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ANALYTICAL METHOD VALIDATION OF RP-HPLC FOR THE ESTIMATION OF ATENOLOL AND AMLODIPINE BESYLATE IN BULK AND TABLET DOSAGE FORMS

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ABSTRACT: The chromatographic separation was Analytical method Development and validation plays an important role in the discovery, Development and manufacture of pharmaceuticals. The methods used ensure the identity, purity, potency, and performance of drug products. There are many factors to consider when developing methods. The majority of the analytical development effort goes into validating a stability indicating HPLC Method. The goal of the HPLC Method is try and separate quantify the main active drugs, any reaction impurities, all available synthetic intermediates and degradants. A new, simple, precise, rapid and accurate RP-HPLC (Reverse Phase - High Performance Liquid Chromatography) method has been developed for the simultaneous estimation of Atenolol (AT) and Amlodipine Besylate (AB) in tablet formulations. The chromatographic separation was achieved on Water's 717 Plus Autosampler HPLC using octadecysilane bonded C_{18}, 5 µm, 250 cm x 4.6 mm column maintained at ambient temperature with mobile
phase, Buffer: Acetonitrile: Methanol, Mobile phase- mix volume of acetonitrile 45 volume or methanole 35 volume a 5 ml Tetrabutyl ammonium hydroxide in 1000 ml with makeup HPLC water of a mixture 20 volume, flow rate 1.5 ml/min, load volume 20 µl and a run time of 10 min. The UV detection was performed at 225 nm. Buffer was prepared with Tetrabutyl ammonium hydroxide and adjusted pH to 3.0 with Ortho-Phosphoric Acid.
Keywords:- RP-HPLC, Validation, Simultaneous estimation, Atenolol, amlodipine besyla

INTRODUCTION

High performance Liquid Chromatography (HPLC)

•HPLC stands for "High-performance liquid chromatography" (sometimes referred to as High-pressure liquid chromatography).

•High performance liquid chromatography is a powerful tool in analysis, it yields high performance and high speed compared to traditional columns chromatography because of the forcibly pumped mobile phase.

•HPLC is a chromatographic technique that can separate a mixture of compounds High – performance liquid chromatography is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, or ion exchange process, depending upon the type of stationary phase.[2-3]

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1.2.1 HPLC Method Development

Most of the drugs in multicomponent dosage forms can be analyzed by HPLC method for the reason that of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tiresome extraction and isolation procedures. HPLC method development is not very difficult when literature reference for the same or similar compounds to be analyzed can be found.

Five stages are to be taken into consideration when starting new HPLC method .

1) **Instrumentation**
2) **Determination of molecular characteristics of sample**
3) **Selection of column**
4) **Selection of mobile phase**
5) **Selection of detector**
6)

1) HPLC Instrumentation

Liquid chromatography (LC) is a physical separation procedure conducted. A sample is separated into its constituent components (or analytes) by distributing among the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column). HPLC is a modern form of LC that uses small-particle column through which the mobile phase is pumped at high pressure.

2) Determination Of molecular characteristics of sample

Method for analyzing drugs in multicomponent dosage forms can be developed ,provided one has knowledge about the nature of the sample , namely , its molecular weight ,polarity ,ionic character and solubility parameter .

The first consideration of HPLC method development is to establish solubility of the sample component. Most of sample preparation rivet the use of organic –aqueous and acid-base extraction techniques. Therefore it is very helpful to understand the solubility and pKa of the analytes .

Solubility in different organic or aqueous solvents determines the best composition of the sample solvent . pKa determine the pH in which the analyte will exist as a neutral or ionic species. When the pH=pKa, for the analyte , at this concentration equal proportion ionic and non-ionic species exists. As a general rule pH caused change in retention occure within ±1.5 units above or below the pKa to ensure practically 100% unionization for retention reason . This information will smooth the progress of an efficient sample extraction scheme and optimum pH in mobile phase to achieve good separation

3.) Selection of column

Column is the heart of HPLC system . Good silica and bonding process will provide the reproducible and symmetrical peaks necessary for exact qualification

4.) Selection of Mobile Phase

The selection of the mobile phase based on the nature and physicochemical properties of the analytes to be determined. Since the mobile Phase governs solute- stationary phase interactions, its choice is decisive.

