

## Research Article

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# Statistical Design for Optimization and Determination of Tizanidine Hcl using Folin-Ciocalteu (Fc) as Chromogenic Reagent

Permender Rathee<sup>1</sup>, Kamal Dua<sup>2</sup>, Sushila Rathee<sup>3</sup> and Vikash Kumar<sup>3\*</sup><sup>1</sup>Quantum Solutions India, Rajiv Gandhi Technology Park, Chandigarh, India<sup>2</sup>Department of Pharmaceutical Technology, School of Pharmacy, International Medical University, Kuala Lumpur, Malaysia<sup>3</sup>PD Memorial College of Pharmacy, Sector-3A, Sarai Aurangabad, Bahadurharh, India**Abstract**

A simple, sensitive spectrophotometric method has been developed for quantitative determination of Tizanidine Hydrochloride in bulk and pharmaceutical formulations with application of factorial design. In this method, Tizanidine Hydrochloride is made to react with Folin-Ciocalteu (FC) reagent under alkaline conditions forming a blue chromogen having absorption maximum at 663 nm. Beer's law was obeyed in the concentration range of 4-36 µg/ml. Results of the analysis were validated as per ICH guidelines and by recovery studies. A 3-factor, 3-level statistical design (Box-Behnken) was used to derive a second-order polynomial equation to construct contour plots for prediction of response. Independent variables studied were the FC-reagent (X1), sodium carbonate (X2) and drug concentration (X3) and the levels of each factor were low, medium, and high. The dependent variable studied was absorbance (Y1). The aims of this study to determination and optimize the Tizanidine HCl using FC as Chromogenic reagent; the design demonstrated the role of the derived equation (polynomial) and two dimensional plots in predicting the values of dependent variable for optimization.

**Keywords:** Spectrophotometry; Tizanidine Hydrochloride; FC reagent; Box-Behnken Design

**Introduction**

Tizanidine, 5-Chloro-N-(4, 5-dihydro-1H-imidazol-2-yl)-2, 1, 3-benzothiadiazol-4-amine, is a new centrally acting skeletal muscle relaxant [1]. It is a  $\alpha_2$  adrenergic agonist [2] which inhibits spinal reflex transmission by descending facilitatory pathways and via supraspinal inhibitory effects. In addition to its muscle relaxant properties and central analgesic effect, it also has gastro protective effect. Hence it is used in combination with NSAIDs for the treatment of local pain. A survey of literature revealed that various methods are available for the estimation of Tizanidine Hydrochloride individually as well as simultaneously; these includes HPTLC [3,4], RP-HPLC [5], HPLC [6,7], LC-MS [8], Spectrophotometric methods [9-14] but none of the spectrophotometric methods has optimized the various parameters. Therefore, it was highly desirable to develop a simple and robust analytical method that provides satisfactory stability and good sensitivity to the complex for routine analysis of Tizanidine HCl in dosage forms by using Folin-Ciocalteu (FC) reagent. The FC reagent has been used as a chromogen for determination of various pharmaceutical agents containing nitrogen [15-21]. The focus of the present study was to optimized the concentration of various reagents spectrophotometrically, involved in the reaction to form colored detectable product by employing 3-factor, 3-level Box-Behnken statistical design, and develop robust and simple spectroscopic method using previously optimized process parameters.

**Material and Methods**

Standard Tizanidine HCl was obtained as gift sample from Jackson Pharmaceuticals Pvt. Ltd., Amritsar (Punjab). FC reagent was procured from Loba Chem. Ltd., Mumbai; Sodium Carbonate was procured from S.D Fine and distilled Water (in house production) was used for making solutions. Tizan (Sun Pharma) and Sirdalud (Novartis) tablets, both containing 2.288 mg of Tizanidine Hydrochloride were procured from local market. All UV spectrophotometric measurements were recorded using Double beam Jasco V-630 UV spectrophotometer

(Jasco V 530, India) with spectral band width of 1.5 nm, wavelength accuracy of  $\pm 2$  nm and matched quartz cells of 10 mm optical path length.

**Preparation of standard solution**

A definite amount of Tizanidine HCl (10 mg) was transferred to a 100 mL volumetric flask. Sufficient quantity of distilled water was added and the solution was sonicated for 10 min. Finally, the volume was maintained with distilled water to give a final concentration of 100 µg mL<sup>-1</sup>. Different sets of working standards at various concentrations were prepared by appropriate dilution of the stock solution.

**Preparation of derivatizing agent**

A working solution of detecting reagent was prepared by dissolving 20% w/v sodium carbonate solution and 2 N FC reagent diluted with two volumes of distilled water. The solution was freshly prepared on daily basis for each experiment.

