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Original Article

Spectrophotometric and High Performance Liquid Chromatographic Determination of Amlodipine Besylate and Nebivolol Hydrochloride in Tablets Dosage Form

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ABSTRACT

Introduction: Absorbance ratio spectrophotometric method and high performance liquid chromatographic method were developed for the determination of the Amlodipine Besylate and Nebivolol Hydrochloride in tablet dosage form. **Methods:** Absorbance ratio spectrophotometric method and high performance liquid chromatographic method were developed for the determination of the Amlodipine Besylate and Nebivolol Hydrochloride in tablet dosage form. A simple liquid chromatographic assay has been developed for the determination of Amlodipine Besylate and Nebivolol Hydrochloride. A C_{18} (250×4.6 mm, 5 µ) column was used with a mobile phase consisting of Water: Acetonitrile (pH adjusted to 3.5 with ortho phosphoric acid) at a flow rate of 1.0 ml min^{-1}. Quantitation was achieved with UV detection at 268 nm based on the peak height ratios. **Results:** Calibration curves were linear in the range of 10–30 µg/ml for Amlodipine Besylate and 10-30 µg/ml for Nebivolol Hydrochloride in absorbance ratio method. Correlation coefficient found to be close to 0.9995 for both the drugs. Accuracy for both the drugs was in the range of 99-101%. Beer's law was obeyed in a concentration range of 10-30 mg ml^{-1} for Amlodipine Besylate and 10-30 mg ml^{-1} for Nebivolol Hydrochloride and the regression line equation was derived with a correlation coefficient of 0.9999 and 0.9998 for Amlodipine Besylate and Nebivolol Hydrochloride respectively. **Discussion:** The proposed procedures were successfully applied to the determination of Amlodipine Besylate and Nebivolol Hydrochloride in bulk and tablet form, with high percentage of recovery, good accuracy and precision.

Key words: Amlodipine Besylate, Nebivolol Hydrochloride, Absorbance ratio method, HPLC, Tablets.

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INTRODUCTION

Amlodipine Besylate (AML), 3-ethyl, 5-methyl, 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1, 4-dihydropyridine-3, 5-dicarboxylate, (Figure 1) It acts as an Antihypertensive Agents, Vasodilator Agents, Calcium Channel Blockers and Antianginals. It is used for the treatment of Hypertension and chronic stable angina.[1,3]

Nebivolol Hydrochloride (NEB), 1-(6-fluoro-3, 4-dihydro-2H-1-benzopyran-2-yl)-2-{[2-(6-fluoro-3, 4-dihydro-2H-1-benzopyran-2-yl)-2-hydroxyethyl] amino}ethan-1-ol, (Figure 2) it acts as an Antihypertensive Agents, Adrenergic beta-Antagonists and Vasodilator Agent.

It is used in treatment of Hypertension, angina, acute myocardial infarction, supraventricular tachycardia, ventricular tachycardia, congestive heart failure, prevention of migraine headaches.[2,4]

Amlodipine Besylate is official in Indian Pharmacopoeia 2010[5] and BP 2009[6] and describes Liquid Chromatographic method and thin layer chromatographic method for its estimation and a literature survey reveals that UV Spectrophtometric,[7-10] RP-HPLC,[11] HPLC[12-15] methods has been developed for its estimation in alone or in combination with other drugs. Nebivolol Hydrochloride is official in Indian Pharmacopoeia 2010.[6] Various Spectrophotometric[16-17] and RP-HPLC[18-21] methods have been reported for the determination of Nebivolol Hydrochloride alone or in combination with other active pharmaceutical agents in dosage forms or in biological fluids.

However, simultaneous determination of Amlodipine Besylate and Nebivolol Hydrochlorude has not been described previously except one simultaneous equation method. The purpose of present study was to develop and validate spectrophotometric and HPLC method for simultaneous determination of Amlodipine Besylate and Nebivolol Hydrochlorude in tablet dosage form.

MATERIALS AND METHODS

Instrumentation

A double beam, UV-visible spectrophotometer (Simadzu-1800, Software–UV Probe, Version 2.42) with 1 cm matched quartz cells was used. The spectral band width was 2 nm and the wavelength scanning speed was medium.

