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Original Article

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR KNOWN AND UNKNOWN IMPURITIES PROFILING FOR CARVEDILOL PHARMACEUTICAL DOSAGE FORM (TABLETS)

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ABSTRACT

Objective: The aim of the research work to develop a simple, sensitive, rugged, robust and specific novel gradient stability indicating reverse phase HPLC method for quantitative determination of known and unknown impurities profiling of Carvedilol pharmaceutical dosage forms (Tablets).

Methods: Chromatographic separation has been achieved on an Inertsil ODS 3V column (150 mm x 4.6 mm, 5µm) with mobile phase consisting Mobile phase-A (Water, Acetonitrile and Trifluroacetic acid in the ratio of 80:20:0.1 v/v/v respectively and pH adjusted to 2.0 with dilute potassium hydroxide solution) and Mobile phase-B (Water and acetonitrile in the ratio of 100:900 v/v respectively) delivered at flow rate of 1.0 ml min^{-1} and the detection wavelength 240 nm. The column compartment temperature maintained at 40 °C.

Results: Resolution between Carvedilol and its impurities has been achieved greater than 1.5. The developed method was validated as per ICH guidelines. Analytical method found Precise, Linear, accurate, specific, rugged and robust.

Conclusion: Developed and validated novel analytical method can be used to for impurity profile analysis of Carvedilol Pharmaceutical dosage form (Tablets).

Keywords: Carvedilol, Method development, Validation, Impurities, Related substances, ICH guidelines

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INTRODUCTION

Carvedilol chemically it is named, (±)-1-(carbazol-4-yloxy)-3-((2-o-methoxyphenoxy) ethyl) amino)-2-propanol. Carvedilol is a racemic mixture where the S (-) enantiomer is a beta adrenoceptor blocker and the R (+) enantiomer is both a beta and alpha-1 adrenoceptor blocker and is currently used to treat heart failure, left ventricular dysfunction and hypertension [1, 2]. Carvedilol shows a greater antioxidant activity than other commonly used beta-blockers [3, 4]. It has been prescribed as an antihypertensive agent, an antiangina agent [5-8]. The dual action of carvedilol is advantageous in combination therapies as moderate doses of 2 drugs have a decreased incidence of adverse effects compared to high dose monotherapy in the treatment of moderate hypertension [9]. Impurity profiling (known and unknown), generation of degradents and identification of degradents of active pharmaceutical ingredients (API) and pharmaceutical dosage form (tablets) is one of the most challenging tasks to pharmaceutical analytical scientists in pharma industry [10]. The presence of degradants, unknown impurities and unknown chemicals at lower levels may affect therapeutic efficacy as well as the safety of pharmaceutical dosage form. As a result, all drug regulators in different countries have established maximum permissible limits for known and unknown impurities both in Active Pharmaceutical Ingredients and pharmaceutical dosage forms [11, 12]. All the major international pharmaceutical regulators require that the study of known and unknown impurities profiles of drug substances and drug products be performed using a suitable stability indicating validated analytical method [13-17].

The comprehensive literature review found that the several RP-HPLC method reported for determination of assay of Carvedilol alone [18-28] and with combination of other drug [29, 30] and very few methods have been reported to determine related substances [31-34] and metabolites [35] of Carvedilol. These methods used a column oven temperature greater than 40 °C which significantly reduces the life of the HPLC column and therefore is not economically feasible for routine testing of the drug substance or drug product for a long time. These methods are not discussed on Carvedilol EP Impurity-D. The unique feature of this new method is separation of Carvedilol EP Impurity-D from main drug as well as from its known and unknown impurities. The method specified for determining of impurities in drug substance [31, 32] and not for the pharmaceutical dosage form (tablets) that involve excipient interference and challenge of extraction of the drug substance from its excipient with accurate quantification of impurities and its profile.

USP monograph method as well as other method [32] is available for determination of know impurities of drug substance and not for pharmaceutical formulation (tablets). USP method for drug substance involve preparation of standard solution using impurities reference standard which is not cost effective. The method which is reported for Pharmaceutical dose i.e. Tablets formulation discussed only two impurities profile [33]. The USP monograph method is available for Pharmaceutical dose i.e. Tablets formulation but has not been discussed regarding the known impurities profile. The related substance method reported for pharmaceutical formulation [34] involves the use of the impurities standard each time for the preparation of a system suitability solution that is not economically feasible.

The other unique feature of the new method is that it is highly sensitive and superior in terms of the limit of quantification of known and unknown impurities compared to other methods [34]. The chemical name of Carvedilol and its impurities are shown in table 1. The structure of Carvedilol and its impurities are shown in fig. 1, 2, 3, 4 and fig. 5.