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- *Practical considerations complete that it should not degrade the equipment or the column packing . For this reason acids , bases and halide solutions should be avoided .*
- *Chemical purity of solvents is an important factor. Since large volumes of solvent are pumped through the column , trace impurity can simply concentrate in the column and ultimately be unfavourable to the results. HPLC grade solvents are recommended .*
- *Volatility should be considered if sample recovery is mandatory.*
- *Viscosity should be less than 0.5 centipoises, otherwise higher pump pressure are required and mass transfer between solvent and stationary phase will reduced . For LC/MS only volatile buffers are used .*
- *The mobile phase should have a pH 2.5 and 7.0 to maximize the lifetime of column .*
- *Reduce cost and toxicity of the mobile phase by means of methanol instead of acetonitrile when possible.[21]*

5.) ***Selection of Detector***

- *There is little running a separation if the detector one uses cannot "see" all the components of interest , or conversely , if it "sees" too much . UV – Visible detectors are the most popular as they can detect a broad range of compounds and have a fair degree of selectivity for some analytes . Unfortunately . UV– Visible detectors are not universal detectors so it is worthwhile to look at the chemical structure of the analytes to see if it has suitable chromaphores, such as aromatic rings, for UV-Visible detection.*

DRUG PROFILE

1 AMLODIPINE BESYLATE

Amlodipine is a long- acting 1,4 –dihydropyridine calcium channel blocker . It acts primarily on vascular smooth muscle cells by stabilizing voltage –gated L-type calcium channel in their inactive conformation. By inhibiting the influx of calcium in smooth muscle cells , amlodipine prevents calcium –depended my ocyte contraction and vasoconstriction. A second proposed mechanism for the drug's vasodilatory effects involves pH-dependent inhibition of calcium influx via inhibition of smooth muscle carbonic anhydrase . Some studies have shown that amlodipine also exerts inhibitory effects on voltage –gated N-type calcium channels.

N-type calcium channel located in the central nervous system may be involved in nociceptive signalling and pain sensation .Amlodipine is used to treat hypertension and chronic stable anagina.

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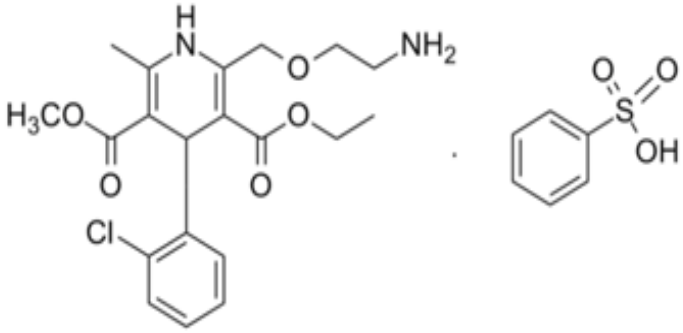

FIG. 1: STRUCTURE OF AMLODIPINE BESYLATE

Molecular weight : *408.876*

Chemical Formula : *C20H25CIN2O5*

IUPS Name: 3-ethyl 5-methyl 2-[(2-aminoethoxy) methyl]-4-(2-chlorophenyl)-6-methyl-1, 4-dihydropyridine- 3

2. Atenolol

Atenolol, 4-[2'-hydroxy-3'-[(1-methyl ethyl) amino] propoxy- benzene acetamide is an odorless white powder, sparingly soluble in water, soluble in ethanol/methanol and practically insoluble in ether is used as an antihypertensive and an antiarrhythmic agent. AT is β1-selective antagonist. It binds at β1 adrenergic receptors in the heart and vascular smooth muscle, inhibiting sympathetic stimulation.

