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ORIGINAL ARTICLE **OPEN ACCESS**

Design of Experiment-Driven Stability Indicating RP- HPLC Method for Simultaneous Estimation of Tetracaine Hydrochloride and Oxymetazoline Hydrochloride

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ABSTRACT
*The aim of the present work is to use experimental design to screen and optimize experimental variables for developing a HPLC method for simultaneous estimation of Tetracaine hydrochloride and Oxymetazoline hydrochloride in presence of excipients in nasal spray solution. Plackett - Burman design was utilized to screen the effect of variable factors. The pH, mobile phase ratio, column type, wavelength and flow rate were selected as independent variables and peak area, theoretical plates and retention time were the dependent variables. From the screening study pH, mobile phase ratio and flow rate were selected for optimization. Box Behnken experimental design with response surface methodology (RSM) has been used to estimate the main, interaction and quadratic effects of these three factors on selected response. The chromatographic conditions obtained from Box Behnken design involve 0.05M Potassium dihydrogen phosphate (pH 3.5): methanol (40:60,%v/v) as mobile phase at a flow rate of 1.0 ml/min. Chromatopak C $_{18}$ (250mm * 4.6mm, 5 µm) column was used as stationary phase and detection was performed at 231 nm. The retention time were found to be 4.24 and 8.15 min for Tetracaine HCl and Oxymetazoline HCl, respectively. Tetracaine HCl and Oxymetazoline HCl (drug and dosage form) was subjected to acid, alkali, neutral, oxidative, thermal and photodegradation. The method was successfully applied to the estimation of Tetracaine HCl and Oxymetazoline HCl in nasal spray solution. The method was found to be simple and rapid with less trial and error experiments by making use of Design of Experiment.*
Keywords: *Plackett - Burman design, Box Behnken design, Tetracaine hydrochloride, Oxymetazoline hydrochloride*

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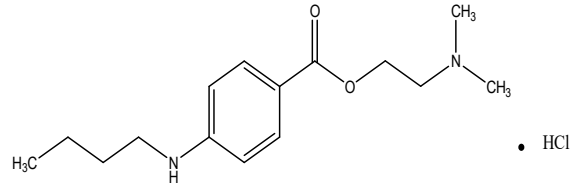
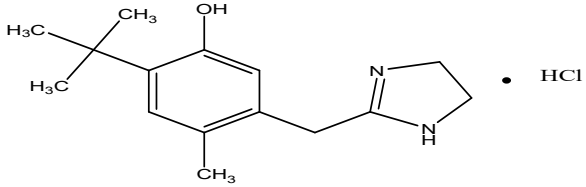
INTRODUCTION

Tetracaine hydrochloride is a local Anesthetics of the ester type and experts its activity by blocking Na^+ ion channels required for the initiation and conduction of neuronal impulses. Chemically, Tetracaine hydrochloride is 2-(dimethyl amino) ethyl 4-(butyl amino) benzoate hydrochloride [1].
Oxymetazoline hydrochloride is an Imidazole derivative with Sympathomimetic activity. Chemically, Oxymetazoline hydrochloride is phenol,3-[(4,5-dihydro-1H-imidazole-2-yl)methyl]-6-(1,1-dimethyl ethyl)-2,4-dimethyl monohydrochloride [2].



Chemical structure of Tetracaine HCl



Chemical structure of Oxymetazoline HCl

Fig. 1. Chemical structures of Tetracaine HCl and Oxymetazoline HCl

The combination is a revolutionary innovation—the world's first-known dental anesthetic administered through the nasal cavity devoid of needle, designed to achieve pulpal (tooth nerve) anesthesia for the restorative treatment of teeth. Based on literature review a number of UV spectrophotometric and chromatographic methods are available for estimation of both the drugs either alone, in combinationor in combination with other drugs [3].

So, there is need to develop and validate stability indicating HPLC method for Simultaneous estimation of Tetracaine hydrochloride and Oxymetazoline hydrochloride [4].
Two level full and fractional factorial designs as well as Plackett Burman designs are used to screen the important factors that influence responses
RSM is a statistical technique used for the development and optimization. Optimization is used after preliminary screening of experimental factors that significantly affect the response using Plackett Burman design [5].
The Box- Behnken design was selected in the present research and used to optimize, validate and analyze Tetracaine hydrochloride and Oxymetazoline hydrochloride chromatographically, because the design provides three levels for each factor and necessitates fewer runs in the three-factor case compared with the central composite design and Doehlert design [6-8].
The method which is used for analysis of stability samples is called as stability indicating assay method.
moreover, the quantification of oxymetazoline hydrochloride in its various drug formulations and/or biological samples was addressed in many reports. Liquid chromatography using various detection modes has been widely applied. Examples of these reports are HPLC with UV detection [11-14] and flow injection analysis [15]. For quantification of Tetracaine hydrochloride HPLC-UV method [16] is reported
two HPLC methods are reported for estimation of Tetracaine hydrochloride and Oxymetazoline hydrochloridein combined dosage form. No stability indicating method is reported for estimation of Tetracaine hydrochloride and Oxymetazoline hydrochloride in combine dosage form.
The aim of the present work was to utilize the experimental design approach for screening and optimizing the experimental variables for developing a stability indicating chromatographic method for determining the content of Tetracaine hydrochloride and Oxymetazoline hydrochloride in bulk and pharmaceutical formulation.

MATERIAL AND METHODS

Instrument

HPLC analysis was carried out on Shimadzu system (software- Spin chrome, Shimadzu) consisted of a binary pump and UV detector SPD – 20A. Rheodyne injection valve with a 20 µL loop used for injection of the samples and the chromatographic separation was carried out in C-18 column (250mm×4.6 mm i.d.,5 µm particle size). The mobile phase consisted of methanol and 0.05 M Potassium dihydrogen phosphate (pH 3.5) in different ratio was used. The freshly prepared mobile phase was filtered through a 0.20 µm pore size nylon membrane filter and pumped in an isocratic mode with a flow rate of 1.0 mL/min. The elution of the analyte was monitored at a wavelength of 231 nm.

Materials and reagents:

Tetracaine hydrochloride was purchase from Balaji drug supplier, (Surat, India). Oxymetazoline hydrochloride was obtained as a gift sample from Anish chemicals (Bhavnagar, India). HPLC grade methanol and water were purchased from Fischer scientific India. Hydrochloric acid, sodium hydroxide, hydrogen peroxide and other solvents used were of analytical grade.

RP-HPLC Method development and Validation for Tetracaine hydrochloride and Oxymetazoline hydrochloride:

Selection of wavelength

Standard solution of Tetracaine hydrochloride and Oxymetazoline hydrochloride 10µg/ml of each were prepared in Methanol as a solvent. Each solution was scanned between200-400nmusing Methanol as a blank.The point at which both drugs show maximum absorbance was selected as wavelength for determination.

Preparation of solutions

Standard stock solution of Tetracaine hydrochloride:

A stock solution of Tetracaine hydrochloride (3000 µg/ml) was prepared by dissolving 30.0 mg of Tetracaine hydrochloride in 10.0 mL of a methanol.

Standard stock solution of Oxymetazoline hydrochloride:

A stock solution of Oxymetazoline hydrochloride (100µg/ml) was prepared by dissolving 1.0 mg of Tetracaine hydrochloridein 10 ml of a methanol.

Preparation of combined Standard stock solution of Tetracaine hydrochloride and Oxymetazoline hydrochloride:

Accurately weighed Tetracaine hydrochloride (30mg) and Oxymetazoline hydrochloride (1mg) transferred into10 ml volumetric flask and dissolved in water to give a stock solution 3000µg/ml of Tetracaine hydrochloride and 100µg/ml of Oxymetazoline hydrochloride.