Table 1: Chemical name of carvedilol and its impurities

Compound name	Chemical name	Molecular weight
Carvedilol	(±)-1-(Carbazol-4-yloxy)-3-[{2-(0-methoxyphenoxy) ethyl} amino]-2-propanol	406.47
Carvedilol EP Impurity-A	1-(4-(2-Hydroxy-3-(2-(2-methoxyphenoxy) ethylamino) propoxy)-9H-carbazol-9-yl)-3-(2-(2-methoxyphenoxy) ethylamino) propan-2-ol	629.74
Carvedilol EP Impurity-B	3,3'-(2-(2-Methoxyphenoxy)ethylazanediyl)bis(1-(9H-carbazol-4-yloxy) propan-2-ol)	645.74
Carvedilol EP Impurity-C	1-(9H-Carbazol-4-yloxy)-3-(benzyl(2-(2-methoxyphenoxy)ethyl) amino)propan-2-ol	496.6
Carvedilol EP Impurity-D	1-(9H-Carbazol-4-yloxy)-3-[4-[2-hydroxy-3-[[2-(2-methoxyphenoxy)ethyl]amino]propoxy] 9H-carbazol-9-yl] propan-2-ol	645.76

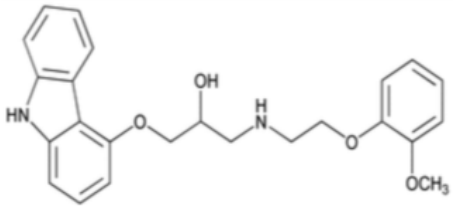

Fig. 1: Carvedilol

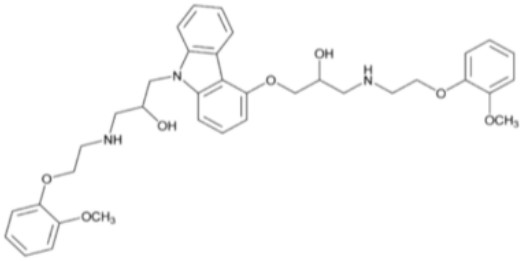

Fig. 2: Carvedilol EP Impurity-A

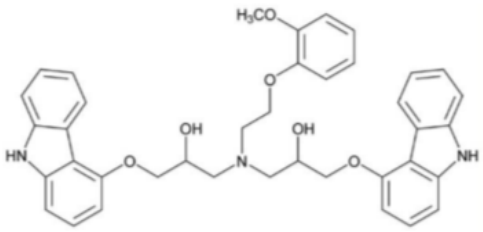

Fig. 3: Carvedilol EP Impurity-B

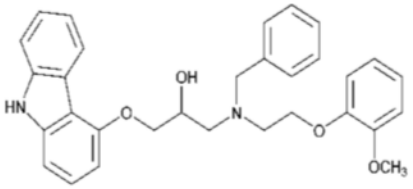

Fig. 4: Carvedilol EP Impurity-C

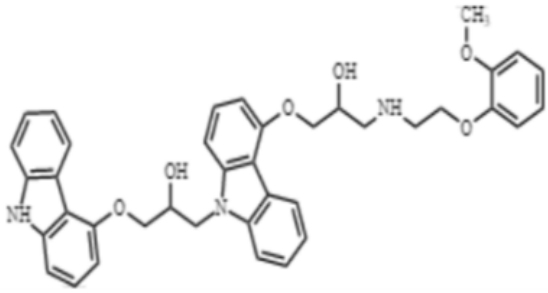

Fig. 5: Carvedilol EP impurity-D

MATERIALS AND METHODS

Regents and materials

Marketed samples of Cholecalciferol tablets were used in developing analytical method and validation of analytical method.

Cholecalciferol associated related substances (impurities) were obtained from OLYMPUS Chemical and Fertilizers from Mumbai, India. Trifluroacetic acid and Acetonitrile was procured from spectrochem Limited, HPLC grade water was obtained from Milli-Q purification system. 0.45 µm PVDF filter used of Merck India make.

Instrumentation

HPLC (Make: Waters) equipped with an integrated autosampler and a quaternary gradient pump was used. The column holder having temperature controlled and an Ultra violet (UV)/Photodiode array detector (PDA) was used for the development and analytical method validation. Chromatographic data was acquired using empower software.

Chromatographic conditions

Inertsil ODS 3V (150 x 4.6) mm, 5 µm column was used. The column holder temperature maintained at 40 °C. The mobile phase consist of a different composition of buffer solution and organic solvents. Mobile Phase-A is mixture of Water, Acetonitrile and Trifluroacetic acid in the ratio 80:20: 0.1 v/v/v and pH of this mixture adjusted to 2.0 with dilute potassium hydroxide solution.

Mobile Phase-B is mixture water and Acetonitrile in the ratio 10:90 v/v.

HPLC gradient programme run mentioned in table 2.