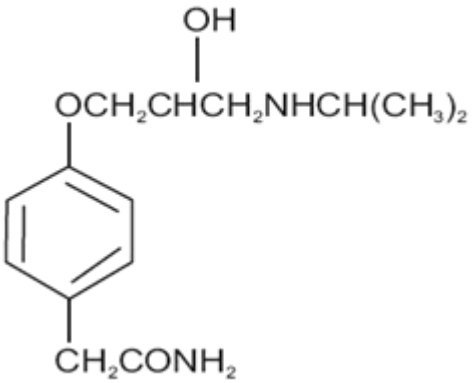

FIG. 2: STRUCTURE OF ATENOLOL

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Molecular Mass : *266.341 g.mol*$^{-1}$

Chemical Formula : $C_{14}H_{22}N_2O_3$

IUPS Name : 4-[2'-hydroxy-3'-[(1-methyl ethyl) amino] propoxy- benzene acetamide

MATERIALS AND METHOD:

INSTRUMENTATION:

High Performance Liquid Chromatograph from Water's 717 Plus Autosampler equipped with auto injector, Water Pump 515 (quaternary) for constant flow and constant pressure delivery and UV detector with deuterium lamp linked to software M power for controlling the instrumentation as well as processing the data generated was used. UV-Visible double beam spectrophotometer from Elico SL210 model with spectral slit width of 1.0 nm and automatic wavelength corrections with 10 mm matched quartz cells linked to UV-Probe software was used for analytical wavelength selection. All weighing were done on electronic balance (Model: Radwag –AS 220 /X). Ultrasonicator (Model: Fast clean 2K9110s09) and pH meter (Model: Lab Tech-DPH-115 PM) was used for solution preparation and pH determination respectively.

REGENTS AND CHEMICALS: AT and AB pure drug samples and combination tablets containing 50 mg AT and 5 mg AB manufactured by Wilcure Remedies Pvt.Ltd., Khandwa Road Indore, M.P., India was used. Acetonitrile and Methanol (HPLC grade) from Standard Company, India, Triethylamine and Ortho-Phosphoric Acid (AR grade) from Rankem and Loba Chemie Purified water (In-house preparation) was used throughout the work.

Selection of Analytical Wavelength:

Preparation of mixed stock solution: An accurately weighed 20 mg each of AT and AB working standards were transferred into a 100 ml volumetric flask. 20 ml of methanol was added into it and sonicated for a while for dissolving the drugs. The flask was made up to 100 ml with methanol so as to get a concentration of 100 µg/ml.

The resulting solution containing 100 µg/ml of AT and 10 µg/ml of AB were scanned in UV-Visible sp0ectrophotometer from 400-200 nm to determine the wavelength of maximum absorption of both the drugs in combination. Both drugs in combination showed maximum absorption at 225 nm. The values are shown in table 1 and the spectra of combination of AT and AB was appended in Fig. 2.

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TABLE 1: **OBSERVATION FROM UV-VISIBLE SPECTRO PHOTOMETER**

SR. NO	Wavelength (nm)	Absorbance of combination
1.	200 nm	0.6628
2.	210 nm	0.8475
3.	220 nm	0.7388
4.	225 nm	0.6711
5.	230 nm	0.6446
6.	250 nm	0.2414
7.	350 nm	0.1584
8.	400 nm	0.044

