4.1. SELECTION OF DRUG COMBINATION

The antiseptic drug combination of Chlorhexidine gluconate and Cetrimide was approved by CDSCO in 2009 for cleansing of physically contaminated wounds and for preoperative disinfection. [1, 2] Chlorhexidine gluconate (CHD) was approved by USFDA on 19 December 2003 as antiseptic agent. Cetrimide [Cetrimonium bromide] (CET) was approved by USFDA on 30 June 2006 as local infective agent. [3] Generic versions of both drugs are widely available. Albeit, for analysis of combination of Chlorhexidine gluconate and Cetrimide, no analytical method was reported till date which constitutes to be main active ingredients for many marketed formulations.

4.2 DRUG PROFILES

Chemical name: Cetrimide [4]

IUPAC Name: trimethyl (tetradecyl) azanium; bromide.

Molecular Formula: $C_{17}H_{38}BrN$

Dose: 3gm in 100ml

Log P value: 0.08

Molecular Weight: 336.40 g/mol

Chemical Structure:

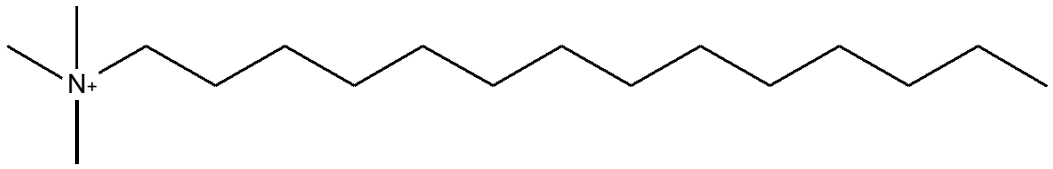


Figure 4.1: Structure of Cetrimide

Appearance: White fine crystalline solid

Melting point: 245-250°C

Solubility: Freely soluble in water

Drug Category: Antiseptic.

Mechanism of action: Cetrimide is a cationic surfactant. It destroys or damages the cell membrane by lowering surface tension. It causes microbial protein denaturation and produces antiseptic action. [5]

Uses: Antiseptic, Disinfectant; deodorant; laboratory reagent.

Marketed Formulation:
Cetrilak lotion containing 5%w/w Cetrimide (Menarine)
Cetrimide antiseptic cream containing 0.5% Cetrimide (Tescon)
Cetrim antiseptic soap containing 0.5% Cetrimide (Derma care)
Cetrimide cream containing 0.5% Cetrimide (Stableline)

Chemical name: Chlorhexidine gluconate [6]

IUPAC Name:(1E)-2-[6-[[amino-[(E)-[amino-(4-chloroanilino) methylidene] amino] methylidene] amino]hexyl]-1-[amino-(4-chloroanilino)methylidene] guanidine.

Molecular Formula: $C_{34}H_{54}Cl_2N_{10}O_{14}$

Molecular Weight: 897.76 g/mol

Chemical Structure:

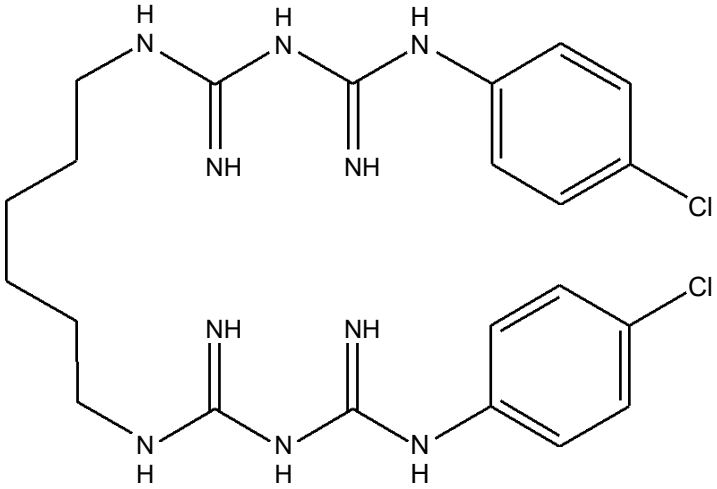


Figure 4.2: Structure of Chlorhexidine gluconate

Appearance: In freebase and stable salt forms, with a white or yellowish appearance

Melting point: 134-136°C

pKa: pKa1 = 7.63; pKa2 = 9.92; pKa3 = 8.22; pKa4 = 10.52

Dose: 0.3 gm in 100 ml

Log P value: 4.51

Solubility: Freely soluble in water

Drug Category: Anti-septic

Mechanism of action: Chlorhexidine is positively charged and reacts with the negatively charged microbial cell surface, thereby destroying the integrity of the cell membrane

Uses: Disinfectant, Topical infective and mouthwash

Marketed Formulation:
Chlorhexidine gluconate disinfectant containing 4% Chlorhexidine gluconate (Radix laboratories)
Gum Paroex mouthwash containing 0.06 % Chlorhexidine gluconate (Sunstar)
Chlorhexidine gluconate mouthwash containing 0.06 % Chlorhexidine gluconate (Hi-tech)
Hibiclens antiseptic containing 4 % Chlorhexidine gluconate (Molnlycke healthcare)

Combination formulations of CHD and CET combination:

Savlon antiseptic solution containing 0.3% CHD and 3% CET (ITC)
Saniquad GHS solution containing 1.5% CHD and 15% CET (Sirmaxo chemicals Pvt. Ltd)
Gersept antiseptic liquid containing 0.3% CHD and 0.6% CET (Green cross remedies)

4.3 LITERATURE REVIEW

Various official as well as nonofficial methods are reported in the literature for estimation CHD in various dosage forms. Official methods reported in pharmacopoeias (USP, EP, IP) describe variant HPLC methods for analysis of CHD. [7, 8-10] For analysis of CHD along with related substances and impurities also HPLC and GC methods are available in literature. [11-13] For analysis of CHD along with p-chloroaniline in surgical scrub product also GCMS method is available in literature. [14] For estimation of CHD in mouthwash formulations, UV spectroscopy method, Fluorometric method as well as radiolabel methods is reported. [15-17]For analysis of CHD in bioanalytical fluids like saliva/urine/serum/blood, HPLC as well as LC-MS-MS methods have been reported. [18-24] However, for estimation of CET only few methods are available owing to its structure showing absence of any prominent chromophoric group. A RP-HPLC method is available in literature for its estimation along with lidocaine as the combination is usually used for pharyngeal irritation treatment. [25] Also titrimetric methods official as well as nonofficial are reported in literature. [26] Despite of sufficient literature available for both drugs individually, no analytical method is available for simultaneous estimation of CHD and CET in literature best to our knowledge. Thereby, it was decided to develop a

UV spectrophotometric as well as chromatographic LC-PDA method for simultaneous estimation of CET and CET.

4.4 SECTION –A

4.4.1 Experimental Approach

Development of UV Spectrophotometric methods for simultaneous estimation of chlorhexidine gluconate and cetrimide.

The methods developed were as follows:

4.4.1.1 Simultaneous equation method

This method also called as Vieordt's method is used for simultaneous estimation of 2 drugs (say X and Y) when both drugs have some absorbance at analytical wavelength of another drug in the sample combination. [27] Let Cx and Cy be the concentrations of X and Y respectively in the diluted sample. Two equations are constructed based upon the fact that at λ1 and λ2, the absorbance of the mixture is the sum of the individual absorbance of X and Y. λmax of both the drugs are normally selected as two analytical wavelengths for estimation. Absorptivity of X and Y is calculated at λmax of both the drugs.
Based on this two equations which can be constructed are
A1 = ax1Cx + ay1Cy..... (1)
A2 = ax2Cx + ay2Cy..... (2)
The concentrations of X and Y can be calculated from following equations.
Cx = (A2 ay1 – A1 ay2) / (ax2 ay1 – ax1ay2)..... (3)
Cy = (A1 ax2 – A2 ax1) / (ax2 ay1 – ax1 ay2)..... (4)
Where; Cx & Cy are concentrations of X and Y respectively in gm/100 ml in the sample solution.
A1 & A2 are the absorbance of the mixture at λmax of both drugs;
ax1 and ax2 = Absorptivity of X at λmax of both drugs
ay1 and ay2 = Absorptivity of Y at λmax of both drugs

4.4.1.2 First derivative spectroscopy method

Derivative spectroscopy on the basis of zero-crossing measurements involves measurement of the absolute value of the total derivative spectrum at an abscissa value corresponding to the zero-crossing wavelength of the derivative spectra of individual components, which should be only a function of the concentration of other component. This method inculcates derivatizing zero order spectra of both drugs to first order. The procedure follows differentiating absorbance of both drugs at zero order with respect to narrow range of wavelength. After differentiation single/ multiple peaks are obtained where the spectra moves through the zero crossing points. Various zero crossing points (ZCP) can be enumerated for both drugs. For example if the study is undertaken for the combination of 2 drugs say X and Y, the best ZCP of X is to be selected for Y and vice

versa. The logic is that, when X drug has zero absorbance at that particular wavelength, the entire absorbance showcased on the spectrum is of Y drug and vice versa. The critical parameters to be optimized include selection of narrow range of wavelength by which differentiation is to be done. Generally Δλ of 5 nm is selected. Also selection of appropriate ZCP's for both drugs is very crucial part of the study for reliable results [28]

4.4.1.3 Absorption ratio spectra method

If the sample mixture contains 2 analytes say X and Y whose spectra are overlapping then Absorption ratio spectra method can be applied for it. In this method, the spectra of mixture is divided by the spectra of any one analyte (say X), for analysis of other corresponding analyte (say Y) in the mixture. Then in this modified spectra two wavelengths are selected (say λ1 and λ2). Now λ2-λ1 will give absorption ratio spectra value for analyte Y. This way also for analyte X, absorption ratio spectra value is to be found out. The above stated is done for whole calibration range and thus if the relationship is linear then it can be applied for analysis of unknown samples.

4.4.1.4 Mean centering of ratio spectra method

Improvement in resolution with better S/N ratio is obtained by application of Mean centring of ratio spectra method. The preliminary steps like derivatizing the sample are also eliminated by use of this method. Consider 2 analytes in the sample mixture say X and Y. In this method first the analysis of mixture (Am) in entire calibration range is to be done at λmax of one drug (say X). This Am values are then divided by molar absorptivity of drug Y. This Am/αY values for entire calibration range are then mean centered using software package Unscrambler X, version 10.5 and fed to model. Similar treatment needs to be done for other drug for analysis of both drugs in the sample mixture. [29, 30-35]

4.4.1.5 Multicomponent analysis method

The multi-component quantitation mode analysis is the mode in which the concentration of each constituent is determine by using absorption spectrum of the mixed sample with pure standards. For example if the sample consists of 2 drugs viz., X and Y, first λmax of both drugs are to be found out. Then by input of this λmax into the system, the absorption spectra of different binary mixture (samples) are to be scanned in the range of 200-400 nm and stored in the memory of the instrument. Multicomponent quantitation mode can be used directly to determine the concentration of unknown samples at particular optimized wavelength.

Chemometric methods

Chemometric methods are one type of multivariate analysis i.e. considering more than one variable at a time. When applied to UV spectrophotometry, many wavelengths are taken as variable and absorbance at each wavelength is considered. Least square approach involves mathematical modelling by which the square of residual (difference between actual and predicted concentration) is minimized to lowest level.

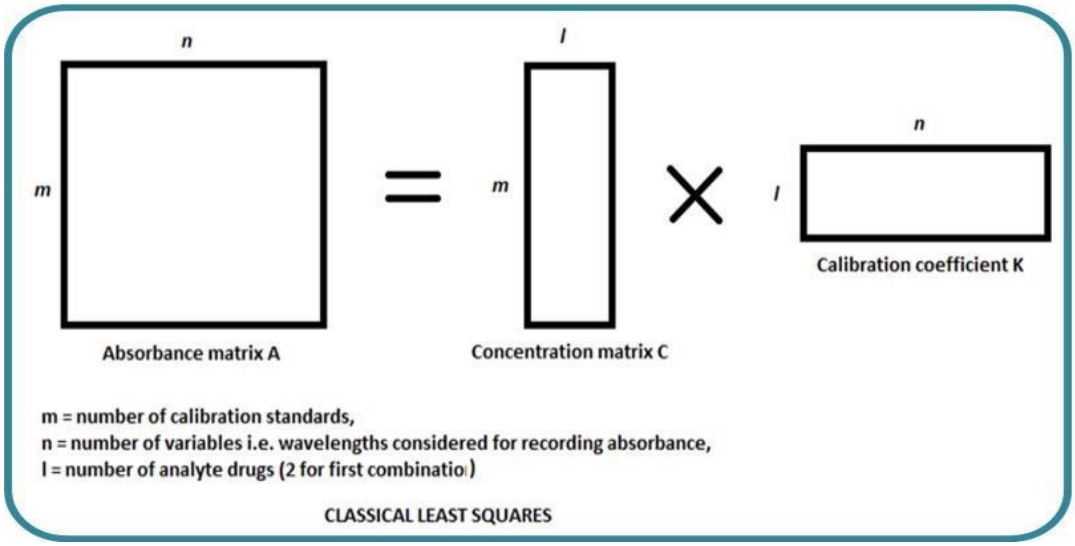
4.4.1.6 Classical least squares

The CLS method assumes the Beer's law model with the absorbance at each wavelength being proportional to the component concentrations. In matrix notation, the Beer's law model for *m* calibration standards containing *l* chemical components with spectra of *n* digitized absorbance is given by A=CK where A is m x n matrix of calibration spectra, C is m x l matrix of component spectra and K is l x n matrix of absorptivity at unit concentration and unit path length. In simplified manner, it can be said that each component will be having specific absorptivity at each particular wavelength being considered. Each row of K matrix represents spectrum of one pure component at its unit concentration. K matrix will have as many rows as components and as many columns as wavelengths. Since CLS is a full-spectrum method, it has following advantages

- Provide significant improvements in precision over methods that are restricted to a small number of frequencies,
- Allow simultaneous fitting of spectral base lines and
- Make available for examination and interpretation least squares estimated pure-component spectra and full-spectrum residuals**.**

Whereas major disadvantage of the CLS method is that it requires full knowledge of concentration matrix C i.e. all interfering chemical components in the spectral region of interest need to be known and included in the calibration. This is because we are representing absorbance matrix A as the sum of absorbance due to each component. Once we have matrices A and C, we can determine K by following equation $K = C^{-1} A$. Unfortunately, computing the inverse of a matrix requires that the matrix be square (having the same number of rows and columns). Unless the calibration set has exactly the same number of calibration standards as chemical components, this will not be true. This does not mean that the above equation cannot be solved. An alternative to computing the true inverse of the C matrix is to compute its "pseudo-inverse", as follows: $K = C' (C C')^{-1} A$ Where C' is the matrix transpose (pivot the matrix so that the rows become the columns) of the concentrations matrix C. This equation is written in the software as follows: **K = pinv (C) * A** Where pinv (C) is the pseudo inverse of concentration matrix C. For predicting the unknown concentration of a mixture from its absorbance matrix, following equation is used: **C = A * pinv (K)** [36]

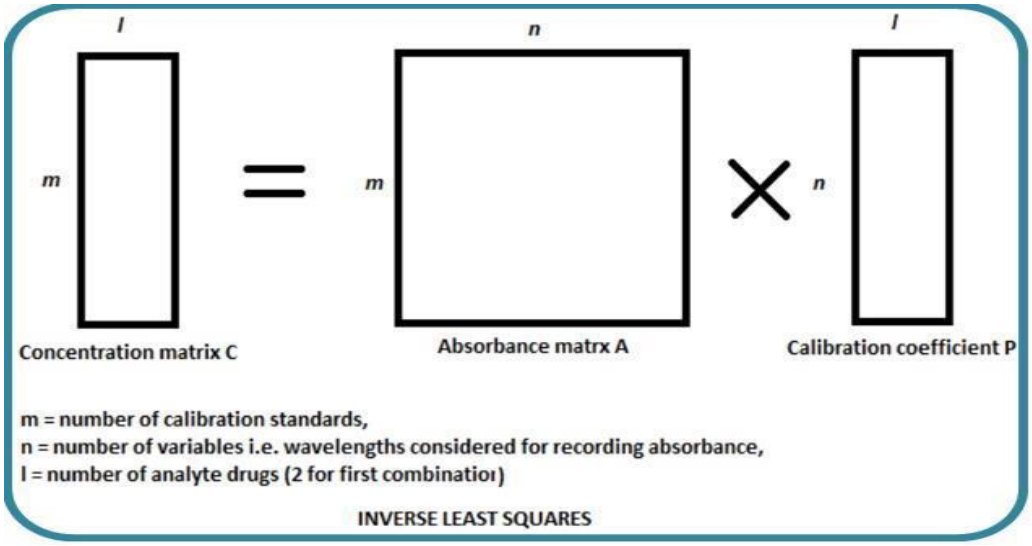
Figure 4.3 Pictorial representation of Classical least squares principle