Table 2: Mobile phase programme for gradient elution

Time (min)	Flow (ml min^{-1})	Mobile phase-A (%)	Mobile phase-B (%)
0	1.0	80	20
10	1.0	80	20
30	1.0	60	40
40	1.0	60	40
50	1.0	80	20
60	1.0	80	20

Diluent

Mixture of 0.02M KH_2O_4 buffer pH 2.5 and Acetonitrile in the ratio 65:35v/v

Standard solution preparations

Solution containing 2µg ml^{-1} of Carvedilol standard prepared in diluent.

Sample solution

Accurately weigh and transferred tablets powder equivalent to 25 mg of Carvedilol into 25 ml volumetric flask, Add about 20 ml of diluent sonicated for 30 min with intermittent shaking and make the volume with diluent. Filtered through 0.45 µm PVDF filter after discarding first five ml of filtrate. (Sample concentration: 1000µg ml^{-1})

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Molecular structures of Carvedilol (fig. 1) shows that all the related compounds of Carvedilol are basic in nature. Carvedilol and its known impurities wavelength scan was performed and impurities shows maximum response at 240 nm wavelength, hence this wavelength is chosen for final quantification of impurities.

Carvedilol, Carvedilol EP Impurity-A, Carvedilol EP Impurity-B, Carvedilol EP Impurity-C and Carvedilol EP Impurity–D molecular structure (fig. 1-5 respectively) shows that these structures contains amine groups due to which showing polar in nature properties.

The development of the method began with the purpose of separating all known, unknown and degradents impurities that are generated during stability.

In initial method development experiments C8 stationary phase column with orthophosphoric acid buffer pH 3.5 and methanol was used as mobile phase. The combination of orthophosphoric acid and methanol indicates early elution of impurities and poor resolution of known impurities. Different development trails runs were carried out to increase the retention time, to reduce the baseline drift and improve the resolution of known and unknown impurities. However a better separation between the impurities has not been achieved. Therefore, column C8 was not considered for further development trials.

Apart from stationary phase, the mobile phase, additives and organic modifiers are also considered in further development trials to obtain good resolution, Gaussian peak shape and sharp peak response for impurities.

With the use of the stationary phase column C18 and with the use of different solvents (mobile phase) a better resolution (greater than 1.5) was achieved between known and unknown impurities and also separated from the main drug (Carvedilol).

After extensive study, the method was finalized on Inertsil ODS 3V (150 x 4.6) mm, 5 µm using the mobile phase of variable composition, Mobile phase-A consist of Water, Acetonitrile and Trifluroacetic acid in the ratio of 80: 20:0.1 v/v/v respectively and pH adjusted to 2.0 with dilute potassium hydroxide solution and Mobile phase-B consist of water and acetonitrile in the ratio of 100:900 v/v respectively. The mobile phase maintained at flow rate of 1.0 ml min−1 and column oven temperature kept at 40 °C. The detector wavelength chosen 240 nm at which all impurities shows maximum response. A representative HPLC chromatogram (fig. 8) shows the peaks well resolved with respect to each other.

Fig. 6-13 represent the Blank, Standard, Placebo, Spike sample, Individual known Carvedilol EP Impurity-A, Carvedilol EP Impurity-B, Carvedilol EP Impurity-C and Carvedilol EP Impurity–D chromatograms respectively.

