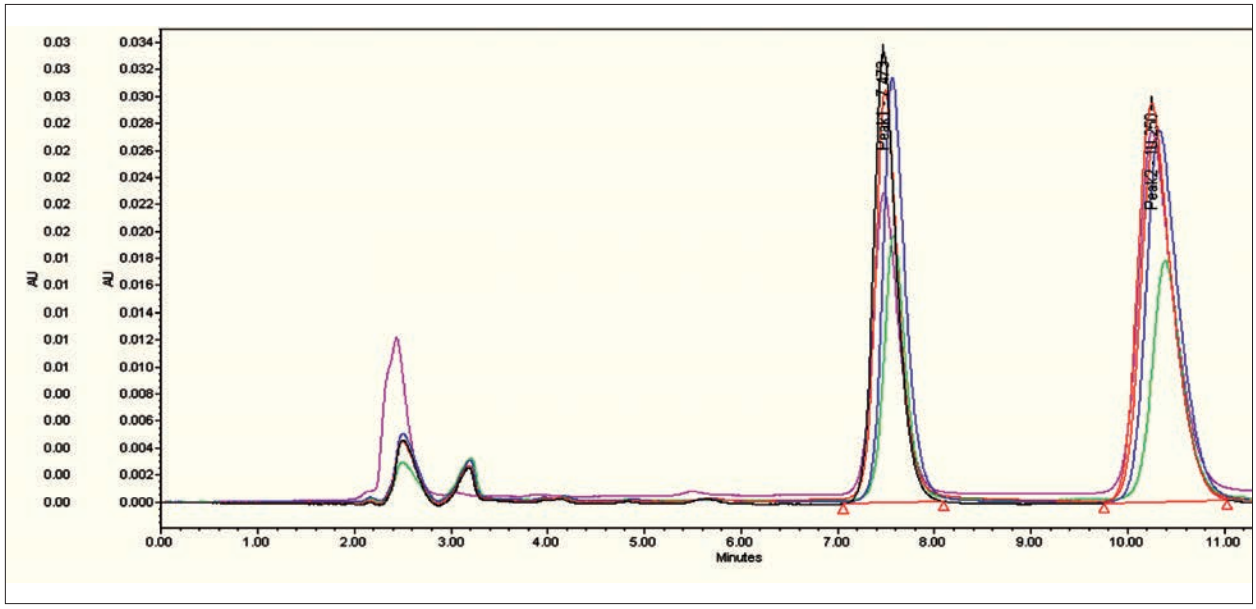

Figure 3: Overlay spectra of the various concentration level chromatograms of the two drugs.

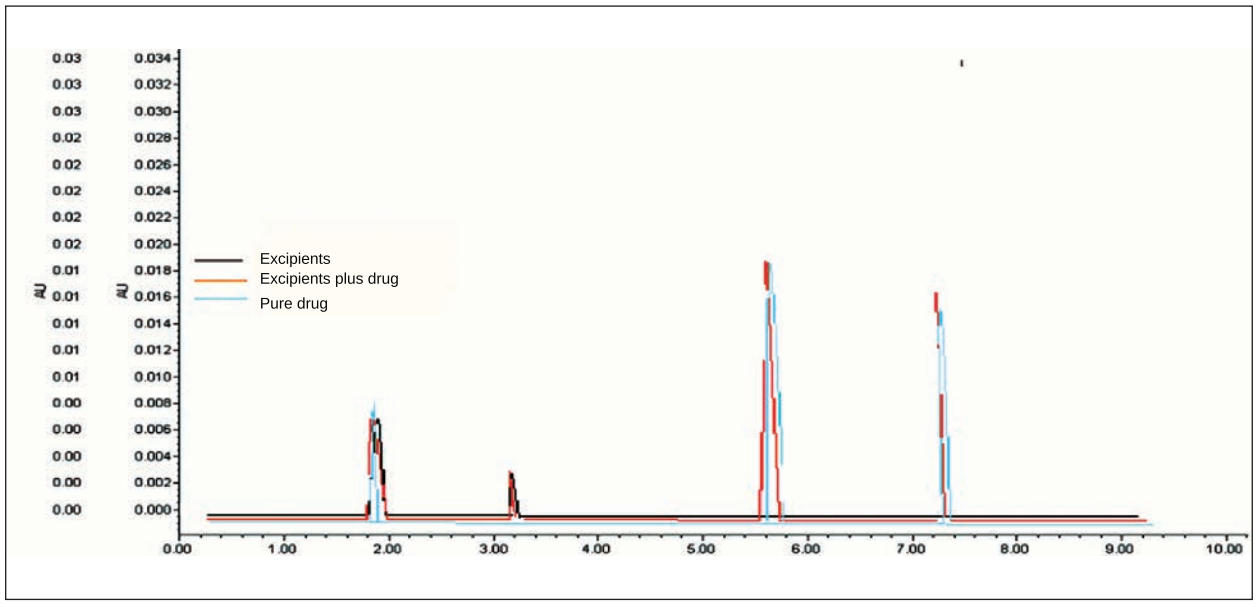

Figure 4: Overlay chromatogram of the excipients, excipient-drug mixture, and pure drug

