Analytical Method Development and validation for Simultaneous Determination of Atenolol and Nitrendipine in Pharmaceutical Dosage Form by RP-HPLC Method

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INTRODUCTION

Atenolol is a competitive β1-selective adrenergic receptor blocking agent without membrane stabilizing or intrinsic sympathomimetic activities. Atenolol inhibits β2-adrenoreceptors, chiefly located in the bronchial and vascular musculature [1]. Chemically it is known as 2-[4-2(RS)-2-hydroxy 3[(1-methylethyl) amino] propoxy] phenyl] acetamide. It is a white powder and molecular weight is 266.3 and freely soluble in methanol and soluble in acetic acid and dimethyl sulfoxide Figure [1]. Atenolol is also used to treat myocardial infarction and arrhythmias, angina and disorders arising from decreased circulation and vascular constriction, including migraine. Atenolol may be used alone or with other antihypertensive agents including thiazide-type diuretics, hydralazine, prazosin, and α-methyldopa [2].

Nitrendipine is a dihydropyridine calcium channel blocker, is used alone or with an angiotension converting enzyme inhibitor, to treat hypertension, chronic stable angio pectoris and prinzmetal’s variant angina. Chemically it is Ethyl methyl (4RS) -2,6-...
dimethyl-4-(3-nitrophenyl)- 1, 4-dihydropyridine-3,5-dicarboxylate. It is a yellow crystalline powder and molecular weight is 360.3 and freely soluble in ethyl acetate, soluble in ethanol and methanol [3] Figure [2].

![Figure 1: Chemical Structure of Atenolol](image1)

![Figure 2: Chemical Structure of Nitrendipine](image2)

Literature survey revealed that several methods have been reported estimation of Atenolol and Nitrendipine individually or in combination with other drugs in pharmaceutical dosage forms and/or in biological fluids [4-20]. However, no HPLC method has been reported so far for the estimation of these two drugs simultaneously in combined dosage forms. Hence, in the present study, a new reversed-phase high performance liquid chromatography method was developed and validated for the simultaneous estimation of Atenolol and Nitrendipine in tablets. The method was validated for accuracy, precision, specificity, detection limit, quantitation limit, robustness and solution stability as per ICH guidelines [21]. Present work describes rapid, accurate, reproducible, and economical methods for simultaneous determination of these drugs in pharmaceutical dosage form.

MATERIALS AND METHODS

Reagents and Chemicals Used:

HPLC grade acetonitrile and methanol was procured from Rankem (Mumbai, India) and Millipore water obtained from (Milli Q) was used in all experiments. Ortho phosphoric acid was procured from Qualigens fine chemicals, (Mumbai, India). Pharmaceutical dosage form containing labeled amount of Atenolol (50mg) and Nitrendipine (10 mg) (Cardif beta-10, Concept Pharmaceuticals Pvt. Ltd.) was purchased from the local pharmacy.

Instrumentation and Conditions:

The chromatographic separation was performed by using Shimadzu LC 2010 AHT HPLC system with UV-Visible detector and Rheodyne injector. Chromatographic analysis was performed on a Phenomenex Luna C-18 with 250×4.6 mm and 5 μm particle size. The mobile phase was composed of mixture of acetonitrile, methanol and water in the ratio of 40:40:20 v/v/v (pH adjusted to 3.0 with orthophosphoric acid). It was filtered through a 0.45 μ membrane filter and degassed for 10 minutes. Before analysis the mobile phase was filtered through a 0.2μm filter and degassed using sonicator at the flow rate of 1.0ml per minutes. Sample solutions were also filtered through a 0.2μm filter and aliquots of 10μL were injected into the chromatographic system. The flow rate of the mobile phase was maintained at 1.5 ml/min. Detection was carried out at 235 nm [Figure 3]. The HPLC system was used in air-conditioned laboratory atmosphere temperature (25±2°C).

Preparation of mobile phase:

The mobile phase was prepared by mixing of methanol: Acetonitrile: water in the ratio of 40:40:20 v/v/v and it is filtered and degassed. The Chromatogram was obtained is shown in [figure-4]

Preparation of Standard Solution:

50mg of Atenolol and 10mg of Nitrendipine working standard was weighed accurately into a 100ml volumetric flask, dissolved with small quantity of methanol (10ml) and was made up to volume with mobile phase. Solution was filtered through 0.45μ membrane filter. First few ml of the filtrate was discarded. 5ml of stick solution was pipette out and transferred into a 50ml volumetric flask and was made up to the volume with mobile phase.