The HPLC (Agilent SHIMADZU) instrument was equipped with a C18 (250×4.6 mm, 5 µ) column, an auto injector, UV-Detector and Chem 32 software.

Materials

Pharmaceutical grade of Amlodipine and Nebivolol Hydrochloride used as reference standard were supplied by Vapi care, Vapi. Combined tablet formulation (Zithium O BD) manufactured by Synochem Pharmaceutical Ltd. and marketed by Alkem Laboratory Ltd., was procured from local market. All other reagents used were AR grade and HPLC grade. AR grade methanol acquired from S.D. Fine Chemicals. Distilled water (HPLC grade-Merk, Renkem),Acetonitrile (HPLC grade-Merk, Renkem), Methanol (HPLC grade-Merk, Renkem),Ortho phosphate (Tri Ethyl Amine), Potassium dihydrogen phoshphate (Merk, Renkem) were used for analysis.

Chromatographic conditions

The mobile phase was prepared by mixing water and acetonitrile in the ratio of (60:40 v/v) and pH 3.5 adjusted with ortho phosphoric acid. It was filtered through 0.45 µ membrane filter. All determinations were performed at ambient temperature (20°C) using C_{18}, 250×4.6 mm, 5 µ, reverse phase column (Agilent SHIMADZU). The column effluent was

monitored at 215 nm, which represents the wavelength of maximum absorbance of AML and NEB. The injection volume was 20 µ l with a flow rate of 1 ml min^{1}.

Standard solutions and calibration graphs for spectrophotometric measurements

Absorbance ratio Method

A stock solution was prepared by dissolving AML and NEB in methanol and dilution was made by methanol to obtain a concentration of 100 µg ml^{1}. The standard solutions were prepared by dilution of the stock solution in methanol to reach concentration ranges of 10–30 µg ml^{-1} and 10-30 µg ml^{-1} for AML and NEB respectively for absorbance ratio method. Each solution was scanned between 200-400 nm. Wavelengths were selected from the overlay spectra of AML and NEB. The absorbance of the solutions was measured at 268 nm and 238 nm against methanol as a reagent blank. The concentrations versus their Absorbance were plotted in order to obtain the calibration graphs.

Standard solutions and calibration graphs for chromatographic procedure (HPLC)

Standard solutions of AML and NEB containing concentration range of 10-30 µg ml^{-1} for AML and 10-30 µg ml^{-1} for NEB were prepared in the mobile phase. Triplicate 20 µ l injections were made for each concentration and the peak height ratio of each concentration were plotted against the corresponding concentrations to obtain the calibration graph.

Sample preparation

A total of 20 tablets containing AML and NEB as the active ingredients were weighed and finely powdered. The powder equivalent to 10 mg of AML and 10 mg of NEB was taken in 100 ml volumetric flask and dissolved in mobile phase. From this 2.0 ml was transferred to 10ml volumetric flask and volume was made up to mark with mobile phase The volume was made up to mark and the solution was filtered through 0.45 micro membrane filter. The appropriately diluted solution was analyzed under optimized chromatographic conditions. The areas of resulting peak were measured at 268 nm. The peak-height ratios were used for the determination of AML and NEB in each sample.

For Absorbance ratio spectrophotometric method, a 1 ml of this solution was diluted to 10 ml with methanol and 2.0 ml of this solution was further diluted to 10 ml with methanol. Absorbance of the resulting solution was measured at 238 nm and 268 nm against methanol. The concentration of AML and NEB can be obtained as,

$$Cx = (Qm–Qy/Qx–Qy) *A1/ax1$$

$$Cy = (Qm–Qx/Qy–Qx) *A1/ay1$$

Qm = Abs of sample at 238 nm (A2)/Abs of sample at 268 nm (A2)

Qx = Absorptivity of AML at 238 nm/Absorptivity of AML at 268 nm

Qy = Absorptivity of NEB at 238 nm/Absorptivity of NEB at 268 nm

Where, Qx and Qy are value of AML and NEB respectively, ax1 and ay1 are absorptivity value at isobestic point for AML and NEB.

