



**RESEARCH ARTICLE**

**Stability Indicating HPLC Method for Estimation of S-amlodipine besylate and Nebivolol hydrochloride in Bulk Drugs and Marketed Formulation**

**Kolasani A<sup>2</sup>, Kumar GVS<sup>1\*</sup>, Puranik SB<sup>3</sup>, Sridhar KA<sup>4</sup>**

*<sup>1,2,3,4</sup>East West College of Pharmacy, Rajiv Gandhi University of Health Sciences, Bangalore, Karnataka, India.*

Manuscript No: IJPRS/V1/I3/00176, Received On: 26/09/2012, Accepted On: 13/10/2012

**ABSTRACT**

A simple, precise and stability indicating reversed phase liquid chromatographic method was developed and validated for estimation of s-amlodipine besylate and nebivolol hydrochloride in bulk drug and marketed formulation. The separation was achieved on Zorbax C8 G (250mm x 4.6mm, 5 $\mu$ m) analytical column with mobile phase comprising of 0.05M Potassium di hydrogen phosphate: Acetonitrile (pH 3.0) (60:40v/v) at isocratic flow of 1.0ml/min with UV detection at 269 nm. The retention times of s-amlodipine besylate and nebivolol hydrochloride was found to be 5.2 and 6.8 minutes respectively. The method was successfully validated in accordance to ICH guidelines for accuracy, precision, specificity, linearity, ruggedness and robustness. The linear regression analysis data for calibration plots showed good linear relationship in the concentration range 0.125-0.375 $\mu$ g/mL for s-amlodipine besylate and 0.25-0.75 for nebivolol hydrochloride. The drugs were exposed to acidic, basic, oxidation, thermal and photolytic stress degradation conditions. The resultant stressed samples were analyzed by the proposed method and was established to provide high resolution among the degradation products and the analytes. All the peaks of degraded product were resolved from the active pharmaceutical ingredient with significantly different retention time and the peak purity of analyte peaks in the stressed samples was confirmed by photodiode array detector. The method could effectively separate the drug from its degradation product; it can be employed as a stability- indicating one.

**KEYWORDS**

s-amlodipine besylate , nebivolol hydrochloride, RP-HPLC, Stability indicating.

**INTRODUCTION**

s-amlodipine besylate (AMB) is chemically (RS)-3-ethyl 5-methyl 2-[(2-amino ethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1, 4-dihydropyridine-3, 5-dicarboxylate. It is indicated for the treatment of essential hypertension. S-Amlodipine is a dihydropyridine calcium antagonist (calcium ion antagonist or slow-channel blocker) that inhibits the trans membrane influx of calcium ions into vascular smooth muscle and cardiac muscle.

Nebivolol hydrochloride (NEB), 1-(6-fluorochroman-2-yl){[2-(6-fluorochroman 2-yl)-2-hydroxy-ethyl]amino}ethanol. **Nebivolol** lowers blood pressure (BP) by reducing peripheral vascular resistance, and significantly increases stroke volume with preservation of cardiac output.

Several methods have been studied for simultaneous determination of AMB and NEB, but there are limited reports on method for combination. So the aim of our study is to develop simple, fast, accurate and specific reversed phase high performance liquid chromatographic method for simultaneous determination of related substances of s-

**\*Address for Correspondence:**

**G.V.S. Kumar**

East West College of Pharmacy,  
Rajiv Gandhi University of Health Sciences,  
Bangalore-560 091,  
Karnataka, India.

**E-Mail Id:** [ewcp.analysis@gmail.com](mailto:ewcp.analysis@gmail.com)

amlodipine besylate and nebivolol hydrochloride in bulk drugs and tablet formulation.

Stability indicating method had been performed on s-amlodipine besylate and nebivolol hydrochloride. The samples were subjected to acid, alkali oxidation, thermal and photolytic conditions. S-amlodipine was found to be degraded at 6<sup>th</sup> hour at 2.5min.

