AN EXPERIMENTAL DESIGN APPROACH FOR OPTIMIZATION OF MODIFIED COLORIMETRIC FIRST-ORDER DERIVATIVE METHOD FOR ESTIMATION OF SERRALYSIN IN BULK AND PHARMACEUTICAL FORMULATION

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ABSTRACT

Objective: The aim of the present work is to use experimental design to screen and optimize experimental variables for developing a colorimetric first-order derivative method for determining content of serralysin (SER) using biuret and Folin–Ciocalteu phenol reagent for stable color development. The method is based on the reaction of peptide bond in the protein with Biuret reagent in alkaline medium and further reaction of remaining tryptophan and tyrosine residues with Folin–Ciocalteu Phenol reagent to form a stable blue-colored complex (first-order derivative λ_{max} 620 nm).

Materials and Methods: A two-level full factorial design was utilized to screen the effect of Volume of NaOH (A), volume of biuret reagent (B), volume of Folin–Ciocalteu phenol reagent (C), and concentration of NaOH (D) on the formation of blue-colored SER - reagent complex (response - absorbance). A box Behnken experimental design with response surface methodology was then utilized to evaluate the main interaction and quadratic effects of these factors on the selected response.

Results: With the help of a response surface plot and contour plot, the optimum values of the selected factors were determined and used for further experiments. These values were volume of NaOH (A) of 1.0 mL, volume of biuret reagent (B) of 0.25 mL, and volume of Folin–Ciocalteu phenol reagent (C) of 10 μL. The proposed method was validated according to the ICH $Q_2(R_1)$ method validation guidelines. The developed colorimetric first-order derivative method was found to be simple, accurate, rapid, sensitive, precise, and economic. Further optimization of the method with experimental design approach makes it convenient for use in laboratory.

Conclusion: The results of present study have clearly shown that an experimental design approach may be effectively applied to the optimization of a modified visible spectrophotometric method for estimation of SER in bulk and in pharmaceutical formulation with the least number of experimental runs possible. The method can be employed successfully for the estimation of SER in both bulk and tablet dosage form.

Keywords: Serralysin, Experimental design, Box–Behnken Model, Colorimetric, First-order derivative, Biuret, Folin–Ciocalteu.

INTRODUCTION

Analytical studies, related to the quality control and routine analysis of commercial products in the research or industry laboratories, use spectrophotometric methods such as derivative spectrophotometry. These spectrophotometric methods are found to be preferable instead of hyphenated analytical instrumentations or techniques such as liquid chromatography-mass spectrometry (LC-MS), gas chromatography-MS, LC-nuclear magnetic resonance, etc., which always require prior steps such as extraction and other tedious analytical process during analysis [1].

Serralysin (SER) (CAS registry number: 70851-98-3, EC 3.4.24.40) has a long history in medicine and is widely used to combat various kinds of inflammation and inflammatory disorders. SER is a proteolytic enzyme isolated from the non-pathogenic enterobacteria *Serratia E 15* found in silkworms [2]. A recent finding suggests that the enzyme reduces capillary permeability induced by histamine, bradykinin, and serotonin; it dissolves the abnormal exudates and proteins by breaking it down and facilitates the absorption of decomposed products through blood and lymphatics [3]. Thus, SER acts as a potent anti-inflammatory agent. The enzyme has a molecular weight of 45–60 KD and is a metalloprotease containing a zinc atom which is important for its proteolytic activity (Fig. 1).

Implementation of quality by design for analytical methods during development allows for enhanced understanding of the analytical method focusing on robustness and ruggedness, thereby facilitating method transfer and providing opportunities for continual improvement [4]. Two-level full factorial design is used for screening the important factors that influence the response. Factorial designs are advantageous over one factor at a time (OFAT) method as it gives wider inductive basis to draw inferences about the process and it also reveals "interactions" of factors. A two-level full factorial design consists of 2 levels of each experimental factor. It provides direction for further experimentation [5].

Response surface methodology (RSM) is a statistical technique used for the development and optimization of complex processes [6-8]. Different types of response surface designs are used for optimization such as central composite design (CCD) and Box–Behnken design (BBD). The beauty of BBD is that it requires fewer test runs and is rotatable [9].

