METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF ANTI-HYPERTENSIVE DRUGS EFONIDIPINE HYDROCHLORIDE ETHANOLATE AND METOPROLOL SUCCINATE IN SOLID DOSAGE FORMS BY RP-HPLC

*Syeda Sana, Dr Husna Kanwal Qureshi**
Deccan School of Pharmacy, Goshamahal, Aghapura, Goshamahal Rd, near Nampally, Hyderabad, Telangana 500001

ABSTRACT: ***A Novel Analytical simple, reproducible and efficient RP-HPLC method was developed for simultaneous estimation of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate in pure form and marketed combined pharmaceutical dosage forms. A column hasDevelosil ODS HG-5 RP C_{18}, 15cmx4.6mm, i.d. Columnin isocratic mode with mobile phase containing Methanol: Acetonitrile in the ratio of 85:15% v/vwas used. The flow rate was 1.0 ml/min and effluent was monitored at 258nm. The retention times and linearity range for Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate was found to be (2.217, 5.789min) and (0-14, 0-28), respectively. The method has been validated for linearity, accuracy and precision, robustness and limit of detection and limit of quantitation. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.08µg/ml and 0.24µg/ml for Efonidipine Hydrochloride Ethanolate and 0.1µg/ml 0.3µg/ml for Metoprolol Succinate, respectively. The % RSD of the Precision was found to be within the limits. The % recovery of the proposed methods was found to be within the limits i.e. 98-102%. The proposed method was found to be accurate, precise and selective for simultaneous estimation of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinatein pure form and marketed combined pharmaceutical dosage forms.***

Keywords:Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate, Accuracy, Precision, RP-HPLC, Validation.

I. INTRODUCTION

Efonidipine is a calcium channel blocker of the dihydropyridine class, commercialized by Shionogi & Co. (Japan). Initially, it was marketed in 1995 under the trade name, Landel. The drug has been shown to block T-type in addition to L-type calcium channels. It has also been studied in atherosclerosis and acute renal failure. This drug is also known as NZ-105, and several studies have been done on its pharmacokinetics in animals.It is a C-nitro compound, a carboxylic ester, a tertiary amino compound and a dihydropyridine.This drug inhibits the L-type and T-type calcium channels, thereby leading to vasodilation and decreased automaticity of the heart[1]. Efonidipineexerts negative chronotropic effects, decreasing heart rate. Acting on SA node cells by inhibiting T-type calcium channel activity, Efonidipine prolongs the late phase-4 depolarization of the sinoatrial node action potential, decreasing heart rate. This is associated with decreased myocardial oxygen demand and increases of blood flow to the coronary arteries and thereby attenuates myocardial ischemia[2]. Efonidipineincreases glomerular filtration rate (GFR) without increasing intra-glomerular pressure and filtration fraction. This increase leads to the prevention of renal damage that is normally associated with hypertension.Efonidipine, an antihypertensive and antianginal agent with a 1,4-dihydropyridine 5-phosphonate structure, acts on both T- and L-type calcium channels, which may produce effects similar to those of mibefradil. Efonidipine has been described as having a chronotropic effect, which may suppress tachycardia[3]. The IUPAC name of Efonidipine Hydrochloride is 2-(N-benzylanilino)ethyl 5-(5,5-dimethyl-2-oxo-1,3,2λ5-dioxaphosphinan-2-yl)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3-carboxylate;ethanol;hydrochloride. The Chemical Structure EthanolateEfonidipine

Hydrochloride Ethanolate is follows[4]

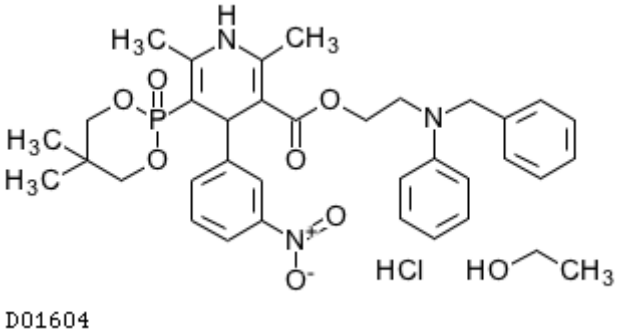

Fig.1. Chemical Structure Ethanolate Efonidipine Hydrochloride Ethanolate

Metoprolol Succinate is the succinate salt form of Metoprolol, a cardioselective competitive beta-1 adrenergic receptor antagonist with antihypertensive properties and devoid of intrinsic sympathomimetic activity[5]. Metoprolol succinate antagonizes beta 1-adrenergic receptors in the myocardium, thereby reducing the rate and force of myocardial contraction, and consequently a diminished cardiac output. This agent may also reduce the secretion of renin with subsequent reduction in levels of angiotensin II thus decreasing sympathetic activation, including vasoconstriction, aldosterone secretion.Metoprolol succinate is an alcohol and a member of phenols.Metoprolol is indicated for the treatment of angina, heart failure, myocardial infarction, atrial fibrillation, atrial flutter and hypertension[6]. Some off-label uses of Metoprolol include supraventricular tachycardia and thyroid storm. Administration of Metoprolol in normal subjects is widely reported to produce a dose-dependent reduction on heart rate and cardiac output[7]. This effect is generated due to a decreased cardiac excitability, cardiac output, and myocardial oxygen demand. In the case of arrhythmias; Metoprolol produces its effect by reducing the slope of the pacemaker potential as well as suppressing the rate of atrioventricular conduction. Metoprolol is used alone or in combination with other medications to treat high blood pressure. It also is used to prevent angina (chest pain) and to improve survival after a heart attack[8]. Metoprolol also is used in combination with other medications to treat heart failure. The IUPAC name of Metoprolol Succinate is Butanedioic acid; 1-[4-(2-methoxyethyl) phenoxy]-3-(propan-2-ylamino) propan-2-ol[9]. The Chemical Structure of Metoprolol Succinate is shown in fig-2.

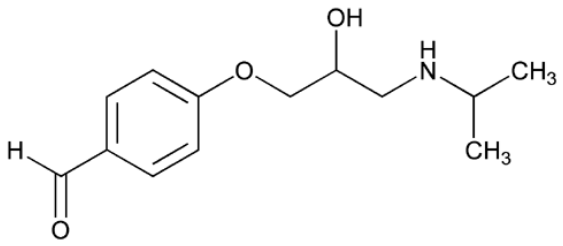

Fig.2. Chemical Structure of Metoprolol Succinate

Very few methods are reported in the literature and no stability indicating method is available in the official compendia using HPLC for analyzing of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate in bulk and pharmaceutical dosage forms[39-42].

The reported methods in the literaturesuffer from one or the other disadvantage such as poor sensitivity, very narrow linearity range, scrupulous control of experimental variables, etc. Since pharmacopoeias do not describe a suitable method for the determination of Efonidipine Hydrochloride Ethanolateand Metoprolol Succinate in bulk and pharmaceutical formulations, in the present work we developed simple, rapid and accurate reverse phase liquid chromatographic method for the simultaneous determination of Efonidipine Hydrochloride Ethanolateand Metoprolol Succinatein bulk and pharmaceutical dosage forms as an alternative method. Apart from this, it can be used for assays of Efonidipine Hydrochloride Ethanolateand Metoprolol Succinate in biological fluids or in pharmacokinetic investigations.

II. MATERIALS AND METHODS

InstrumentsUsed

Table-1: List of Instrument used

S. No.	Instruments/Equipment/Apparatus
1.	HPLC with Empower2 Software with Isocratic and UV-Visible Detector (Waters).
2.	T60-LAB INDIA UV – Vis Spectrophotometer
3.	Electronic Balance (SHIMADZU ATY224)
4.	Ultra Sonicator(Wensar wuc-2L)
5.	Thermal Oven
6.	Develosil ODS HG-5 RP C_{18}, 15cmx4.6mm, i.d. Column
7.	P^H Analyzer (ELICO)
8.	Vacuum filtration kit (BOROSIL)

Chemicals / ReagentsUsed

Table-2: List of Chemicals used

S.No.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai

Efonidipine Hydrochloride Ethanolate Standard Solution Preparation

Weigh accurately 10 mg of standard Efonidipine Hydrochloride Ethanolate and transfer into a clean & dry 100 ml of volumetric flask. Add i.e. 10ml mobile phase and further do sonication in order to dissolve. Finally make up to the volume up to mark with the mobile phase[10]. The final resulted solution contained about 100 µg/ml of Efonidipine Hydrochloride Ethanolate.

Metoprolol Succinate Standard Solution Preparation

Weigh accurately about 10 mg of standard Metoprolol Succinate and transferred into a clean and dry 100 ml volumetric flask. Add 10ml mobile phase and further do sonication in order to dissolve. Finally make up the volume with the same mobile phase i.e. same solvent system[11]. The volume was made up to the mark with same

solvent. The final solution contained about 100µg/ml of Metoprolol Succinate.

Initialization of the HPLC instrument

First switch on the HPLC instrument. The selected column was washed with the HPLC grade water for 45 minutes. Then selected column was saturated with the mobile phase for 45 minutes. Then keep the mobile phase for stabilization. The mobile phase was run to obtain the peaks. After completion of stabilization,after 20 minutes the standard drug solution was injected in HPLC[12].

Optimization of Chromatographic Conditions:

The different HPLC chromatographic conditions were used to find out the optimum chromatographic condition for best elution of drugs[13].

Table-3: Different Chromatographic used and their Optimizations

S.No.	Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Result
1	Symmetry C 18, 5µm, 25mmx4.6mm i.d.	Water: Methanol =30:70	1.0 ml/min	258nm	Peaks didn't Separate	Method rejected
2	Waters C18,5µm, 25cmx4.6mmi.d.	Water : ACN = 55:45	1.0 ml/min	258nm	Early elution of peak	Method rejected
3	Waters C18,5µm, 25cmx4.6mm.i.d.	ACN: methanol= 60: 40	1.0 ml/min	258nm	Lowresolution peak	Method rejected
4	Develosil ODS HG-5 RP C18, 5µm, 15mmx4.6mm i.d.	ACN: methanol 90:10	1.0 ml/ min	258nm	Resolutioninc reasesbutPeak shapes notgood	Method rejected
5	Develosil ODS HG-5 RP C18, 5µm, l5mmx4.6mm i.d.	Methanol : Acetonitrile = 85:15	1.0 ml/min	258nm	Nice and Good Peaks	Method Accepted

Mobile Phase Optimization:
Initially the mobile phase tried was Methanol: Water and Acetonitrile: Water with varying proportions. Finally, the mobile phase was optimized to Acetonitrile: Water in proportion 85:15% v/v respectively.

Optimization of Column:
The method was performed with various columns like Symmetry, Hypersil and Sunfire C18 (4.6×150mm, 5µ), Develosil ODS HG-5 RP C18, 15cmx4.6mm, i.d. Column was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

Preparation of Mobile Phase:
Accurately measured 850ml (85%) of Methanol, 150ml of Acetonitrile (15%) were mixed and degassed in digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.
Diluent Preparation:
The Mobile phase was used as the diluent.
Validation Parameters
System Suitability

Accurately weigh and transfer 10 mg of Efonidipine Hydrochloride Ethanolateand 10mg of Metoprolol Succinate working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.1ml of the Efonidipine Hydrochloride Ethanolate and 0.2ml of the Metoprolol Succinate stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.
Procedure:
The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.
Specificity:
Preparation of Standard Solution:
Accurately weigh and transfer 10 mg of Efonidipine Hydrochloride Ethanolateand 10mg of Metoprolol Succinate working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.1ml of the Efonidipine Hydrochloride Ethanolate and 0.2ml of the Metoprolol Succinate stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.
Preparation of Sample Solution:
Take average weight of Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight ofEfonidipine Hydrochloride Ethanolate and Metoprolol Succinate sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.
Further pipette 0.1ml of the Efonidipine Hydrochloride Ethanolate and 0.2ml of the Metoprolol Succinate stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.
Procedure:
Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Linearity:
Accurately weigh and transfer 10 mg of Efonidipine Hydrochloride Ethanolateand 10mg of Metoprolol Succinate working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Preparation of Level – I (6ppm of Efonidipine Hydrochloride Ethanolate&12ppm of Metoprolol Succinate):

Pipette out 0.06ml of Efonidipine Hydrochloride Ethanolate and 0.12ml of Metoprolol Succinate stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (8ppm of Efonidipine Hydrochloride Ethanolate& 16ppm of Metoprolol Succinate):

Pipette out 0.08ml of Efonidipine Hydrochloride Ethanolate and 0.16ml of Metoprolol Succinate stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (10ppm of Efonidipine Hydrochloride Ethanolate& 20ppm of Metoprolol Succinate):

Pipette out 0.1ml of Efonidipine Hydrochloride Ethanolate and 0.2ml of Metoprolol Succinate stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (12ppm of Efonidipine Hydrochloride Ethanolate& 24ppm of Metoprolol

Succinate):

Pipette out 0.12ml of Efonidipine Hydrochloride Ethanolate and 0.24ml of Metoprolol Succinate stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (14ppm of Efonidipine Hydrochloride Ethanolate&28ppm of Metoprolol Succinate):

Pipette out 0.14ml of Efonidipine Hydrochloride Ethanolate and 0.28ml of Metoprolol Succinate stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Procedure:
Inject each level into the chromatographic system and measure the peak area.
Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Accuracy:

For Preparation of 80% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Efonidipine Hydrochloride Ethanolateand 10mg of Metoprolol Succinate working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.08ml of the Efonidipine Hydrochloride Ethanolate and 0.16ml of the Metoprolol Succinate stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For Preparation of 100% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Efonidipine Hydrochloride Ethanolateand 10mg of Metoprolol Succinate working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.1ml of the Efonidipine Hydrochloride Ethanolate and 0.2ml of the Metoprolol Succinate stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For Preparation of 120% Standard Stock Solution:
Accurately weigh and transfer 10 mg of Efonidipine Hydrochloride Ethanolateand 10mg of Metoprolol Succinate working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.12ml of the Efonidipine Hydrochloride Ethanolate and 0.24ml of the Metoprolol Succinate stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:
Inject the Three replicate injections of individual concentrations (80%, 100%, 120%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Efonidipine Hydrochloride Ethanolateand Metoprolol Succinate and calculate the individual recovery and mean recovery values.