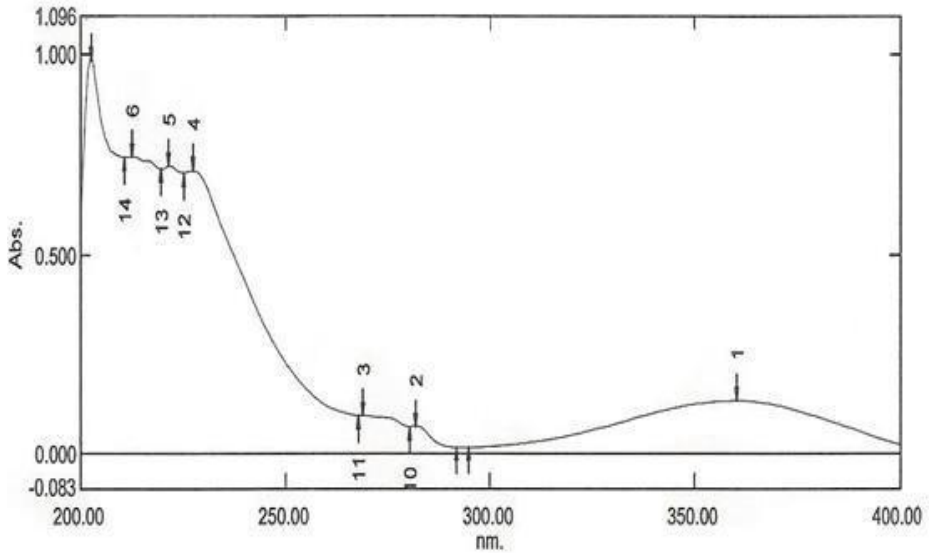

FIG. 2: COMBINEDSPECTRA OF ATENOLOL AND AMLODIPINE BESYLATE

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Optimized HPLC conditions:

Chromatographic System-

- Stainless steel column 25 cm X 4.6 mm, packed with octadecysilane bonded to (column C_{18}) Porous silica (5 µm).
- Column temperature At a Room temperature.
- Mobile phase- mix volume of acetonitrile 45 volume or methanole 35 volume a 5 ml Tetrabutyl ammonium hydroxide in 1000 ml with makeup HPLC water of a mixture 20 volume.
- Phosphate adjust to pH 3.0 with adjust for Ortho-Phosphoric Acid
- Flow rate 1.5 ml per minute.
- Spectrophotometer set at 232 nm.
- Injection volume 20 µl.

Standard preparations:

Standard Stock Solution: Standard stock solution was prepared by transferring 14 mg of AB working standard into 20 ml volumetric flask, added methanol and sonicated for 5 min for dissolving the drug and volume was made up with mobile phase.

Working Standard Solution: Working standard solution was prepared by transferring 10 mg of AT into 100 ml volumetric flask, added methanol and sonicated for 5 min for dissolving the drug. 2 ml of AB stock solution was added and volume was made up with mobile phase.

Sample preparation for Tablet Analysis: Tablet powder equivalent to weight of one tablet was accurately weighed and transferred into 100 ml volumetric flask, 60 ml of mobile phase was added and sonicated for 15 min; volume was made up with mobile phase. Solution was filtered through 0.45µ nylon filter. 5 ml of the filtrate was diluted to 25 ml with mobile phase. 10 µl of the blank, placebo, standard and sample solution were injected separately into the chromatographic system and chromatograms were recorded and the peak areas were measured. A typical chromatogram of AT and AB was appended in Fig. 3.

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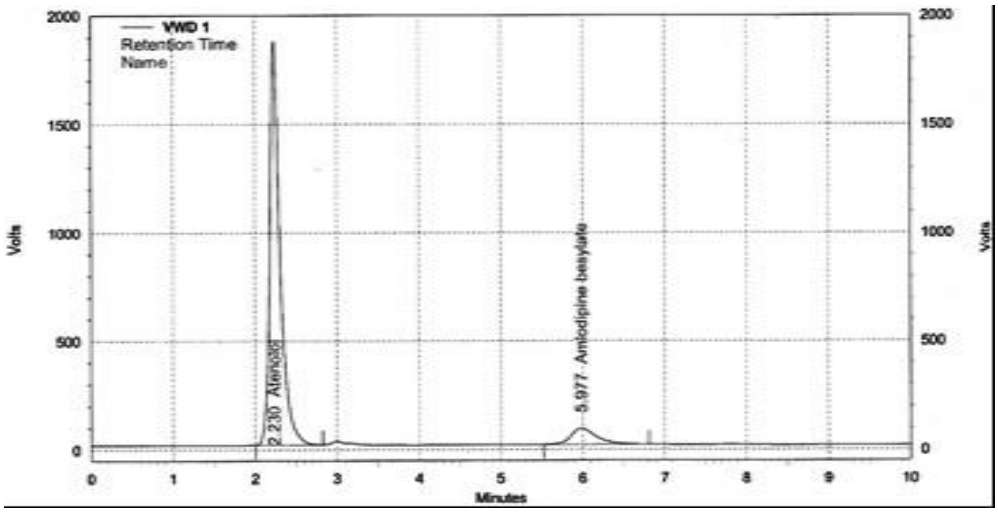