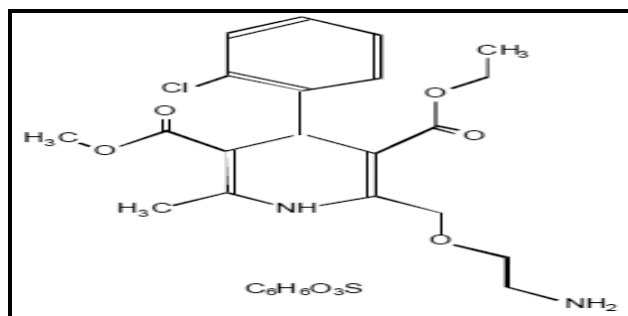


Figure 1: S-Amlodipine besylate

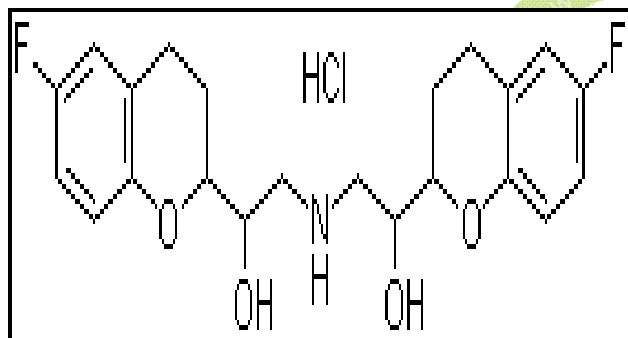


Figure2: Nebivolol hydrochloride

## MATERIALS AND METHODS

### Chemicals and Reagents

Pure samples of AMB and NEB were obtained respectively from Emcure pharmaceuticals ltd, Pune, India and Hetero pharmaceuticals limited, Hyderabad, India. The commercial pharmaceutical preparation NEBICARD-SM containing 0.25mg and 0.5mg AMB and NEB respectively (Marketed by Torrent pharmaceutical Ltd) were procured from local pharmacy. Acetonitrile; Methanol and HCl was procured from SD fine- chem Limited, Mumbai, India; High purity deionised water was obtained from [Millipore, Milli-Q] purification system.

### HPLC Instrumentation and Conditions

HPLC system LC SHIMADZU UFLC-2000 ProminenceLC-20AD Binary Gradient System, Shimadzu Corporation, Japan. The column compartment having temperature control and Photodiode Array/ Ultraviolet (PDA/UV) Detector was employed throughout the analysis. Chromatographic data was acquired using Empower software.

### Chromatographic Conditions

Zorbax C-8 (250mmX4.6mm, 5µm) column was used. Mobile phase consisting of 0.05M KH<sub>2</sub>PO<sub>4</sub> buffer (0.05M KH<sub>2</sub>PO<sub>4</sub> buffer was prepared by dissolving 6.8045 g in 1000 ml of Millipore water or double distilled water and pH adjusted to 3.0 with ortho phosphoric acid) : ACN, (60:40 v/v). The flow rate was 1.0 mL/min. UV detection was performed at 269 nm at ambient temperature using 20 µL injection volumes.

### Standard and Sample Preparation

The standard stock solutions of AMB and NEB of 1000µg/mL and 500 µg/mL were prepared separately by dissolving working standards in small proportion of mobile phase and later diluted to desired volume with mobile phase. Standard calibration solutions of AMB and NEB having concentration in the range of 0.125 µg/mL and 0.25-0.375 µg/mL respectively were prepared by diluting stock solution with mobile phase.

