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# Rapid Separation of Five Anti-Hypertensive Agents - Atenolol, Metoprolol, Hydrochlorothiazide, Amlodipine and Nebivolol: Application to Estimation of Metoprolol Succinate in Tablet Dosage Form

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**ABSTRACT**

*One of the key goals of High Performance Liquid Chromatography technique is to achieve a consistent and reproducible separation. A simple, precise, selective and sensitive HPLC method with UV detection was developed for separation of five anti-hypertensive agents, atenolol hydrochloride, metoprolol succinate, hydrochlorothiazide, amlodipine besylate and nebivolol hydrochloride and validated for determination of metoprolol succinate. RP-HPLC method was developed by using Welchrom $C_{18}$Column (4.6 mm i.d. X 250mm, 5µm), Shimadzu LC-20AT ProminenceLiquid Chromatograph. The mobile phase composed of10 mM Phosphate buffer (pH3.0,adjusted with triethylamine): acetonitrile(50:50, v/v).The flow rate was set to 1.0 ml/min with the responses measured at 235nm using Shimadzu SPD-20A Prominence UV-Visible detector. The retention times of atenolol hydrochloride, Metoprolol succinate, hydrochlorothiazide, amlodipine besylate and nebivolol hydrochloride were found to be 2.303 min, 2.827 min, 3.500 min, 4.253 minand 4.957 min respectively. The separation was achieved within 6 min. The statistical validation of the developed method was carried out according to ICH guidelines. Metoprolol succinate was found to give linear response in the concentration range of 2-10µg/ml. Recovery studies were performed to ascertain the accuracy by standard addition method and average recovery was found to be 99.8-100.75%. The LOD and LOQ were found to be 0.1840 µg/ml and 0.5578 µg/ml respectively. The developed method can be used for routine quality control analysis of metoprolol succinatein pharmaceutical tablet dosage form. It can also be extended for the determination of other most commonly prescribed anti-hypertensive agents namely atenolol hydrochloride, hydrochlorothiazide, amlodipine besylate and nebivolol hydrochloride. This method provides a fast, simple method with excellent peak symmetry and high resolution.*

**Key words:** Metoprolol, atenolol, hydrochlorothiazide, amlodipine, nebivolol.

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## INTRODUCTION

Atenolol(ATEN), is a relatively cardio-selective β-adrenergic blocking agent used primarily in the treatment of angina pectoris and hypertension, heart failure and heart attacks. ATEN is chemically 2-[4-[2-hydroxy-3-(1-methylethylamino)propoxy]phenyl]ethanamide (fig. 1(a)).

Metoprolol succinate(METO), 1-(Isopropylamino)-3-[4-(2-methoxyethyl)phenoxy]-2-propanol succinate (fig. 1(b)) is a selective $\beta_1$-receptor blocker used in treatment of several diseases of the cardiovascular system, especially hypertension.

Hydrochlorothiazide(HCTZ),6-chloro-1,1-dioxo-3,4-dihydro-2H-1,2,4-benzothiazine-7-sulfonamide (fig. 1(c)) is a diuretic drug of the thiazide class that acts by inhibiting the kidneys ability to retain water. This reduces the volume of the blood, decreasing blood return to the heart and thus cardiac output and by other mechanisms, is believed to lower peripheral vascular resistance. Hydrochlorothiazide is often used for the treatment of congestive heart failure, symptomatic edema, hypertension, diabetes insipidus, renal tubular acidosis, and for prevention of kidney stones.

Amlodipine(AMLO), is a long-acting calcium channel blocker ofdihydropyridine(DHP) class used in the management of hypertension, coronary artery disease and in the treatment of angina pectoris. Amlodipine acts by relaxing the smooth muscle in the arterial wall, decreasing total peripheral resistance thereby reducing blood pressure. AMLO is chemically 3-ethyl-5-methyl-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-3,5-pyridinedicarboxylate monobenzenesulphonate (fig. 1(d)).

Nebivolol(NEBI), 1-(6-fluorochroman-2-yl)-{[2-(6-fluorochroman-2-yl)-2-hyroxy-ethyl]amino}ethanol (fig. 1(e)), is a$\beta_1$-receptor blocker. It has a nitric oxide potentiating, vasodilatory effect and is used in treatment of hypertension. Nebivolol lowers blood pressure by reducing peripheral vascular resistance and increases stroke volume with preservation of cardiac output.

Literature survey reveals that for the determination of above said five anti-hypertensive agents in biological fluids like plasma, blood, urine and pharmaceutical dosage forms by spectrophotometry[1-4], spectrofluorimetry[5],TLC[6-7], HPTLC[8], High Performance Liquid Chromatography (RP-HPLC) with UV detection[9-21], fluorimetric detection[22], ion-pair HPLC[23], RP-UPLC[24] and Liquid Chromatography-Mass Spectrometry[25-26].In fact there is a need for the development of a novel, simple, rapid, efficient RP-HPLC analytical method with reproducibility for determination of metoprolol in pharmaceutical dosage forms.

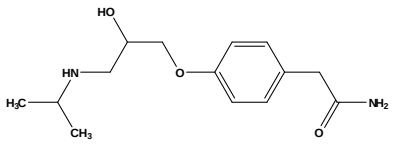

Fig.1(a). Atenolol

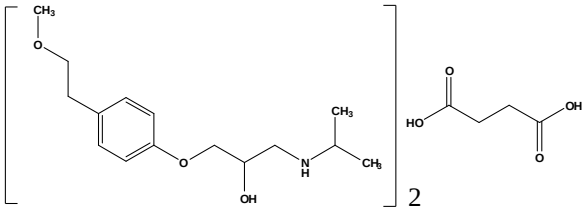

Fig.1(b). Metaprolol succinate

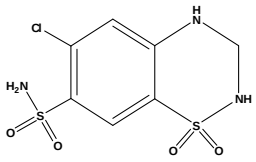

Fig.1(c). Hydrochlorothiazide

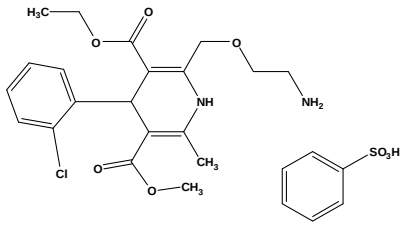

Fig.1(d). Amlodipine besylate

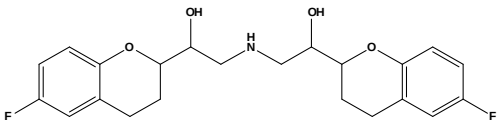

Fig.1(e). Nebivolol

Fig.1. Structures of Anti-hypertensive agents investigated in the present study

When developing a new method one of the most prominent goals is to achieve a consistent reproducible separation. The selection of a highly reproducible HPLC method is essential if this goal is to be attained. Literature survey revealed thatthere was noRP-HPLC method reported till date for separation and subsequent estimation of the METOin pharmaceutical dosage forms.Thus the present study illustrates development of a novel, simple, rapid and efficient RP-HPLC analytical method with successful separation of five most commonly used anti-hypertensive agents with short retention time.This method also provides rapid separation with good resolution, excellent peak shape, use of smaller sample volumes and buffer volumes, providing cost savings. The established method for determination of METO was validated with respect to specificity, linearity, precision, accuracy, robustness, LOD and LOQ according to ICH guidelines[27].

## MATERIALS AND METHODS

**Chemicals and Reagents:**
The reference standards of ATEN, METO, HCTZ, AMLO, NEBI were kindly supplied as gift sample by Hetero Drugs Ltd., Hyderabad, Andhra Pradesh, India. All the chemicals were analytical grade. Potassium dihydrogen orthophosphate from Rankem Ltd., Mumbai, India, while acetonitrile (HPLC grade) and triethylamine (HPLC grade) from Merck Pharmaceuticals Private Ltd., Mumbai, India. O-Phosphoric acid used was of HPLC grade and purchased from Merck Specialties Private Ltd., Mumbai, India. Commercial tablets of METO formulation was procured from local market. METOLARtablets containing metoprolol succinate (50mg) are manufactured by Cipla Ltd., Mumbai, India.