FIG. 3: TYPICAL CHROMATOGRAM OF ATENOLOL AND AMLODIPINE BESYLATE

ANALYTICAL VALIDATION OF METHOD FOR ATENOLOL AND AMLODIPINE BESYLATE TABLET

Validation:

Method validation is the process by which it is established that performance characteristics of the method meet the requirements for the intended analytical applications. Methods need to be validated or revalidated before their introduction into routine use. The International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use2 has developed a text on the validation of analytical procedures. The United States Food and Drug Administration (USFDA) have proposed guidelines on submitting samples and analytical data for methods validation5-7. The United States Pharmacopoeia (USP) has published specific guidelines for method validation for compound evaluation[8]. The document includes definitions for eight validation$_{14}$

Definition:

- Documented evidence that the manufacturing process consistently produces product that meets predetermined specifications.
- Manufacturing process validation consists of successfully manufacturing at least three full-scale batches in succession, which pass all in-process and product quality attributes.

- **ACCURACY**: "The accuracy of an analytical method is the closeness of the test results obtained by that method to the true value." The accuracy of the method shall be determined by adding known amount of analytic to cover both above and below (80, 100, and 120%) the normal levels expected in the sample.

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PREPARATION: Prepared the placebo of amlodipine besylate & atenolol tablet Except the amlodipine besylate & atenolol tablet active ingredient add the quantity of amlodipine besylate & atenolol tablet (as per below mention) and dissolve in inactive ingredient then add in each 200 gm placebo.

S. No.	Preparation With amlodipine besylate & atenolol tablet		The % amlodipine besylate & atenolol added
	Qty. of placebo	Qty. of amlodipine besylate & atenolol added	
1	200 gm	96 gm	80 %
2	200 gm	120 gm	100 %
3	200 gm	144 gm	120 %

METHOD:

Standard solution preparation: (Amlodipine Besylate)

Weight accurately 20 mg amlodipine Besylate working standard and transfer in to 100 ml volumetric flask and dissolve in Mobile phase make up to 100 ml.

Standard solution preparation: (Atenolol)

Weight accurately 20 mg Atenolol working standard and transfer in to 100 ml volumetric flask and dissolve in Mobile phase make up to 100 ml.

Test solution (1) Weight Accurately at About 0.2 gm sample and transfer in 100 ml volumetric flask and dissolve in Mobile phase make up to 100 ml. Take 10 ml of above solution in mobile phase make up to 100 ml.

Calculate the content Amlodipine besylate ($C_{20}H_{25}CIN_2$, O_5).

Calculate the content Atenolol ($C_{14}H_{22}N_2O_{3)}$.

Test solution (2). Weight Accurately at about 0.2 gm sample and transfer in 100 ml volumetric flask and dissolve in Mobile phase make up to 100 ml. Take 10 ml of above solution in mobile phase make up to 100 ml.

Calculate the content Amlodipine besylate ($C_{20}H_{25}CIN_2$, O_5).

Calculate the content Atenolol ($C_{14}H_{22}N_2O_{3)}$.

Test solution (3). Weight Accurately at about 0.2 gm sample and transfer in 100 ml volumetric flask and dissolve in Mobile phase make up to 100 ml. Take 10 ml of above solution in mobile phase make up to 100 ml.

Calculate the content Amlodipine besylate ($C_{20}H_{25}CIN_2$, O_5).

Calculate the content Atenolol ($C_{14}H_{22}N_2O_{3)}$.

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1) **TEST SOLUTION 1:** (80 %) Analyse sample as per above method.
2) **TEST SOLUTION 2:** (100 %) Analyse sample as per above method.
3) **TEST SOLUTION 3:** (120 %) Analyse sample as per above method.

Now do the analysis of all three samples as per the method of analysis and determined the recovery of Atenolol and Amlodipine Besylate.

ACCEPTANCE LIMITS: In terms of % recovery: 90.0 % to 110.0 %

Specificity (Sensitivity or Selectivity): Specificity was performed to detect the presence of interference peak (blank and placebo peaks) at the retention time of the analyte peak. The interference of placebo was detected by preparing samples by taking the placebo equivalent to about the weight in portion of test preparation as per the test method and were injected into the HPLC system. The interference of blank was detected by injecting mobile phase as per the test method.