Range

- Linearity range: 30 – 70 μg/ml
- Target range: 40, 50, 60 μg/ml
- Working range: 0.188 μg/ml – 70 μg/ml (AMB) and 0.31μg/ml – 70 μg/ml (NBH)
- Target concentration: 50 μg/ml

Precision

Standard stock solution of 0.3 ml, 0.5 ml, and 0.7 ml was taken out and diluted to 10 ml to make 30 μg/ml, 50 μg/ml, and 70 μg/ml, respectively. Three replicates of each dilution were injected into the HPLC system.

Repeatability

Repeatability was accessed by six replicate injections of 50 μg/ml solution of the drug prepared for the standard stock solution. Volumes of 20 μl were injected. % RSD was found to be 0.039 and 0.505 for AMB and NBH, respectively.

Intra-day precision

The same procedure was followed and three replicates were injected, thrice a day. % RSD was found to be 0.436 and 0.681, respectively, for AMB and NBH.

Inter-day precision

The same procedure was followed and three replicates were injected in three days. % RSD was found to be 0.403 and 0.683, respectively, for AMB and NBH.

Accuracy

Recovery studies were performed with two brands Nodon-AM (Cadila Pharmaceuticals Ltd.) and Amlopress-NB (Cipla Pharmaceuticals Ltd.).

Powdered Nodon-AM tablets (Cadila Pharmaceuticals Ltd.), equivalent to 10 mg of AMB, were transferred to a 10 ml volumetric flask and ultrasonication was done for 10 minutes with approximately a 5 ml mobile phase. The solution was then diluted up to the mark with the mobile phase and filtered through a 0.45 μ filter. This solution, 0.3 ml, was spiked in three different 10 ml volumetric flasks with 0.1, 0.2, and 0.3 ml of previously analyzed standard stock solution. Finally the volume was made up to the mark with the mobile phase and estimation of the drug content was done by the proposed method.

The same procedure was followed for Amlopres-NB tablets. Recovery study of Amlodipine and Nebivolol in the Nodon-AM tablet and Amlopres-NB is given under Tables 1 and 2 respectively.

Limit of quantification and limit of detection

Limit of quantification (LOQ) and limit of detection (LOD) were calculated on the standard deviation of the response, and the slope detection limit could be expressed as:

$LOD = 3.3 \times (SD/S)$

$LOQ = 10 \times (SD/S)$

Where, S.D = the standard deviation of response

S = the slope of the calibration curve

LOD and LOQ for AMB were found to be 0.062 and 0.188 μg/ml, respectively, and the LOD and LOQ for NBH were found to be 0.10 and 0.31 μg/ml, respectively.

System suitability testing

For system suitability testing, six replicates of 1000 μg/ml were injected. The tailing factor, asymmetry factor, and R.T. of each replicate were established. The number of theoretical plates (N) and the height equivalent to a theoretical plate (HETP) were observed through the software.

Bench top stability

The stability of the standard solution and sample solutions were determined by an assay after 24 and 48 hours, at room temperature, against fresh standard solutions. It showed that the drug was stable and did not show much variation in the time span, up to 48 hours.

Estimation of AMB and NBH in the tablet dosage form

Twenty tablets of NODON-AM were individually weighed and the average weight was calculated. All the 20 tablets were crushed in a pestle and mortar and the powdered Nodon-AM tablet, equivalent to 10 mg of AMB and 10 mg of NBH, was taken in a 10 ml volumetric flask, and to this, 5 ml of mobile phase was added and it was ultrasonicated for 10 minutes. The volume was made up to the mark with the mobile phase and filtered. The content of drug in the tablet was found by using the equation:

$$\frac{\text{Area of test}}{\text{Area of standard}} \times \frac{\text{Weight of std.}}{\text{Dilution of std.}} \times \frac{\text{Dilution of test}}{\text{Weight of test}} \times \frac{\text{Potency. of std}}{100} \times \text{Av. Wt. of tab.}$$

The same procedure was followed for Amlopress-NB tablets and results are presented in Table 3.

