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DEVELOPMENT AND VALIDATION OF BIOANALYTICAL RP-HPLC METHOD FOR DETERMINATION OF CARVEDILOL AND DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR DETERMINATION OF CARVEDILOL IN BULK DRUG AND FORMULATION

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ABSTRACT

A new method is established for estimation of Carvedilol by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Carvedilol by using Agilent column (4.6×150mm) 5µ, flow rate was 1.0 ml/min, mobile phase ratio was di-potassium hydrogen phosphate: MeoH (25:75% v/v),detection wavelength was 270 nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, photo diode array detector 996, Empower-software version-2. The retention times were found to be 5.242 mins. The % purity of Carvedilol was found to be 98.56%. The system suitability parameters for Carvedilol such as theoretical plates and tailing factor were found to be 4343.2, 1.6. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study of Carvedilol was found in concentration range of 20µg-100µg and correlation coefficient (r^2) was found to be 0.999, % recovery was found to be 98.96%, % RSD for repeatability was 0.3, % RSD for intermediate precision was 0.8. The precision study was precision, robustness and repeatabilty. LOD value was 0.7 and LOQ value was 0.13. Hence the suggested RP-HPLC method can be used for routine analysis of Carvedilol in API and Pharmaceutical dosage form.

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INTRODUCTION

The aim of present study is to develop and validate simple, precise, accurate RP-HPLC bioanalytical method for determination of carvedilol drug in plasma. Apart from this that developed method should also be used for determination of carvedilol in API and pharmaceutical formulations also. Simple, precise, accurate RP-HPLC bioanalytical method for determination of carvedilol drug in plasma is so far not available which is applicable for all above mentioned reasons and moreover developed method can also be used for stability study of drugs also.[1-7] Chromatographic methods of ion separation have become particularly important in recent years. As is well known , chromatographic analysis was first introduced into science in 1903 by the eminent Russian botanist M. Tsvet.[8]

AIM AND OBJECTIVE

Literature review reveals that there is no analytical method reported for the analysis of selected drug in blood plasma Carvedilol by estimation by RP-HPLC. Spectrophotometer, HPLC and HPTLC are the reported analytical methods for compounds either individually or in combination with other dosage form. Hence, it was felt that, there is a need of new analytical method development for the estimation of blood Plasma Carvedilol in pharmaceutical dosage form.[1-8]

Present work is aimed to develop a new, simple, fast, rapid, accurate, efficient and reproducible RP-HPLC method for the analysis of Carvedilol. The developed method will be validated according to ICH guidelines.

EXPERIMENTAL WORK

Chemicals and standards used

Table.No.1 List of chemicals and standards used.

S.No	Chemicals	Manufacturer Name	Grade
1.	Water	Merck	HPLC grade
2.	Methanol	Merck	HPLC grade
3.	di-potassium hydrogen phosphate	Merck	HPLC grade
4.	Ortho phosphoric acid	Merck	G.R
5.	KH_2PO_4	Merck	G.R
6.	K_2HPO_4	Merck	G.R
7.	0. 22µ Nylon filter	Advanced lab	HPLC grade
8.	0.45µ filter paper	Millipore	HPLC grade
9.	Tancodep-2	Torrent pharmaceuticals	Tablet form
10	Carvedilol	In – House	In- House

Instruments used

Table.No.2 List of instruments used.

S.No	Instrument name	Model number	Soft ware	Manufacturers Name
1	HPLC-auto sampler –UV detector	Separation module2695, UV.detector2487	Empower-software version-2	Waters
2	U.V double beam spectrometer	UV 3000+	U.V win soft ware	Lab India
3	Digital weighing balance (sensitivity 5mg)	ER 200A	-	Ascoset
4	pH meter	AD 102U	-	ADWA
5	Sonicator	SE60US	-	Enertech

Method development for the simultaneous estimation of Carvedilol by using RP-HPLC.

1. Selection of mobile phase
2. Selection of detection wavelength
3. Selection of column
4. Selection of solvent delivery system
5. Selection of flow rate
6. Selection of column temperature
7. Selection of diluent
8. Selection of test concentration and injection volume

Selection of mobile phase

- di-potassium hydrogen phosphate:MeoH (25:75%v/v)
- Below 2: siloxane linkages are cleaved.
  Above 8: dissolution of silica.
- pH selected: 3 ±0.05
- pH controls the elution properties by controlling the ionization characteristics.
- Reasons: To decrease the retention and improve separation. Good Response, Area, Tailing factor, Resolution.

Selection of wavelength:

10 mg of Carvedilol was dissolved in mobile phase. The solution was scanned from 200-400 nm the spectrum was obtained. The overlay spectrum was used for selection of wavelength for Carvedilol. The isobestic point was taken as detection wavelength.

Selection of column

- Heart of HPLC made of 316 grade stainless steel packed with stationary phase.

Silica based columns with different cross linking's in the increasing order of polarity are as follows:

←------- Non-polar----------moderately polar--------Polar----------→

C_{18}< C_8< C_6< Phenyl < Amino <Cyano< Silica

- In reverse phase chromatography, hydrophobic interaction between drug molecule and the alkyl chains on the column packing material.
- Column is selected based on solubility, polarity and chemical differences among analysts and Column selected: i.e. Agilent C18 column (4.6 x150 mm) 5 µ.

Selection of solvent delivery system

- Always preferable solvent delivery system.
- More chance of getting reproducible result on retention time of analytes.
- More economic than gradient technique.

Selection of flow rate

Acceptable limit: - Not more than 2.5 ml/min

- Flow rate selected was 1.4ml/min
- Flow rate is selected based on

Reasons:

- ❖For earlier elution of analyte and elution of all impurities within 6.0 min.
- ❖Information from the reference method in literature.

Selection of diluent

- Selection of diluent is based on the solubility of the analyte
- Diluent selected: di-potassium hydrogen phosphate:MeoH(25:75%v/v)

Selection of column temperature:

- Preferable temperature is ambient or room temperature.

Reasons:

❖To elute all impurities along with analyte with in 10.0 min of run time.
❖Less retention time
❖Good peak shape
❖Higher theoretical plates.
❖Good resolution.

Selection of test concentration and injection volume

Test concentration is finalized after it is proved that API is completely extractable at the selected test concentration.

- Test concentration is fixed based upon the response of API peak at selected detector wavelength.
- And the test concentration selected is 10 ppm.
- Injection volume selected was 15µL.

Reason: good peak area, retention time, peak symmetry.

Chromatographic trials for estimation of Carvedilol by RP- HPLC in blood plasma.

Trial-1

Chromatographic conditions

Column	:	Xterra , RP18 4.6x150mm ,5µm
Mobile phase ratio	:	MeOH: H_2O (60:40%v/v)
Detection wavelength	:	270nm
Flow rate	:	1ml/min
Injection volume	:	10µl
Run time	:	8.0min
Retention time	:	1.791min

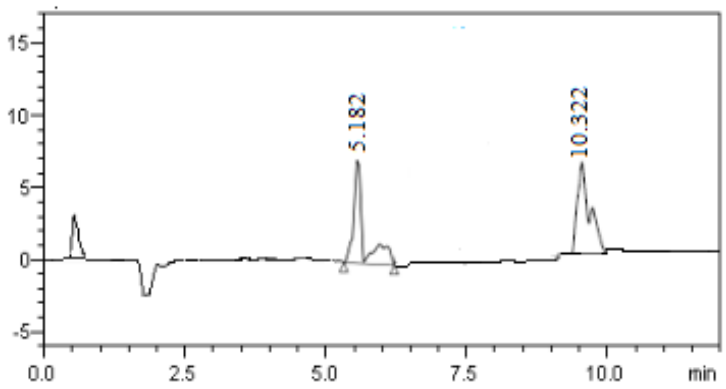


Fig.No.1 Chromatogram showing trial 1 injection.

Observation:

The trial shows no good peak separation, so more trials were required for obtaining peaks.

Trial - 2

Chromatographic conditions

Column	:	Inertsil ODS 4.5×150mm 5.0 µm
Mobile phase ratio	:	ACN: Hdi-potassium hydrogen phosphate (80:20%v/v)
Detection wavelength	:	270nm
Flow rate	:	1ml/min
Injection volume	:	20µl
Column temperature	:	Ambient
Auto sampler temperature	:	Ambient
Run time	:	8 min

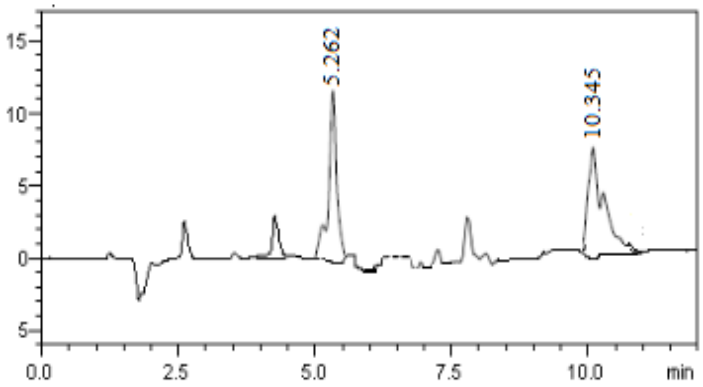


Fig.No.2 Chromatogram showing trial-2 injection.

