Original Article

Robust Analytical Method for Iron Estimation by Experimental Design Approach

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ABSTRACT

Aim: To perform Iron estimation by UV-Visible spectroscopy using an Experimental design approach. **Objectives:** The robust analytical method was developed for the estimation of iron (III) using 1, 10-phenanthroline reagent. **Methods:** The analytical method is an exploration of the chemical reaction of iron with 1, 10- phenanthroline reagent to form a colored complex which was measured in the UV-Visible region at 509 nm. To monitor the effect of diverse factors like the concentration of reagent (A), volume of reagent (B), pH (C) and time (D) on the formation of iron 1, 10 – phenanthroline complex the full factorial design (two-level) was used. From the Pareto chart, Normal plot and half normal plot, it was studied that a combination of all factors was initiate to be significant. Then significant variables are optimized by response surface methodology (RSM) via Box-Behnken design. The evaluation of design was performed to study the effect on the selected response by quadratic effects and main interaction effects. The contour plot and surface plot used for the determined response of the selected factors for their optimum value. **Results:** The prime reaction state, Beer's law were obeyed in 2.0-10.0 μg/ml concentration range with a correlation coefficient of 0.998. **Conclusion:** The method was successfully applied for the estimation of iron in iron sucrose injection. The optimized method was used for the quantitative analysis of iron sucrose injection.

Key words: Iron sucrose, 1, 10- Pheanthroline, Full factorial design, Box-Behnken design.

INTRODUCTION

Iron is necessary for oxidative metabolism, wound healing, reproduction, cellular growth, execution of several metabolic processes.[1] Iron is employed in the production of oxygen-carrying hemoglobin, myoglobin and proteins which are required for the basic metabolic process in the cell.[2] Iron deficiency anemia are the most frequent forms of nutritional deficiency generally, anemia is distinct as decrease of hemoglobin value.[3] It possesses severe health complications as it causes general weakness, laziness, tiredness, sub-optimal work performance and in certain circumstances psychological obstruction, reduced aptitude and atypical immune response.[4] Optimization states to improving the routine of a method, a practice, or produce to get the highest output from it. The term optimization has been generally used in analytical chemistry as a means of determining situations at which to apply a process that creates the best probable response.[5]

The experimental design is a statistical technique utilized for planning, analyzing and statistical data obtained from primary investigational trials. The experimental design gives exhaustive information from the lowest numeral of trials. Identification of interacting variables characterized the effect of critical factors, evaluation of the effect of preparation and system factors on critical quality attributes.[6]

The conventional optimization approach, varying one variable/factor at a time (OVAT, also called OFAT).[7] One factor at a time (OFAT) does not include interactive outcomes between the variables deliberate as a consequence. OFAT does not include the comprehensive effects of a factor on

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the response. One factor optimization is the rise in the numeral of trial essential to conduct the research, which adds to more interval time and cost with an increase in the consumption of reagent and materials.[8]

In a mandate to overcome these tricky, multivariate statistic techniques have been used for analytical processes optimization. Full (two-level) factorial, fractional factorial designs and Plackett–Burman designs are generally used to monitor and identify the causes and interactions that have an important effect on the studied responses among a large set of factors.[9] Response Surface Methodology (RSM) is appropriate multivariate methods widely used in analytical optimization. RSM is a group of arithmetical and numerical methods useful for designing trials, emerging models and estimating the effects of the variables in which a response is a concern partial by some variables.[10] Mainly, RSM consists of Box-Behnken Design (BBD), Central Composite Design (CCD), D-optimal design one-factor design. Box-Behnken design and CCD are the widely used statistical techniques.[11]

The contrast between the response surface designs (Doehlert matrix, central composite and full factorial design (three-level)) and Box-Behnken design has proved that the Box-Behnken design is somewhat more effective compared to central composite design but to a great extent effective contrast to full factorial designs (three-level). The Box-Behnken is a noble design for RSM since it permits: (i) assessment of the factors of the quadratic model; (ii) constructing sequential designs; (iii) recognition of non-existence of fit model; and (iv) use of blocks.[12]

The various method is available for iron detection such as high-performance liquid chromatography (HPLC)[13,14] colorimetric,[15,16] Atomic absorption.[17,18] As well as Inductively coupled plasma mass spectrometry (ICP-MS).[19] Numerous methods were reported for iron estimation with a derivatizing agent such as ammonium thiocyanate,[20] thioglycolic acid[21] and 1, 10-phenanthroline.[16] The limitation of an iron derivatizing agent such as the color concentration of ferric thiocyanate complex is unstable and decreases its intensity with 1 % for every degree centigrade increase in temperature and time.[22] Thioglycolic acid possesses very low sensitivity, In case of 1,10 -phenanthroline non-uniformity was found about reagent concentration, volume, pH and reaction time like parameters in reported analytical methods.[23-27]