**Instruments:**
Quantitative HPLC was performed on a isocratic high performance liquid chromatograph (Shimadzu LC-20AT Prominence Liquid Chromatograph) with a LC-20AT VP pump, manual injector with loop volume of 20 µl (Rheodyne), programmable variable wavelength Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom $C_{18}$ Column (4.6 mm i.d. X 250mm, 5µm particle size). The HPLC system was equipped with "Spinchrom" software. In addition an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), a sonicator (spectra lab, model UCB 40), UV-Visible Spectrophotometer (Systronics model 2203) were used in this study.

**Chromatographic conditions:**
METO was analyzed by various reversed phase columns like $C_8$and $C_{18}$ columns. Among $C_8$ and $C_{18}$ columns, $C_{18}$ (4.6 mm i.d. X 250 mm,5µm particle size) column was selected. Various combinations of acetonitrile, phosphate buffer and methanol with triethylamine as column modifier were tested. The mixture of 10mM Phosphate buffer (pH adjusted to 3.0 using triethylamine) and Acetonitrile in ratio of 50:50, v/v was selected as mobile phase and UV detection wavelength was 235nm with a flow rate of 1ml/min. Injection volume was 20µl, with ambient temperature, run time was 6min. and retention time was 2.827 min.

**Preparation of solutions and Reagents**

**a. Mobile phase:**
A 10mM Phosphate buffer was prepared by dissolving 6.056 g of potassium dihydrogen orthophosphate in 445 ml of HPLC grade water. To this 55ml of 0.1M phosphoric acid was added and pH was adjusted to 3.0 with triethylamine. The above prepared buffer and acetonitrile were mixed in the proportion of 50:50, v/v and was filtered through 0.45 µm nylon membrane filter and degassed by sonication.

**b. Stock and Working Standard Solutions:**
Accurately weigh 100 mg of METO, dissolve in a 100ml volumetric flask with mobile phase. This is stock standard solution of METO with concentration of 1000 µg/ml. Prepare five working standard solutions for calibration by adding defined volumes of the stock standard solution and diluting with mobile phase. The concentrations of METO are 2.0, 4.0, 6.0, 8.0, 10.0 µg/ml, respectively. Similarly 10 µg/ml of each standard Anti-Hypertensive agent were prepared from 1000 µg/ml stock standard solutions of ATEN, HCTZ, AMLO and NEBI respectively into each10ml volumetric flask.

**c. Tablet Sample preparation:**
Accurately weighed and grind 20 tablets of METO (METOLAR) in a mortar and triturated to a fine powder. From this, tablet powder which is equivalent to 50 mg of METO was taken and the drug was extracted into 50 ml of mobile phase in a beaker, stir and place in an ultrasonic bath until dissolution is complete. Transfer this solution into a 100 ml volumetric flask, rinse the beaker with mobile phase a few times, and transfer into the same volumetric flask. Add mobile phase to bring to volume. The resulting solution was filtered using 0.2 µm filter and degassed by sonication. This solution was further suitably diluted for chromatography.

**Selection of detection wavelength:**
The overlain UV spectra of various diluted solutions of ATEN, METO, HCTZ, AMLO and NEBI in mobile phase were recorded using UV spectrophotometer. The isobestic point of maximum absorbance was observed at 235nm. This wavelength was used for detection of METO and other anti-hypertensive agents.

**Calibration curve for Metoprolol succinate:**
Replicates of each calibration standard solutions (2,4,6,8,10 µg/ml) were injected using a 20µl fixed loop system and the chromatograms were recorded. Calibration curves were constructed by plotting concentration of METO on X-axis and peak areas of standard METO on Y-axis and regression equations were computed for METO.

**VALIDATION OF THE PROPOSED METHOD**
The developed method of analysis was validated as per the ICH for the parameters like system suitability, specificity, linearity, precision, accuracy, robustness and system suitability, limit of detection (LOD) and limit of quantitation (LOQ).

**System suitability:**
System suitability tests are an integral part of chromatographic method which was used to verify reproducibility of the chromatographic system. To ascertain its effectiveness, certain system suitability test parameters were checked by repetitively injecting the drug solution at the concentration level 10µg/ml for METO to check the reproducibility of the system. At first the HPLC system was stabilized for 40 min. One blank followed by six replicates of a single calibration standard solution of METO was injected to check the system suitability. To ascertain the system suitability for the proposed method, the parameters such as theoretical plates, peak asymmetry, retention time and parameters were taken.

**Specificity:**
The effect of wide range of excipients and other additives usually present in the formulations of METO in the determinations under optimum conditions was investigated. The specificity of the RP-HPLC method was established by injecting the mobile phase and placebo solution in triplicate and recording the chromatograms. The common excipients such as lactose anhydrous, microcrystalline cellulose, purified talc and magnesium stearate have been added to the placebo solution and injected and tested. The chromatogram for placebo indicating the specificity of developed method is presented in fig. 2.

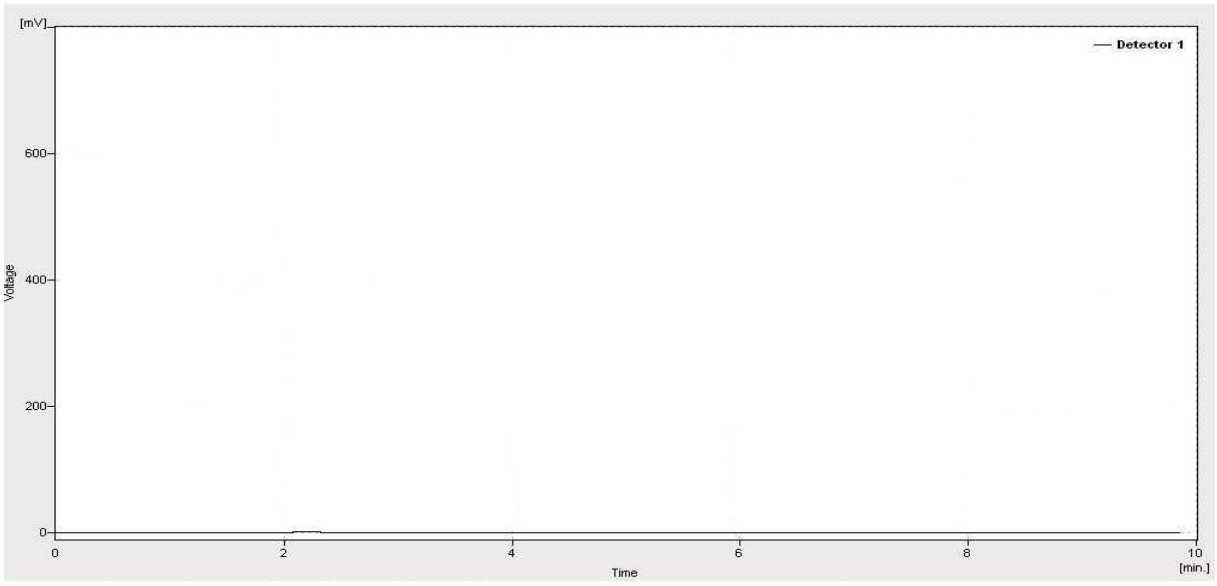


**Fig 2: Chromatogram of placebo**

**Linearity:**
The linearity graphs for the proposed assay methods were obtained over the concentration range of 2-10 µg/ml of METO. Method of least square analysis was carried out for getting the slope, intercept and correlation coefficient, regression data values. A calibration curve was plotted between concentration and area response and statistical analysis of the calibration curve was performed.