Selectivity. Selectivity was determined in the presence of common excipients used in the tablet formulation. Sample containing 100% atenolol and amlodipine was injected first. Then the samples mixed with three different placebo formulations were injected to find out the selectivity of the method.

- **PRECISION**: "The degree of repeatability or reproducibility of the results in a service of experiments runs during a single session by single operator with identical reagents and equipment".

PREPARATION: Prepared the placebo of amlodipine besylate & atenolol tablet Except the Volvopin-AT active ingredient add the quantity of amlodipine besylate & atenolol tablet (as per below mention) and dissolve in inactive ingredient then add in each 200 gm placebo.

S. No.	Preparation With amlodipine besylate & atenolol			The % of amlodipine besylate & atenolol added
	Sample name	Qty. of placebo	Qty. of amlodipine besylate & atenolol added	
1	Sample I	200 gm	120 gm	100 %
2	Sample II	200 gm	120 gm	100 %
3	Sample III	200 gm	120 gm	100 %

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METHOD:

Standard solution preparation: (Amlodipine Besylate)

Weight accurately 20 mg amlodipine Besylate working standard and transfer in to 100 ml volumetric flask and dissolve in Mobile phase make up to 100 ml.

Standard solution preparation: (Atenolol)

Weight accurately 20 mg Atenolol working standard and transfer in to 100 ml volumetric flask and dissolve in Mobile phase make up to 100 ml.

Sample solution preparation

Weight Accurately at about 0.2 gm sample and transfer in 100 ml volumetric flask and dissolve in Mobile phase make up to 100 ml. Take 10 ml of above solution in mobile phase make up to 100 ml.

Calculate the content Amlodipine besylate ($C_{20}H_{25}CIN_2$, O_5).

Calculate the content Atenolol ($C_{14}H_{22}N_2O_{3)}$.

TEST SOLUTION: (100 %) Prepared three test samples and follow the above method of analysis on all three test solution and determined the six replicates absorbance of one sample and determine the SD and RSD of the absorbance

S. No.	HPLC Area Standard Amlodipine Besylate			HPLC Area Standard Atenolol		
	I	II	III	I	II	III
1.	32768856	32685152	32745251	3386031	3385102	3345781
2.	32765351	32578121	32735205	3378526	3375121	3358454
3.	32568750	32685151	32678515	3365812	3365845	3356781
4.	32678512	32578652	32665715	3375012	3256785	3365784
5.	32705826	32745785	32675812	3365785	3356945	3336546
6.	32745258	32678521	32685121	3355781	3356875	3375894

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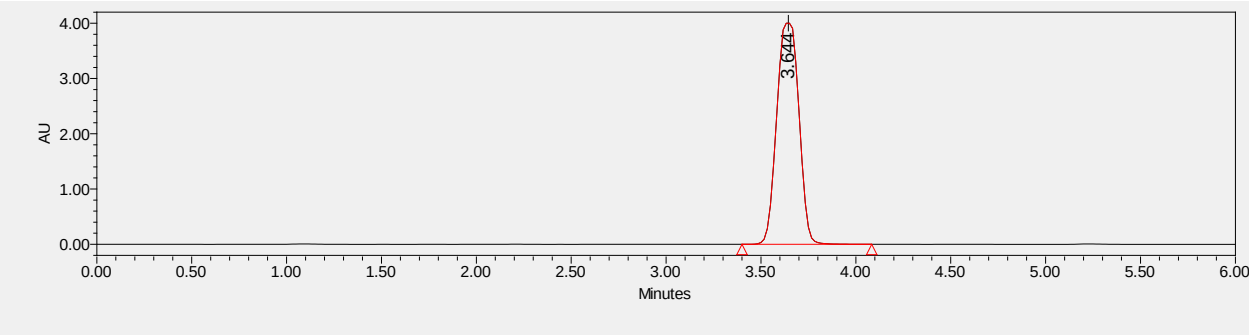
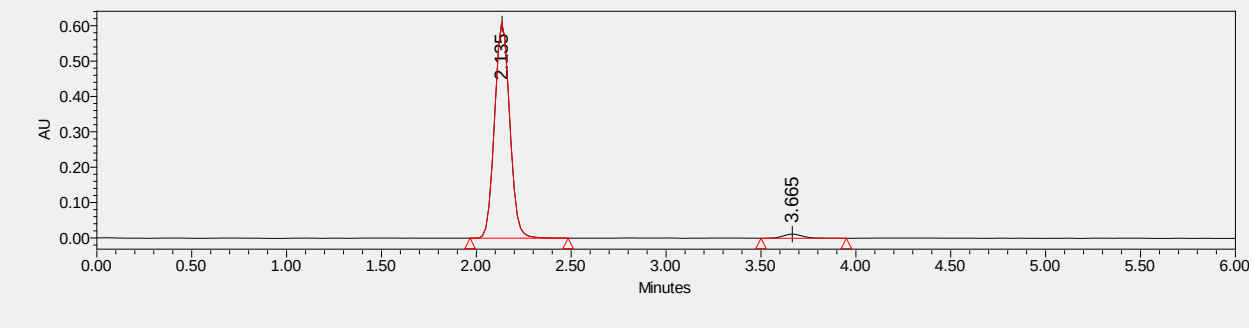
Mean	32705425.5	32658563.67	32697603.17	3371157.833	3349445.5	3356540
SD	69048.06432	60932.44756	30811.48758	9867.858961	42616.4466	12775.40897
RSD	0.211121131	0.186574181	0.09423164	0.292714238	1.272343336	0.380612445