### Analysis of Dosage Form

Twenty tablets were weighed, their mean weight determined, and crushed in mortar. An amount of powdered mass equivalent to one tablet content was transferred into a 50ml volumetric flask containing 10 ml of mobile phase, mechanically shaken for 10 min, ultrasonicated for 5 min, and then diluted to volume with mobile phase (sample stock solution). About 1 ml of sample stock solution was centrifuged at 10,000 rpm, and diluted to 100 ml with mobile phase and (sample solution). Further 5ml of the

above stock solution diluted to 100 with the mobile phase A small portion of sample solution was filtered through 0.45 $\mu$  nylon filter and used for injection on HPLC.

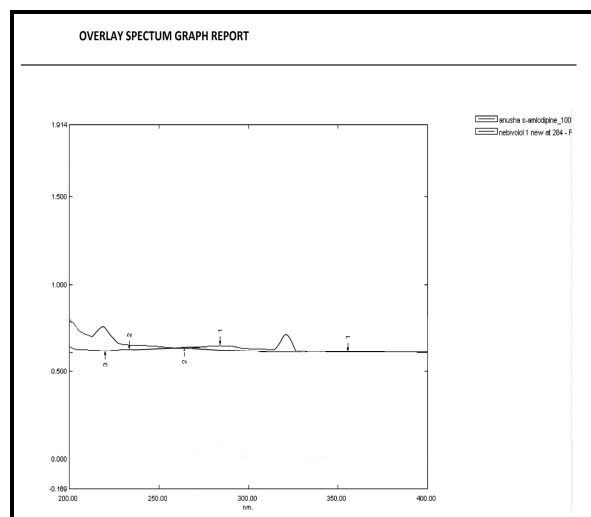


Figure 3: Overlay spectra of AMB and NEB

### Method Validation<sup>10-17</sup>

The optimized chromatographic conditions were validated by evaluating specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness and system suitability parameters in accordance with the ICH guideline Q2 (R1).

### Linearity and Range

Linearity was determined by plotting the standard curve in the concentration range of 0.125-0.375 $\mu$ g/mL and 0.25-0.75  $\mu$ g/mL for both s-amlodipine besylate and nebivolol hydrochloride Fig. 5. The linearity of the methods was evaluated by linear regression analysis, using least square method Table-1.

Table 1: Linearity data for AMB and NEB

Linearity (n=5)	AMB	NEB
Range	0.125-0.375 $\mu$ g/mL	0.25-0.75 $\mu$ g/mL
Mean 'r <sup>2</sup> ' value	0.998	0.999
Regression equation	Y=392037x+1693	Y=720412x-9302

### Accuracy

This parameter is performed to determine the closeness of test results with that of the true value which is expressed as % recovery. These studies were performed for both s-amlodipine besylate and nebivolol hydrochloride at three different levels (50%, 100% and 150%), the mixtures were analyzed by the proposed method. The experiment was performed in triplicate and recovery (%), standard deviation (SD) and relative standard deviation RSD (%) of the spiked drugs was calculated. Results are presented in Table-2.

### Precision

The precision (system, method) of the proposed method was evaluated by carrying out six independent assays of the sample. RSD (%) of six assay values obtained was calculated. The intermediate precision was carried out by analyzing the sample at different days and different analysts and the data is presented in Table-3.

### Specificity

Specificity of the method was evaluated by injecting the stressed samples (sample heated to 60°C, sample treated with 0.1N HCl in acid condition, and sample treated with 0.1N NaOH in alkali condition, 3% v/v H<sub>2</sub>O<sub>2</sub> in oxidative condition, thermal at 48hrs and photolytic at 48hrs) and working standard solutions separately into HPLC. The subjected drug peaks of AMB and NEB were evaluated with photo diode detector for purity angle Fig.6.

### Limit of Detection and Limit of Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) for AMB and NEB were determined from standard deviation of the response and the slope.

$$\text{LOD} = \sigma/S \times 3.3; \text{LOQ} = \sigma/S \times 10$$

### Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method

parameters and provides an indication of its reliability during normal usage Table-4.

