IJPSR (2026), Volume 17, Issue 2 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 12 August 2025; received in revised form, 14 October 2025; accepted, 26 October 2025; published 01 February 2026

AQBD DRIVEN DEVELOPMENT AND VALIDATION OF A HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF FURAZOLIDONE AND METRONIDAZOLE IN PHARMACEUTICAL DOSAGE FORM

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Keywords:

Analytical QualitybyDesign**,** RP-HPLC, 3^2 factorial design, independent variable, dependent variable

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ABSTRACT: A Quality by Design approach to analytical method development involves selection of predetermined objective, determined critical parameters, evaluation, appropriate method selection and risk assessment. Analytical Quality by Design (AQbD) helps to understand the selected method more precisely than traditional method. An important component of AQbD is understanding of independent variables (Factors) and dependent Variables (Response) and their interactions were analysed. Present study entails AQbD driven development of reversed-phase (RP) high-performance liquid chromatography (HPLC) for simultaneous estimation of Furazolidone and Metronidazole in pharmaceutical dosage form. The risk based HPLC method development and validation for combination of pharmaceutical dosage form helps to optimize the separation process of the APIs. The chromatographic conditions were optimized using Stat-Ease 360 software ,with 32 factorial design applied to analyse the effect of mobile phase composition and flow rate and effect was evaluated on retention time, number of theoretical plates (NTP), symmetry factor and resolution. The analysis was performed on HiQSil C18H5 column (4.6mm ×250mm) using acetonitrile: phosphate buffer (pH adjusted to 3 with 0.1% OPA) (55:45 v/v) as mobile phase with flow rate 0.8 ml/min. The method achieved retention time 4.7 min for Furazolidone and 3.5 min for Metronidazole respectively. The developed method was found to be linear in the concentration range of 10-50 µg/ml with regression coefficient (r2) 0.9988 for Furazolidone and concentration range 10-50 µg/ml with regression coefficient (r2) 0.9971 for Metronidazole detection wavelength at 324 nm. The % RSD for intraday and inter-day precision was 1.34% and 1.7% for Furazolidone, and 1.34% and 1.38% for Metronidazole respectively. The robustness value was less than 2% for both APIs. The assay results were 98.6% for Furazolidone and 98.65% for metronidazole. All method validation parameters fell within the prescribed limits outlined by ICH Q2(R1). Consequently, a QbD approach was employed in the development of the RP-HPLC analytical method to enhance understanding of the interactions between independent and dependent variables.

INTRODUCTION: Furazolidone, 3-[(E)-(5-nitrofuran-2-yl) methylidene amino]-1, 3-oxazolidin-2-one is a broad-spectrum anti-infective, antibacterial and antiprotozoal in nature.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.17(2).588-01

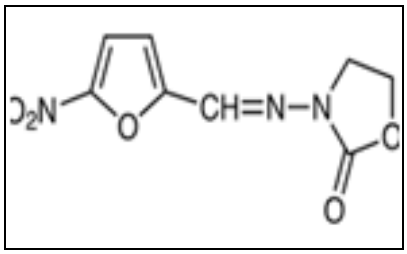
This article can be accessed online on www.ijpsr.com

DOI link: https://doi.org/10.13040/IJPSR.0975-8232.17(2).588-01

Molecular formula is C8H7N3O5. Molecular mass is 225.158 g/mol, melting point 256 to 257ºC, pKa is -2.4. Furazolidone was soluble in formic acid, DMSO (dimethyl sulfoxide), DMF (dimethyl formamide) [1].

Furazolidone indicated as a secondary agent in the treatment of cholera caused by *Vibrio cholerae* (comma), in the treatment of bacterial diarrhoea caused by susceptible organism and also in the treatment of giardiasis caused by *Giardia lamblia*. Furazolidone is active *in-vitro* against

Campylobacter jejuni, Enterobacter aerogenes, Escherichia coli, Proteus species, Salmonella species, Shigella species, and staphylococci. Furazolidone interferes with several bacterial enzymes, neither significantly alters normal bowel flora nor result in excessive Fungal growth. Furazolidone also acts as a monoamine oxidase inhibitor (MAOI). MAOIs prevent the inactivation of tyramine by hepatic and gastrointestinal monoamine oxidase [2]. Metronidazole, 2-(2-Methyl -5-nitro-1H-imidazol-1-yl) ethanol is an antibiotic used to treat wide variety of infections such as gastrointestinal (GI) tract, reproductive system, skin, heart, bone, joint, lung, blood and nervous system. It is also used to treat certain sexually transmitted diseases (STDs). Molecular formula is C6H9N3O3, molecular mass 171.16 g/mol, melting point is 159 to163 ºC. pKa is 2.62. Metronidazole was soluble in water, ethanol, methanol, chloroform, DMSO (dimethyl sulfoxide) [3]. It is antibacterial and antiprotozoal in nature works by stopping the growth of certain bacteria and parasites.



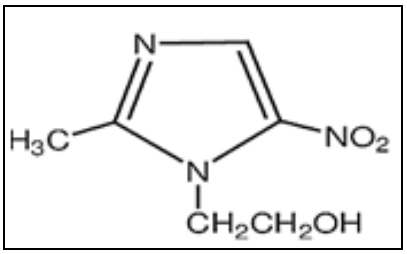


FIG. 1: STRUCTURE OF FURAZOLIDONE FIG. 2: STRUCTURE OF METRONIDAZOLE

Analytical Quality by Design (AQbD) is a systematic approach to pharmaceutical development that begins with predetermined objectives and emphasises product and process understanding and process control, based on sound science and Quality risk management. Analytical Quality by Design (AQbD) is same as QbD principles applied to development of analytical method and outcome of AQbD is well understood, fit for the intended purpose for robustness throughout the lifecycle. AQbD is well describe in ICH Q14 this guideline complements to ICH Q2(R1) [4, 5]. **Fig. 3** presents an overview of AQbD approach.

FIG. 3: SCHEMATIC REPRESENTATION OF QBD APPROACHIN ANALYTICAL METHOD DEVELOPMENT

According to literature survey, few HPLC and HPTLC methods have been reported for the simultaneous estimation of the Furazolidone and Metronidazole combination [6], but lacks AQbD driven RP-HPLC method development and validation for the combination. Thus, aim of the present study is to develop and validate AQbD driven RP-HPLC method for simultaneous estimation of the Furazolidone and Metronidazole combination and optimization of developed method using Stat-Ease360 software, with 3^2 factorial design followed by validation as per ICH Q2(R1) guideline [7-10].

MATERIALS AND METHODS:

Chemicals and Reagents: Furazolidone and Metronidazole Active Pharmaceutical Ingredients were procured from Aarti Drugs Limited (Mumbai), Analytical grade solvents and reagents such as Acetonitrile (HPLC), Methanol (HPLC), Water (HPLC), Potassium Dihydrogen Orthophosphate (AR), Sodium Dihydrogen Orthophosphate (AR), Triethylamine (AR), Sodium Hydroxide (AR), Ortho-phosphoric acid (AR) were obtained from Loba Chemie Pvt. Ltd (Mumbai). The marketed formulation FURA-M, was purchased from local retail pharmacy (Ambernath).