Preparation of synthetic mixture of Tetracaine HCl and Oxymetazoline HCl

Accurately weighed Tetracaine hydrochloride (30mg) and Oxymetazoline hydrochloride (1mg) transferred into10 ml volumetric flask and dissolved in water togiveastocksolution3000µg/mlof Tetracaine hydrochloride and 100µg/ml of Oxymetazoline hydrochloride. From the stock solution 1.0 ml was transferred into 10 ml volumetric flask and citric acid, hydroxyl ethyl cellulose and benzyl alcohol was added as mentioned in formula and diluted up to mark with water to obtain working standard solution300µg/ml of Tetracaine hydrochlorideand10µg/ml of Oxymetazoline hydrochloride.

Preparation of working standard solution:

From the stock solution of combined drug (300µg/ml Tetracaine hydrochloride ,100 µg/ml Oxymetazoline hydrochloride), take 1ml of that solution and dilute upto10 ml with methanol. This gave concentration of300µg/ml Tetracaine hydrochloride and10 µg/ml Oxymetazoline hydrochloride.

Preparation of Mobile phase:

0.05 M Potassium Dihydrogen Phosphate Buffer was prepared and pH adjusted 3.5 and sonicated for 20 min.

Preparation of 0.05 M Potassium dihydrogen phosphate:

An accurately weighed 6.8 gm of Potassium dihydrogen phosphate was transferred into 1000 ml volumetric flask and dissolved in distilled water and volume was made up to mark with distilled water.

2.3.3 Selection of optimized chromatographic condition

Optimized Chromatographic Condition by trial and error method:

Column: C18, 250 mm × 4.6 mm,5 µm.
Flow Rate: 1.0 ml/min.
Wavelength: 231 nm
Injection Volume: 20 µL
Run Time: 10 min
Mobile Phase

A. 0.05 M potassium dihydrogen phosphate pH 3.5
B. Methanol

Mobile Phase Ratio: 40: 60 % v/v

Design of Experiment:

Screening of mobile phase by applying Design of Experiment.

- Here, Plackett Burman Design was applied for developing Quality in the method.
- pH, mobile phase ratio, flow rate, wavelength and column type was selected as independent variables and theoretical plates, retention time and peak area were the dependent variables. 2 Levels which were using for the particular Factor which were assigned as +1, -1 And it's values are given into the Table 1:

Table 1:List of Factors and Levels with their Assigned & Actual value

Levels	pH (X_1)	Mobile phase ratio (X_2)	Wavelength (X_3)	Flow Rate(X_4)	Column (X_5)
-	2.5	35:65	222	0.5	C_8
+	4.5	45:55	235	1.5	C_{18}

Optimization of mobile phase by applying Design of Experiment.

A three-level Box-Behnken design with three center points was used to evaluate the main, interaction and quadratic effects of pH(X_1), mobile phase ratio (X_2), and Flow Rate(X_3). 3 Levels which were using for the particular Factor which were assigned as +1,0, -1 and it's values are given into the Table

Table 2 List of Factors and Levels with their Assigned & Actual value:

Levels	pH(X_1)	mobile phase ratio (X_2)	Flow Rate(X_3)
-	2.5	35:65	0.5
0	3.5	40:60	1.0
+	4.5	45:55	1.5

Procedure for Preparation of Samples for Forced degradation Study:

Acid degradation:

1ml of standard stock solution of Tetracaine HCl (300 µg/ml) and Oxymetazoline HCl (10 µg/ml) was transferred into volumetric flask. To this 2ml of 0.01NHCl solution was added and mixed well. The volumetric flask was kept in dark place for 3 hrs. After time period, mixture was neutralized with 2mlof 0.01N NaOH and then diluted tovolume10 ml with methanol.

Base degradation:

1ml of standard stock solution of Tetracaine HCl (300 µg/ml) and Oxymetazoline HCl (10 µg/ml) was transferred into volumetric flask. To this 2 ml of 0.01NNaOH solution was added and mixed well. The

volumetric flask was kept in dark place for 2 hrs. After time period, mixture was neutralized with 2 ml of 0.01NHClandthendilutedtovolume10ml with methanol.

Neutral degradation:

1mlof standard stock solution of Tetracaine HCl (300 µg/ml) and Oxymetazoline HCl (10 µg/ml) was transferred into volumetric flask. To this2 ml of water was added and mixed well. The volumetric flask was kept in dark place for 4hrs.Aftertimeperiod,mixture was dilutedtovolume10ml with methanol.

Oxidation degradation:

1mlof standard stock solution of Tetracaine HCl (300 µg/ml) and Oxymetazoline HCl (10 µg/ml) was transferred into volumetric flask. To this 2ml of 3%H_2O_2 solution was added and mixed well. The volumetric flask was kept in dark place for 6hrs.After time period, diluted to volume10 ml with methanol.

Thermal degradation:

1ml of standard stock solution of Tetracaine hydrochloride (300 µg/ml) and Oxymetazoline HCl (10 µg/ml) was transferred into volumetric flask. The volumetric flask was placed in heating mantle at 50°C for 60 min. After time period, the content was cooled to ambient temperature and diluted up to 10 ml with methanol.

Photolytic degradation:

1mlofstandardstocksolutionof Tetracaine hydrochloride (300 µg/ml) and Oxymetazoline HCl (10 µg/ml) was transferred in to petri dish. The petri dish was put in UV chamberfor2hrs. After time period, the content was diluted with methanol up to 10 ml.

Preparation of Calibration curve:

Calibration curve for Tetracaine HCl and Oxymetazoline HCl consists of different concentrations of standard Tetracaine hydrochloride solution ranging from 150–750 µg/ml and Oxymetazoline hydrochloride 5 - 25µg/ml. The solutions were prepared by withdrawing 0.5ml,1.0 ml,1.5 ml, 2.0 ml and 2.5ml of the combine working standard solution of Tetracaine hydrochloride (3000 µg/ml) and Oxymetazoline hydrochloride (100 µg/ml) in to 10ml volumetric flasks. Makeup volume to 10ml with methanol. These solutions contained 150 µg/ml, 300µg/ml, 450µg/ml,600µg/ml, 750µg/ml of Tetracaine hydrochloride and 5µg/ml, 10µg/ml, 15µg/ml,20µg/ml,25 µg/ml of Oxymetazoline hydrochloride respectively.

Method Validation [12]

The method validation parameters studied were specificity, linearity, accuracy, precision, limit of detection and limit of quantification as per ICH Q2R1guidelines

Specificity:

For the determination of specificity 300µg/ml solution of the standard Tetracaine hydrochlorideand10µg/ml solution of the standard Oxymetazoline hydrochloride was injected. Synthetic mixture of same concentration was also injected .Both chromatograms were compared and check for any interference of excipient peak. Chromatogram of blank was also recorded to check any interference. Single standard solutions of both drugs were injected for selectivity and peak information.

Linearity (Calibration Curve) (n=5):

The calibration curves were plotted over a wide range and linear response was observed over a range of 150-750 µg/ml for Tetracaine hydrochloride and 5-25 µg/ml for Oxymetazoline hydrochloride. The solutions of each concentration were injected under the operating chromatographic conditions as described earlier. Chromatograms were recorded. These operations were done five times and mean responses were calculated. %RSD was calculated.

Accuracy:

It was determined by calculating the recovery of Tetracaine hydrochloride and Oxymetazoline hydrochloride from formulation by standard addition method. To a fixed amount of test 80%,100%and120% amount of standard was added and the amount of standard added was calculated using regression equation. Known amount of standard solutions of Tetracaine hydrochloride (540, 600, 660 µg/ml) and Oxymetazoline hydrochloride (18,20 and 22µg/ml) were added to a pre-quantified sample solution of Tetracaine hydrochloride and Oxymetazoline hydrochloride (300 and 10 µg/ml, respectively). Each solution was injected in triplicate and the percentage recovery was calculated by measuring the responses and fitting these values into the regression equations of the respective calibration curves.