**Precision:**
Intra-day and inter-day precision study of METO was carried out by estimating corresponding responses 3 times on the same day and on 2 different days for the concentration of 10µg/ml. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0.

**Accuracy (Recovery studies):**
The accuracy of the method was determined by calculating recovery of METO by the standard addition method. Known amount of METO at 80%, 100% and 120% was added to a pre quantified tablet sample. The recovery studies were carried out in the tablet in triplicate each in the presence of placebo.

**Robustness:**
The Robustness was evaluated by the analysis of METO under different experimental conditions such as making small changes in flow rate (± 0.2 ml/min), detection wavelength (±5 nm) and Mobile phase composition (±5%).

**LOD and LOQ:**
Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of Detection and Limit of Quantitation were calculated using following formula LOD= 3.3(SD)/S and LOQ= 10 (SD)/S, where SD=standard deviation of response (peak area) and S= slope of the calibration curve.

## RESULTS AND DISCUSSION

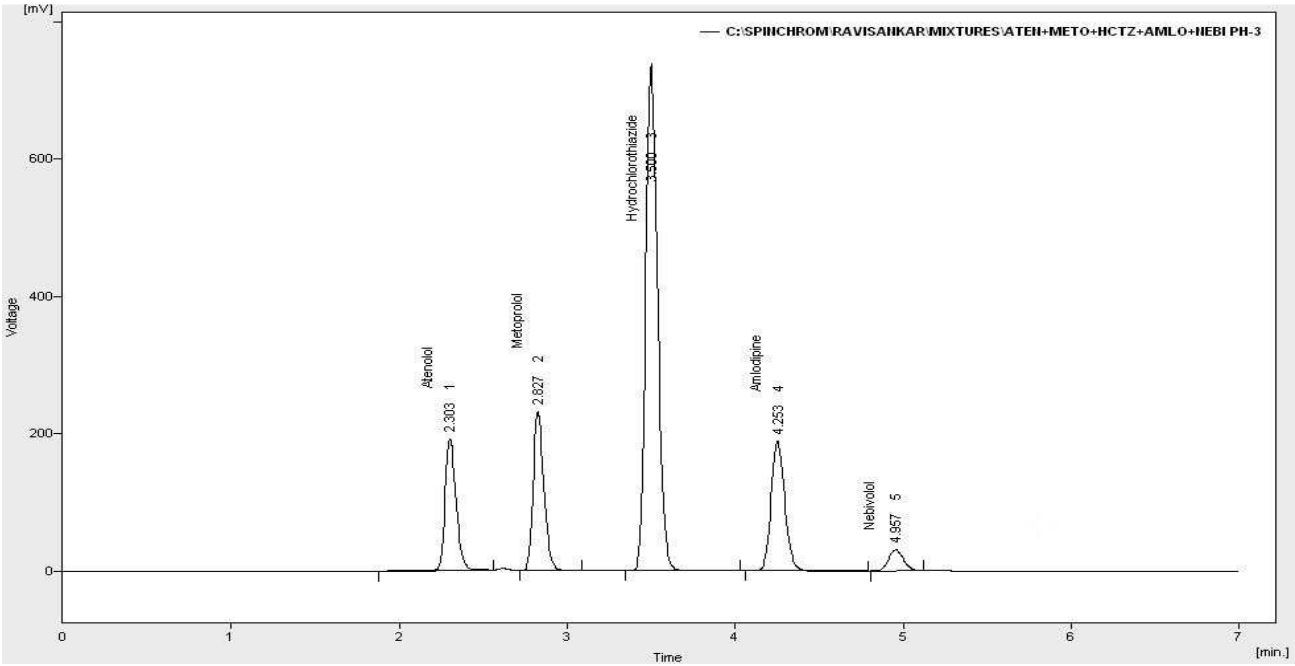


**Fig. 3: A typical chromatogram of Mixture of five standard Anti-Hypertensive agents**

The goal of this work was to create one method to separate five anti-hypertensive agents applicable to the determination of ATEN, METO, HCTZ, AMLO and NEBIand in various combinations. Preliminary experiments were carried out to achieve the best chromatographic conditions for the simultaneous determination of the drug substances. Several column types and lengths were tried considering other chromatographic parameters. $C_{18}$ column with a 4.6 mm inner diameter and 5µm particle size was chosen. Spectroscopic analysis of drugs showed that ATEN, METO, HCTZ, AMLO and NEBI had maximum UV absorbance($\lambda_{max}$) at237 nm, 243 nm, 235 nm, 240 nm and 282 nm respectively. UV overlain spectra of these drugs showed that these drugs absorbed appreciably at 235 nm, so that this wavelength was selected as the detection wave length. Chromatographic conditions were optimized by changing the mobile phase composition & buffers used in the mobile phase.Different experiments were performed to optimize the mobile phase but adequate separation of drugs could not be achieved. By altering the pH of buffer from 4.5 to 3.0, a best separation was achieved. Different proportions of solvents were tested. Eventually the best separation was obtained by the isocratic elution system using a mixture of phosphate buffer (pH 3.0): acetonitrile (50:50, v/v). pH of buffer was adjusted to 3.0 using triethylamine.Flow rate used was 1 ml/min. A

typical chromatogram for simultaneous estimation of the five drugs obtained by using a fore mentioned mobile phase. Under these conditions ATEN, METO, HCTZ, AMLO and NEBI were eluted at 2.303min, 2.827 min, 3.500 min,4.253 minand 4.957 min respectively. Fig 3 shows a separation of all five anti-hypertensive compounds in 6 minutesand results are summarized in Table1.

**TABLE 1: CHROMATOGRAM RESULTS OF PROPOSED COMBINATION OF FIVE ANTI-HYPERTENSIVE AGENTS**

| Name of the Compound | Retention time($t_R$), min | Peak No. | Asymmetry[#] | Efficiency[#] (theoretical plates) | Resolution[#] |
|---|---|---|---|---|---|
| ATEN | 2.303 | 1 | 1.062 | 5465 | - |
| METO | 2.827 | 2 | 1.084 | 8231 | 4.210 |
| HCTZ | 3.500 | 3 | 1.026 | 10604 | 5.182 |
| AMLO | 4.253 | 4 | 1.092 | 12373 | 5.229 |
| NEBI | 4.957 | 5 | 1.188 | 13611 | 4.368 |

*[#]acceptance criteria for Asymmetry> 2.0, for Efficiency > 3000 and for Resolution > 2.0.*

For the comparative evaluation of retention times and peak areas of anti-hypertensive drugs, chromatograms of these five anti-hypertensive drug standards were recorded individually. The representative individual standard chromatograms of the five anti-hypertensive drug standards are shown in fig numbers 4 to 8 and the results are presented in Table 12.