Instruments: HPLC studies were conducted using Jasco HPLC (Model no. A061361868) with (UV detector) operated using chromnav HPLC software, A column C18, HiQSil C18H5 (4.6mm×250mm)

employed at ambient temperature. Other equipment such as UV Spectrophotometer (Serial no. A24256, Shimadzu), Analytical weighing balance (CAH-223, Contech), Ultrasonicator (TUC-20ASK Lab Instruments), pH meter (LI120, Elico) FTIR (Jasco FTIR-4100) were used.

Calibration of Instruments: Calibration of analytical weighing balance, HPLC system, ultrasonicator, pH meter was done as per standard operating procedure. HPLC system was calibrated for flow rate accuracy, linearity, wavelength Accuracy, precision, carryover effect.

QbD Software: Stat-Ease 360 Software were used for method optimization by generating contour plots and 3-D contour plots.

Identification of APIs: Characterization of procured APIs samples was done by UV-Visible spectra and FTIR, additionally melting point of the sample were determined. A standard solution of Furazolidone and Metronidazole, each at a concentration of 10 µg/ml, were prepared using the solvent systems specified in the Indian Pharmacopoeia 2010. Dimethylformamide (DMF) was used as the solvent for Furazolidone, whereas 0.1 N hydrochloric acid (HCl) was employed for Metronidazole. The UV spectra of these solutions were recorded accordingly. Additionally, both compounds were scanned using an FT-IR spectrophotometer to identify their characteristic functional groups. The melting points of Furazolidone and Metronidazole were determined using a melting point apparatus, with values of 256°C and 159°C, respectively [11].

Preparation of Buffer:

Preparation of Phosphate Buffer pH 3: Dissolve 1.36 g of potassium dihydrogen orthophosphate and 2 ml of triethylamine in 800 ml of water, adjust the pH to 3.0 with orthophosphoric acid and add sufficient water to produce 1000 ml [12].

Preparation of Mobile Phase and Diluent: Acetonitrile mixed with phosphate buffer pH 3 (55:45) v/v and sonicated for 30 min to degas. The mentioned mobile phase was used as diluent for preparation of standard stock solution and sample stock solution.

Preparation of Standard Stock Solution: Accurately 10 mg Furazolidone and Metronidazole were weighed and transferred into 10 ml volumetric flask, the 3/4th of diluent was introduced into the flask. Sonicated for 10 minutes with intermittent shaking to dissolve. Subsequently, the solution was filled up to mark by Acetonitrile: phosphate buffer (55:45) v/v and mixed thoroughly (1000 µg/ml). withdrawn 1ml from standard stock solution and transferred in 10 ml volumetric flask and volume was made up to 10 ml with solvent system. (100 µg/ml Furazolidone and 100 µg/ml Metronidazole respectively).

Preparation of Sample Stock Solution: Twenty tablets (FURA-M) were weighed and powdered, then weight equivalent to a tablet (100 mg Furazolidone and 400 mg Metronidazole) was transferred into 100 ml volumetric flask. The 50 ml of solvent system was introduced to it and sonicated for 25-30 minutes with intermittent shaking. Subsequently, the cooled solution was filled up to mark by solvent system and mixed thoroughly. Finally, the sample was filtered using whatman filter paper of 0.45 micron. Labelled the solution (1000 µg/ml Furazolidone and 4000 µg/ml Metronidazole),withdrawn 1 ml from resultant stock solution and transferred into 10 ml volumetric flask and made up the volume to 10 ml (100 µg/ml Furazolidone and 400 µg/ml Metronidazole).

Stability of Analyte Solution: The analyte solution was stable at room temperature and in refrigerator (2-8ºC). The mobile phase was clear and free from visible particles. Later stability of stored sample results was evaluated for day 1 and day 2, and results were compared with earlier results, subsequently found that analyte solution was stable for 2 days storage at room temperature (RT).

Experimental Work: Experimental work involves Analytical method development, Analytical method optimization using QbD, Analytical method development and validation as per ICH Q 2(R1) [13-16].

Analytical Method Development: The steps involved in analytical method development for estimation of Furazolidone and Metronidazole in combined dosage form include selection of solvent system, wavelength analysis, HPLC trials.

Selection of Solvent System: Based on the pharmacopeial information and literature survey, several trials were performed. The details of solvent system are given in **Table 1.**

TABLE 1: SOLVENT SYSTEM TRIALS

Sr. no.	Solvent system/mobile phase	Ratio (V/V)	Remark
1.	Acetonitrile: Water	70:30	Furazolidone-solution become turbid. Metronidazole -soluble
2.	Acetonitrile: phosphate Buffer pH7	70:30	Furazolidone -soluble but later precipitation observed. Metronidazole -slightly soluble.
3.	Acetonitrile: phosphate Buffer pH6	70:30	Turbidity/ cloudy nature of mobile phase
4.	Acetonitrile: phosphate Buffer pH3	70:30	Furazolidone-Solution becomes clear. Metronidazole-Soluble
5.	Acetonitrile: water: formic acid (0.1%) pH 4	70:30:0.1	Furazolidone- not soluble Metronidazole- soluble

Wavelength Analysis: A standard solution containing 10 µg/ml concentration of Furazolidone and Metronidazole was prepared in two different solvent system acetonitrile: phosphate buffer pH7 and acetonitrile: phosphate buffer pH3. The same solution was scanned in the wavelength range of 200-400 nm against respective mobile phase as a blank.

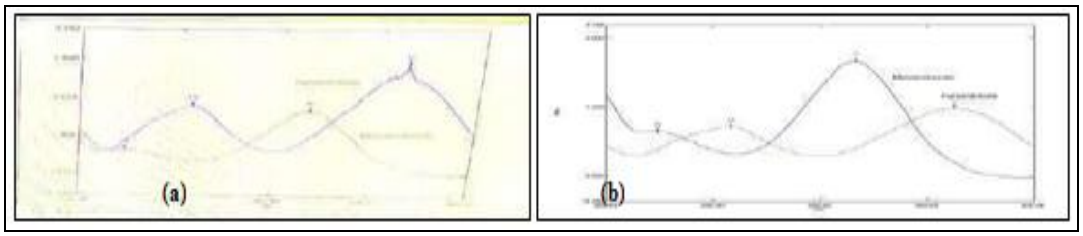


FIG. 4: (A) OVERLAY OF METRONIDAZOLE (10PPM) AND FURAZOLIDONE (10 PPM) IN ACETONITRILE: PHOSPHATE BUFFER PH7 (B) OVERLAY OF METRONIDAZOLE (10 PPM) AND FURAZOLIDONE (5 PPM) IN ACETONITRILE: PHOSPHATE BUFFER PH 3

As shown in **Fig. 4,** both API exhibits absorbance at each other's λ max wavelengths: 315nm for the acetonitrile: phosphate buffer atp H7, and 324 nm for the acetonitrile: phosphate buffer at pH3. Based on the UV scans, these respective λ max values were selected for the HPLC trials.

HPLC Trials:

TABLE 2: HPLC TRIALS

Sr. no.	Mobile phase	Selected wavelength	Observation
Trial 1:	Acetonitrile: Phosphate buffer pH7 (70:30) v/v	315nm	Peak shape was not proper, poor resolution
Trial 2:	Acetonitrile: Phosphate buffer pH3(70:30)v/v	324nm	Peak shape was proper with resolution
Trial 3:	Acetonitrile:Phosphate buffer pH3(60:40)v/v	324nm	Peak shape was proper with better resolution

Observation: Based on the observation, the chromatogram obtained with mobile phase composition [Acetonitrile: Phosphate buffer pH3 (60:40)v/v] showed better resolution and hence used for further method optimization step.