**Calibration solution for analysis**

Aliquots of (0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2 and 3.6 mL) stock solution were transferred to a 10 mL of volumetric flask using a micropipette. 3 mL of reagent solution were added to each flask and heated at  $42 \pm 2^\circ\text{C}$  for 10 min. Each reaction mixture was cooled at room temperature and the volume was made up to mark with distilled water to give final concentrations of 4, 8, 12, 16, 20, 24, 28, 32, 36 µg

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mL<sup>-1</sup>. The absorbance was measured at 663 nm against a reagent blank. The calibration curve was constructed by plotting absorbance versus concentration (µg/ml) and correlation coefficient was also measured by taking mean of concentrations measured in triplicates.

### Sample solution for determination

For the analysis of Tizanidine Hydrochloride in tablets, two different commercial brands of 2.288 mg strength (Tizan- Sun Pharma, Sirdalud-Novartis) were taken. Twenty tablets each of Tizan and Sirdalud were weighed and powdered. The tablets powder equivalent to 10 mg of Tizanidine Hydrochloride was accurately weighed for both brands and dissolved volumetrically with 100 mL of water by heating at 42°C to give a concentration of 100 µg mL<sup>-1</sup>. The flask was cooled and the sample was filtered using filter paper (Whatmann). The filtrate (2.5 mL) was transferred to another 10 mL volumetric flask containing 3 mL of the reagent solution, heated at 45 ± 5°C for 20 min, volume made up to 10 mL with distilled water and analyzed three times and a mean of the triplicate measurements was taken.

### Validation

Linearity was determined by preparing different concentrations of sample solution (4–36 µg mL<sup>-1</sup>). The precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by assaying samples, at the same concentration and during the same day. The intermediate precision was studied by comparing the assays on different days (3 days). Three sample solutions ranging 8, 16 and 24 µg mL<sup>-1</sup> were prepared and analyzed. The accuracy of the method was determined as recovery from 20 µg mL<sup>-1</sup> standard solution spiked with 80, 100 and 120% extra Tizanidine HCl. Specificity was determined by observing that the placebo samples were free from any interfering substances. Placebo samples were prepared by dissolving expected ingredients other than drugs in equal proportions and then assayed in order to verify that none of the excipients of the tablets interfered with the quantity of drugs. Limit of detection (LOD) and Limit of Quantification were calculated against blank. All solutions were prepared and used in triplicate.

### Design

Box-Behnken statistical design was used to optimize the validation parameters and systemically investigate the effect of wide range of independent and dependent variables. FC reagent concentration (X<sub>1</sub>), sodium bicarbonate concentration (X<sub>2</sub>) and drug concentration (X<sub>3</sub>) were three independent variables (factors) considered in the method development of Tizanidine HCl, while the absorbance was a dependent variable (response). Different concentration ranges for FC reagent (1.0-10.0 mL), NaHCO<sub>3</sub> (1.0-10.0 mL) and drug (4-36 µg mL<sup>-1</sup>) were selected. The process parameters were studied by conducting the 17 runs at different levels of all factors. Data collected for responses in each run were analyzed using Design Expert 7.1 software (Statease, USA) and fitted into a multiple linear regression model.

### Results

#### Optimization of parameters

Folin-Ciocalteu reagent [22] is a commonly used agent for the determination of primary amines and amino acids. It reacts with groups of Tizanidine HCl in the presence of sodium bicarbonate [23,24] to form colored reaction product and it measures the amount of the substance needed to inhibit the oxidation of the reagent [25]. The reaction conditions were optimized on the basis of maximum absorbance by UV-Visible spectrophotometer.

To identify the optimum levels of different process parameters influencing the absorbance, an experimental design of 17 runs containing central points was made according to the Box-Behnken statistical design for three selected parameters. The individual and interactive effects of these process variables were studied by conducting the process at different levels of all factors. All the responses observed in 17 runs were simultaneously fitted to first order-, second order- and quadratic models using Design Expert. It was observed that the best fitted model was the quadratic model (results of experimental data and simulated values are enlisted in Table 1).