Precision

Repeatability

Preparation of Efonidipine Hydrochloride Ethanolate and Metoprolol SuccinateProduct Solution for Precision:

Accurately weigh and transfer 10 mg of Efonidipine Hydrochloride Ethanolateand 10mg of Metoprolol Succinate working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)
Further pipette 0.1ml of the Efonidipine Hydrochloride Ethanolate and 0.2ml of the Metoprolol Succinate stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Intermediate Precision:
To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:

Day 1:
The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for

the area of six replicate injections was found to be within the specified limits.

Day 2:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Robustness:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results. .

For Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Efonidipine Hydrochloride Ethanolateand 10mg of Metoprolol Succinate working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the Efonidipine Hydrochloride Ethanolate and 0.2ml of the Metoprolol Succinate stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Effect of Variation of Flow Conditions:

The sample was analyzed at 0.8ml/min and 1.0ml/min instead of 0.9ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded

Effect of Variation of Mobile Phase Organic Composition:

The sample was analyzed by variation of mobile phase i.e. Acetonitrile and Water was taken in the ratio and 35:65, 45:55% instead (40:60), remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Stability Studies

Following protocol was strictly adhered to for stability studies of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate Active Pharmaceutical Ingredient (API). The APIs of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate was subjected to different stability conditions in various ways to observe the rate and extent of degradation occur in the course of storage after administration to body. This is one type of accelerated stability studies that helps us determining the fate of the drug that is likely to happen after long time storage, within a very short time as compare to the real time or long term stability testing. The various degradation pathways studied are acid hydrolysis, basic hydrolysis, thermal degradation, photolytic degradation and oxidative degradation.

Acid Hydrolysis: An accurately weighed 25 mg of pure drug was transferred to a clean & dry 25 ml volumetric flask. To which 0.1N Hydrochloric acid was added & make up to the mark & kept for 24 hrs. from that 4 ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of HCl (after all optimized conditions).

Basic Degradation Studies: An accurately weighed 10 mg. of pure drug was transferred to a clean & dry 10 ml volumetric flask. To which 0.1N Sodium hydroxide was added & make up to the mark & kept for 24 hrs. from that 4s ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of NaOH (after all optimized conditions).

Thermal Degradation Studies: Accurately weighed 10 mg of pure drugs were transferred to a clean & dry 100 ml of volumetric flask and make up to the mark with the mobile phase and maintained at 50 ^{0}C for 24hrs.Then finally injected into the HPLC system against a blank of mobile phase (after all optimized chromatographic conditions).

Photolytic DegradationStudies: Approximately 10 mg of pure drug was taken in a clean & dry Petridis. It was kept in a UV cabinet at 254 nm wavelength for 24 hours without interruption. Accurately weighed 1 mg. of the UV exposed drug was transferred to a clean & dry 10 ml. volumetric flask. First the UV exposed drug was dissolved in methanol & make up to the mark. Then injected into the HPLC system against a blank of mobile phase (after all optimized conditions).

Oxidation with (3%) H_2O_2Studies: Accurately weighed 10 mg. of pure drug was taken in a clean & dry 100 ml. volumetric flask. 30 ml. of 3% H_2O_2 and a little methanol was added to it to make it soluble & then kept as such in dark for 24 hours. Final volume was made up to 100 ml. using water to prepare 100ppm solution. The above sample was injected into the HPLC system.

III. RESULTS AND DISCUSSION

Selection of Wavelength

Determination of Wavelength of Maximum Absorbance for Efonidipine Hydrochloride Ethanolate

Standard Efonidipine Hydrochloride Ethanolate solution (1ml) was transferred to separate 10 ml volumetric flask. The volume was adjusted to 10 ml with same solvent mixture. The absorbance of the final solution was scanned in the range 400 to 200 nm against solvent mixture as blank.The result is shown in Figure-3.

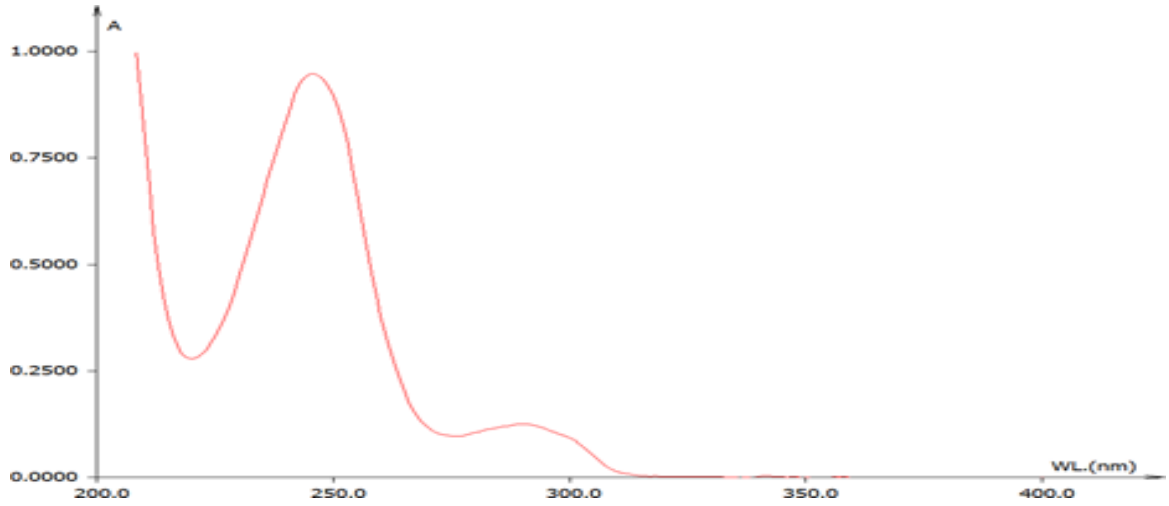

Fig.3. UV Spectrum for Efonidipine Hydrochloride Ethanolate

Determination of Wavelength of Maximum Absorbance for Metoprolol Succinate

Standard Metoprolol Succinate solution (1 ml) was transferred to separate 10 ml volumetric flask. The volume was adjusted to 10 ml with same solvent mixture. The absorbance of the final solution was scanned in the range 400 to 200 nm against solvent mixture as blank.The result is represented in fig-4.

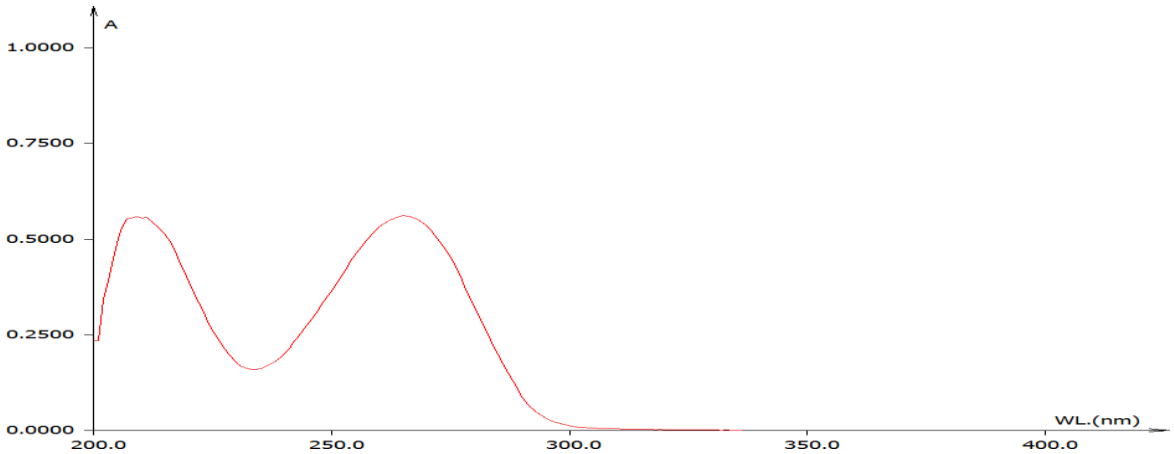

Fig.4. UV Spectrum for Metoprolol Succinate

Selection of Wavelength

Selectivity of HPLC method that uses UV detector depends on proper selection of Wavelength. A wavelength which gives good response for the drug to be detected is to be selected. From the UV spectra 258 nm was selected as the wavelength for study. The λ max of this method can be determined as 258 nm.

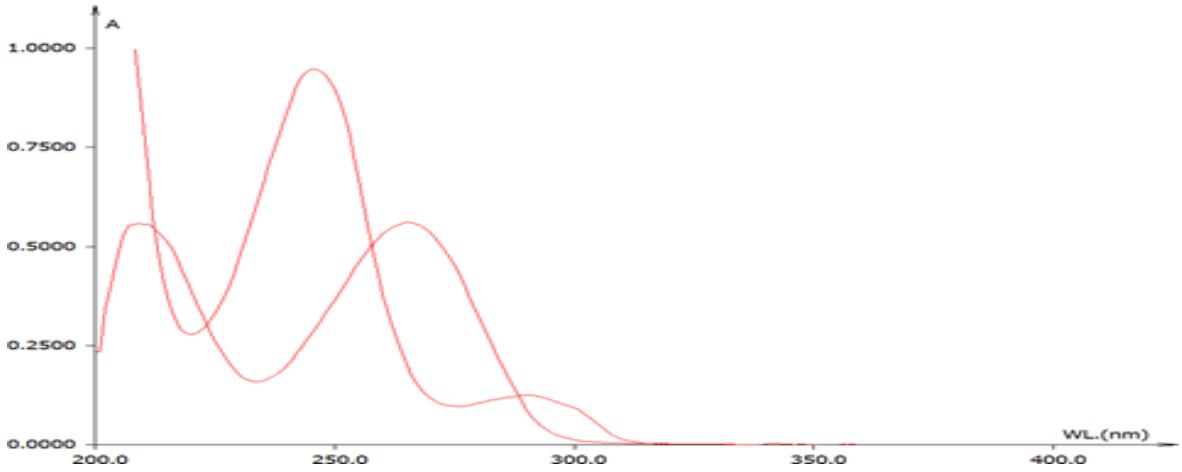

Fig.5. Isobestic point Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate (258nm)

Trials for the Method Development:

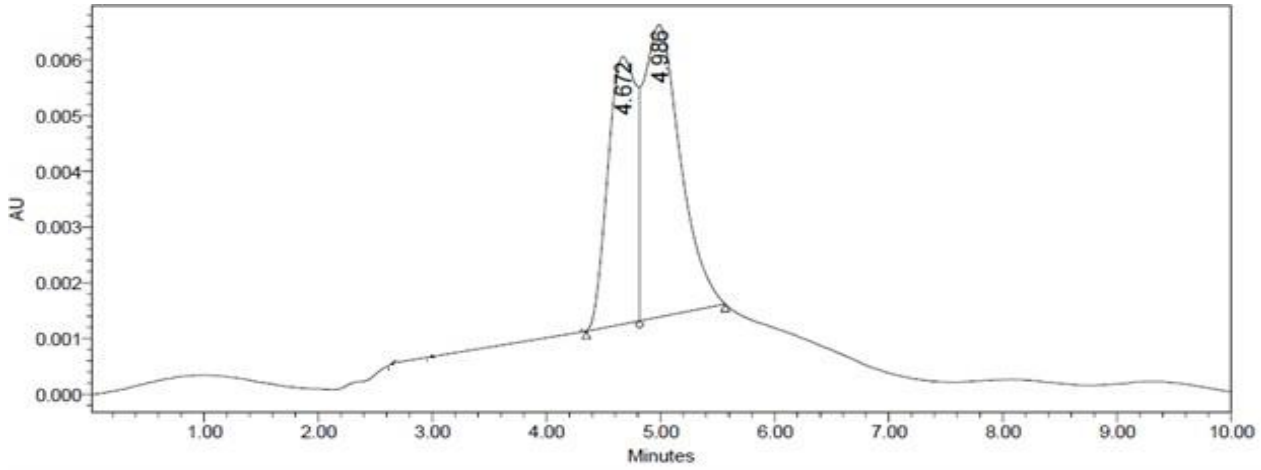

Fig.6. Chromatogram for Trial-1

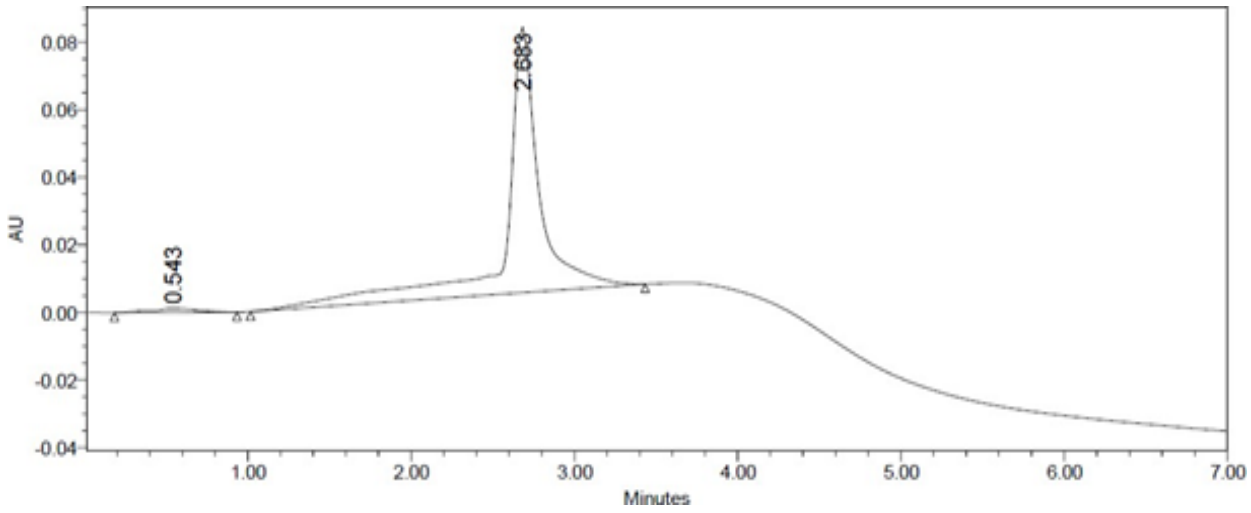

Fig.7. Chromatogram for Trial-2

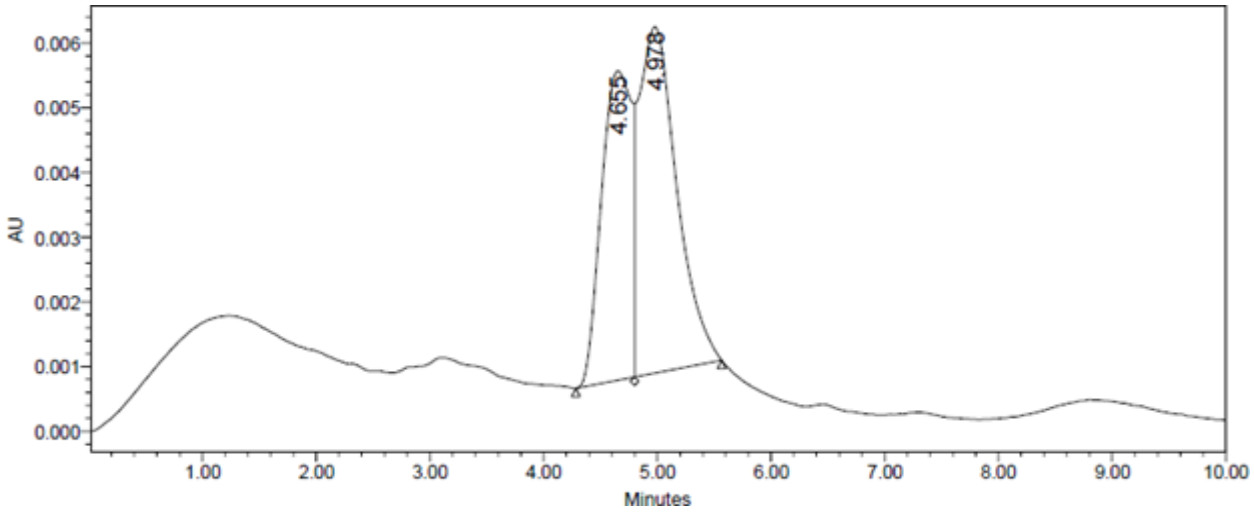

Fig.8. Chromatogram for Trial-3

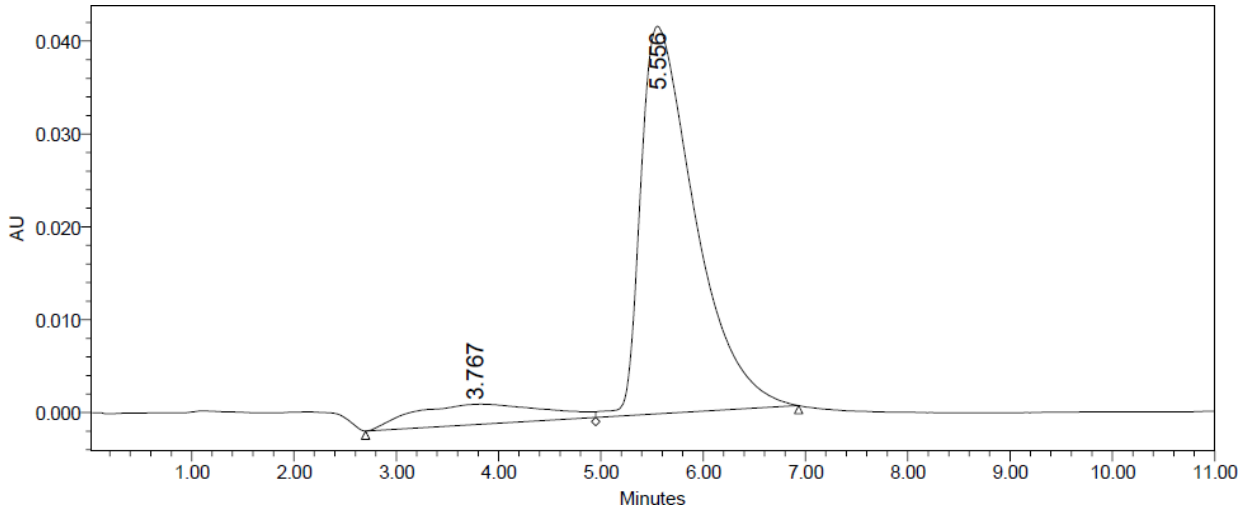

Fig.9.Chromatogram for Trial-4

Optimized Chromatographic Method:

Table-4: Summary of Optimized Chromatographic Conditions

Mobile phase	Methanol: Acetonitrile (85:15% v/v)
Column	Develosil ODS HG-5 RP C_{18}, 15cmx4.6mm, i.d. Column.
Column Temperature	Ambient
Detection Wavelength	258 nm
Flow rate	1.0 ml/ min.
Run time	15 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase- Methanol: Acetonitrile (85:15% v/v)
Injection Volume	10µl
Type of Elution	Isocratic

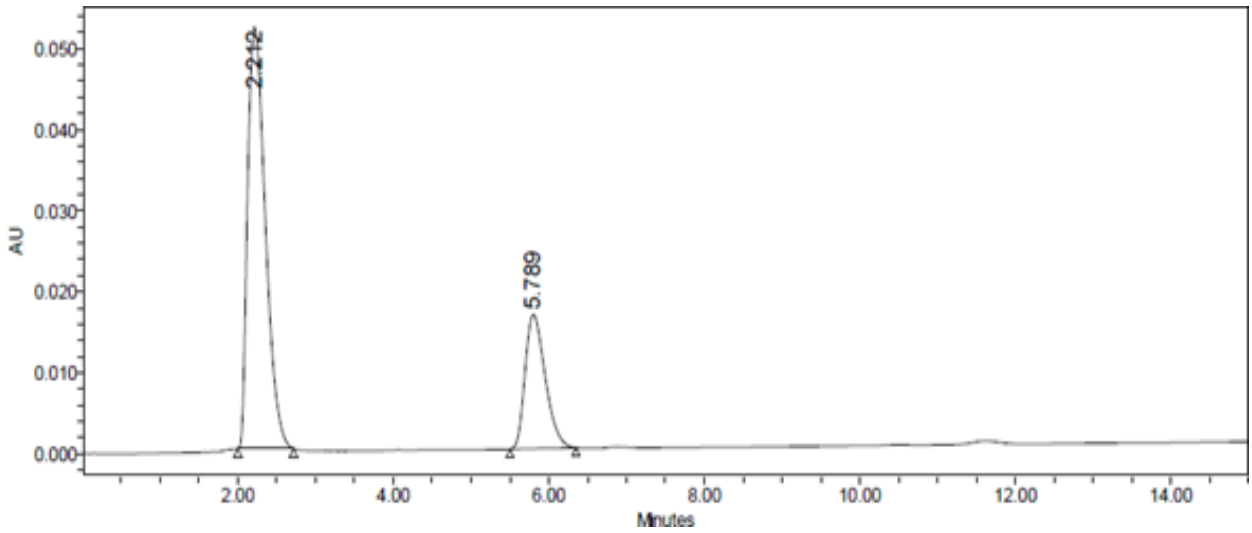

Fig.10. Chromatogram of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate in Optimized Chromatographic Condition

Method Validation

1. System SuitabilityParameter:

It is an integral part of so many analytical procedures. The parameters are based on the idea that the equipment, electronics, analytical operations and the samples to be analyzed constitute as an integral system which can be examined. Finally system suitability test parametersare established[35-36]. The obtained data is shown in the following table-14.

Table-14: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs < 2	3.65
2	Asymmetry	T < 2	Efonidipine Hydrochloride Ethanolate = 0.35 Metoprolol Succinate = 0.23
3	Theoretical plates	N < 2000	Efonidipine Hydrochloride Ethanolate = 3771 Metoprolol Succinate =2437

2. Linearity and Range:

To evaluate the linearity, serial dilution of analyte were prepared from the stock solutions.Diluted with mobile phase to get a series of concentration ranging from 6-14µg/ml and 12-28µg/ml for Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate, respectively[14]. The prepared solutions were sonicated. From these solutions, 10µl injections of each concentration were injected into the HPLC system and chromatographed under the

optimized conditions. Calibration curves were constructed by plotting the mean peak area (Y-axis) against the concentration (X-axis) [15]. The Linearity data is presented in table-5 & 6. The Calibration Curves of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinateare represented in fig-11 & 12.