**TABLE 2: INDIVIDUAL CHROMATOGRAM RESULTS OF STANDARDS OF FIVE ANTI-HYPERTENSIVE AGENTS**

| Name of the Compound | Retention time ($t_R$), min. | Assymmetry | Efficiency (theoretical plates) |
|---|---|---|---|
| Atenolol hydrochloride | 2.310 | 1.064 | 5497 |
| Metoprolol succinate | 2.827 | 1.082 | 8231 |
| Hydrochlorothiazide | 3.473 | 1.028 | 10443 |
| Amlodipine besylate | 4.293 | 1.090 | 12238 |
| Nebivolol hydrochloride | 4.957 | 1.182 | 13739 |

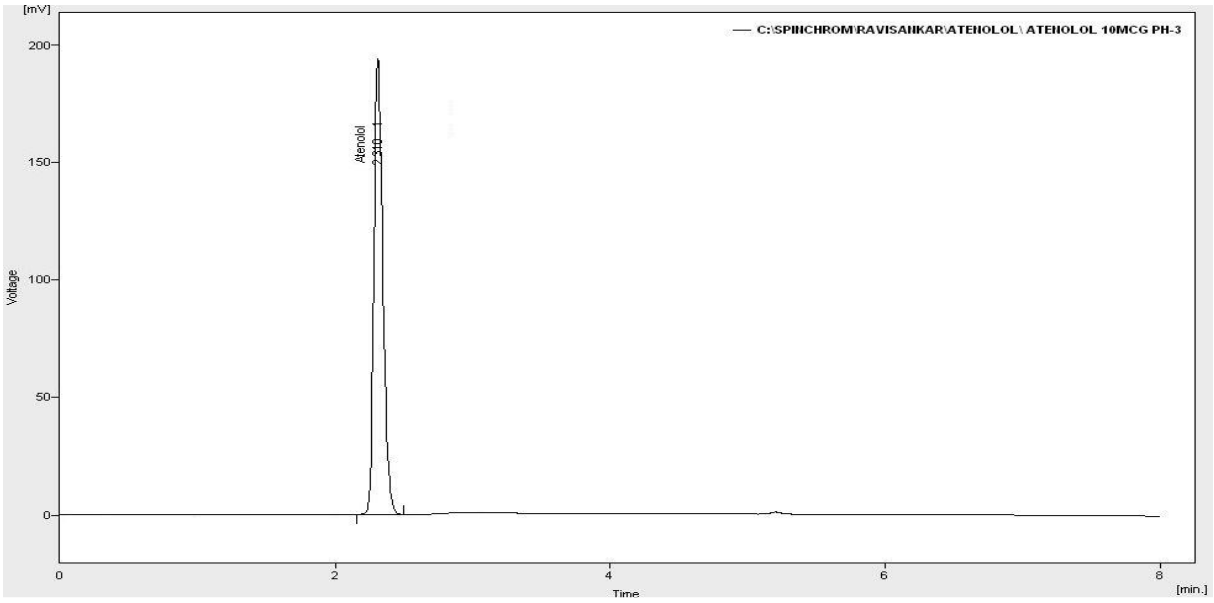


**Fig. 4: Standard chromatogram of Atenolol standard (10 µg/ml)**

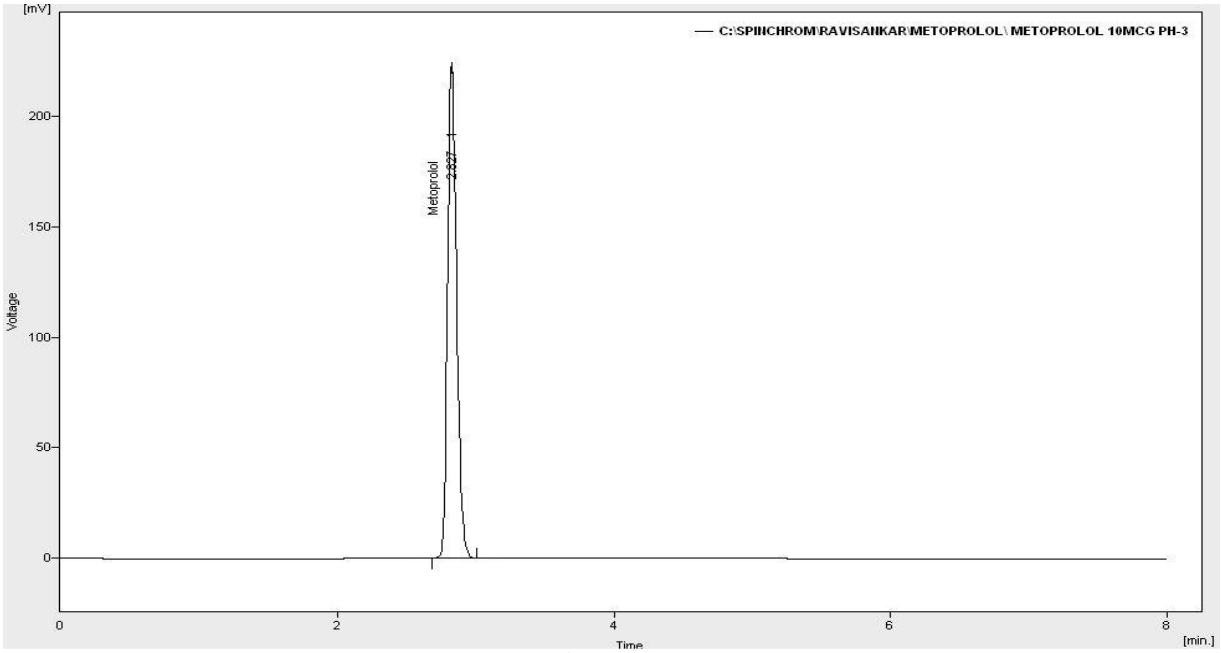


**Fig. 5: A typical chromatogram of Metoprolol succinate standard (10 µg/ml)**

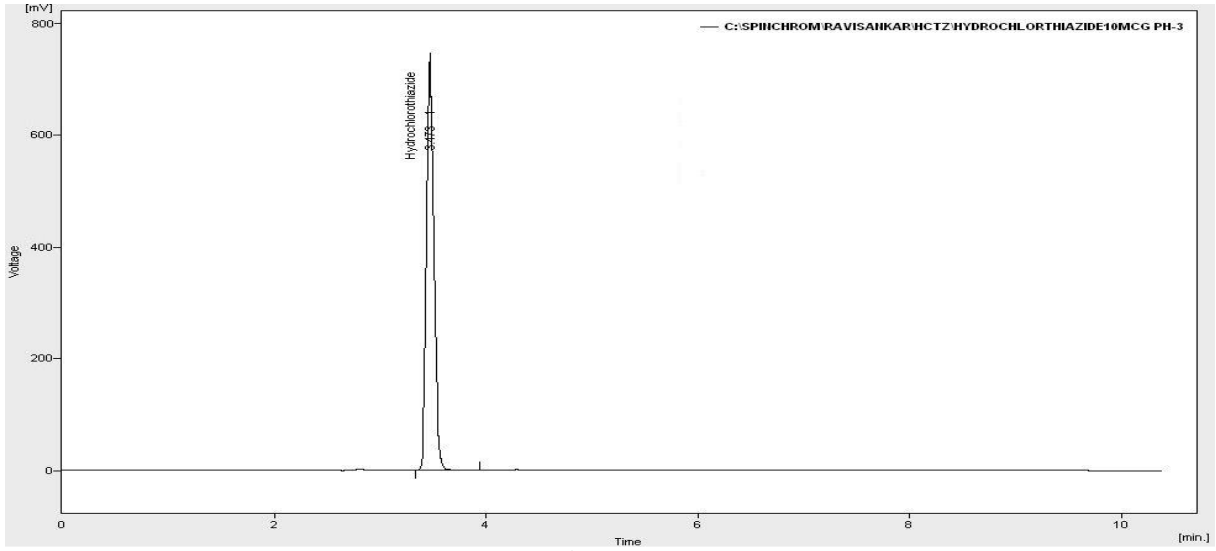


**Fig. 6: Standard chromatogram of Hydrochlorothiazide standard (10 µg/ml)**

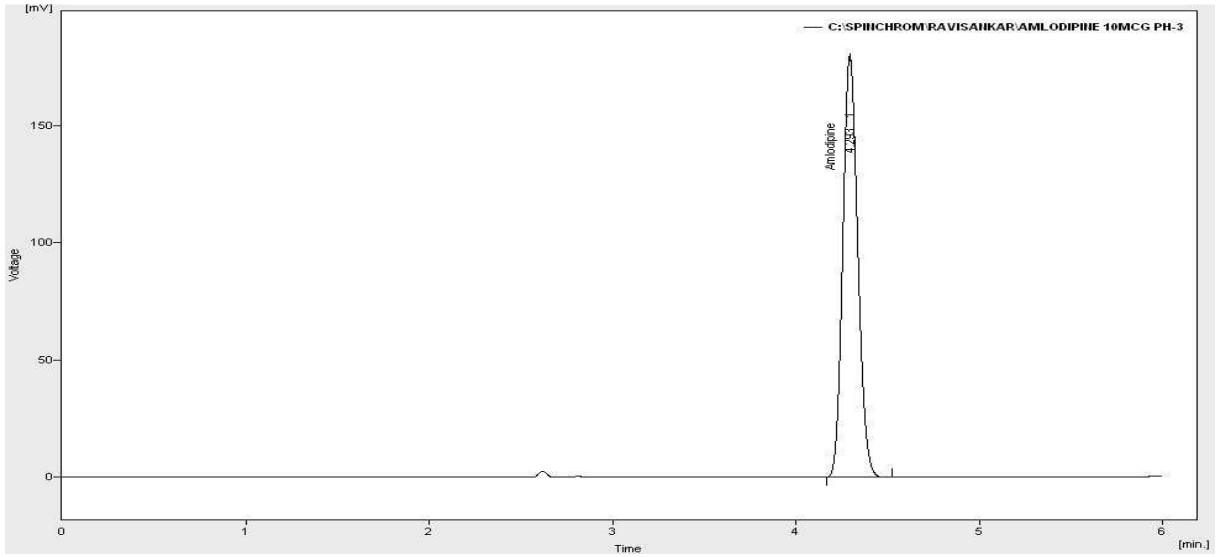


**Fig. 7: Standard chromatogram of Amlodipine standard (10 µg/ml)**

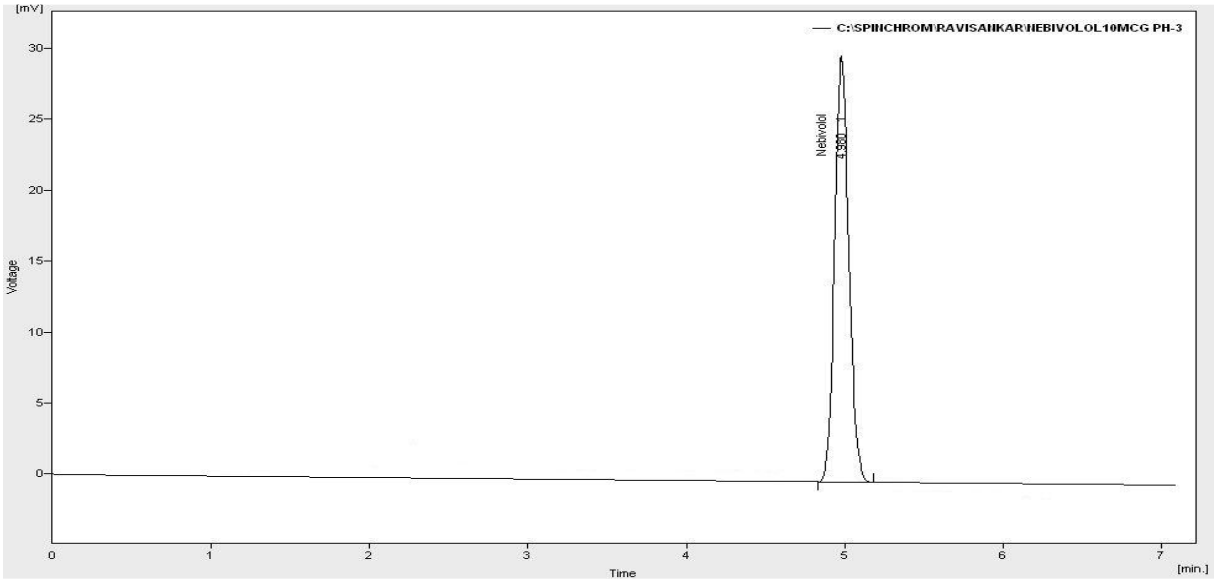


**Fig. 8: Standard chromatogram of Nebivolol standard (10 µg/ml)**

The mobile phase consisting of phosphate buffer (pH 3.0): acetonitrile (50:50, v/v) at1ml/min flow rate was optimized which gave sharp peak, minimum tailing factor with short runtime for METO. The retention time for METO was 2.827 min. UV spectra of METO showed that the drug absorbed maximum at 235 nm, hence this wavelength was selected as the detection wavelength. System suitability parameters and optimized chromatographic conditions were shown in Table no 3.

**TABLE 3: OPTIMIZED CHROMATOGRAPHIC CONDITIONS AND SYSTEM SUITABILITY PARAMETERS OF PROPOSED RP-HPLC METHOD FOR METOPROLOL SUCCINATE**

| Parameter | Chromatographic conditions |
|---|---|
| Instrument | SHIMADZU LC-20AT prominence liquid chromatograph |
| Column | WELCHROM $C_{18}$ Column (4.6 mm i.d. X 250mm, 5µm particle size) |
| Detector | SHIMADZU SPD-20A prominence UV-Vis detector |
| Diluents | 10mM Phosphate Buffer(pH3.0) : Acetonitrile (50:50, v/v) |