#### Fitting of data to the model

A three-factor, three-level Box-Behnken statistical experimental design as the RSM requires seventeen experiments. The independent variables and the response for all seventeen experimental runs are given in Table 2. The ranges of Y<sub>1</sub> for all batches were 0.200-0.876 respectively. All the responses observed for seventeen formulations were simultaneously fitted to quadratic models using Design Expert® (Version 7.1.3, Stat-Ease Inc., and Minneapolis, MN). It was observed that the best-fitted model was quadratic model and the comparative values of R<sup>2</sup>, SD and % CV are given in Table 3 along with the regression equation generated for each response. All statistically significant (p<0.05) coefficients are included in the equations. A positive value represents an effect that favors the optimization, while a negative value indicates an inverse relationship between the factor and the response. It is evident that all the three independent variables viz. the amount of

Factor	Levels used, Actual (Coded)		
	Low (-1)	Medium (0)	High (+1)
Independent variables			
X <sub>1</sub> =FC-reagent (ml)	1.00 (-1)	5.50 (0)	10.00 (+1)
X <sub>2</sub> =Sodium carbonate (ml)	1.00 (-1)	5.50 (0)	10.00 (+1)
X <sub>3</sub> =Drug concentration (µg ml <sup>-1</sup> )	4.00 (-1)	20.00 (0)	36.00 (+1)
Constraints			
Dependant variables			
Y <sub>1</sub> =Absorbance			
0.200 ≤ Y <sub>1</sub> ≤ 0.872			

**Table 1:** Variables in Box Behnken design.

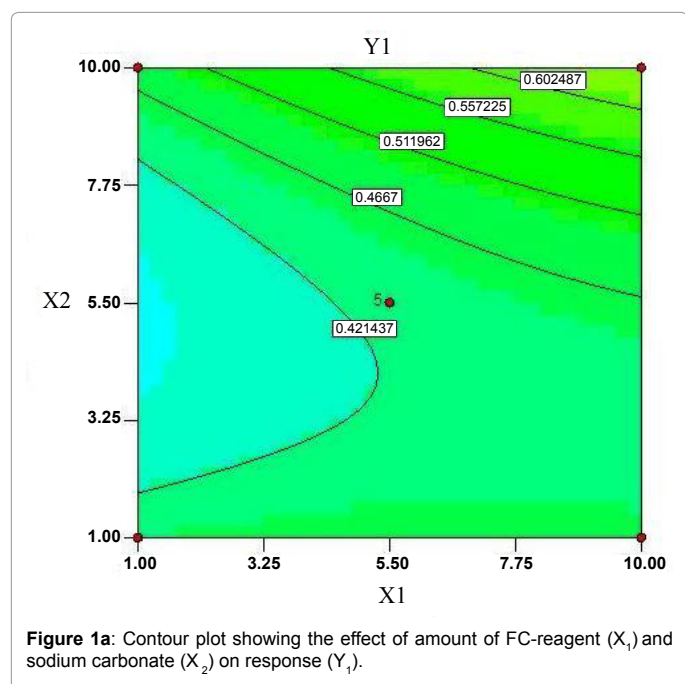
		Independent Variable			Dependent Variables	
Runs	Batches	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	Y <sub>1</sub> (ACTUAL)	Y <sub>1</sub> (PREDICTED)
1	B1	0	1	-1	0.352	0.67
2	B2	-1	-1	0	0.476	0.24
3	B3	1	1	0	0.621	0.25
4	B4	-1	1	0	0.492	0.23
5	B5	1	-1	1	0.758	0.11
6	B6	0	-1	1	0.675	0.67
7	B7	-1	0	-1	0.201	0.697
8	B8	1	-1	0	0.453	0.23
9	B9	0	0	0	0.432	0.67
10	B10	0	0	0	0.408	0.24
11	B11	0	0	0	0.421	0.24
12	B12	0	1	1	0.871	0.16
13	B13	0	-1	-1	0.286	0.23
14	B14	0	0	0	0.465	0.24
15	B15	-1	0	1	0.568	0.11
16	B16	-1	-1	0	0.432	0.16
17	B17	1	0	-1	0.253	0.20

**Table 2:** X<sub>1</sub>=FC Reagent (ml) X<sub>2</sub>=Sodium carbonate (ml) X<sub>3</sub>=Drug Conc. (µg ml<sup>-1</sup>) Y<sub>1</sub>=Absorbance.

Quadratic model	R <sup>2</sup>	Adjusted R <sup>2</sup>	Predicted R <sup>2</sup>	SD	% CV
Response (Y <sub>1</sub> )	0.9898	0.9898	0.9768	0.027	5.58

Regression equations of the fitted quadratic model  
 $Y_1 = 0.34 + 0.044X_1 + 0.056X_2 + 0.22X_3 + 0.038X_1X_2 + 0.035X_1X_3 + 0.032X_2X_3 + 0.011X_1^2 + 0.090X_2^2 + 0.024X_3^2$

**Table 3:** Summary of results of regression analysis for response Y<sub>1</sub> for fitting to quadratic model.



FC-reagent (X<sub>1</sub>), sodium carbonate (X<sub>2</sub>) and drug concentration (X<sub>3</sub>) has interactive effects on the response viz. Y<sub>1</sub>.