Preparation of Sample Solution:

Twenty tablets were weighed accurately and powdered. Powder equivalent to 50mg of atenolol was weighed.
Figure 4: Chromatogram for Mobile Phase (Blank)

Figure 5: Chromatogram for Placebo

Figure 6: Chromatogram for Atenolol and Nitrendipine
and transferred to 100ml volumetric flask and dissolved in small quantity of methanol (10ml) by sonicating the flask for 15 mins and was made up to volume with mobile phase. The solution was filtered through 0.45μ membrane filter. Take 5ml of the above filtrate and diluted to 50ml with mobile phase.

**Optimization of Chromatographic Conditions:**

The initial literature search indicated that many UV, HPLC and HPTLC methods are available for individual drugs and their combination with different drugs or formulations. Based on literature search, attempts were made to develop a simple method which has less retention time and high selectivity, top priority was given for complete separation of Atenolol and Nitrendipine. Several mobile phase were tested until good resolution, theoretical plate and tailing factors obtained between two drugs.

In preliminary experiments all the two drugs i.e., Atenolol and Nitrendipine were subjected to separation by reverse phase HPLC equipped with the Phenomenox Luna C-18 (250mm X 4.6 mm X 5μm) column and with flow rate 1.5mL/min and detection wavelength of 235nm. Column temperature was maintained at ambient. Injection volume is 20μL.

The mobile phase consists of buffer pH 4, acetonitrile and methanol (35:30:35%v/v/v). These drugs were able to be separated on the chromatogram but peak shape was not good and broad, retention time was more and tailing was more than specified limit. The effect of mobile phase composition was checked with different ratio and different composition and different solvents. It improved peak purity and peak shape and its pass all the system suitability parameters. Finally a method developed with methanol: acetonitrile: water (40:40:20%v/v/v). The chromatogram obtained was better than the previous one in all aspects with good peak shape, tailing factor, resolution and theoretical plate as per ICH and USP requirement. The retention times of Atenolol and Nitrendipine peaks are about 2.621 and 5.169 minutes respectively. The chromatograms were shown in [Figure 6].

**RESULTS:**

**Validation:**

The method was successfully validated as per ICH guideline Q2B (R1): validation of analytical procedures: text and methodology, international conference on harmonization, Food and Drug Administration, USA, November 2005. The method was validated and parameters were linearity, range, accuracy, precision, LOQ, LOD, robustness, system suitability and solution stability.

**Specificity**

Specificity was tested against standard compounds and against potential interferences in the presence of placebo. The comparison of the chromatograms of the synthetic placebo mixture and the spiked drug solution revealed that there was no interference of placebo with the peaks of Atenolol and Nitrendipine in sample solution. No interference from placebo was observed at the retention time of the drugs. Therefore, it was concluded that the method is specific. The Chromatogram was shown in [Figure 5].

**Linearity:**

Appropriate aliquots of standard stock solutions of Atenolol and Nitrendipine were diluted mobile phase to obtain final concentrations in the range of 30-70 μg/ml of Atenolol and 6-14 μg/ml of Nitrendipine. The solutions were injected in triplicates for each concentration using a 20 μl fixed loop system and chromatograms were recorded. Calibration curves were constructed by plotting average content of the drug versus respective concentrations and regression equations were computed for Atenolol and Nitrendipine. The plots of average content Vs respective concentration of Atenolol and Nitrendipine were found to be linear in the range of 30-70 μg/ml and 6-14 μg/ml with coefficient of correlation 0.9989 and 0.9992 for Atenolol and Nitrendipine respectively. The data of linearity curve was summarized in the Tables 1, 2 and [Figures 7 and 8].

| Table 1: Linearity data for Atenolol |
| S. No. | Concentration | Peak Area |
|-------|---------------|-----------|
| 1     | 30 μg/ml      | 1218292   |
| 2     | 40 μg/ml      | 1723148   |
| 3     | 50 μg/ml      | 2157782   |
| 4     | 60 μg/ml      | 2589426   |
| 5     | 70 μg/ml      | 3021724   |
|       | Correlation Coefficient (R²) | 0.9989 |

| Table 2: Linearity Date for Nitrendipine |
| S. No. | Concentration | Peak Area |
|-------|---------------|-----------|
| 1     | 6 μg/ml       | 519916    |
| 2     | 8 μg/ml       | 667884    |
| 3     | 10 μg/ml      | 847280    |
| 4     | 12 μg/ml      | 1017826   |
| 5     | 14 μg/ml      | 1188728   |
|       | Correlation Coefficient (R²) | 0.9992 |
Precision:
To check the system precision (repeatability) for peak response obtained with five replicates of standard at specified concentration. The %RSD found to be within 2.0%. To check repeatability (method precision) of the method six individual sample preparations from same batch were prepared and injected the %RSD with six samples found to be within 2.0%. The results obtained were presented in Table 6 and 7.