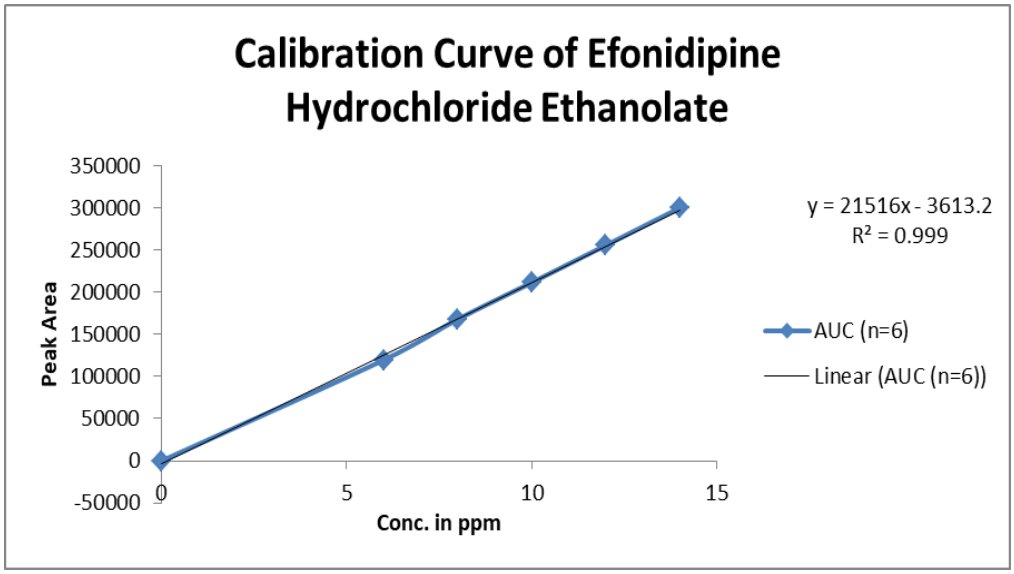

Fig.11.Standard Curve for Efonidipine Hydrochloride Ethanolate

Table-5: Linearity Results for Efonidipine Hydrochloride Ethanolate

CONC. (µg/ml)	AUC (n=6)
6	119571
8	167873
10	211264
12	255428
14	299987

Linearity Plot:

The plot of Concentration

(x) versus the Average Peak Area (y) data of Efonidipine Hydrochloride Ethanolate is a straight line.

Y = mx + c

Slope (m) = 21516

Intercept (c) = 3613.2

Correlation Coefficient (r) = 0.999

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater[16].

Conclusion: Correlation Coefficient[17] (r) is 0.99, and the intercept is 3613.2. These values meet the validation criteria.

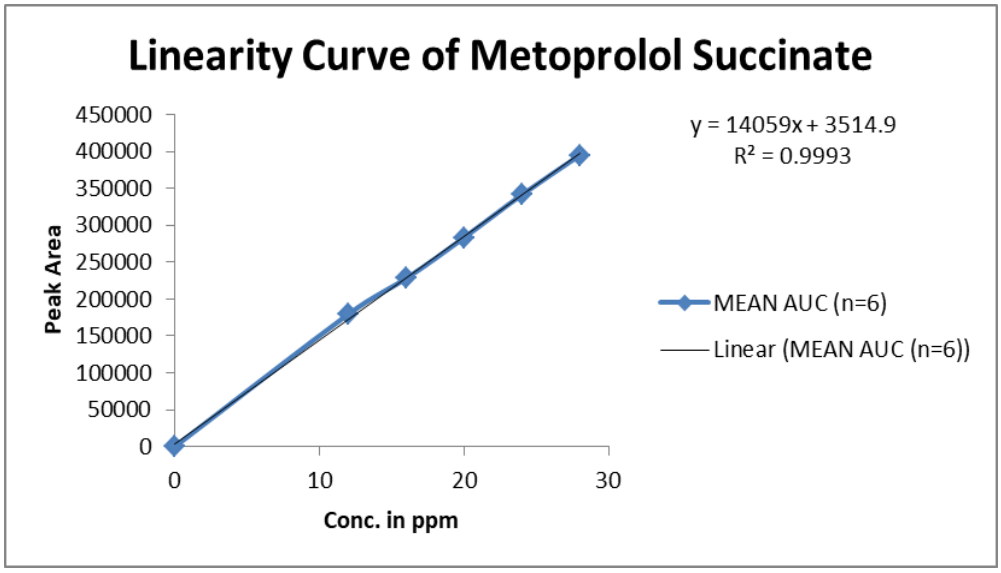

Fig.12.Standard Curve for Metoprolol Succinate

Table-6: Linearity Results for Metoprolol Succinate

CONC.(µg/ml)	MEAN AUC (n=6)
12	179371
16	227893
20	283264
24	341428
28	394987

Linearity Plot:

The plot of Concentration (x) versus the Average Peak Area (y) data of Metoprolol Succinate is a straight line.

Y = mx + c

Slope (m) = 14059

Intercept (c) = 3514.9

Correlation Coefficient (r) = 0.9993

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 3514.9. These values meet the validation

criteria.

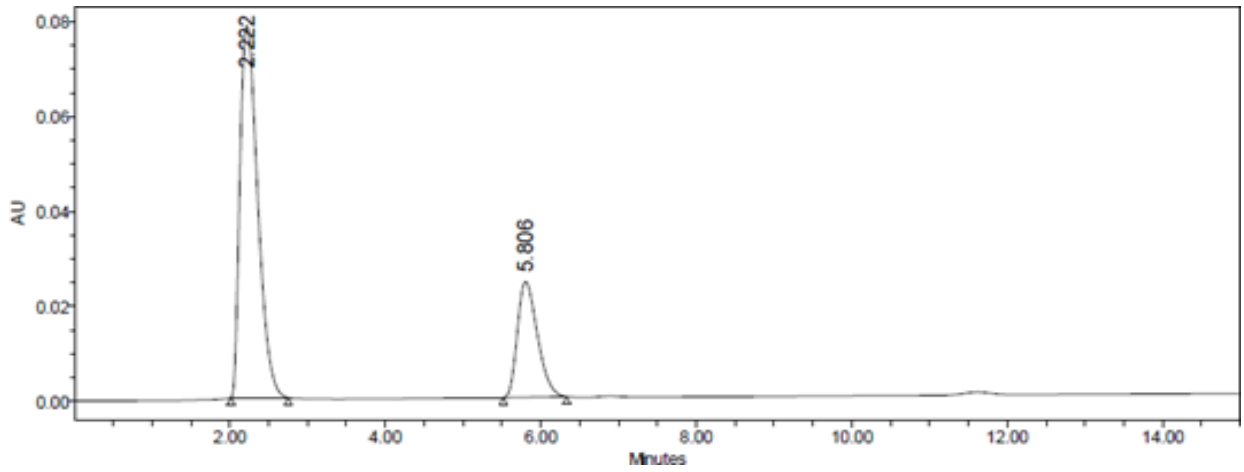

Fig.13. Chromatogram for Linearity-1

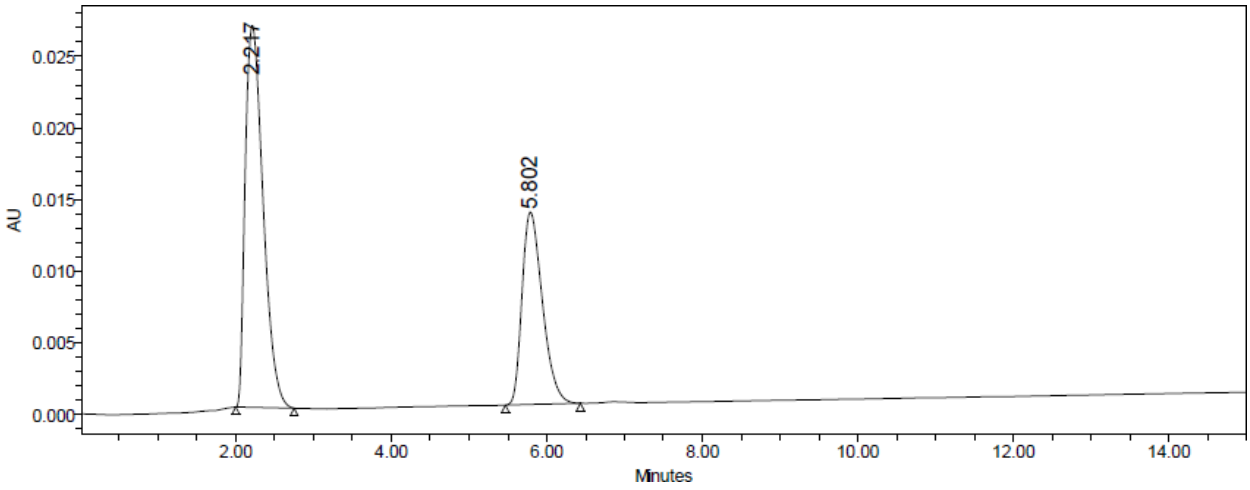

Fig.14.Chromatogram for Linearity-2

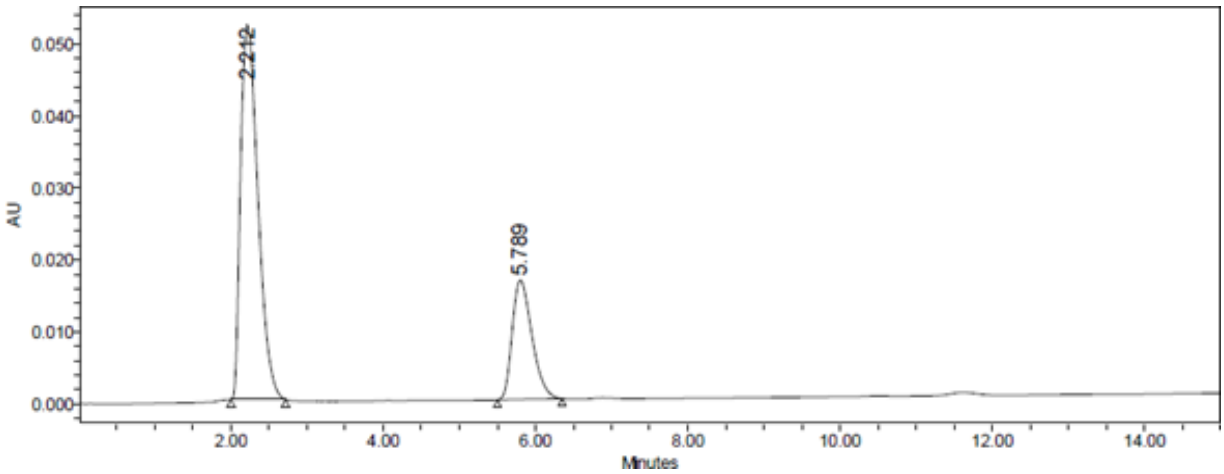

Fig.15.Chromatogram for Linearity-3

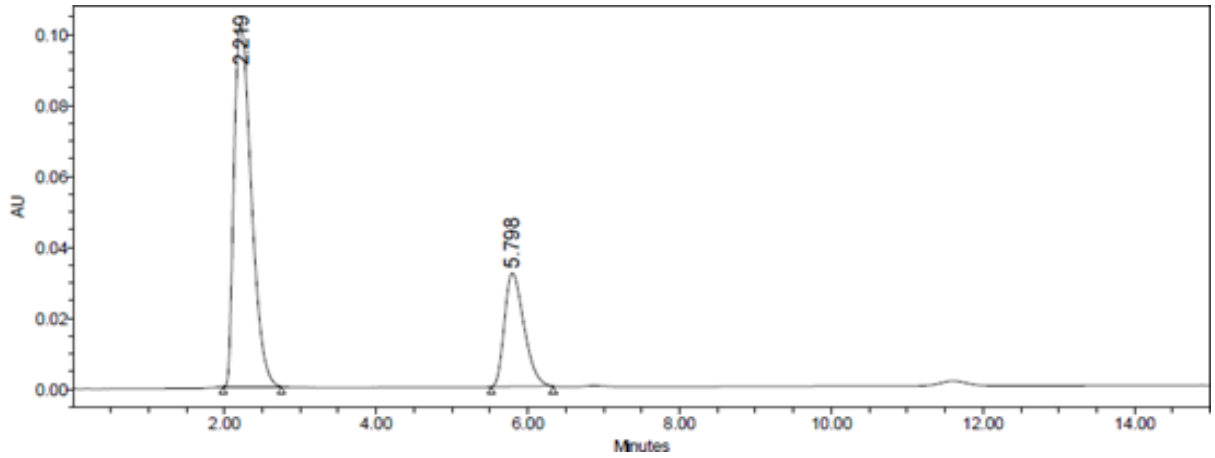

Fig.16.Chromatogram forLinearity-4

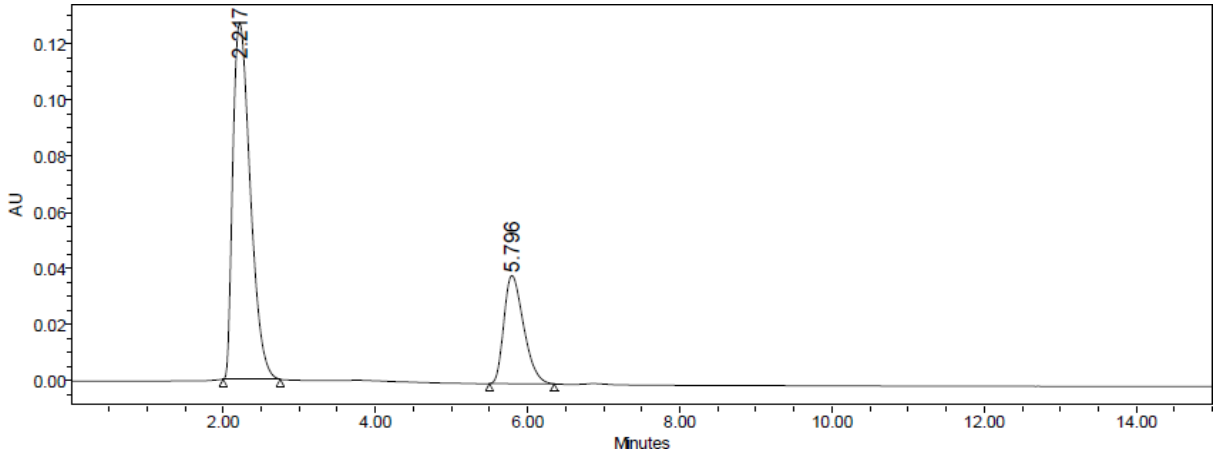

Fig.17.Chromatogram for Linearity-5

3. Accuracy:

The accuracyof the method was determined by recovery studies and the percentage recovery was calculated[18]. The recoveries of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate were found to be in the range of 98-102%. The proposed Liquid Chromatographic methodwas applied to the determination of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate, respectively[19]. The results for Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate comparable with the corresponding labeled amounts.