Observation

In this trial no peak was observed, still more trials was required for good peaks.

Trial-3

Chromatographic conditions

Column	:	Xterra RP18 4.6x150mm 5µm
Mobile phase ratio	:	ACN: pH 6.8phosphate buffer (75:25 % v/v)
Detection wavelength	:	270 nm
Flow rate	:	1.0ml/min
Injection volume	:	10µl
Column temperature	:	Ambient
Auto sampler temperature	:	Ambient
Run time	:	10.0 mins

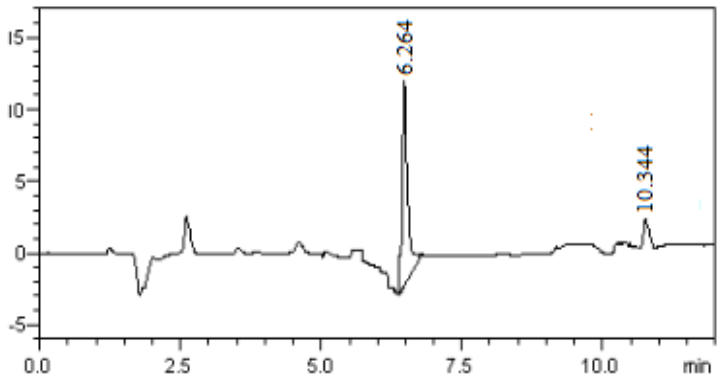


Fig.No.3 Chromatogram showing trial-3 injection.

Observation

In this trial Carvedilol was eluted but there is no proper separation. Still more trials was required for better separation in peak.

Trial-4

Chromatographic conditions

Column	:	Agilent C18 4.6×250mm 5µm
Mobile phase ratio	:	ACN: pH 3phosphate buffer (60:40% v/v)
Detection wavelength	:	270nm
Flow rate	:	1.0ml/min
Injection volume	:	10µl
Column temperature	:	Ambient
Auto sampler temperature	:	Ambient
Run time	:	20 min

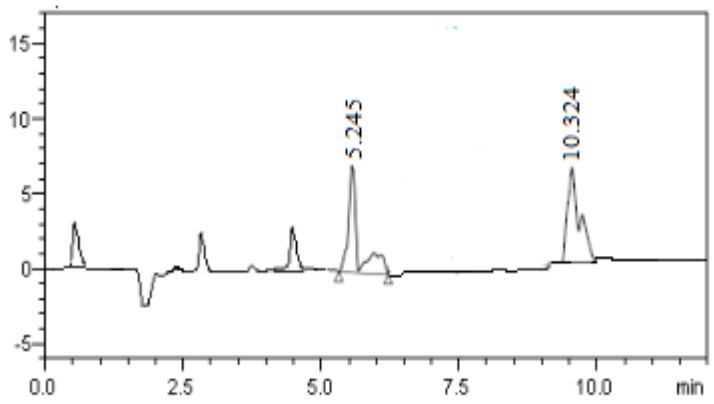


Fig.No.4 Chromatogram showing trial-4 injections.

Observation:
The separation was good; peak shape was good, still more trials were required to decrease the retention times of peak.

Trial -5 (optimised method):
Chromatographic conditions

Column	:	Agilent (4.6×150mm) 5µ
Mobile phase ratio	:	di-potassium hydrogen phosphate buffer: MeoH (25:75% v/v)
Detection wavelength	:	270 nm
Flow rate	:	1.0 ml/min
Injection volume	:	10µl
Column temperature	:	Ambient
Auto sampler temperature	:	Ambient
Run time	:	10min

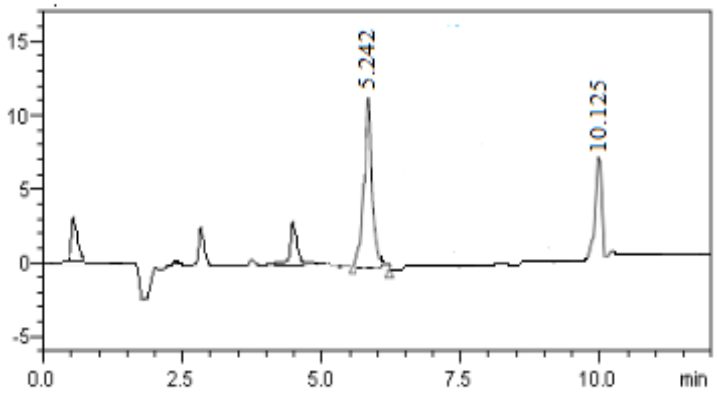


Fig.No.5 Chromatogram showing trial-5 injection.

Observation
The separation was good, peak shape was good, so we conclude that there is no required for decrese the retention times of peak, so it is taken as final method.

Procedure
Diluent preparation
Mobile phase was used as the diluent.

Preparation of mobile phase:
A mixture of methanol and 0.01M di-potassium hydrogen phosphate buffer in ratio of (75:25) and adjusted to pH 7.0 using o-phosphoric acid, filtered, degassed and used. 0.01M Di-potassium hydrogen phosphate buffer (pH 7.0) prepared in 100 ml volumetric flask, add 17.41 gm of dipotassium hydrogen phosphate and dissolve it in some of amount of HPLC grade water, and make up to volume with HPLC grade water. Adjust the pH 7.0 of resultant buffer by orthophosphoric acid, as required.

Preparation of the individual standard preparation
10 mg Amount of standard was mixed with 10 ml methanol to +2ml of rat plasma(untreated) then vertically shaked for 30 min then centrifuged at 5000rpm for 1 hr .Then it was filterated using membrane filters to get clear organic solution .Then it was filled in to the sample vials of HPLC and loaded on to HPLC for Run.

Preparation of the Carvedilol sample solution
Sample solution preparation
Blood samples are collected from the animals rats and then centrifuged at 5000rpm for 1 hr to separate the plasma from blood. Then the separated was mixed with methanol then loaded on to the HPLC for Run.

System suitability

- Tailing factor for the peaks due to Carvedilol in standard solution should not be more than 1.5.
- Theoretical plates for the Carvedilol peaks in standard solution should not be less than 2000.

Assay calculation

$$Assay\ \% = \frac{samplearea}{Standardarea} \times \frac{dilutionsample}{dilutionofstandard} \times \frac{P}{100} \times \frac{Avg.wt}{Lc} \times 100$$

Where:
Avg.wt = average weight of tablets
P= Percentage purity of working standard
LC= Label Claim of Carvedilol mg/ml.

ANALYTICAL METHOD VALIDATION [9]

Validation parameters

- Specificity
- Linearity
- Range
- Accuracy
- Precision
- Repeatability
- Intermediate Precision
- Detection Limit
- Quantitation Limit
- Robustness

Specificity

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The specificity was performed by injecting blank.

Linearity

Preparation of stock solution

10 mg of Carvedilol working standard was accurately weighed and was transferred into a 10ml clean dry volumetric flask, add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Preparation of Level – I (50ppm of Carvedilo)

0.5 ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level – II (75ppm ofCarvedilol)

0.7 ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level – III (100ppm of Carvedilol)

1.0 ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level – IV (125 ppm of Carvedilol)

1.2 ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level – V (150 ppm of Carvedilol)

1.5 mlof stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

Procedure

Each level was injected into the chromatographic system and peak area was measured.Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and the correlation coefficient was calculated.

Acceptance criteria

- Correlation coefficient should be not less than 0.999.

Range

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 50µg/ml-150µg/ml of Carvedilol.

Accuracy

Preparation of standard stock solution

10mg of Carvedilol working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette out 1 ml of the above stock solution into a 10 ml volumetric flask and was diluted up to the mark with diluent.

Preparation of sample solutions

For preparation of 50% solution (with respect to target assay concentration)

5 mg of Carvedilol working standard was accurately weighed and transferred into a 10 ml clean dry volumetric flask add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock Solution).Further pipette out 10 ml of the above stock solution into a 100ml volumetric flask and was diluted up to the mark with diluent.

For preparation of 100% solution (with respect to target assay concentration)

10 mg of Carvedilol working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette out 1ml of above stock solution into a 10 ml volumetric flask and was diluted up to the mark with diluent.

For preparation of 150% solution (with respect to target assay concentration)

15 mg of Carvedilol working standard into a 10ml clean dry volumetric flask add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette out 1ml of the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

Procedure

The standard solution of accuracy 50%, 100% and 150% was injected into chromatographic system. Calculate the amount found and amount added for Carvedilol and calculate the individual % recovery and mean % recovery values.

Acceptance criteria

❖The % recovery for each level should be between 98.0 to 102.0%

Precision

Repeatability

Preparation of stock solution

10 mg of Carvedilol working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette out 1ml of the above stock solution into a 10ml volumetric flask and was diluted 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.

Acceptance criteria

❖The % RSD for the area of five standard injections results should not be more than 2.

IntermediatePrecision/Ruggedness

To evaluate the intermediate precision (also known as ruggedness) of the method, precision was performed on different days by using different make column of same dimensions.

Preparation of stock solution

10 mg of Carvedilol working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2ml of diluent and sonic ate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette out 1ml of the above stock solution into a 10ml volumetric flask and was diluted 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.

Acceptance criteria

❖The % RSD for the area of five sample injections results should not be more than 2%.