4.4.1.7 Inverse Least Squares

The inverse least squares method assumes that concentration is a function of absorbance. The inverse Beer's law model for *m* calibration standards with spectra of n digitized absorbances is given by **C = A P** Where **C** and **A** are as before, **P** is the *n* X *l* matrix of the unknown calibration coefficients relating the *l* concentrations to the spectral intensities. The inverse representation of Beer's law has the significant advantage that the analysis based on this model is invariant with respect to the number of chemical components, *l*, included in the analysis. Unlike CLS, ILS does not require that we provide concentration values for all of the components present. Instead the model allows us to pick up only that portion of spectral absorbance that correlates well to the concentration. Once we have matrices **A** and **C**, we can determine **P** by following equation: **P = pinv (A) * C.** Pseudo inverse of matrix A is calculated instead of inverse as explained in Classical Least Squares method. (We can calculate P for as many components as we provide in matrix C i.e. if all the components in matrix C are not known, P will be calculated only for those components which are known and present in matrix C.) For predicting the unknown concentration of a mixture from its absorbance matrix, following equation is used: **C = A * P** [37]

Figure 4.4 Pictorial representation of Inverse least squares principle

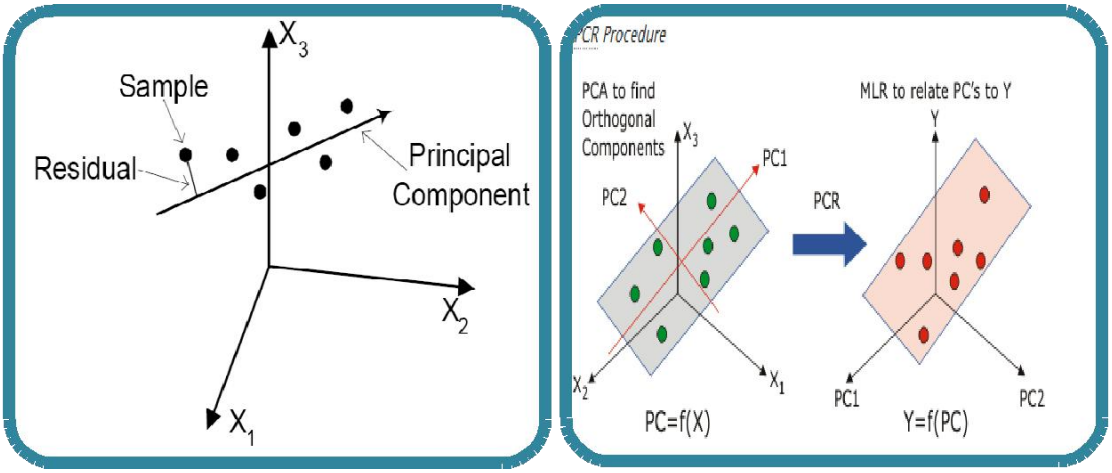


4.4.1.8 Principal Component Regression [36]

PCR is a method by which the dimensionality or complexity of the data is reduced. With reference to application for spectrophotometric analysis, absorbance matrix concerns to a large pool of data containing *n* variables (wavelengths, one column for each) and *m* samples (calibration standards, one row for each). Each sample can be presented as a point in *n* dimensional space according to its absorbance values at different wavelengths. Thus, one sample is described by *n* variables. PCR is a tool which reduces number of variables to only a few components, referred to as principal components (PCs). They are computed in such a way that the first PC is the one that carries most information (or in statistical terms: most explained variance). The second PC will then carry the maximum share of the residual information (i.e. not taken into account by the previous PC), and so on. All the PCs will be orthogonal to each other and they are ranked in a manner so that first PC will explain maximum variance in the data. Theoretically, there can be as many PCs as the number of actual variables (n*)*. But only first few PCs are considered because they explain almost all the variance in the data. After reducing the dimensionality of data to a few PCs, next step is regression to relate these PCs with concentration. There will be individual regression for each drug present in the sample. Thus, PCR deals with only one response variable (concentration) at a time.

Properties of Principal Components: Each variable has a *loading* on each PC. It reflects how much the variable contributed to that PC. Each sample has a *score* on each PC. It reflects the sample location along that PC. Each sample has *residual* on each PC, that is, the difference between its actual location and its location approximated by the model. This is called sample residual.

Figure 4.5 Pictorial representation of Principal component regression principle



Sample Residual

Total residual variance is the average residual variance of all samples for one particular PC.

Explained variance is the complement of residual variance, expressed as a percentage of the global variance in the data.

Similarly, residual variance and explained variance can be determined for variables also.

Residual variance expresses how much variation in the data *remains to be explained* once the current PC has been taken into account. It should be near to zero.

Explained variance, often measured as a percentage of the total variance in the data, is a measurement of the proportion of variation in the data accounted for by the current PC. It should be near to 100.

4.*4.1.9 Partial least squares or Projection to Latent Structures* [36]

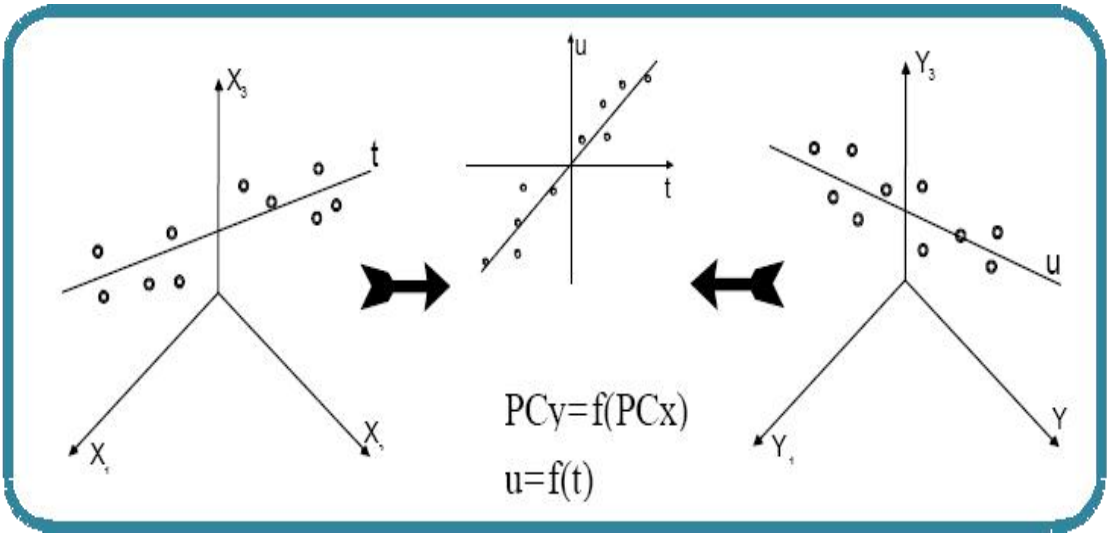
PLS models both the X and Y matrices simultaneously to find the latent variables in X that will best predict the latent variables in Y. This method is called projection to latent structures. These PLS latent variables are similar to principal components, and will be referred to as PCs or factors. In PCR, we find PCs for only X matrices (variables) and then these PCs are related directly to Y (sample concentration). Here in PLS, PCs are computed for X and Y both. And then PC of X is related with PC of Y. PLS can handle multiple responses in Y at a time in contrast to PCR. The difference between PCR and PLS lies in the algorithm. PLS uses the information lying in both X and Y in order to fit the model. It switches between X and Y iteratively to find the relevant PCs. So PLS often needs fewer PCs to reach the optimal solution because the focus is on the prediction of the Y-variables (not on achieving the best projection of X as in PCR).

Full cross validation method is used for determining the optimum number of factors. The algorithm used for PLS was NIPALS i.e. nonlinear iterative partial least squares. For

determining the optimum number of principal factors for PLS the parameters considered were

1) Total explained Y variance
2) Total residual Y variance
3) RMSEP values for validation

Figure 4.6 Pictorial representation of Partial least squares principle



4.4.1.10 Statistical analysis

Statistical analysis of developed methods can be done by using ANOVA test. If the null hypothesis are rejected for ANOVA, further posthoc analysis can be done using tests like Tukey Honest significant difference test (Tukey HSD test), Scheffe multiple comparison test, Least square difference (LSD) test and Duncan's multiple range test (DMRT test). [27]

4.4.2 Experimental

4.4.2.1 Chemicals and materials

Gift samples of standard CHD and CET API were obtained from MIL Laboratories Pvt. Ltd, Baroda. Savlon antiseptic solutions manufactured by ITC were procured from a local pharmacy. (Labelled claim was 0.3 gm CHD and 3 gm CET per 100 ml) methanol, acetonitrile analytical reagent grade (Fischer Scientific Pvt. Ltd, Mumbai, India) and double distilled water (DDW) were used as the solvent and diluents for UV spectrophotometric method.

4.4.2.2 Equipments and analysis conditions

Shimadzu UV-1700 double beam spectrophotometer connected to a computer loaded with Shimadzu UV Probe 2.10 software was used for all the spectrophotometric

measurements. Shimadzu UV- 1800 double beam spectrophotometer was also employed for ruggedness study. 1cm quartz cells were used to measure the absorbance spectra of the reference and test solutions over the range of 200-400 nm. All the samples were weighed on electronic analytical balance (A×120, Shimadzu). The chemometric models were developed by using the software packages like MATLAB from Math works, Design expert 7.0 and SAS JMP 13.

4.4.2.3 Preparation of calibration samples and quality control samples

For all UV spectrophotometric methods, 10mg each of CHD and CET were weighed accurately and transferred into two separate 10 ml volumetric flasks containing 1 ml acetonitrile. DDW was added up to the mark to produce a stock solution containing 1000 µl/ml of CHD and CET respectively. For all UV spectrophotometric methods, 2.5 ml each of CHD and CET transferred into two separate 25 ml volumetric flasks containing 2.5 ml acetonitrile. DDW was added up to the mark to produce a stock solution containing 100 µl/ml of CHD and CET respectively. Considering the ratio of CHD and CET in commercial formulation to be 1:10 appropriate aliquots of CHD and CET working standard solutions were taken in different 6 ml volumetric flasks each and diluted up to the mark with solvent to obtain final concentrations of 3-18 µl/ml and 30-180 µl/ml respectively.

4.4.2.4 Methodology

4.4.2.4.1 Vieordt's method [27]

In this method for selection of analytical wavelengths, standard solutions of CHD and CET were scanned between 200-400 nm wavelength ranges which implicated λmax of 217nm for CET and 260 nm for CHD. The zero order overlain spectra of CHD and CET are shown in Figure 4.7. Calibration curve of absorbance versus concentration were prepared. The calculations were done by using the formula stated below for simultaneous analysis of both analytes in the mixture.

$$C_{CET} = \frac{(A2\ ay1 - A1\ ay2)}{(ax2\ ay1 - ax1 ay2)}$$

$$C_{CH} = \frac{(A1\ ax2 - A2\ ax1)}{(ax2\ ay1 - ax1\ ay2)}$$

Where; Cx & Cy are concentrations of CET and CHD respectively in gm/100 ml in the sample solution.
A1 & A2 are the absorbance of the mixture at λmax of both drugs ie., at 217 and 260 nm respectively;
ax1 and ax2 = Absorptivity of CET at λmax of both drugs ie., at 217 and 260 nm respectively;
ay1 and ay2 = Absorptivity of CHD at λmax of both drugs ie., at 217 and 260 nm respectively;

4.4.2.4.2 First Derivative spectroscopy method [23]

In this method zero order spectra of the two drugs were derivatized to first order and superimposed over each other. Then on basis of zero crossing points (ZCP) of the corresponding drugs the two wavelengths for analysis were chosen. The first order derivatized spectra of CHD and CET are shown in figure 4.8. As per the study, CET was analyzed at 222 nm which was ZCP of CHD whereas CHD was analyzed at 275 nm which was ZCP of CET. The parameters Δλ and scaling factor were set to be 5.

4.4.2.4.3 Multicomponent analysis method

This method is developed using the UV 1700 instrument without PC control with UV probe. λ max of each single drug was determined first. λmax selected were 260 nm and 217 nm for CHD and CET respectively. Mixture solutions were prepared in previously decided ratio for both drugs. The entire range of mixture solutions was scanned in the Multicomponent mode. This analytical data was then saved in the inbuilt software of the instrument for analysis of unknown samples.

4.4.2.4.4 Absorption ratio spectra method [29]

In this method, the spectra of mixture are divided by the spectra of any one analyte (CET), for analysis of other corresponding analyte (CHD) in the mixture. Then in this modified spectra two wavelengths are selected (λ1=225 nm and λ2=263). Now λ2-λ1 will give absorption ratio spectra value for analyte CHD. This way for CET also, absorption ratio spectra value is found out. The two wavelengths selected for CET are (λ1=200nm and λ2=217 nm). Here λ2-λ1 will give absorption ratio spectra value for analyte CET. The above stated was done for a series of different concentrations. The relationship is linear and thus applied for analysis of unknown samples.

4.4.2.4.5 Mean centering of ratio spectra method [29]

Mean centering of ratio spectra method is an improvement for resolution of two analytes in a mixture. Also it eliminates the need for preliminary steps like derivatizing the sample and thus S/N ratio is improved in it. In this method first the analysis of mixture (Am) in entire calibration range was done at a λmax of CET (217 nm). This Am values are then divided by molar absorptivity (αCHD=505446 1/mol/cm). This Am/αCHD values for entire calibration range are then mean centered using software package Unscrambler X, version 10.5 and used fed to model for analysis of CET. This way for CHD also Am/ αCET is done (where λmax of CHD is 260 nm and αCET= 364450 1/mol/cm) and then mean centered and fed for analysis of CHD. These values can now act as a predictor for future analysis of unknown sample.

Chemometrics methods were developed using the software packages like Design expert 7.0 and Matlab from Math works and SAS JMP 13. For data manipulation in CLS and ILS methods, Matlab software was utilized and for data manipulation by PLS and PCR methods, SAS JMP 13 software was utilized. In all chemometrics methods first calibration and validation sets were defined by application of full factorial design. Once

defined, calibration and validation sets were prepared and their absorbances were taken in wavelength range 220-260 nm with interval of 1 nm, thereby at twenty wavelengths. For production of absorption matrix A, calibration set consisting of 30 sets was utilized. Other six sets were considered for validation of the model.

4.4.2.4.6 Classical least squares method [37]

The mathematical model for this method can be represented by A=CK where A is m x n matrix of calibration spectra, C is m x l matrix of component spectra and K is l x n matrix of absorptivity at unit concentration and unit path length.(m=20, n=30, l=2).The calibration coefficient matrix (K) was calculated as K= pin v(C)*A and using the K value of calibration coefficient unknown was computed using formula C= A* pinv (K).

4.4.2.4.7 Inverse least squares method [37]

The mathematical model for this method can be represented by C=AP where P is l x n matrix of unknown calibration, C and A are same as defined for CLS method mentioned above at unit concentration and unit path length. (m=20, n=30, l=2).The calibration coefficient matrix (P) was calculated as P= pin v (A)*C and using the P value of calibration coefficient unknown was computed using formula C= A* P.

4.4.2.4.8 Partial least squares method [36]

PLS computes factors for A and C both and then correlates them. It models both the A and C matrices simultaneously to find the latent variables in A that will best predict the latent variables in C. Full cross validation method is used for determining the optimum number of factors. The algorithm used for PLS was NIPALS i.e. nonlinear iterative partial least squares. PLS uses the information lying in both X and Y in order to fit the model. It switches between X and Y iteratively to find the relevant PCs. So, PLS often needs fewer PCs to reach the optimal solution because the focus is on the prediction of the Y-variables.

4.4.2.4.9 Principal component regression

PCR computes factors for A and C both and then correlates them. It models both the A and C matrices simultaneously to find the latent variables in A that will best predict the latent variables in C. There will be individual regression for each drug present in the sample. Thus, PCR deals with only one response variable (concentration) at a time.