### Polynomial Equations Analysis

The model proposes the following polynomial equation for Absorption

$$Y_1 = 0.34 + 0.044X_1 + 0.056X_2 + 0.22X_3 + 0.038X_1X_2 + 0.035X_1X_3 + 0.032X_2X_3 + 0.011X_1^2 + 0.090X_2^2 + 0.024X_3^2$$

Where Y<sub>1</sub> is absorbance, X<sub>1</sub> is FC-reagent, X<sub>2</sub> is sodium carbonate and X<sub>3</sub> is the concentration of drug. The Model F-value of 75.70 implies the model is significant (P<0.0001). The “Lack of Fit F-value” of 2.42 implies the Lack of Fit is not significant (P=0.2068). In this case X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub>, X<sub>1</sub>X<sub>2</sub>, X<sub>1</sub>X<sub>3</sub>, X<sub>2</sub>X<sub>3</sub>, X<sub>2</sub><sup>2</sup> are significant model terms and X<sub>1</sub>X<sub>3</sub> and X<sub>2</sub>X<sub>3</sub> had a more pronounced effect on absorption than any other parameters. The “Pred R-Squared” of 0.8895 is in reasonable agreement with the “Adj R-Squared” of 0.9768. “Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 31.319 indicates an adequate signal.

Therefore this model can be used to navigate the design space. The 2D plots showed the effect of different independent variables on absorbance (Y<sub>1</sub>).

### Plots analysis

Two dimensional contour plots were prepared for the response (Y<sub>1</sub>) and are shown in Figure 1a-c respectively. The plots are known to study the interaction effects of the factors on the response as well as

these plots are utile in studying the effects of two factors on the response at one time. Interactive effect of X<sub>1</sub>X<sub>2</sub> showed that absorbance increased with increasing concentration of either FC-reagent or NaHCO<sub>3</sub> at constant level. Figure 1b shows that increase in drug concentration (X<sub>3</sub>) from 4 to 36 (μg mL<sup>-1</sup>) enhances the absorbance (Y<sub>1</sub>) from 0.078 to 0.908 with the concentration of FC-reagent (X<sub>1</sub>). Also the interactive effect of X<sub>2</sub> and X<sub>3</sub> were observed and absorbance was found to increase with increasing the concentration of either drug or sodium carbonate (Figure 1c).

### Method validation

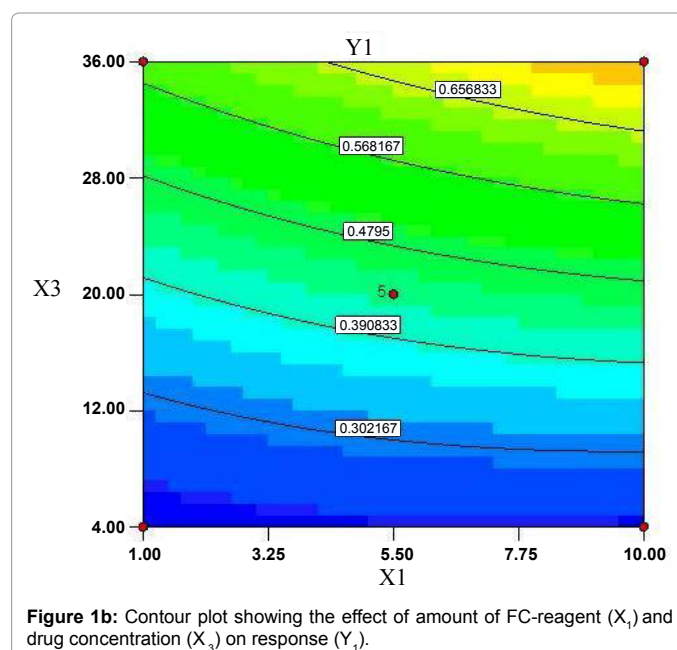
Using the above-optimized parameters, absorption spectra in the range (200-600 nm) were obtained for the Tizanidine HCl. Linear regression equation was  $y = 0.0239x - 0.0591$  with mean correlation coefficient of 0.9994. Accordingly, the precision of the method was determined satisfactorily. The RSD for intra- and inter-day precision were in the range of 0.139-0.513% and 0.141-0.987%, respectively. The average recovery of the proposed spectrophotometric method was 98.5% (RSD=0.57%, SEM=0.119). The method was found to be specific as placebo samples did not exhibit any absorption under the experimental conditions. LOD and LOQ were found to be 0.207 and 0.627 μg mL<sup>-1</sup>, respectively. All the measurements were taken in triplicate and mean of readings were used.