The analytical methods reported for Iron estimation (HPLC, colorimetric, atomic absorption, ICP-MS) tested robustness as a part of extensive method development. The determination of the robustness method nowadays most extensively useful in the pharmaceutical domain because of the stringent rules set by regulatory authorities. Hence, the main propose of this research was to optimize and report a robust analytical process for the iron estimation in mass and therapeutic dosage form using 1, 10- phenanthroline as the derivatizing reagent. The screening of the reaction parameters by (two-level) full factorial design was exploited to estimate the key effect of independent issues on the response. Then optimization of reaction parameters by Box-Behnken design. The range of particular parameters was found to make the iron estimation method robust one. The optimized analytical method is used for the estimation of iron from in and pharmaceutical formulation.

MATERIALS AND METHODS

Chemical and reagents

The hydroxylamine hydrochloride, ferrous ammonium sulphate, 1,10- phenanthroline reagent, sodium acetate was purchased from Merck chemicals. Fercee injection manufactured by alkem laboratories Mumbai, The Fercee injection was purchased from the local market.

Instrumentation

A Shimadzu UV–visible spectrophotometer (1700) with a pair of matched 1 cm quartz cells, UV-Probe 2.21 software was used for absorbance measurement. The experimental design study was performed using Minitab 17 (trial version) software.

Reagents and solutions

1, 10 - phenanthroline solution (0. 3 % w/v)

An accurate weight quantity of about 300.0 mg of 1,10-phenanthroline was dissolved containing 0.1 ml of hydrochloric acid (1M) in double-distilled water.

Sodium acetate (1M)

An accurate weight quantity of about 8.2034 g of sodium acetate was dissolved in 100 mL of double-distilled water.

Hydroxylamine hydrochloride (10 % w/v)

An accurate weight quantity of about hydroxylamine hydrochloride (10 gm) was dissolved in double-distilled water (100 ml).

ferrous ammonium sulphate hexahydrate (100 ppm) (Standard stock solution)

An accurately weighed quantity of about 70.0 mg of ferrous ammonium sulphate hexahydrate was dissolved up to 100.0 ml water containing 2.5 ml (1M) concentrated sulfuric acid.

Working standard solution (4 µg/ml) of ferrous ammonium sulphate hexahydrate

From the standard stock solution, 0.4 ml aliquot about was diluted with water to 10.0 ml.

Methods

Full factorial two-level design

Full factorial two-level design was used to detect the effect of the factor such as reagent concentration (A), volume of reagent (B), pH (C), time (D) on formation ferric ions (Fe^{2+}) and 1, 10 -phenanthroline complex on basis of initial trials. The different levels of the nominated factors as low and high levels given in Table 1. The design matrix of the full factorial designs (two-level) is generated using Minitab as revealed in Table 2. The absorbance of the complex is considered as a response. The trials were performed and data generated as shown in Table 2.

Table 1: Experimental factors and response variable for two-level full factorial design.

Experimental factors	Code	Level	
		Low	High
1,10-phenanthroline reagent concentration (%w/v)	A	0.2	0.4
Volume of 1,10-phenanthroline reagent (ml)	B	1	2
pH	C	2	9
Time (min)	D	10	30

Table 2: Design matrix of two-level full factorial design.

Run	A (Conc. of reagent	B (Volume of reagent)	C (pH)	D (time)	Response
1	0.4	1	9	10	0.1235
2	0.4	2	9	30	0.1515
3	0.2	2	2	30	0.1207
4	0.4	2	9	10	0.1663
5	0.3	1.5	5.5	20	0.1450
6	0.2	2	9	10	0.1345
7	0.2	1	9	30	0.1228
8	0.4	1	9	30	0.1685
9	0.2	2	5.5	30	0.1532
10	0.3	1.5	2	20	0.1528
11	0.4	2	9	10	0.1355
12	0.2	1	2	10	0.1671
13	0.2	1	2	30	0.1608
14	0.2	2	2	10	0.1607
15	0.3	1.5	5.5	20	0.1728
16	0.2	1	2	10	0.1150
17	0.4	2	2	30	0.1638
18	0.3	1.5	5.5	20	0.1385
19	0.4	1	2	10	0.1630
20	0.4	1	2	30	0.1128

Optimization by Box-Behnken design

BBD (three-level) with four center point was exploit to estimate the quadratic effects and significant interaction of 1,10-phenanthroline reagent concentration (%w/v) (A), Volume of 1,10-phenanthroline reagent (mL) (B), pH (C), Time (D) factors. Entire the investigation were achieved in triplicate. The design matrix for optimization of method parameters as revealed in Table 3. The absorbance of the solution was added as a response after experimentation.