4.4.2.5 Applicability of the method

The developed UV methods were applied for analysis of their formulation available in market. “Savlon antiseptic solution” manufactured by ITC. Kolkata [38] was procured from local pharmacy. Formula for 100 ml antiseptic solution is represented in Table 4.1. 0.5 ml of the sample formulation was withdrawn in a 50 ml volumetric flask and diluted up to the mark using acetonitrile: DDW (1:9) to produce a clear solution. The resulting

solution was again diluted using acetonitrile: DDW (1:9) by withdrawing 1.25 ml and making up to 25 ml to give the final solution for analysis. The final solution was analyzed and absorbance was recorded. Concentrations of both analytes were then calculated from the calibration graph. Six replicate samples were used for analysis.

Table 4.1 Formula for 100 ml solution of formulation for checking applicability of developed methods [38]

Ingredients	**Quantity**
Chlorhexidine gluconate	0.3 gm
Cetrimide	3 gm
N-propyl alcohol	2.84%
Benzyl benzoate	0.06%
Water	Q.S

4.4.2.6 Method Validation [39]

Linearity and range: The proposed spectrophotometric method showed good linearity in the concentration range of 3-18 µg/ml for CHD and 30-180 µg/ml for CET.
Precision: Inter-day and intra-day precision for the method were measured in terms of % RSD. The experiment was repeated 3 times in a day (Intraday precision) and the average % RSD values of the results were calculated. Similarly the experiment was repeated on 3 different days (Inter day precision) and the average % RSD value for absorbance of CHD and CET were calculated. The low value of SD obtained confirms the precision of the method.
LOD and LOQ: Calibration curve was repeated for 9 times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were measured as follows. LOD=3.3 * SD/slope of calibration curve, LOQ=10 * SD/slope of calibration curve where SD = Standard deviation of intercepts
Accuracy: Accuracy of the method was confirmed by recovery study from marketed formulation at 3 level of standard addition (80%, 100%, and 120%) of label claim. Recovery greater than 98 % with low SD justified the accuracy of the method
The stability of the working stock solutions was studied at room temperature and in refrigerator (8-25ºC).
Robustness and ruggedness: The robustness of the method was determined by using acetonitrile of 3 different manufacturers for the preparation of stock solution of standard drugs. The ruggedness of method is determined using different models of UV spectrophotometer and different analysts. The average value of % RSD for determination of CHD and CET less than 2% revealed the robustness and ruggedness of the method.
For chemometric methods validation, the predicted concentrations of the compounds in each sample were compared with the actual concentrations in the training samples and the root mean square error of prediction (RMSEP) was calculated for each method. The RMSEP value was used as a diagnostic test for examining the errors in the predicted concentrations.

The PRESS (predicted residual error sum of squares) value was calculated as the difference between the real and the calculated concentrations, squares and summed over all references for each component. The selected model was that with the fewest number of factors such that its RMSEP values were not significantly greater than that for the model.

The RMSEP value can be quantified with following formula:

$$RMSEP = \sqrt{\frac{\sum_{i=1}(\quad - \quad)^2}{N}}$$

4.4.2.7 Statistical analysis [40]

Statistics may be defined as the collection, presentation, analysis and interpretation of numerical data. Analysis of Variance (ANOVA) is a technique of separating the total variability in a set of data into components parts, represented by a statistical model. If more than two assay methods are to be compared, the correct statistical procedure to compare the means is One-way ANOVA. P-value in One-way ANOVA is the probability of that random sampling would lead to a difference between sample means as large (or larger) than you observed. P value threshold is fixed to the value same as alpha (probability level) i.e. 0.05. On that basis we either reject or accept the null hypothesis. If in one way ANOVA we reject the null hypothesis, post hoc analysis is to be done using tests like Tukey Honest significant difference test (Tukey HSD test), Scheffe multiple comparison test, Least square difference (LSD) test and Duncan's multiple range test (DMRT test). Here we are applying Tukey HSD test and Scheffe multiple comparison test.

4.4.3 Results and discussion

In this study 9 UV spectrophotometric methods were developed in which 4 methods were Chemometric assisted methods and 5 were classical spectrophotometric methods. The methods were developed as specified in methodology and validated as per ICH guidelines.

4.4.3.1 Method optimization and development

4.4.3.1.1 Vierordt's method:

In this method as represented in Figure 4.7, λmax for CHD was chosen as 260 nm and λmax for CET was chosen 217nm. The linearity range selected for CHD was 3-18 µg/ml and for CET was 30-180 µg/ml considering the ratio of drug combination in the

formulation. All validation parameters were within limits as prescribed by ICH guidelines as represented in Table 4.6, Table 4.7, Table 4.9 and Table 4.10.

Figure 4.7 a: Vieordt's method absorption spectra representing λmax of CHD=260 nm and λmax of CET=217nm

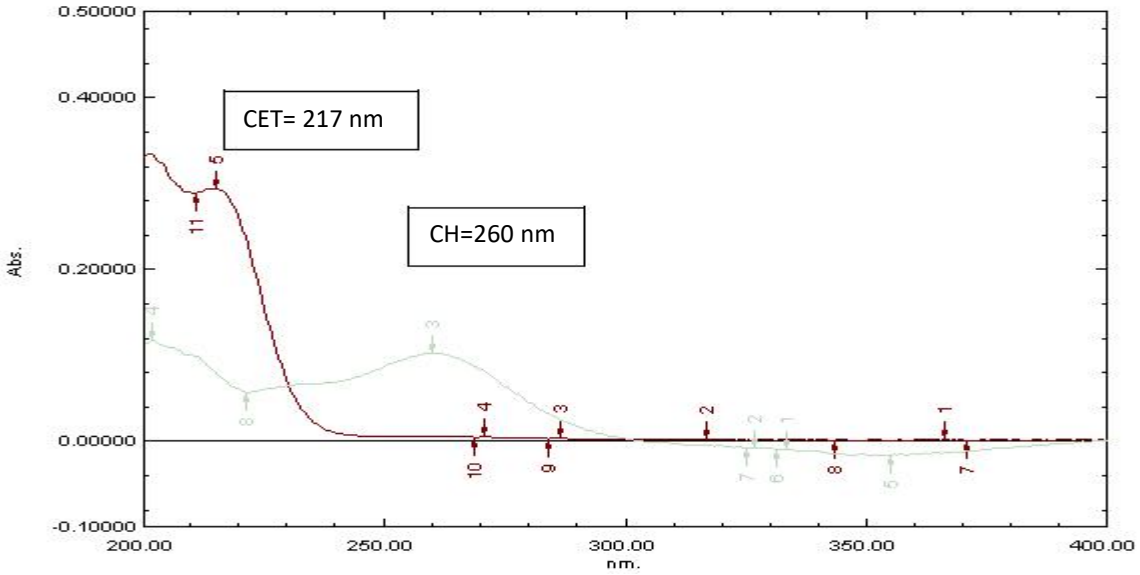
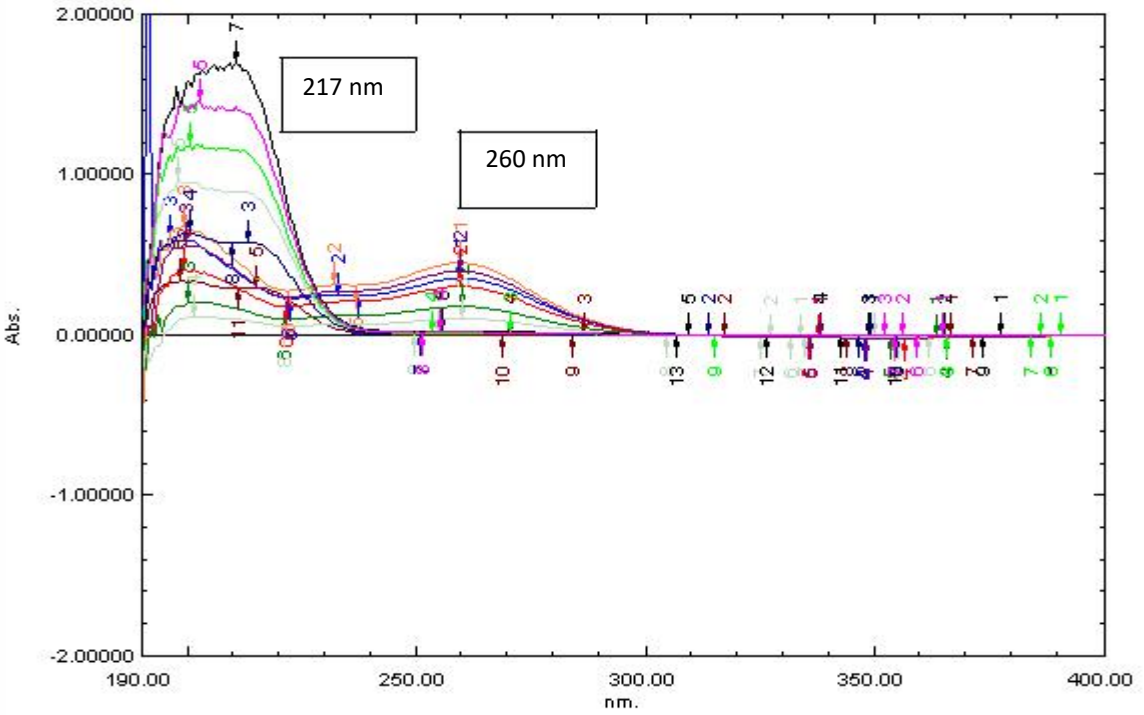


Figure 4.7 b: Vieordt's method absorption spectra representing λmax of CHD=260 nm and λmax of CET=217nm (Overlain spectra)



4.4.3.1.2 First derivative spectroscopic method:

In this method as represented in figure 4.8, detection λ for CHD was chosen as 275 nm as it is the zero crossing point of CET and detection λ for CET was chosen 222nm as it is the zero crossing point of CHD. The linearity range selected for CHD was 3-18 µg/ml and for CET was 30-180 µg/ml. All validation parameters were within limits as prescribed by ICH guidelines as represented in Table 4.6, Table 4.7, Table 4.9 and Table 4.10.

Figure 4.8 a: First derivative spectroscopic method representing detection λ of CHD=275 nm (ZCP of CET) and detection λ of CET=222nm (ZCP of CHD)

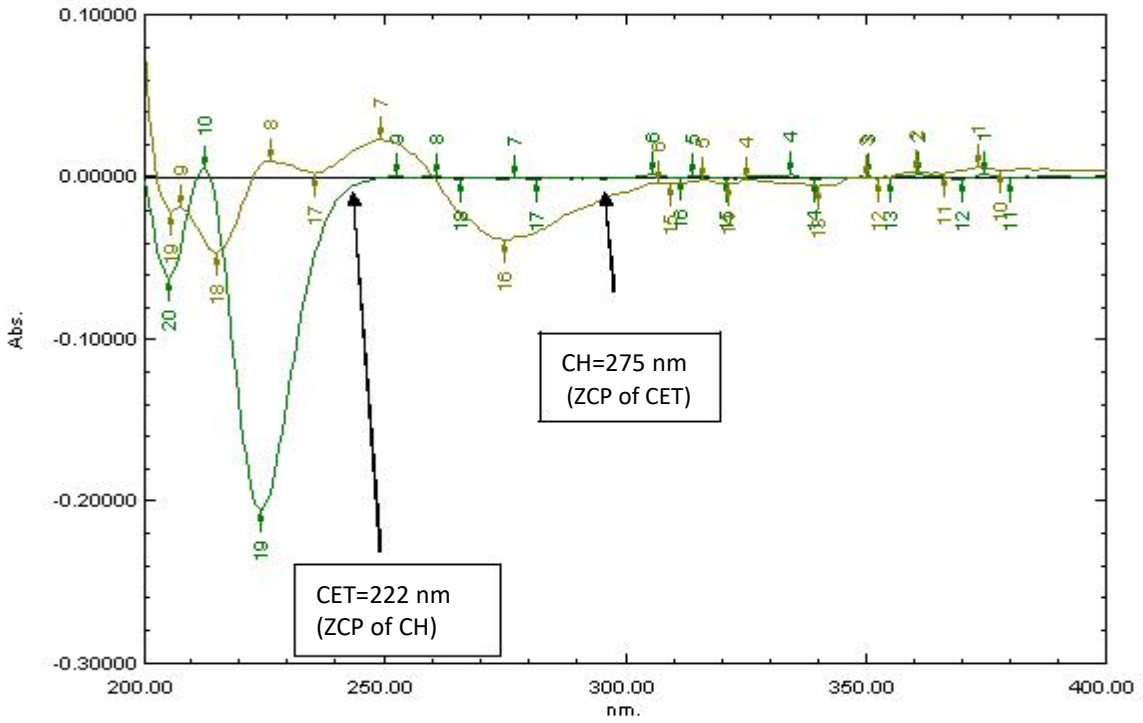
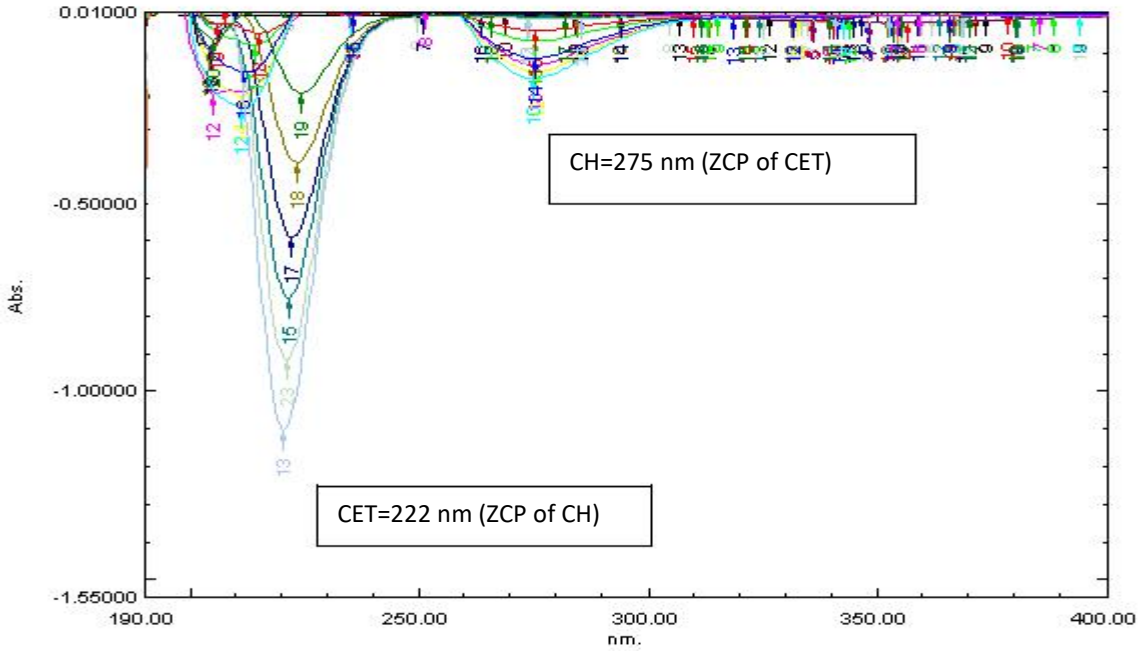


Figure 4.8 b: First derivative spectroscopic method representing detection λ of CHD=275 nm (ZCP of CET) and detection λ of CET=222nm (ZCP of CHD) (Overlain spectra)



4.4.3.1.3 Multicomponent analysis method:

In this method also the λmax for CHD was chosen as 260 nm and λmax for CET was chosen 217nm. The linearity range selected for CHD was 3-18 µg/ml and for CET was 30-180 µg/ml. The predicted vs Actual concentration by Multicomponent mode is shown in figure 4.9 and 4.10. All validation parameters were within limits as prescribed by ICH guidelines as represented in Table 4.6, Table 4.7, Table 4.9 and Table 4.10.