Accuracy:
The accuracy of an analytical method is established across its range. Accuracy is performed in three different levels for Atenolol and Nitrendipine. The known quantity of Atenolol and Nitrendipine at 80%, 100% and 120% level is analysed for each level. The % recovery values for these drugs were found to be in between 99.52% to 100.71% and %RSD values were found to be less than 2.0%. The accuracy results were tabulated in the Table 4 and 5.

LOD and LOQ:
These methods were evaluated on the basis of signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit. A typical signal-to-noise ratio required for LOQ is 10:1. According to a formula given by miller, the limit of detection (LOD) and limit of quantification (LOQ) were calculated. The results are given in Table 3.

Robustness:
The robustness of an analytical method 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. Robustness was done by changing the flow rate (±0.2ml/min) and the Wave length (±20nm). The results obtained were presented in Table 8 and 9.

System Suitability:
According to USP system suitability tests are an integral part of chromatographic method validation. The tests were used to verify that the reproducibility of the chromatographic system is adequate for analysis. To ascertain its effectiveness system suitability tests were carried out on freshly prepared standard solution. 20μL of solution was injected into the optimized chromatographic system. For system suitability six replicates of working standard samples were injected and the parameters like retention time (RT), theoretical plate (N), peak area, tailing factor and resolution of sample were calculated these results are presented in the Table 11.

Solution Stability:
Solution stability study of Atenolol and Nitrendipine was done by preparing sample and standard solution (Initial, 24Hr and 48Hr). It was done for check whether it’s stable up to given hours. The results are shown in Table 10.

DISCUSSION:
To optimize the mobile phase various proportions of buffers with methanol and acetonitrile were tested. Mobile phase composition was changed and the method development was started by Phenomenox Luna C-18 (250mm X 4.6 mm X 5μm) column and with flow rate 1.5mL/min and detection wavelength of 235nm. Column temperature was maintained at ambient. Injection volume is 20μL. The mobile phase consists of methanol: acetonitrile: water (40:40:20%v/v/v) pH 3.0 was adjusted with orthophosphoric acid was used. The retention times of Atenolol and Nitrendipine peaks are about 2.621 and 5.169 minutes respectively.

Quantitative linearity was observed over the concentration range of 30-70 μg/ml of Atenolol and 6-14 μg/ml of Nitrendipine. The regression equations of concentration of Atenolol and Nitrendipine are found to be $y = 44731x - 94497$ and $y = 84378x + 4543.8$ of

![Figure 7: Linearity graph for Atenolol](image_url)

![Figure 8: Linearity graph for Nitrendipine](image_url)

| Parameters       | Atenolol | Nitrendipine |
|------------------|----------|--------------|
| L.O.D. (3.3 × σ/m) | 1.964 µg/ml | 0.342 µg/ml |
| L.O.Q. (10 × σ/m)  | 5.952 µg/ml | 1.036 µg/ml |

Table 3: Data for Limit of Detection and Quantitation
### Table 4: Recovery Data for Atenolol

| Level | Peak Area | % Recovery | Mean Recovery | S.D.  | % RSD |
|-------|-----------|------------|---------------|-------|-------|
| 80%   | 1701948   | 99.136     | 99.865        | 0.691 | 0.692 |
|       | 1715893   | 99.948     | 100.512       |       |       |
|       | 1725574   | 100.056    |               |       |       |
| 100%  | 2135756   | 99.057     | 99.525        | 0.691 | 0.573 |
|       | 2159557   | 100.161    |               |       |       |
|       | 2142193   | 99.356     |               |       |       |
| 120%  | 2558129   | 99.251     | 100.014       | 0.665 | 0.665 |
|       | 2589522   | 100.469    |               |       |       |
|       | 2585762   | 100.323    |               |       |       |

### Table 5: Recovery Data for Nirendipine

| Level | Peak Area | % Recovery | Mean Recovery | S.D.  | % RSD |
|-------|-----------|------------|---------------|-------|-------|
| 80%   | 654681    | 99.350     | 100.460       | 0.995 | 0.990 |
|       | 663958    | 100.758    |               |       |       |
|       | 667352    | 101.273    |               |       |       |
| 100%  | 858422    | 101.603    | 100.714       | 0.770 | 0.764 |
|       | 846931    | 100.242    |               |       |       |
|       | 847384    | 100.296    |               |       |       |
| 120%  | 1029824   | 100.667    | 99.750        | 0.807 | 0.809 |
|       | 1014218   | 99.141     |               |       |       |
|       | 1017312   | 99.444     |               |       |       |