Linearity study

Aliquots portion of about 0.2-1.0 mL of ferrous ammonium sulphate hexahydrate (standard stock solu-

Table 3: Design matrix for optimization of method parameters by Box-Behnken design.

Run	A (concentration of reagent)	B (volume)	C (pH)	D (time)	Response
1	0.4	1	5.5	20	0.1332
2	0.3	2	5.5	30	0.1184
3	0.2	2	5.5	20	0.1257
4	0.4	2	5.5	20	0.1284
5	0.3	2	2	20	0.1216
6	0.3	1	2	20	0.1228
7	0.3	2	9	20	0.1108
8	0.3	1	5.5	30	0.1108
9	0.3	1.5	9	30	0.1190
10	0.3	1	9	20	0.1650
11	0.3	2	5.5	10	0.1144
12	0.4	1.5	5.5	30	0.1158
13	0.3	1	5.5	10	0.1250
14	0.2	1.5	9	20	0.1284
15	0.3	1.5	5.5	20	0.1465
16	0.4	1.5	2	20	0.1342
17	0.2	1	5.5	20	0.1622
18	0.3	1.5	5.5	20	0.1172
19	0.3	1.5	2	30	0.1384
20	0.4	1.5	5.5	10	0.1211
21	0.2	1.5	2	20	0.1486
22	0.2	1,5	5.5	10	0.1206
23	0.3	1.5	5.5	20	0.1106
24	0.2	1.5	5.5	30	0.1273
25	0.3	1.5	2	10	0.1238
26	0.4	1.5	9	20	0.1345
27	0.3	1.5	9	10	0.1301

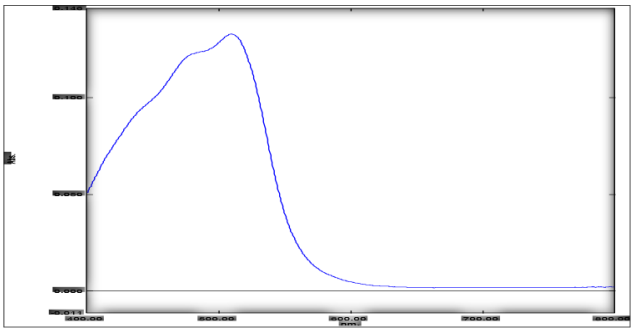


Figure 1: UV-Visible spectrum of complex.

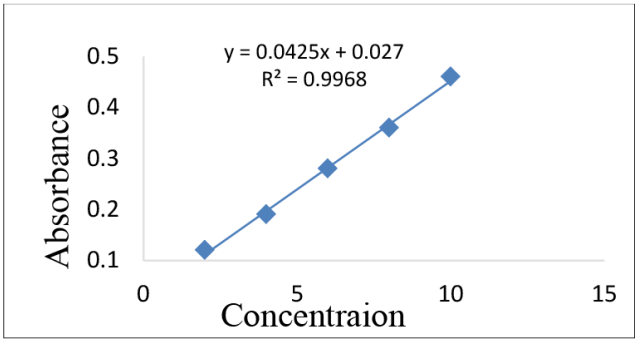


Figure 2: Calibration curve.

tion) were transferred into volumetric flasks (10 mL). To each flask, 1.0 mL of hydroxylamine hydrochloride, 1.5 mL of 1, 10-phenanthroline and 1mL of sodium acetate were added and the solution was kept as such for 15 min. Then, the final volume was adjusted with water. The solution of complex scanned in UV-Visible range against blank (Figure 1) for each concentration. The study was performed and the calibration curve of concentration of iron against absorbance was plotted (Figure 2). The wavelength 509.50 nm shows the maximum absorbance for iron 1, 10- phenanthroline complex.

Determination of Iron from Iron sucrose injection

Sample preparation Iron sucrose (100 ppm)

The ampoule "fercee" was broken. Aliquot of about 3.5 ml solution of iron sucrose was transfer to a 100.0 ml volumetric flask containing 2.5 ml (1M) sulfuric acid. The sample was sonicated (10 min.) Then, the solution was diluted up to the mark with water. Then the solution was filtered using Whatman paper No 41.