The BBD is advantageous because it does not contain any points at the extremes of the cubic region created by the two-level factorial combinations [6,7,10]. BBD is a second-order quadratic model for non-linear responses which is mainly used when the factor space is within the experimental domain [11]. The BBD was selected in the present investigation and used to optimize and validate a modified spectrophotometric method for estimating SER because the design provides three levels for each factor and requires fewer runs as compared to CCD.
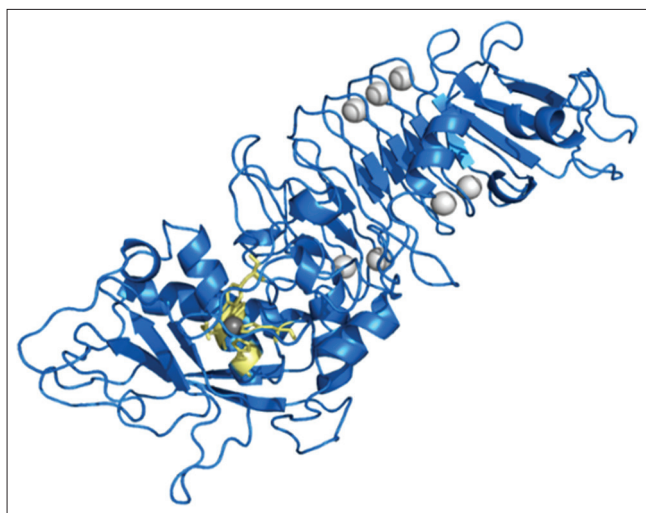
Fig. 1 Serralysin structure

Numerous studies have been carried out for quantitative and qualitative estimation of SER [12]. Thin-layer chromatography [13], SDS-PAGE gel electrophoresis [14], and X-ray powder diffractometry [15] are some of the techniques reported for successful qualitative estimation of SER. Quantitative instrumental methods use chromatography, field flow fractionation, electrophoresis, etc., for separation, and physical properties such as absorption, fluorescence, conductivity, and light scattering are used to measure the analyte accurately and precisely [12]. For quantitative analysis, caseinolytic assay method [16-27], spectroscopy [28-35], fluorescence spectrometry [36], enzyme-linked immunosorbent assay (ELISA) [37-39], radioimmunoassay [40], ultraviolet (UV) microplate method [41], high-performance LC (HPLC) [42], and LC-MS method [43] are reported.

In the reported spectrophotometric methods, the absorbance of SER was measured in the UV region wavelengths. The present study uses a colorimetric first-order derivative analysis of SER in bulk and tablet formulation. For protein quantitation, the biuret method [44-47] and the Lowry method [48,49] have been widely used as standard procedures. The biuret method has a more linear correlation between protein concentration and absorbance but lacks sensitivity. Therefore, the sensitive Lowry method is more preferred. However, the necessity of preparing chemicals for each protein measurement due to poor stability of the combined reagents, instability of color development, the standard curve being non-reproducible, especially at low concentrations and non-linearity between absorbance and protein concentration are some of the problems associated with the Lowry method. In 1976, S. Ohnishi and Barry proposed a simplified method of quantifying protein using the biuret and phenol reagents where the protein sample was first treated with biuret reagent and then with 2 N Phenol reagent for color development. The method was superior to any of the widely used colorimetric method for protein estimation [50].

In the present work, combined application of the biuret method and the Lowry method in enhanced alkaline conditions is employed. The aim of the present work was to utilize the experimental design approach for screening and optimizing the experimental variables for developing modified colorimetric first-order derivative method for estimation of SER in bulk and pharmaceutical formulation.

EXPERIMENTAL

Instrument

A Jasco UV visible spectrophotometer V-730 (Spectra Manager Software) with 1 cm matched quartz cells was used for measuring absorbance.

MATERIALS AND METHODS

SER was purchased from Analab Fine chemicals, Mumbai (Maharashtra). Cipzen forte tablets (10 mg) and Bidanzen (5 mg) were purchased from the local medical shop. Analytical grade chemicals (biuret reagent, sodium hydroxide, Folin–Ciocalteu's phenol reagent) and distilled water were used in the experiments.

Reagents and standards

Stock biuret reagent from Analab Fine chemicals and Folin–Cioclteu's phenol reagent from MERCK were used for the work. Different concentrations of sodium hydroxide (0.5 N, 1.0 N, and 1.5 N) were prepared according to Indian Pharmacopoeia.

Standard solution for spectrophotometric method

A stock solution of SER (2000 μg/mL) was prepared by dissolving 0.2 g in 100 mL of distilled water.

Sample solution for spectrophotometric method

Twenty tablets of each brand were weighed and powdered. A quantity of powder equivalent to 10 mg of SER was transferred to 10 mL distilled water. The solution was sonicated in an ultrasonic bath for 10 min, mixed and filtered using Whatman filter paper.