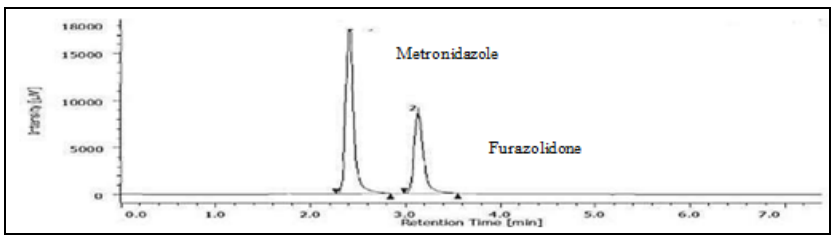


FIG. 5: CHROMATOGRAM FOR METRONIDAZOLE AND FURAZOLIDONE (10PPM) [ACETONITRILE: PHOSPHATE BUFFER PH3 (60:40)V/V

Analytical Method Optimization [17-19]:

Software Aided Method Optimization: The Stat-Ease 360 software was used for method optimization using QbD. The 3^2 factorial design applied to analyse the effect of mobile phase composition and flow rate and effect. The was evaluated on retention time, number of theoretical plates (NTP), symmetry factor and resolution. To obtained ideal and best chromatographic condition CQA (Critical Quality Attribute) were identified. CQA consist of CMA (Critical Method Attributes) and CAA (Critical Analytical Attributes). The 9 analytical trials (9 runs) were carried out using the factor (ratio of mobile phase, flow rate of mobile phase) and responses (retention time, symmetry factor, number of theoretical plates, and resolution). The experimental run given by software were completed using HPLC system and results of responses were fed in to the software for further statistical evaluation as shown in **Table 4.**

TABLE 3: DESIGN SUMMARY, DESIGN TYPE: 3^2 FACTORIAL DESIGN, RUNS: 9 EXPERIMENTAL RUNS

Factor	Name	Type	Subtype	Level-1	Level0	Level+1
A	Mobile phase composition	Numeric	Continuous	55:45	60:40	65:35
B	Flow rate	Numeric	Continuous	0.8ml/min	1ml/min	1.2ml/min

TABLE 4: RESULT FOR 3^2 FACTORIAL DESIGN PLAN AND RESPONSES

Std	Run	Factor 1 Mobile phase composition Acetonitrile: phosphate buffer pH 3	Factor 2 Flow rate ml/min	Response 1 Retention time 1 min	Response 2 NTP1	Response 3 Symmetry 1	Response 4 Resolution	Response 5 Retention time 2 min	Response 6 NTP2	Response 7 Symmetry 2
4	1	-1	0	2.925	4002	1.05	3.86	4.15	4643	1.045
1	2	-1	-1	3.65	4624	1.104	5.025	5.162	4484	1.005
9	3	1	1	2.35	4830	1.162	4.241	3.07	6314	1.195
8	4	0	1	2.4	4300	1.154	4.62	3.125	5285	1.212
2	5	0	-1	3.542	5735	1.175	5.239	4.633	6599	1.102
6	6	1	0	2.817	4421	1.2	3.614	3.433	5260	1.225
3	7	1	-1	3.475	5482	1.109	4.141	4.3	6596	1.129
5	8	0	0	2.825	4852	1.133	4.09	3.725	4913	1.154
7	9	-1	1	2.542	4466	1.002	3.462	3.65	5704	1.225

TABLE 5: SOLUTION GIVEN BY STAT-EASE 360 SOFTWARE

Solution

Number	Mobile phase composition	Flow rate	Ratantion time 1	NTP1	Symmetry factor	Rasolution	Ratention time 2	NTP2	Symmetry factor 2	Desirability	
1	0.904	-1.000	3.641	4729.265	1.101	5.000	5.162	4946.000	1.033	0.969	Selected
2	0.972	-1.000	3.639	4734.077	1.102	5.298	5.154	4960-439	1.033	0.909	
3	0.962	1.000	3.637	4737, 951	1.103	5.104	5.147	4971,93	1.034	0.969	
4	0.949	1.000	3.636	4743,127	1.105	5.113	5.138	4985,997	1.034	0.968	
5	0.941	1.000	3.634	4746,356	1.106	5.118	5.132	4995,089	1.035	0.968	
6	0.935	1.000	3.629	4760,465	1.109	5.140	5.108	5034,674	1.036	0.967	
7	0.817	1.000	3.617	4795,679	1.118	5.189	5.050	5132,378	1.040	0.964	
8	0.780	1.000	3.612	4810,303	1.121	5.206	5.026	5172,497	1.042	0.962	
9	0.716	1.000	3.603	4835,363	1.127	5.333	5.985	5241,975	1.045	0.958	
10	0.150	1.000	3.520	5179,494	1.163	5.112	5.543	6096,627	1.084	0.839	
11	0.350	1.000	3.509	5258,894	1.161	5.958	5.475	6273,079	1.094	0.796	

Statistical Analysis and Final Optimization: After completion of trials given by Stat-Ease 360 software with 3^2 factorial design the experimental data of responses were integrated into design and subsequently, further statistical analysis was performed to determine best chromatographic conditions. The contour plots and 3-D contour plots were generated based upon analysis (ANOVA), Based on the observation and interactions of the method parameter (ratio of mobile phase composition and flow rate) and responses (retention time, NTP, symmetry factor and resolution) chromatographic condition and method were finalized. The statistical analysis was carried out with Stat-Ease 360 software ANOVA test. The ANOVA test helps to identify the significance level of model generated for each response and best trial, which has desirability close to 1 selected for HPLC analysis.

Response 1: Retention time 1

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.98	5	0.3953	541.80	0.0001	significant
A-Mobile phase composition	0.0376	1	0.0376	51.53	0.0056	
B-Flow rate	1.90	1	1.90	2601.69	< 0.0001	
AB	0.0001	1	0.0001	0.0990	0.7736	
A^2	0.0028	1	0.0028	3.85	0.1444	
B^2	0.0378	1	0.0378	51.82	0.0055	
Residual	0.0022	3	0.0007			
Cor Total	1.98	8				

FIG. 6: ANOVA FOR RESPONSE 1

Response 2: NTP1

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.905E+06	2	1.452E+06	8.30	0.0187	significant
A-Mobile phase composition	1.203E+06	1	1.203E+06	6.88	0.0394	
B-Flow rate	1.701E+06	1	1.701E+06	9.73	0.0206	
Residual	1.049E+06	6	1.749E+05			
Cor Total	3.954E+06	8				

FIG. 7: ANOVA FOR RESPONSE 2

Response 3: Symmetry factor1

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.1782	7	0.0255	4679.99	0.0113	significant
A-Mobile phase composition	0.0112	1	0.0112	2066.33	0.0140	
B-Flow rate	0.0144	1	0.0144	2654.08	0.0124	
AB	0.0003	1	0.0003	47.02	0.0922	
A^2	0.0819	1	0.0819	15038.73	0.0032	
B^2	0.0049	1	0.0049	906.16	0.0211	
A^2B	0.0024	1	0.0024	432.00	0.0306	
AB^2	0.0028	1	0.0028	507.00	0.0283	
A^3	0.0000	0				
B^3	0.0000	0				
Residual	5.444E-06	1	5.444E-06			
Cor Total	0.1782	8				