Working solution (5 µg/ml) Iron sucrose solution

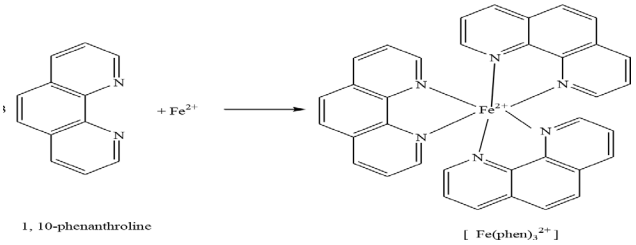
From the standard stock solution of iron sucrose, 0.5 ml aliquot was diluted to 10.0 ml with water.

Procedure

An appropriate aliquot of 0.5 mL was transfer to 10.0 mL volumetric flask to which added 1.0 mL of hydroxylamine hydrochloride, 1.0 mL of sodium acetate, 1.5 mL of 0.3% w/v of 1, 10 -phenanthroline. The solution was kept as such for 15 min. Finally, the sample diluted with a solvent to mark. The solutions absorbance was noted at 509.50 nm against a blank.

RESULTS AND DISCUSSION

A spectrophotometric method using the experimental design process was optimized for iron content determination using 1, 10- phenanthroline as a derivatizing reagent. The 1, 10-phenanthroline ($C_{12}H_8N_2$, ortho-phenanthroline or o-Phen) is hetero-tricyclic nitrogen containing compound that reacts with iron to form colored complexes.[27] This property offers a tremendous sensitive method for determining metal ions in aqueous solution. An intense orange-red color was produced by the reaction of ferric ions (Fe^{2+}) with three molecules of the 1,10-phenanthroline to form a colored complex between ferric ion and 1,10-phenanthroline [$Fe(phen)_3^{2+}$] which shows maximum absorption at 509.50 nm.



Therefore, we selected 1, 10- Pheanthroline as the derivatizing reagent for iron estimation in the present work.

The experimental variables involved in the proposed method were screened (by two-level full factorial) and optimized by Box-Behnken design. Screening designs are frequently used to regulate which factors have a noteworthy influence on the response.

(Two-level) a full factorial design was exploited to scrutinize the effect of a factor on formation of ferric ions (Fe^{2+}) and 1, 10 - phenanthroline complex. The major target was to identify notable factor with the lowest scores as conceivable.

Full factorial two-level design

As the (two-level) full factorial design was screened the Pareto charts (Figure 3), half normal and normal plot (Figure 4 and 5) were resulting from multivariate regression analyses. The bar lengths of Pareto chart were relative to the entire value of the estimated effects.[28]

Figure 3-5 Pareto rank study had shown that, the arithmetically significant factors ($P<0.05$) for nominated response Y were the concentration of reagent (A),

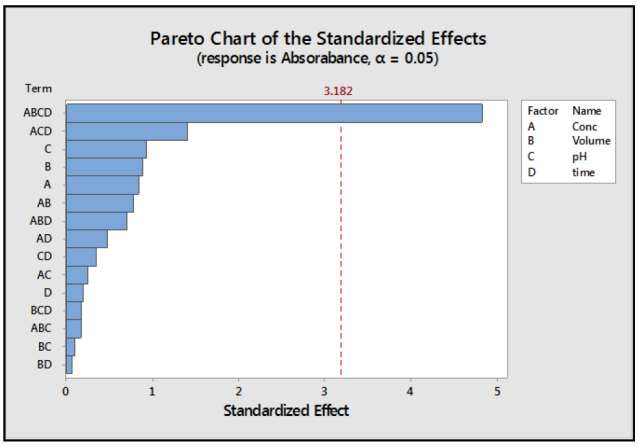


Figure 3: Pareto chart of the effect.

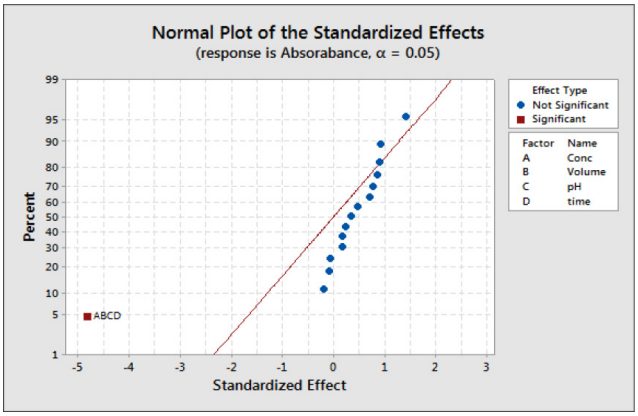


Figure 4: Normal plot of the effect.