Figure 4.9: Predicted Vs Actual concentration of CHD in Multicomponent analysis method

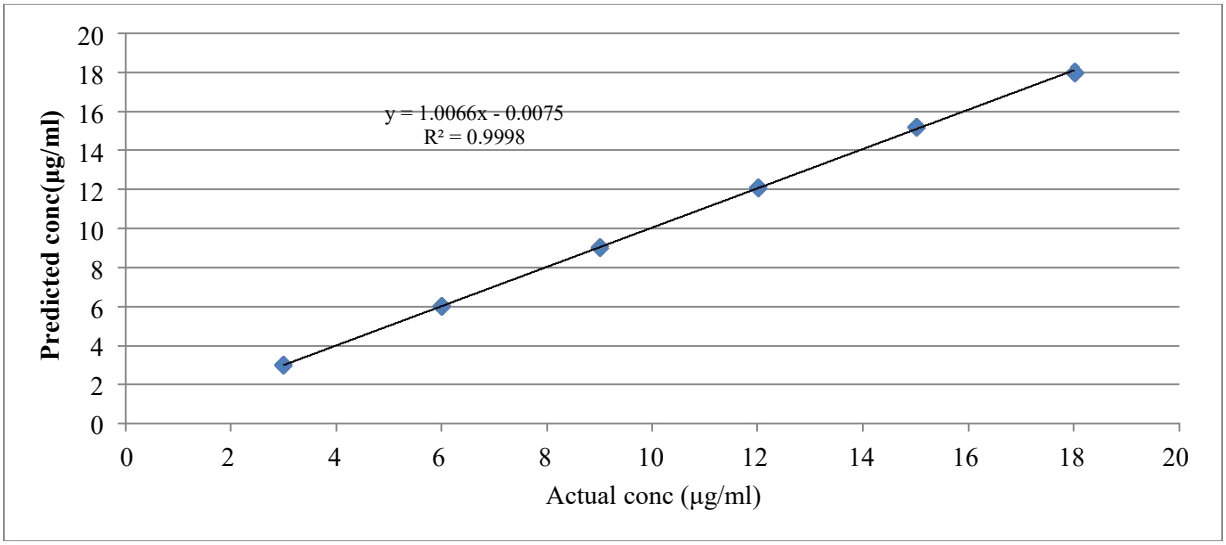
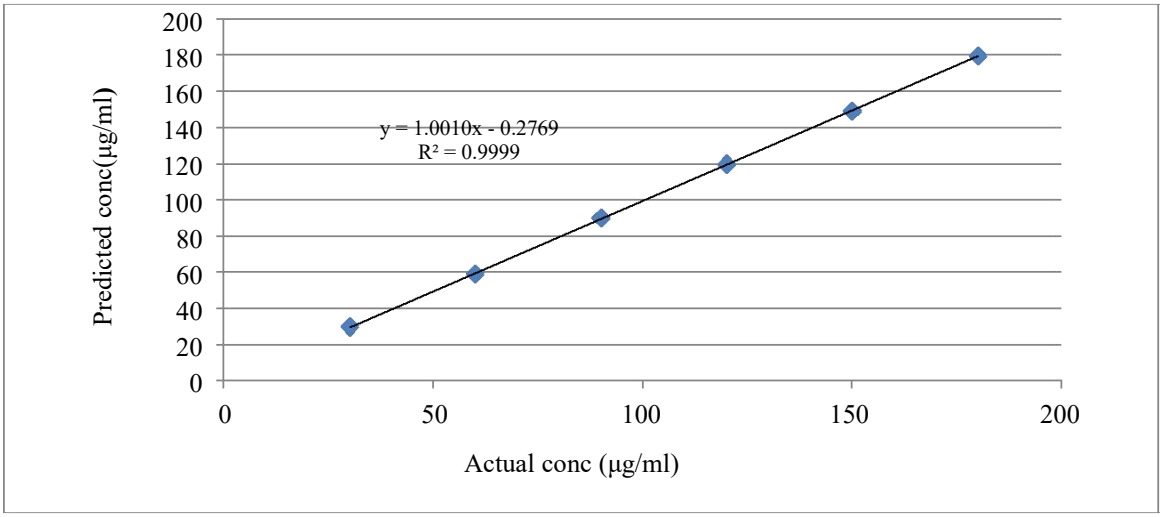


Figure 4.10: Predicted Vs Actual concentration of CET in Multicomponent analysis method



4.4.3.1.4 Absorption ratio spectra method:

In this method as represented in figure 4.11, Δλ for CET was chosen as a difference between 217 nm and 200 nm while for CHD it was chosen a difference between 263 and 225 nm. The linearity range selected for CHD was 3-18 µg/ml and for CET was 30-180 µg/ml. All validation parameters were within limits as prescribed by ICH guidelines as represented in Table 4.6, Table 4.7, Table 4.9 and Table 4.10.

Figure 4.11 a: Absorption ratio spectra method representing values of 217-200 nm (λ2- λ1) for CET and 263-225 nm (λ2- λ1) for CHD

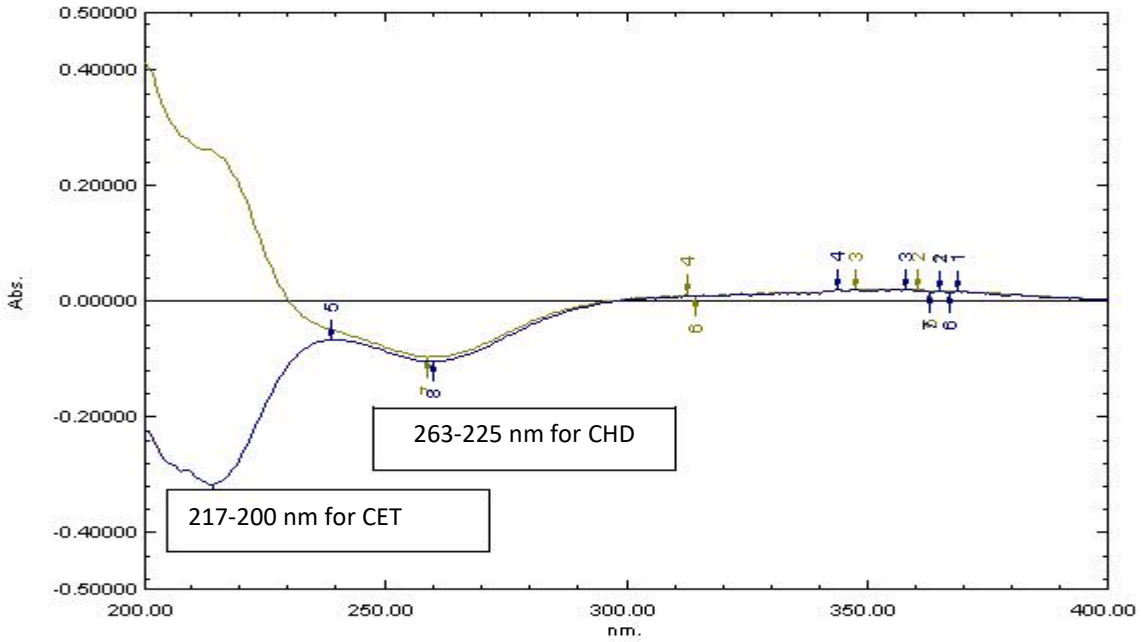
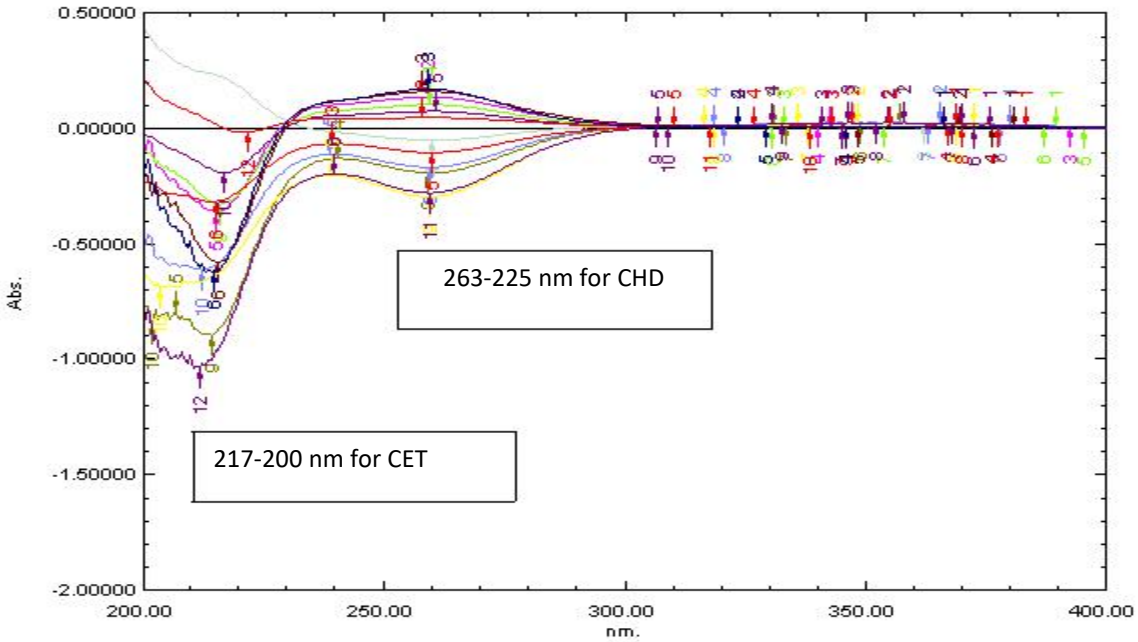


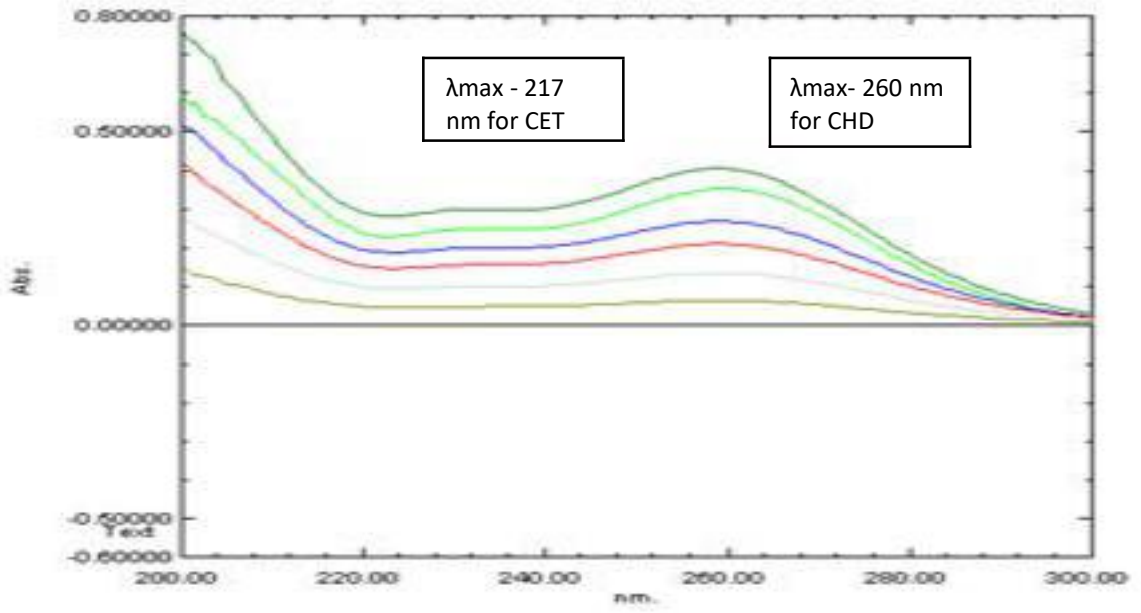
Figure 4.11 b: Absorption ratio spectra method representing values of 217-200 nm (λ2- λ1) for CET and 263-225 nm (λ2- λ1) for CHD (Overlain spectra)



4.4.3.1.5 Mean centering of ratio spectra method

As shown in Figure 4.12, spectra of mixture was taken and then it was first divided by molar absorptivity of CHD =505446 1/mol/cm (αCHD). The absorbance (Am) obtained in spectra of mixture at 217 nm which is the λmax of CET was recorded for whole range. Then Am/ αCHD values are calculated for entire calibration range and then mean centered using Unscrambler X, version10.5 software package. These values now can be used for analysis of CET in future for prediction of unknown samples. Similar treatment is to be done for analysis of CHD using 364450 1/mol/cm as molar absorptivity value of CET and 260 nm as λmax of CHD. The linearity range selected for CHD was 3-18 µg/ml and for CET was 30-180 µg/ml. All validation parameters were within limits as prescribed by ICH guidelines as represented in Table 4.6, Table 4.7, Table 4.9 and Table 4.10.

Figure 4.12: Spectra of mixture for mean centering of ratio spectra method



4.4.3.1.6 Classical least square method:

The quality of Multicomponent analysis by chemometrics is dependent on the wavelength range used. The range selected for our study was 220 to 260 where both for the study drugs gave significant absorbance. In this method full factorial design was used for preparation of total number of sets. Thus, total 36 sets were obtained from which 30 were included in the calibration set and 6 were constituted in the validation set. This allowed modeling of data with the optimum amount of information and avoidance of over fitting or under fitting. Full cross validation is implied for the model. The cross validation procedure consists of systematically removing one of a group of training samples in turn and using only the remaining ones for the construction of latent factors and applied regression. Then, absorbances of all 36 sets were taken in wavelength range 220-260 nm with interval of 1 nm, thereby at 20 wavelengths. After that using the data matrix, CLS model was developed for obtaining the K matrix (Table 4.4) and thus using it the unknown set can be quantified. Table 4.2 and 4.3 represent the Concentration matrix for calibration and validation set for application of CLS method.

The RMSEP values and all other validation parameters represent the validity of the developed method as represented in Table 4.6, Table 4.7, Table 4.8, Table 4.9 and Table 4.10. The predicted Vs actual concentration were obtained from the model as shown in figure 4.13 and 4.14 for CHD and CET respectively where regression coefficient approaching 1 represents model to be valid and reliable.

Table 4.2 Concentration matrix for Calibration set of Chemometrics methods ILS, CLS, PLS, PCR

CHD (µg/ml)	CET(µg/ml)
18	120
12	180
6	60
12	120
3	30
6	30
12	30
9	90
15	150
18	180
6	120
12	90
15	90
3	90
6	90
3	120
15	120
18	30
9	60
9	120
18	60
15	30
9	180
6	180
3	180
18	90
12	150
18	150
9	150
3	150

Table 4.3 Concentration matrix for Validation of Chemometrics methods ILS, CLS, PLS, PCR

CHD (µg/ml)	CET(µg/ml)
15	60
3	60
12	60
9	30

15	180
6	150

Table 4.4 K matrix for Classical least square method

Wavelength	CHD	CET
220	0.0084	0.0098
222	0.4758	-0.0341
224	-0.5226	0.0764
226	0.0801	-0.0356
228	-0.2863	-0.0818
230	0.5389	-0.0125
232	-0.5208	0.1615
234	-0.0538	-0.0493
236	-0.1855	-0.0536
238	-1.1708	0.1854
240	1.2131	1.2131
242	0.0231	-0.0197
244	0.3230	-0.0788
246	-0.0203	0.0876
248	-0.0434	-0.0297
250	-0.1463	-0.0784
252	0.1021	0.1196
254	0.3675	-0.0889
256	-0.4314	0.1360
258	-0.1100	0.0652
260	0.4162	-0.0403