FIG. 8: ANOVA FOR RESPONSE 3

Response 4: Resolution

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.86	5	0.5713	23.14	0.0133	significant
A-Mobile phase composition	0.0205	1	0.0205	0.8318	0.4290	
B-Flow rate	0.7225	1	0.7225	29.27	0.0124	
AB	0.6914	1	0.6914	28.01	0.0132	
A^2	0.7021	1	0.7021	28.44	0.0129	
B^2	0.7200	1	0.7200	29.17	0.0124	
Residual	0.0741	3	0.0247			
Cor Total	2.93	8				

FIG. 9: ANOVA FOR RESPONSE 4

Response 5: Retention time 2

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.94	5	0.7880	220.81	0.0005	significant
A-Mobile phase composition	0.7769	1	0.7769	217.69	0.0007	
B-Flow rate	3.01	1	3.01	843.56	< 0.0001	
AB	0.0199	1	0.0199	5.57	0.0904	
A^2	0.0355	1	0.0355	9.94	0.0512	
B^2	0.0974	1	0.0974	27.29	0.0136	
Residual	0.0107	3	0.0036			
Cor Total	3.95	8				

FIG. 10: ANOVA FOR RESPONSE 5

Response 6: NTP2

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.765E+07	5	3.531E+06	32.47	0.0082	significant
A-Mobile phase composition	3.284E+06	1	3.284E+06	30.20	0.0119	
B-Flow rate	3.039E+06	1	3.039E+06	27.94	0.0132	
AB	1.932E+06	1	1.932E+06	17.76	0.0244	
A^2	3.574E+06	1	3.574E+06	32.86	0.0103	
B^2	5.825E+06	1	5.825E+06	53.56	0.0053	
Residual	3.263E+05	3	1.088E+05			
Cor Total	1.798E+07	8				

FIG. 11: ANOVA FOR RESPONSE 6

Response 7: Symmetry factor 2

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	0.1285	2	0.0643	6.81	0.0286	significant
A-Mobile phase composition	0.0689	1	0.0689	7.30	0.0355	
B-Flow rate	0.0596	1	0.0596	6.31	0.0457	
Residual	0.0566	6	0.0094			
Cor Total	0.1852	8				

FIG. 12: ANOVA FOR RESPONSE 7

TABLE 6: MODELS USED FOR EACH RESPONSE

Response	Retention time 1	NTP1	Symmetry Factor 1	Resolution	Retention time 2	NTP 2	Symmetry Factor 2
Model	Quadratic	Linear	Cubic	Quadratic	Quadratic	Quadratic	Linear

Contour and 3-D Contour Plots: The Analysis of variance test (ANOVA) was applied to each response to study significance of model generated for response. Contour plot and 3-D contour plot were analysed to visualize the effect of Factors and their interaction with responses. The **Fig. 13** to **Fig. 25.** The results for the contour and 3-D contour plots were specified in result and discussion. **Table 7.** Optimized chromatographic condition using Stat-Ease 360 software

TABLE 7: OPTIMIZED CHROMATOGRAPHIC CONDITION USING STAT-EASE 360 SOFTWARE

Column used	HiQSilC18H5 4.6mmx250mm
Wavelength	324nm
Mobile phase composition	Acetonitrile: phosphate buffer(pH3) 55: 45V/V
Flow rate	0.8ml/min
Injection Volume	20 µl

HPLC Method Validation: 20-23 Validation of AQbD aided optimized analytical method was performed as per ICHQ2(R1) using following Parameters.

Specificity: To demonstrate specificity blank solution, standard solution, sample solution were injected. The interference from blank was check at Furazolidone and Metronidazole retention time **Fig. 23, 24, 25.**

Linearity and Range: The standard stock solution containing Furazolidone (1000 µg/ml) and Metronidazole (1000 µg/ml) was prepared to ascertain the linearity. The linearity was carried out over 5 different concentrations of Furazolidone and Metronidazole in the Range of 10-50 µg/ml in triplicate. Calibration curve was plotted and the regression coefficient (R2) were calculated by constructing linearity curve for concentration to peak area **Fig 26**.

Accuracy: The accuracy of method performed by standard addition method and confirmed by recovery analysis using marketed formulation FURA-M and standard solution at 80%, 100%, and 120%. Triplicate injections were given for each level of accuracy and percentage recovery of Furazolidone and Metronidazole was found out. The result specified in **Table 9, 10.**

Precision: The precision of developed analytical method were determined by method repeatability (Intraday) and intermediate precision (Interday). The intraday precision was evaluated by analysing six replicates of calibration standards of sample on same day. Meanwhile the intraday precision was assessed by running calibration standards of sample on different days. The result specified in **Table 11**.

Limit of Detection (LOD) and Limit of Quantification (LOQ): Limit of Detection and Quantification was determined using signal-to-noise Method. The detection limit was referred to as the lowest concentration level resulting in a peak area of three times the baseline noise. The quantitation limit was referred to as the lowest concentration level, providing a peak area with a signal-to-noise ratio higher than ten. The results obtained are shown in **Table 12.**

$$LOD = 3{:}3\ \sigma/S,\ LOQ = 10\ \sigma/S$$

Robustness: For robustness study 100 µg/ml Furazolidone and 400 µg/ml Metronidazole stock solution were used. The evaluation of method robustness was carried out by deliberate variation in method parameters such as flowrate, pH, wavelength respectively. **Table 13** specified robustness data of purposed method.

System Suitability: The chromatographic parameters for system reproducibility were checked using system suitability and system reproducibility **Fig. 28, 29**.

RESULT AND DISCUSSION:

Statistical Analysis and Experimental Data by Stat-Ease 360 Software: Analysis of variance test (ANOVA) was applied to each response and analyse the model significance for Response. Contour plot and 3-D contour plot were analysed to visualize the effect of factors and their interaction with responses. The ANOVA test for response Specified in **Table 6-12**. The contour and 3-D contour plots were generated using 32 factorial design. **Fig. 13, Fig. 19** represents the contour and 3-D contour plots.

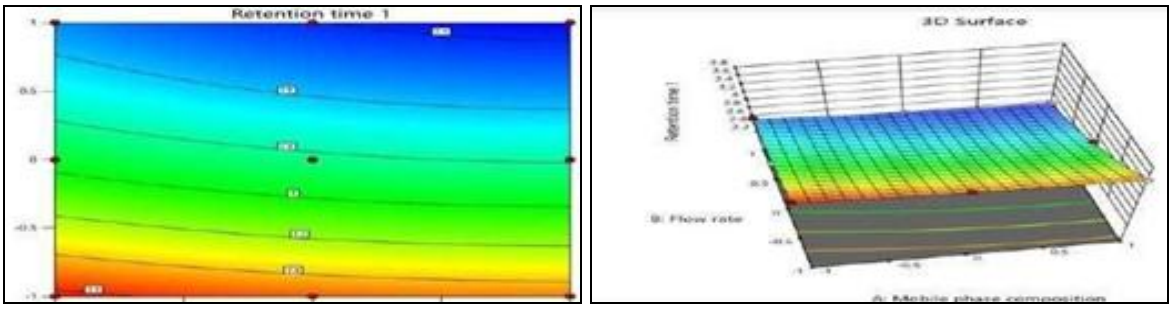


FIG. 13: CONTOUR AND 3-D CONTOUR PLOT FOR RETENTION TIME 1

Fig. 13 illustrates the retention time of Metronidazole is influenced by both the composition of the mobile phase and the flow rate during chromatography. The retention time of Metronidazole has significant effect on method, need to optimize carefully to achieve best results.