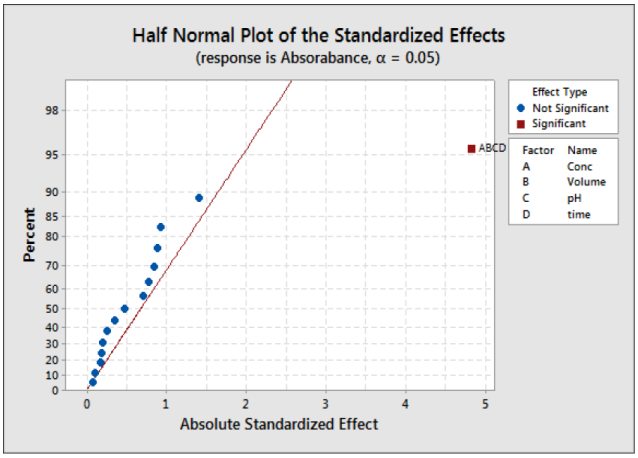


Figure 5: Half normal plot of the effect.

volume of 1,10-phenanthroline reagent (B) pH (C) and time (D). The route of the bar shows whether the factor has a shortest or converse effect on the response. The concentration of reagent (A), volume of 1,10- phenanthroline reagent (B) pH (C) and time (D) have shortest associations with the absorbance Y and their effects are arithmetically noteworthy ($P<0.05$). Not a single factor was found significant but a combination of all factors was found to be significant. It is the advantage of experimental design.

Box-Behnken experimental design

For the average response Y, a full quadratic model was acquired by multivariate regression analysis. The following equation as

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^3 + \beta_{44}X_4^4 + \beta_{12}X_1X_2 + \beta_{34}X_3X_4$$

Y-Selected response;

$\beta_{0 =}$ arithmetic mean response;

β_1, β_2, β_3, $\beta_{4 =}$ regression coefficients of the factors X_1, X_2, X_3 and X_4 respectively.

After the experiment and processing data through Minitab the standards of regression coefficients and their connected *P*-values were obtained (Tables 4 and 5). It was observed that Volume of 1,10-phenanthroline reagent (ml) (B) and pH (C) expressively exaggerated the absorbance (Y) of the iron 1,10-phenanthroline reagent complex ($P<0.05$) as revealed in Table 4.

The volume was found a significant factor as $P=0.019$ whereas an interaction between volume of 1, 10-phenanthroline reagent (ml) (B) and pH (C) ($P=0.040$) also

Table 4: Analysis of variance for a response.

Source	DF	Adj MS	Adj MS	F-value	*P*-value
Model	14	0.003463	0.000247	1.66	0.191
Linear	4	0.001004	0.000251	1.69	0.217
Conc	1	0.000173	0.000173	1.17	0.302
Volume	1	0.000828	0.000828	5.57	0.036
pH	1	0.000000	0.00000	0.00	0.970
Time	1	0.0000002	0.000002	0.02	0.902
Square	4	0.001116	0.000279	1.88	0.179
Conc*conc	1	0.000245	0.000245	1.65	0.223
Volume*volume	1	0.000013	0.000013	0.09	0.772
pH*pH	1	0.000220	0.000220	1.48	0.247
Time*Time	1	0.000311	0.000311	2.09	0.174
2-way interaction	6	0.001342	0.000224	1.51	0.257
Conc*volume	1	0.000251	0.000251	1.69	0.218
Conc*pH	1	0.000105	0.000105	0.71	0.417
Cocn*Time	1	0.000036	0.000036	0.24	0.632
Volume*pH	1	0.000702	0.000702	4.72	0.050
Volume*Time	1	0000083	0.000083	0.56	0.470
pH*time	1	0.000165	0.000165	1.11	0.313
Error	12	0.001784	0.000149		
Lack-of-fit	10	0.001054	0.000105	0.29	0.928
Pure Error	2	0.000730	0.000365		
Total	26	0.005247			

Table 5: Estimated regression coefficient for a response.