Limit of detection (LOD)

LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

$$LOD = 3.3 \text{ X } \frac{\sigma}{S}$$

Where

σ - Standard deviation (SD)

S - Slope

Limit of quantification

LOQ's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula. Again, the standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

$$LOQ = 10 \text{ X } \frac{\sigma}{S}$$

Where

σ - Standard deviation

S - Slope

Robustness

As part of the robustness, deliberate change in the flow rate, mobile phase composition was made to evaluate the impact on the method.

a) The flow rate was varied at 0.8 ml/min,1.0ml/min to 1.2 ml/min. Standard solution 60 ppm of Carvedilol prepared and analysed using the varied flow rates along with method flow rate.

b) The organic composition in thermobile phase was varied from 65% to75 %standard solution 60 µg/ml of Carvedilol was prepared and analysed using the varied mobile phase composition along with the actual mobile phase composition in the method.

System suitability

10 mg of Carvedilol working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette out 1ml of Carvedilol from the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

RESULTS AND DISCUSSION

The present investigation reported in the thesis was aimed to develop a new method development and validation for the estimation of Carvedilol by RP-HPLC method. Literature reveals that there are no analytical methods reported for the estimation Carvedilol by RP-HPLC method. Hence, it was felt that, there is a need of new analytical method development for the estimation of Carvedilol in pharmaceutical dosage form.

Method Development

The detection wavelength was selected by dissolving the drug in mobile phase(blood plasma) to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400nm. The overlay spectrum of Carvedilol was obtained and the isobestic point of Carvedilol showed absorbance's maxima at 270 nm. The spectrums are shown in Fig. 6.

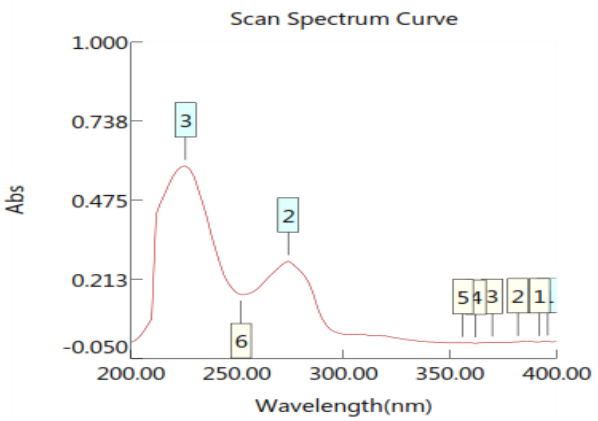


Fig.No.6 Spectrum showing overlapping spectrum of Carvedilol.

The chromatographic method development for the estimation of Carvedilol were optimized by several trials for various parameters as different column, flow rate and mobile phase, finally the following chromatographic method was selected for the separation and quantification of Carvedilol in API and pharmaceutical dosage form by RP-HPLC method.

Optimized chromatographic conditions for Carvedilol by RP-HPLC method

Column	:	Agilent (5µm, 4.6x150mm)
Column temperature	:	Ambient
Wavelength	:	270 nm
Mobile phase ratio	:	di-potassium hydrogen phosphate: MeoH (25:75% v/v)
Flow rate	:	1.0 ml/min
Auto sampler temperature	:	Ambient
Injection volume	:	10µl
Run time	:	10.0 minutes

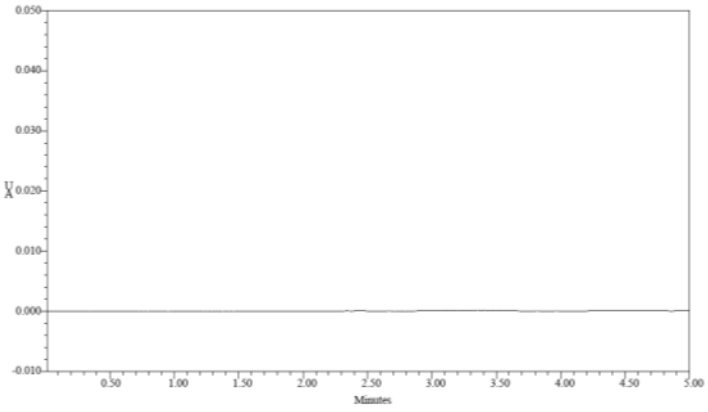


Fig.No.7 Chromatogram showing blank preparation (mobile phase).

Assay calculation for Carvedilol

The assay study was performed for the Carvedilol. Each three injections of sample and standard were injected into chromatographic system. The chromatograms are shown in Fig.8 and results are tabulated in Table.NO.4.

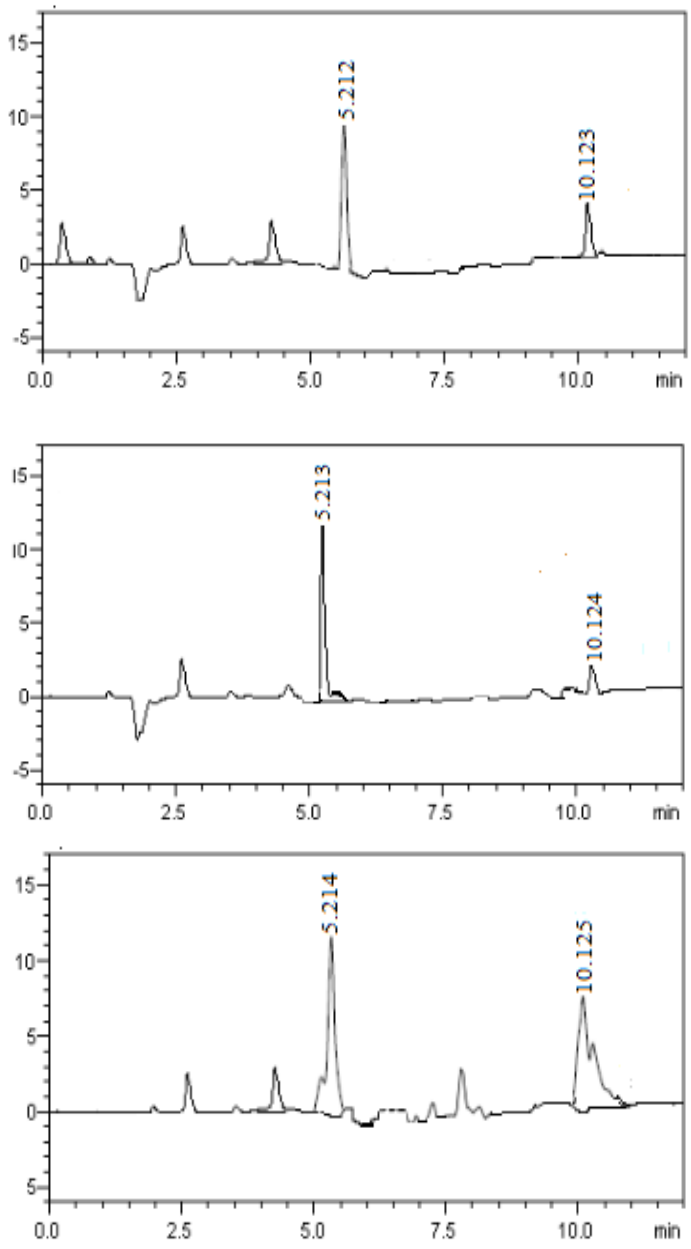


Fig.No.8 Chromatogram showing assay of sample injection-1,2,3.

Peak name Carvedilol

Table No. 3 Study of Carvedilol.

	Peak Name	RT	Area	Height
1	Carvedilol	5.212	823128	159724
2	Carvedilol	5.213	823892	158616
3	Carvedilol	5.214	823703	158823
Mean			823574.4	
Std. Dev.			397.8	
% RSD			0.0	

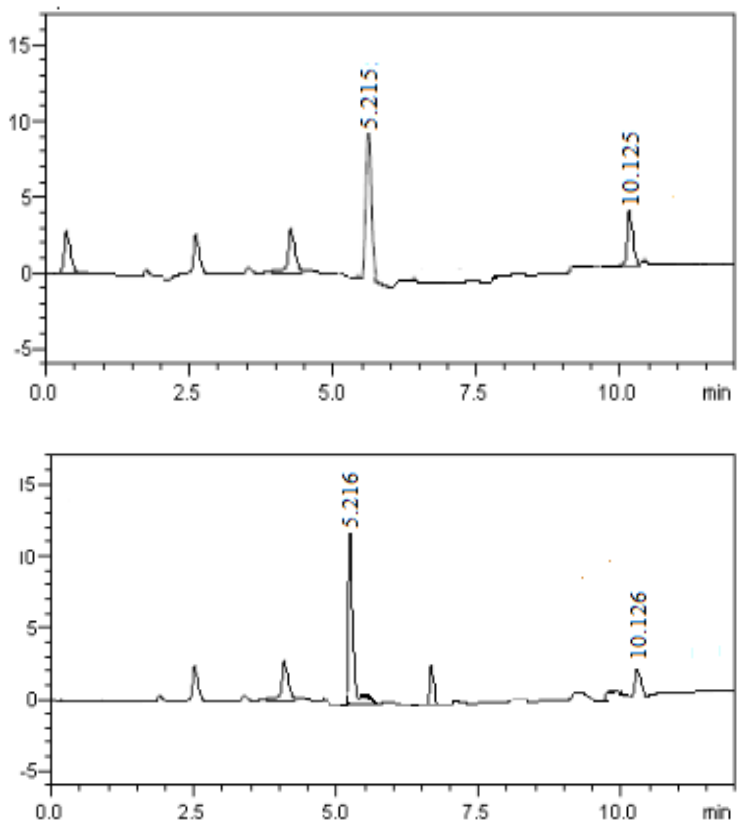


Fig.No.9 Chromatogram showing standard of sample injection -1,2.