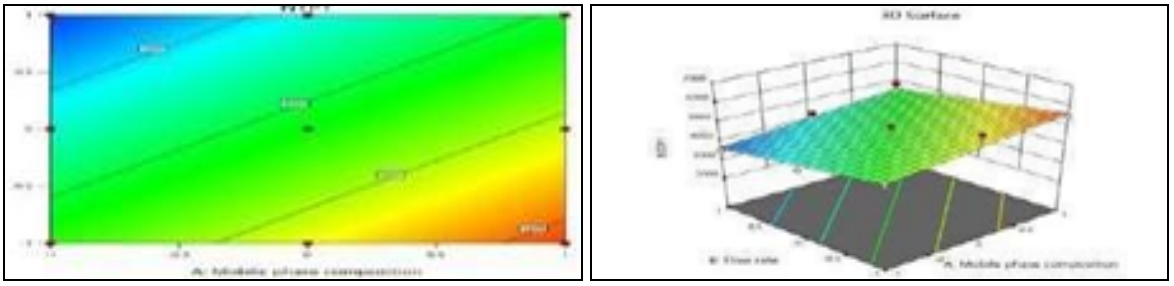


FIG. 14: CONTOUR AND 3-D CONTOUR PLOT FOR NTP 1

Fig. 14 describes the number of theoretical plates (NTP) for Metronidazole is significantly influenced by the composition of mobile phase and flow rate during chromatographic method development.

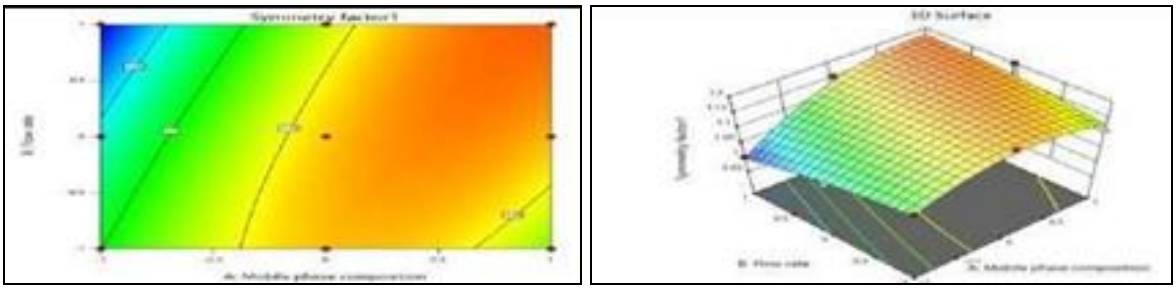


FIG. 15: CONTOUR AND 3-D CONTOUR PLOT FOR SYMMETRY FACTOR 1

Fig. 15 represents Symmetry factor for Metronidazole and their interaction with mobile phase and flow rate. The (-1) level of mobile phase composition and (0) level of flow rate 1.1. hence mobile phase composition and flow rate significantly impact on symmetry factor.

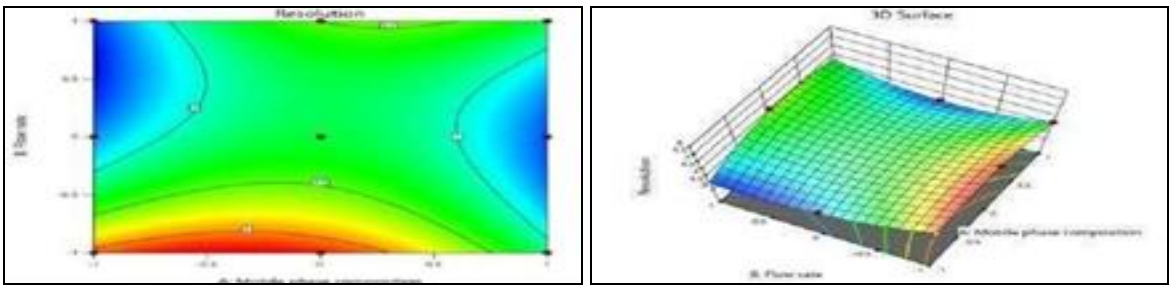


FIG. 16: CONTOUR AND 3-D CONTOUR PLOT FOR RESOLUTION

Fig. 16 represents the resolution parameter is significantly influenced by both mobile phase and flow rate. The resolution is maximized at higher mobile phase composition while flow rate can both enhance and diminish the overall separation quality.

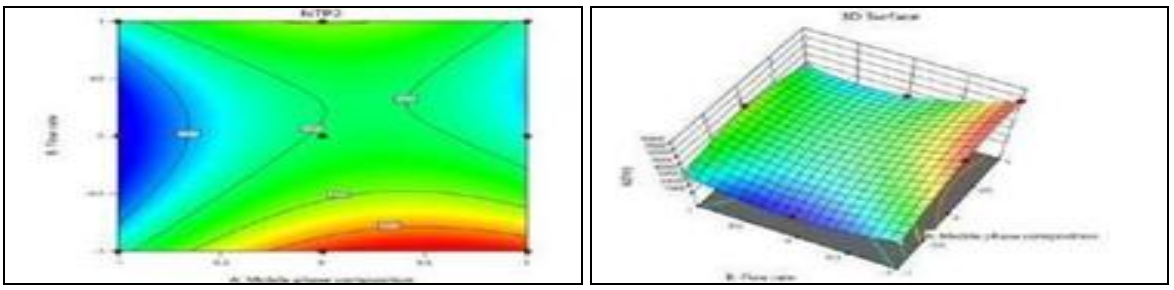


FIG. 17: CONTOUR AND 3-D CONTOUR PLOT FOR RETENTION TIME 2

Fig. 17 indicates the Retention time for Furazolidone is affected by the composition of mobile phase and flow rate as the mobile phase composition and flow rate are adjusted to different levels indicates a decrease in retention time with change in both the mobile phase composition and flow rate.

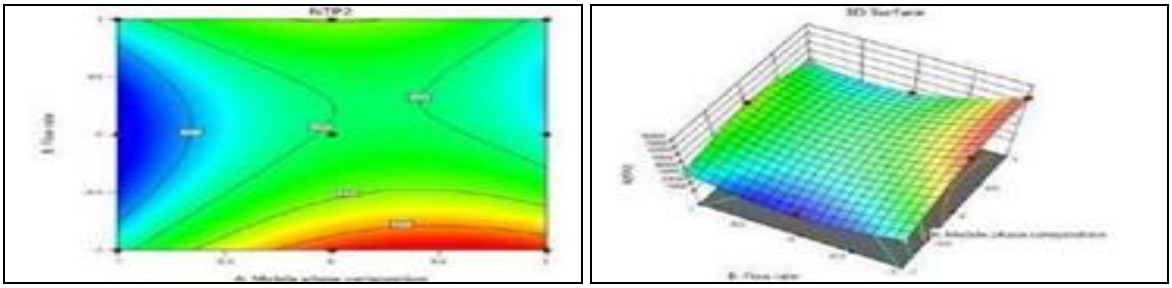


FIG. 18: CONTOUR AND 3-D CONTOUR PLOT FOR NTP 2

Fig. 18 illustrates the number of theoretical plates (NTP) for Furazolidone is significantly influenced by both the composition of mobile phase and flow rate. The NTP represent a measure column efficiency therefore; maximizing the NTP improves separation, resolution, and sensitivity of detection in chromatographic analysis.