Term	Coef	SE coef	*t*-value	*P*-value
Constant	0.1248	0.0104	12.00	0.0000
Conc	-0.00712	0.00520	-1.37	0.196
Volume	-0.01413	0.00520	-2.72	0.019
pH	0.00237	0.00520	0.46	0.657
Time	-0.00044	0.00520	-0.08	0.934
Conc*conc	0.00885	0.00780	1.13	0.279
Volume*volume	0.00738	0.00780	0.95	0.362
pH*pH	0.00727	0.00780	0.93	0.269
Time*Time	-0.01054	0.00780	-1.35	0.201
Conc*volume	0.01787	0.00900	1.99	0.070
Conc*pH	0.00513	0.00900	0.57	0.580
Conc*Time	-0.00300	0.00900	-0.33	0.745
Volume*pH	-0.02075	0.00900	-2.31	0.040
Volume*Time	0.00455	0.00900	0.51	0.622
Volume*Time	0.00455	0.00900	0.51	0.622
pH*Time	-0.00642	0.00900	-0.71	0.489

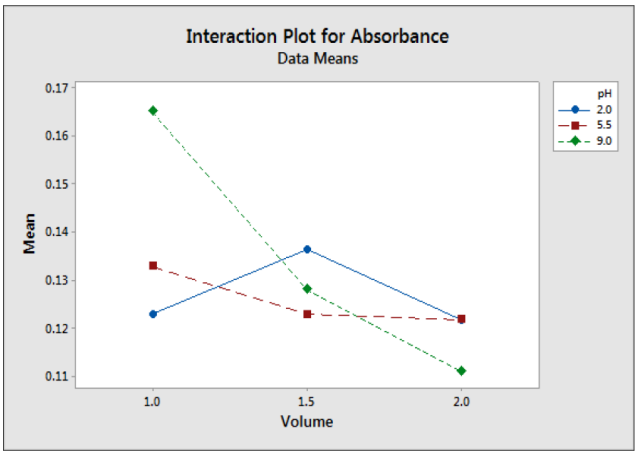


Figure 6: Interaction plot of Volume against ph.

found significant as in Table 5 (Figure 6). An interaction between the 1, 10 –pheanthroline reagent concentration (% w/v) (A), Volume of 1, 10-phenanthroline reagent (ml) (B) (*P*=0.070) near to significant as in Table 5 (Figure 7).

No interaction was found between Concentration (% w/v) and pH (C) (P=0.580), Concentration and Time (D) (*P*=0.745), Volume (ml) (B) and Time (D) (*P*= 0.622), pH (C) and Time (D) (*P*=0.489) as revealed in Table 5.

The contour plots and response surface were also examined to envisage the parameter effects on the chosen response Y. Figure 8 shows that concentration of 1, 10 -phenanthroline reagent (A) in 0.2 - 0.3% w/v range and a volume 1, 10 -phenanthroline reagent (B) 1-1.5 mL

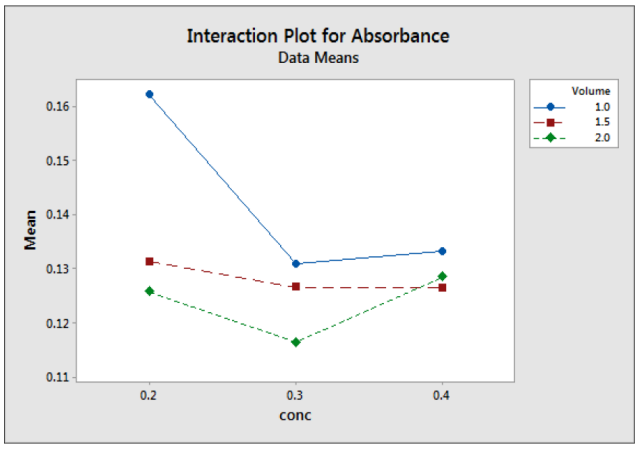


Figure 7: Interaction of concentration against volume.

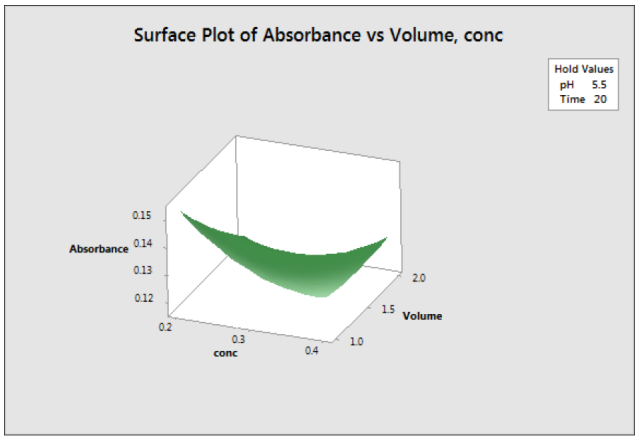


Figure 8: Surface plot.

has the maximum absorbance Figure 9 shows pH (C) was in 5-8 range and a volume 1, 10 -phenanthroline reagent (B) 1-1.5 mL gives maximum absorbance.