| Mobile phase | 10mM Phosphate Buffer (pH 3.0) : Acetonitrile (50:50, v/v) |
| Flow rate | 1ml/min. |
| Detection wave length | UV at 235nm. |
| Run time | 6 minutes |
| Column back pressure | 128-130 kgf |
| Temperature | Ambient temperature(25$^{o}$C) |
| Volume of injection loop | 20µl |
| Retention time ($t_R$) | 2.827 min |
| Theoretical plates[th.pl] (Efficiency) | 8,231 |
| Theoretical plates per meter[t.p/m] | 164,621 |
| Tailing factor (asymmetry factor) | 1.082 |

The calibration data in Table 4 show linear peak area response for METO.The calibration curve for METO was found to be linear over the range of 2-10 µg/ml. The data of regression analysis of the calibration curve is shown in Table 5.

**TABLE 4: CALIBRATION DATA OF THE PROPOSED HPLC METHOD FOR ESTIMATION OF METOPROLOL SUCCINATE**

| S. | Concentration, µg/ml | Retention time, ($t_R$)min | Peak area, mV |
|---|---|---|---|
| 1 | 0 | - | 0 |
| 2 | 2 | 2.813 | 209.904 |
| 3 | 4 | 2.810 | 427.096 |
| 4 | 6 | 2.813 | 636.383 |
| 5 | 8 | 2.813 | 869.278 |
| 6 | 10 | 2.827 | 1062.739 |

**TABLE 5: LINEAR REGRESSION DATA OF THE PROPOSED HPLC METHOD OF METOPROLOL SUCCINATE**

| Parameter | Method |
|---|---|
| Detection wavelength( $\lambda_{max}$) | UV at 235nm |
| Linearity range (µg/ml) | 2-10µg/ml |
| Regression equation (Y = a + bX) | Y= -1.559 + 107.1X |
| Slope(b) | 107.1 |
| Intercept(a) | -1.559 |
| Standard error of slope ($S_b$) | 0.987211 |
| Standard error of intercept ($S_a$) | 5.977864 |
| Standard error of estimation ($S_e$) | 8.259607 |
| Regression coefficient ($R^2$) | 0.9997 |
| % Relative standard deviation* i.e., Coefficient of variation(CV) | 1.127212 |
| Percentage range of errors* (Confidence limits)<br>0.005significance level<br>0.001 significance level | <br>1.183296<br>1.846085 |

**Average of six determinations*

The developed method was applied to the assay of METO tablets and results are shown in Table 6. The amount was between 100.586 and 101.682%.