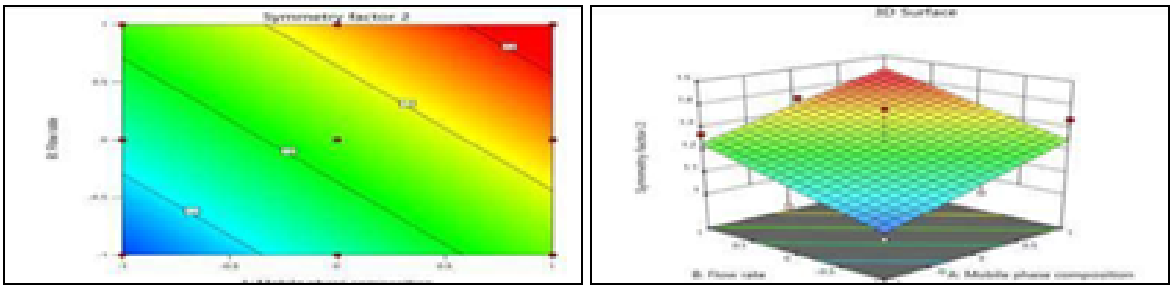


FIG. 19: CONTOUR AND 3-D CONTOUR PLOT FOR SYMMETRY FACTOR 2

Fig 19 describes the symmetry factor for Furazolidone across mobile phase composition and flow rate. Symmetry factor of Furazolidone improves with changes in mobile phase composition, potentially enhance the separation of chromatograms.

Optimization by Desirability Function: The desirability plots indicates that the optimized condition provided by stat ease 360 base on selected goals exhibits the desirability result close to 1, i.e. 0.9688. It also shows the prediction of results of responses values which was statistically and experimentally proven during method development and validation. After contour and 3-D contour plot was obtained further analysis was done for predicted and actual value for selection of Model for the respective responses were suitable for the selected design. The desirability function depends on a desirability scale ranging from d=0, indicating a completely undesirable response, to d=1, representing a fully desirable response.

This scale was used to confirm the optimized conditions provided by the Stat-Ease 360 software, three replicate injections of Furazolidone and Metronidazole (10 µg/ml) were analysed to determine the retention time, number of theoretical plates, symmetry factor and resolution. The region shaded dark blue represents lower values, and shaded in dark red represents higher values and the region shaded in light blue, green and yellow represents intermediate values.

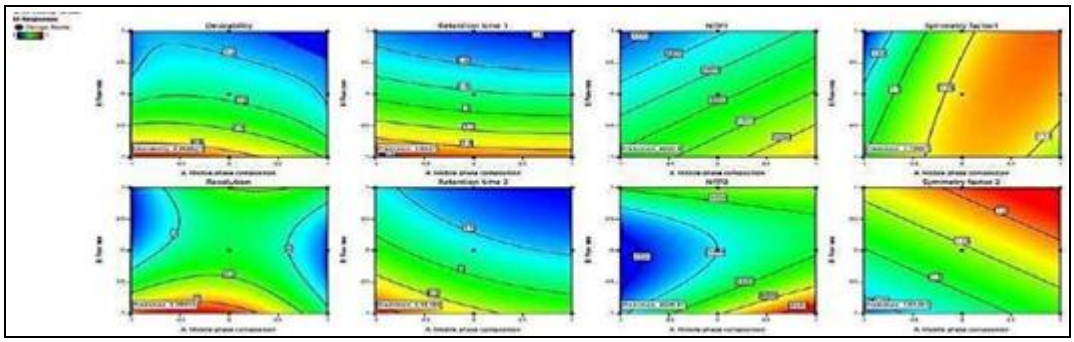


FIG. 20: DESIRABILITY PLOTS FOR FINAL OPTIMIZED METHOD

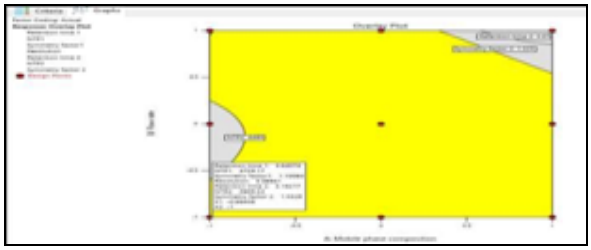


FIG. 21: METHOD OPERABLE DESIGN SPACE

The Method Operable Design Space (MODS), illustrated in **Fig. 21**, represents the optimal conditions under which the experimental design yields reliable results. The design space offers insight into variations in critical method parameters (CMPs) can impact the outcomes while still maintaining the quality and effectiveness of the method. i.e. it also shows that even if significant changes are made in CMPs we will obtain desired results. Method operable design space not only promotes the optimization of method but also enhances the relatability and adaptability of method.

TABLE 8: OPTIMIZED CHROMATOGRAPHIC CONDITION USING STAT-EASE 360 SOFTWARE

Column used	HiQSilC18H5 4.6mmx250 mm
Wavelength	324nm
Mobile phase composition	Acetonitrile: phosphate buffer (pH3)55:45V/V
Flow rate	0.8ml/min
Injection volume	20 µl

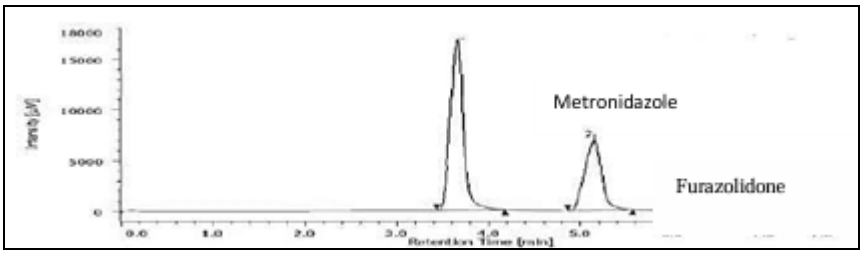


FIG. 22: CHROMATOGRAM FOR OPTIMIZED CHROMATOGRAPHIC CONDITION METRONIDAZOLE (10 PPM) AND FURAZOLIDONE (10 PPM)

HPLC Method Validation:

Specificity: The developed method was specific for the Furazolidone and Metronidazole as the interference from blank absent at Furazolidone and Metronidazole retention time.