Robustness of the method was investigated under a variety of conditions like change in flow rate by  $\pm 0.2$  ml/minute, and change in wavelength by  $\pm 2$  nm. The mixed standard solution is injected in five replicates and sample solution of 100% concentration is prepared and injected in triplicate for every condition and % RSD of assay was calculated for each condition.

### System Suitability

The system suitability parameters with respect to theoretical plates, tailing factor and resolution between impurity AMB<sub>1</sub> peak and NEB peak were established. Results are presented in Table-5.

### Force Degradation Study

Both the drugs AMB and NEB were subjected to stress testing as per ICH recommended test conditions. The drugs were subjected to acid hydrolysis by using 0.1N hydrochloric acid and alkali hydrolysis by using 0.1N sodium hydroxide solution; oxidation by using 30% v/v solution of hydrogen peroxide; thermal and photolysis. The objective of stress study was to generate the degradation products under various stress conditions and to verify that the degradation peaks are well resolved from the main peaks.

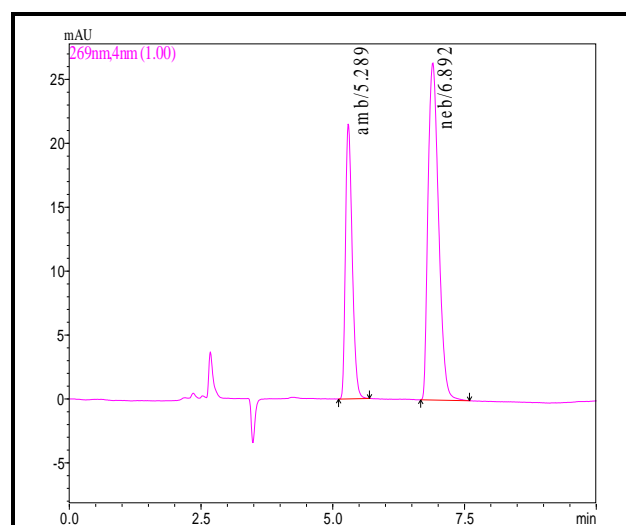


Figure 4: A typical chromatogram of standard solutions of AMB and NEB

## RESULTS AND DISCUSSION

### Method Development

A variety of mobile phases were investigated in the development of a stability-indicating LC method for the analysis of AMB and NEB in tablet dosage form. The suitability of mobile phase was decided on the basis of selectivity and sensitivity of the assay, stability studies and separation among impurities formed during forced degradation studies.

The maximum absorption wavelength of the reference drug solution and of the forcefully degraded drug solution was found to be 269 nm. This was observed from the UV absorption spectra Fig. 3 and was selected as detection wavelength for LC analysis. As the main objective of this chromatographic method was separation of degraded impurities from both the drugs.

During the optimization of the method, different columns (Inertsil C8, 250mm×4.6mm, 5 $\mu$ m; Zorbax C8 250mm×4.6mm, 5 $\mu$ m; Symmetry C18 250mm×4.6mm, 5 $\mu$ m; Enable C18 G 250mm x 4.6mm, 5 $\mu$ m) and two organic solvents (acetonitrile and methanol) were tested. The chromatographic conditions were also optimized by using different buffers like phosphate, acetate and citrate for mobile phase preparation.

After a series of screening experiments, it was concluded that phosphate buffers gave better peak shapes than their acetate and citrate counter parts. With methanol as solvent both the peaks shows less theoretical plates and more retention time compared to acetonitrile.

While assessing the effect of pH on the retention time of analytes and degradants, the peak of NEB with phosphate buffer (pH 2.2) and acetonitrile in the ratio of 60:40 (% v/v), revealed from the PDA analysis, was not pure which suggested co-elution of some impurity peak(s). Further on increase of pH of mobile phase to pH 3.0 potassium di hydrogen phosphate buffer helped to sharpen the NEB peak, probably due to increase in hydrophobic interactions between stationary phase and less



unionized analyte. After several trials, using Zorbax C8 G (250mm x 4.6mm, 5 $\mu$ m) analytical column and the mobile phase consisting 0.05M KH<sub>2</sub>PO<sub>4</sub> buffer (pH 3.0) and ACN (60:40% v/v), and the flow rate of 1.0ml/min was considered optimum to achieve adequate retention time and sharp peaks of both the drugs and their impurities. System suitability parameters (Tailing factor, HETP, Resolution, Theoretical Plates, Asymmetry) for analyte peaks were evaluated and presented in the table-5.