The contour plots show a nonlinear effect of these factors on designated response Y (as concluded from the surface diagram as per Figure 10). The effect of concentration of 1, 10-phenanthroline reagent (A) 0.2- 0.3% w/v and volume of 1, 10 -phenanthroline reagent (B) 1-1.5 ml give the maximum absorbance. Figure 11 shows the pH effect (c) in the range of 5-9 and volume of 1, 10-phenanthroline reagent 1.5-1.8 ml give the maximum absorbance.

Model authentication

The experimental results, predicted values obtained by the polynomial model equation had shown that the predicted value match reasonably with R-Sq of 65.67% and R-Sq (adj) of 26.19% for designated response R. For designated response R in the model equation a residual plot was as revealed in Figure 12. The distribution of the residuals for the response has approxi-

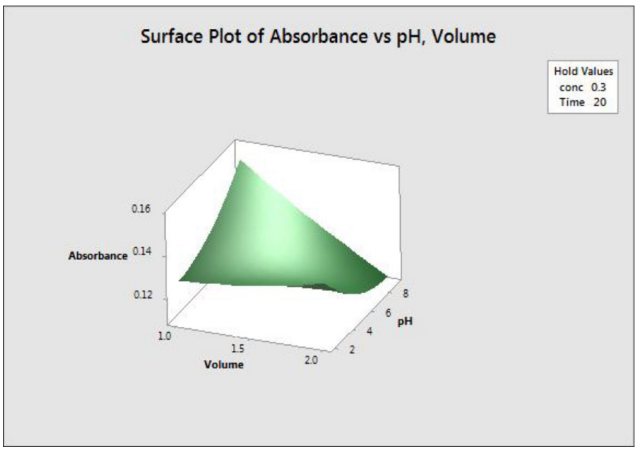


Figure 9: Surface plot.

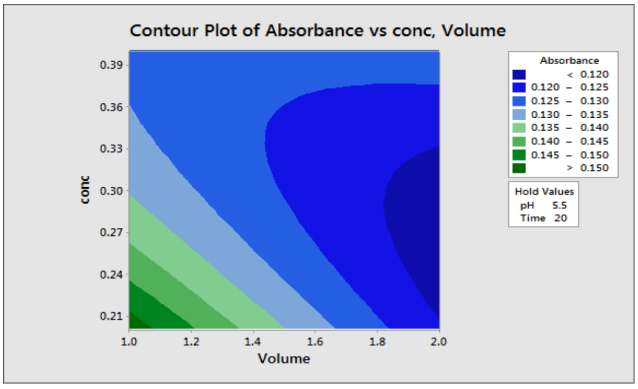


Figure 10: Contour plot of Absorbance Vs Conc, Volume.

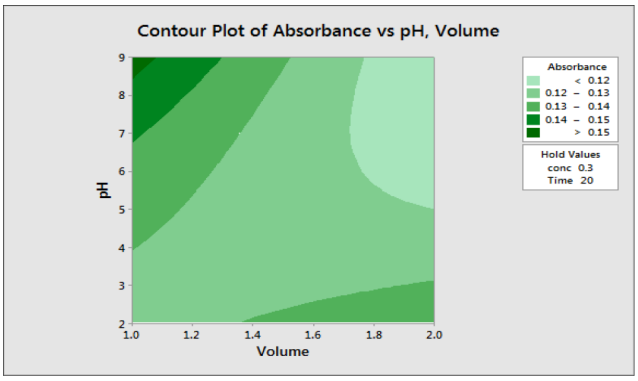


Figure 11: Contour plot of Absorbance Vs. pH, Volume.

mately followed the fixed normal distribution whereas the response unsystematically dispersed in the residual plot.[7]

Optimized method parameters for experimentation

From the interaction plot and surface plot, the method parameters which make the method rugged one was chosen. The method parameters volume of reagent (1.5 + 0.1 ml) and pH of the solution (2-9) with the absorbance 0.123-0.135 (Figure 6 Interaction plot of

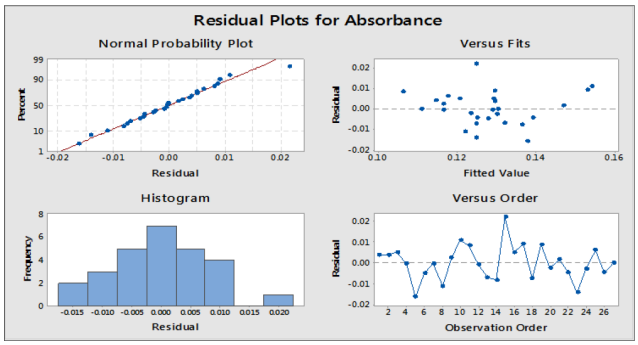


Figure 12: Residuals plot.