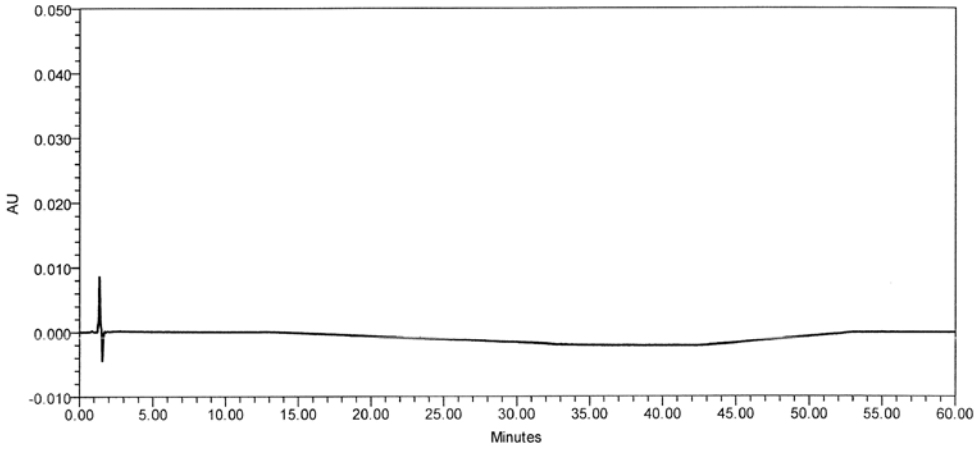

Fig. 6: Typical HPLC chromatogram of blank

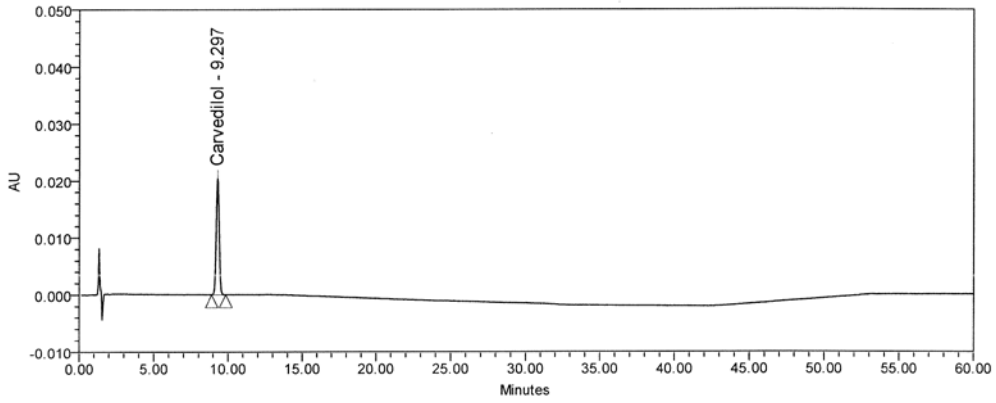

Fig. 7: Typical HPLC chromatogram of standard

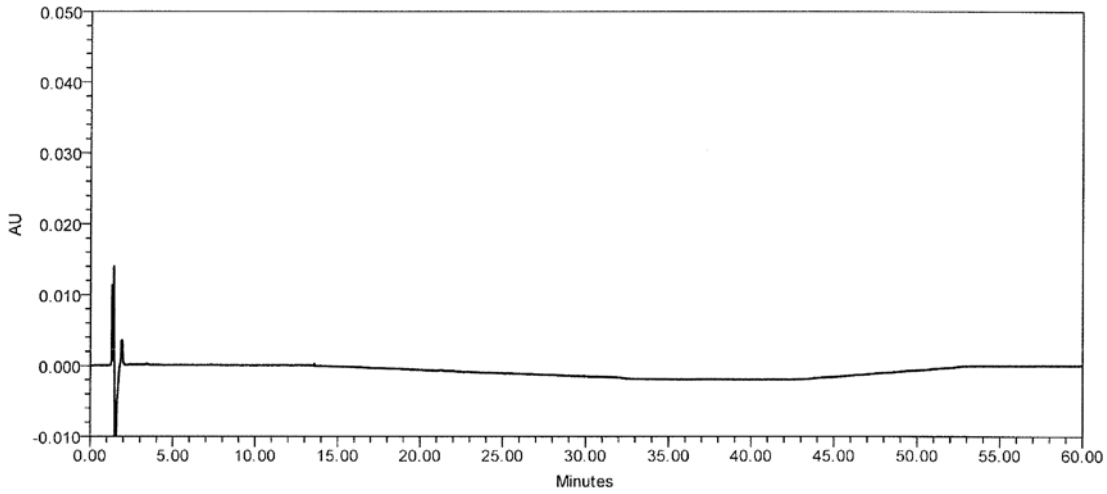

Fig. 8: Typical HPLC chromatogram of placebo solution

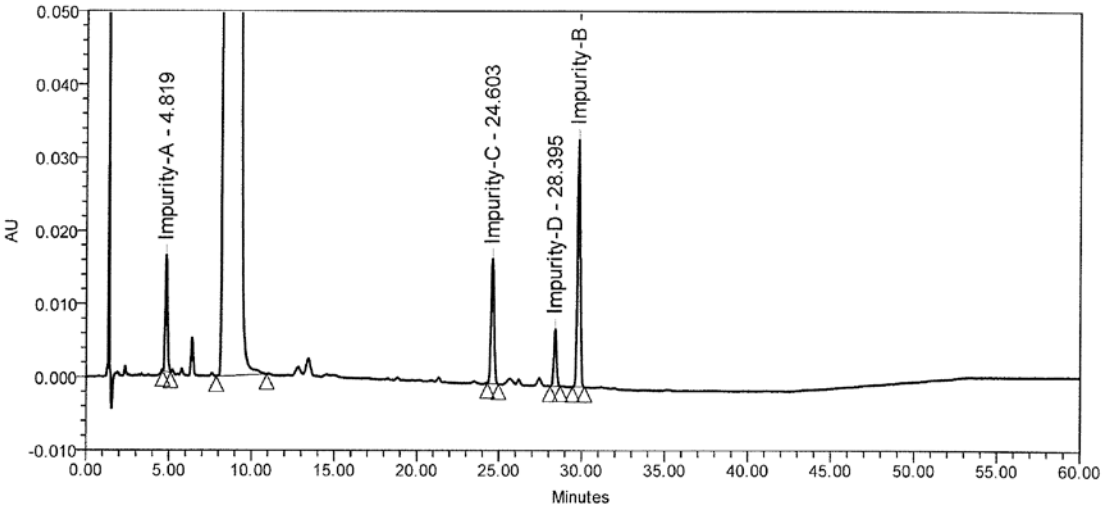

Fig. 9: Typical HPLC chromatogram of spike sample

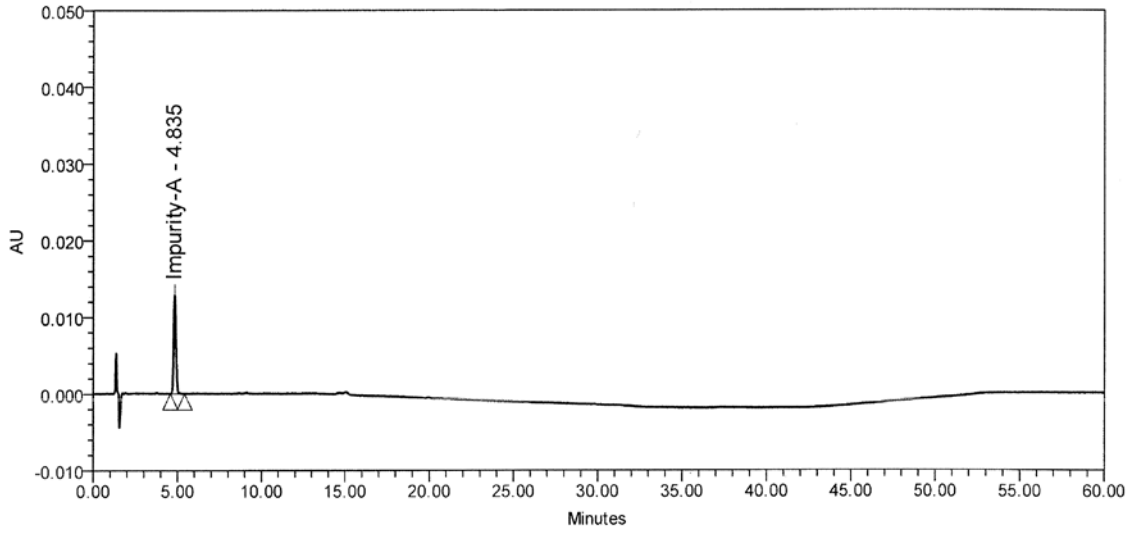

Fig. 10: Typical HPLC chromatogram of carvedilol EP impurity-A

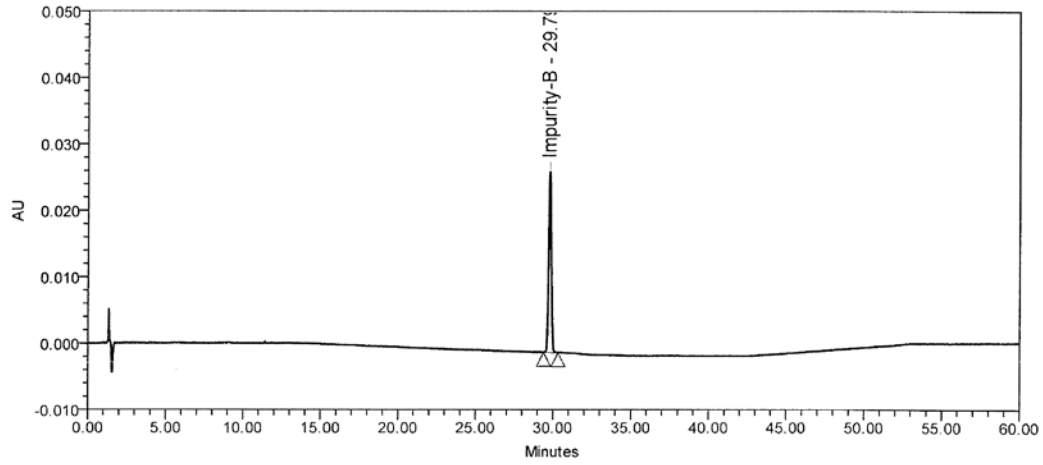

Fig. 11: Typical HPLC chromatogram of carvedilol EP Impurity-B

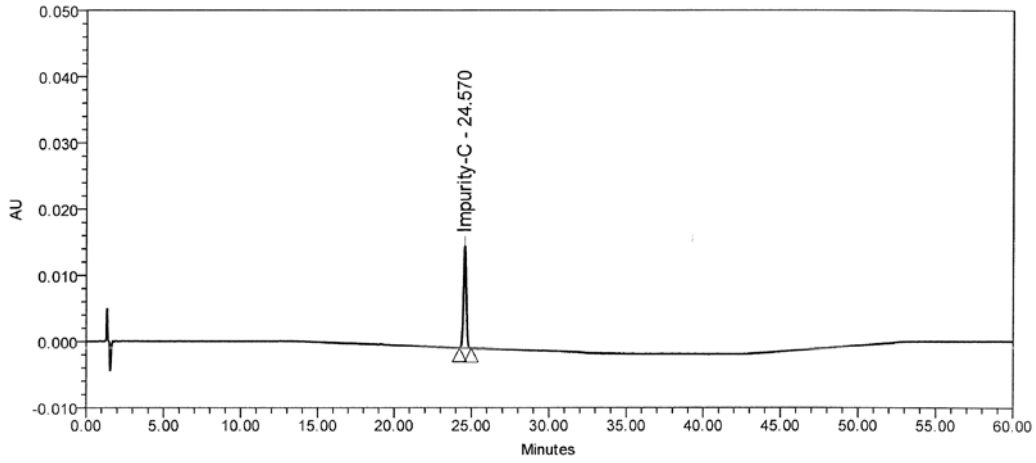

Fig. 12: Typical HPLC chromatogram of carvedilol EP Impurity-C

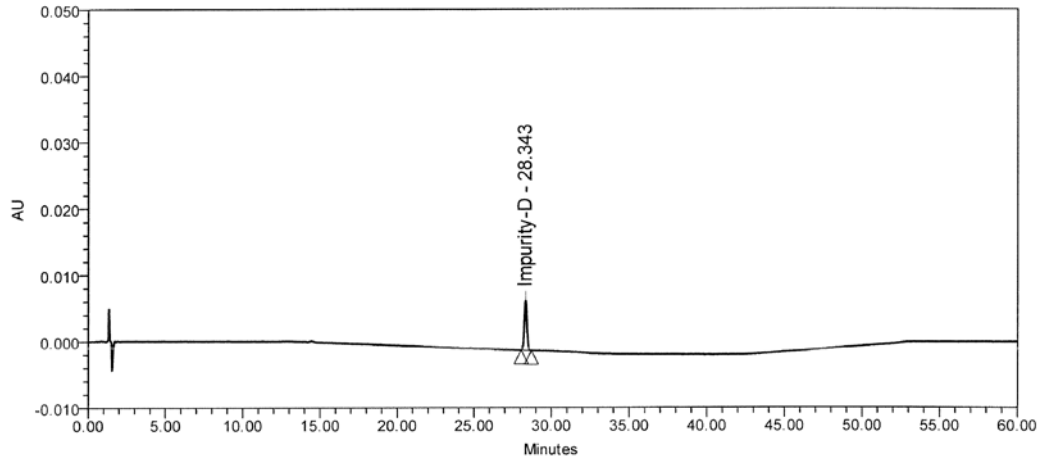

Fig. 13: Typical HPLC chromatogram of carvedilol EP Impurity-D

Solution stability of sample solution and standard solution

The stability of the sample solution and standard solution was verified by injecting the sample solution and standard solution at initial and different regular intervals into the proposed method at room temperature.