### Method Validation

The calibration plot for the method was linear over the concentration range of 0.125-0.375 $\mu$ g/mL and 0.25-0.75 for AMB and NEB. The determination of coefficients ( $r^2$ ) was 0.998 and 0.999 for AMB and NEB, respectively. Values of the method Accuracy was calculated by recovery studies for AMB and NEB at three levels and found to be 98.81% to 102.75% and 99.25% and 102.42% respectively. For precision and intermediate precision, % RSD of AMB and NEB were within 2.0% thus confirm good precision of the analytical method development. In Specificity there was no any interference at the retention time of AMB and NEB in the chromatogram of placebo solution. In peak purity analysis with photo diode detector, purity angle was less than purity threshold for both the analytes. The LOD and LOQ of AMB and NEB were found to be 0.0025 ng/mL, 0.009 ng/mL and 0.013 ng/mL, 0.027 ng/mL respectively. Robustness of the method was performed by making deliberate changes in flow rate and wave length and it was by calculating established % RSD values and was within acceptance criteria range of 2.0%.

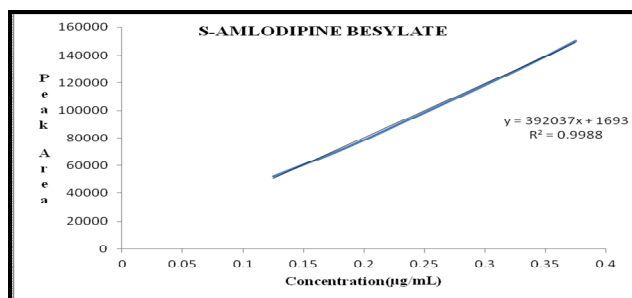


Figure 5: Calibration curve of AMB

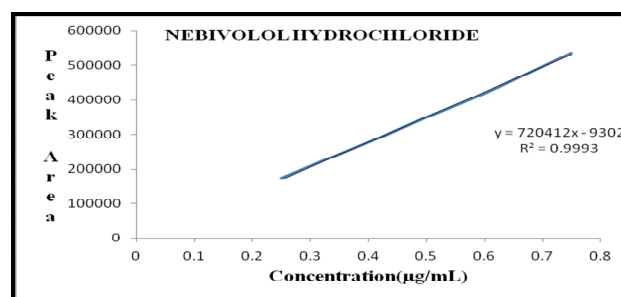


Figure 5: Calibration curve of NEB

Table 2: Results of recovery analysis of AMB and NEB

Drugs	Levels	Mean recovery	$\pm$ SD	% RSD
AMB	L <sub>1</sub>	102.75%	0.73	0.71
	L <sub>2</sub>	98.81%	0.92	0.94
	L <sub>3</sub>	100.49%	1.0	1.03
NEB	L <sub>1</sub>	100.12%	0.90	0.90
	L <sub>2</sub>	99.25%	0.80	0.80
	L <sub>3</sub>	102.42%	0.54	0.54

Table 3. Results of Intermediate precision (intra-day and inter-day) experiment using proposed method

Compound	Intra-day precision		Inter-day precision	
	% of Label	% RSD	% of Label	% RSD
AMB	100.5	1.08	100.9	0.71
NEB	100.3	0.61	100.1	0.65

Table 5: System suitability data for AMB and NEB using proposed method

Compound	Retention time (minutes)	Tailing factor	Theoretical plate/meter	Resolution
AMB	5.34	1.42	7558.829	5.242
NEB	6.96	1.37	5581.943	