Precision:

Repeatability:

1.5ml solution of Tetracaine hydrochloride(3000 µg/ml) and Oxymetazoline hydrochloride(100µg/ml) was transferred to a 10ml of volumetric flask.The volume was adjusted upto mark with methanol to get

450µg/ml solution of Tetracaine hydrochloride and 15µg/ml solution of Oxymetazoline hydrochloride.The areas of solutions were measured six times and%RSD was calculated.

Intraday Precision:

1.0, 1.5 and 2.0ml of working standard solution of Tetracaine hydrochloride(3000µg/ml) and Oxymetazoline hydrochloride (100 µg/ml) were transferred to a series of 10 ml volumetric flask.The volume was adjusted upto mark with methanol to get 300, 450 and 600 µg/ml solution of Tetracaine HCl and 10, 15 and 20µg/ml solution of Oxymetazoline HCl. The area of peaks were measured three different times on the same day and %RSD was calculated.

Interday Precision:

Aliquots 1.0, 1.5 and 2.0ml of working standard solution of Tetracaine HCl(3000µg/ml) and Oxymetazoline HCl (100 µg/ml)were transferred to a series of 10 ml volumetric flask. The volume was adjusted upto mark with methanol to get 300, 450 and 600 µg/ml solution of Tetracaine HCland10, 15 and 20µg/ml solution of Oxymetazoline HCl. The area of peaks were measured three times on the three different days and %RSD was calculated.

Robustness:

In this parameter, small changes are made into HPLC system like pH change, mobile phase ratio change, wavelength change and flow rate change. After this changes, %RSD is calculated.

LOD and LOQ:

The LOD (Limit of Detection) and LOQ (Limit of Quantification) was estimated from the set of 5calibration curves used to determine method linearity.

System Suitability Test:

Systemsuitabilityisthecheckingofasystemtoensuresystemperformance before or during the analysis of unknowns. Parameters such as Theoretical Plates,Tailing factors, Resolution (% RSD, retention time and area for six repetitions) were determined and compared against the specifications set for the method.

Analysis of sample by RP-HPLC method:

Marketed formulation is KOVANAZE nasal spray solution.1ml nasal spray contains 30mg/ml Tetracaine HCl and 1 mg/ml Oxymetazoline HCl were taken and dilute upto10mlwith water. This solution had 3000µg/ml Tetracaine HCl and 100µg/ml Oxymetazoline HCl.From this solution 1ml was taken and dilute upto10 with water.The solution had 300 µg/ml Tetracaine HCl and 5 µg/ml Oxymetazoline HCl. This solution was injected in HPLC. From the peak area, concentrations of both drugs were determined from regression equation.% assay of that formulation was calculated.

RESULT AND DISCUSSION

Chromatographic method was optimized for determining the content of Tetracaine HCl and Oxymetazoline HCl. The experimental design approach was utilized for screening and optimizing the experimental variables of the chromatographic method.

Screening designs are normally used when a large number of factors are likely to affect a particular response. A Plackett Burman design was utilized to evaluate the main effect of four independent factors on the selected response (dependent variables). The primary purpose was to identify significant main effects with the least number of runs as possible. The effects of all the factors included in the experimental design on the selected response Y are shown in Table Pareto ranking analysis revealed that, the factors thatwere statistically significant for selected response were the pH, mobile phase ratio and flow rate.

Table 3 Data of screening study of Tetracaine HCl and Oxymetazoline HCl

	Tetracaine HCl						**Oxymetazoline HCl**			
pH	Mobile phase ratio	Wavelength (nm)	Flow rate (ml/min)	Column	Peak area (mV) (Y_1)	Retention time (min) (Y_2)	Theoretical plates (Y_3)	Peak area (mV) (Y_1)	Retention time (min) (Y_2)	Theoretical plates (Y_3)
2.5	35:65	222	1.5	C_8	1673.4	2.51	2307	126.69	4.57	3447
4.5	35:65	222	0.5	C_{18}	8064.0	7.98	1643	647.01	16.03	1624
4.5	45:55	222	0.5	C_8	4256.5	9.27	2320	24.346	18.86	4617
2.5	45:55	235	0.5	C_8	3878.7	8.60	2398	135.38	13.14	1820
4.5	35:65	235	1.5	C_8	1266.3	2.63	1955	42.48	5.41	5011
2.5	45:55	222	1.5	C_{18}	1536.7	3.08	1567	113.52	7.38	2515
2.5	35:65	235	0.5	C_{18}	4034.3	7.60	2106	201.46	11.79	1604
4.5	45:55	235	1.5	C_{18}	1283.3	3.33	1274	30.56	9.43	2585

Polynomial equation:
Y (Retention time) = 3.24 + 0.705 X_1 + 1.778 X_2 - 10.94 X_4
Y (Peak area) = 4523.1 + 468.37 X_1 -2041.36 X_2 -2533.99X_3 -7236.95 X_4 + 1921.7 X_5
Y (Theoretical plates) = 1032 - 452 X_1 - 104 X_2 - 682X_4

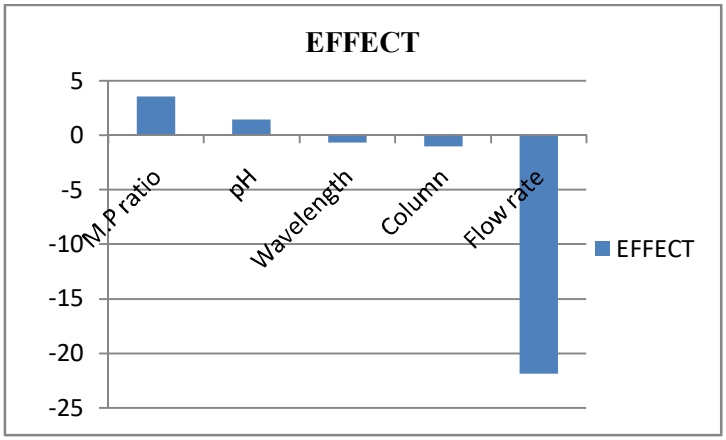


Fig. 2 Pareto chart for retention time

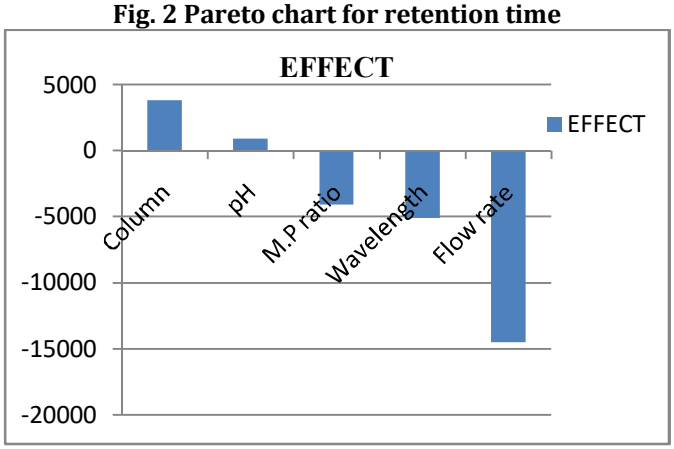


Fig.3 Pareto chart for Peak area

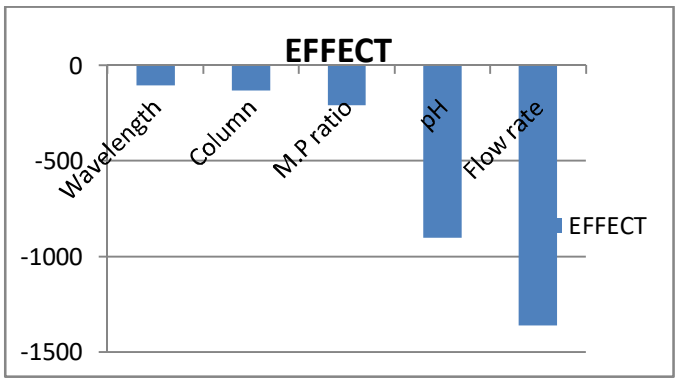


Fig. 4 Pareto chart for Theoretical plates

From the screening study the factors **pH, mobile phase ratio** and **flow rate** were selected for optimization of mobile phase which were significantly affect the responses.