### Recovery studies

Recovery studies were carried (I.C.H Q2B guidelines) out by adding 1, 2 and 3 mg of drug to different samples of tablet powder containing the equivalent of 10 mg of drug. From the amount found, percentage recovery was calculated. Precision of the method was studied by carrying out intraday, interday analysis and expressed as % Relative Standard Deviation. The results of recovery studies were found to be satisfactory and are reported in Table 4.

### Discussion

The mixed acid in the FC reagent involves the following chemical species; 3H<sub>2</sub>O.P<sub>2</sub>O<sub>5</sub>.13WO<sub>3</sub>.5MoO<sub>3</sub>.10H<sub>2</sub>O and 3H<sub>2</sub>O.

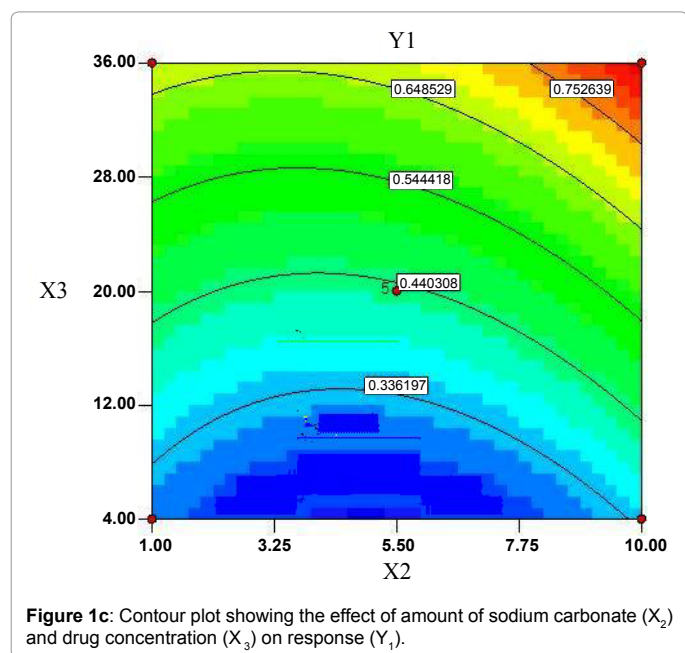


**Figure 1b:** Contour plot showing the effect of amount of FC-reagent (X<sub>1</sub>) and drug concentration (X<sub>3</sub>) on response (Y<sub>1</sub>).

Brand	Labeled amount	Amount found <sup>a</sup>	%Labeled amount	% RSD
Tizan	2.288	2.2732 ± 0.0048	99.355 ± 0.2631	0.2648
Sirdalud	2.288	2.2742 ± 0.0043	99.399 ± 0.3373	0.3394

a: Average of Four Readings

**Table 4:** Results of estimation of Tizanidine Hydrochloride in Tablet dosage forms.



**Figure 1c:** Contour plot showing the effect of amount of sodium carbonate ( $X_2$ ) and drug concentration ( $X_3$ ) on response ( $Y_1$ ).

$P_2O_5 \cdot 14WO_3 \cdot 4MoO_3 \cdot 10H_2O$ . Tizanidine Hydrochloride probably affects the reduction of 1, 2, or 3 oxygen atoms from tungstate and / or molybdate in FC reagent (Phosphomolybdo tungstate), thereby producing one or more of the possible reduced species which have an intense blue color [22]. The proposed method for determination of Tizanidine Hydrochloride showed molar absorptivity of  $5.642 \times 10^3$  litre  $mol^{-1} cm^{-1}$  and Sandell's sensitivity of  $0.0514 \mu g/cm^2/0.001$  absorbance units. Linear regression of absorbance on concentration gave the equation  $y = 0.0239x - 0.0591$  with a correlation coefficient ( $r$ ) of 0.9994. Percent relative standard deviation for intraday and interday analysis was found to be 0.741 and 0.645 respectively. Limit of detection and limit of quantitation were found to be 0.207 and 0.627 respectively. The higher percentage recovery value indicates that there is no interference of the excipients present in the formulation. This method is useful for the determination of Tizanidine Hydrochloride in bulk and pharmaceutical formulations.

## Concluding Remarks

Tizanidine HCl can be determined in pharmaceutical tablets based on reaction with FC reagent in the presence of sodium bicarbonate. Simplicity, accuracy and rapidness are a most attractive advantage of proposed method. The results obtained confirm the optimization and suitability of the proposed methods for the precise analysis and of Tizanidine HCl in quality control laboratories.

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