Figure 4.13 Predicted vs. Actual conc. for CHD for CLS model

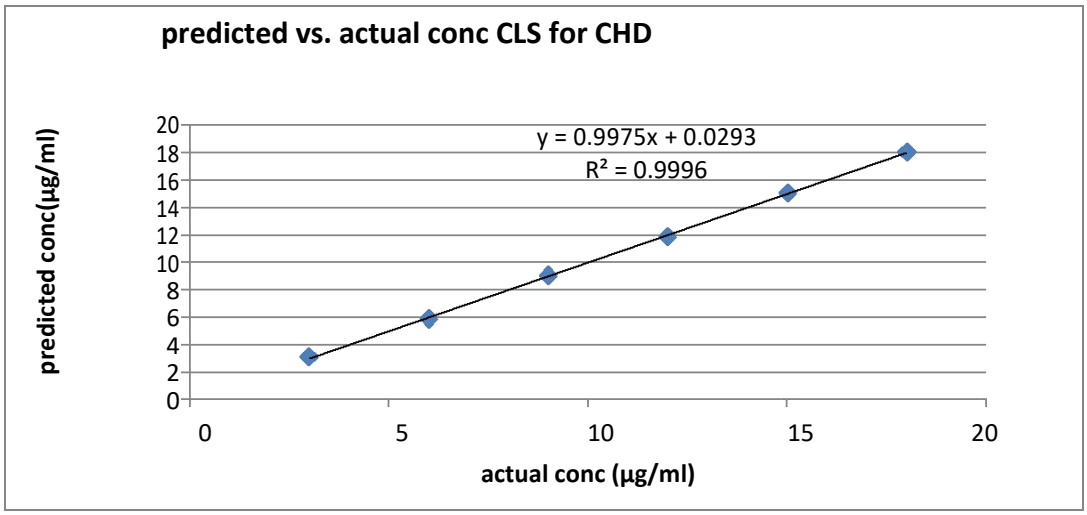
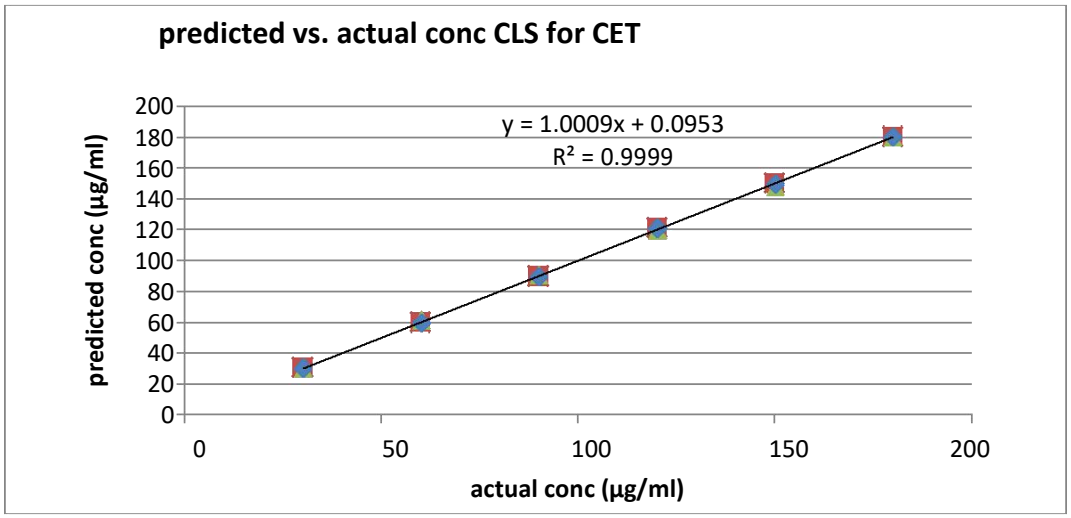


Figure 4.14 Predicted vs. Actual conc. for CET for CLS model



4.4.3.1.7 Inverse square method:

Keeping all other parameters same as for CLS model mentioned in section 4.4.3.1.6, ILS model was developed for obtaining the P matrix (Table 4.5) for quantification of unknown. The RMSEP values and all other validation parameters represent the validity of the developed method as represented in Table 4.6, Table 4.7, Table 4.8, Table 4.9 and Table 4.10. The predicted Vs actual concentration were obtained from the model as shown in figure 4.15 and 4.16 for CHD and CET respectively where regression coefficient approaching 1 represents model to be valid and reliable.

Table 4.5 P matrix for Inverse square method

Wavelength	CHD	CET
220	27.9202	19.6099
222	175.165	6.3011
224	59.8893	3.8674
226	12.4329	28.974
228	259.7824	45.1507
230	13.379	26.9688
232	200.822	2.4336
234	102.696	26.018
236	43.0846	72.7414
238	124.917	41.8134
240	702.6883	29.9677
242	134.998	29.8982
244	340.1333	61.888
246	315.161	16.0759
248	159.9956	107.8174
250	201.7889	75.4349
252	267.7467	83.4908
254	82.4908	10.124
256	88.6634	34.9325
258	228.38	45.8436
260	261.759	9.5344

Figure 4.15 Predicted Vs. Actual conc. for CHD for ILS model

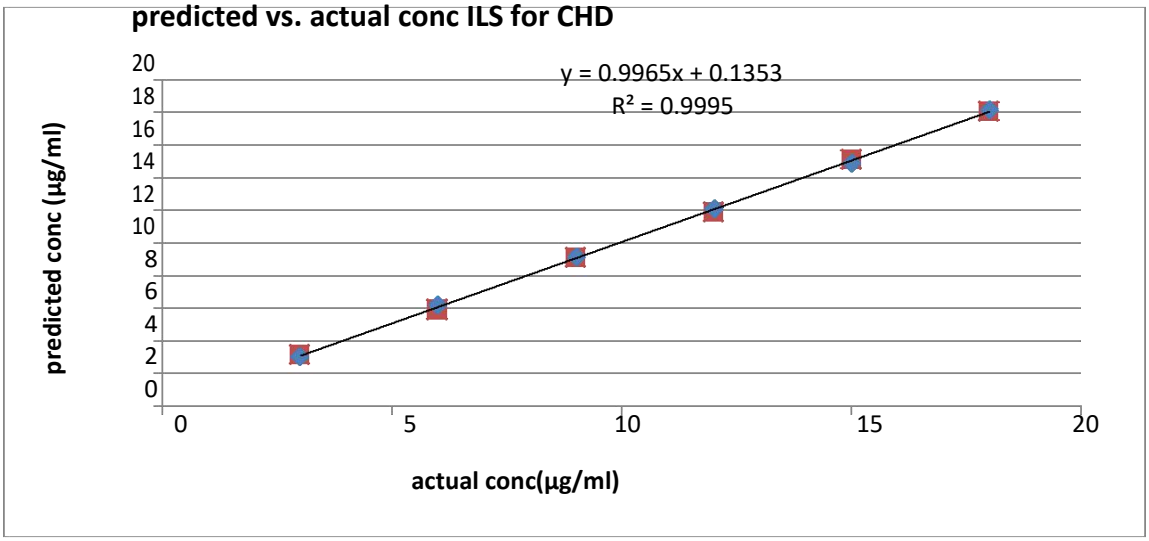
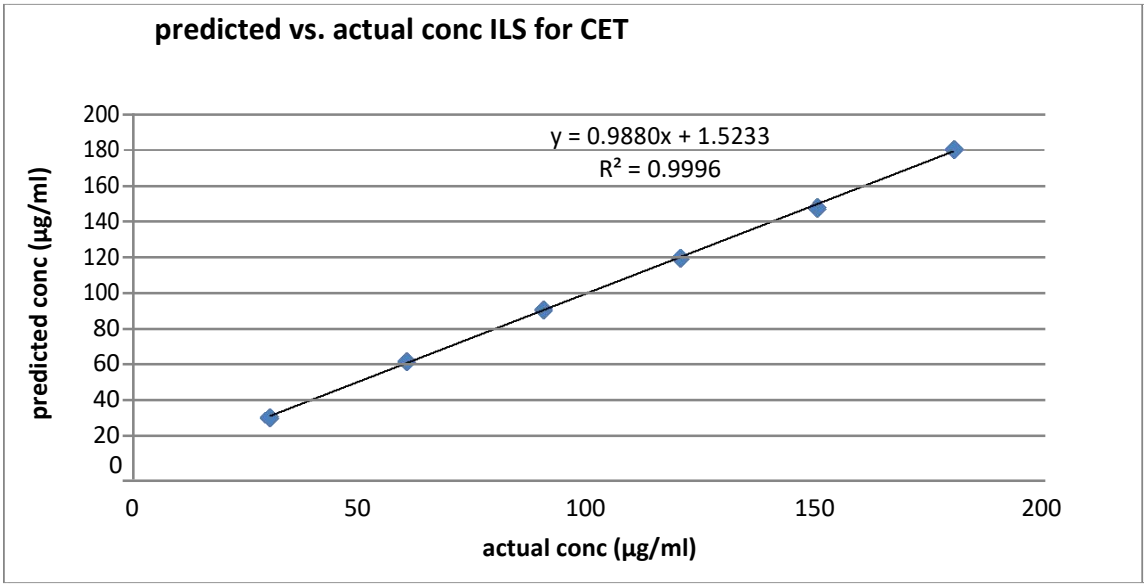


Figure 4.16 Predicted Vs. Actual conc. for CET for ILS model



4.4.3.1.8 Partial least square method and
4.4.3.1.9 Principal component regression method

In both of methods also like CLS and ILS, 36 sets were obtained using full factorial design as represented in section 4.4.3.1.6. Using the data matrix, PLS model was developed. Figure 4.17 and figure 4.18 represents the explained variance for PLS model showing that first principal components constitutes maximum information (about 81 %) and second principal component carries the remaining information about the model. The predicted Vs actual concentration were obtained from the PLS model as shown in figure

4.21 and 4.22 for CHD and CET respectively where regression coefficient approaching 1 represents model to be valid and reliable. Figure 4.19 and figure 4.20 represents explained variance for PCR model. Also the predicted Vs actual concentration were obtained from the PCR model as shown in figure 4.23 and 4.24 for CHD and CET respectively where regression coefficient approaching 1 represents model to be valid and reliable. The RMSEP values and all other validation parameters represent the validity of the developed method as represented in Table 4.6, Table 4.7, Table 4.8, Table 4.9 and Table 4.10. PLS turns out to be a better model than PCR as it requires few components than PCR to achieve the same level of prediction and thus it's more compact model than PCR.

Figure 4.17 Pictorial representation of explained variance with 4 principal components for PLS model

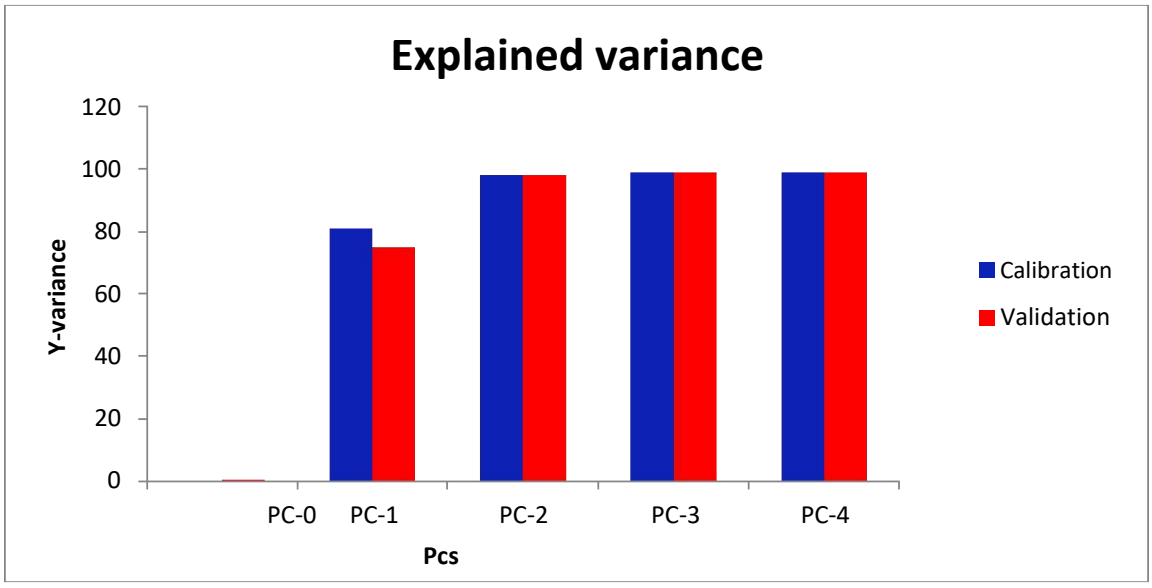


Figure 4.18 Pictorial representation of residual variance with 4 principal components for PLS model showing maximum information being carried by first 2 PC's

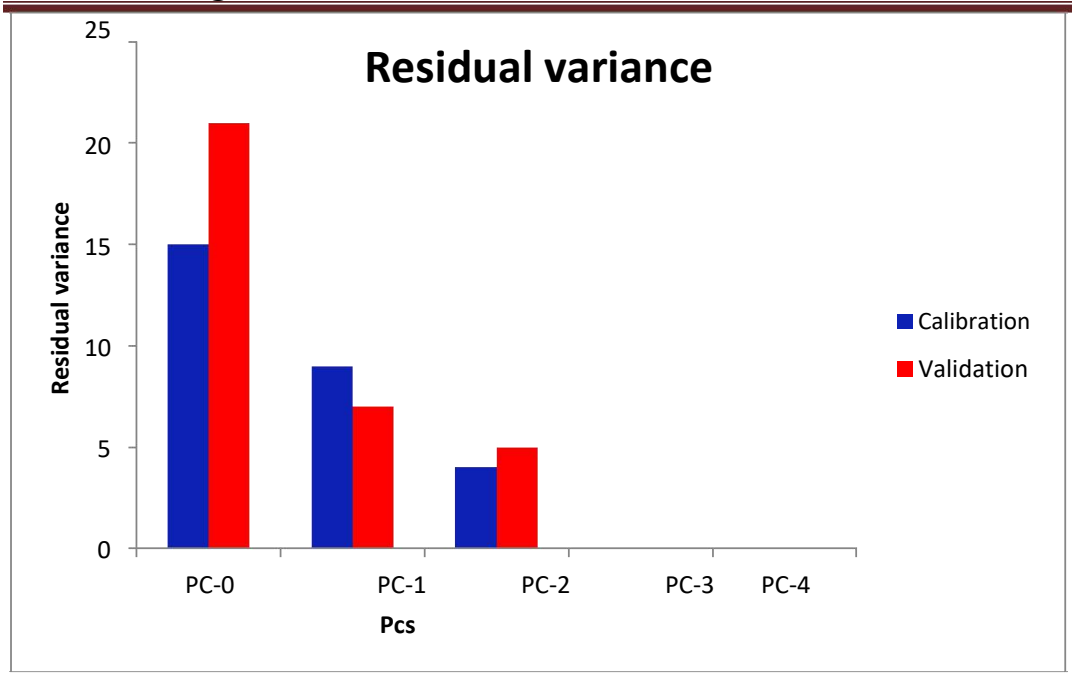


Figure 4.19 Pictorial representation of explained variance with 4 principal components for PCR model

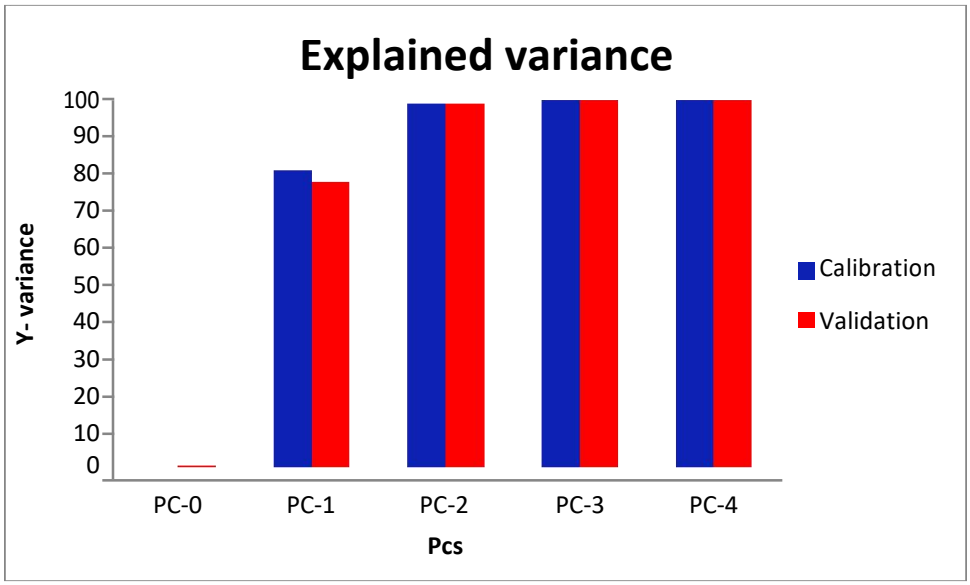


Figure 4.20 Pictorial representation of residual variance with 4 principal components for PCR model showing maximum information being carried by first 2 PC's