Table 4: Results of Robustness data for change in flow rate and wavelength

Changing Factor	Level	AMB (n=3) Mean % assay % RSD	NEB (n=3) Mean % assay % RSD
Flow rate	0.8 MI	96.94% (0.55%)	100.29% (1.04%)
	1.2 MI	98.88% (0.68%)	99.92% (0.97%)
Wavelength	267	99.64% (0.78%)	98.82% (0.94%)
	271	98.92% (0.65%)	99.90% (1.09%)

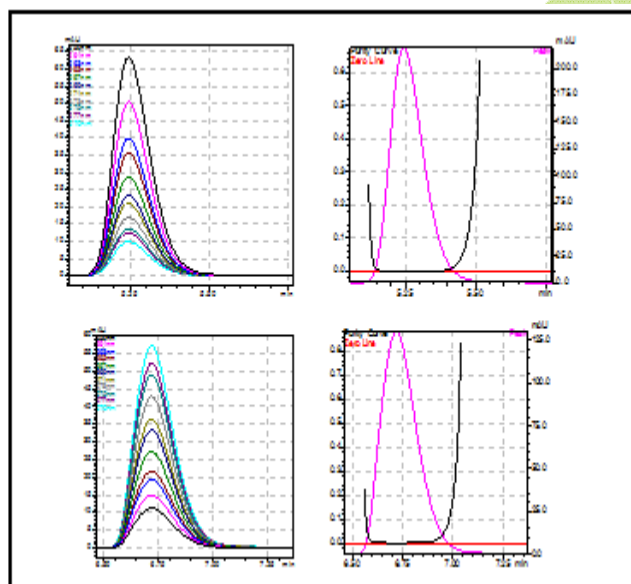


Figure 6. Chromatograms for specificity (AMB and NEB Peak profile and Peak purity)

### Results of Forced Degradation Studies

Subsequently, different forced degradation samples were analyzed. Both the drug peaks in acid, alkaline, oxidation, thermal and photo-degraded solutions passed the purity test. Results of forced degradation study showed that impurity AMB<sub>1</sub> was formed as a result of hydrolysis of AMB during oxidation stress studies at retention time 2.5 min. Fig. 7.

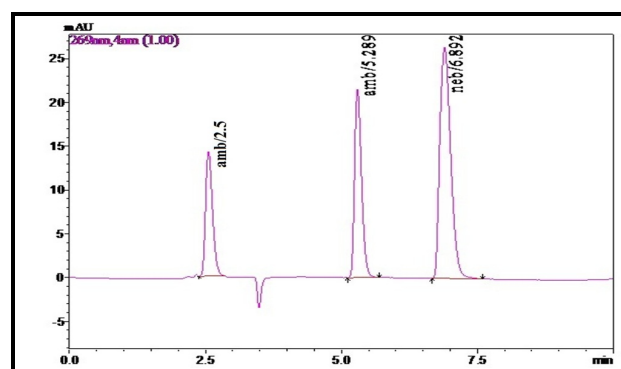


Figure 7: The simple chromatogram of Acid degraded sample

### CONCLUSION

The developed HPLC technique is precise, specific, accurate and Stability-indicating. Statistical analysis proves that the method is suitable for the analysis of both AMB and NEB in bulk and pharmaceutical formulation without any interference from the excipients. The method can be used to determine the purity of drug available from various sources by detecting any related impurities. The method has been found to be better than previously reported methods, because of use of a less economical and readily available mobile phase, lack of extraction procedures, no internal standard, and use of the same mobile phase for washing of the column. All these factors make this method suitable for quantification of AMB and NEB in bulk drugs and in pharmaceutical dosage forms.