CONCLUSIONS

In the present study, the reversed phase HPLC (RP-HPLC) method has been developed for the estimation

Table 1: Recovery study of Amlodipine and Nebivolol in the Nodon-AM tablet

Conc. (µg/ml)	Conc. found before Spiking (µg/ml) C_1	Conc. of Std. added C_2	Conc. found after Spiking (µg/ml) C_3	% Recovery (C_3-C_1) *100/C_2	Mean ± SD	RSD
			AMLODIPINE			
30	29.999	10	40.048	100.49	100.07 ± 0.1068	0.1067
			39.935	99.36		
			40.072	100.73		
	29.982	20	49.938	99.78		
			49.967	99.925		
			49.989	100.35		
	30.002	30	60.007	100.016		
			60.023	100.07		
			59.976	99.913		
			NEBIVOLOL			
30	29.992	10	40.122	100.3	100.083 ± 0.1260	0.1258
			39.968	99.76		
			39.936	99.44		
	30.037	20	49.982	99.725		
			49.963	99.63		
			50.129	100.46		
	29.977	30	60.009	100.106		
			59.992	100.05		
			60.063	100.28		

Table 2: Recovery study of Amlodipine and Nebivolol in Amlopress-NB

Conc. (µg/ml)	Conc. found before Spiking (µg/ml) C_1	Conc. of Std. added C_2	Conc. found after Spiking (µg/ml) C_3	% Recovery (C_3-C_1) *100/C_2	Mean ± SD	RSD
			AMLODIPINE			
30	30.103	10	40.142	100.39		
			39.953	98.5		
			39.995	98.92		
	30.007	20	49.880	99.36		
			50.030	100.115	99.532 ± 0.4906	0.4929
			49.650	98.21		
	29.985	30	60.061	100.253		
			60.110	100.416		
			59.873	99.626		
			NEBIVOLOL			
30			39.626	99.73		
	29.653	10	40.015	100.62		
			39.770	101.17		
			49.634	100.265		
	29.581	20	49.886	101.525	100.5 ± 0.5180	0.5154
			49.832	101.255		
			60.052	100.29		
	29.965	30	59.925	99.866		
			59.900	99.783		

Table 3: Estimation of Amlodipine Besylate and Nebivolol Hydrochloride in tablet dosage form

S.No.	Nodon-AM		Amlopress-NB	
	Conc. of AMB	Conc. of NBH	Conc. of AMB	Conc. of NBH
1.	4.9350	4.8687	4.9824	4.8771
2.	4.8956	4.8268	4.9179	4.8479
3.	4.9789	4.9743	5.0725	5.0352
Mean(mg) ± SD	4.9365 ± 0.04167	4.8899 ± 0.07600	4.9909 ± 0.0755	4.9200 ± 0.1007
% Assay	98.73%	97.79%	99.81%	98.4%

Table 4: Summary of system suitability parameters

Parameter	AMB	NBH	Standard Limit
Number of theoretical plates (N)	8272	9529	> 2000
Height equivalent to theoretical plate (HETP)	3.022×10^{-3}	2.62×10^{-3}	-
Retention time	7.478	10.251	-
Capacity factor	6.48	9.25	1-10
Tailing factor	1.24	1.34	</ = 1.5
Asymmetry factor	1.23	1.345	</ = 2.0

Table 5: Summary of validation parameters for AMB and NBH

Parameters	Results		
		AMB	NBH
Specificity	Resolution of excipients with drug peak > 1.5, hence, method is specific		
Linearity	Method shows linearity between 30 and 70 µg/ml		
Range (µg/ml)	Linearity range	30 – 70	30 – 70
	Target range	30, 50, 70	30, 50, 70
	Working range	25 – 100	25 – 100
	Target concentration	50	50
Accuracy (% recovery)		98.21 – 101.73	99.44 – 101.525
Precision (RSD)	Intra-day	0.436	0.681
	Inter-day	0.403	0.683
	Repeatability	0.03925	0.05058
LOQ and LOD		0.188 and 0.062 µg/ml	0.31 and 0.010 µg/ml

of AMB and NBH in tablet dosage form. The proposed method is simple, precise, and accurate and does not suffer from any interference due to common excipients. It is evident from the study that the method is simple, precise, specific, and accurate. Summary of system suitability and validation parameters are presented in Tables 4 and 5 respectively. The newly developed methods can be used in the pharmaceutical industry for the routine analysis of Amlodipine Besylate and Nebivolol Hydrochloride simultaneously, in the tablet dosage form.

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