VALIDATION PROCEDURE

System suitability

The typical values for evaluating system suitability of a chromatographic procedure include the RSD <1%, tailing factor <2 and theoretical plates >2000. The determination of system suitability of analytical method was accomplished by assaying six samples of AML and NEB. The sample concentration of AML and NEB used in this analysis was 10-30 µg/ml and 10-30 µg/ml, respectively. The retention time, peak area, theoretical plates and tailing factor were evaluated for system suitability.

Sensitivity

The limit of detection (LOD) and quantification limit (LOQ) were determined by gradually diluting the sample and analysing by the proposed method. The signal/noise ratio (S/N) was determined for each tested strength. The typical S/N ratio recommended by the International Conference on Harmonisation (ICH) is 3/1 and 10/1 for LOD and LOQ, respectively.

Calibration curve

The above-mentioned calibration standards were analysed for determining linearity. The sample strengths ranged from 10-30 µg/ml for AML and NEB, respectively for HPLC method and 10-30 µg/ml of AML and NEB for Absorbance ratio method. The regression analysis was accomplished by slope, intercept and correlation coefficient (r^2).

Accuracy and precision

The accuracy was determined by percent recovery method. Furthermore, precision (inter-day variance and intra-day variance) were determined by assaying samples over a period of 1 day and 3 days, respectively. The standard concentrations used for these study were 10, 20, and 30 µg/ml for AML and 10, 20, and 30 µg/ml for NEB for HPLC method and for Absorbance ratio method the concentrations used were 4, 12, 20 µg/ml and 5, 15 and 25 µg/ml for AML and NEB.

Robustness

The influence of slight deliberate changes in chromatographic conditions such as column temperature, flow rate of mobile phase and pH of mobile phase on the retention time and peak area were observed one by one. The test was performed in triplicate for each set of conditions. The standard concentrations of AML and NEB used in this analysis were 20 µg/ml and 25 µg/ml, respectively.

RESULTS AND DISCUSSION

Absorbance ratio method

Tablet powder was dissolved in methanol. Figure 3. shows the absorption (zero-order) UV spectra of (a) AML and NEB standard solution. However, the application of the absorbance ratio spectrophotometric technique allowed complete elimination of the back-ground absorption due to the excipients.

Chromatographic procedure (HPLC)

A reversed phase HPLC method was developed to provide a specific procedure suitable for rapid quality control of AML and NEB tablet dosage form. A mobile phase consisting of acetronitrile and water in the ratio 40:60 (v/v) and pH 5.0 adjusted with ortho phosphoric acid, was chosen after several trials with methanol: water and acetonitrile: water. The apparent pH of the aqueous phase was adjusted to 5 using orthophosphoric acid. The above described chromatographic system allowed an adequate resolution (R_s 6.415) between AML (t_r 2.769) and NEB (t_r 5.236) in a reasonable time (R_s, resolution; t_r, retention time). The applied analytical conditions produced the peaks with suitable peak symmetry (<2).

The typical conditions for system suitability of an analytical method encompass the relative standard deviation (RSD) < 1%, peak symmetry <2 and theoretical plates >2000. The results of system suitability of present chromatographic method are described in Table 1. The peak area, retention time, tailing factor and theoretical plates were within the recommended limits. Therefore, the method was considered a suitable for quantitative determinations a linear calibration graph (Y=78031x+35060,

Table 1: Results for system suitability test

Parameters	Data obtained	
	AML	NEB
Theoretical plates per column	7063	5814
Symmetry factor/Tailing factor	1.124	1.348
Resolution	6.415	

Table 2: Regression and analytical parameters for estimation of two drugs by absorbance ratio and HPLC method