The stability of the sample solution was checked on the basis of the formation of additional peaks and no increase in the known and unknown impurity of 0.04 % from its original level.

On verifying the formation of additional peaks, it was found that no additional peaks were formed and no increase of present known and

unknown impurity by 0.04% level from its initial level to till 48 h indicating that the sample solution is stable for about 48 h at room temperature.

The stability of the standard solution was evaluated by monitoring the peak area at different time intervals. % RSD of the peak area was monitored from its initial level to 48 h and found less than 5.0%, indicating that the standard solution is stable for about 48hours at room temperature.

Linearity and range

The test solutions concentration for Carvedilol is 1.0 mg ml^{-1}. Considering the impurities limit levels 0.2 %, response function (peak area) was determined by preparing standard solution of each component (Carvedilol, Carvedilol EP Impurity-A, Carvedilol EP Impurity-B, Carvedilol EP Impurity-C and Carvedilol EP Impurity-D) at different concentration level ranging from lower limit of quantification to 150 % limit level.

The graph of the peak area of the analytes relative to their respective concentrations is plotted and a linear ship was observed and they fitted straight lines responding to equation. Y-intercept bias at 100 % linearity level found less than 5.0 %. The correlation coefficients (r) found greater than 0.99 the acceptance threshold for the quantification of impurity content in bulk drug.

Method found Linear in the range from Limit of Quantification (LOQ) to 150% level, considering the specification level 0.2 % w. r. t. sample concentration. The residuals plot follow the random patterns with the residuals passing the normal distribution test ($p<0.05$), all of which proves that the method is linear in the tested range. The Regression statistics of Linearity experimental results are shown in table 3.