Table 6: Optimized method parameters.

Sr. No.	Parameter	Range investigated using experimental design	Parameter used in experimentation
	Concentration (% w/v)	0.2 – 0.4	0.3
	Volume of reagent (ml)	1 – 2	1.5
	pH	2 – 9	2
	Time (min)	---	15

Volume against pH). The method to be considered rugged here concerning method parameters concentration of reagent (0.2-0.4%) and volume of solution (1.5ml) give the absorbance nearer to 0.130 (Figure 7. The interaction plot of concentration against volume) the time is not a critical factor to affect the process in this case. Therefore, it was kept 15 min as this time was required for color formation (depending on preliminary work). In Table 6, the optimized method parameters for experimentation are shown.

Linearity study

A linear relationship was found in the 0.2 – 10 µg/mL concentration range. The graph had a noble correlation coefficient (r^2 = 0.9968) with the intercept (0.027) deliberate by using the regression equation (0.0425X + 0.027).

CONCLUSION

The UV-Visible spectrophotometric technique for the estimation of iron was optimized by a QbD approach. The four independent factors for iron detection were the volume of reagent, concentration of reagent, pH and time were successfully screened by full factorial

design (two-level). All factors in combination were found significant for iron determination. Then a significant factor optimized by RSM via Box-Behnken design. Due to interaction graphs possible to calculate the range of parameter which will make the analytical method robust one. The parameters are 0.3% w/v concentration of reagent, reagent volume 1.5 ml, pH 2 and time 15 min were optimized. The factors were capable to provide the rugged method for iron estimation. The method was used to determine iron content from the iron pharmaceutical formulation. As compared to one factor at a time, we systematically define the effect of each parameter on iron 1, 10-phenanthroline complex.

ABBREVIATIONS

OFAT: One factor at time; **RSM:** Response surface methodology; **CCD:** Central composite design; **HPLC:** High-performance liquid chromatography; **ICP-MS:** Inductively coupled plasma mass spectrometry; **QbD:** Quality by design.

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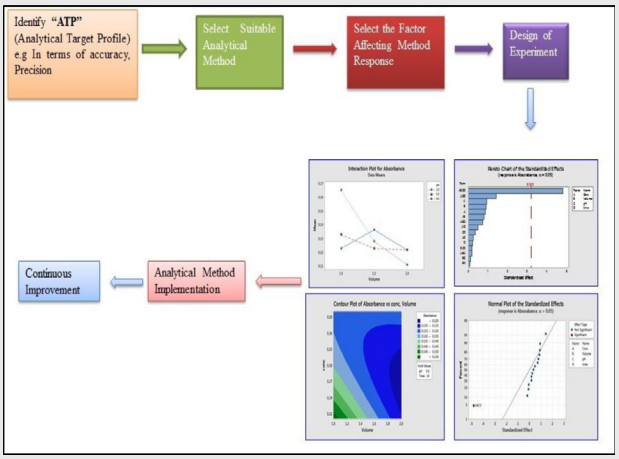
PICTORIAL ABSTRACT



SUMMARY

- The main propose of this study was to establish a relatively simple, precise and inexpensive visible method for the iron determination. An intense orange-red color was produced by the reaction of ferric ions (Fe^{2+}) with three molecules of the 1,10-phenanthroline to form a colored complex between ferric ion and 1,10-phenanthroline [$Fe(phen)_3^{2+}$] which shows maximum absorption at 509.50 nm. (Two-level) full factorial design was exploited to study the effect of concentration of reagent, volume of reagent, pH and time on formation ferric ions (Fe^{2+}) 1, 10 -phenanthroline complex on basis of preliminary experiments. The reaction parameter optimizes by BBD. A Box-Behnken design (three-level) with three center points was used to optimize the quadratic effect and main interaction of reagent concentration (A), volume of reagent (B), pH (C), time (D) on absorbance Y. One factor optimization is the rise in the extent of the experiment essential to conduct the investigation, which causes an increase of interval and cost, more consumption of reagent and materials. The experimental designing method was organized, less time consuming, cost-effective as compared to the conventional methods. Therefore, the proposed method for estimation of iron using an experimental design approach could be applicable for routine quality control analysis of iron containing formulation.

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