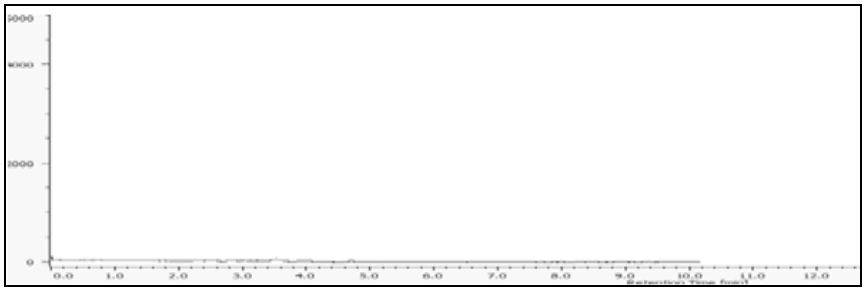


FIG. 23: CHROMATOGRAM OF BLANK FOR SPECIFICITY

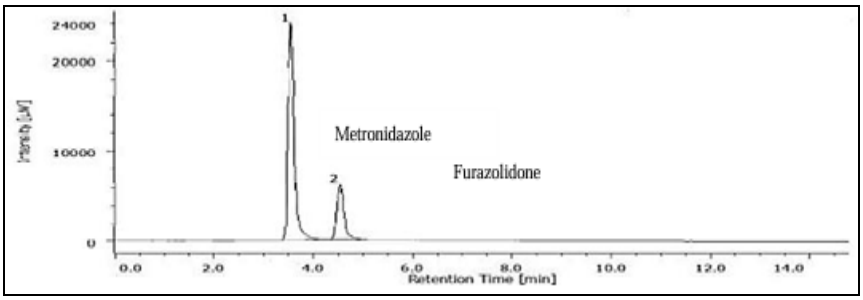


FIG. 24: CHROMATOGRAM OF SAMPLE STOCK SOLUTION METRONIDAZOLE (40 PPM) AND FURAZOLIDONE (10 PPM)

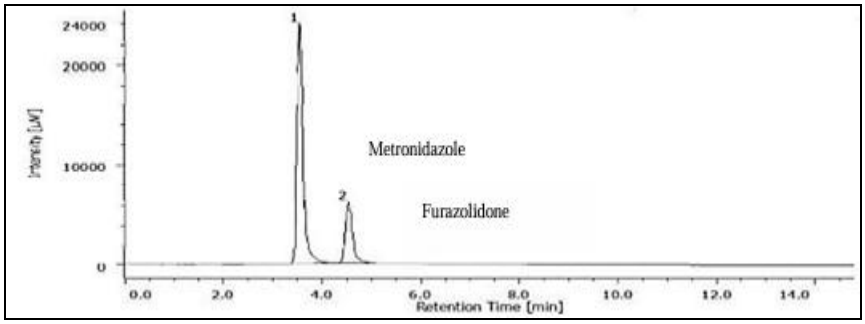


FIG. 25: CHROMATOGRAM OF STANDARD STOCK SOLUTION METRONIDAZOLE (40 PPM) AND FURAZOLIDONE (10 PPM)

Linearity and Range: The developed method was linear at concentration range of 10-50 µg/ml Furazolidone and 10-50 µg/ml Metronidazole with correlation coefficient r^2 0.9988 and 0.9971 respectively.

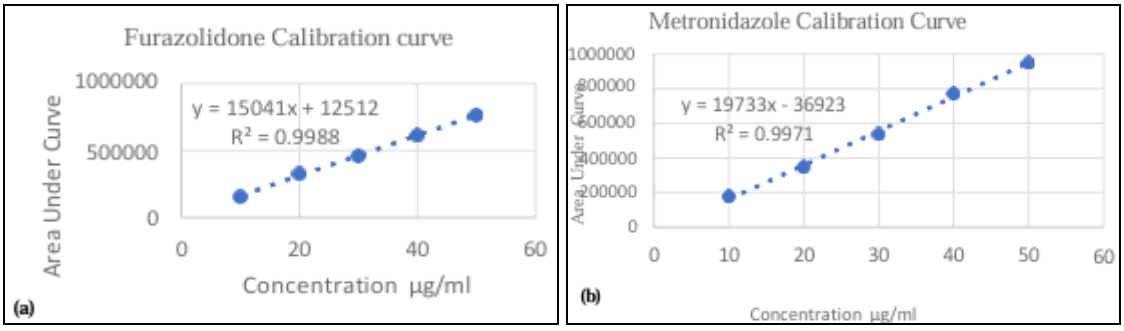


FIG. 26: CALIBRATION CURVE OF (A) FURAZOLIDONE AND (B) METRONIDAZOLE

Accuracy: Accuracy was performed at 80%, 100%, 120% levels of recovery in triplicates and percent recovery was observed 100.9% for Furazolidone and 99.11% for Metronidazole. The result of accuracy are given in **Table 9, 10**. The Percent Recovery Should Be 98% To 102% [24].

TABLE 9: OBSERVATIONS FOR ACCURACY DETERMINATION FOR FURAZOLIDONE

%level	Amount Spiked (µg/ml)	Amount Recovered (µg/ml)	%Recovery	Mean% Recovery
	9	9.24	102.6	
80 %	9	9.30	103.3	102.8
	9	9.24	102.6	
	10	9.99	100.1	
100%	10	9.87	98.7	100.2
	10	10.2	102	
	11	10.67	97	
120%	11	11.24	102.1	99.6
	11	10.97	99.7	

TABLE 10: OBSERVATIONS FOR ACCURACY DETERMINATION FOR METRONIDAZOLE

%level	Amount Spiked (µg/ml)	Amount Recovered (µg/ml)	%Recovery	Mean% Recovery
	36	35.8	99	
80 %	36	35.5	98	99
	36	36.1	100	
	40	40.4	101	
100%	40	40.2	101	101.3
	40	40.8	102	
	44	43.2	98	
120%	44	43.6	99	98.6
	44	43.9	99	

Precision: The percentage RSD of intraday and interday precision was 1.34 for intraday and 1.7 interday for Furazolidone while 1.34 Intraday and 1.38 interday for Metronidazole.

TABLE 11: PRECISION TABLE FOR FURAZOLIDONE AND METRONIDAZOLE

Sr. no.	Name of the API	Intraday Precision	Interday Precision
1.	**Furazolidone**		
	Average	234882	226523
	SD	3097.59	4013.14
	%RSD	1.34	1.7
2.	**Metronidazole**		
	Average	317630	313050
	SD	4175.22	4251.38
	%RSD	1.34	1.38

Limit of Detection (LOD) and Limit of Quantification (LOQ):

TABLE 12: LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

APIs	LOD (µg/ml)	LOQ (µg/ml)
Furazolidone	1.24	3.77
Metronidazole	1.89	5.73

Robustness:

TABLE 13: RESULTS OF ROBUSTNESS PARAMETER

Sr. no.	Condition	%RSD of Furazolidone	%RSD of Metronidazole
1	pH2.9(-1)	1.6	0.73
2	pH3.1(+1)	1.8	1.53
3	Flow Rate 0.7ml/min (-1)	0.6	1.6
4	Flow Rate 0.9ml/min (+1)	0.4	1.6
5	Wavelength 322nm (-2)	0.62	1.27
6	Wavelength 326nm (+2)	0.87	0.8

Assay: FURA-M tablet has labelled claim of 100mg Furazolidone and 400mg Metronidazole. An assay was performed with the FURA-M tablet formulation. The percent assay was found to be 98.6% for Furazolidone and 98.65% for Metronidazole [25].