Optimization phase (BOX- BEHNKEN DESIGN):

Table 4: Box Behnken design of Tetracaine HCl and Oxymetazoline HCl

				Tetracaine HCl			Oxymetazoline HCl		
Sr.no.	**pH**	**Mobile phase ratio**	**Flow rate (ml/min)**	**Peak area (mV)**	**Retention time (min)**	**Theoretical plates**	**Peak area (mV)**	**Retention time (min)**	**Theoretical plates**
1	2.5	35:65	1	4063.8	3.87	2762	271.35	5.93	2746
2	4.5	35:65	1	5988.2	4.06	1545	945.52	8.78	3015
3	2.5	45:55	1	4047.7	4.50	2048	205.74	10.47	2999
4	4.5	45:55	1	5356.7	5.03	810	104.96	9.22	1418
5	2.5	40:60	0.5	9223.0	8.24	2066	417.60	15.03	2603
6	4.5	40:60	0.5	10513	8.22	697	475.96	19.25	3237
7	2.5	40:60	1.5	3148.2	2.80	1856	132.31	5.19	2597
8	4.5	40:60	1.5	3715.4	3.03	509	209.16	6.94	2611
9	3.5	35:65	0.5	11763	8.96	2198	606.07	14.53	4874
10	3.5	45:55	0.5	11460	9.17	1815	450.91	21.53	3341
11	3.5	40:60	1.5	3887.7	2.83	1812	171.48	5.48	2738
12	3.5	45:55	1.5	3864.6	3.09	1636	151.91	7.46	2889
13	3.5	40:60	1.0	5915.0	4.17	2055	409.58	7.98	2489
14	3.5	40:60	1.0	5530.0	4.15	2237	236.10	7.99	3250
15	3.5	40:60	1.0	5627.3	4.17	2117	285.54	7.98	3057
16	3.5	40:60	1.0	5816.1	4.17	2117	204.97	7.97	3372
17	3.5	40:60	1.0	5829.9	4.17	2052	236.68	7.97	3231

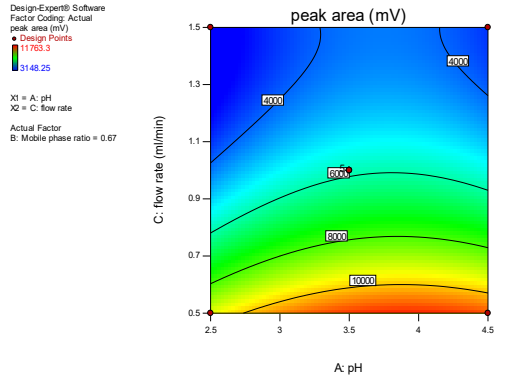


Fig. 4 Contour plot of response of Peak area by flow rate, pH and ratio

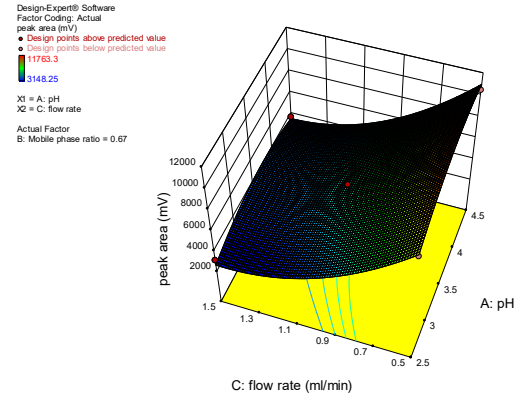


Fig. 5 3D plot of response of peak area by flow rate, ratio and pH:

Peak area = +5841.25 + 636.39* A -3543.07*C -1035.61*A^2 + 1844.39* C^2

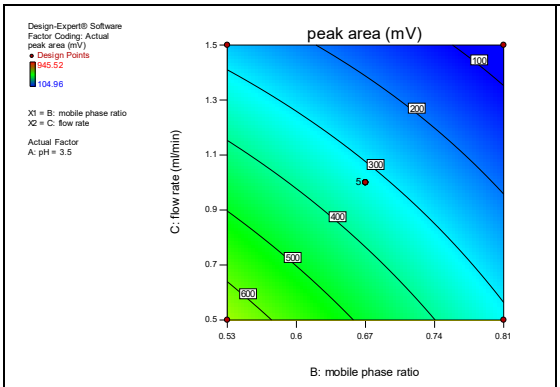


Fig. 6 Contour plot of response of retention time by flow rate, pH and ratio

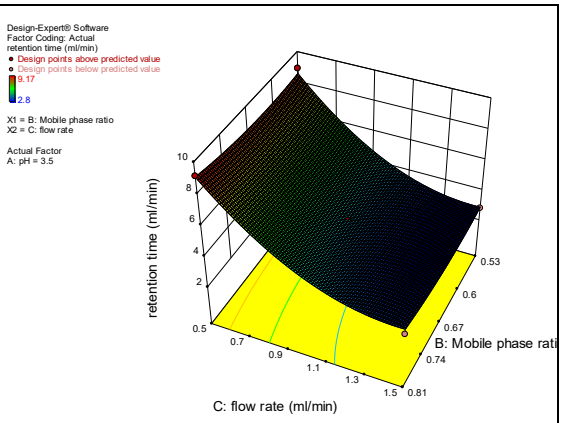


Fig. 7 3D plot of response of retention time by flow rate, ratio and pH

Retention time = 4.17 + 0.26 * B – 2.85 *C + 0.32 B^2 + 1.53 C^2

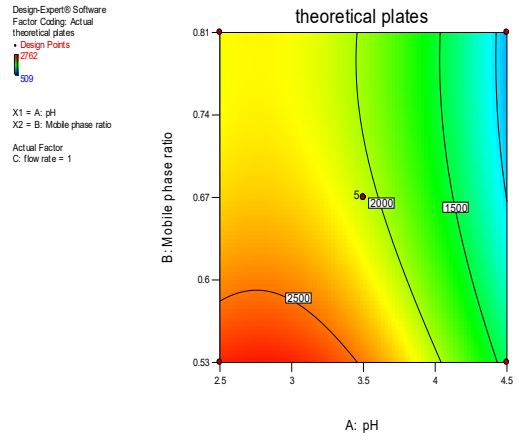


Fig. 8 Contour plot of response of Theoretical plates by flow rate, pH and mobile phase ratio

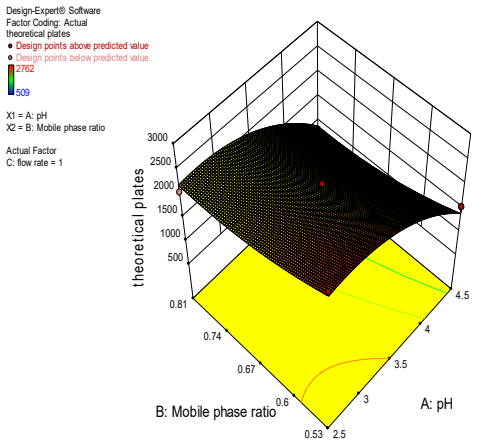


Fig. 9 3D plot of response of Theoretical plates by flow rate, ratio and pH

Theoretical plates = +2071.40 -646.38 * A -251.00 * B -120.38 * C -431.70 * A^2 +151.55 * B^2 -357.70 * C^2

Validation of DoE model by assessing the % relative error between the predicted and experimental response