Table.No.4 Showing assay results.

	Peak Name	RT	Area	Height	USP Plate Count	USP Tailing
1	Carvedilol	5.215	819454	162382	4384.6	1.6
2	Carvedilol	5.216	826308	161456	4343.4	1.6
Mean			822880.5		4364.0	
Std. Dev.			4846.5			
% RSD			0.6			

The retention time of Carvedilol was found to be 5.216 mins. The system suitability parameters for Carvedilol such as theoretical plates and tailing factor were found to be 4545.4, 1.5. The % purity Carvedilol in pharmaceutical dosage form was found to be 99.45%.

VALIDATION REPORT

Specificity

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The study was performed by injecting blank. The chromatograms are shown in Fig.No.10-12.

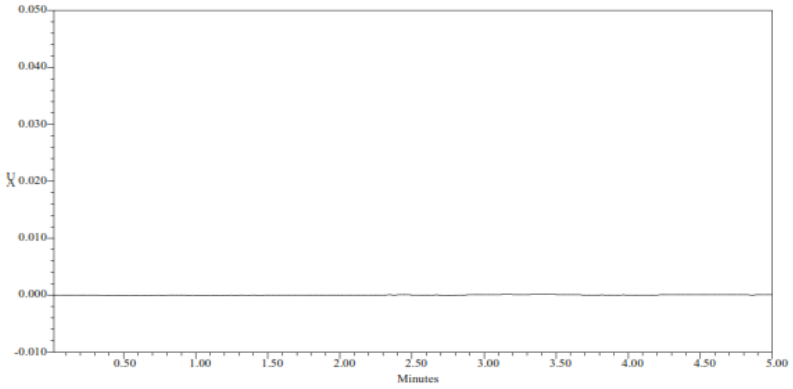


Fig.No.10 Chromatogram showing blank (mobile phase preparation).

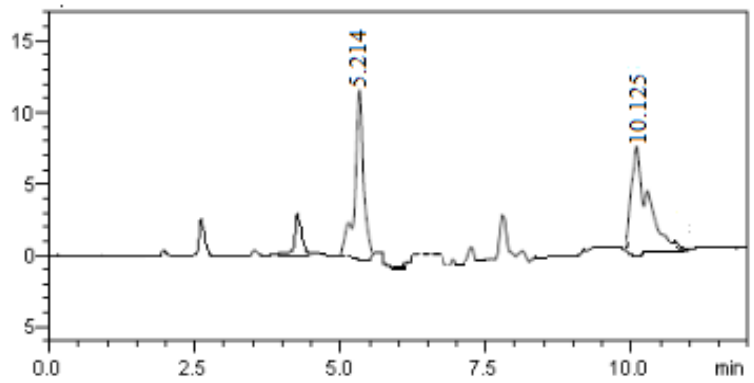


Fig.No.11 Chromatogram showing standard injection.

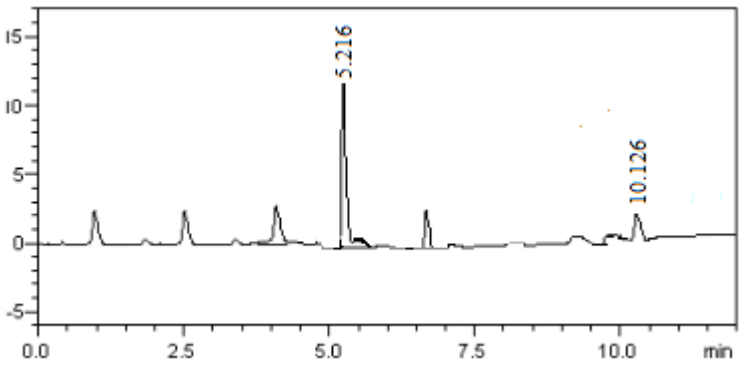


Fig.No.12 Chromatogram showing sample injection.

The specificity test was performed for Carvedilol. It was found that there was no interference of impurities in retention time of analytical peak.

Linearity

The linearity study was performed for the concentration of 20-100 ppm Carvedilol. Each level was injected into chromatographic system. The area of each level was used for calculation of correlation coefficient. The results are tabulated in Table. No 5-6. Calibration graph for Carvedilol was shown in Fig. No.14.

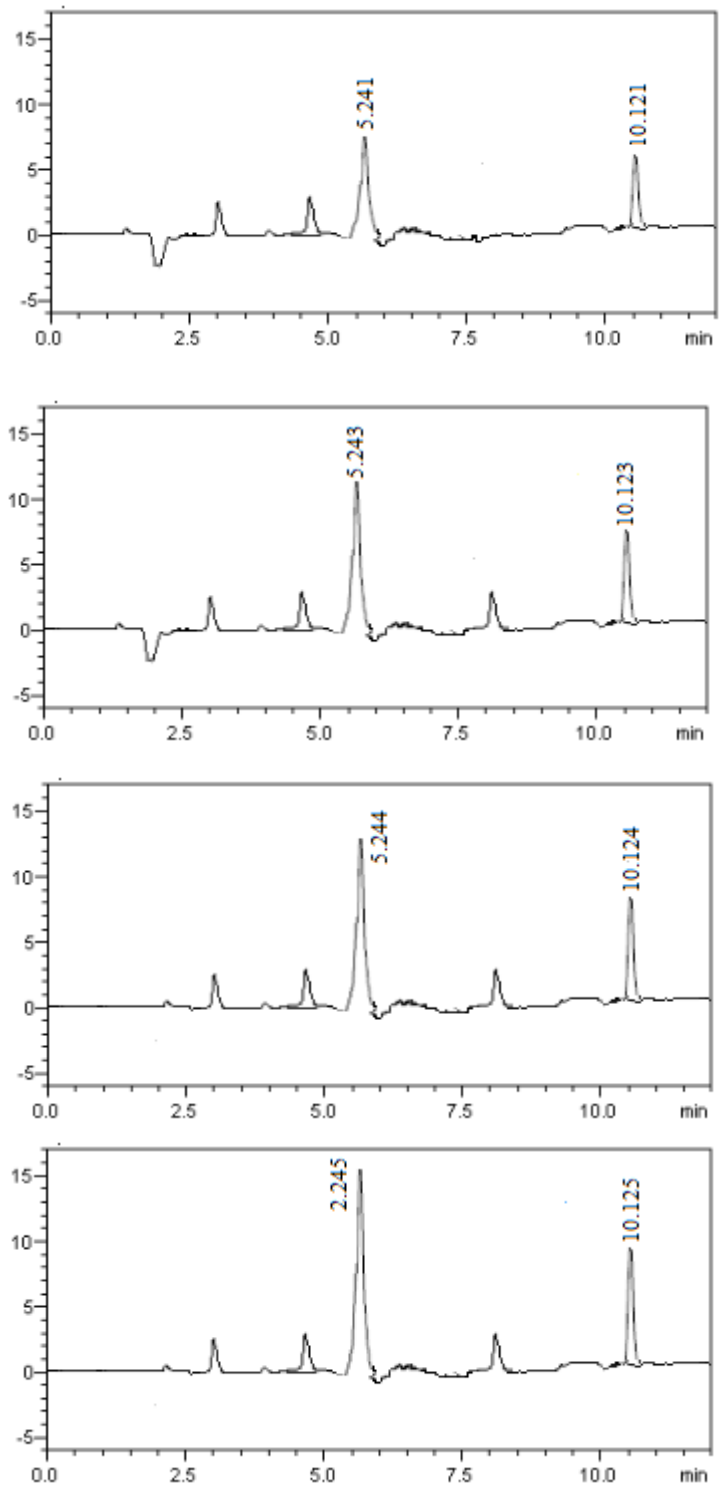


Fig.No.13 Chromatograms showing linearity level-1 to level 5 (20ppm-100 ppm of Carvedilol) injections.