Table-7: Accuracy Readings for Efonidipine Hydrochloride Ethanolate

Sample ID	Concentration (± g/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	8	7.997368	115949	99.9671	Mean= 100.7003%
S_2 : 80 %	8	8.106622	117485	101.3328	S.D. = 0.6884036

S_3 : 80 %	8	8.064087	116887	100.8011	% R.S.D.= 0.683616%
S_4 : 100 %	10	9.904901	142767	99.04901	Mean= 100.36157%
S_5 : 100 %	10	10.02966	144521	100.2966	S.D. = 1.346221
S_6 : 100 %	10	10.17391	146549	101.7391	%R.S.D.= 1.3413706%
S_7 : 120 %	12	12.01807	172476	100.1506	Mean= 100.183756%
S_8 : 120 %	12	11.88079	170546	99.00657	S.D. = 1.19411
S_9 : 120 %	12	12.16729	174574	101.3941	% R.S.D. = 1.19191%

Recovery Study: Metoprolol Succinate

Table-8: Accuracy Results for Metoprolol Succinate

Sample ID	Concentration (± g/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	16	16.08685	229679	100.5428	Mean= 100.54488%
S_2 : 80 %	16	15.93079	227485	99.56745	S.D. =0.97847%
S_3 : 80 %	16	16.2439	231887	101.5244	% R.S.D.=0.9731%
S_4 : 100 %	20	20.07632	285767	100.3816	Mean= 99.97095%
S_5 : 100 %	20	19.98769	284521	99.93847	S.D. = 0.395406
S_6 : 100 %	20	19.91856	283549	99.59279	% R.S.D.= 0.39552%
S_7 : 120 %	24	23.75432	337476	98.97634	Mean= 100.27718%
S_8 : 120 %	24	24.11494	342546	100.4789	S.D. = 1.21262
S_9 : 120 %	24	24.33032	345574	101.3763	% R.S.D. = 1.20927%