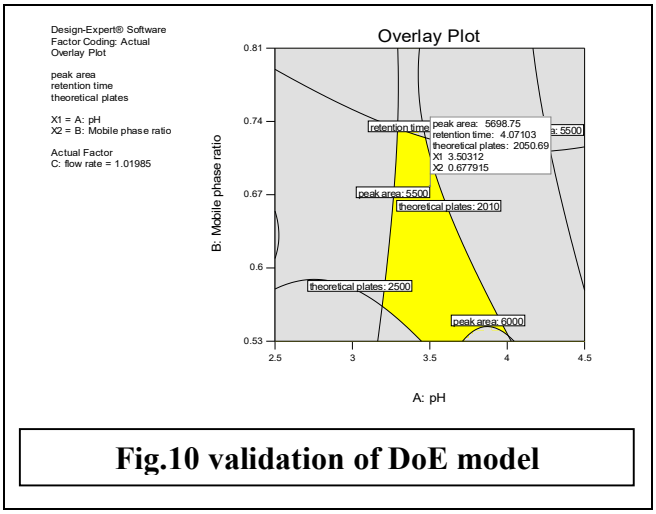


Fig.10 validation of DoE model

Optimized condition generated by DoE software:

- **Stationary phase**: Chromatopak C_{18} column (250 mm X 4.6 mm i.d., 5µm).
- **Mobile phase**: 0.05M KH_2PO_4 (pH 3.5): Methanol (40:60% v/v).
- **Flow rate**: 1.0 ml/min.
- **Wavelength**: 231 nm.
- **Injection volume**: 20 µL

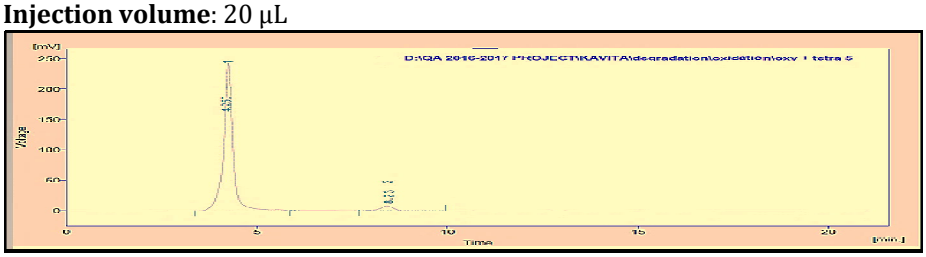


Fig. 11 Chromatogram of standard Tetracaine HCl (300 µg/ml) and Oxymetazoline HCl (10 µg/ml)

Table 5: Data for standard Tetracaine HCl (300 µg/ml) and Oxymetazoline HCl (10 µg/ml)

Drug	Retention time (min)	Area (mV)	Resolution
Tetracaine hydrochloride	4.25	4146.65	9.08
Oxymetazoline hydrochloride	8.23	206.398	

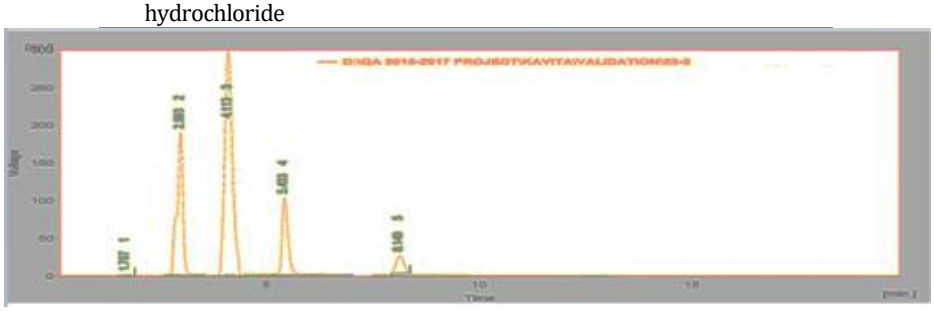


Fig. 12 Chromatogram of formulation

Table 6: Data for Marketed formulation

Sr. no.	Drug and excipients	Retention time (min)	Resolution
1.	Citric acid	2.99	-
2.	Tetracaine hydrochloride	4.11	4.17
3.	Benzyl alcohol	4.43	4.58
4.	Oxymetazoline hydrochloride	8.14	9.12

Forced degradation study of Tetracaine hydrochloride and Oxymetazoline hydrochloride:

Acid degradation:

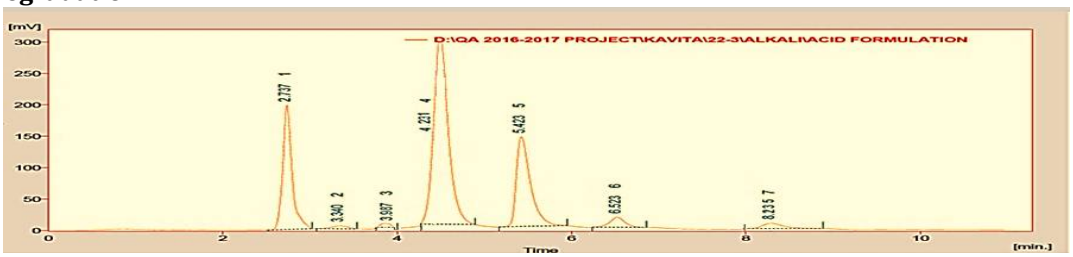


Fig. 13 Chromatogram of formulation in 0.01N HCl for 3 hrs at RT

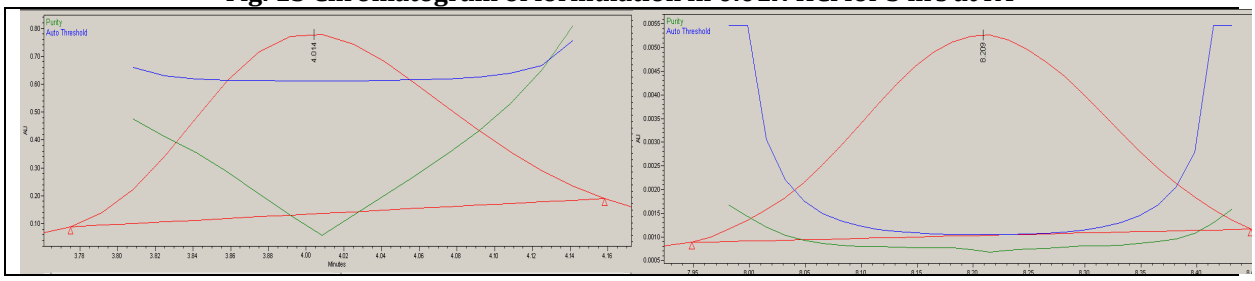


Fig. 14 Purity plot of Tetracaine hydrochloride and Oxymetazoline hydrochloride in acidic condition

Alkali degradation:

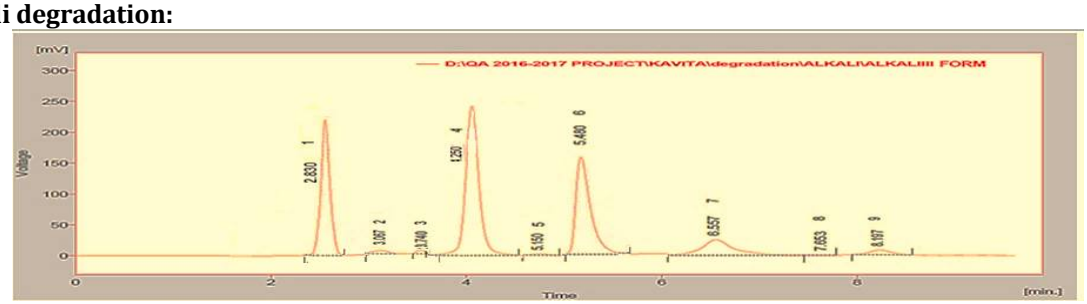


Fig. 15 Chromatogram of formulation in 0.01N NaOH for 2 hrs at RT

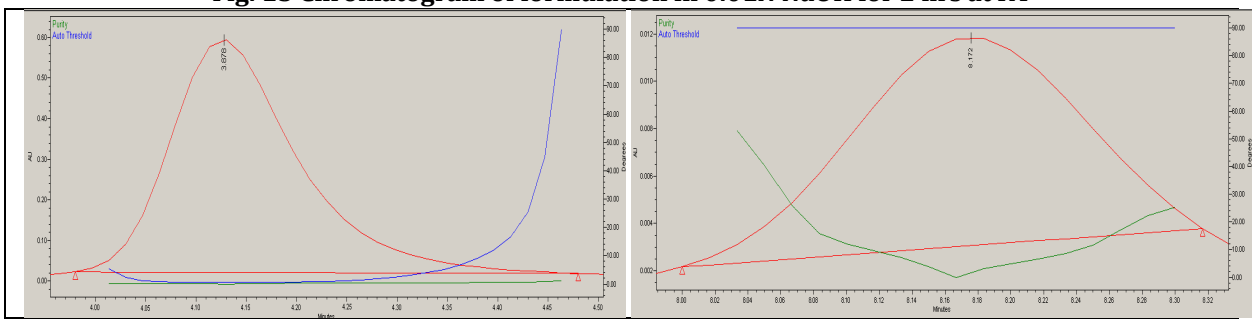


Fig. 16 Purity plot of Tetracaine HCl and Oxymetazoline HCl in base condition

Neutral degradation:

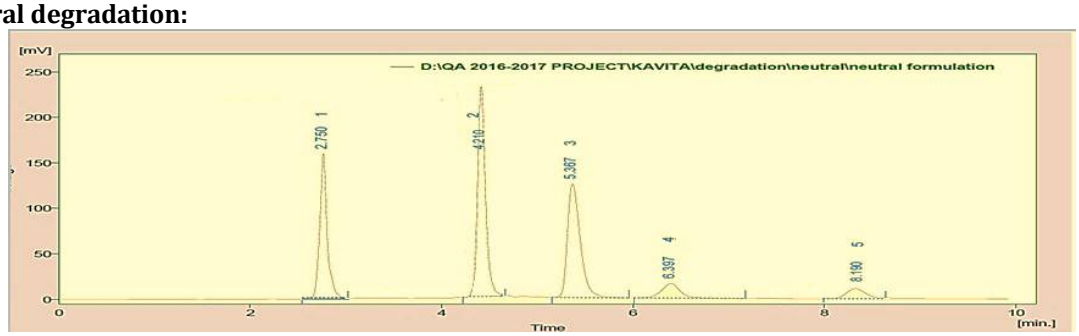


Fig. 17 Chromatogram of formulation in water for 4 hrs at RT

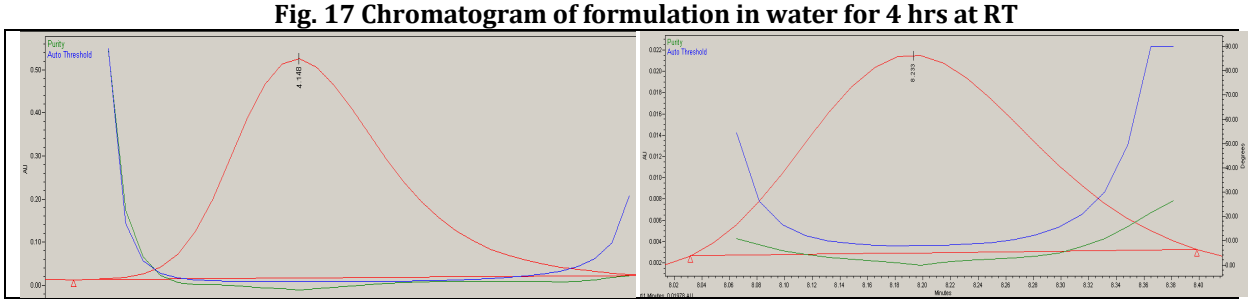


Fig. 18 Purity plot of Tetracaine hydrochloride and Oxymetazoline hydrochloride in neutral condition

Oxidative degradation:

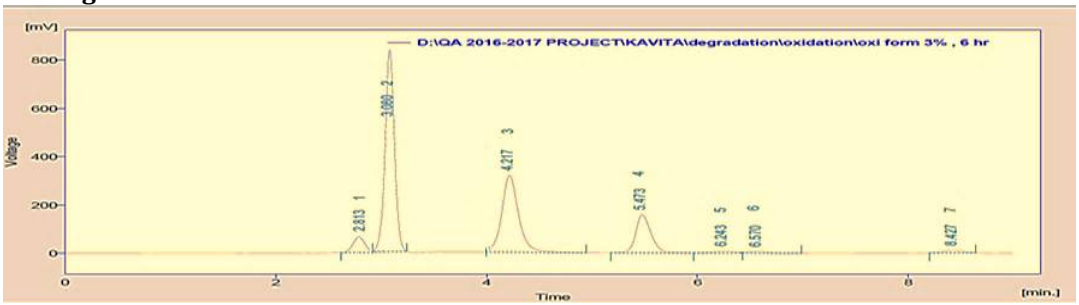


Fig. 19 Chromatogram of formulation in 3% H_2O_2 for 6 hrs

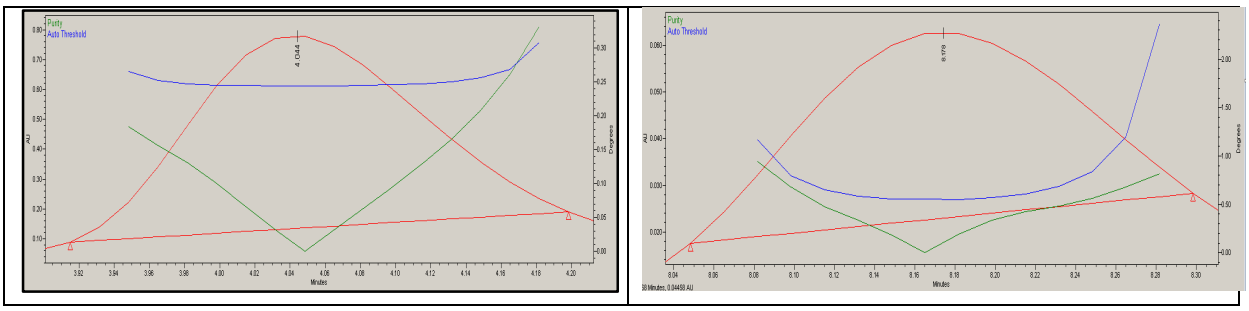


Fig. 20 Purity plot of Tetracaine HCl and Oxymetazoline HCl in oxidative condition

Thermal degradation:

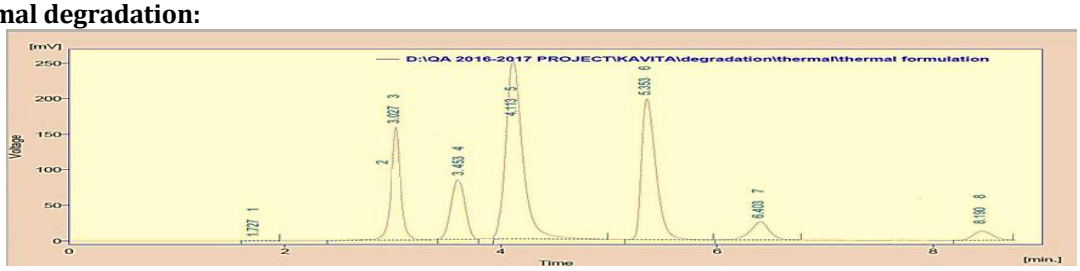


Fig. 21 Chromatogram of formulation at 50º C for 1 hr

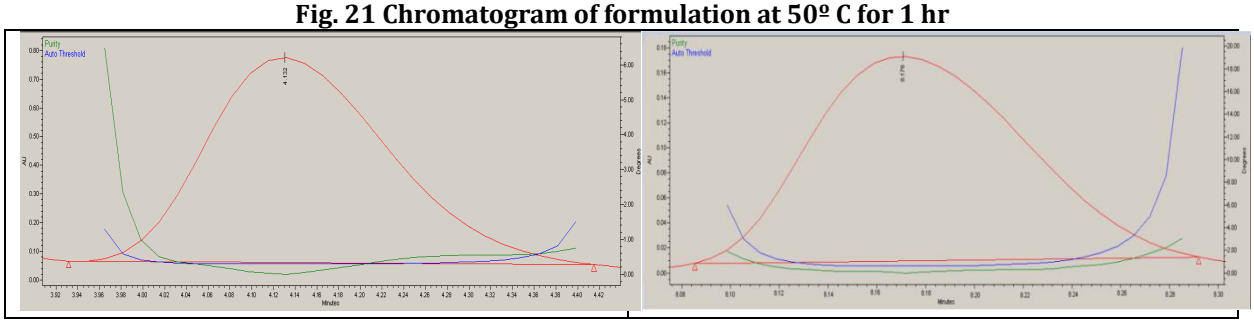


Fig. 22 Purity plot of Tetracaine HCl and Oxymetazoline HCl in Thermal condition

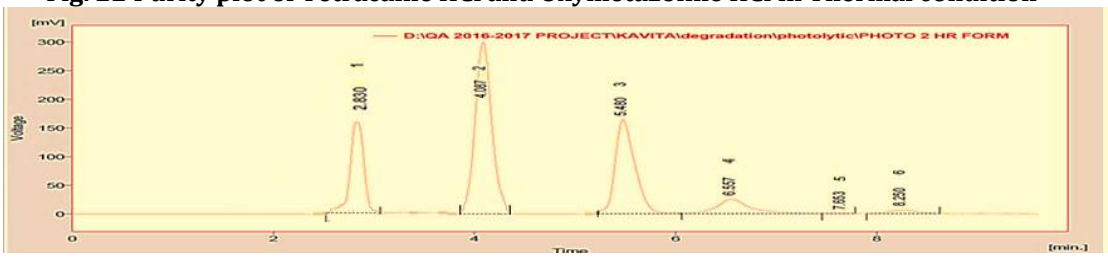


Fig. 23 Chromatogram of formulation in UV chamber for 2 hrs

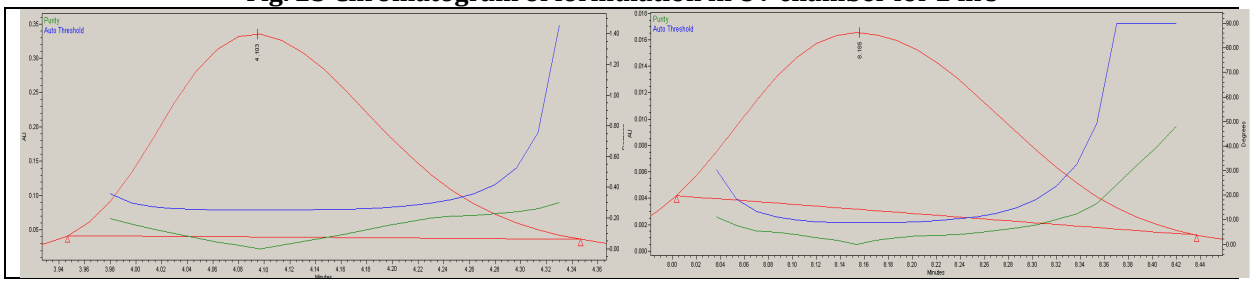


Fig. 24 Purity plot of Tetracaine HCl and Oxymetazoline HCl in Photolytic condition

Summary of Degradation Study

Table 7 Summary of Degradation Study

Stress condition	Tetracaine hydrochloride		Oxymetazoline hydrochloride	
	% Degradation of API	% Degradation of formulation	% Degradation of API	% Degradation of formulation
Acid	18.49	17.49	22.23	18.21
Alkali	24.29	19.50	25.73	17.59
Neutral	18.47	16.69	22.23	25.55
Oxidation	17.92	15.18	32.76	28.38
Thermal	20.01	17.19	28.36	25.69
UV light	26.36	23.73	46.60	42.81

System suitability testing

Table 8: Data of system suitability of Tetracaine HCl and Oxymetazoline HCl

Sr. No	Theoretical Plates		Retention time		Tailing Factor		Resolution
	Tetracaine HCl	Oxymetazoline HCl	Tetracaine HCl	Oxymetazoline HCl	Tetracaine HCl	Oxymetazoline HCl	
1	2610	4120	4.19	8.36	1.042	1.121	9.08
2	2602	4247	4.19	8.30	1.075	1.045	9.11
3	2602	4234	4.19	8.30	1.124	1.020	8.98
4	2686	4298	4.18	8.26	1.079	1.043	9.07
5	2623	4126	4.19	8.36	1.127	1.171	9.05
6	2642	4205	4.18	8.25	1.103	1.072	9.08
Result	>2000	> 2000	% RSD = 0.1233	% RSD = 0.5686	<1.5	<1.5	> 2
Limit	> 2000		% RSD < 2		< 1.5		> 2

Theoretical plates of Tetracaine hydrochloride and Oxymetazoline hydrochloride greater than 2000.The tailing factor of six replicate of Tetracaine hydrochloride and Oxymetazoline hydrochloride is less than 1.5 Resolution of the peak is greater than 2.0

Specificity:

Chromatogram of blank, standard solution of Tetracaine hydrochloride and Oxymetazoline hydrochloride and formulation it can be seen that there was no interference of excipients during validation study.

Linearity and range

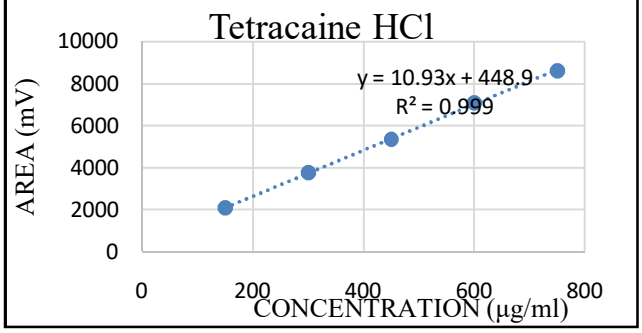


Fig. 3.25 Calibration curve Tetracaine hydrochloride (150 – 750 µg/ml)

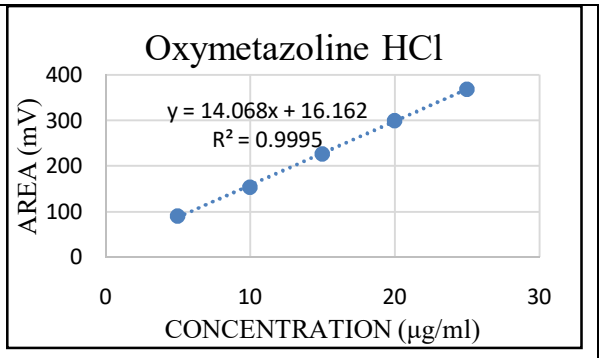


Fig. 3.26 Calibration curve of Oxymetazoline HCl (5 – 25 µg/ml)

For Tetracaine hydrochloride, regression equation was found to be y = 10.933x + 448.93 and correlation coefficient R^2 was found to be 0.9997.

For Oxymetazoline hydrochloride, regression equation was found to be y = 14.068x + 16.162 and correlation coefficient R^2 was found to be 0.9995

Precision

Repeatability

Table 9: Repeatability data of Tetracaine HCl and Oxymetazoline HCl

Conc. of Tetracaine hydrochloride (µg/ml)	Area (mV)	Conc. of Oxymetazoline hydrochloride (µg/ml)	Area (mV)
300	5778.67	10	247.26
	5788.78		247.17
	5770.90		241.96
	5758.18		250.35
	5722.47		246.21
	5831.41		248.20
MEAN	5775.0706	MEAN	246.8607
SD	35.8996	SD	2.7790
% RSD	0.6216	% RSD	1.1257

The % RSD for Tetracaine hydrochloride and Oxymetazoline hydrochloride was found to be 0.621 and 1.125 respectively.