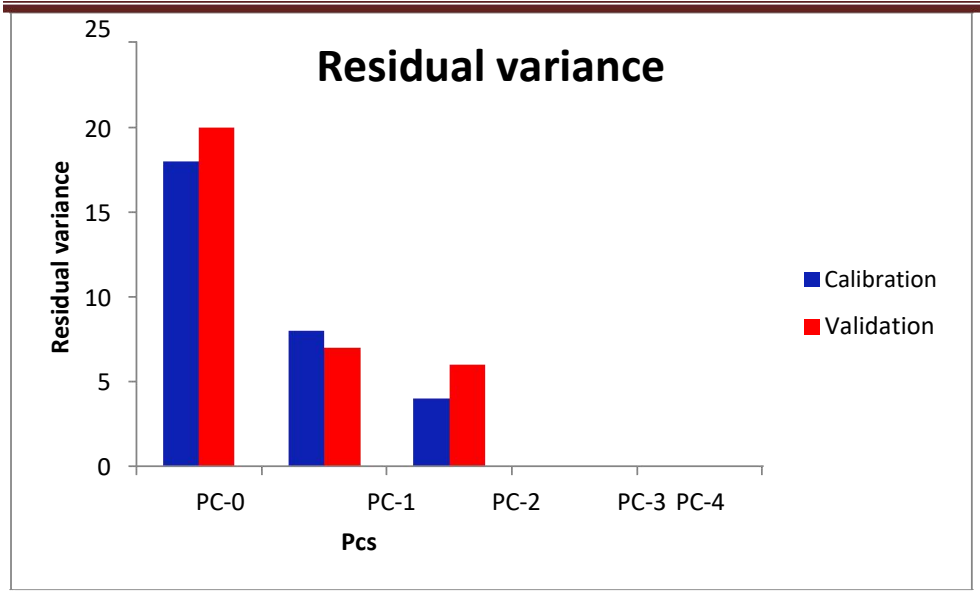


Figure 4.21 Predicted Vs. Actual conc. For CHD for PLS model

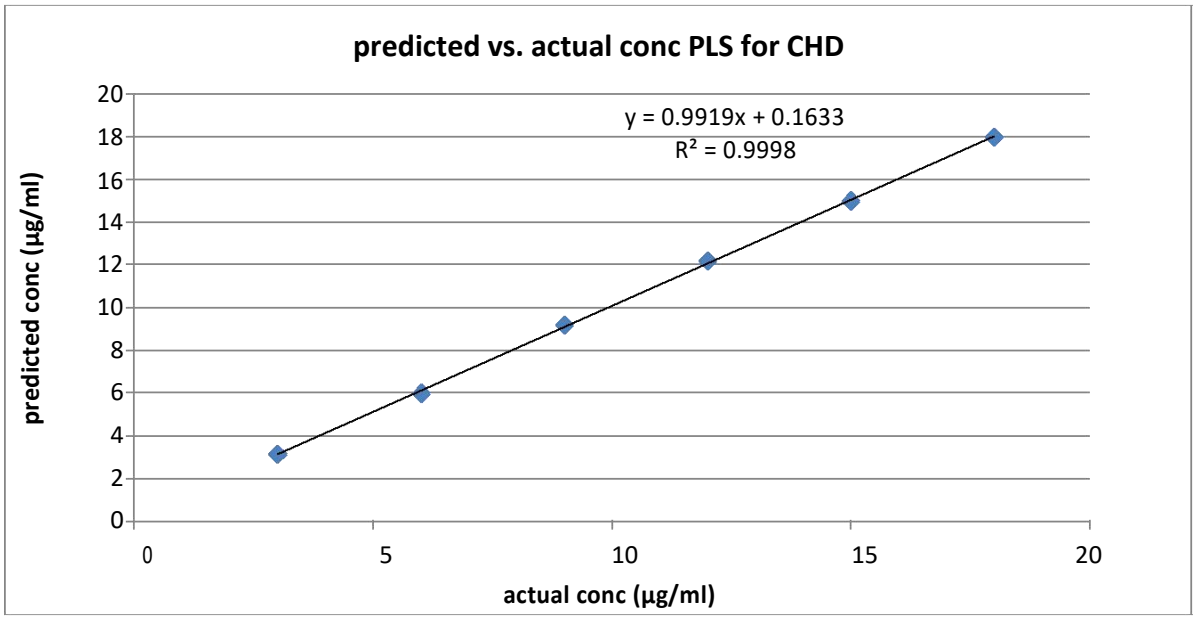


Figure 4.22 Predicted Vs. Actual conc. For CET for PLS model

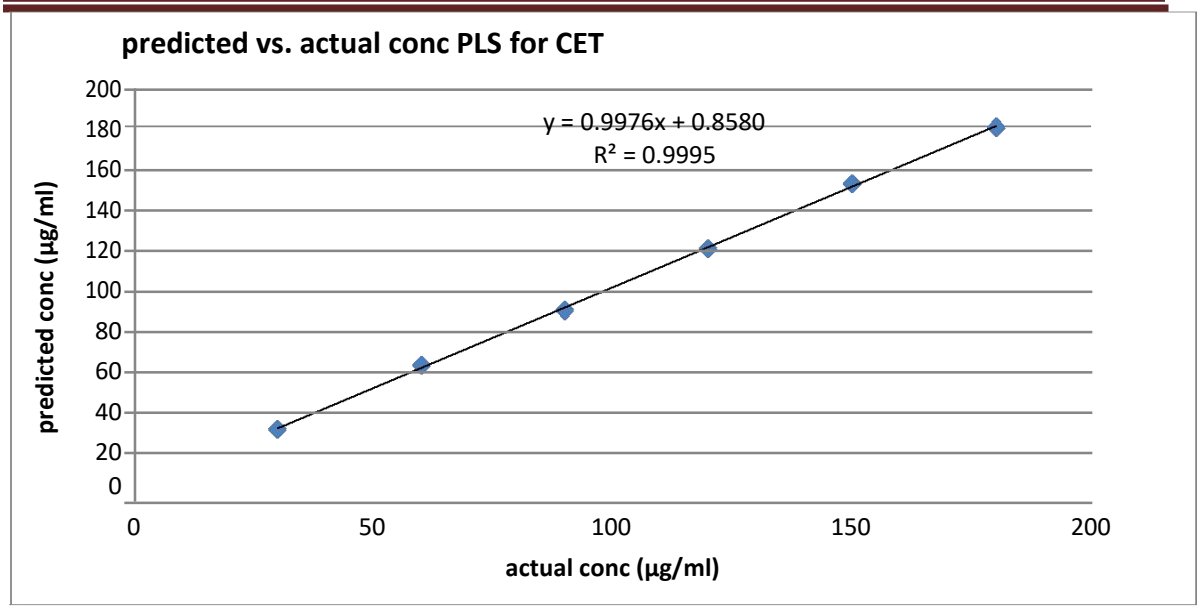


Figure 4.23 Predicted Vs. Actual conc. of CHD for PCR model

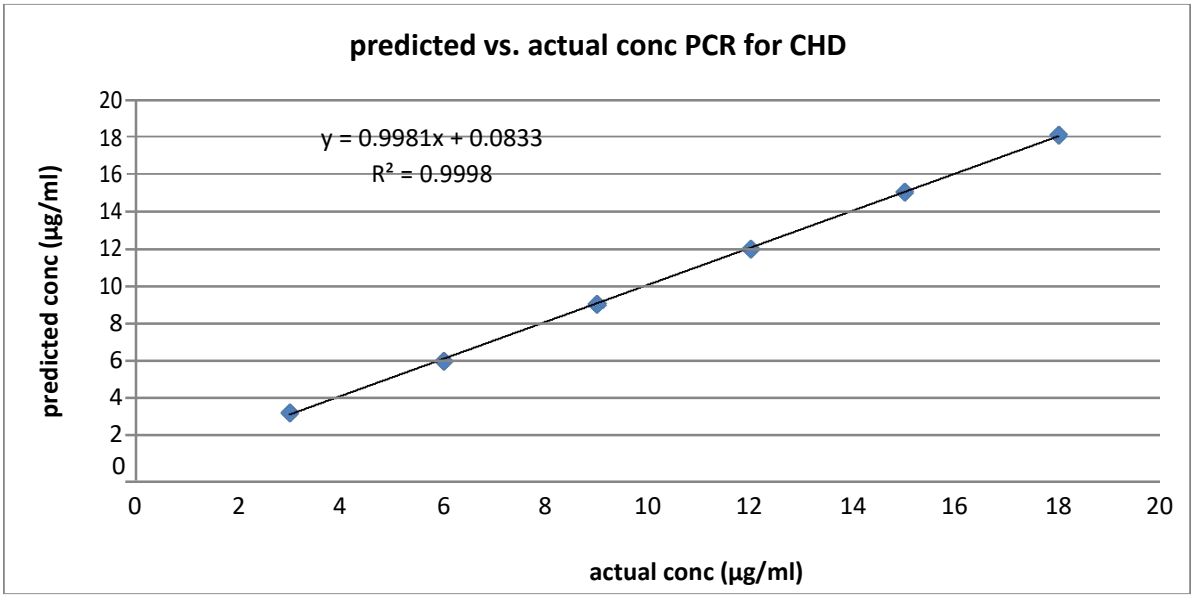
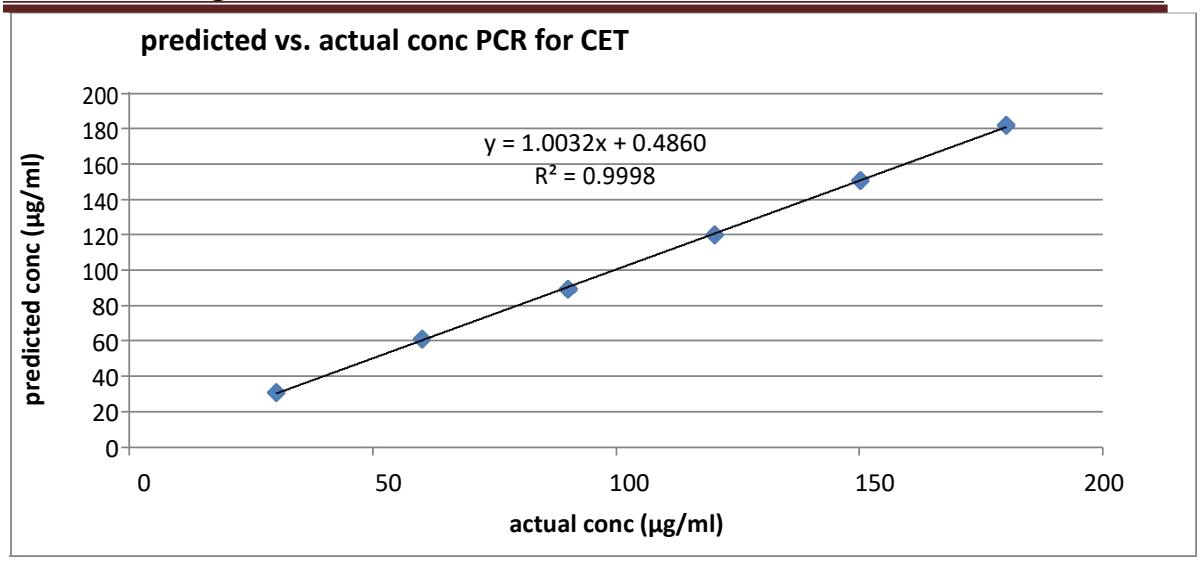


Figure 4.24 Predicted Vs. Actual conc. For CET for PCR model



4.4.3.2 Method validation using ICH Q2 (R1) guideline

The validation of developed UV methods was done as per ICH guidelines. The summary of validation parameters is represented in Table 4.6.

The linearity for determination of CHD and CET for all developed methods were evaluated by analysing a series of concentrations of compound. The calibration range was established with respect to the practical range necessary, according to the marketed formulation, to give accurate, precise and linear results which are 3-18 µg/ml for CHD and 30-180 µg/ml for CET.

The precision was performed at three concentration levels for compound in triplicate. The three levels were 3, 9, 18 µg/ml of CHD and 30, 90, 180 for CET. The experiment was repeated three times in a day for intra-day precision and on three different days for inter-day precision. (Table 4.7)

The accuracy was performed by standard addition method. Known amounts of CHD and CET were added to a known concentration of the commercial formulation at three levels of standard addition (80%, 100%, and 120%). As discussed in section 4.4.2.4, from the working stock solution of formulation, 1 ml each of 50 µg/ml was transferred to 9 different 10 ml volumetric flasks. To it 40 µg/ml (80%), 50 µg/ml (100%), 60 µg/ml (120%) of standard CHD and 400 µg/ml (80%), 500 µg/ml (100%), 600 µg/ml (120%) of standard CET was added in 3 different flasks in triplicate and diluted up to mark giving final concentrations 9, 10, 11 µg/ml of CHD and 90, 100, 110 µg/ml of CET respectively in triplicate. The resulting mixtures were analyzed and the percentage recovery was calculated. (Table 4.9) For determining the limit of detection (LOD) and limit of quantitation (LOQ), the approach based on the standard deviation (S.D.) of the y-intercept and the slope was used and the values were obtained.

Robustness study was performed by making small but deliberate changes like changing brand of acetonitrile for preparation of calibrators and variation in detection wavelength by ±1%. For ruggedness study change in UV instrument was done. UV 1800 was used for the study. From these results it is concluded that the methods has capacity to

withstand some extent of human or system errors and are thus robust and rugged methods. The stability of the working stock solutions was studied at room temperature and in refrigerator (8-25ºC).

The specificity of the method was assessed by analyzing the formulation, whether any interference of the excipients was there or not. (Table 4.10)

For system suitability study, six replicates of the solution 4 µg/ml CPH was used to evaluate the system suitability and their %RSD were found. The peak purity curves were also assessed.

Table 4.6: Summary of Validation parameters of UV-spectroscopy developed methods

Parameters	**Results**																	
Analytical Methods	Vieordt's method		First Derivative spectroscopy method		Multicomponent analysis method		Classical least squares		Inverse least square		Partial least squares		Principal component regression		Absorption ratio spectra method		Mean centering of ratio spectra method	
	CHD	CET	CHD	CET	CHD	CET	CHD	CET	CHD	CET	CHD	CET	CHD	CET	CHD	CET	CHD	CET
Analytical wavelength (nm)	260	217	275	222	260	217	220-260	220-260	220-260	220-260	220-260	220-260	220-260	220-260	217-200	263-225	260	217
Linearity range (µg/ml)	3-18	30-180	3-18	30-180	3-18	30-180	3-18	30-180	3-18	30-180	3-18	30-180	3-18	30-180	3-18	30-180	3-18	30-180
Correlation coefficient (R^2)	0.9998	0.99990	0.99991	0.99990	0.99998	0.99999	0.99996	0.99998	0.99994	0.9998	0.9999	0.9997	0.9999	0.9998	0.9998	0.9997	0.9999	0.9998
LOD(µg/ml	0.0	0.15	0.12	0.09	0.17	0.12	0.64	0.12	0.23	0.1	0.5	0.2	0.0	0.1	0.1	0.2	0.1	0.1

)	8									0	6	3	7	2	4	5	2	5
LOQ(µg/ml)	0.26	0.46	0.36	0.29	0.53	0.37	1.92	0.37	0.70	0.30	1.69	0.71	0.03	0.14	0.43	0.75	0.37	0.46

Table 4.7: Results of Intraday and Interday precision

Methods	**Precision**	**%RSD**
Vieordt's method	Intraday	0.25
	Interday	1.35
First Derivative spectroscopy method	Intraday	0.98
	Interday	0.46
Multicomponent analysis method	Intraday	1.56
	Interday	1.02
Absorption ratio spectra method	Intraday	0.15
	Interday	0.79
Mean centering of ratio spectra method	Intraday	0.65
	Interday	1.25
Classical least squares	Intraday	0.56
	Interday	0.65
Inverse least squares	Intraday	1.02
	Interday	0.84
Partial least squares	Intraday	0.46
	Interday	0.78
Principal component regression	Intraday	0.57
	Interday	0.35

Table 4.8: RMSEP and PRESS values for developed Chemometrics methods

Method	**Drug**	**RMSEP**	**PRESS**
Classical least squares	CHD	0.105	0.02205
	CET	0.114	0.025992
Inverse least square	CHD	0.099	0.019602
	CET	0.109	0.023762
Partial least squares	CHD	0.114	0.025992
	CET	0.104	0.021632
Principal component regression	CHD	0.120	0.0288
	CET	0.078	0.012168

Table 4.9: Accuracy (Recovery study) results of developed UV spectrophotometry methods

Method	Drug	%Spiking	%Recovery+SD
Vieordt'method	CHD	80	100.44±1.02
		100	99.39±0.08
		120	101.56±1.01
	CET	80	99.32±0.03
		100	100.10±0.97
		120	99.29±0.09
First Derivative spectroscopy method	CHD	80	101.43±1.03
		100	100.34±0.09
		120	99.49±0.67
	CET	80	101.45±0.09
		100	100.21±1.01
		120	100.89±1.01
Multicomponent analysis method	CHD	80	100.49±0.09
		100	101.11±0.790
		120	99.27±0.67
	CET	80	100.02±0.09
		100	99.23±0.09
		120	100.34±1.00
Absorption ratio spectra method	CHD	80	101.22±1.08
		100	100.34±1.09
		120	100.56±0.09
	CET	80	101.10±0.56
		100	100.88±0.46
		120	99.70±0.09
Mean centering of ratio spectra method	CHD	80	99.44±0.07
		100	101.12±0.37
		120	100.23±1.01
	CET	80	100.56±1.08
		100	100.39±0.09
		120	101.00±1.07
Classical least squares	CHD	80	100.75±0.45
		100	101.12±0.86
		120	100.78±0.26
	CET	80	99.76±1.02
		100	100.86±0.48
		120	101.82±0.89
Inverse least squares	CHD	80	100.46±0.59

		100	99.55±1.02
		120	100.36±1.00
	CET	80	101.22±0.46
		100	100.79±0.23
		120	100.23±0.21
Partial least squares	CHD	80	100.69±0.68
		100	101.04±0.47
		120	99.45±0.25
	CET	80	101.45±0.95
		100	100.63±0.12
		120	100.48±0.04
Principal component regression	CHD	80	99.12±0.04
		100	100.85±0.58
		120	101.02±0.04
	CET	80	100.65±0.01
		100	99.57±0.38
		120	99.36±0.16

4.4.3.3 Applicability of developed method for analysis of formulation

The applicability of all methods developed was checked on marketed formulation of the drug combination as described in section 4.4.2.4. The results of Assay are represented in Table 4.10. Data are obtained from six replicate injections.