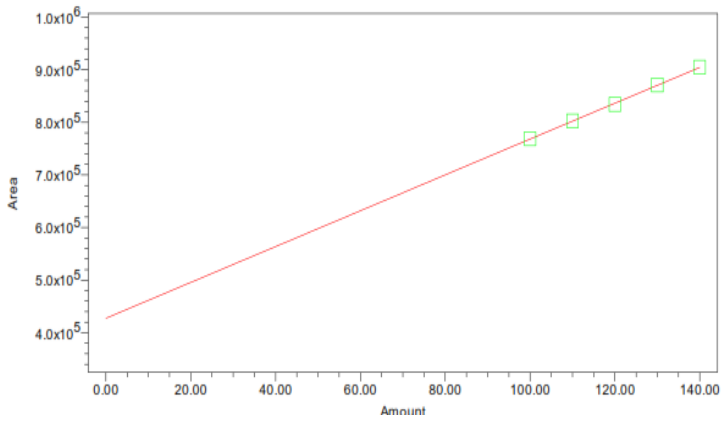
Table No.5 Linearity Results for Carvedilol:

Peak name Carvedilol

S.NO	Peak Name	RT	Area	Height
1	Carvedilol	5.241	9059571	171899
2	Carvedilol	5.242	1033622	178806
3	Carvedilol	5.243	1200310	160124
4	Carvedilol	5.244	1436425	152938
5	Carvedilol	5.245	1608820	146161

Table No.6 Linearity Results for Carvedilol:

S.No	Linearity Level	Concentration	Area
1	I	20 ppm	905957
2	II	40 ppm	1033632
3	III	60 ppm	1200130
4	IV	80 ppm	1403642
5	V	100 ppm	1608820
Correlation Coefficient			0.999



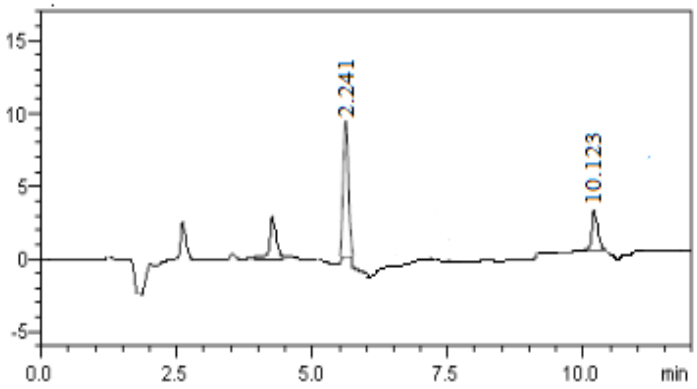
Carvedilol $r^2 = 0.999$

Fig.No.14 Showing calibration graph for Carvedilol.

The linearity study was performed for concentration range of 20µg-100µgCarvedilol and the correlation coefficient was found to be 0.999 (NLT 0.999).

Accuracy

The accuracy study was performed for 50%, 100% and 150 % for Carvedilol. Each level was injected in triplicate into chromatographic system. The area of each level was used for calculation of % recovery. Chromatograms are shown in Fig.No.15-17 results are tabulated in Tables.



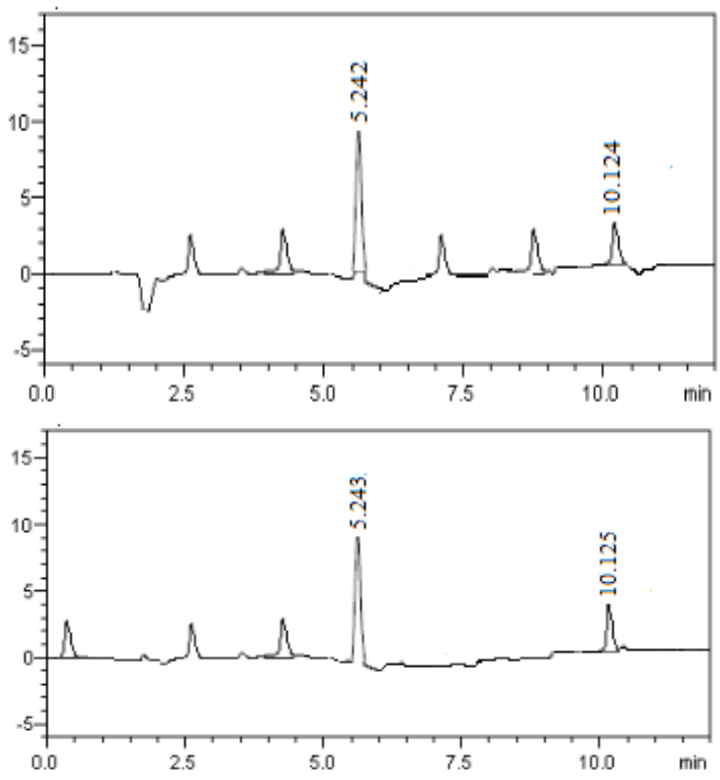
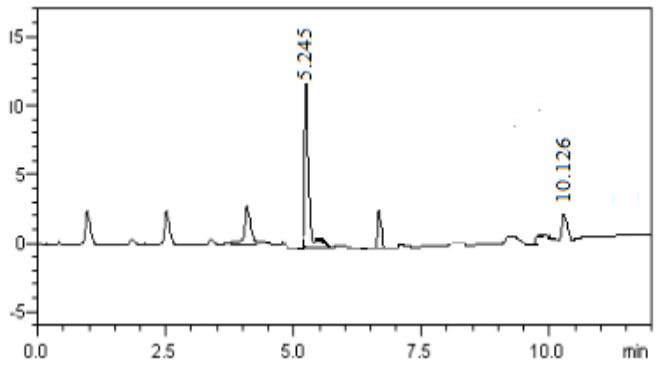


Fig.No.15-17. Chromatograms showing accuracy-50% injection-1,2,3.

	PeakName	RT	Area	Height 1
1	Carvedilol	5.241	1494887	222732
2	Carvedilol	5.242	1495328	223172
3	Carvedilol	5.243	1490342	223832
Mean			1143519.1	
Std. Dev.			2760.1	
% RSD			0.18	

Accuracy -100%



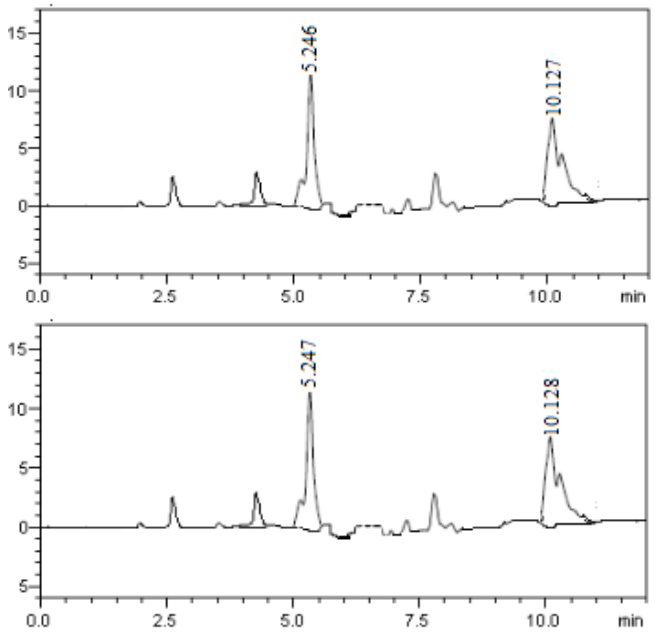
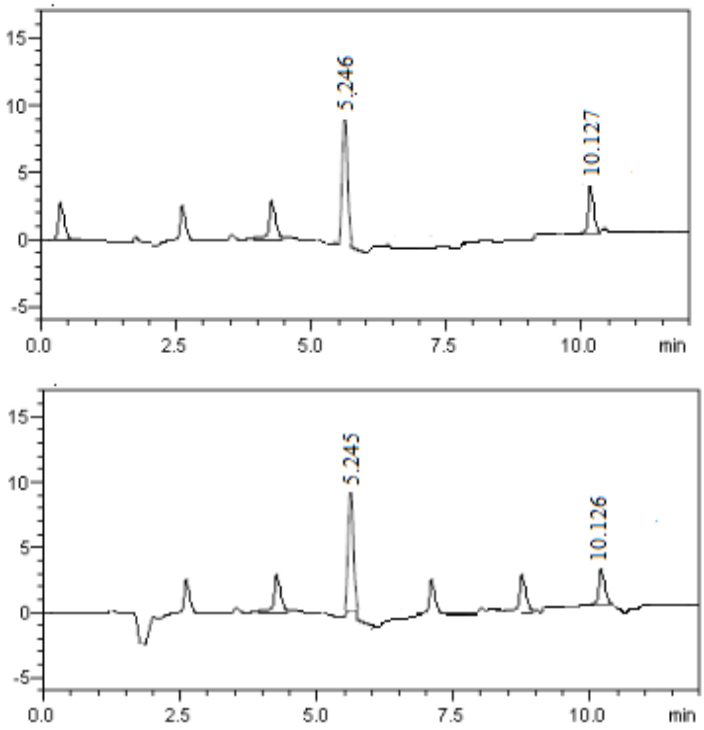


Fig.No.18-20 Chromatogram showing accuracy -100% injection-1,2,3.

Peak name Carvedilol.

	Peak Name	RT	Area	Height
1	Carvedilol	5.245	2938710	492043
2	Carvedilol	5.246	2939239	496109
3	Carvedilol	5.247	2937077	488205
Mean			2938342.1	
Std. Dev.			1127.5	
% RSD			0.17	

Accuracy 150%



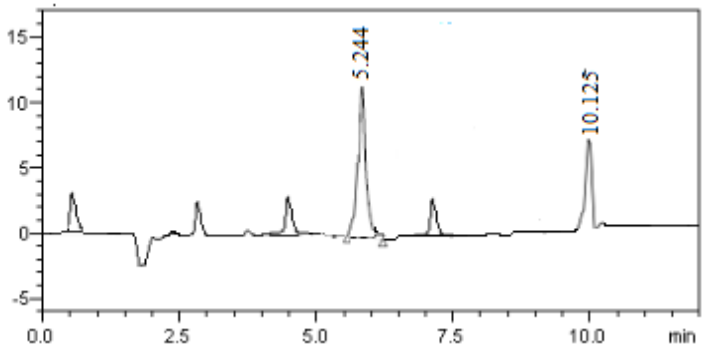


Fig.No.21-23 Chromatogram showing accuracy -150 % injection-1,2,3.