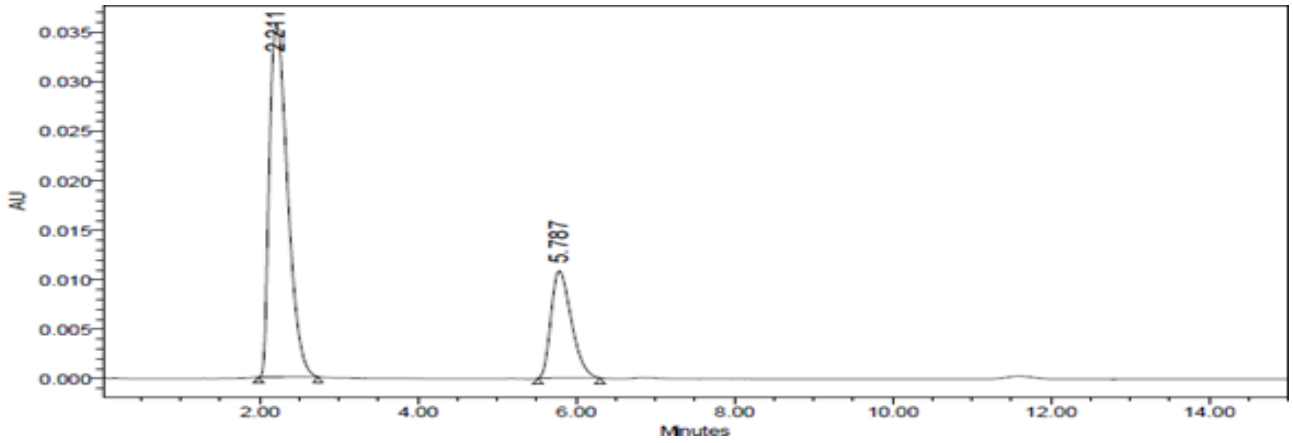

Fig.18.Chromatogram for Accuracy-1 replicate-1

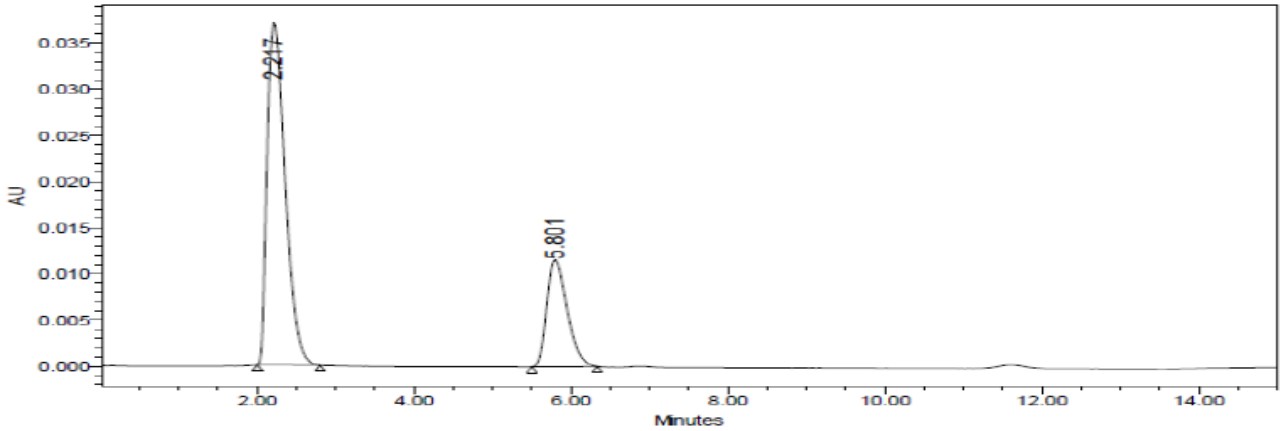

Fig.19.Chromatogram for Accuracy-1 replicate-2

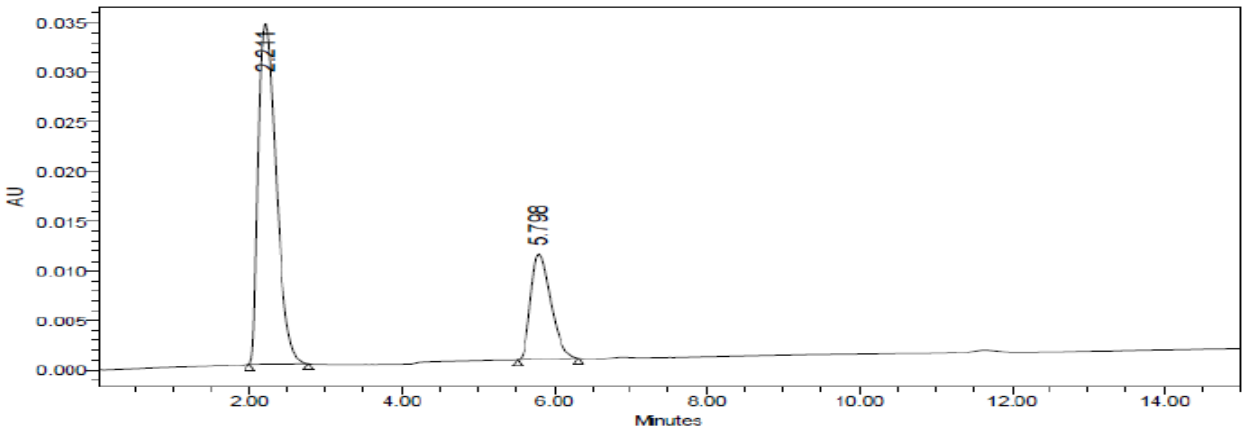

Fig.20. Chromatogram for Accuracy-1 replicate-3

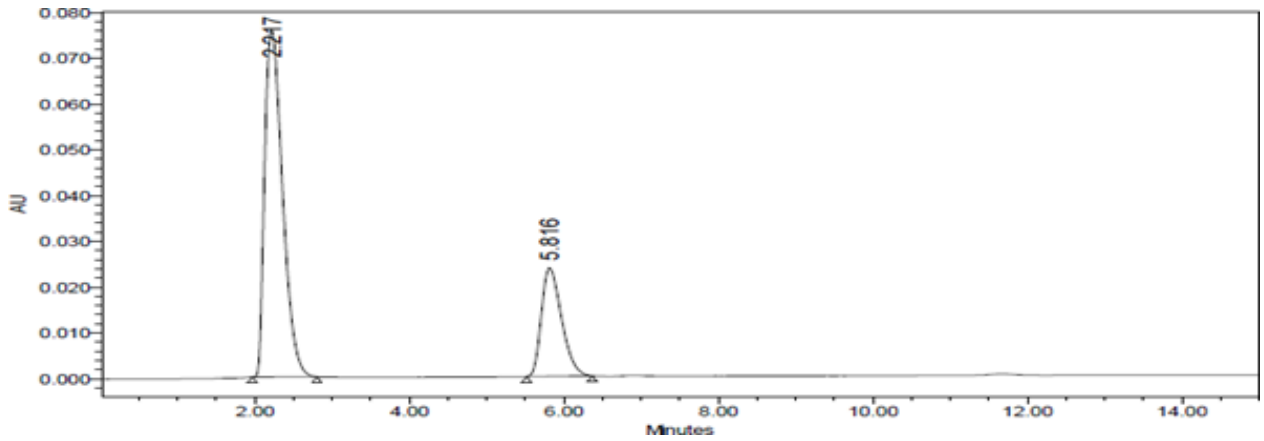

Fig.21. Chromatogram for Accuracy-2 replicate-1

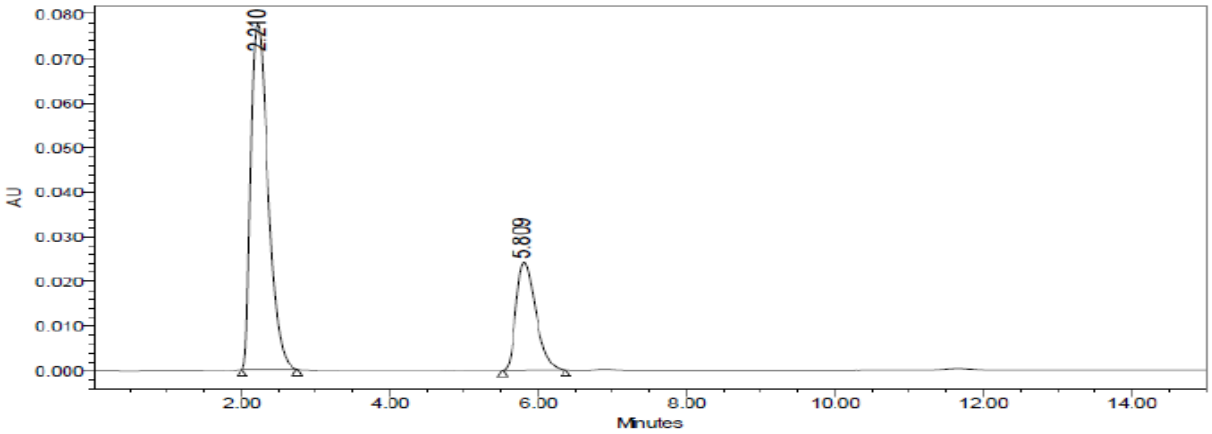

Fig.22. Chromatogram for Accuracy-2 replicate-2

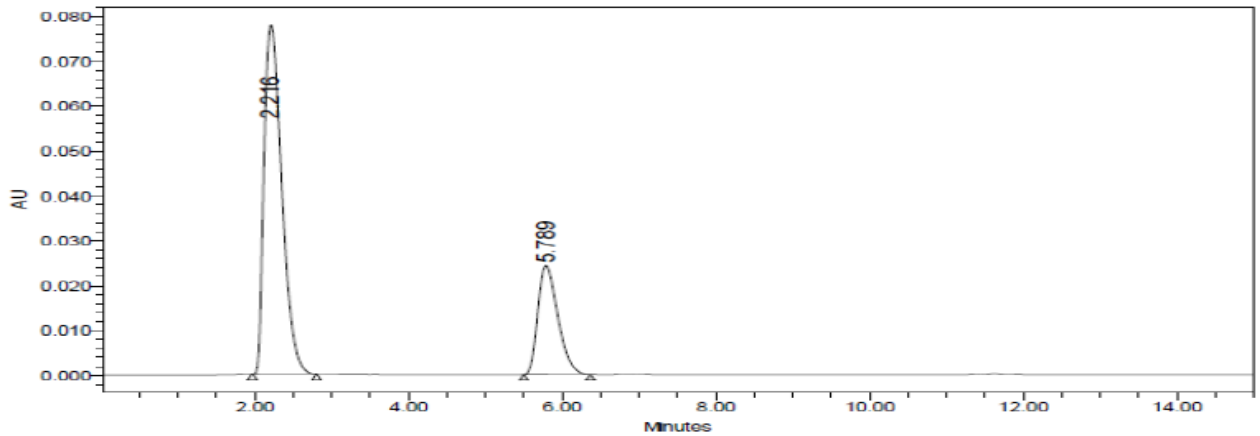

Fig.23. Chromatogram for Accuracy-2 replicate-3

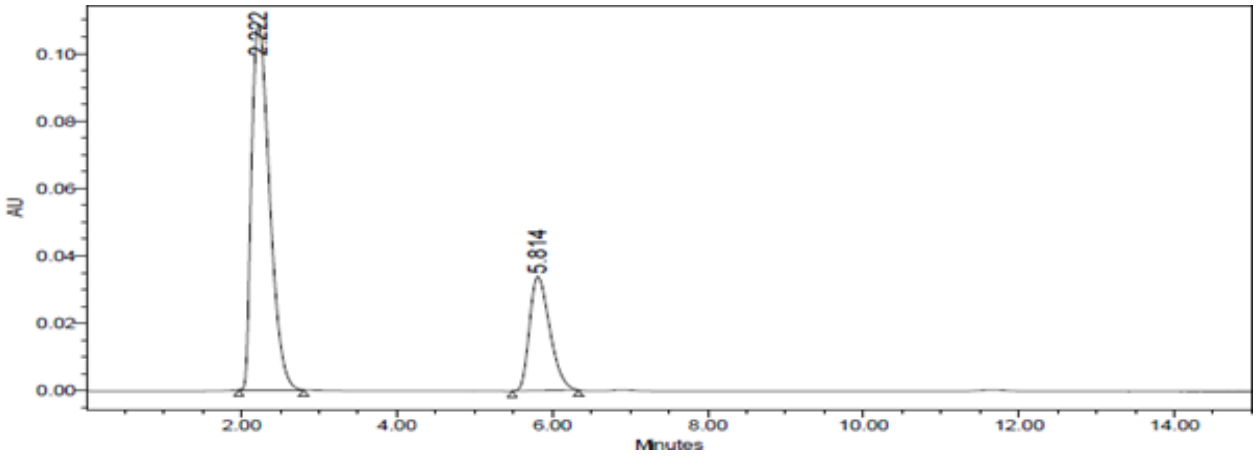

Fig.24. Chromatogram for Accuracy-3 replicate-1

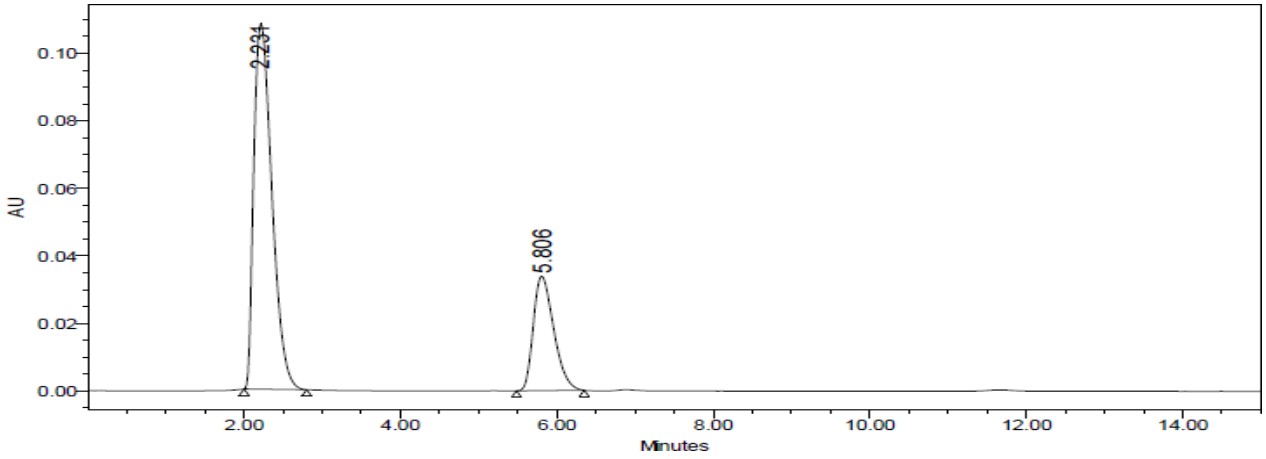

Fig.25. Chromatogram for Accuracy-3 replicate-2

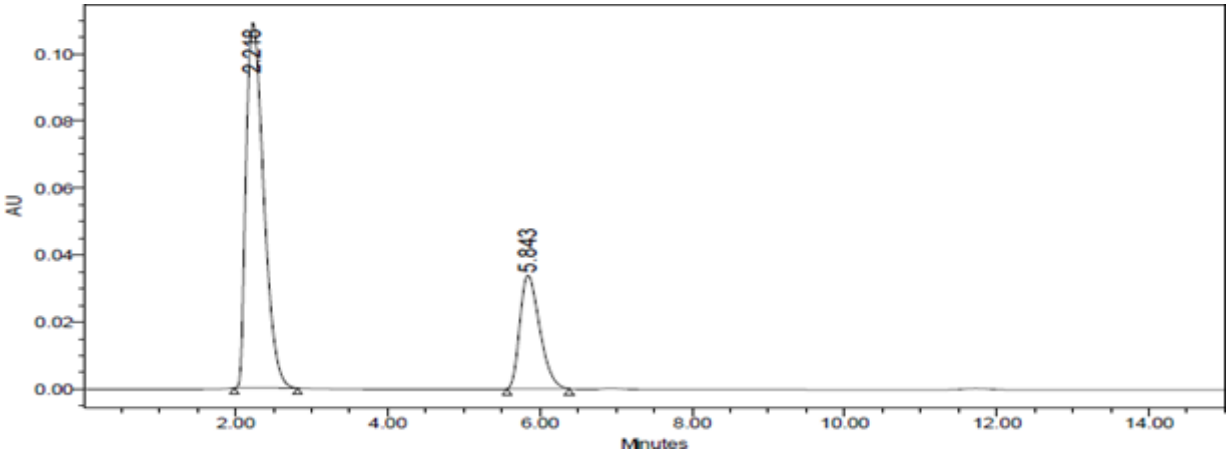

Fig.26. Chromatogram for Accuracy-3 replicate-3

4. Precision:

The precisionof an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions[20].

Repeatability: Obtained Six (6) replicates of 100% accuracy solution as per experimental conditions. Recorded

the peak areas and calculated % RSD.

Table-9: **Data showing repeatability analysis for Efonidipine Hydrochloride Ethanolate & Metoprolol Succinate**

HPLC Injection Replicates	Drug Concentration [Efo + Met] (µg/ml)	AUC for Efonidipine Hydrochloride Ethanolate	AUC for Metoprolol Succinate
Replicate – 1	10 + 20	113568	241022
Replicate – 2	10 + 20	113241	240137
Replicate – 3	10 + 20	115408	242911
Replicate – 4	10 + 20	117412	245245
Replicate – 5	10 + 20	112541	241941
Replicate – 6	10 + 20	112546	240444
Average		**114119.3333**	**241356.6667**
Standard Deviation		**1925.83838**	**1416.95812**
% RSD		**1.68756**	**0.58708**