Table 4.10: Assay results of UV spectrophotometry methods

Methods	**%Assay+SD**	**%Assay+SD**
	CHD	CET
Vieordt's method	100.35±0.04	100.12±0.25
First Derivative spectroscopy method	100.11±0.54	99.94±0.06
Multicomponent analysis method	99.86±1.04	99.86±1.08
Classical least squares	100.51±0.09	100.34±0.65
Inverse least square	101.08+0.34	100.51+0.09
Partial least squares	100.56±1.00	100.27±0.21
Principal component regression	100.89 +1.09	100.78 +0.58
Absorption ratio spectra method	99.72±0.78	99.72±1.01
Mean centering of ratio spectra method	100.67+0.03	100.67+0.45

4.4.3.4 Statistical analysis using One-way Anova test and further by Tukey HSD test and Scheffe test.

Statistical analysis was done by using One-way ANOVA test as explained previously. P value threshold is fixed to the value same as alpha (probability level) i.e. 0.05.Results of

% Assay obtained by all developed UV methods were subjected to One-way ANOVA. The analysis was done 6 times by each method (count = 6). Data analysis was done using Microsoft Excel 2007. From the statistical analysis it was found that p-value for CH was less that α value at 0.05 level of significance and also F calculated value for CHD is more than F critical value, which infers that we have to reject null hypothesis whereas for CET p-value is more than α value at 0.05 level of significance and also F calculated value for CET is less than F critical value which infers that we do not have to reject null hypothesis. Thus from the methods, one or more methods give significant different results for CHD whereas for CET , all methods give results with insignificant difference from each other. (Table 4.11 and Table 4.12) For further investigation for CHD to identify about which pairs of methods are giving significantly different results post hoc analysis of the data was done using Tukey's Honest significant difference test (Tukey HSD test) and Scheffe multiple comparison test for both drugs. Table 4.13 and table 4.14 represent Anova table for Tukey's and Scheffe's test. In Scheffe test no significant difference was obtained in any of the methods. (Table 4.15) So, for further clarification Tukey HSD test was applied in which it was found that there is significant difference in assay results of Inverse least square methods and Absorption ratio spectra method. (Table 4.16)

Table 4.11 One-way ANOVA table for CET

SUMMARY				
Groups	**Count**	**Sum**	**Average**	**Variance**
Vieordt's method	6	600.72	100.12	0.16552
First Derivative spectroscopy method	6	599.66	99.94333333	0.53910667
Multicomponent analysis method	6	599.19	99.865	0.44439
Classical least squares method	6	602.08	100.3466667	0.23662667
Inverse least square method	6	603.06	100.51	0.16824
Partial least squares method	6	601.66	100.2766667	0.02378667
Absorption ratio spectra method	6	598.34	99.72333333	0.07330667
Mean centering of ratio spectra method	6	604.06	100.6766667	0.24306667
Principal component regression	6	602.45	100.40	0.24857979

Source	**Sum of Squares ss**	**Degrees of Freedom ν**	**Mean square Ms**	**F statistic**	**P-value**
Treatment	6.5985	7	0.9426	3.4163	0.0059
Error	11.0369	40	0.2759		
Total	17.6353	47			

Table 4.12 One-way ANOVA table for CHD

SUMMARY				
Groups	**Count**	**Sum**	**Average**	**Variance**

Vieordt's method	6	602.12	100.35333	0.5145867
First Derivative spectroscopy method	6	600.66	100.11	1.12044
Multicomponent analysis method	6	599.19	99.865	0.44439
Classical least squares method	6	603.08	100.51333	0.4406267
Inverse least square method	6	606.48	101.08	0.48716
Partial least squares method	6	603.4	100.56667	0.3497467
Absorption ratio spectra method	6	598.34	99.723333	0.0733067
Mean centering of ratio spectra method	6	604.06	100.67667	0.2430667
Principal component regression	6	600.02	100.00	0.1559897

Source	Sum of Squares ss	Degrees of Freedom ν	Mean square Ms	F statistic	P-value
Treatment	2.9089	7	0.4156	1.8572	0.1028
Error	8.9502	40	0.2238		
Total	11.8591	47			

Table 4.13 ANOVA table for posthoc analysis by Tukey's HSD test and Scheffe's test for CET

Source	Sum of squares ss	Degrees of freedom vv	Mean square ms	F statistic	P-value
Treatment	0.3849	8	0.0481	0.6670	0.7104
Error	0.6492	9	0.0721		
Total	1.0340	17			

Table 4.14 ANOVA table for posthoc analysis by Tukey's HSD test and Scheffe's test for CHD

Source	Sum of squares ss	Degrees of freedom vv	Mean square ms	F statistic	P-value
Treatment	0.7432	8	0.0929	1.1127	0.4346
Error	0.7514	9	0.0835		
Total	1.4946	17			

Table 4.15 Q values for CH and CET by posthoc analysis by Scheffe's test

Sr.No	Method comparison	T value (CET)	Significance	Q value (CHD)	Significance
1	Vieordt's method Vs First Derivative spectroscopy method	0.6578	Insignificant	0.4211	Insignificant
2	Vieordt's method Vs Multicomponent analysis method	0.8036	Insignificant	0.8450	Insignificant
3	Vieordt's method Vs Classical least squares method	0.0931	Insignificant	0.5595	Insignificant
4	Vieordt's method Vs Inverse least square method	0.3972	Insignificant	0.5595	Insignificant
5	Vieordt's method Vs Partial least squares method	0.0372	Insignificant	0.2769	Insignificant
6	Vieordt's method Vs Absorption ratio spectra method	0.0372	Insignificant	1.0902	Insignificant
7	Vieordt's method Vs Mean centering of ratio spectra method	1.0674	Insignificant	0.3692	Insignificant
8	Vieordt's method Vs Principal component regression	0.7075	Insignificant	1.2574	Insignificant
9	First Derivative spectroscopy Vs Multicomponent analysis method	0.1458	Insignificant	0.4240	Insignificant
10	First Derivative spectroscopy Vs Classical least squares method	0.7509	Insignificant	0.9806	Insignificant
11	First Derivative spectroscopy Vs Inverse least square method	1.0550	Insignificant	0.9806	Insignificant
12	First Derivative spectroscopy Vs Partial least squares method Absorption ratio spectra method	0.6206	Insignificant	0.6979	Insignificant
13	First Derivative spectroscopy Vs Mean centering of ratio spectra method	0.6205	Insignificant	0.6691	Insignificant
14	First Derivative spectroscopy	0.4096	Insignificant	0.7902	Insignificant

	Vs				
15	First Derivative spectroscopy Vs Principal component regression	0.6206	Insignificant	1.4045	Insignificant
16	Multicomponent analysis method Vs Classical least squares method	1.3653	Insignificant	1.4045	Insignificant
17	Multicomponent analysis method Vs Inverse least square method	0.8967	Insignificant	1.1219	Insignificant
18	Multicomponent analysis method Vs Partial least squares method	1.2008	Insignificant	0.2451	Insignificant
19	Multicomponent analysis method Vs Absorption ratio spectra method	0.7664	Insignificant	1.2142	Insignificant
20	Multicomponent analysis method Vs Mean centering of ratio spectra method	0.7664	Insignificant	2.1025	Insignificant
21	Multicomponent analysis method Vs Principal component regression	0.2637	Insignificant	0.000	Insignificant
22	Classical least squares method Vs Inverse least square method	1.511	Insignificant	0.2826	Insignificant
23	Classical least squares method Partial least squares method	0.3041	Insignificant	1.6497	Insignificant
24	Classical least squares method Absorption ratio spectra method	0.1303	Insignificant	0.1903	Insignificant
25	Classical least squares method Mean centering of ratio spectra method	0.1303	Insignificant	0.6979	Insignificant
26	Classical least squares method Principal component regression	1.1605	Insignificant	1.3670	Insignificant
27	Inverse least square method Vs Absorption ratio spectra method	0.6144	Insignificant	0.0923	Insignificant
28	Inverse least square method Vs Partial least squares method	0.4344	Insignificant	0.9806	Insignificant
29	Inverse least square method Vs Absorption ratio spectra	0.4344	Insignificant	1.4593	Insignificant

	method				
30	Inverse least square method Mean centering of ratio spectra method	1.4646	Insignificant	2.3476	Insignificant
31	Partial least squares method Vs Principal component regression	0.3103	Insignificant	0.8883	Insignificant
32	Partial least squares method Vs Mean centering of ratio spectra method	0.000	Insignificant	0.7902	Insignificant
33	Partial least squares method Vs Absorption ratio spectra method	1.0302	Insignificant	1.6785	Insignificant
34	Absorption ratio spectra method Vs Mean centering of ratio spectra method	1.0531	Insignificant	1.4045	Insignificant
35	Absorption ratio spectra method Vs Mean centering of ratio spectra method	0.7447	Insignificant	1.4045	Insignificant
36	Mean centering of ratio spectra method Vs Principal component regression	1.7748	Insignificant	1.1219	Insignificant

Table 4.16 Q values for CHD and CET by posthoc analysis by Tukey's test representing significance

Sr.No	Method comparison	Q value (CET)	Significance	Q value (CHD)	Significance
1	Vieordt's method Vs First Derivative spectroscopy method	1.1365	Insignificant	0.5955	Insignificant
2	Vieordt's method Vs Multicomponent analysis method	0.1316	Insignificant	1.1950	Insignificant
3	Vieordt's method Vs Classical least squares method	0.5617	Insignificant	0.7913	Insignificant
4	Vieordt's method Vs Inverse least square method	0.0527	Insignificant	0.7913	Insignificant
5	Vieordt's method Vs Partial least squares method	0.0527	Insignificant	0.3915	Insignificant
6	Vieordt's method Vs Absorption ratio spectra method	1.5095	Insignificant	1.5417	Insignificant

7	Vieordt's method Vs Mean centering of ratio spectra method	1.0005	Insignificant	0.5221	Insignificant
8	Vieordt's method Vs Principal component regression	0.2062	Insignificant	1.7783	Insignificant
9	First Derivative spectroscopy Vs Multicomponent analysis method	1.0619	Insignificant	0.5996	Insignificant
10	First Derivative spectroscopy Vs Classical least squares method	1.4920	Insignificant	1.3867	Insignificant
11	First Derivative spectroscopy Vs Inverse least square method	0.8776	Insignificant	1.3867	Insignificant
12	First Derivative spectroscopy Vs Partial least squares method Absorption ratio spectra method	0.8776	Insignificant	0.9870	Insignificant
13	First Derivative spectroscopy Vs Mean centering of ratio spectra method	0.5792	Insignificant	0.9462	Insignificant
14	First Derivative spectroscopy Vs	1.9308	Insignificant	1.1176	Insignificant
15	First Derivative spectroscopy Vs Principal component regression	1.2682	Insignificant	2.3738	Insignificant
16	Multicomponent analysis method Vs Classical least squares method	1.6982	Insignificant	1.9863	Insignificant
17	Multicomponent analysis method Vs Inverse least square method	1.0839	Insignificant	1.9863	Insignificant
18	Multicomponent analysis method Vs Partial least squares method	1.0839	Insignificant	1.5866	Insignificant
19	Multicomponent analysis method Vs Absorption ratio spectra method	0.3730	Insignificant	0.3467	Insignificant
20	Multicomponent analysis method Vs Mean centering of ratio spectra method	2.1370	Insignificant	1.7171	Insignificant
21	Multicomponent analysis method Vs Principal component regression	0.4300	Insignificant	2.9733	Insignificant

22	Classical least squares method Vs Inverse least square method	0.1843	Insignificant	0.0000	Insignificant
23	Classical least squares method Partial least squares method	0.1843	Insignificant	0.3997	Insignificant
24	Classical least squares method Absorption ratio spectra method	1.6412	Insignificant	2.3330	Insignificant
25	Classical least squares method Mean centering of ratio spectra method	0.8688	Insignificant	0.2692	Insignificant
26	Classical least squares method Principal component regression	0.6143	Insignificant	0.9870	Insignificant
27	Inverse least square method Vs Absorption ratio spectra method	0.6143	Insignificant	0.3997	**Significant**
28	Inverse least square method Vs Partial least squares method	2.0712	Insignificant	2.3330	Insignificant
29	Inverse least square method Vs Absorption ratio spectra method	0.4388	Insignificant	0.2692	Insignificant
30	Inverse least square method Mean centering of ratio spectra method	0.0000	Insignificant	0.9870	Insignificant
31	Partial least squares method Vs Principal component regression	1.4569	Insignificant	1.9333	Insignificant
32	Partial least squares method Vs Mean centering of ratio spectra method	1.0531	Insignificant	0.1305	Insignificant
33	Partial least squares method Vs Absorption ratio spectra method	1.4569	Insignificant	1.3867	Insignificant
34	Absorption ratio spectra method Vs Mean centering of ratio spectra method	1.0531	Insignificant	2.0638	Insignificant
35	Absorption ratio spectra method Vs Mean centering of ratio spectra method	1.1365	Insignificant	3.3200	Insignificant
36	Mean centering of ratio spectra method Vs Principal component regression	0.1316	Insignificant	1.2562	Insignificant

4.5 SECTION –B

4.5.1 Experimental

Development and validation of RP-HPLC method for Chlorhexidine gluconate and Cetrimide drug combination

4.5.1.1 Chemicals and materials

Chlorhexidine gluconate and Cetrimide was kindly supplied as a gift sample by Mil Laboratories Pvt Ltd, Vadodara. Methanol and acetonitrile used were of HPLC grade and were purchased from Fisher Scientific Pvt. Ltd. Double distilled water was prepared at the laboratory premises. All other reagents and chemicals used were of analytical grade.

4.5.1.2 Equipments and analysis conditions

The liquid chromatographic system was of Waters, Ahmedabad and consisting of following components a gradient pump, PDA detector, a manual injection facility with 20 µl fixed loop. The chromatographic analysis was performed using Empower 3 software on a Hypersil BDS C18 column (250×4.6 mm, 5 µm particle size).

4.5.1.3 Preparation of mobile phase buffer

20 mM phosphate buffer was prepared by dissolving 0.272 g of potassium dihydrogen orthophosphate in sufficient water to produce 100 ml. The pH was adjusted to 3 using orthophosphoric acid. The buffer was filtered through 0.22 µ membrane filter, stored at ambient temperature.

4.5.1.4 Preparation of mobile phase: (phosphate buffer (pH 3): ACN: methanol (15:30:55) v/v/v):

The appropriate volumes of phosphate buffer, acetonitrile and methanol were transferred into a reagent bottle, mixed thoroughly, sonicated for 10 min and filtered through 0.22 µm membrane filter and used as mobile phase.