Peak name Carvedilol

	PeakName	RT	Area	Height
1	Carvedilol	5.246	4454604	492677
2	Carvedilol	5.245	4451189	496588
3	Carvedilol	5.244	4452482	486638
Mean			4452758.3	
Std. Dev.			1724.1	
% RSD			0.03	

Table.No.7 Showing accuracy results for Carvedilol.

%Concentration (at specification level)	Average area	Amount added (mg)	Amount found (mg)	% Recovery	Mean recovery
50%	1143519	5	4.86	98.81%	
100%	2938342	10	9.88	99.08%	98.96%
150%	4452758	15	15.0	100.0%	

The accuracy study was performed for % recovery of Carvedilol . The % recovery was found to be 98.96% (NLT 98% and NMT 102%)

Precision

❖Repeatability
❖Intermediate Precision

Repeatability

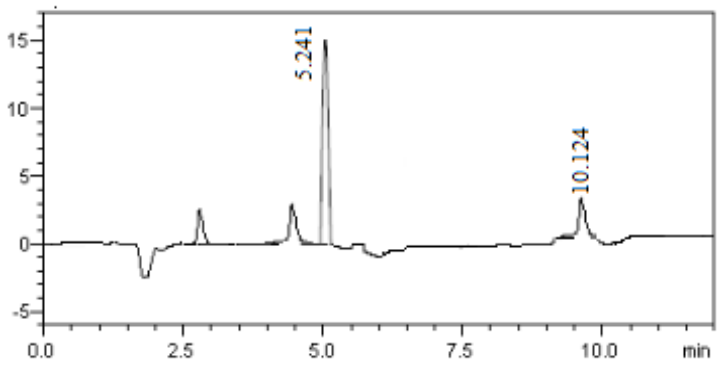
The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

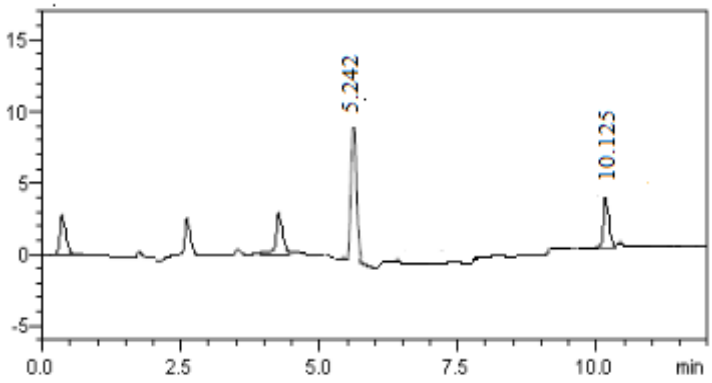
Intermediate precision/Ruggedness

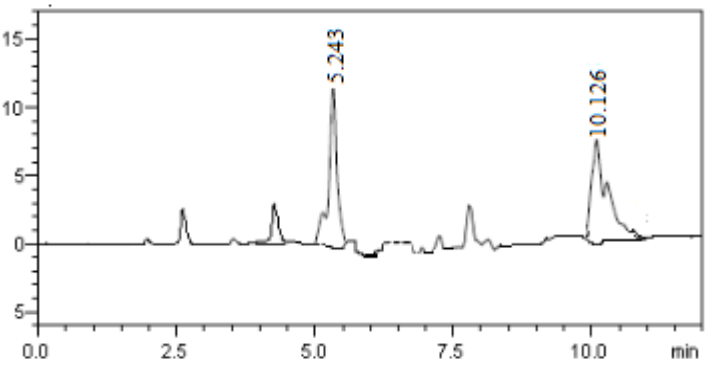
The standard solution was injected for five times and measured the area for all fiveinjections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

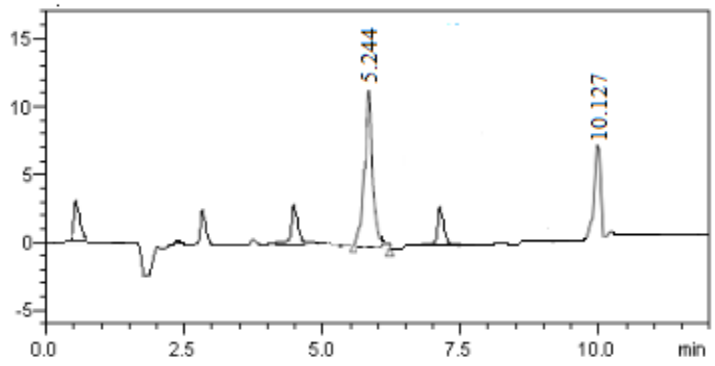
Repeatability

The precision study was performed for five injections of Carvedilol. Each standard injection was injected into chromatographic system.The area of each Standard injection was used for calculation of % RSD.
The chromatograms are shown in Fig.No.24-28 and results are tabulated in Table.No.8


5.241
10.124
15
10
5
0
-5
0.0
2.5
5.0
7.5
10.0
min


5.242
10.125
15
10
5
0
-5
0.0
2.5
5.0
7.5
10.0
min


5.243
10.126
15
10
5
0
5
0.0
2.5
5.0
7.5
10.0
min


5.244
10.127
15
10
5
0
-5
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2.5
5.0
7.5
10.0
min

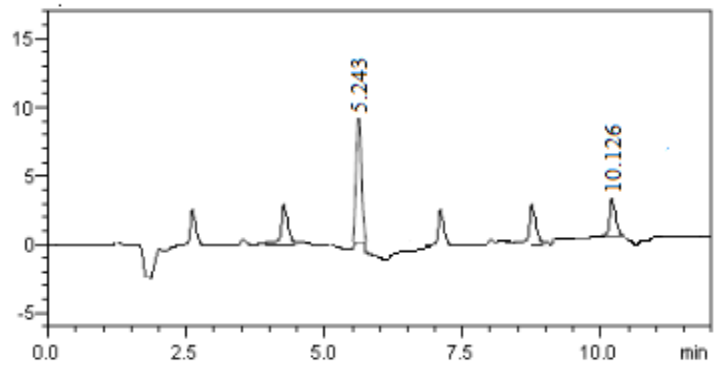


Fig.No.24-28 Chromatograms showing precision injections -1 to 5.

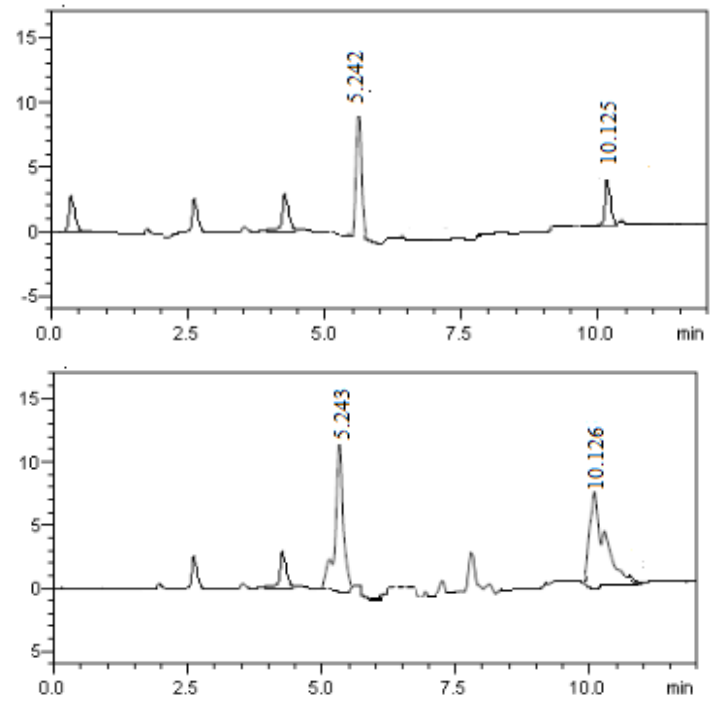
Table No.8 Showing% RSD results for Carvedilol.

	Peak Name	RT	Area	Height
1	Carvedilol	**5.241**	824170	158772
2	Carvedilol	**5.242**	826053	157336
3	Carvedilol	**5.243**	823442	156124
4	Carvedilol	**5.244**	818967	155674
5	Carvedilol	**5.243**	823476	156033
Mean			823221.9	
Std. Dev.			2604.2	
% RSD			0.3	

The Method precision study was performed for the %RSD of Carvedilol was found to be 0.3 (NMT 2).

Intermediate precision/Ruggedness

The intermediate precision study was performed for five injections of Carvedilol. Each standard injection was injected into chromatographic system. The area of each standard injection was used for calculation of % RSD. The chromatograms are shown in Fig.No.29-33 and results are tabulated in Table.9.



