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HPLC METHOD VALIDATION FOR 4-(2,3-EPOXYPROPOXY) CARBOZOLE CONTENT IN CARVEDILOL

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ABSTRACT

High Performance Liquid Chromatography (HPLC) method validation was performed for the determination of 4-(2,3-epoxypropoxy) carbazole content in carvedilol. The validation method utilizes an Inertsil ODS-3V (250X4.6mm), 5µ column at 40 ^{o}C, isocratic elution with Buffer: Acetonitrile (50:50) as the mobile phase. The mobile-phase flow rate was 1.0 mL min^{-1}. The linearity, range, system precision, method precision, method ruggedness are found to be satisfactory. Therefore the method is assumed to be selective for the determination of 4-(2,3-epoxypropoxy)carbazole content in carvedilol by HPLC. This study showed that 4-(2,3-epoxypropoxy)carbazole peak was well resolved from the other known impurities and carvedilol, the purity angle of 4-(2,3-epoxypropoxy)carbazole is less than the purity threshold and there is no blank interference at the retention time of 4-(2,3-epoxypropoxy)carbazole. Therefore the method is determined to be specific, as judged by resolving 4-(2,3-epoxypropoxy)carbazole content in carvedilol by HPLC.

KEYWORDS: HPLC,Carvedilol,Carbozole.

INTRODUCTION

Carvedilol, which is chemically known as (±)-1-9H-(carbazol-4-yloxy)-3-[[2-(2-methoxyphenoxy)ethyl]amino]-2-propanol, is an antihypertensive agent with β- and α_1-adrenergic receptor blocking activities.[1-3] Carvedilol has much greater antioxidant activity than other commonly-used β-blockers.[4-5] It has been prescribed as an antihypertensive agent and an angina agent.[6-7] and for treatment of congestive heart failure.[8] High-performance liquid chromatography (HPLC) with fluorescence detector.[9-14], mass spectrometer.[15-16] or electrochemical detection has been used for the analysis of carvedilol and its enantiomers in

biological samples. Determination of cavedilol by capillary electrophoresis has also been reported.[17] The literature survey revealed that there is no method validation that has been reported for 4-(2,3-epoxypropoxy)carbazole content in carvedilol by HPLC.[18] Hence, it was considered worthwhile to validate suitable method for an assay of 4-(2,3-epoxypropoxy)carbazole (**Figure 1**) in carvedilol in the present work.

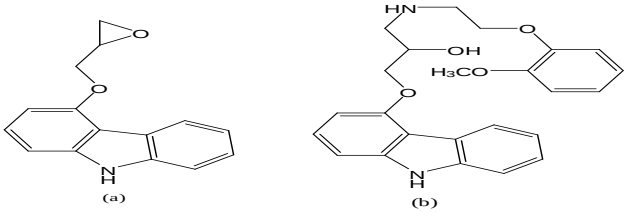

Figure 1. Structure of (a) Carvedilol (b) 4-(2,3-Epoxypropoxy)carbazole.

MATERIALS AND METHODS

Chemicals, Reagents and Samples

The chemicals, equipment and samples which were used in the present stduy was given in the following Tables 1-3.

Table 1: List of chemicals.

Name	Grade	Make	Lot No/ B.No.	Assay/Purity (%)
Potassium dihydrogen orthophosphate	GR	Merck	MH8M581870	99.5
Acetonitrile	HPLC	Rankem	R162J09	99.8
Water	Milli-Q	-	-	-

Table 2: List of equipments.

Name of the Instrument	Make	Model
HPLC	Waters	2695 model pump with 2489 UV detector
HPLC	Waters	2695 model pump with 2489 UV detector
HPLC	Waters	2695 model pump and 2998 PDA detector
HPLC	Waters	2695 model pump with 2489 UV detector
Electronic balance	Stratories	CP224S

Table 3: List of the samples.

Name	Grade	Lot No/B.No	Assay/Purity (%)
Carvedilol-impurity-A	IH*	A019/091	98.11
Carvedilol-impurity-B	IH*	A012/123	99.14
Carvedilol-impurity-C	IH*	A012/171	98.45
4-(2,3-epoxypropoxy) carbozole	IH*	A019/073	99.43
Carvedilol-sample	IH*	B.No: PP04	--
Carvedilol-sample	IH*	B.No:PP05	--
Carvedilol-sample	IH*	B.No: PP06	--

Chromatographic conditions

The chromatographic condition used in the present study were given in the following Table 4.

Table 4: Chromatographic conditions.

Column	**Inertsil ODS-3V, (250 X 4.6) mm, 5µ (or) its equivalent Make: GL sciences, Part No: 5020-01802**
Flow	1.00 mL/min
Column oven temperature	40°C
Detector wavelength	UV at 240 nm
Injection volume	20 µL
Run time	30 minutes
Elution	Isocratic
Diluent	Buffer: Acetonitrile (50:50)

Buffer preparation

Weighed and transfered about 1.36 grams of potassium dihydrogen orthophosphate in to 1000 mL of milli-Q and filtered the buffer using 0.22µ membrane filter paper.

Mobile phase preparation

Mobile phase was prepared by mixing buffer and acetonitrile in the ratio of 50:50 (v/v).

Preparation of Standard and System Suitability Solution

Weighed and transfered about 10.00 mg of -(2,3-epoxypropoxy) carbozole into a 100 mL volumetric flask. Added 5.0 mL of diluent to dissolve and made up to the volume with diluent. Transfered 7.5 mL of above solution in to a 100 mL volumetric flask and made upto mark with diluent. Further dilutions have been used were 1.0 mL to 100.0 mL with diluent.

Evaluation of system suitability

Injected blank in duplicate (Table 5), followed by standard solution (five times) in ultra performance liquid chromatography and evaluated the chromatogram. The system is suitable for analysis if and only if, the percentage relative standard deviation of five replicate injections in standard solution for 4-(2,3-epoxypropoxy) carbozole peak should not be more than 1.00.

Sample preparation

Accurately weighed and transfered about 50.00 mg of carvedilol in to 10.0 mL volumetric flask added 2.0 mL of diluent to dissolve and made upto mark with diluent. Prepared the sample solution in duplicate (Labeled as Sample preparation-1 and 2).

Procedure

If the system suitability passes, inject the sample preparations 1 and 2 (Order of injections as specified) and record the chromatograms. The approximate retention time for 4-(2,3-epoxypropoxy) carbozole is about 12.8 minutes.

Table 5: Order of Injection.

Name of the preparations	No. of injections	Purpose
Blank	2	Blank
Standard preparation	5	System suitability / Quantification
Sample preparation-1	1	Sample analysis
Sample preparation-2	1	Sample analysis

Calculations

4-(2,3-Epoxypropoxy) carbozole content was calculated with individual injection and report the average of two injections using the following formulae given below.

4-(2,3-epoxypropoxy) carbozole = *[(AT/AS) × (WS/10) × (1/100) × (1/100) × (P/100)] × 1000000*

AT = Area of 4-(2,3-epoxypropoxy) carbozole peak area obtained in sample preparation, AS = Average area of the 4-(2,3-epoxypropoxy) carbozole peak area obtained in system suitability solution preparation, WT = Weight of sample in mg, WS = Weight of standard in mg, P = Purity/ assay of 4-(2,3-epoxypropoxy) carbozole standard and Acceptance criteria: 4-(2,3-epoxypropoxy) carbozole (Not more than-15 ppm).

RESULTS AND DISCUSSION

Selectivity/Specificity

Each known impurity (Impurity A, Impurity B, Impurity C and 4-(2,3-epoxypropoxy) carbozole) solution was prepared individually and a solution of 4-(2,3-epoxypropoxy) carbozole spiked with the carvedilol at 0.20% level and as well as Carvedilol sample was also prepared. All these solutions were analyzed by using the PDA detector as per the HPLC method described in the protocol.

Table 7: Summary of retention time (RT), for carvedilol and its related impurities and the peak purity values of 4-(2,3-epoxypropoxy) carbozole.

Peak name	Retention time (minutes)	Peak purity	
		Purity angle	Purity threshold
Impurity-A	1.915	---	---
Carvedilol	2.843	---	---
Epoxypropoxy carbozole	14.180	0.679	1.635
Impurity-C	14.721	---	---
Impurity-B	15.511	---	---

This study showed that 4-(2,3-epoxypropoxy) carbozole peak was well resolved from the other known impurities and Carvedilol, the purity angle of 4-(2,3-epoxypropoxy) carbozole is less than the purity threshold and there is no blank interference at the retention time of 4-(2,3-epoxypropoxy) carbozole peak. Therefore the method is selective for the determination 4-(2,3-epoxypropoxy) carbozole content in Carvedilol by HPLC. The criteria for acceptance which include a) Peak should be homogeneous and there should be no coeluting peaks. b) Peak purity of analyte should pass. For peak purity of analyte, purity angle should be less than the purity threshold. c) No blank interference should be at the retention time of 4-(2, 3-epoxypropoxy) carbozole.

Limit of Detection (LOD)

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified. The limit of detection was determined as the lowest concentration for which the response is approximately three times greater than the baseline noise. The limit of detection is determined by calculating the signal to noise ratio and by comparing test results from samples with known concentrations of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. The result obtained for 4-(2,3-epoxypropoxy) carbozole peak is listed in table 2.

Table: Limit of detection for 4-(2,3-epoxypropoxy) carbozole

Component name	4-(2,3-epoxypropoxy) carbozole
Purity (%)	99.43
Weight taken (mg)	10.1
Dilution for LOD solution	Weight taken → 100 mL; 7.5mL → 100 mL; 1.0 mL → 100 mL; 1.0 mL→10 mL; 3.3 mL to 10 mL with diluent.
Conc. (mg/mL) (w.r.to purity of 4-(2,3-epoxypropoxy) carbozole)	0.0000025
LOD with respect to sample conc. (PPM)	0.50
Signal to Noise ratio	3.1:1
Reported LOD (PPM)	0.5

As shown in the table-2, the S/N ratio (LOD) value obtained was about 3.1:1 for 4-(2,3-epoxypropoxy) carbozole (0.5 PPM). The acceptance criteria of signal to noise ratio should be ≥ 2:1.

Limit of Quantitation (LOQ)

The Limit of quantitation (LOQ) values was determined from the same experiment as mentioned in the limit of detection section. Based on the limit of detection, roughly three folds of limit of detection solution was prepared and analyzed for the determination of limit of quantitation. The limit of quantitation is determined by calculating the signal to noise ratio and by comparing test results from samples with known concentrations (approx 3.0 folds to limit of detection) of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably quantified. The result obtained for 4-(2,3-epoxypropoxy) carbozole is listed in Table 8. The acceptance criteria is that signal to noise ratio should be ≥ 10:1 and the quantitation limit should be less than level of specification, preferably much less.

Table 8: Limit of quantitation for 4-(2,3-epoxypropoxy) carbozole

Component name	**4-(2,3-epoxypropoxy) carbozole**
Purity (%)	99.43
Weight taken (mg)	10.1
Dilution for LOQ solution	Weight taken → 100 mL; 7.5mL → 100 mL; 1.0 mL → 100 mL; 1.0 mL→10 mL with diluent.
Conc. (mg/mL) (w.r.to purity of 4-(2,3-epoxypropoxy) carbozole)	0.0000075
LOQ with respect to sample conc. (PPM)	1.50
Signal to Noise ratio	10.2:1
Reported LOQ (PPM)	1.5

Precision at LOQ

The repeatability expresses the precision under the same operating conditions over a short interval of time. It expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample. The precision at LOQ was performed by analysing six replicate injections of LOQ level solution (concentration). A result of peak area of 4-(2,3-epoxypropoxy) carbozole is summarized in Table 9. The acceptance criteria has been given to the percentage relative standard deviation for the peak area of 4-(2, 3-epoxypropoxy) carbozole at LOQ level and it should be less than 15.00. The percentage relative standard deviation for the peak area of 4-(2,3-epoxypropoxy) carbozole

obtained was 0.81 at the LOQ level and it indicates acceptable precision of the limit of quantitation.

Table 9: Summary of peak areas at LOQ level

Injection No.	4-(2,3-epoxypropoxy) carbozole peak area at LOQ level
1	1836
2	1808
3	1796
4	1813
5	1806
6	1827
Average peak area	1814
SD	14.6788
%RSD	0.81

Linearity

The linearity of the HPLC method was demonstrated for 4-(2, 3-epoxypropoxy) carbozole solutions ranging from LOQ to 250.0% of the specification limit. Results obtained are shown in Table 10 shows the line of best fit for peak area versus concentration for 4-(2, 3-epoxypropoxy) carbozole. The acceptance criteria is based on the working standards such as 1) No apparent non-linearity should be observed graphically for 4-(2, 3-epoxypropoxy) carbozole, 2) The correlation co-efficient (R) should not be less than 0.9884 and 3) Report the slope and intercept values. As shown in the Figure 8, the linearity results for 4-(2, 3-epoxypropoxy) carbozole in the specified concentration range were found satisfactory, with a correlation coefficient (R) greater than 0.9900.

Table 10: Linearity for 4-(2, 3-epoxypropoxy) carbozole.

Component name	**4-(2, 3-epoxypropoxy) carbozole**		
Purity (%)	99.43		
Weight taken (mg)	10.1		
Stock solution : Weight taken (mg)→100 mL; 7.5 mL→100.0 mL with diluent.			
Levels	Dilution	Conc.(PPM) (w.r.to purity & sample conc)	Average peak area of 4-(2, 3-epoxypropoxy) carbozole
LOQ level	1.0 mL of stock solution→100mL →1.0 mL→10mL with diluent	1.51	2035
30.0 % level	0.30 mL of stock solution→100mL with diluent	4.52	5645
50.0 % level	0.50 mL of stock solution→100mL with	7.53	9723

	diluent		
100.0 % level	1.00 mL of stock solution→100mL with diluent	15.06	19468
120.0 % level	1.20 mL of stock solution→100mL with diluent	18.08	24279
200.0 % level	2.00 mL of stock solution→100mL with diluent	30.13	44256
250.0 % level	2.50 mL of stock solution→100mL with diluent	37.66	47793

Regression statistics

Slope (1345.0723), Intercept (-114.0464), Correlation coeffcient (R) (0.9884) and Coefficient of determination (R^2) (0.9942)

Accuracy

The accuracy of the method was determined using four solutions containing Carvedilol spiked with the 4-(2,3-epoxypropoxy) carbozole at approximately LOQ, 100%, 120.0% and 250.0% of the working concentration.

The percentage recovery results obtained for 4-(2,3-epoxypropoxy) carbozole are listed in Table 11. Report percentage recovery and percentage relative standard deviation for each level. The percentage recovery calculated should be in the range of 95.54 to 100.66.

The percentage relative standard deviation of the recoveries obtained for 4-(2,3-epoxypropoxy) carbozole should be less than 15.00. The percentage recovery values obtained for 4-(2,3-epoxypropoxy) carbozole were in the range of 0.76 to 1.68. The percentage relative standard deviation values of recoveries obtained for 4-(2,3-epoxypropoxy) carbozole were in the range of 0.76 to 1.68. The acceptance criteria was sucessfully fulfilled.

Table 11: Summary of percentage recoveries for 4-(2,3-epoxypropoxy)carbozole.

Level	Theoretical conc. (PPM)	Measured conc. (PPM)	% Recovery	Average	SD	% RSD
LOQ	1.51	1.50	99.34	99.12	1.6663	1.68
	1.51	1.52	100.66			
	1.51	1.47	97.35			
100.0 %	15.06	14.65	97.28	97.83	0.739	0.76
	15.06	14.69	97.54			
	15.06	14.86	98.67			
120.0 %	18.08	18.14	100.33	99.54	0.8991	0.90
	18.08	18.03	99.72			
	18.08	17.82	98.56			
250.0 %	37.66	37.12	98.57	97.21	1.5396	1.58
	37.66	36.73	97.53			
	37.66	35.98	95.54			

Range

Range of the method is determined from the linearity and accuracy data. The range of the 4-(2,3-epoxypropoxy) carbozole impurity was found in between 1.51 PPM (10.1 %) to 37.66 PPM (251.1%), i.e. LOQ to 250.0 % level. The range should be about LOQ to 250.0 % with respect to the working concentration.

Precision

The repeatability expresses the precision under the same operating conditions over a short interval of time. It expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample.

System precision

The system precision was performed by ten replicate injections of a standard solution at 100.0 % of the specified limit with respect to the working concentration. Result of peak area for 4-(2,3-epoxypropoxy) carbozole for ten replicate injections is summarized in Table 12. The percentage relative standard deviation of peak area of ten replicate injections for peak area of 4-(2,3-epoxypropoxy) carbozole should not be more than 10.00. The percentage relative standard deviation for the peak area of the 4-(2,3-epoxypropoxy) carbozole was 0.22 at the working concentration.

Method precision

The precision of the method was determined by analyzing a sample of carvedilol spiked with 4-(2,3-epoxypropoxy) carbozole at 100% of the specification limit (Six replicate spiked sample preparations). Results obtained are summarized in Table 13.

The percentage relative standard deviation for 4-(2,3-epoxypropoxy) carbozole content (PPM) level in 6 preparations should not be more than 15.00. The percentage relative standard deviation for the 4-(2,3-epoxypropoxy) carbozole (carvedilol spiked with 4-(2,3-epoxypropoxy) carbozole at 15.0 ppm level six times at the specification level) was 1.10 at the working concentration. The acceptance criteria was sucessfully fulfilled.

Table 13: Summary of results for precision of the method.

Preparation No (Spiked samples)	4-(2,3-epoxypropoxy) carbozole (PPM)
1	14.6
2	14.7
3	14.8
4	15.0
5	15.0
6	14.9
Average (PPM)	14.8
SD	0.1633
% RSD	1.10
Preparation No (Spiked samples)	4-(2,3-epoxypropoxy) carbozole (PPM)

Ruggedness (Intermediate precision):

Evaluating the variability of the results obtained for 4-(2,3-epoxypropoxy) carbozole with the analysis of Carvedilol solution spiked with 4-(2,3-epoxypropoxy) carbozole six times at the specification limit by different analysts, using different columns on different days and assessed the method ruggedness. Results are summarized in Table 14.

The overall percentage relative standard deviation for 4-(2,3-epoxypropoxy) carbozole content (PPM) level in 12 preparations (method precision and intermediate precision) should not be more than 15.0.

The percentage relative standard deviation for 4-(2,3-epoxypropoxy) carbozole content [carvedilol spiked with 4-(2,3-epoxypropoxy) carbozole (method preparation and intermediate precision) at the specification level] obtained were in the range of 1.10 to 1.14 at the working concentration.

The overall percentage relative standard deviation for 4-(2,3-epoxypropoxy) carbozole content (ppm level) in 12 preparations obtained was 2.04. The acceptance criteria was sucessfully fulfilled.

Table 14: Results of ruggedness data.

Sample ID	Analyst (1) /Day (1)/instrument (1) [4-(2,3-epoxypropoxy) carbozole impurity(PPM)] (Method precision)	Analyst (2) /Day (2)/ instrument (2) [4-(2,3-epoxypropoxy) carbozole -impurity(PPM)] (Intermediate precision)
Sample-1	14.6	15.4
Sample-2	14.7	15.3
Sample-3	14.8	15.1
Sample-4	15.0	15.6
Sample-5	15.0	15.4
Sample-6	14.9	15.2
Average (PPM)	**14.8**	15.3
SD	**0.1633**	**0.1751**
%RSD	**1.10**	**1.14**
Over all % RSD (12 preparations)	**2.04**	

CONCLUSION

A robust and sensitive HPLC method was validated for the determination of 4-(2,3-epoxypropoxy) carbozole content in carvedilol. The method employs isocratic elution HPLC with PDA detection. The injection precision, linearity, LOQ, LOD, selectivity, accuracy, ruggedness and stability were evaluated and found to be satisfactory. The method can be use routinely to ensure the quality of manufactured carvedilol.

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