Amlodipin standard 2



ACCEPTANCE LIMITS: RSD of each sample of six replicates absorbance should not exceed 2%

- **LINEARITY AND RANGE**: The linearity of analytical is ability to elicit test results and directly proportional to the concentration of the analyte in sample within range. A minimum five concentrations are recommended for the establishment of linearity.

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METHOD:

Standard solution preparation: (Amlodipine Besylate)

Weight accurately 20 mg amlodipine Besylate working standard and transfer in to 100 ml volumetric flask and dissolve in Mobile phase make up to 100 ml.

Standard solution preparation: (Atenolol)

Weight accurately 20 mg Atenolol working standard and transfer in to 100 ml volumetric flask and dissolve in Mobile phase make up to 100 ml.

Now do the five dilutions as below of above solution as follow:

1. Take 1.0 ml of this solution
2. Take 1.5 ml of this solution
3. Take 2.0 ml of this solution
4. Take 2.5 ml of this solution
5. Take 3.0 ml of this solution

Take above mention solution in 100 ml volumetric flask and make up with mobile phase up to 100 ml.

S. No.	**Qty. of standard**	**% of *amlodipine besylate & atenolol* STD.**
1	1.0 ml	80 %
2	1.5 ml	90 %
3	2.0 ml	100 %
4	2.5 ml	110 %
5	3.0 ml	120 %

TEST SOLUTION Prepared the placebo of amlodipine besylate & atenolol tablet Except the amlodipine besylate & atenolol active ingredient add the quantity amlodipine besylate & atenolol (as per below mention) and dissolve in inactive ingredient then add in each 200 gm placebo.

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Now do the five dilutions as below of above solution as follow:

6. Take 120 gm of this solution
7. Take 125 gm ml of this solution
8. Take 130 gm of this solution
9. Take 135 gm of this solution
10. Take 140 gm of this solution

Weight Accurately at about 0.2 gm sample and transfer in 100 ml volumetric flask and dissolve in Mobile phase make up to 100 ml. Take 10 ml of above solution in mobile phase make up to 100 ml.

Calculate the content Amlodipine besylate ($C_{20}H_{25}CIN_2$, O_5).

Calculate the content Atenolol ($C_{14}H_{22}N_2O_{3)}$.

S. No.	Qty. of sample	Qty. of of amlodipine besylate & atenolol presence in sample	% of amlodipine besylate & atenolol
1	120 gm	96.0 gm	80 %
2	125 gm	112.5 gm	90 %
3	130 gm	130.0 gm	100 %
4	135 gm	148.5 gm	110 %
5	140 gm	168.0 mg	120 %

Adopt the above method of analysis on all five concentration test solution and determined the absorbance of amlodipine besylate & atenolol added and plotted graph of concentration against absorbance shall be linear (straight line) and determined the correlation coefficient.