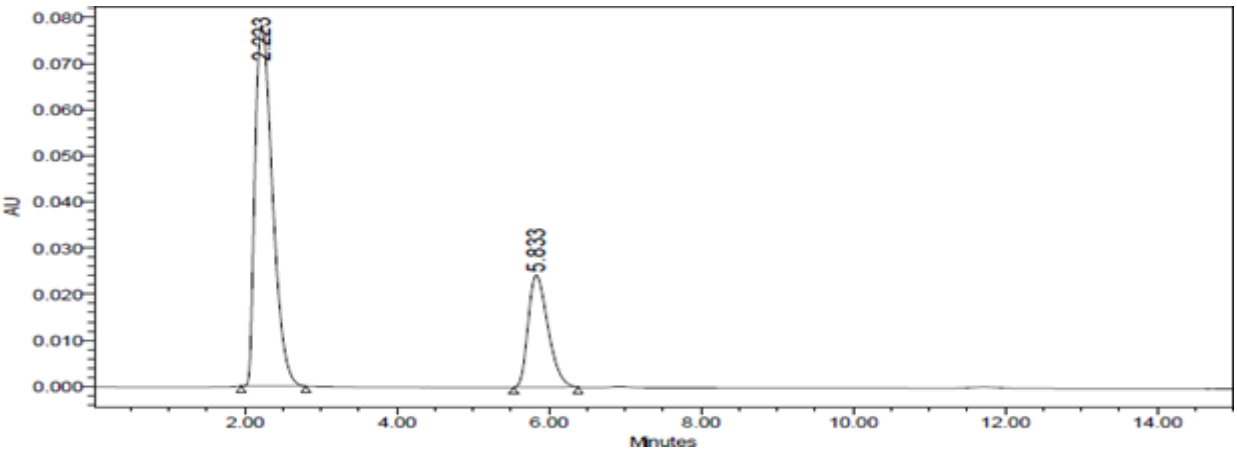

Fig.27.Chromatogram for Repeatability-1

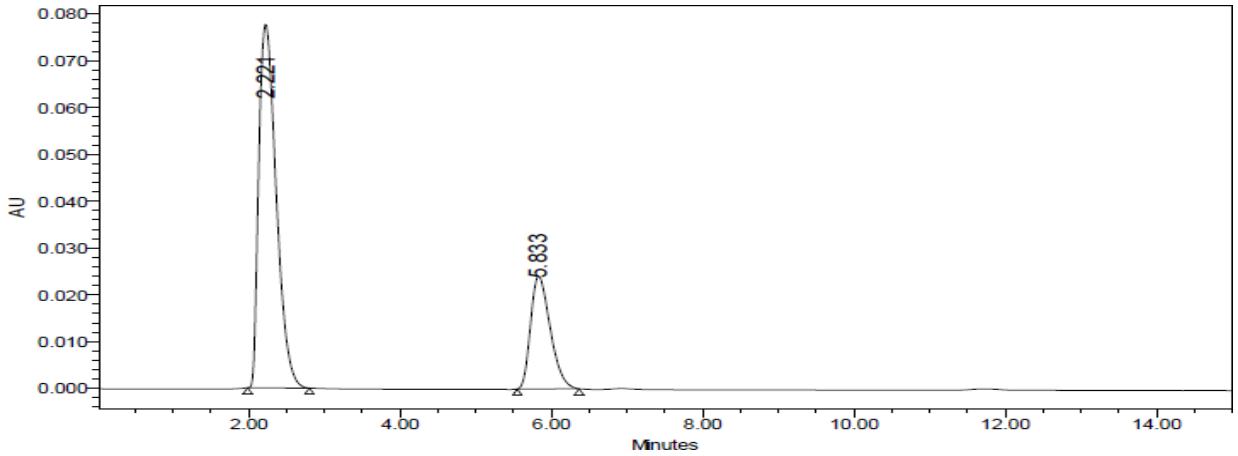

Fig.28. Chromatogram for Repeatability-2

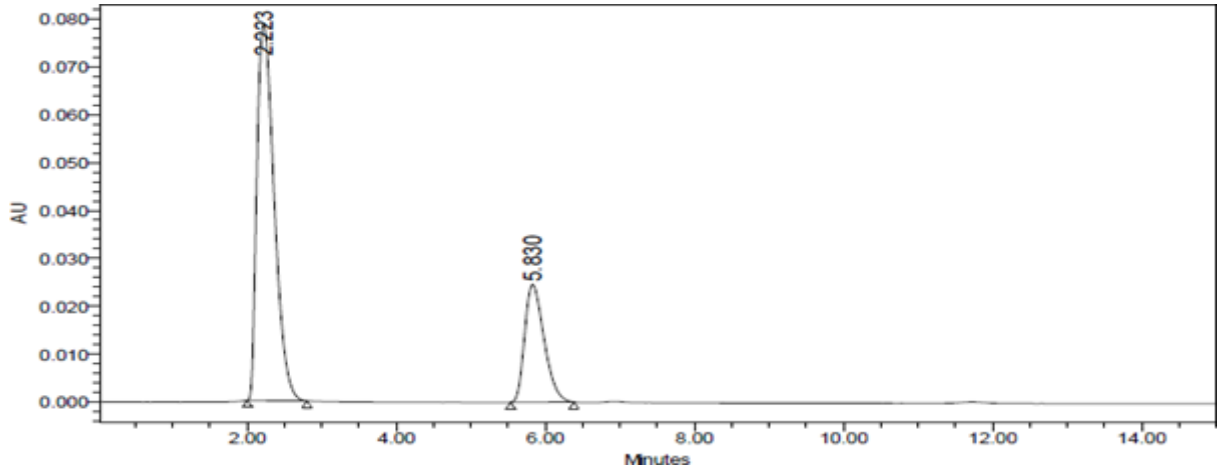

Fig.29. Chromatogram for repeatability-3

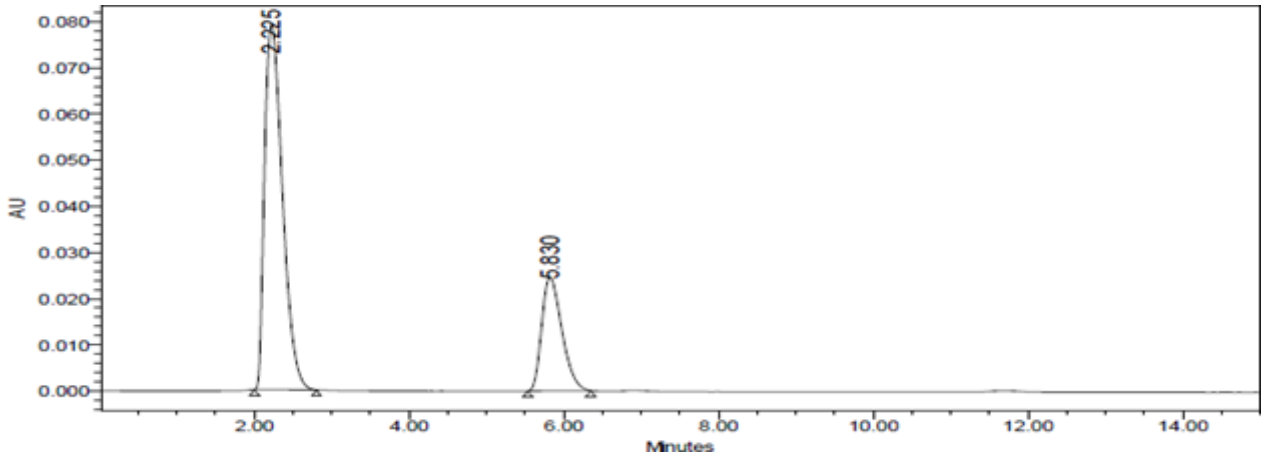

Fig.30. Chromatogram for repeatability-4

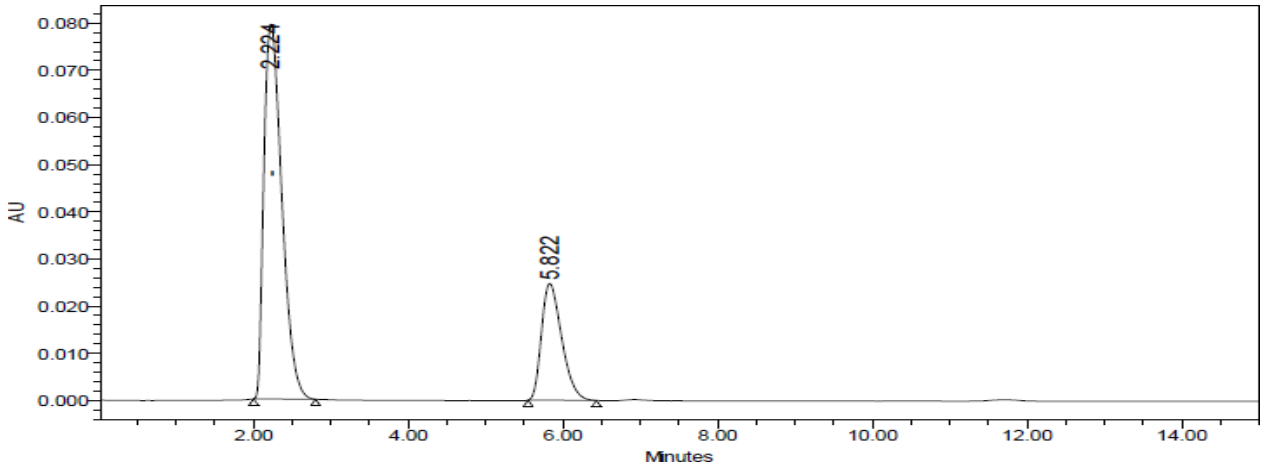

Fig.31. Chromatogram for repeatability-5

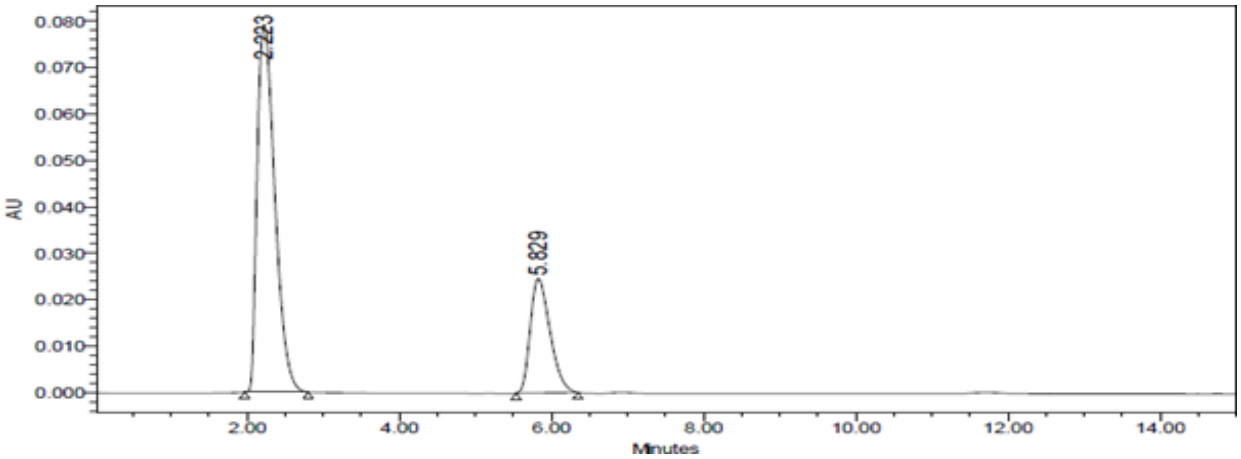

Fig.32. Chromatogram for Repeatability-6

4.1. Intermediate Precision:

4.1.1. Intra-Assay & Inter-Assay:

The intra & inter day variationof the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Efonidipine Hydrochloride Ethanolate and Metoprolol Succinaterevealed that the proposed method is precise[21-24].

Table-10: Results of Intra-Assay & Inter-Assay

Conc. of Efonidipine Hydrochloride Ethanolate(API) (µg/ml)	Observed Conc. of Efonidipine Hydrochloride Ethanolate (µg/ml) by the Proposed Method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
8	8.09	0.97	8.03	0.96
10	10.05	0.45	10.04	0.47

12	11.98	0.37	11.90	0.12

Table-11: Data for Metoprolol Succinate Intra-Assay & Inter-Assay Analysis

Conc. of Metoprolol Succinate (API) (µg/ml)	Observed Conc. of Metoprolol Succinate (µg/ml) by the Proposed Method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
16	15.97	0.27	16.09	0.59
20	20.14	1.29	19.95	0.64
24	24.08	0.61	23.94	0.26

Result and Discussion:

The Intraday and interday related studies shows that the % RSD was found to be within limit i.e. (≤2%). So it is indicated that the developed method is within the limits[25]. Hence finally we concluded that the developed method was found to be precise.

5. Limit of detection (LOD) & Limit of quantification (LOQ):

The detection limit(LOD) and quantitation limit (LOQ) may be expressed as[26]:

L.O.D. = 3.3 (SD/S).

L.O.Q. = 10 (SD/S)

Where, SD = Standard deviation of the response

S = Slopeof the calibration curve

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.08 & 0.24 µg/ml respectively for Efonidipine Hydrochloride Ethanolate[27].

The LOD was found to be 0.1 µg/ml and LOQ was found to be 0.3 µg/ml for Metoprolol Succinate which represents that sensitivity of the method is high[28].

6. Method Robustness:

Influence of small changes in chromatographic conditionssuch as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±2nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness[32] of the method are also in favour of (Table-12, % RSD < 2%) the developed RP-HPLC method for the analysis of Efonidipine Hydrochloride Ethanolate (API)[29-31].

Table-12: Result of Method Robustness Test

Change in Parameter	% RSD
Flow (1.1 ml/min)	1.05
Flow (0.9 ml/min)	0.67
Temperature (27^0C)	0.58
Temperature (23^0C)	0.61
Wavelength of Detection (280 nm)	0.38
Wavelength of detection (270 nm)	0.17

Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^0C), Wavelength of detection (±2nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness of the method are also in favour of (Table-13, % RSD < 2%) the developed RP-HPLC method for the analysis of Metoprolol Succinate (API)[33-34].

Table-13: Result of Method Robustness Test

Change in Parameter	% RSD
Flow (1.1 ml/min)	0.09
Flow (0.9 ml/min)	0.07
Temperature (27^0C)	0.28
Temperature (23^0C)	0.74
Wavelength of Detection (235 nm)	0.86
Wavelength of detection (240 nm)	0.67

7. Estimation of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate in Pharmaceutical DosageForm

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Finally the weighed tablets are powdered and triturated well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drugs were transferred to a clean and dry 100ml of volumetric flask and add 70 ml of mobile phase and the resulted solution was sonicated for 15 minutes by using ultra sonicator, Then the final volume was make up to the mark with the mobile phase. The final solution was filtered through a selected membrane filter (0.45 µm) and in order to sonicate to degas the mobile phase (Solvent system). From this above stock solution (1 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system (Mobile phase).

The prepared solutions were injected in five replicates into the HPLC system and the observations were recorded.

A duplicate injection (Blank Solution) of the standard solution also injected into the HPLC system and the chromatograms and peak areas were recorded and calculated. The obtained data are shown in Table 15.

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Average weight} = \text{mg/tab}$$

Where:

AT = Test Preparation Peak Area

AS = Standard preparation Peak Area

WS = Working standard weight taken in mg

WT = Sample weight taken in mg

DS = Standard solution dilution

DT = Sample solution dilution

P = Working standard percentage purity

The assay was performed as explained in the previous chapter (Above). The results which are obtained are following[37]:

Table-15: Recovery Data for estimation Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate in Efnocar-MX Tablet

Brand Name of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate	Labelled Amount of Drug (mg)	Mean (± SD) Amount (mg) found by the Proposed Method(n=6)	Assay % (± SD)
Efnocar-MX Tablet (Zuventus Healthcare Ltd)	40mg/25mg	39.586 (± 0.628)/24.685 (± 718)	99.749 (±0.852)/99.459 (± 0.698)

Result & Discussion: The %purity of Efonidipine Hydrochloride Ethanolate & Metoprolol Succinate for Tablets was found to be 99.749% and 99.459%respectively.