Parameter	HPLC method		Absorbance ratio method			
	AML	NEB	AML		NEB	
Wavelength(nm)	215	215	238	268	238	268
Concentration range(µg/mL)	10-30	10-30	10-30	10-30	10-30	10-30
Intercept	78031	235144	0.0317	0.0055	0.0029	0.0055
Slope	35060	43731	0.0194	0.0034	0.0046	0.0088
Correlation coefficient (r2)	0.9999	0.9998	0.9966	0.9987	0.9973	0.9968
Regression equations	78031x+35060	43731x+235144	0.0317x+0.0194	0.0055x+0.0034	0.0029x-0.0046	0.0055x-0.0088
Repeatability (%RSD, n=6)	0.434	0.430	0.277	0.726	0.556	0.570
Intraday precision (%RSD, n=3)	0.581-0.925	0.867-1.078	0.985-1.088	1.072-1.178	1.155-1.234	1.111-1.158
Interday precision (%RSD, n=3)	1.054-1.496	1.456-1.646	1.395-1.876	1.547-1.950	0.511-1.776	1.637-1.851
Accuracy (%recovery)	99.87-100.12	99.49-100.46	98.9-101.10	99.65-100.34	100.30-101.41	99.25-100.84
LOD (µg/ml)	0.213	0.434	0.127	0.213	0.582	0.560
LOQ (µg/ml)	0.647	1.317	0.387	0.387	1.765	

Table 3: Data for Intraday and Interday precision

Drug	HPLC method			Absorbance ratio method			
	Amt. (µg/ml)	% RSD		Amt. (µg/ml)	% RSD		
		Intraday	Interday		Intraday		Interday	
					238 nm	268 nm	238 nm	268 nm
AML	10	0.719	1.273	10	1.088	1.178	1.876	1.789
	20	0.925	1.496	20	1.061	1.135	1.586	1.950
	30	0.581	1.054	30	0.985	1.072	1.395	1.547
NEB	10	1.078	1.646	10	1.155	1.158	1.774	1.851
	20	0.867	1.456	20	1.234	1.121	1.741	1.637
	30	0.936	1.513	30	1.169	1.111	0.511	1.643

r=0.9999; n=3, for AML and Y=43731x+235144,r^2= 0.9998; n=3) was obtained over the working concentration range of 10-30 µg ml^{-1} for AML and 10-30 µg ml^{-1} for NEB.

The specificity and selectivity of the HPLC system were ascertained by a separate chromatographic analysis of either the excipient mixtures or sample; no interfering peaks at the retention times of AML and NEB peaks were observed.

The LOD and LOQ in accordance with the ICH guidelines is 3/1 and 10/1, respectively. LOD and LOQ values for AML and NEB for Absorbance ratio method and HPLC method are given in Table 2.

Each calibration curve was constructed with five standard strengths (Figure 3). For Absorbance ratio method, the calibration curve of AML was made with 10, 15, 20, 25 and 30 µg/ml concentrations (Figure 4a and 4b). Similarly, the concentrations used in the formation of calibration curve of NEB were 10, 15, 20, 25 and 30 µg/ml (Figure 4c and 4d). Typical chromatogram is given in Figure 5. For HPLC method, the calibration curve of AML was made with 10,15,20,25 and 30 µg/ml concentrations (Figure 6a). Similarly, the concentrations used in the formation of calibration curve of NEB were 15, 20, 25, 30 and 35 µg/ml (Figure 6b). The regression analysis is displayed in Table 2. The correlation coefficient (r^2) was close to 0.9999 for both AML and NEB.

The results of accuracy and precision (inter-day variance and intra-day variance) are shown in Table 3 and 4. For accuracy, all the recovery values were within ± 5%. By Absorbance ratio method, the mean recovery value of AML and NEB was 99.65-100.34 % and 99.25-100.84 % at 238 nm and 100.05-100.62% and 99.19-101.54% at 268 nm. In HPLC method the recovery values of AML and NEB was 99.87% and 100.12% and 99.49-100.46 respectively. For inter-day and intra-day variance assess-

Table 4: Results of recovery study by HPLC and UV methods. (n=3)

Method	Drug	Amt. present (µg/ml)	Amt. added (µg/ml)	Amt. recovered (µg/ml)	% Recovery
HPLC Method	AML	10	8	-	100.04
		10	10	19.92	99.63
	NEB	10	12	23.91	99.64
		10	20	19.96	99.83
		10	25	24.91	99.65
		10	35	29.90	99.68
UV method	AML 238 nm	12	9.6	22.32	100.83
		12	12	24.5	98.9
		12	14.4	27.2	101.10
	NEB 238 nm	15	15	27.1	102..50
		15	27	30.2	102.66
		15	30	32.1	100.55
	AML 268 nm	12	9.6	22.02	100.30
		12	12	24.56	101.41
		12	14.4	26.9	100.76
	NEB 268 nm	15	15	27.3	98.33
		15	27	30.5	100.0
		15	30	33.76	101.44