ACCEPTANCE LIMITS: The correlation coefficient value must between 0.99 to 1.00 over the working range for amlodipine besylate & atenolol.

Robustness. The robustness of the method was assessed by altering the some experimental conditions such as, by changing the flow rate from 0.9 to1.1 ml/min, amount of acetonitrile (10% to15%,) the temperature of the column (28 °C to 32 °C) and PH of the mobile phase.

PLACEBO INTERFERENCE: The Placebo interference of analytical is ability to elicit the interference of the non-active ingredient used in preparation of amlodipine besylate & atenolol.

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- **METHOD:**

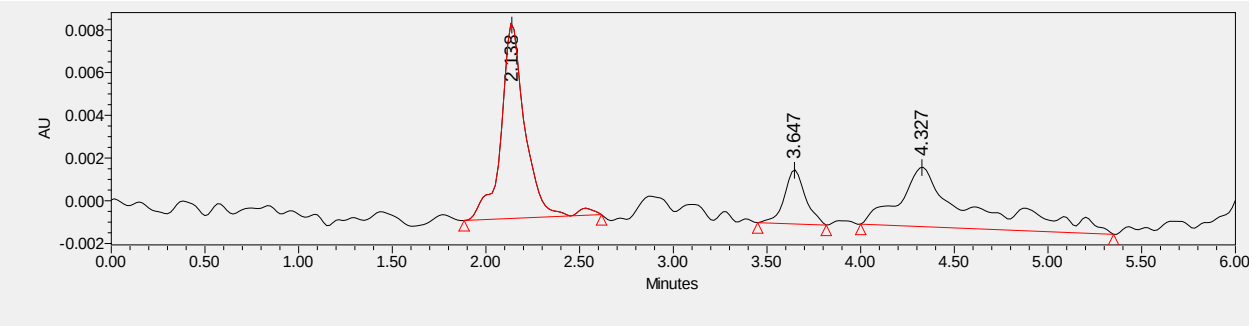
Sample solution preparation

Weight Accurately at about 0.2 gm sample and transfer in 100 ml volumetric flask and dissolve in Mobile phase make up to 100 ml. Take 10 ml of above solution in mobile phase make up to 100 ml.

Calculate the content Amlodipine besylate ($C_{20}H_{25}CIN_2$, O_5).

Calculate the content Atenolol ($C_{14}H_{22}N_2O_{3)}$.

Found the absorbance of placebo:-……



Placebo sample

ACCEPTANCE LIMITS: The absorbance of the placebo must not be found.

11.0 ACCEPTANCE CRITERIA

S. No.	**PERFORMANCE PARAMETER**		**ACCEPTANCE LIMITS**
1	Accuracy	For amlodipine besylate & atenolol.	In terms of % recovery : 90.0 % to 110.0 %
2	Precision	For amlodipine besylate & atenolol.	RSD of each sample of six replicates absorbance should not exceed 2%

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3	Linearity and Range	For amlodipine besylate & atenolol.	The correlation coefficient value must between 0.99 to 1.00 over the working range for Bromhexine hydrochloride IP
4	Placebo interference	For amlodipine besylate & atenolol.	The absorbance must be zero.

RESULTS AND DISCUSSION: A new RP-HPLC method was developed for the simultaneous estimation of AT and AB in tablet formulation and validated as per ICH norms for the following parameters: system suitability, linearity and range, precision (repeatability), intermediate precision (ruggedness), specificity, accuracy and robustness. The observations and results obtained for each of the parameters lies well within the acceptance criteria. So the developed method was simple, specific, linear, precise, accurate, robust and rugged and could be extensively used for the simultaneous estimation of AT and AB in tablet dosage form.

CONCLUSION: From the results obtained, it was observed that the developed method was proven to be specific, precise, linear, accurate, rugged and robust and is suitable for its intended purpose. So the work performed gives documented evidence, that the analytical method for the simultaneous estimation of AT and AB by RP-HPLC in tablet dosage forms will consistently analyze these drugs quantitatively and can be used for routine analysis in quality control and R&D laboratory.

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