**TABLE 6: ASSAY RESULTS OF METOPROLOL SUCCINATE FORMULATION**

| S. No | Formulations | Labelled amount | Amount found | % Assay ±SD* |
|---|---|---|---|---|
| 1 | METOLAR (CIPLA Ltd., Mumbai) | 50mg | 50.56 mg | 101.134 ± 0.548% |

***Average of 6 determinations; SD is standard deviation.*

The USP monograph for this product specifies that there should be not less than(NLT) 98.0% and not more than(NMT) 102.0% of the Active Pharmaceutical Ingredient (API) in the drug product on dried basis. The assay results demonstrated that formulation met the USP criteria. The representative standard and sample chromatograms of METO are shown in Fig. 5 and fig. 9 respectively. The regression equation was found to be Y = -1.5598 + 107.1X with correlation coefficient was $R^2$ =0.9997 which indicates this method had good linearity. The representative chromatograms of the calibration standards of METO are shown in Fig. 10 to 14. The calibration plot is shown in Fig.15.

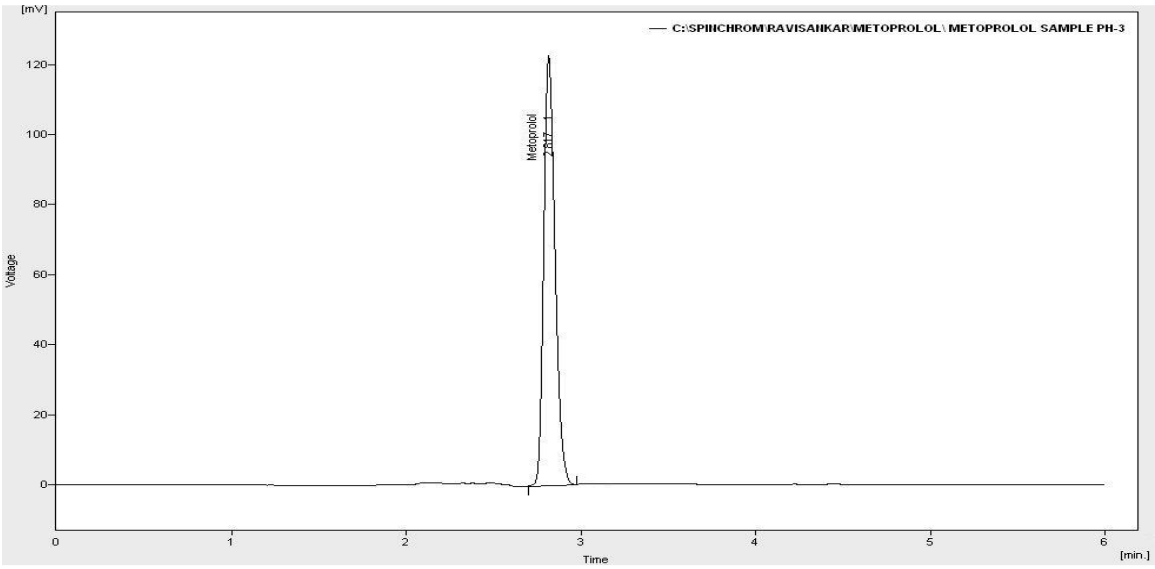


**Fig. 9: Chromatogram of market formulation (METOLAR 50 mg tablets) of Metoprolol succinate**

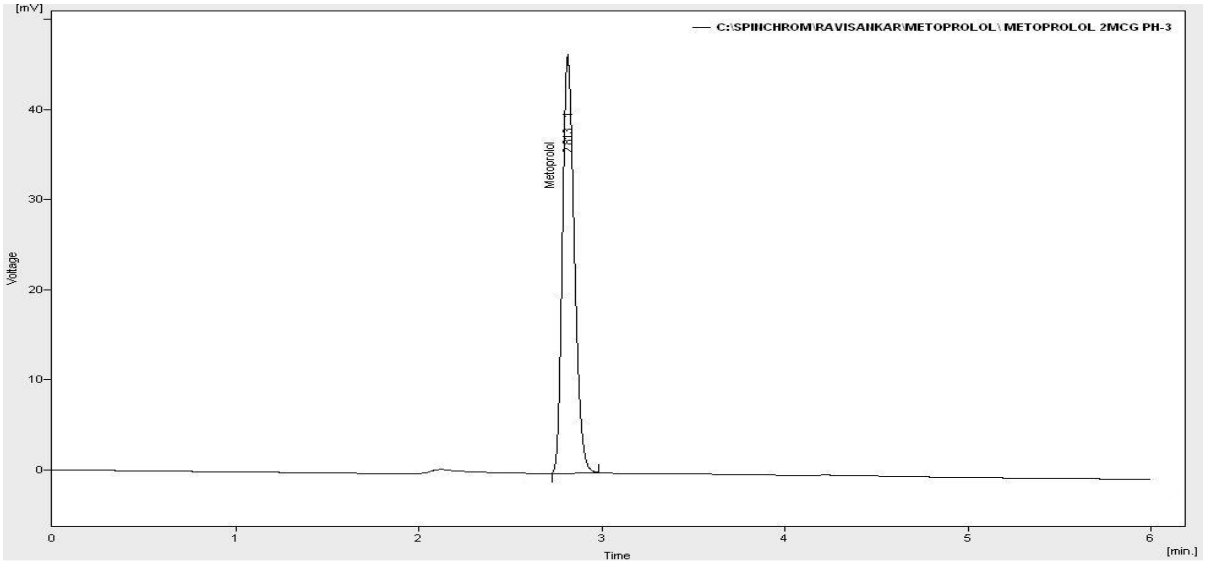


**Fig. 10: Standard chromatogram of Metoprolol succinate (2 µg/ml)**

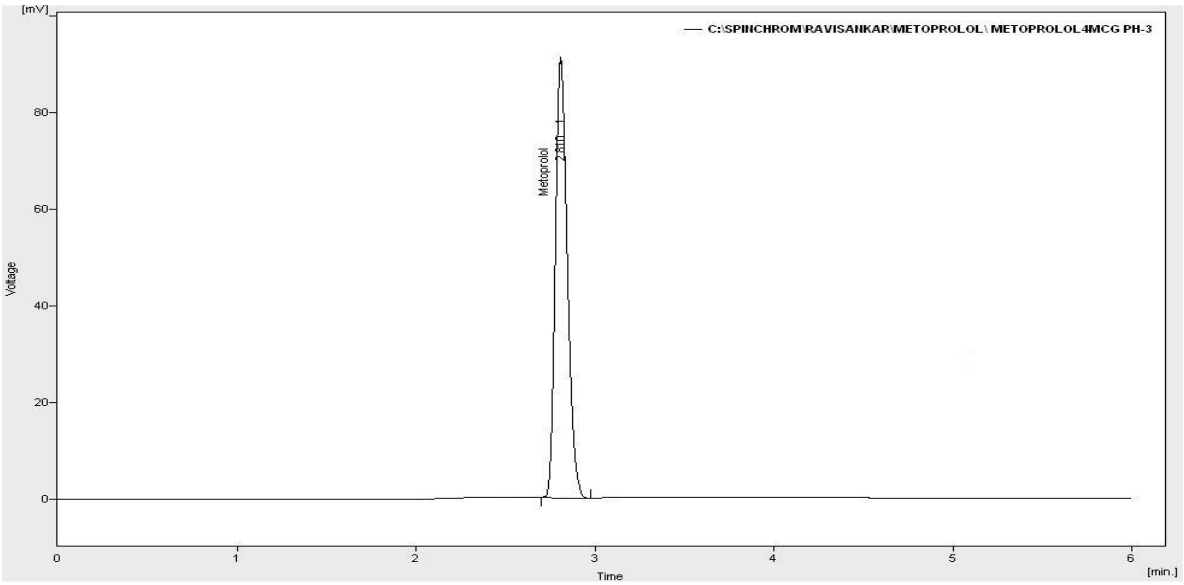


**Fig. 11: Standard chromatogram of Metoprolol succinate(4µg/ml)**

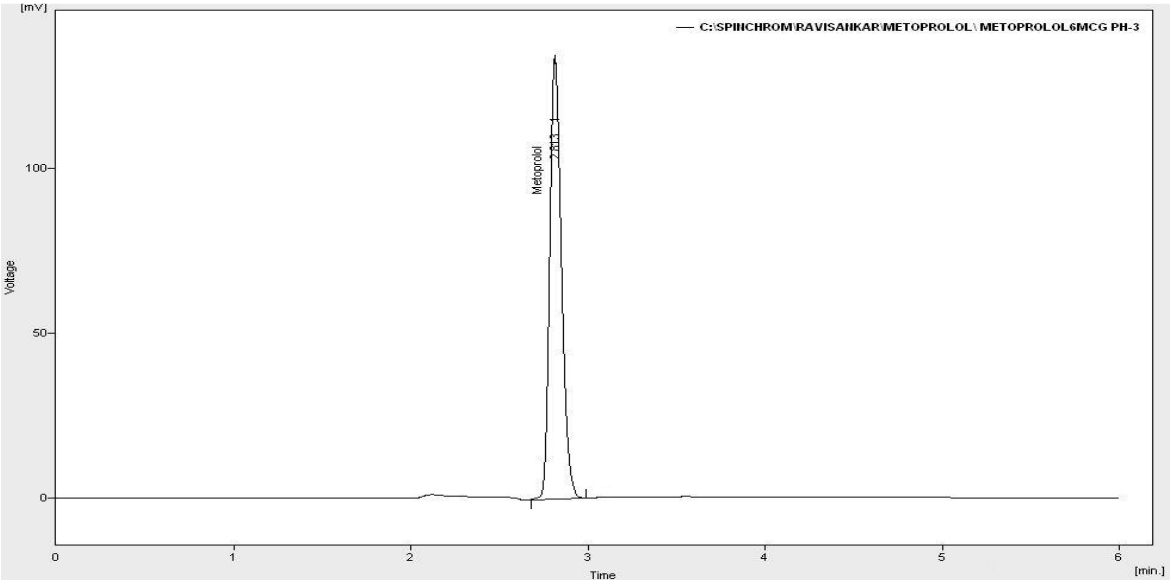


**Fig. 12: Standard chromatogram of Metoprolol succinate(6 µg/ml)**

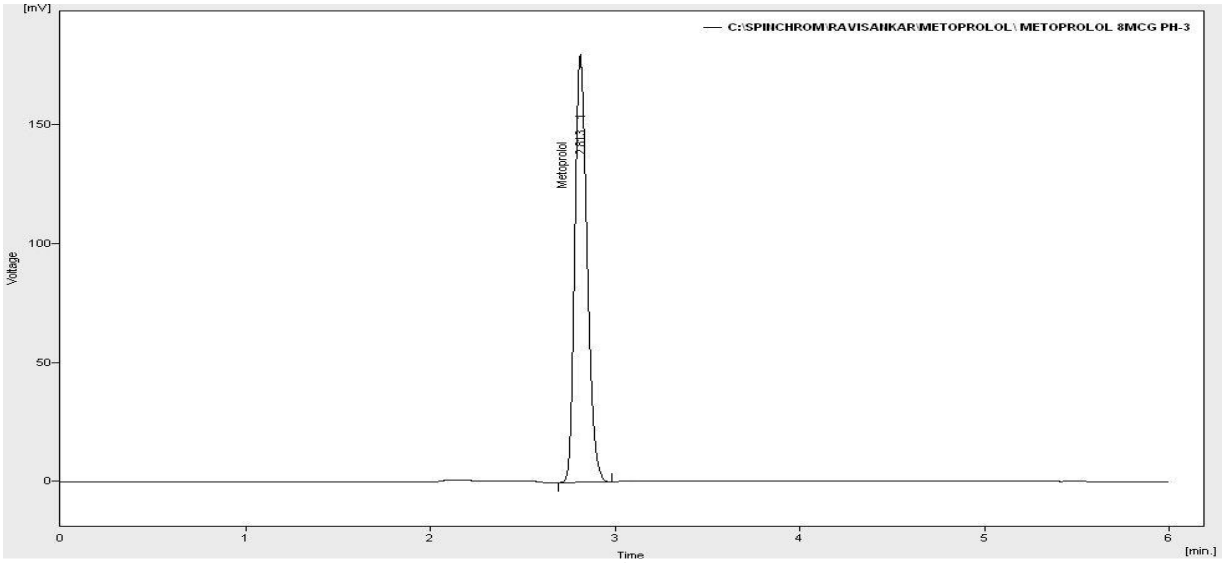


**Fig. 13: Standard chromatogram of Metoprolol succinate (8 µg/ml)**

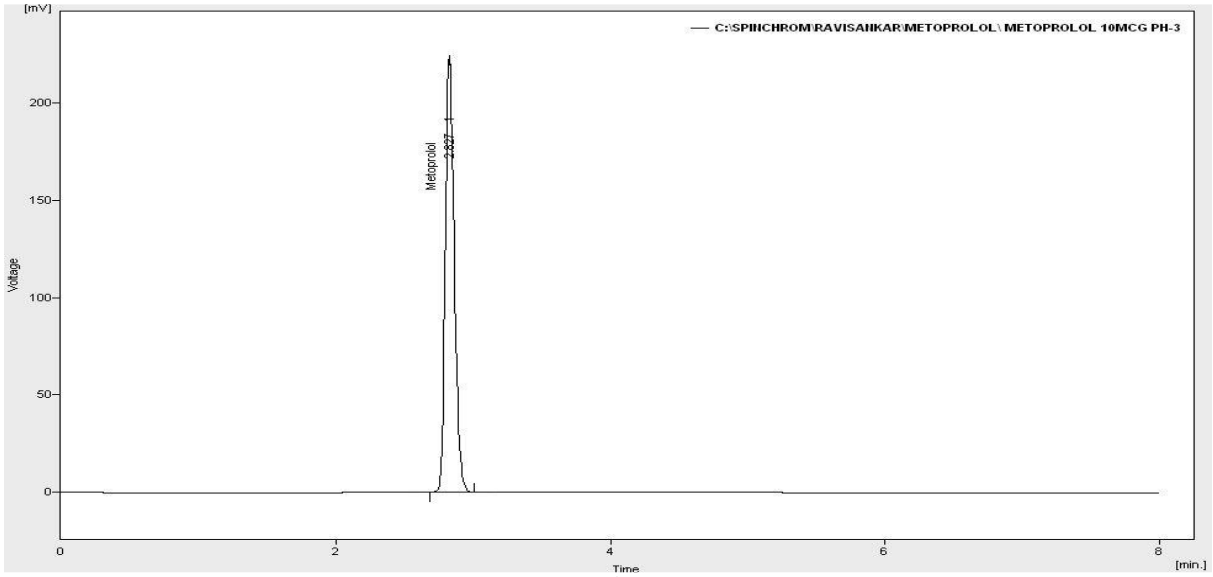


**Fig. 14: Standard chromatogram of Metoprolol succinate (10 µg/ml)**

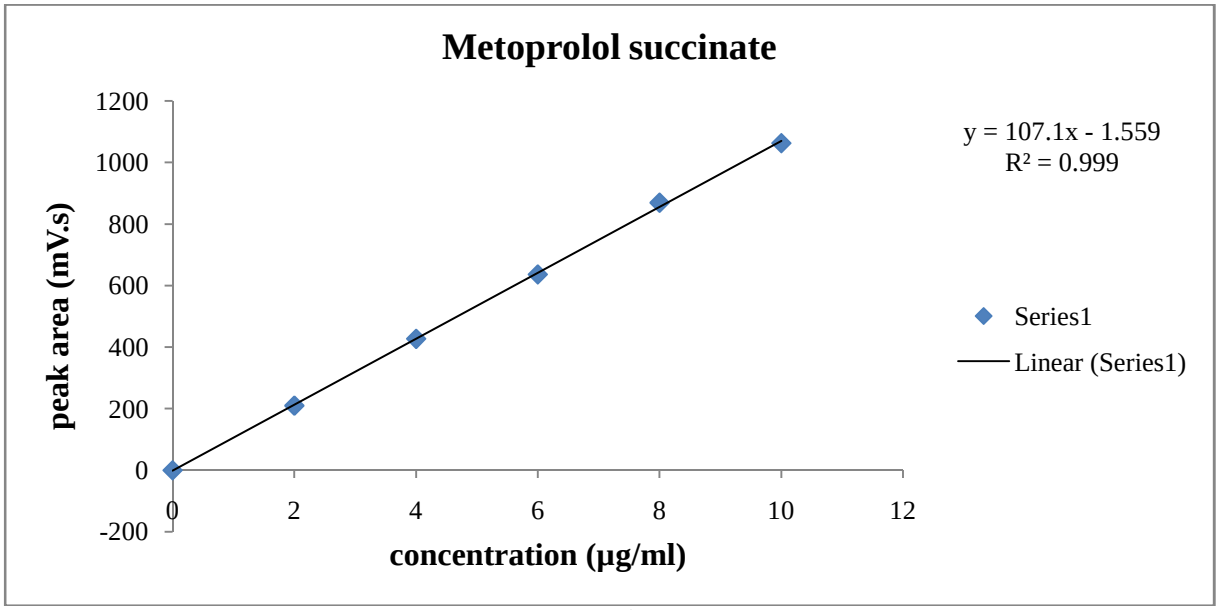


**Fig. 15: Calibration plot of Metoprolol succinate**

The specificity was studied for the examination of the presence of interfering components, while the comparison of chromatograms there was no interference from placebo with sample peak. They do not disturb the elution or quantification of METO; furthermore the well-shaped peaks were also indicative of the specificity of the method. Therefore, it was concluded that the method was specific. The specificity results are summarized in Table 7.

**TABLE 7: SPECIFICITY STUDY**

| Name of the solution | Retention time, ($t_R$)min. |
|---|---|
| Mobile phase | No peaks |
| Placebo | No peaks |
| Metoprolol succinate, 10 µg/ml | 2.827 min. |