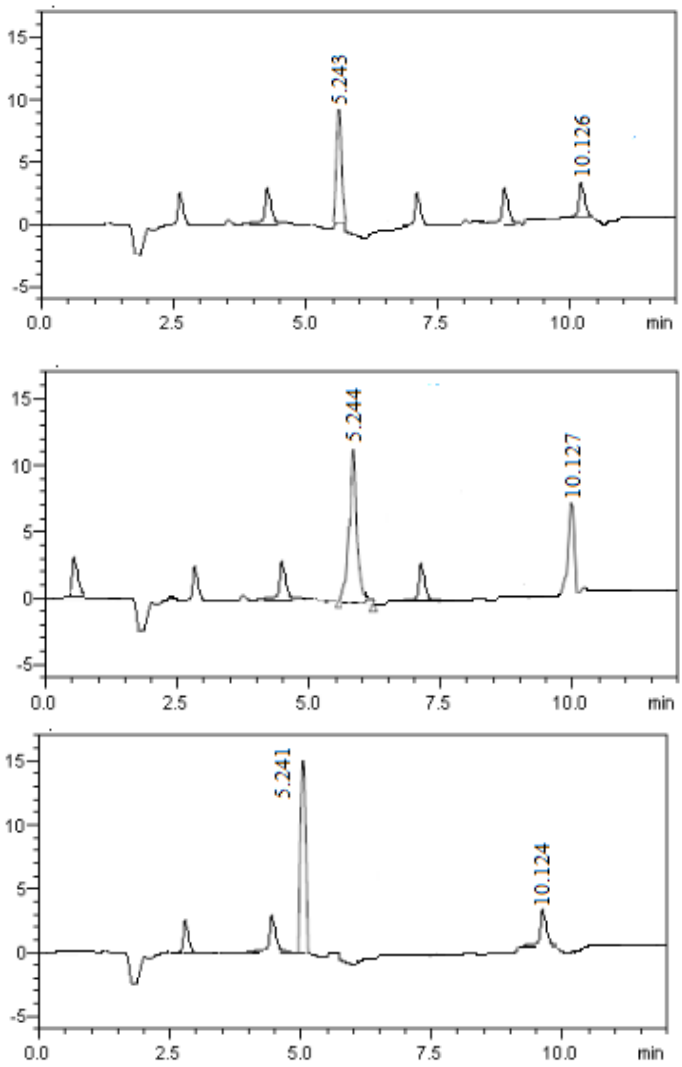


Fig. No.29-33 Chromatograms showing intermediate precision injections -1to 5.

Table No.9 Showing results for intermediate precision of Carvedilol.

	Peak Name	RT	Area	Height
1	Carvedilol	5.242	830760	160374
2	Carvedilol	5.243	832532	160030
3	Carvedilol	5.243	823385	159662
4	Carvedilol	5.244	840724	161107
5	Carvedilol	5.241	829385	160286
Mean			831357.4	
Std. Dev.			6263.2	
% RSD			0.8	

The intermediate precision was performed for %RSD of Carvedilol was found to be 0.8 (NMT 2).

Detection limit

LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

$$\mathbf{LOD = 3.3\ X\ \frac{\sigma}{S}}$$

Where

σ - Standard deviation (SD)

S - Slope

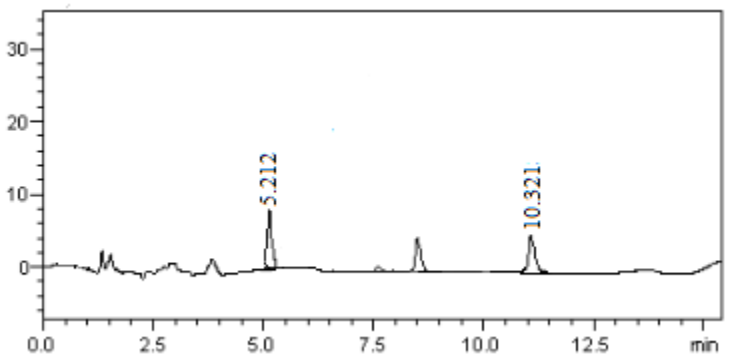


Fig.No.34 Showing results for Limit of Detection.

Table No.10 Showing results for Limit of Detection.

Drug name	Standard deviation(σ)	Slope(s)	LOD(µg)	S/N Ratio
Carvedilol	2604.5	17757	0.439	0.7

The LOD was performed for Carvedilol was found to be 0.7.

Quantitation limit

LOQ's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula. Again, the standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

$$\mathbf{LOQ = 10\ X\ \frac{\sigma}{S}}$$

Where

σ - Standard deviation

S - Slope

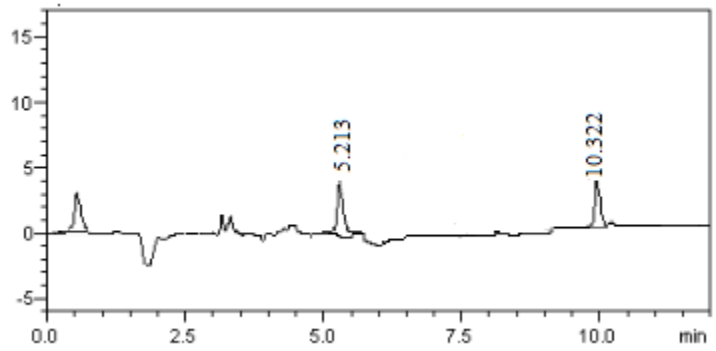


Fig No.35 Showing results for Limit of Quantitation.

Table No.11 Showing results for Limit of Quantitation.

Drug name	Standard deviation(σ)	Slope(s)	LOQ(µg)	S/N Ratio
Carvedilol	2604	17757	1.466	0.13

The LOQ was performed for Carvedilol was found to be 0.13.

Robustness

The robustness was performed for the flow rate variations from 0.8ml/min to 1.2ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Carvedilol. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%.The chromatograms are shown in Fig.No.36-39 and results are tabulated in Table.No.12-13.

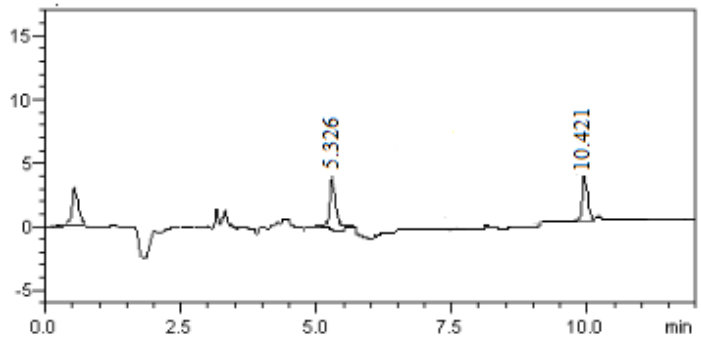


Fig.No.36 Chromatogram showing less flow rate 0.8ml/min.

	PeakName	RT	Area	Height	USP Plate Count	USP Tailing
1	Carvedilol	5.326	759146	162231	4517.0	1.7

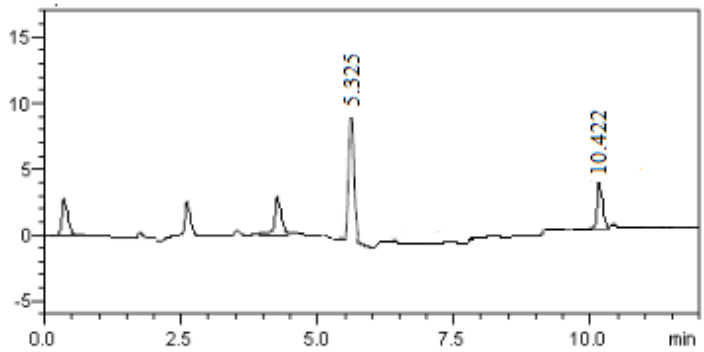


Fig.No.37 Chromatogram showing more flow rate 1.2 ml/min.

	PeakName	RT	Area	Height	USP Plate Count	USP Tailing
1	Carvedilol	5.325	756888	157802	4209.0	1.6

The results are summarized on evaluation of the above results, it can be concluded that the variation in flow rate affected the method significantly. Hence it indicates that the method is robust even by change in the flow rate ±0.2ml/min.The method is robust only in less flow condition.

Table No.12 Showing system suitability results for Carvedilol.

S. No	Flow rate (ml/min)	System suitability results	
		USP Plate Count	USP Tailing
1	0.8	4517	1.7
2	**1.0**	**4343**	**1.6**
3	1.2	4209	1.6

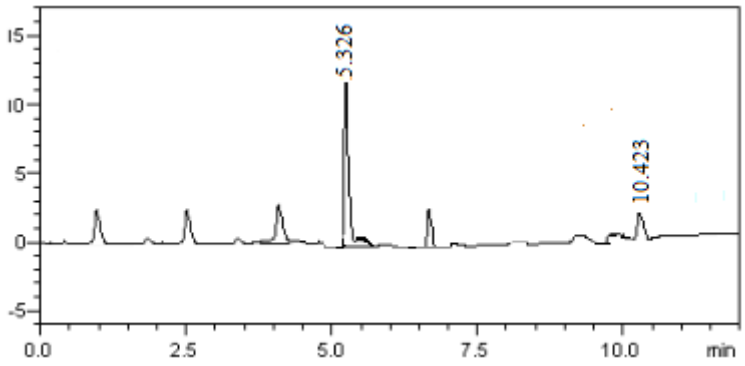


Fig.No.38 Chromatogram showing more organic phase ratio.