MATERIALS AND METHODS

Two-level factorial design

A two-level full factorial design was employed to evaluate the effects of four independent factors, namely, volume of NaOH (A), volume of biuret reagent (B), volume of Folin–Ciocalteu phenol reagent (C), and concentration of NaOH (D) on absorbance R. On the basis of preliminary experiments, the ranges of values used in the design are the following: Volume of NaOH (A): 0.5–1.5 mL, volume of biuret reagent (B): 0.1-0.5 mL, volume of Folin–Ciocalteu phenol reagent (C): 0–10 μL and concentration of NaOH (D): 0.5–1.5 N. The different levels of the selected factors are presented in Table 1 and the design matrix of the two-level full factorial design is provided in Table 2. All the experimental runs were performed in triplicate and results are presented in Table 2.

Optimization by RSM

A three-level BBD with three center points (Table 3) was used to evaluate the main, interaction, and quadratic effects of volume of NaOH (A), volume of biuret reagent (B), and volume of Folin–Ciocalteu phenol reagent (C) on absorbance R. On the basis of results obtained by applying two-level full factorial design, it may be concluded that addition of Folin–Ciocalteu's phenol reagent (C), significantly affects the response absorbance. However, the reproducibility was lost if volume of Folin–Ciocalteu's phenol reagent is increased beyond 12 μL. Based on this fact, the volume of Folin–Ciocalteau's phenol reagent was optimized within the range 8–12 μL. The experimental conditions that were maintained constant include concentration of NaOH (0.5 N) and the diluting solvent (distilled water). All the experimental runs were performed in triplicate and the results are presented in Table 3.

Linearity study

An aliquot of the standard stock solution (3.0–6.0 ml) was transferred to 10 ml volumetric flask. The total volume of each flask was brought to 10 ml with distilled water. 1.0 ml of 0.5N NaOH was added to each flask. The solutions were kept aside for 5 min. 0.25 ml of biuret reagent was then added in each flask and solution was thoroughly mixed on vortex for 5 min reaction time. Then, 10 μL of Folin–Ciocalteu's phenol reagent was added and reaction mixture was again mixed for 1 min. The blue-colored reaction solution was scanned in the range of 400–800 nm against reagent blank. The spectra were converted in first-order derivative form and absorbances were measured at λ_{max} 620 nm. (Fig. 2)

Assay of SER in pharmaceutical formulation

Aliquots of about 3 and 10 ml of the standard stock and sample solutions were transferred to 10 ml volumetric flask. The total volume of standard was brought to 10 ml with distilled water. 1 ml of 0.5 N

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

Factor	Name	Units	Type	Minimum	Maximum
A	Volume of NaOH	ml	Numeric	0.5	1.5
B	Volume of biuret reagent	ml	Numeric	0.1	0.5
C	Volume of Folin–Ciocalteu's phenol reagent	µL	Numeric	0	10
D	concentration of NaOH	N	Numeric	0.5	1.5
Response R	Absorbance	Abs units	Goal maximum		

Table 2: Experiment 2^4 and response value

Standard	Run	Factor 1	Factor 2	Factor 3	Factor 4	Response 1
		A: Volume of NaOH	B: Volume of biuret reagent	C: Volume of FC reagent	D: Concentration of NaOH	Absorbance
		mL	mL	µL	N	A
7	1	0.5	0.5	12	0.1	0.00050993
6	2	1.5	0.1	12	0.1	0.000391224
3	3	0.5	0.5	8	0.1	0.000693873
10	4	1.5	0.1	8	2	0.000441652
11	5	0.5	0.5	8	2	0.000816232
5	6	0.5	0.1	12	0.1	0.000350717
8	7	1.5	0.5	12	0.1	0.000620997
15	8	0.5	0.5	12	2	0.000756431
2	9	1.5	0.1	8	0.1	0.000488967
4	10	1.5	0.5	8	0.1	0.000685252
16	11	1.5	0.5	12	2	0.00046393
14	12	1.5	0.1	12	2	0.000471261
9	13	0.5	0.1	8	2	0.000561577
12	14	1.5	0.5	8	2	0.00048388
1	15	0.5	0.1	8	0.1	0.000545019
13	16	0.5	0.1	12	2	0.000456169

Table 3: Optimization method parameters for Box–Behnken experimental design

Run	A: Volume of NaOH	B: Volume of biuret reagent	C: Volume of Folin–Ciocalteu's phenol reagent	Predicted response	Observed response R
1	0.5	0.25	12	0.087	0.080
2	1	0.4	8	0.077	0.072
3	1	0.25	10	0.096	0.095
4	0.5	0.4	10	0.068	0.071
5	0.5	0.25	8	0.077	0.079
6	1	0.25	10	0.096	0.092
7	1	0.1	8	0.052	0.048
8	1	0.1	12	0.063	0.068
9	1	0.4	12	0.078	0.082
10	1	0.25	10	0.096	0.098
11	1	0.25	10	0.096	0.097
12	1.5	0.25	12	0.083	0.081
13	1.5	0.4	10	0.075	0.073
14	1.5	0.1	10	0.048	0.045
15	0.5	0.1	10	0.054	0.056
16	1	0.25	10	0.096	0.096
17	1.5	0.25	8	0.082	0.090