Table 5: Assay of marketed formulation by HPLC and UV methods

Assay	Marketed formulation	Drug	Label claimed (mg/tab)	Amt. found (mg/tab)	% Label claimed	SD
HPLC Method	A30708	AML	5	4.94	98.8	0.245
		NEB	5	4.96	99.2	0.161
	ND60388	AML	5	5.03	100.6	0.488
		NEB	5	5.01	100.2	0.436
	N40250	AML	5	4.94	98.8	0.688
		NEB	5	4.99	99.8	0.381
UV method	A30708	AML	5	4.98	99.6	0.245
		NEB	5	5.02	100.4	0.545
	ND60388	AML	5	5.05	101.0	0.121
		NEB	5	5.04	100.8	0.150
	N40250	AML	5	4.92	98.4	0.714
		NEB	5	4.91	98.2	0.280

ment %RSD was calculated. All the samples exhibited RSD values <1% confirming that the analytical method was precise.

Robustness study was carried out by making minor changes in conditions like composition of mobile phase, flow rate of mobile phase and pH of mobile phase. No substantial variances were observed in the retention time and peak area of each component when the chromatographic conditions were slightly changed one by one. Moreover, the RSD for each value was <1%. Thus, the proposed method was considered as robust.

Statistical evaluation of the developed procedures

The HPLC method was chosen as the analytical reference method. Absorbance ratio spectrophotometric procedures were compared with HPLC. The slopes, intercepts and linearity of each calibration graph were calculated and summarized in Table 2. The order of linearity for the calibration graphs in the ranges stated in Table 2 for the different analytical method was: Absorbance ratio/HPLC. The concentration ranges, detection limits and quantitation limits are summarized in Table 2. The lowest detection limit calculated was obtained for absorbance ratio method indicating the highest sensitivity. Relative sensitivities, based on detection limits, were calculated with respect to the chromatographic method. The order of sensitivity for this method was: Absorbance ratio\ HPLC. Commercially available tablets were analyzed using the HPLC and the Absorbance ratio spectrophotometric methods. The results obtained were summarized in Table 5. No significant differences were

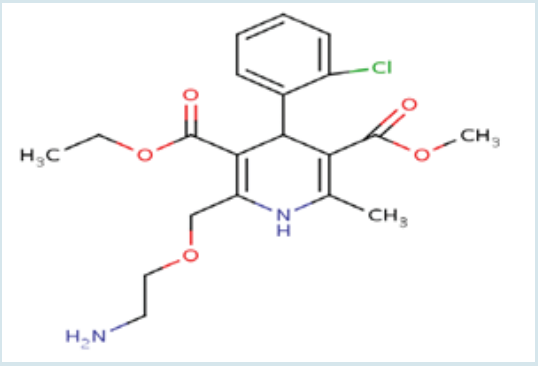

Figure 1: Structure of Amlodipine Besylate.

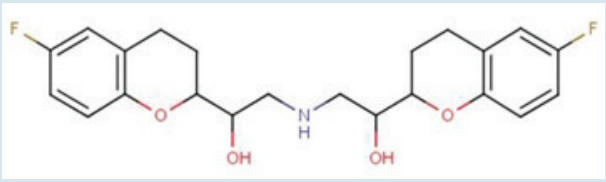

Figure 2: Structure of Nebivolol Hydrochloride.

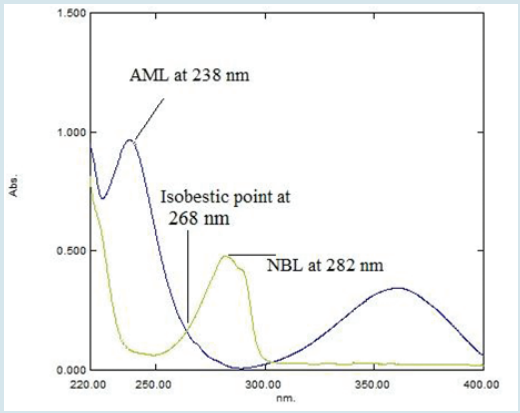

Figure 3: Overlay UV-spectra of AML and NEB.

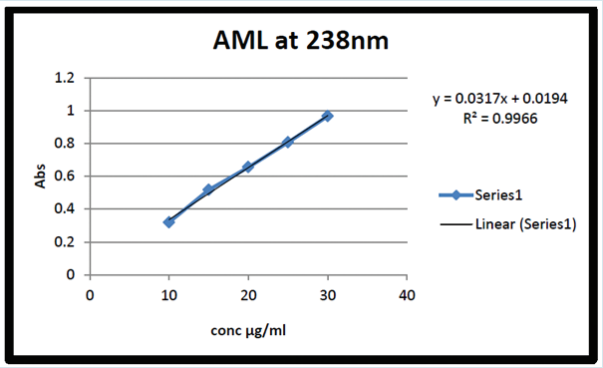

Figure 4a: Calibration curve for AML at 238 nm.

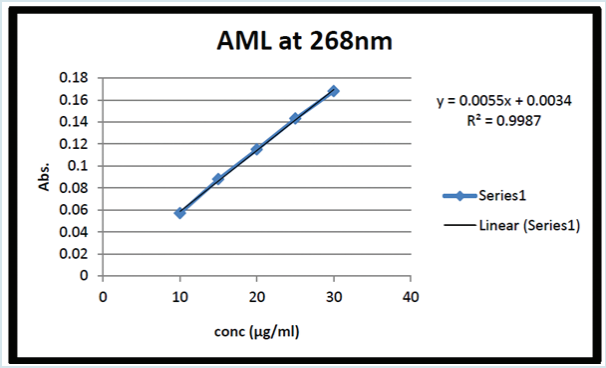

Figure 4b: Calibration curve of AML at 268 nm.

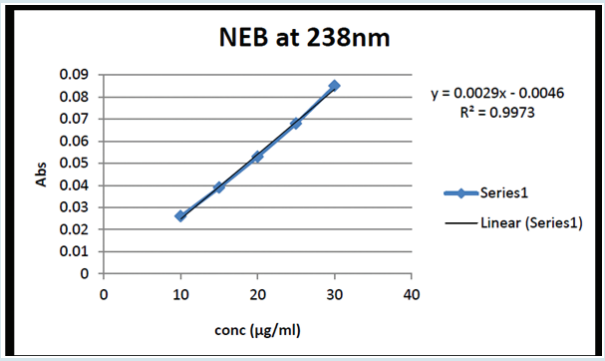

Figure 4c: Calibration curve for NEB at 238 nm.

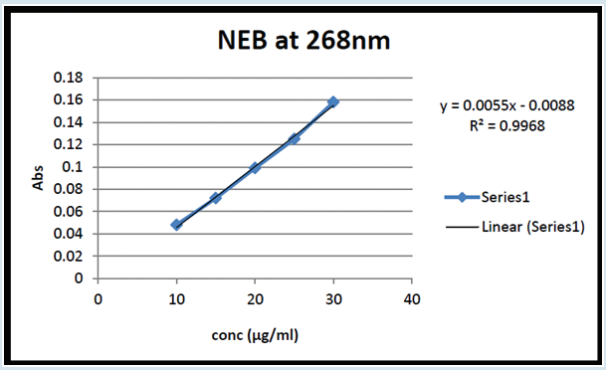

Figure 4d: Calibration curve for NEB at 268 nm.

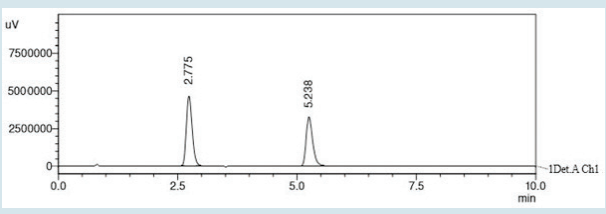

Figure 5: Typical Chromatogram of AML and NEB.