	PeakName	RT	Area	Height	USP Plate Count	USP Tailing
1	Carvedilol	5.327	756154	157815	4623.5	1.6

Fig.No.39 Chromatogram showing less organic phase ratio.

	PeakName	RT	Area	Height	USP Plate Count	USP Tailing
1	Carvedilol	5.327	759234	162213	4864.4	1.6

On evaluation of the above results, it can be concluded that the variation in±5%. Organic composition in the mobile phase affected the method significantly. Hence it indicates that the method is robust even by change in the mobile phase ±5%.

Table No.13 Showing system suitability results for Carvedilol.

S.No	Change in organic composition in the mobile phase	System suitability results	
		USP Plate Count	USP Tailing
1	5 % less	4626	1.6
2	***Actual**	**4545**	**1.6**
3	5 % more	4867	1.6

FORCED DEGRADATION STUDIES

Forced degradation of Test sample was performed under acidic, alkaline, heat, photolytic and oxidative stress conditions.

Stock solution preparation

Twenty Tablets were weighed and powdered. Tablet powder having weight equivalent to 20 mg of was weighed accurately and taken in a 10 mL volumetric flask. To it 5 mL of the mobile phase was added and sonicated for 15 minutes to dissolve the drugs. The volume was made up to 10 mL with mobile phase. The resulting solution was then filtered through a 0.45 µm membrane filter to prepare a stock solution of the tablet sample. Further dilution was done by diluting 0.1 mL of stock solution to 10 mL mobile phase. The concentration of Carvedilol in the solution was 10 µg/mL, respectively.

Acid hydrolysis

Forced degradation in acidic media was performed by adding 2 mL 0.1 M HCl to 10 mL of stock solution and the mixture is heated at 60°C for approximately 26hrs and the solution is neutralized by addition of 0.1 M NaOH. The prepared solution is injected and chromatograms were recorded.

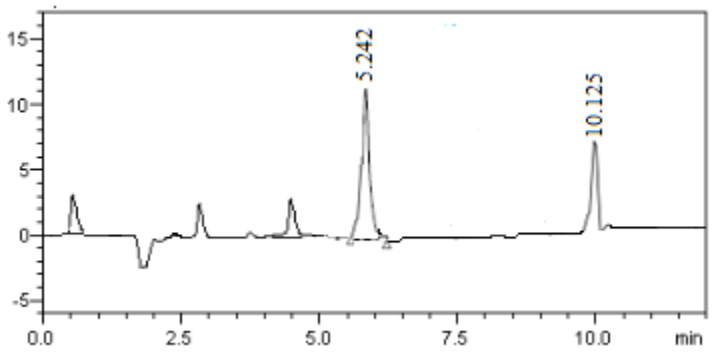


Fig. No. 40 Chromatogram of Acid degradation sample.

Observation:

The study indicates that the drugs under study were degraded and assay results shows that mostly Carvedilol (8.4%) was degraded following by the drug were well separated from the degradation products.

Alkaline hydrolysis

Forced degradation in basic media was performed by adding 2 mL 0.1 M NaOH to 10 mL of stock solution and the mixture is heated at 60°C for approximately 26hrs and the solution is neutralized by addition of 0.1 M HCl. The prepared solution is injected and chromatograms were recorded.

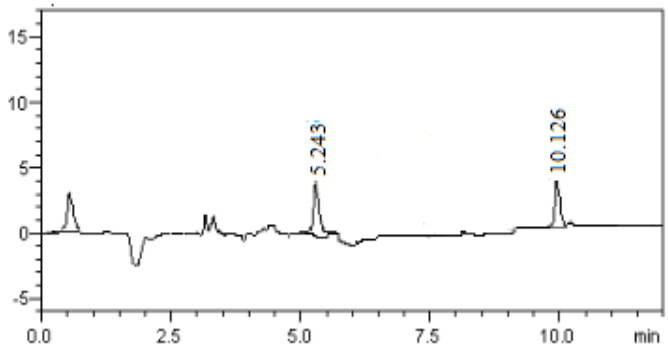


Fig. No. 41 Chromatogram of Alkali degradation sample.

Observation:

The results of alkaline hydrolysis indicates that some degradation occurred and assay results shows that Carvedilolwas degraded by 13.4% the drug were well separated from the degradation products.

Oxidative Degradation

To study the effect of oxidizing conditions, an aliquot of stock solution was added to 1 mL 30 % H_2O_2 solution. The prepared solution 10□l is injected and chromatograms were recorded.

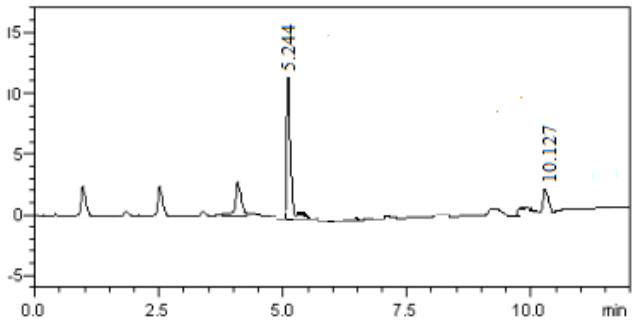


Fig. No. 42 Chromatogram of Oxidative degradation sample.

Observation:

From the observation it was found that the Carvedilol were found stable i.e, no significant peaks were found. So it is stable in the above condition.

Thermal Degradation

To study the effect of temperature an aliquot of stock solution was kept at 70^{0}C for 26 hrs. 10□l of resulting solution was injected into HPLC and chromatograms were recorded.

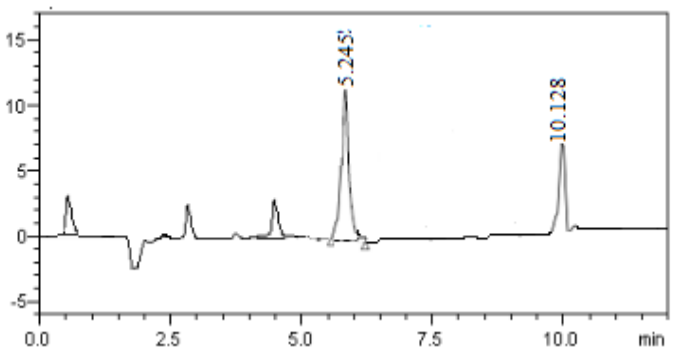


Fig. No. 43 Chromatogram of Thermal degradation sample.

Observation:

From the observation it was found that theCarvedilol were found to be degraded by 13.3% respectively.

Photolysis

To study the effect of photolysis, an aliquot of stock solution was exposed to UV light for 4hrs.
10ml of resulting solution was injected into HPLC and chromatograms were recorded.

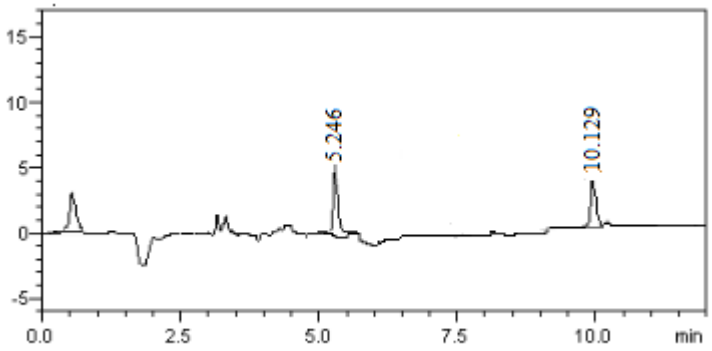


Fig. No. 44 Chromatogram of Photolytic degradation of sample.

Observation:

From the observation it was found that the Carvedilol were found stable i.e, no significant peaks were found. So it is stable in the above condition.

Summary on degradation studies

Table No. 14 Results of Stress degradation studies.

Stress Condition	Sample-1 (Carvedilol)		
	Area	%Assay	%Degradation
Acidic	120474	91.6	8.7
Alkaline	124265	92.0	12.7
Photolytic	1123268	874	13.6
Thermal	102475	95.2	14.4
Oxidative	115736	94.3	12.5

SUMMARY AND CONCLUSION

For routine analytical purpose it is desirable to establish methods capable of analyzing huge number of plasma samples in a short time period with good robustness, accuracy and precision without any prior separation step. HPLC method generates large amount of quality data, which serve as highly powerful and convenient analytical tool. A new method was established for estimation of Carvedilol by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Carvedilol by using Agilent column (4.6×150mm) 5µ, flow rate was 1.0 ml/min, mobile phase ratio was di-potassium hydrogen phosphate: meoH (25:75% v/v), detection wavelength was 270 nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, photo diode array detector 996, Empower-software version-2. The retention times were found to be 5.242 mins. The % purity of Carvedilol was found to be 98.56%. The system suitability parameters for Carvedilol such as theoretical plates and tailing factor were found to be 4343.2, 1.6. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study of Carvedilol was found in concentration range of 20µg-100µg and correlation coefficient (r^2) was found to be 0.999, % recovery was found to be 98.96%, % RSD for repeatability was 0.3, % RSD for intermediate precision was 0.8. The precision study was precision, robustness and repeatabilty. LOD value was 0.7 and LOQ value was 0.13. Hence the suggested RP-HPLC method can be used for routine analysis of Carvedilol in API and Pharmaceutical dosage form.

ACKNOWLEDGEMENT

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