NaOH was added to each flask. The solutions were kept aside for 5 min. 0.25 ml of biuret reagent was then added in each flask and solutions were mixed on vortex mixer for 5 min reaction time. Then, 10 µL of Folin–Ciocalteu's phenol reagent was added and reaction mixture was again mixed for 1 min. The spectra for standard and sample solutions were converted into first-order derivative spectra and absorbances were measured at 620 nm. The content of SER in the pharmaceutical dosage form was calculated using the following formulae:

$$X_{Est} = \frac{(A_{Sample} \times W_{Std} \times W_{Avg})}{A_{Std} \times W_{Sample}}$$

$$\% \text{Label Claim} = \frac{X_{Est}}{X_{LABEL CLAIM}} \times 100$$

where X_{Est} = content of SER per tablet of average weight; A_{Sample} = absorbance of sample solution; A_{Std} = absorbance of standard solution; W_{Std} = weight of standard (mg); W_{Sample} = weight of sample (mg); and $X_{LABEL CLAIM}$ = labeled content of SER per tablet (mg).

RESULTS AND DISCUSSION

A colorimetric derivative spectrophotometric method was developed and optimized for determining the content of SER using the biuret reagent and Folin–Ciocalteu's phenol reagent for stable and reproducible color development. The use of both the reagents together improves the absorbance. The conversion in first-order derivative spectra is needed for better linearity. The experimental design approach was utilized for screening and optimizing the experimental variables of the first-order derivative spectrophotometric method.

Two-level full factorial design

Factorial design is a tool that allows us to experiment on many factors simultaneously. In proposed work, a two-level full factorial design was utilized to evaluate the main effect of four independent factors on the selected response R. The primary purpose was to identify significant main effects with the minimum number of experimental runs as possible.

The effects of all factors included in the experimental design on selected response R are shown in Table 2. An effect was considered to be significant at $p \leq 0.05$. The significant factor is the influencing factor. The effect of each factor was interpreted as the average change in response R as the factor changed from low to high level (Table 1). Pareto ranking analysis revealed that the factors that were statistically significant ($p \leq 0.05$) for selected response R were the volume of NaOH (A), volume of biuret reagent (B) and volume of Folin–Ciocalteau's phenol reagent (C). The Pareto charts (Fig. 3) gives vital few effects on left and trivial many at right. The length of each bar in Pareto chart is equivalent to the magnitude of the regression coefficients of the factor. The volume of NaOH (A), volume of biuret reagent (B), and volume of Folin–Ciocalteu's phenol reagent (C) have direct relationships with the absorbance R and their effects are statistically significant ($p \leq 0.05$). The Concentration of NaOH (D) also has a direct effect on the selected response R, but this is not statistically significant ($p > 0.05$).
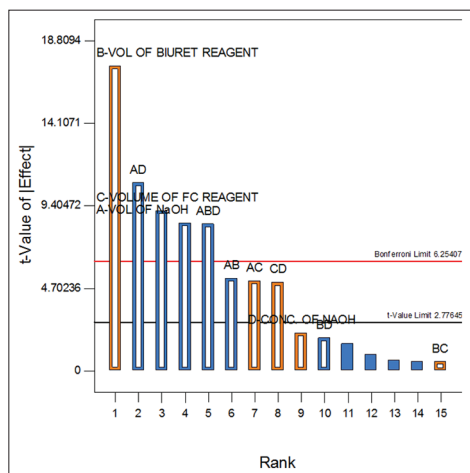
Box–Behnken experimental design

Using multivariate regression analysis, a fitted full quadratic model was obtained for the average response R, given by following equation:

$$R = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$
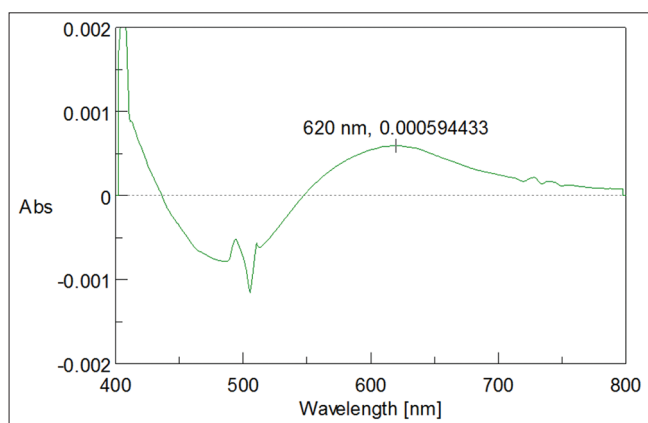
Fig. 2: First-order derivative spectra of standard serralysin in visible range after reaction with biuret reagent and Folin–Ciocalteau's reagent