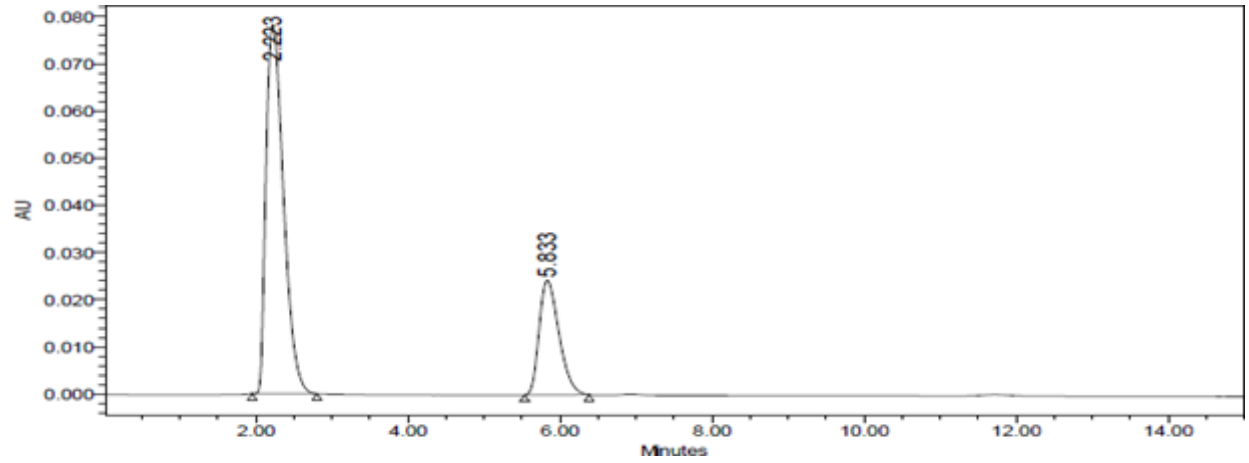

Fig.33.Chromatogram for Standard Solution

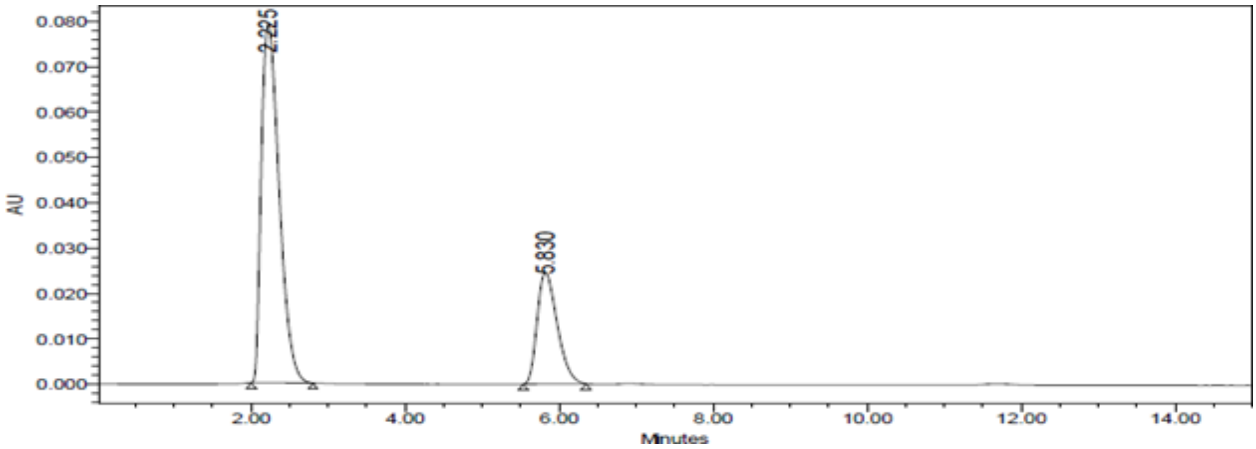

Fig.34. Chromatogram for Sample Solution

STABILITY STUDIES

Results of Degradation Studies:

The results of the stress studies indicated the **specificity** of the method that has been developed. Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate were stable only in photolytic stress conditions and little bit in thermal stress conditions[38]. The results of forced degradation studies are given in the following Table-16.

Table-16: Results of Forced Degradation Studies of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate API

Stress Condition	Time (hours)	Assay of Active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	95.62	4.38	100.00

Basic Hydrolysis (0.IN NaOH)	24Hrs.	97.13	2.87	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	96.24	3.76	100.00
UV (254nm)	24Hrs.	95.43	4.57	100.00
3% Hydrogen peroxide	24Hrs.	96.16	3.84	100.00

IV. SUMMARY

The analytical method was developed by studying different parameters.
First of all, maximum absorbance was found to be at 258nm and the peak purity was excellent.
Injection volume was selected to be 10µl which gave a good peak area.
The column used for study was Develosil ODS HG-5 RP C_{18}, 15cmx4.6mm, i.d. Column because it was giving good peak.
An ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 0.9ml/min because of good peak area and satisfactory retention time.
Mobile phase is Methanol and Acetonitrile in the ratio of 85:15% v/v was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study.
Run time was selected to be 15min because analyze gave peak around 2.212, 5.789 ±0.02min of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinate respectively and also to reduce the total run time.
The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range.
The analytical method was found linearity over the range6-14µg/ml of Efonidipine Hydrochloride Ethanolateand 12-28 µg/ml of Metoprolol Succinateof the target concentration.
The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

V. CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinatein bulk drug and pharmaceutical dosage forms.
This method was simple, since diluted samples are directly used without any preliminary chemical derivatization or purification steps.
Methanol and Acetonitrile (85:15% v/v)was chosen as the mobile phase. The solvent system used in this method was economical.
The %RSD values were within 2 and the method was found to be precise.
The results expressed inTablesfor RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods.
This method can be used for the routine determination of Efonidipine Hydrochloride Ethanolate and Metoprolol Succinatein bulk drug and in Pharmaceutical dosage forms.

REFERENCE

1. https://go.drugbank.com/drugs/DB09235
2. https://pubchem.ncbi.nlm.nih.gov/compound/Efonidipine-hydrochloride-ethanolate
3. https://pubchem.ncbi.nlm.nih.gov/compound/Efonidipine
4. https://en.wikipedia.org/wiki/Efonidipine
5. https://go.drugbank.com/salts/DBSALT000863
6. https://go.drugbank.com/drugs/DB00264
7. https://pubchem.ncbi.nlm.nih.gov/compound/Metoprolol-succinate

8. https://pubchem.ncbi.nlm.nih.gov/compound/Metoprolol
9. https://en.wikipedia.org/wiki/Metoprolol
10. "Practical Pharmaceutical Chemistry", 4th edition, Part 2, by Beckett and Stenlake, CBS Publishers and Distributors, P.No.157-174.
11. Govt. of India, Ministry of Health and Family Welfare. Vol. 2. Delhi: Publication by Controller of Publication; 2007. Indian Pharmacopoeia; pp. 484–554.
12. British Pharmacopoeia.(International Ed.) 1993; Vol. 1:429, 483. Published on the Recommendation of the Medicines Commissions Pursuant to Medicines Act 1968, 1993.
13. United States Pharmacopoeia 29 NF 24, Published on the Recommendation of the Medicines Commissions Pursuant to Medicines, page no. 587.
14. "Principles of Instrumental Analysis", 5th edition, Harcourt Publishes Int Company, Skoog, Holler and Nieman, Chapter 28, p.726-766.
15. "HPLC Columns" Theory, Technology and Practice. Uwe D. Neue, Wiley-VC
16. Handbook of HPLC, Vol.78, by Elena Katz et al. Marcel Dekker Inc.
17. "Instrumental Methods of Chemical Analysis", 5th Edition, Galen W. Ewing, McGraw Hill Book Company 1988.
18. "HPLC in Pharmaceutical Industry", Fong and Long, Marcel Dekker Series
19. "Instrumental Method of Chemical Analysis" by Chatwal Anand, Himalaya Publishing House, p.no.615-623.
20. Dr. Kealey and P.J Haines, Analytical Chemistry, 1st edition, Bios Publisher, (2002), P1-7.
21. Skoog, West, Holler, Crouch, "Fundamentals of analytical chemistry", eighth edition, 2009 (Indian edition), Cengage learning India Pvt ltd, New Delhi, Page no. 271-280.
22. A.V Kasture, K.R Mahadik, S.G Wadodkar, H.N. More, "A textbook of pharmaceutical analysis, Instrumental methods", Nirali Prakashan, vol.2, 9th edition, page no. 5-7, 28-30.
23. Settle FA, In: Handbook of Instrumental Techniques for Analytical Chemistry. 1st Ed, Singapore, Pearson Education Inc.2004.
24. Willard HH and Dean AJ. Instrumental Methods of Analysis. CBS Publishers and distributors, 7th Ed, 1986, 513-515.
25. Connors AK. In: A Text Book of Pharmaceutical Analysis. A Wiley Interscience Publication, 3rdEd, 2005, 373-400.
26. Ahuja S. In: High Pressure Liquid Chromatography of Comprehensive Analytical Chemistry. Elsevier Publications. 2006.
27. Principles and Methods. In: Amesham Biosciences of Reversed Phase Chromatography. 6-8.
28. Synder LR, Kirkland JJ and Glajch JL. In: Practical HPLC Method Development, 2nd Ed, John Wiley and Sons Inc. Canada. 1997.
29. Mohammad T et al., HPLC Method Development and Validation for Pharmaceutical Analysis- A Review. International Pharmaceutica Sciencia. 2012, 2(3), 14.
30. Snyder LR, Kirkland JJ and Glajch JL. In: Practical HPLC Method Development. 2nd ed, 2001.
31. Vibha G et al., Development and validation of HPLC method - a review. International Research Journal of Pharmaceutical and Applied Sciences. 2012, 2(4), 22-23.
32. Bliesner DM. In: Validating Chromatographic Methods. John Wiley & sons Inc. 2006, 88-92.
33. Validation of Analytical Procedures: Methodology. ICH-Guidelines Q2B, Geneva. 1996, 11. (CPMP/ICH/281/95).
34. Development and validation of HPLC method - A Review, Vibha Gupta et al, International Research Journal of Pharmaceutical and Applied Sciences, 2012; 2(4):17-25.
35. A Review: HPLC Method Development and Validation, Santosh Kumar Bhardwaj *et al. International Journal of Analytical and Bioanalytical Chemistry, accepted 20 November 2015.
36. Method Development: A Guide to Basics Quantitative & Qualitative HPLC, LC, GC chromacademy.
37. Lalit V Sonawane* Bioanalytical Method Validation and Its Pharmaceutical Application- A Review Pharmaceutica Analytical Acta 2014, 5:3Center for Drug Evaluation and Research (CDER) Reviewer Guidance.
38. ICH Topic Q 2 (R1) Validation of Analytical Procedures: Text and Methodology.
39. S Divya Teja Banda1, Dr. Mohd Javed Naim2, RP-HPLC Method Development and Validation for the Simultaneous Estimation of Chlorthalidone and Metoprolol Succinate in Bulk and Formulation, Nat. Volatiles & Essent. Oils, 2021; 8(6): 5303-5311.
40. Grishma H. Patel*, Shreya D. Adeshra, Dhananjay B. Meshram, Rp-hplc method development and validation for simultaneous estimation of Efonidipine hydrochloride Ethanolate and Telmisartan in their synthetic mixture, International Journal of Pharmaceutics and Drug Analysis, Vol: 9, Issue: 3, 2021; 190-195.
41. Ms. Priyal Shah1 , Dr. Anuradha Gajjar2, Development and Validation of Stability Indicating RPHPLC Method for Efonidipine Hydrochloride Ethanolate, International Journal of Innovative Research in Technology, © November 2021| IJIRT | Volume 8 Issue 6 | ISSN: 2349-6002, Pages: 340-345.
42. Dipika M Solanki, Dhara V Patel*, Dhananjay B Meshram, Development and validation of UV Spectrophotometric method for simultaneous estimation of Efonidipine hydrochloride Ethanolate and Chlorthalidone in their Synthetic Mixture, Drug Analytical Research, v. 6, n. 1, p. 27-34, Jan./June 2022, Pages: 27-34.