Precision was studied to find out intra and inter day variations in the test methods of METO for the three times on the same day and different day. The intra-day and inter-day precision obtained was % RSD (< 2) indicates that the proposed method was quite precise and reproducible and results are shown in the Table 8 and Table 9 respectively.

**TABLE 8: RESULTS OF PRECISION STUDY (INTRA-DAY)**

| Sample | Concentration (µg/ml) | Injection no. | Peak area (mV.s) | %RSD |
|---|---|---|---|---|
| Metoprolol succinate | 10 | 1 | 536.743 | 0.563803 |
| | | 2 | 532.975 | |
| | | 3 | 533.405 | |
| | | 4 | 538.885 | |
| | | 5 | 530.643 | |
| | | 6 | 532.643 | |

*% RSD is percentage of relative standard deviation (acceptance criteria < 2.0).*

**TABLE 9: RESULTS OF PRECISION STUDY (INTER-DAY)**

| Sample | Concentration (µg/ml) | Injection no. | Peak area (mV.s) | %RSD |
|---|---|---|---|---|
| Metoprolol succinate | 10 | 1 | 540.332 | 0.672324 |
| | | 2 | 538.408 | |
| | | 3 | 542.086 | |
| | | 4 | 546.819 | |
| | | 5 | 536.273 | |
| | | 6 | 539.197 | |

*% RSD is percentage of relative standard deviation (acceptance criteria < 2.0).*

Recovery studies of the drug was carried out for the accuracy parameter at three different concentrations levels i.e., multiple level recovery studies. A known amount of METO standard was added into pre-analyzed sample and subjected them to the proposed HPLC method. The percentage recovery was found to be within the limits listed in Table 10. Generally the mean percentage recovery of METO at each level was not less than 99% and not more than 101%. In this case percentage recovery of METO was found to be in the range of 99.11 to 99.8%.

**TABLE 10: RECOVERY DATA OF THE PROPOSED METOPROLOL SUCCINATE BY RP-HPLC METHOD**

| Recovery level | Amount added (mg) | Total amount (mg) | Amount found (mg) | Mean % Recovery ± SD* | % RSD # |
|---|---|---|---|---|---|
| 80% | 40 | 90 | 89.6 | 99.75±0.9013 | 0.9036 |
| | | | 89.8 | | |
| | | | 90.3 | | |
| 100% | 50 | 100 | 100.2 | 99.80±1.7776 | 1.7812 |
| | | | 98.9 | | |
| | | | 100.6 | | |
| 120% | 60 | 110 | 109.2 | 99.11±1.5485 | 1.5624 |
| | | | 108.7 | | |
| | | | 110.5 | | |

*SD is standard deviation; % RSD is percentage of relative standard deviation (acceptance criteria < 2.0).*

Robustness was done by small changes in the chromatographic conditions like mobile phase flow rate, $\lambda_{max}$, mobile phase composition. It was observed that there were no marked changes in the chromatograms. The parameters lie within the limits indicates that the method had robustness and was suitable for routine use. The Robustness results are presented in Table 11.