Fig. 3: Pareto chart of effects

where R= Selected response; β_0 = arithmetic mean response; and β_1, β_2, and β_3= regression coefficients of the factors A, B, and C, respectively.

Tables 4 and 5 have shown the values of regression coefficients and their associated p-values. It may be observed that volume of NaOH (A), volume of biuret reagent (B), and volume of Folin–Ciocalteu's phenol reagent (C) significantly affected the absorbance (R) of colored complex ($p < 0.05$). An interaction was observed between the volume of NaOH (A) and volume of biuret reagent (B) ($p = 0.3351$), between volume of NaOH (A) and volume of Folin–Ciocalteu's phenol reagent (C) ($p = 8039$) and it was also observed between volume of Biuret reagent (B) and volume of Folin–Ciocalteu's phenol reagent (C) ($p = 0.3724$), but these interactions are found to be insignificant as $p > 0.05$. Volume of biuret reagent was found to affect the response more. An interaction plot is shown in Fig. 4.

Response surface and contour plots were also analyzed to visualize the effects of the parameters on the selected response R. Fig 4 shows the effects of volume of NaOH (A), volume of biuret reagent (B), and volume of Folin–Ciocalteu's phenol reagent (C) on the selected response R.

It may be interpreted from the surface diagram that volume of biuret reagent (B) was in the range of 0.24-0.26 ml and that a volume of NaOH (A) in the range of 1.0–1.1 ml gives maximum absorbance (R) (Fig. 5a).

A volume of NaOH (A) was in the range of 1.0–1.1 ml and that a volume of Folin–Ciocalteu phenol reagent (C) in the range of 10–12 µL gives maximum absorbance (R) (Fig. 5b).

A volume of Folin–Ciocalteu phenol reagent (C) was in the range of 10-12 µL and that a volume of biuret reagent (B) in the range of 0.25–0.28 ml gives maximum absorbance (R) (Fig. 5c).

Model validation

The experimental results and the predicted values obtained using polynomial model show that the predicted values match the selected response R reasonably with an R^2 of 96.65% and an R^2 (adj) of 95.35%.

Fig. 6 shows the residual plots of the selected response R in model equation. The distribution of the residuals of the response approximately follows the fitted normal distribution, whereas the
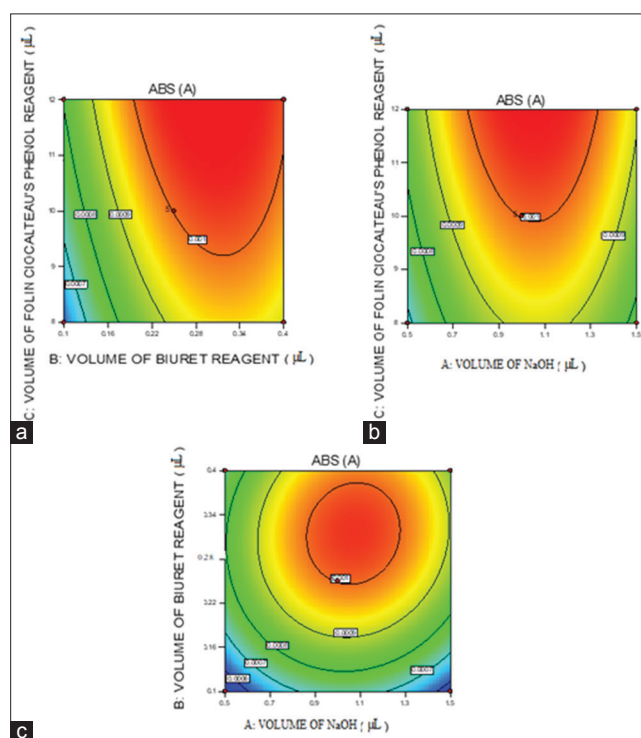
Fig. 4: (a-c) Interaction plot of response R