### Table 6: Repeatability Data for Atenolol and Nirendipine

| S. No | Atenolol | Nitrendipine |
|-------|----------|--------------|
| Peak Area | Amount (%) | Peak Area | Amount (%) |
| 1 | 2156778   | 100.42       | 844122     | 100.97 |
| 2 | 2154088   | 100.29       | 839816     | 100.45 |
| 3 | 2157194   | 101.44       | 832488     | 99.57  |
| 4 | 158254    | 101.49       | 831982     | 99.51  |
| 5 | 2154682   | 100.32       | 838819     | 100.33 |
| 6 | 2118996   | 98.65        | 824978     | 98.67  |

R.S.D 0.710 0.822
### Table 7: Data for Intermediate precision (Day to Day analysis)

| S. No | Atenolol Day 1 | Atenolol Day 2 | Nitrendipine Day 1 | Nitrendipine Day 2 |
|-------|---------------|---------------|-------------------|-------------------|
| 1     | 100.51        | 101.38        | 101.08            | 101.26            |
| 2     | 100.33        | 100.59        | 100.46            | 100.88            |
| 3     | 99.63         | 101.10        | 99.34             | 100.56            |
| 4     | 100.53        | 101.38        | 99.62             | 100.68            |
| 5     | 100.42        | 101.27        | 100.25            | 99.35             |
| 6     | 100.02        | 100.07        | 99.58             | 100.03            |
| R.S.D | 0.351         | 0.539         | 0.661             | 0.675             |

### Table 8: Data for robustness - Changes in flow rate

| Conditions Parameters | Atenolol | Nitrendipine |
|-----------------------|----------|--------------|
|                       | AUC      | USP Tailing  | R.S.D  | AUC      | USP Tailing | R.S.D  |
| 1.3 ml/min            | 2456250  | 1.215        | 0.285  | 954340   | 1.154       | 0.195  |
| 1.7 ml/min            | 1894820  | 1.218        | 0.226  | 748793   | 1.157       | 0.373  |

### Table 9: Data for robustness Changes in Wavelength

| Conditions Parameters | Atenolol | Nitrendipine |
|-----------------------|----------|--------------|
|                       | AUC      | USP Tailing  | R.S.D  | AUC      | USP Tailing | R.S.D  |
| 230 nm                | 2024016.667 | 1.219        | 0.263  | 854352.666 | 1.157       | 0.449  |
| 240 nm                | 2215540  | 1.222        | 0.874  | 792688   | 1.157       | 0.191  |

### Table 10: Data for Sample solution stability for atenolol and nitrendipine

| Time     | Atenolol | % Assay | Nitrendipine | % Assay |
|----------|----------|---------|--------------|---------|
| Initial  | 2141458  | 100.528 | 822782       | 100.480 |
| 24hrs    | 2140682  | 100.491 | 811536       | 99.107  |
| 48hrs    | 2117894  | 99.421  | 804888       | 98.295  |
| SD       | 13386.29 | 0.628   | 9044.92      | 1.104   |
| %RSD     | 0.6274   |         | 1.112        |         |

### Table 11: Data for System suitability for atenolol and nitrendipine

| Drugs     | RT (min) | AUC       | No. of Theoretical plates (n) | Tailing factor |
|-----------|----------|-----------|-------------------------------|----------------|
| Atenolol  | 2.622    | 2158821.33| 5489.67                      | 1.222          |
| Nitrendipine | 5.162 | 846381.66 | 4069.35                      | 1.160          |
Atenolol and Nitrendipine respectively, where y is the peak area and x is the concentration of drugs (μg/mL). The correlation coefficient of Atenolol and Nitrendipine was found to be 0.9989 and 0.9992 respectively.

The numbers of theoretical plates obtained were 5489.67 and 4069.35 for Atenolol and Nitrendipine respectively which indicates the efficiency of the column. The high percentage recovery indicates that the proposed method is highly accurate. There is no interference of filters with standard and sample solutions as the difference in responses is within the limit. The %RSD was found to be less than 2.0%.

CONCLUSION:
A simple, specific, accurate, precise, reproducible and efficient reverse phase high performance liquid chromatography method has been developed which can be used accurately for quantitative estimation of Atenolol and Nitrendipine for routine analysis of individual and combination of drugs. Method was validated as per ICH Q2 (R2) so it can be used by analytical department.

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