### ACKNOWLEDGEMENT

The author thanks Emcure Pharma Ltd, Pune, Mumbai and Hetero Pharma Ltd, Hyderabad for providing the gift samples of s-amlodipine besylate and nebivolol hydrochloride. We thank to the EWCP management for providing the facilities and chemical.

### REFERENCES

1. Jain Mayank *et al*; "A validated HPLC method simultaneous estimation of Amlodipine Besylate and Nebivolol hydrochloride in pharmaceutical dosage forms". Indian Journal of Pharmaceutical Sciences, 2009, 357-59.

2. Shah D.A *et al*; "Stability indicating RP-HPLC estimation of Nebivolol hydrochloride in Pharmaceutical Formulations". Indian Journal of Pharmaceutical Sci., 2008, 336-42.
3. Meyyanathan SN *et al*; "A validated RP – HPLC method for simultaneous estimation of Nebivolol and Hydrochlorothiazide in tablet". Indian Journal of Pharmaceutical Sciences, 2008, 9, 687 – 9.
4. Rajan KB *et al*; "Simultaneous high performance liquid chromatographic determination of atenolol and amlodipine in pharmaceutical dosage form. Pakistani Journal of Pharmaceutical Sciences, 2007, 20(4), 274-9.
5. Prasad RCHMM *et al*; "RP-HPLC method of simultaneous estimation of amlodipine besylate and metoprolol in combined dosage form". International Journal of Pharmaceutical Research and Development, 2010, 2(9), 69-76.
6. Chitlange SS, Kiran B, Sakarkar DM, "Stability indicating RP-HPLC method for simultaneous estimation of valsartan and amlodipine in capsule formulation". Asian Journal of Research in Chemistry, 2008, 1(1), 15-19.
7. Devi R, Ramakrishna S, "New spectrophotometric methods for simultaneous determination of amlodipine besylate and atorvastatin calcium in tablet dosage form". International Journal of Pharmaceutical Sciences, 2010, 2(4), 215-9.
8. Mustafa C, *et al*; "HPLC method development for the simultaneous analysis of amlodipine and valsartan in combined dosage forms and invitro dissolution studies". Brazilian Journal of Pharmaceutical Sciences, 2010, 46(4), 761-8.
9. Wankhede SB *et al*; "Simultaneous estimation of amlodipine besylate and olmesartan medoxomil in pharmaceutical dosage form". Indian journal of Pharmaceutical Sciences, 2009, 71(5), 563–7.
10. Pournima P, *et al*, "Spectrophotometric method for the simultaneous determination of olmesartan medoxomil and amlodipine besylate from tablet dosage form". International Journal of Pharmaceutical Sciences Research and development, 3(2), 74-79.
11. Santaji N, *et al*, "Rapid simultaneous determination of telmisartan, amlodipine besylate and hydrochlorothiazide in a combined poly pill dosage form by stability-indicating ultra performance liquid chromatography". Sci Pharm., 2011, 79, 69-84.
12. Naidu KR, Kale UN, Shingare MS, "Stability indicating RP-HPLC method for simultaneous determination of amlodipine and benazepril hydrochloride from their combination drug product". Journal of Pharmaceutical and Biomed Analysis, 2005, 39(1-2), 147-55.
13. Priyanka PR *et al*; "RP-HPLC method for the simultaneous estimation of losartan potassium and amlodipine besylate in tablet formulation", International Journal of ChemTech Research, 2009, 1(3), 464-9.
14. Shah DA *et al*; "Stability Indicating RP-HPLC Estimation of atorvastatin calcium and amlodipine besylate in pharmaceutical formulations", Indian Journal of Pharmaceutical Sciences, 2008, 70(6), 754-60.
15. International Conference on Harmonization, ICH Q1 A(R2); "Stability Testing of New Drug Substances and Products", 2003.
16. ICH Q2B- "Validation of Analytical Procedures: Methodology", International Conference on Harmonization, Nov, 1996.
17. ICH-Q2B "Validation of Analytical Procedures: 1996. Methodology", International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland.