



“Stability indicating method development for the estimation of diloxanide furoate in marketed formulation”

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Abstract: Analytical method development and validation plays an important role in the discovery, development and manufacture of pharmaceuticals. These methods used to ensure the identity, purity, potency and performance of drug products. In the present study, a successful attempt was made for the HPLC quantitative estimation of Diloxanide furoate in bulk formulation. The method was developed by experimentation based on thorough literature survey and ascertained by statistical parameters of sampling. The entire work was performed on waters HPLC with U.V. Vis detector. The result obtained shows the developed method to be precise, simple, rapid and accurate. Thus these can be used for routine analysis of Diloxanide furoate in bulk drug dosage form. It was thus, concluded that the proposed method is new, simple, accurate, safe, free from pollution, precise and can be successfully employed in the routine analysis.

Index Terms - Diloxanide furoate, HPLC Method validation, Method development, Stability study.

I. INTRODUCTION

Introduction: Analytical method development and validation plays an important role in the discovery, development and manufacture of pharmaceuticals. These methods used to ensure the identity, purity, potency and performance of drug products. There are many factors to consider when developing methods. 1. High-performance liquid chromatography is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, or ion exchange processes, depending upon the type of stationary phase used. 7

2. Material Methods

2.1 Characterization and identification of Diloxanide furoate

2.1.1 Physicochemical characteristics

It refers to the evaluation by sensory characters-taste, appearance, odor, feel of the drug, etc.

2.1.2 Solubility

Solubility of the drug was determined by taking some quantity of drug (about 1-2 mg) in the test tube separately and added the 5 ml of the solvent (Water, Ethanol, Methanol, 0.1 N HCl, 0.1 N NaOH, Chloroform and Acetonitrile). Shake vigorously and kept for some time. Note the solubility of the drug in various solvents (at room temperature). Solubility of Diloxanide furoate was established by I.P. method. Results of solubility shown in Table 2.1.

2.1.3 Determination of melting point

A small quantity of powder was placed into a fusion tube. That tube was placed in the melting point determining apparatus (Chemline, CL-725) containing castor oil. The temperature of the castor oil was gradual increased automatically and read the temperature at which powder started to melt and the temperature when all the powder gets melted.

2.1.4 Identification using FTIR spectrum

IR absorption spectra of Diloxanide furoate was obtained by KBr pellet method. Spectra of pure Diloxanide furoate shown in the Figure 7.

2.2 Chemicals and solvents

2.2.1 Instruments

- A Labindia (3000 plus) spectrophotometer with 1cm quartz cells.
- The HPLC system consisted of a waters pump, a U.V. Visible detector, a Thermo C18 (250 X 4.60 mm), 5µm column, and data ace software.

2.3 In house physical mixture of Diloxanide furoate

Diloxanide furoate 200 mg

2.4 Analytical method development by HPLC: -

2.4.1 Mobile Phase Selection

Initially to estimate Diloxanide furoate number of mobile phase in different ratio were tried⁴⁶. Results were shown in Table 7.5. Taking into consideration the system suitability parameter like RT, Tailing factor, No. of theoretical plates and HETP, the mobile phase found to be most suitable for analysis was 20mM KH₂PO₄: Acetonitrile, pH 3.8 (with orthophosphoric acid) 25:75 v/v. The mobile phase was filtered through 0.45µ filter paper to remove particulate matter and then degassed by sonication. Flow rate employed for analysis was 1.0 ml/min.

2.4.2 Selection of wavelength

10 mg of Diloxanide furoate was weighed accurately and transferred to a 100ml volumetric flask, and the volume was adjusted to the mark with the mobile phase. From above solutions of 0.1 ml was transferred to 10 ml volumetric flasks, and make up the volume up to mark⁴⁷. Resulting solution was scanned over UV range (200-400nm), maximum absorbance was found at Lambda max 254.0 nm.

2.4.3 Selection of Separation Variable

Standard drug solution of Diloxanide furoate was prepared in different mobile phase and chromatograph was recorded by using different column (5 and 10 µm) at different chromatographic condition like different flow rate and temperature. Considering the theoretical facts and after several trials separation variables were selected which were constant during whole experiment.

2.4.4 System Suitability Parameters

Separation variables were set and mobile phase was allowed to saturate the column at 1.00 ml/min⁴⁸. After complete saturation of column, three replicates of working standard of Diloxanide furoate 10 µg/ml was injected separately. Peak report and column performance report were recorded for all chromatogram.

2.4.5 Preparation of Standard Stock Solution

10mg of Diloxanide furoate was weighed accurately and transferred to separate 10ml volumetric flask, and the volume was adjusted to the mark with the methanol to give a stock solution of 1000ppm.

2.4.6 Preparation of Sub stock Solution From stock solutions of Diloxanide furoate.

1 ml was taken and diluted up to 10 ml. from this solution 0.5, 1.0, 1.5, 2.0, 2.5 ml solutions were transferred to 10ml volumetric flasks and make up the volume up to 100 ml with methanol, gives standard drug solution of 5, 10, 15, 20, 25 µg/ ml concentration.

2.4.7 Preparation of the Calibration Curves of the Drug

Standard drug solutions were injected 3 times and the mean peak area of drug was calculated and plotted against the concentration of the drug. The regression equation was found out by using this curve. A typical chromatogram and the calibration curve are shown in figure.

2.4.8 Analysis of tablet formulation for analysis of the tablet formulation

Weight equivalent to weight 10 mg of Diloxanide furoate was transferred to 10 ml volumetric flask and dissolved in mobile phase⁵⁰⁻⁵¹. The solution was shaking vigorously for 20 mins and filtered through Whatman filter paper no.41, then volume was made up to mark with mobile Experimental Work Page 31 phase. From the above solution 1 ml of solution was taken and diluted to 10 ml with mobile phase to get a solution containing 100µg/ml. From the above solution 1 ml of solution was taken and diluted to 10 ml with mobile phase to get a solution containing 10 µg/ml of Diloxanide furoate. The amounts of Diloxanide furoate in tablet formulation were calculated by extrapolating the value of area from the calibration curve. Analysis procedure was repeated six times with tablet formulation. Result is shown in Table 7.8.

2.5 Validation

2.5.1 Linearity:

Linearity of analytical procedure is its ability (within a given range) to obtain test, which are directly proportional to area of analyte in the sample. The calibration plot was constructed after analysis of five different (from 5 to 25 µg/ml) concentrations and areas for each concentration were recorded three times, and mean area was calculated⁵². The regression equation and correlation coefficient of curve are given and the standard calibration curve of the drug is shown in figure 7.4. From the mean of AUC observed and respective concentration value, the response ratio (response factor) was found by dividing the AUC with respective concentration.

2.5.2 Accuracy

Recovery studies were performed to validate the accuracy of developed method. To preanalysed sample solution, a definite concentration of standard drug (80%, 100%, and 120%) was added and then its recovery was analyzed⁵³.

2.5.3 Precision

(A) Repeatability

Standard dilutions were prepared and three replicates of each dilution were analyzed in same day for repeatability and results were subjected to statistical analysis⁵⁴. Standard dilutions were prepared and three replicates of each dilution were analyzed in different days and by different analysts. Statistical analysis was carried out. Experimental Work Page 32

(B) Intermediate Precision

(a) Day to Day

(b) Analyst to Analyst

The intermediate precision expresses with in laboratories variation: different days, different analysts, and different equipment etc⁵⁵. The standard dilution was prepared and three replicate of each dilution were analyzed by different analysts for all the developed methods. The statistical analysis method was carried out and the data is presented in Table 7.13. 4.4 System Suitability Parameters Separation variables were set and mobile phase was allowed to saturate the column at 1.00 ml/min⁴⁸. After complete saturation of column, three replicates of working standard of Diloxanide furoate 10 µg/ml was injected separately. Peak report and column performance report were recorded for all chromatogram. The intermediate precision expresses with in laboratories variation: different days, different analysts, and different equipment etc⁵⁵. The standard dilution was prepared and three replicate of each dilution were analyzed by different analysts for all the developed methods. The statistical analysis method was carried out and the data is presented in table.

2.5.4 Robustness

As per ICH norms, small, but deliberate variations, by altering the pH and / or concentration of the mobile phase were made to check the method capacity to remain unaffected. The effect of change in pH of mobile phase, flow rate, mobile phase ratio on the retention time, theoretical plates, area under curve and percentage content of Diloxanide furoate was studied.

2.5.5 Detection limit and quantitation limit

The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve.

2.6 Forced degradation studies

In order to determine whether the method is stability indicating, forced degradation studies were conducted on Diloxanide furoate powder and the analysis was carried out by HPLC with a U.V. detector. 20µl of each of forced degradation samples were injected.

2.6.1 Acid degradation:

50 mg of Diloxanide furoate sample was taken into a 50 ml round bottom flask, 50 ml of 0.1 M HCl solution was added and contents were mixed well and kept for constant stirring for 8 h at 80°C. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation using calibration curve of Diloxanide furoate.

2.6.2 Alkaline hydrolysis:

50 mg of Diloxanide furoate sample was taken into a 50 ml round bottom flask, 50 ml of 0.1 M NaOH solution was added and contents were mixed well and kept for constant stirring for 8 h at 80°C. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation using calibration curve of Diloxanide furoate.

2.6.3 Oxidative degradation:

50 mg of Diloxanide furoate sample was taken into a 50 ml round bottom flask, 50 ml of 3% hydrogen peroxide solution was added, and contents were mixed well and kept for constant stirring for 24 hr at room temperature. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage

Degradation using calibration curve of Diloxanide furoate.

2.6.4 Thermal degradation:

50 mg of Diloxanide furoate sample was taken in to a petri dish and kept in oven at 50°C for 4 weeks. Samples were withdrawn and diluted to get 10 µg/ml subjected to HPLC and calculate the percentage degradation

S. No.	Melting Point of Diloxanide furoate
1.	112-114°C

using calibration curve of Diloxanide furoate.

3. Result:

3.1 Identification of Diloxanide furoate

3.1.1 Physical evaluation It was found that Diloxanide furoate was white fine powder, odorless and bitter in tastes which are similar to that given in pharmacopeia.

3.1.2 Results of Solubility

S. No.	Solvent	Solubility of Diloxanide furoate
1	Water	Soluble
2	Ethanol	Slightly Soluble
3	Acetonitrile	Soluble
5	0.1 N HCl	Freely soluble
6	0.1 N NaOH	Soluble
7	Chloroform	Insoluble
8	Methanol	Soluble

3.1.3 Melting point Table 7.2: Melting point of Diloxanide furoate

3.1.4 Structure Identification of Diloxanide furoate by FTIR

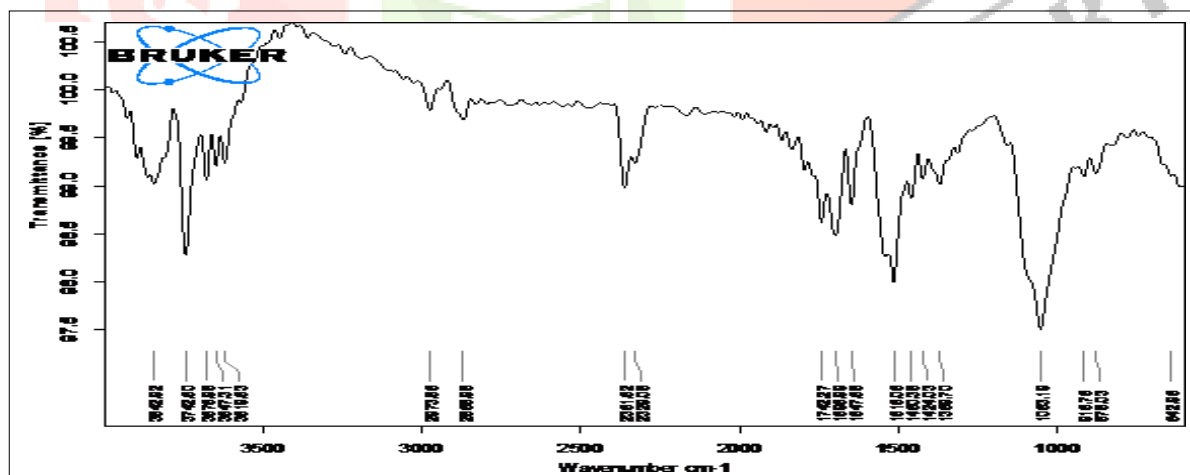


Figure 7.2: FTIR spectra of Diloxanide furoate

3.2 Chemical Reagent and Instrument Table

Chemicals/Reagents	Grade	Company
Acetonitrile	HPLC	Merck
Methanol	HPLC	Merck
Potassium dihydrogen phosphate	AR	Rankem
Water	HPLC	Milli-Q
Triethanolamine	AR	Thomas Baker
Orthophosphoric acid	AR	Hi Media

Table 3.2 (Chemical Reagent used method development)

HPLC	
Pump	515 Isocratic pump
Injector	Rheodyne injector with a 20-microlitre fixed loop
Detector	UV Vis detector
Software	Data ace software
Column	Thermo C-18 column (4.6 x 25 0mm, 5 μ particle size)
Balance	Citizen (Cx-265)
Millipore	Mili- Q
Sonicator	PCI (Mumbai)
U.V. Vis. spectroscopy	Labindia 3000 Plus

3.3 Results of HPLC method development

3.3.1 Determination of λ_{max}

Accurately weighed 10 mg of drug was transferred into 10 ml volumetric flasks and dissolved in 10 ml of methanol and vortex it to get complete dissolution. From that 0.1 ml of stock solution dissolve in 10 ml of methanol which gives 10 μ g/ml of standard solution of Diloxanide furoate.

3.3.2 Selection of Mobile Phase

Initially to estimate Diloxanide furoate in fix dosage form number of mobile phase in different ratio were tried. A result was shown in Table 3.3

Mobile Phase	Ratio	Retention Time
Methanol : Water	50 : 50 v/v	Tailing in peak
Acetonitrile : Methanol	50 : 50 v/v	Tailing in peak
20mM KH ₂ PO ₄ : Acetonitrile (pH-3.8 with OPA)	25 : 75 v/v	Most suitable

3.3.3 Selection of Diluent

Diluent used for preparation of sample were compatible with mobile phase and no any significant affect retention and resolution of analyte. After various trials mobile phase was used as diluents.

Table 3.4: Selection of separation variable

Variable	Condition
Column	C18
Dimension	250mm x 4.60mm
Particle Size	5 μ
Bonded Phase	Octadecylsilane (C ₁₈)
Mobile Phase	20mM KH ₂ PO ₄ 25% : Methanol%
20mM KH ₂ PO ₄	25%
Methanol	75%
Diluent	Acetonitrile
Flow rate	1.0 ml/min
Temperature	Ambient
Sample Size	20 μ l
Detection wavelength	254 nm
Retention time	7.213 \pm 0.3 min.

Table 3.4 (Selection of separation variable)

3.3.5 Preparation of the calibration curves of the drug

Each of the working standard solutions were injected 6 times and the mean peak area ratio of each drug to that of internal standard were calculated and plotted against the concentration of the drug. The regression of the concentration of each drug over the mean peak area ratio was obtained and these regression equations were used for the assay of tablets containing these drugs. A typical chromatogram and the calibration curves are shown later in figure 3.4.

Table 3.4 (Linearity of Diloxanide furoate)

Standard Concentration $\mu\text{g/ml}$	Area under Curve (AUC)						Mean
	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6	
0	0	0	0	0	0	0	0
5	186.547	199.856	198.785	186.658	194.745	186.624	192.203
10	385.658	386.585	379.965	375.452	373.325	376.658	379.607
15	579.985	585.658	571.125	569.958	565.452	563.321	572.583
20	755.652	756.658	763.321	759.985	775.458	768.854	763.321
25	933.254	945.587	942.125	946.657	948.856	960.745	946.204

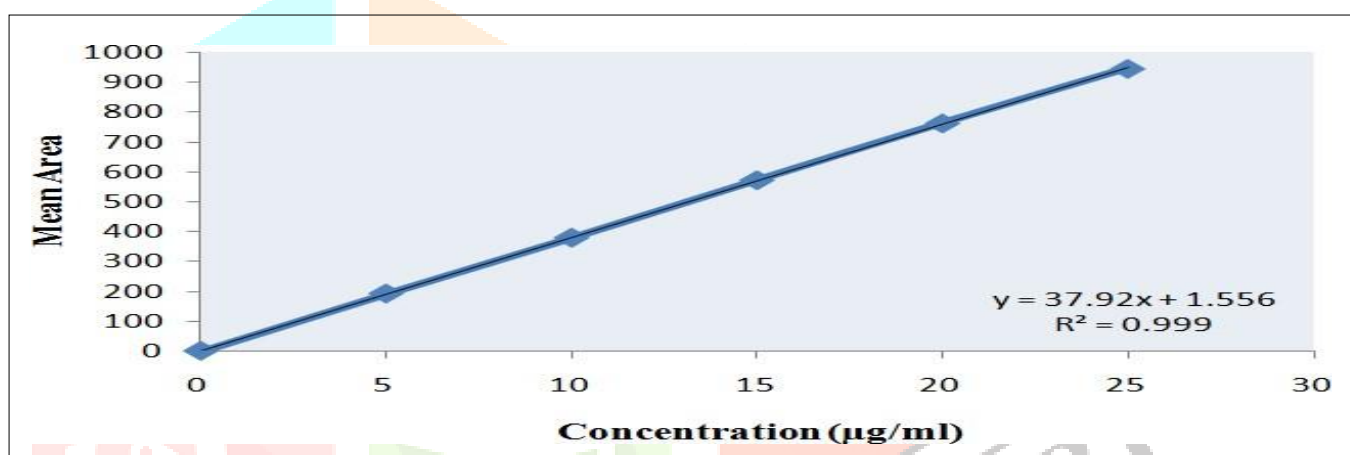


Figure 3.4: Calibration Curve of Diloxanide furoate

$$\text{Regression Equation } Y = mx + c$$

$$\text{AUC} = 37.92 \text{ conc.} + 1.556$$

$$Y = \text{AUC}$$

$$m = \text{Slope} = 37.92$$

$$x = \text{Conc. in } \mu\text{g/ml}$$

$$c = \text{Intercept } 1.556$$

$$r^2 = 0.999$$

3.3.6 Analysis of tablet sample

Physical mixture amount equal to 10 mg of Diloxanide furoate was taken in 100 ml volumetric flask. The volume is made up to the mark by methanol and filtered by whatmann filter paper (no.41) and the filtrate was used to prepare samples of 10µg/ml concentration.

S. No	Analysis	<u>Diloxanide furoate</u>
1.	Amount present	500mg
2.	Amount found	499.65
3.	Mean	99.93
4.	S.D.	0.112
5.	% RSD	0.118

3.4 Validation of developed method

3.4.1 Linearity

From the mean of AUC observed and respective concentration value, the response ratio (response factor) was found by dividing the AUC with respective concentration. The Curve was plotted between response ratio Vs. Concentration.

Table 3.4.1 (Response ration data for linearity of Diloxanide furoate)

Replicates	Concentration (µg/ml)	Mean AUC	Response Ratio
Rep-1	5	192.203	38.440
Rep-2	10	379.607	37.960
Rep-3	15	572.583	38.172
Rep-4	20	763.321	38.166
Rep-5	25	946.204	37.848
Mean		38.118	
S.D.		0.227	
R.S.D.		0.597	

Figure 3.4.1(Response Ration graph for linearity of Diloxanide furoate)

Conc. of drug in sample (mg)	10	10	10
Std drug added (mg)	8	10	12
Replicate 1	7.85	9.95	11.98
Replicate 2	8.01	9.98	11.96
Replicate 3	7.98	9.92	11.89
Mean	7.947	9.950	11.943
SD	0.085	0.030	0.047
%RSD	1.070	0.302	0.396
Mean % Recovery	99.333	99.500	99.528

3.4.2 Accuracy

Recovery studies were performed to validate the accuracy of developed method.

To preanalysed sample solution, a definite concentration of standard drug was added and then its recovery was analyzed.

3.4.3 Precision Repeatability

Standard dilutions were prepared and three replicates of each dilution were analyzed in same day for repeatability and results were subjected to statistical analysis.

Table 3.4.1 Result of repeatability for Diloxanide furoate

Conc. of drug in sample µg/ml	5	10	15	20	25
Replicate 1	4.98	9.98	14.55	19.52	25.45
Replicate 2	4.85	9.95	14.65	19.65	25.47
Replicate 3	4.92	9.82	14.85	20.02	24.32
Mean	4.917	9.917	14.683	19.73	25.08
SD	0.053	0.069	0.125	0.212	0.537
%RSD	1.081	0.7	0.849	1.074	2.143

Standard dilutions were prepared and three replicates of each dilution were analyzed in different days and by different analysts. Statistical analysis was carried out.

3.4.4 Intermediate Precision: (A) Day to Day

Table 3.4.2: Result of intermediate precision for Diloxanide furoate

Replicate	5 µg/ml	10 µg/ml	15 µg/ml	20 µg/ml	25 µg/ml
Replicate 1	4.85	9.85	14.85	19.85	24.85
Replicate 2	4.95	9.78	14.96	19.77	24.65
Replicate 3	4.85	9.69	14.69	19.65	24.75
Mean	4.883	9.773	14.833	19.757	24.750
SD	0.058	0.080	0.136	0.101	0.100
%RSD	1.182	0.821	0.915	0.510	0.404

Intermediate Precision: (B) Analyst to Analyst

Table 3.4.3: Result of intermediate precision for Diloxanide furoate

Replicate	5 µg/ml	10 µg/ml	15 µg/ml	20 µg/ml	25 µg/ml
Replicate 1	4.72	9.95	14.78	19.96	24.78
Replicate 2	4.85	9.85	14.65	19.98	24.65
Replicate 3	4.96	9.65	14.85	19.92	24.85
Mean	4.843	9.817	14.760	19.953	24.760
SD	0.120	0.153	0.101	0.031	0.101
%RSD	2.480	1.556	0.688	0.153	0.410

3.4.4 Detection Limit and Quantitation Limit

The LOD and LOQ of developed method were calculated based on the standard deviation of response and slope of the linearity curve.

Table 3.4.1: LOD and LOQ of Diloxanide furoate

Name	LOD ($\mu\text{g/ml}$)	LOQ ($\mu\text{g/ml}$)
Diloxanide furoate	0.45	1.25

3.4.5 Results of forced degradation studies

Table 3.4.5. Results of Forced degradation studies of Diloxanide furoate

Stress conditions	Drug recovered (%)	Drug decomposed (%)
Standard drug	99.98	0
Acidic hydrolysis	82.23	17.77
Alkaline hydrolysis	93.32	6.68
Oxidative degradation	86.65	13.35
Photolytic degradation	81.74	18.26

Summary & Conclusion

Modern medicines for human use are required to comply with specific standards and regulation set forth by the concerned authorities. The efficacy and safety of medicinal products can only be assured by analytical monitoring of its quality. Pharmaceutical analysis is an art and science of determining the concentration of drug constituents present in marketed formulation. It is considered as an application of procedures necessary to determine and estimate the identity, strength, quality and purity of drug. Therefore, the quality control laboratory is considered as the backbone of the Pharma industries with ever-increasing need for the development of analytical techniques for drug formulation.

In the present study, a successful attempt was made for the HPLC quantitative estimation of Diloxanide furoate in bulk formulation.

The method was developed by experimentation based on thorough literature survey and ascertained by statistical parameters of sampling. The entire work was performed on waters HPLC with U.V. Vis detector.

The result obtained shows the developed method to be precise, simple, rapid and accurate. Thus these can be used for routine analysis of Diloxanide furoate in bulk drug dosage form.

It was thus, concluded that the proposed method is new, simple, accurate, safe, free from pollution, precise and can be successfully employed in the routine analysis. The simplicity, rapidity reproducibility and economy of the proposed methods completely fulfill the objective of this research work.

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