Table 3: Regression statistics of linearity experimental results

Compound	Concentration µg ml^{-1}	Multiple R	Regression equation	F	P-value
Carvedilol	0.04 to 3.00	0.9998	y = 139510.1x-60.0	15.2	0.0009
EP Imp.-A	0.04 to 3.11	0.9999	y = 67384.6x-156.8	15.1	0.0009
EP Imp.-B	0.04 to 3.06	0.9998	y = 156349.4x-455.1	15.1	0.0009
EP Imp.-C	0.04 to 3.14	0.9998	y = 100096.5x-161.1	15.1	0.0009
EP Imp.-D	0.04 to 2.93	0.9998	y = 48648.3x-195.9	14.9	0.0009

Determination of limit of quantification and detection (LOQ and LOD)

Calibration curve method (established from Linearity experiment) used for the determination of Limit of Quantification (LOQ) and Limit of Detection (LOD).

By applying Residual standard deviation (σ) method LOQ and LOD values were predicted. For predicted levels precision and accuracy was established.

The visuals method also considered to assess the signal to noise ratio of analyte peak.

$$LOQ = 10\sigma / s$$

$$LOD = 3.3\sigma / s$$

Where,

σ = residual standard deviation of response

s = slope of the calibration curve

Predicted and precise Limit of Quantification (LOQ) and Limit of Detection (LOD) values are shown in table 4.

Table 4: Limit of quantification, detection, response factor (LOQ, LOD and RF)

Compound	LOQ		LOD		RF
	µg ml^{-1}	%w/w*	µg ml^{-1}	%w/w*	
EP Impurity-A	0.04	0.004	0.013	0.001	2.07
EP Impurity-B	0.04	0.004	0.013	0.001	0.89
EP Impurity-C	0.04	0.004	0.013	0.001	1.39
EP Impurity-D	0.04	0.004	0.013	0.001	2.87
Carvedilol	0.04	0.004	0.013	0.001	1.00

Note: * %w/w calculated w. r. t. sample concentration (1000 µg ml^{-1})

Determination of Response Factor (RF)

The Linear calibration curves for all impurities and main drug (Carvedilol) were derived using the peak areas against their concentrations. The linear regression equation containing slope for all impurities and main drug and their concentration range were summarized in table 3.

The Response Factor (RF) was determined as the ratio of slope of the regression line of main drug component (Carvedilol) to that for each impurity and is listed in table 4.

Accuracy

Accuracy was evaluated by the simultaneous quantification of analytes (impurities) in sample solutions prepared by adding the known amount of impurities to test sample.

The experiment was performed corresponding to four concentration levels at LOQ, 50 %, 100 % and 150 % by considering the impurities specification level i.e. 0.2 %w/w with respect to sample solution concentration.

The samples were prepared in triplicate at each level. The quantification of added impurities was calculated as per methodology by applying RF (response factor) of respective impurity.

The accuracy experimental results shows that approximately 80 % to 120 % recoveries were obtained for all known impurities and % RSD for triplicate test sample preparation of recovery results found less than 10 %. Therefore, based on the recovery data (table 5 to table 9) the quantification of impurities that are mentioned in this report has been demonstrated to be accurate, precise for intended purpose and is adequate for routine analysis.

Table 5: Recovery for carvedilol

Carvedilol	Preparation	% Recovery	Mean	%RSD
LOQ	1	110.0	104.2	5.0
	2	102.5		
	3	100.0		
50%	1	94.0	93.9	0.2
	2	93.7		
	3	93.9		
100%	1	96.5	96.6	0.1
	2	96.6		
	3	96.6		
150%	1	98.1	98.0	0.1
	2	98.0		
	3	98.0		

Table 6: **Recovery for carvedilol EP impurity-A**

Impurity-A	Preparation	% Recovery	Mean	%RSD
LOQ	1	97.5	93.3	4.1
	2	90.0		
	3	92.5		
50%	1	106.7	107.0	0.5
	2	107.7		
	3	106.7		
100%	1	108.2	107.9	0.3
	2	107.7		
	3	107.7		
150%	1	109.9	110.8	0.7
	2	110.9		
	3	111.5		

Table 7: **Recovery for carvedilol EP impurity-B**

Impurity-B	Preparation	% Recovery	Mean	%RSD
LOQ	1	97.6	97.6	4.9
	2	102.4		
	3	92.9		
50%	1	109.5	108.3	1.3
	2	108.6		
	3	106.7		
100%	1	107.6	107.3	0.3
	2	107.1		
	3	107.1		
150%	1	109.6	110.4	0.7
	2	110.5		
	3	111.1		

Table 8: **Recovery for carvedilol EP impurity-C**

Impurity-C	Preparation	% Recovery	Mean	%RSD
LOQ	1	92.9	93.7	3.9
	2	97.6		
	3	90.5		
50%	1	104.8	103.8	0.9
	2	103.8		
	3	102.9		
100%	1	107.1	106.8	0.2
	2	106.7		
	3	106.7		
150%	1	109.8	110.4	0.5
	2	110.5		
	3	110.8		

Method precision and Intermediate precision

Method precision experiment was evaluated by preparing six spike samples preparation by spiking the known impurities (EP Impurity-A, EP Impurity-B, EP Impurity-C and EP Impurity-D) at 0.2 % w/w level with respective of test concentration (1000 µg ml^{-1}).