Table 4: Analysis of variance for selected response

Source	Sum of squares	df	Mean square	F	p>F	Inference
Model	4.372E-007	9	4.858E-008	22.46	0.0002	Significant
A-volume of NaOH	1.295E-008	1	1.295E-008	5.98	0.0443	
B-volume of biuret reagent	1.252E-007	1	1.252E-007	57.88	0.0001	
C-volume of Folin–Ciocalteau's phenol reagent	4.221E-008	1	4.221E-008	19.51	0.0031	
AB	2.317E-009	1	2.317E-009	1.07	0.3351	
AC	1.438E-010	1	1.438E-010	0.066	0.8039	
BC	1.964E-009	1	1.964E-009	0.91	0.3724	
A^2	1.406E-007	1	1.406E-007	65.00	< 0.0001	
B^2	9.384E-008	1	9.384E-008	43.38	0.0003	
C^2	1.157E-009	1	1.157E-009	0.53	0.4884	
Residual	1.514E-008	7	2.163E-009			
Lack of fit	9.025E-009	3	3.008E-009	1.97	0.2611	Not significant
Pure error	6.118E-009	4	1.529E-009			
Total	4.524E-007	16				

Table 5: Estimated regression coefficient for selected response

Factor	Coefficient estimate	df	SE	95% CI low	95% CI high
Intercept	1.002E-003	1	2.080E-005	9.525E-004	1.051E-003
A-volume of NaOH	4.023E-005	1	1.644E-005	1.344E-006	7.911E-005
B-volume of biuret reagent	1.251E-004	1	1.644E-005	8.622E-005	1.640E-004
C-volume of Folin–Ciocalteau's phenol reagent	7.264E-005	1	1.644E-005	3.376E-005	1.115E-004
AB	2.407E-005	1	2.326E-005	−3.092E-005	7.906E-005
AC	−5.996E-006	1	2.326E-005	−6.099E-005	4.899E-005
BC	−2.216E-005	1	2.326E-005	−7.715E-005	3.283E-005
A^2	−1.827E-004	1	2.267E-005	−2.363E-004	−1.292E-004
B^2	−1.493E-004	1	2.267E-005	−2.029E-004	−9.569E-005
C^2	−1.658E-005	1	2.267E-005	−7.017E-005	3.702E-005

SD=4.651E-005, Press=1.540E-007, R^2=0.9665, adjusted R^2=0.9535, predicted R^2=0.6597. SD: Standard deviation, SE: Standard error, CI: Confidence interval
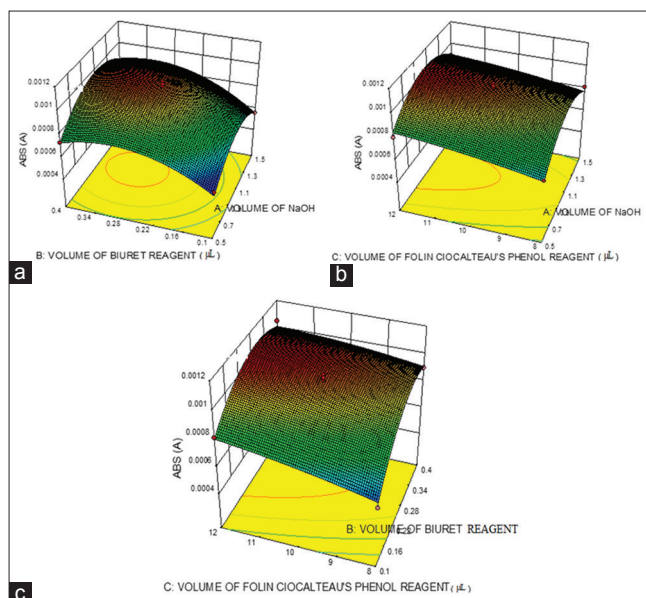

Fig. 5: Response surface plot of response R (a) A: Volume of NaOH versus B: Volume of Biuret reagent, (b) A: Volume of NaOH versus C: Volume of Folin–Ciocalteu's phenol reagent, (c) B: Volume of biuret reagent versus C: Volume of Folin–Ciocalteu's phenol reagent

residuals of the response are randomly scattered in the residual plots.

Reaction mechanism

The proposed method involves copper chelation chemistry. The assay is performed in two distinct steps. In the first step, the protein reacts with Biuret reagent to form a tetradentate copper complex from four peptide bonds and one atom of copper. Second, a reaction with Folin–Ciocalteu's phenol reagent in which a phosphomolybdic-phosphotungstic acid solution is added. This compound (called Folin-phenol reagent) becomes reduced and produces intense blue color. It is believed that the color enhancement occurs when the tetradentate copper complex transfers electrons to the phosphomolybdic-phosphotungstic acid complex. Addition of NaOH makes the solution more alkaline and this is observed to enhance the color intensity and its stability.