TABLE 14: RESULTS OF ASSAY PARAMETER

Name of the Drug	Labelclaim (mg)	Estimated amount (mg)	% Assay
Furazolidone	100mg	98.6	98.6%
Metronidazole	400mg	399.8	98.65%

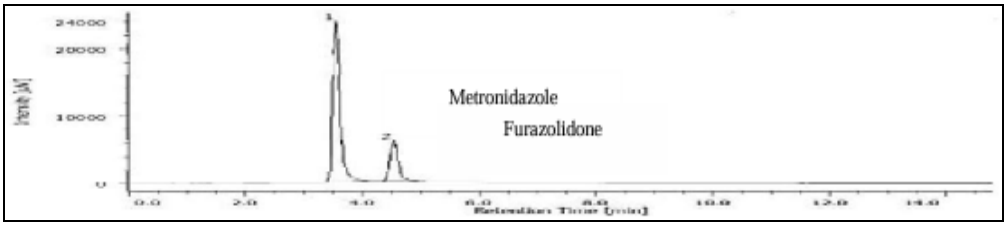


FIG. 27: CHROMATOGRAM OF FORMULATION

System Suitability: The sample solution was stored in an amber-colored container. It was kept at room temperature swell as in a refrigerator (2–8°C). The mobile phase was clear and free from visible particles. The stability study of both the standard and sample solutions met the acceptance criteria for system suitability and repeatability [26-27]. This indicates that the mobile phase remained stable for at least 2 days when stored under the specified conditions **Fig. 28, 29.**

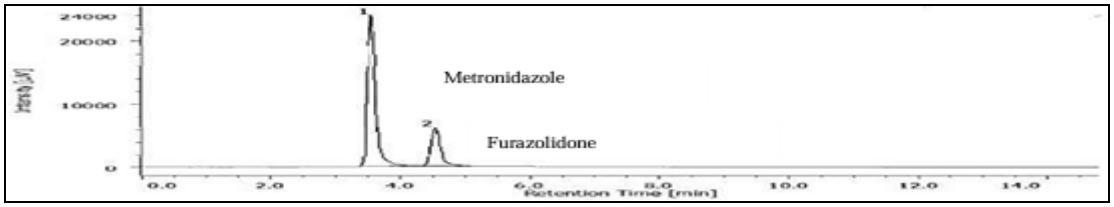


FIG. 28: CHROMATOGRAM OF SYSTEM SUITABILITY DAY 1

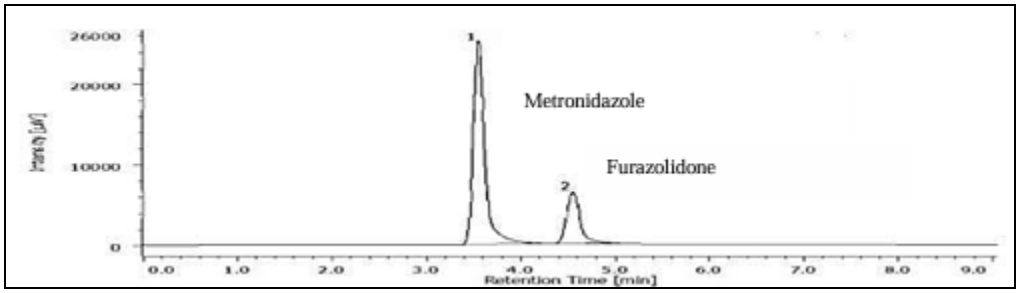


FIG. 29: CHROMATOGRAM OF SYSTEM SUITABILITY DAY 2

CONCLUSION: Analytical method development using AQbD is a well characterised approached to method development. The main goal of AQbD was to develop more reliable method which provides high degree of assurance that consistently produce data meeting predetermined criteria when operated within boundaries. AQbD can be applied to develop and evaluation of analytical method. AQbD tools are as follows ATP, CQA consisting of CMA and CAA, initial risk assessment, method optimization with DoE, MODS, and control strategy with risk assessment, method validation and continuous Method Monitoring (CMM) and continuous improvement. QbD requires not only the right ATP but also risk assessment and usage of right tools and performing the appropriate quantity of work within proper time lines. It provides regulatory flexibility in future. Any modification in the method would be covered by internal change control procedure only. Different trials were done to determine the solubility criteria for Furazolidone and Metronidazole. After determining solubility, to ascertain the detection wavelength UV scan was performed in solvent system followed by overlay in the same solvent system using UV visible spectrophotometer.

Determination of solubility plays crucial role in selecting the ideal mobile phase for HPLC analysis. Thereafter, trials were done on HPLC to develop an analytical method. The post analysis of chromatogram and observation the method is examined under Stat-Ease 360 software for AQbD method optimization. The 32 factorial design used for the optimization of developed method. The software gives trials to perform and from that trial best trial were selected for further evaluation of parameters. The software generates contour plots and 3-D contour plots based on the ANOVA analysis. The contour plots and 3-D contour plots were obtained for interaction between CAAs (i.e. retention time of APIs, number of theoretical plates, symmetry factor and resolution) and CMAs (i.e. composition of mobile phase and flow rate of mobile phase composition). The contour plot and 3-D contour plots of both the APIs represent that the use of acetonitrile: phosphate buffer pH 3 in the ratio of 55: 45 with 0.8 flow rate will get the best chromatographic condition and within the set range or predicted values of responses. The desirability plots showed the experimental design which produced desire valid results. On the basis of assigned goals within the software and after analysis of the responses the software gave best solution chromatographic method whose desirability value is 1 or close to 1, i.e highest desirability, the optimized method was statistically proven by desirability plots which was further experimentally proven in method development and validation state. The optimized method was a mobile phase consisting of acetonitrile: phosphate buffer pH 3 (55:45) v/v with flow rate 0.8 ml/min using (Jasco HPLC) chromnav software. The validation of developed and optimized method was performed for specificity, linearity and range, accuracy, precision, LOD and LOQ, robustness, system suitability solution stability.

The result of all specified validation parameters meets the acceptance criteria. The present study will provide validated, cost effective, less time consuming AQbD -driven RP-HPLC method for simultaneous estimation of Furazolidone and Metronidazole which will meet the intended Requirements. The Regulatory authorities encourage the adoption of QbD principles, including the establishment of design spaces, by integrating MODS, companies align with these expectations, thereby facilitating smoother regulatory submissions. The risk assessment helps to improve method performance. The documentation and maintaining design space helps

for audit preparation. The developed HPLC method for the simultaneous estimation of Furazolidone and Metronidazole has been validated both statistically and experimentally. As a result, the applicability of this method is significantly enhanced.

ACKNOWLEDGEMENTS: Authors express their sincere gratitude to Saraswathi Vidya Bhavan's College of Pharmacy, Dombivli, Maharashtra for providing all necessary facilities to accomplished the entitled work. Authors were grateful to Dr. Sonal Desai (SSR College of pharmacy, silvassa UT of Dadra and Nagar Haveli, India) for extended help during QbD trials.

CONFLICTS OF INTEREST: Authors declares that there are no conflicts of interest.

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How to cite this article:
Ojaswi G and Dhaneshwari G: AQBD driven development and validation of a HPLC method for simultaneous estimation of furazolidone and metronidazole in pharmaceutical dosage form. Int J Pharm Sci & Res 2026; 17(2): 588-01. doi: 10.13040/IJPSR.0975-8232.17(2).588-01.