**TABLE 11: ROBUSTNESS RESULTS OF METOPROLOL SUCCINATE**

| S. no | Parameter[a] | Optimized | Used | Peak area | Retention time ($t_R$), min | Plate count | Peak asymmetry |
|---|---|---|---|---|---|---|---|
| 1. | Flow rate (±0.2 ml/min) | 1.0 ml/min | 0.8 ml/min | 556.634 | 2.980 | 8482 | 1.130 |
| | | | 1.0 ml/min | 540.332 | 2.827 | 8231 | 1.082 |
| | | | 1.2 ml/min | 522.754 | 2.638 | 8078 | 1.084 |
| 2. | Detection wavelength (±5nm) | 235 nm | 230nm | 518.584 | 2.824 | 8210 | 1.120 |
| | | | 235nm | 540.332 | 2.827 | 8231 | 1.082 |
| | | | 240nm | 526.540 | 2.821 | 8254 | 1.116 |
| 3. | Mobile phase composition (±5%) | 50:50, v/v | 55:45, v/v | 518.478 | 2.972 | 8486 | 1.112 |
| | | | 50:50, v/v | 540.332 | 2.827 | 8231 | 1.082 |
| | | | 45:55, v/v | 524.626 | 2.678 | 8142 | 1.107 |

[a] *three parameters were slightly changed at three levels (-1,0,+1).*

The limit of detection (LOD) and limit of quantitation (LOQ) was calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approximating the LOD and LOQ. The limit of detection (LOD) was 0.184088µg/mland the limit of quantitation (LOQ) was 0.557844 µg/ml shows that this method was very sensitive. The results are presented in Table 12.

**TABLE 12: LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ)**

| | |
|---|---|
| Limit of Detection(LOD) | 0.184088µg/ml |
| Limit of Quantitation(LOQ) | 0.557844 µg/ml |

## CONCLUSION

The developedmethod successfully separated all five anti-hypertensive drugs with a relatively short retention time, provides decorous resolution, excellent peak shape, gave consistent and highly reproducible results. The method overall proved to be economical, simple, rapid, precise, very sensitive, cost- effective, time saving, robust and accurate. It can be reliably used for determination of the said five anti-hypertensive drugs in short period and even in small concentrations. By using this method one can elute all the five drugs within six minutes. This method was completely validated shows phenomenal results and also free from interference of the other additives used in the formulations. The ease in constitution of mobile phase and economy of the components of mobile phase make this method the best choice in routine analysis of ATEN, METO, HCTZ, AMLO, NEBI in their pharmaceutical dosage forms. This method would also be applied for the combinations of any two or three of the above said anti-hypertensive drugs, irrespective of their concentration levels.

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