Spectral characteristics

The scanning speed of 1000nm/min, data interval 0.5 nm, and UV response 0.96 seconds were selected as the instrumental parameters by OFAT method. The colored complex formed was observed to give a peak at 620 nm in the first-order derivative UV spectra when run against reagent blank prepared in similar manner except SER.

Choice of solvent for preparation of SER solution

Distilled water was selected as solvent for preparing SER solution considering solubility and stability.

Reagent solution

The Biuret reagent was used without dilution. The Folin–Ciocalteu's reagent 2N was used in the assay.

Linearity study

A calibration graph of the SER concentration versus the absorbance was constructed for the method under optimized conditions. A linear relationship was observed in the concentration range of 0.1–4.0 mg/ml. The graph shows a good correlation coefficient (r^2=0.9995) with the intercept (0.001) calculated using regression equation (Table 6)

Assay results of SER in pharmaceutical formulations

The results obtained from the analysis of marketed formulations are provided in Table 7. The percent purities of marketed formulations, namely, Bidanzen (5 mg) and Cipzen forte (10 mg) tablet were found to be 99.50±0.34 and 100.60±0.41, respectively.
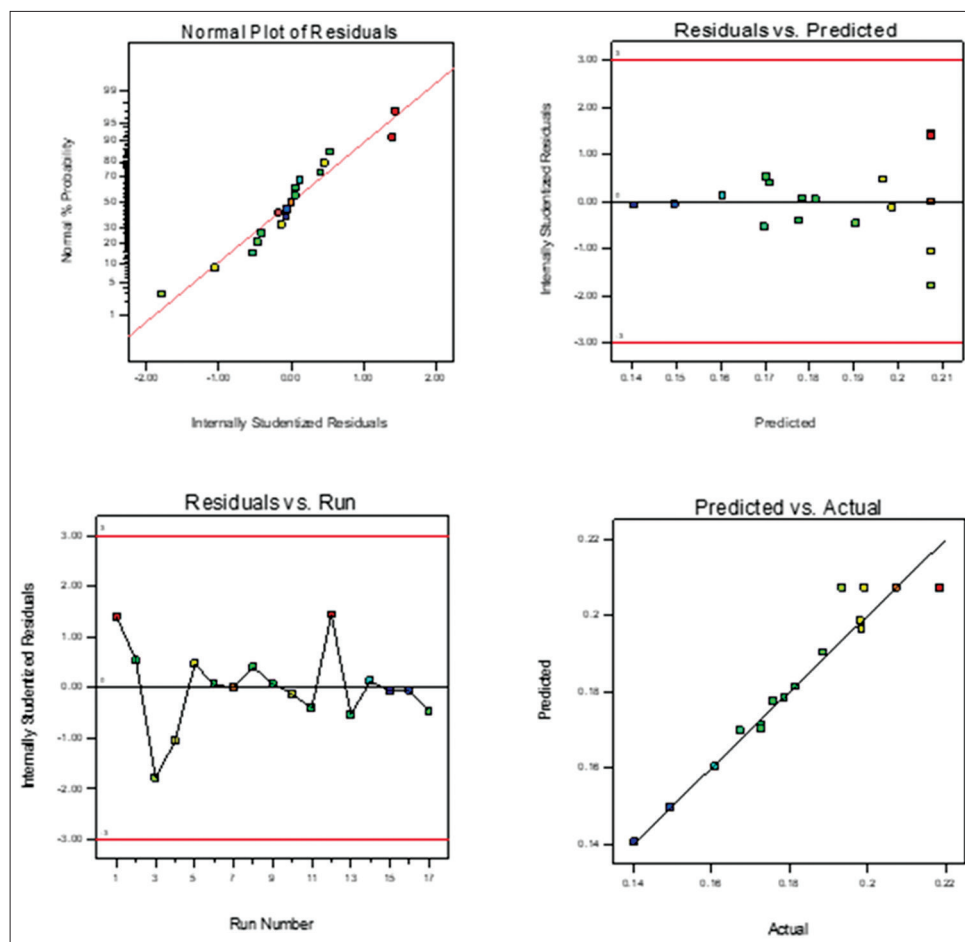
Fig. 6 Residual plots of response R

Table 6: Optical characteristics and statistical data of regression equations for the color reaction

Parameter	SER color complex
pH	Alkaline (~12.7)
Colour	Intense blue
Derivative λ_{max}	620 nm
Beer Lambert's law limit (mg/ml)	0.1–4.0
Sandell's sensitivity (mg/ml cm)	1.1
Slope	0.0009
Intercept	5E-05
Regression coefficient	0.9903