Limit: % RSD should be < 2.

Intraday Precision

Table 10: Data for Intraday Precision of Tetracaine HCl and Oxymetazoline HCl

TETRACINE HYDROCHLORIDE			OXYMETAZOLINE HYDROCHLORIDE		
CONC. (µg/ml)	MEAN AREA ± SD	% RSD	CONC. (µg/ml)	MEAN AREA ± SD	% RSD
300	3717.84 + 33.1477	0.8915	10	152.093 + 0.9683	0.6366
450	5779.45 + 08.9611	0.1550	15	246.883 + 0.5822	0.2358
600	7024.90 + 42.3589	0.6029	20	265.376 + 4.0987	1.5444

Interday Precision

Table 11: Data of Interday Precision

TETRACAINE HYDROCHLORIDE			OXYMETAZOLINE HYDROCHLORIDE		
CONC. (µg/ml)	MEAN AREA ± SD	% RSD	CONC. (µg/ml)	MEAN AREA ± SD	% RSD
300	3778.68 + 74.4399	1.9699	10	151.18 + 0.9488	0.6276
450	5817.027 + 49.9956	0.8594	15	249.05 + 1.6895	0.6783
600	7045.457 + 85.8573	1.2612	20	266.37 + 2.0302	0.7621

Accuracy

Table 12: Accuracy data for Tetracaine hydrochloride

Amount of Tetracaine HCl (µg/ml)	% of Tetracaine HCl std spiked	Conc. After spiking drug (µg/ml)	Amount found (µg/ml)	% Recovery	% recovery (mean ± SD)	% RSD
300	80	540	536.80	99.40	99.39 ± 0.0251	0.025
			536.89	99.42		
			536.65	99.37		
	100	600	603.45	100.57	100.253 ± 0.395	0.394
			598.87	99.81		
			602.32	100.38		
	120	660	662.45	100.37	100.373 ± 0.075	0.074
			661.98	100.30		
			662.97	100.45		

Table 13: Accuracy data for Oxymetazoline hydrochloride

Amount of Oxymetazoline HCl (µg/ml)	% of Oxymetazoline HCl std spiked	Conc. After spiking drug (µg/ml)	Amount found (µg/ml)	% Recovery	% recovery (mean ± SD)	% RSD
10	80	18	18.05	100.27	100.9 ± 0.57	0.564
			18.25	101.38		
			18.19	101.05		
	100	20	19.82	99.10	99.68 ± 0.728	0.730
			19.89	99.45		
			20.10	100.50		
	120	22	22.00	100.00	99.74 ± 0.250	0.251
			21.94	99.72		
			21.89	99.50		

LOD AND LOQ

Table 14: Data for LOD and LOQ

PARAMETER	TETRACAINE HCl	OXYMETAZOLINE HCl
SD of intercept	30.3640	6.9805
Mean of slope	10.88	14.95
LOD (µg/ ml)	9.20	1.53
LOQ (µg/ ml)	27.90	4.66

Robustness

Table 15: Data for robustness when change in flow rate

Drug	Concentration (µg/ml)	Peak area (mV)			Mean peak area (mV)	SD	% RSD
		0.9 ml/min	1.0 ml/min	1.1 ml/min			
Tetracaine HCl	300	3454.76	3590.69	3501.01	3515.48	69.11	1.9659
	450	5115.13	5198.35	5095.32	5136.26	54.67	1.0644
	600	6873.35	7011.01	6934.59	6939.65	68.96	0.9938
Oxymetazoline HCl	10	108.57	110.21	108.13	108.97	1.09	1.0059
	15	149.82	151.34	149.83	150.33	0.87	0.5818
	20	220.22	215.47	217.81	217.83	2.37	1.0903

Table 16: Data for robustness when change in wavelength

Drug	Concentration (µg/ml)	Peak area (mV)			Mean peak area	SD	% RSD
		230 nm	231 nm	232 nm			
Tetracaine HCl	300	3560.92	3595.69	3563.51	3573.37	19.37	0.5420
	450	5590.78	5582.67	5576.86	5583.43	6.991	0.1252
	600	7058.96	7011.01	6924.31	6998.09	68.24	0.9752
Oxymetazoline HCl	10	106.838	110.21	108.13	108.392	1.70	1.5695
	15	154.82	151.34	146.76	148.93	2.29	1.5439
	20	211.71	215.47	212.24	213.14	2.03	0.9548

Table 17 Data for robustness when change in mobile phase ratio

Drug	Concentration (µg/ml)	Peak area (mV)			Mean peak area (mV)	SD	% RSD
		39:61	40:60	41:59			
Tetracaine HCl	300	3501.09	3595.69	3516.05	3537.61	50.851	1.4374
	450	5542.07	5582.67	5555.72	5560.15	20.659	0.3715
	600	7019.27	7011.01	7062.23	7030.83	27.499	0.3911
Oxymetazoline HCl	10	108.17	110.21	109.03	109.137	1.0241	0.9384
	15	148.05	151.34	151.97	150.453	2.1050	1.3991
	20	213.04	215.47	215.86	214.79	1.5280	0.7114

% RSD was less than 2.

Analysis of drug in synthetic mixture

Table 18 Data for Tetracaine HCl and Oxymetazoline HCl in their combined dosage form

TETRACAINE HCl			OXYMETAZOLINE HCl		
Amt taken(µg/ml)	Amt found(µg/ml)	% Assay	Amt taken(µg/ml)	Amt found (µg/ml)	% Assay
300	300.39	100.13	5	5.02	100.4
	299.87	99.95		5.06	101.2
	300.09	100.03		5.09	101.8
	299.92	99.97		5.04	100.8
	300.69	100.23		5.03	100.6
MEAN	300.192	100.062	MEAN	5.048	100.96
SD	0.3447	0.1171	SD	0.0277	0.5549
% RSD	0.1148	0.1170	% RSD	0.5497	0.5497

Solution stability

Table 19: Data of Solution stability

Sr. no.	Time	Absorbance	
		Tetracaine HCl	Oxymetazoline HCl
1	0	0.992	0.796
2	2	0.986	0.791
3	4	0.983	0.783
4	6	0.978	0.779
5	12	0.967	0.768
6	24	0.945	0.757
Average		0.975167	0.779
S.D		0.017011	0.0145
% RSD		1.744397	1.8637

Summary of Validation Parameters

Table 20: Summary of Validation parameters

PARAMETERS	TETRACAINE HYDROCHLORIDE	OXYMETAZOLINE HYDROCHLORIDE
Linearity (µg/ml)	150 - 750	5 – 25
Accuracy (% Recovery)	99.39- 100.37 %	99.68 - 100.9 %
Precision (% RSD)		
Repeatability	0.6216	1.1257
Intraday (n=3)	0.155 - 0.891	0.235 - 1.544
Interday (n=3)	0.859 - 1.969	0.627 - 0.762
Robustness (% RSD)		
Change in flow rate	0.993 - 1.965	0.581 - 1.090
Change in mobile phase ratio	0.371 - 1.437	0.711 - 1.399
Change in wavelength	0.125 - 0.975	0.954 - 1.569
LOD	9.20	1.53
LOQ	27.90	4.66

CONCLUSION

The stability indicating RP- HPLC method was developed and validated as per ICH guideline for simultaneous determination of Tetracaine hydrochloride and Oxymetazoline hydrochloride in nasal spray solution using Design of Experiment (DoE) approach. The proposed method is applicable for routine analysis of Tetracaine hydrochloride and Oxymetazoline hydrochloride in nasal spray solution. The developed method is accurate, precise, specific, stability indicating and robust.

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