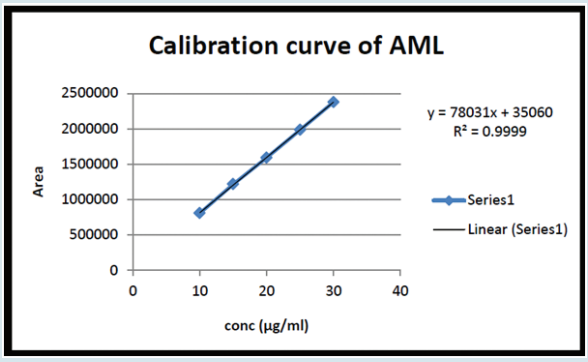

Figure 6a: Calibration curve for AML in HPLC method.

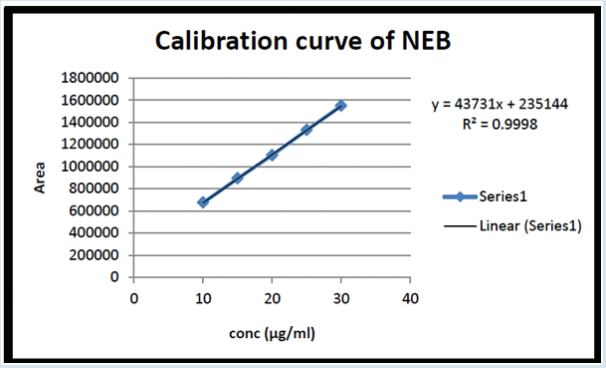

Figure 6b: Calibration curve for NEB in HPLC method.

found between the results obtained by the HPLC and the spectrophotometric procedures, for the same batch at the 95% confidence level. Statistical comparison was done on assay results obtained from UV and HPLC methods for marketed formulation by using student's t-test. Calculated values for t-test were 1.05 and 0.078 for AML and NEB respectively which is less than $t_{critical}$ value (4.30) indicating that there was no significant difference between the HPLC method and UV method.

CONCLUSION

The HPLC method and the spectrophotometric (Absorbance ratio) method were found to be reproducible and accurate in the analysis of AML and NEB in pharmaceutical tablets. Under the experimental conditions, mentioned above, the Absorbance ratio method was the most sensitive method; however, better selectivity was obtained with the the HPLC method. All the proposed methods were linear with good reproducibility and sensitivity. In general, all the proposed methods can be used for the routine analysis of AML and NEB in bulk and tablet dosage form.

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CONFLICT OF INTEREST

No conflict of interest.

ABBREVIATIONS USED

HPLC: High Performance Liquid Chromatography; **UV:** Ultra violet; **HPTLC:** High performance thin layer chromatography; **AML:** Amlodipine Besylate; **NEB:** Nebivolol Hydrochloride; **ICH:** International Conference of Harmonization; **S/N:** Signal to noise; **RSD:** Relative standard deviation.

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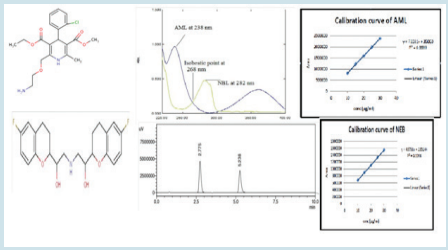
PICTORIAL ABSTRACT



SUMMARY

- Absorbance ratio spectrophotometric method is developed for the determination of Amlodipine Besylate And Nebivolol Hydrochloride in tablet dosage form at 268 nm and 238 nm.
- A simple liquid chromatographic assay method has been developed for the determination of Amlodipine Besylate And Nebivolol Hydrochloride. C_{18} (250×4.6 mm, 5 µm) column was used with a mobile phase consisting of Water:Acetonitrile; 60:40 v/v (pH adjusted to 3.5 with ortho phosphoric acid) at a flow rate of 1.0 ml min^{-1}. UV detection was at 268 nm based on the peak height ratios.
- The proposed procedures were successfully applied to the determination of Amlodipine Besylate And Nebivolol Hydrochloride in bulk and tablet form, with high percentage of recovery, good accuracy and precision.

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