Intermediate precision has been assessed by different analysts on different HPLC systems, on different columns on different days. The experiment was conducted same as method precision experiment by spiking the known impurities (EP Impurity-A, EP Impurity-B, EP Impurity-C and EP Impurity-D) at 0.2 %w/w level with respective of test concentration.

From the Method precision and Intermediate precision experiment results the method was found precise. Results (%w/w) were calculated for known, unknown and total impurities for method precision and intermediate precision experiment. %RSD was calculated for (%w/w) known, unknown and total impurities and found less than 10%. Overall %RSD for (%w/w) known, unknown and total impurities was calculated for method precision and intermediate precision experiment results (n=12 results, six from method precision and six from intermediate precision) and found less than10.0 %.

The results for method precisions and intermediate precision were listed in table 10 to table 12 reveal that the method has good reproducibility with acceptable precision.

Table 9: **Recovery for carvedilol EP impurity-D**

Impurity-D	Preparation	% Recovery	Mean	%RSD
LOQ	1	92.1	96.5	7.9
	2	92.1		
	3	105.3		
50%	1	102.0	100.3	1.5
	2	100.0		
	3	99.0		
100%	1	102.0	102.0	0.0
	2	102.0		
	3	102.0		
150%	1	104.3	104.7	0.3
	2	104.7		
	3	105.0		

Table 10: Comparison of method precisions and Intermediate precision results

Name	MP*	IP*	MP*	IP*
	Imp-A	Imp-A	Imp-B	Imp-B
Spike sample-1	0.20	0.20	0.20	0.21
Spike sample-2	0.20	0.20	0.20	0.21
Spike sample-3	0.20	0.20	0.20	0.21
Spike sampel-4	0.20	0.20	0.20	0.21
Spike sample-5	0.20	0.20	0.20	0.21
Spike sample-6	0.20	0.20	0.20	0.21
Mean	0.20	0.20	0.20	0.21
%RSD	0.0	0.0	0.0	0.0
Overall Mean	0.20		0.21	
Overall %RSD	0.0		2.5	

MP*: Method precision IP*: Intermediate precision.

Table 11: Comparison of method precisions and intermediate precision results

Name	MP*	IP*	MP*	IP*
	Imp-C	Imp-C	Imp-D	Imp-D
Spike sample-1	0.21	0.21	0.19	0.21
Spike sample-2	0.21	0.21	0.19	0.21
Spike sample-3	0.21	0.21	0.18	0.21
Spike sampel-4	0.21	0.22	0.19	0.21
Spike sample-5	0.21	0.22	0.19	0.21
Spike sample-6	0.21	0.21	0.19	0.21
Mean	0.21	0.21	0.19	0.21
%RSD	0.0	2.4	2.2	0.0
Overall Mean	0.21		0.20	
Overall %RSD	1.8		5.8	

MP*: Method precision IP*: Intermediate precision.

Table 12: Comparison of method precisions and intermediate precision results

Name	MP*	IP*	MP*	IP*
	Unknown imp.(SM*)	Unknown imp.(SM*)	Total impurities	Total impurities
Spike sample-1	0.12	0.12	0.92	0.95
Spike sample-2	0.11	0.12	0.91	0.95
Spike sample-3	0.12	0.12	0.91	0.95
Spike sampel-4	0.12	0.12	0.92	0.96
Spike sample-5	0.11	0.12	0.91	0.96
Spike sample-6	0.11	0.12	0.91	0.95
Mean	0.12	0.12	0.91	0.95
%RSD	4.8	0.0	0.6	0.5
Overall Mean	0.12		0.93	
Overall %RSD	3.8		2.3	

MP*: Method precision IP*: Intermediate precision. SM*: Single max

Robustness

The robustness of the method has been demonstrated by verifying the system suitability parameters which meet the predefine acceptance criteria.

By making deliberate change in chromatographic conditions, i.e. change inflow rate by ±0.1 ml min^{-1}, change in column oven temperature by+5 °C, change in organic composition of mobile phase-A and mobile phase-B by+2% absolute and change in wavelength by+5 nm, system suitability parameters were verified for each above mentioned conditions.

System suitability criteria of method was meet at different robustness condition, Hence the method is robust over an acceptable working range of its HPLC operational conditions.

CONCLUSION

Analytical Method validation experimental results found within predefine acceptance criteria which prove that the method is linear in proposed working range, accurate and precise. There is no interference observed from blank, excipient for known and unknown impurities which prove that the method is specific and stability indicating in nature. The method is found robust for change in flow rate, change in column oven temperature, change in organic composition of mobile phase-A and mobile phase-B and change in wavelength.

Hence the proposed reverse phase HPLC analytical method can be used for impurity profiling of known, unknown impurities and degradent (which is formed during stability) analysis as well as routine analysis for Carvedilol tablets.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declare none

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