SER: Serralysin

Table 7: Determination of serralysin in pharmaceutical formulation

Label claim	Amount of drug founded (mg)±%RSD ($n=5$)			
	Proposed method	Reported method	t^a	F^b
Bidanzen (5 mg)	99.50±0.34	100.43±0.32	1.7548	0.7275
Cipzen forte (10 mg)	100.60±0.41	100.98±0.62	1.2811	0.7152

RSD: Ratio of the standard deviation

Accuracy

The accuracy of the proposed method was determined through a recovery study. A known amount of pure SER was used to spike to pre-analyzed tablet formulation. Analysis of SER was carried out at three concentration levels (80%, 100%, and 120%) within the specified linearity range. Determination of the SER content was carried out using the formula mentioned in the section titled "assay of SER in pharmaceutical formulations." The percent recovery of the proposed method was calculated using the formula as below.

$$\%Recovery = \frac{E}{T+P} \times 100$$

where E = total amount of ser estimated (mg); T = amount of SER from pre-analysis of the tablet powder (mg); and P = amount of pure SER added (mg). The average percent recoveries for Bidanzen (5mg) and Cipzen forte (10 mg) tablet are found to be 101.02±0.64 and 100.70±0.41, respectively (Table 8).

Precision

The interday precision and intraday precision of the method were determined. A repeatability study (intraday precision) was performed by analyzing a sample of SER repeatedly within a day. An interday precision study was performed by analyzing a sample of SER repeatedly at different days. The results of interday and intraday precision were expressed as %RSD and they were found to be 0.93 and 1.15, respectively. The %RSD value indicates that the method has good precision.

Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD and LOQ of the analytical method developed for estimating the SER content were calculated using the following formulae.

LOD = $(3.3 \times \sigma)/S$

Table 8: Results of recovery studies by the standard addition method

Amount of drug in tablet (mg)	Amount of pure drug spiked (mg)	Total found (mg)	Recovery of pure drug added (%)	% RSD ($n=3$)
Bidanzen (5 mg)				
5	3	8.12	104	0.95
5	5	10.58	111.60	0.43
5	7	12.24	103.42	0.76
Mean			106.34	0.71
Cipzen forte (10 mg)				
10	8	18.25	103.12	1.31
10	10	20.41	104.10	0.37
10	12	22.31	102.58	0.29
Mean			103.26	0.65

RSD: Ratio of the standard deviation

$$LOQ = ((10 \times \sigma)/S$$

where σ = standard deviation of the response; and S = slope of calibration curve.

The LOD and limit of quantitation were found to be 1.2045 and 4.2136 mg/mL, respectively.

Stability of solution

The solution used in the precision study was stored for 24 h at room temperature. During the storage period, the absorbance of the solution was measured after different time intervals (0, 15, 30 min, 1, 2, 3, 4, 5, 6, and 24 h), and it was found that the color intensity of the solution was stable up to 6 h.

Statistical analysis of data

The validity of the proposed method for determining the SER content in pharmaceutical formulations was tested by analyzing the SER using a previously developed HPLC method. The results of the statistical comparison are given in Table 7. At the 95% confidence level, the calculated t-value and F-value do not exceed the theoretical values for either Bidanzen (5 mg) or Cipzen forte (10 mg) tablet. Therefore, there is no significant difference between the proposed and reported methods, indicating that the proposed method is as accurate and precise as the reported method.

CONCLUSION

A two-level full factorial design was used to design an experimental program to extract data regarding influencing factors that had significant effects on the selected response (R). One-way ANOVA and Pareto ranking analyses showed that the volume of NaOH (A), volume of biuret reagent (B) and volume of Folin–Ciocalteu's phenol reagent (C) were statistically significant factors ($p<0.05$) affecting the formation of the colored complex of SER and absorbance (R). A Box–Behnken experimental design with RSM was then utilized to evaluate the main, interaction, and quadratic effects of these three factors on the selected response Y. With the help of a response surface plot and contour plot, the optimum values of the selected factors were identified. The predicted values of the response matched the experimental values reasonably well, with an R^2 of 96.65% and an R^2 (adj) of 95.35% for the selected response R. In order to gain a better understanding of the effects of the variables on the selected response R, the predicted model was presented in the form of a response surface and contour plot design. It was concluded that the experimental design concept could be effectively used to optimize a visible spectrophotometric method for estimating the SER content in bulk and pharmaceutical formulations with the least number of experimental runs possible.

AUTHOR'S CONTRIBUTION

Both the authors have contributed for the concept, experimental work, data analysis, and manuscript preparation of the presented research work.

CONFLICTS OF INTEREST

We declare that there is no conflict of interest.

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