4.5.1.5 Preparation of Stock solutions (1000 µg/ml)

10mg each of CHD and CET were weighed accurately and transferred into separate 10 ml volumetric flasks containing 1 ml acetonitrile. DDW was added up to the mark to produce a stock solution containing 1000 µl/ml of CHD and CET respectively.

4.5.1.6 Preparation of working standards and calibration curve solutions

For preparation of working standard solution, 2.5 ml each of CHD and CET transferred into a 25 ml volumetric flask containing 2.5 ml acetonitrile. DDW was added up to the mark to produce a stock solution containing 100 µl/ml of CHD and CET respectively. Considering the ratio of CHD and CET in commercial formulation to be 1:10 appropriate aliquots of CHD and CET working standard solutions were taken in different 6 ml volumetric flasks each and diluted up to the mark with mobile phase to obtain final concentrations of 3-18 µl/ml and 30-180 µl/ml respectively.

4.5.1.7 Method Development

For development of liquid chromatographic method, various parameters were considered like

1) Selection of appropriate λmax for detection. For this parameter, whole UV range was scanned by PDA detector
2) Mobile phase selection was based on variation of one factor at a time method (on trial and error basis). The pH of buffer was selected based on pKa values of drugs.
3) For selection of diluents of final samples, various solvents were tried.
4) The flow rate was also selected based on one factor at a time method (on trial and error basis).

4.5.1.8 Applicability of the method

The developed HPLC method was applied for analysis of its formulation available in market. "Savlon antiseptic solution" manufactured by ITC was procured from local pharmacy. 0.5 ml of the sample formulation was withdrawn in a 50 ml volumetric flask and diluted up to the mark using acetonitrile: DDW in ratio of (1:9) to produce a clear solution. The resulting solution was again diluted by withdrawing 1.25 ml and making up to 25 ml with acetonitrile: DDW in ratio of 1:9 to give the final solution for analysis. The final solution was analyzed and chromatogram was recorded. Concentrations of both analytes were then calculated from the calibration graph. Six replicate samples were used for analysis.

4.5.1.9 Method Validation [39]

Method validation was done as per ICH guidelines encasing parameters like linearity, range, precision, accuracy, LOD, LOQ, ruggedness, specificity and selectivity.

Robustness

Since the mobile phase consisted of two organic solvents to be used for optimum resolution and symmetry of the two drugs of our study, probability of changes in SST parameters due to slight inadvertent variation in analytical factors existed, thus checking robustness of developed method was very crucial. As, conventional one factor variation at one time (OFAT) can predict the effect of one factor on the performance of the developed method but cumulative impact of the various factors on method performance can be studied by DOE. The experiment conducted on application of DOE is called a run and the corresponding response got for the factors considered in the run is called observation. The entire set of runs is the design. For study of robustness a model based on Box Behnken design of Response surface methodology was utilized which is excellent choice for fitting a quadratic model devoid of embedded factorial or fractional factorial design. In it the treatment combinations are at the middle of edges of the process space and at the centre. This design is rotatable or nearly rotatable and requires 3 levels of each factors selected.

System suitability parameters were checked for the developed method and we found them to be within the prescribed limits assuring the suitability and effectiveness of chromatographic system.

4.5.1.10 Statistical analysis [41, 42]

Reliability on quality of medicines is based on the results of analytical methods used for it's testing. Thus, normal distribution in the data for these methods is quite essential. For accomplishment of this task, statistical analysis can be undertaken. In order to verify whether a statistical procedure follows a normal distribution or not, 3 common types of Normality tests are performed namely:

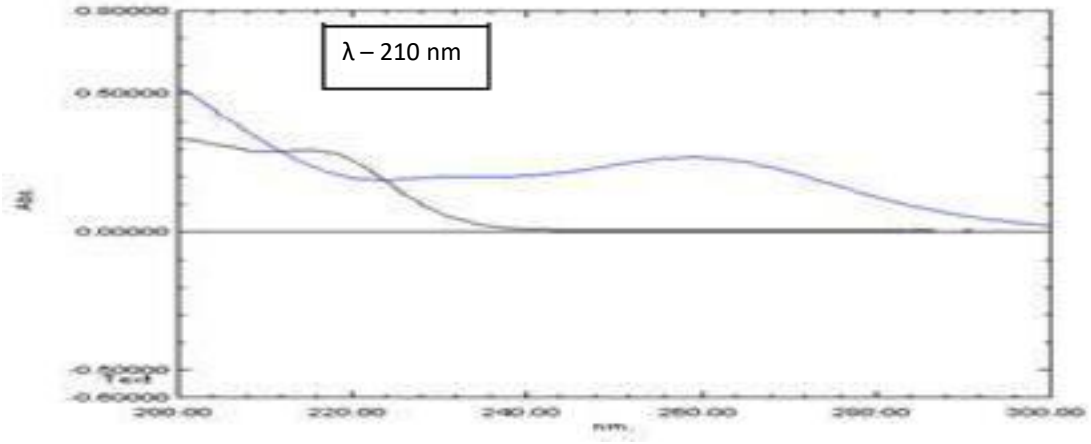
1) Anderson-Darling Test
2) Ryan-Joiner Test
3) Kolmogorov-Smirnov Test.

4.5.2 Results and discussion

4.5.2.1 Determination of suitable wavelength

The UV spectrum of CHD and CET is presented in figure 4.25. The spectrum indicates that detection wavelength 210 nm to be optimal for development of analytical method for combination of CHD and CET by RP-HPLC method.

Figure 4.25: Selection of wavelength maxima for combination dosage form =210nm



4.5.2.2 Method optimization and development

For optimization of chromatographic conditions for combined dosage form, various combinations of solvents, dilutions solvents, pH were tried as shown in Table 4.17. The final chromatographic conditions selected for analysis of sample were as represented in Table 4.18. Diluent for sample preparation was mobile phase. Figure 4.26 represents the overlay chromatogram of the combination of CHD and CET combination. Figure 4.27 represents the calibration curve for CHD and figure 4.28 represents calibration curve for CET. The system suitability parameters were within limits as prescribed by ICH guidelines for optimized HPLC method. (Table 4.19)

Table 4.17 Mobile phase optimization trials for RPHPLC method for CHD and CET combination

Mobile phase	**Ratio (%v/v/v)**	**Column =** BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	**Flow rate (ml/min)**	**Cetrimide and Chlorhexidine gluconate**	
				Rt (min)	Peak shape
0.1% FA: ACN	60:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	3.6,3.8	Broad peak
ammonium formate buffer 20 mm(pH=3.5, pH adjusted with formic acid:ACN	60:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm))	1.0	5.5,6.3	Merge peak
ammonium acetate buffer 10 mm(pH=4.6, pH adjusted withGAA:ACN	20:50:30	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	6.5	Broad peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN	60:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	5.9,6.5	Split peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	25:25:50	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm))	1.0	4.5,5	Merge peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	20:20:60	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	4.7,4.9	Merge peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	30:30:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	3.8,4.2	Split peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	30:15:55	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm))	1.0	4.5,5	Split peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	20:30:50	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	3.8,4.2	Asymmetric peak

		)			
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	40:10:50	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	4.7,4.9	Split peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	35:50:15	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm))	1.0	6.5	Broad peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	15:50:35	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	2.6,3.5	Symmetric peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	20:40:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	2.5,3.2	Asymmetric peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	15:60:25	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm))	1.0	6.5	Merge peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	15:30:55	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	2.7,3.1	Split peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	25:30:45	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	0.8	3.9,4.4	Symmetric peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	20:30:50	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm))	0.8	3.2,4.2	Symmetric peak
phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol	**15:30:55**	**BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)**	**0.8**	**3.2,3.8**	**Sharp and Symmetric peak**

Table 4.18 Optimized chromatographic parameters for RP-HPLC method of CHD and CET combination

Method parameter	Optimized value
Column	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)
Mobile phase	phosphate buffer 20 mm,pH=3, adjusted with OPA : ACN:methanol (15:30:55)
Retention time	3.2,3.8 min for CET and CHD
Detection wavelength	210 nm
Flow rate	0.8 ml/min
Temperature	Ambient

Figure 4.26: Optimized Peak and calibration curve for Cetrimide and Chlorhexidine gluconate

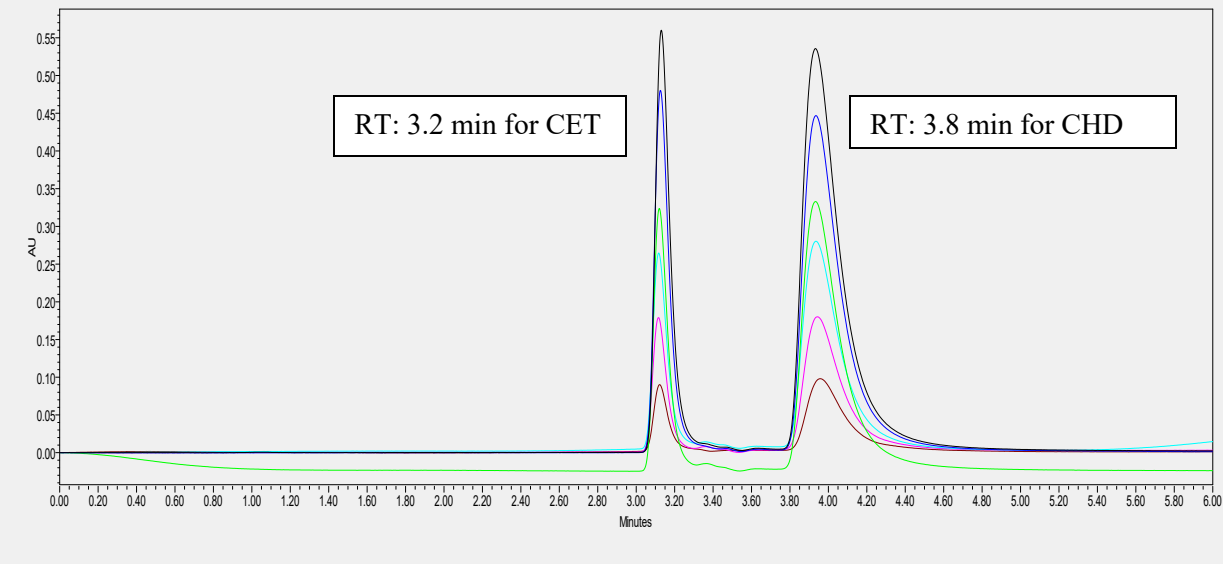


Figure 4.27: Calibration curve of Chlorhexidine gluconate

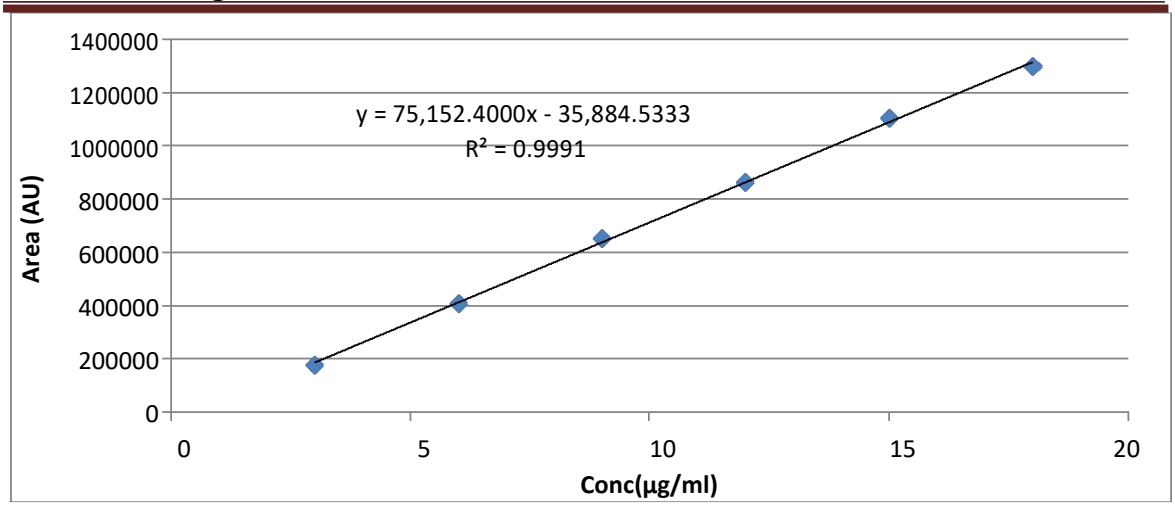


Figure 4.28: Calibration curve of Cetrimide

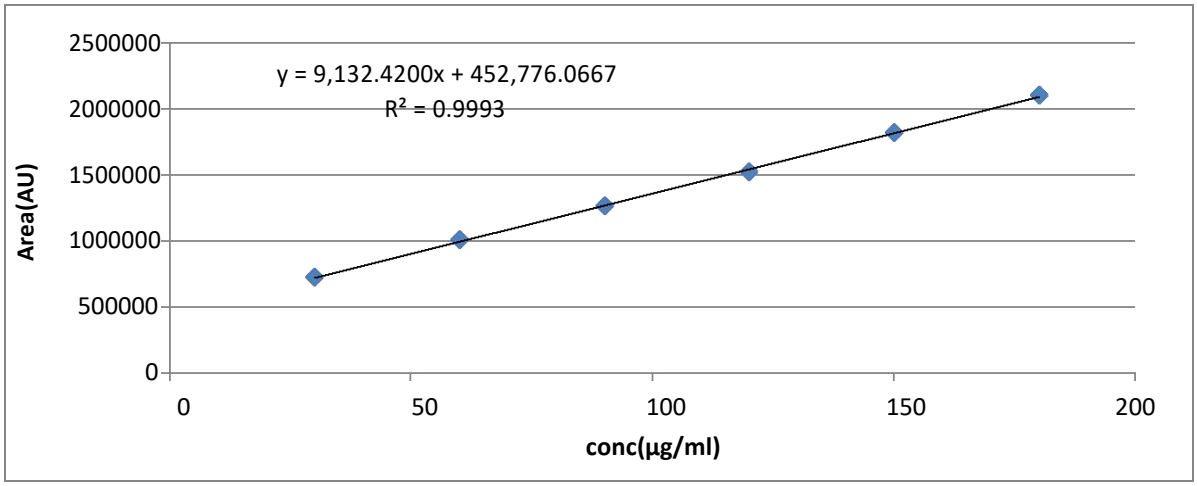


Table 4.19: System suitability test (SST) parameters (*Data obtained from 6 replicate Injections)

Parameter	Data obtained for Cetrimide	Parameter	Data obtained for Chlorhexidine gluconate
Retention time (min) ± SD	3.2 ± 0.04	Retention time (min) ± SD	3.8 ± 0.05
Theoretical plate ± SD	8100± 98.49	Theoretical plate ± SD	2103± 23.38
Tailing factor ± SD	1.23 ± 0.02	Tailing factor ± SD	1.19 ± 0.01
-	-	Resolution	3.2± 0.26

4.5.2.3 Method validation using ICH Q2 (R1) guideline and DOE

Linearity and range: The proposed RP-HPLC method showed good linearity in the concentration range of 3-18 µg/ml for CHD and 30-180 µg/ml for CET.

LOD and LOQ: Calibration curve was repeated for 9 times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were measured as follows. LOD=3.3 * SD/slope of calibration curve, LOQ=10 * SD/slope of calibration curve where SD = Standard deviation of intercepts

Precision: Inter-day and intra-day precision for the method were measured in terms of % RSD. The experiment was repeated 3 times in a day (Intraday precision) and the average % RSD values of the results were calculated. The precision was performed at three concentration levels for compound in triplicate. The three levels were 3, 9, 18 µg/ml of CHD and 30, 90, 180 for CET. Similarly the experiment was repeated for three different days for inter-day precision. (Table 4.20) The average % RSD values were calculated. The low value of SD obtained confirms the precision of the method.

The accuracy was performed by standard addition method. Known amounts of CHD and CET were added to a known concentration of the commercial formulation at three levels of standard addition (80%, 100%, and 120%). As discussed in section 4.4.2.4, from the working stock solution of formulation, 1 ml each of 50 µg/ml was transferred to 9 different 10 ml volumetric flasks. To it 40 µg/ml (80%), 50 µg/ml (100%), 60 µg/ml (120%) of standard CHD and 400 µg/ml (80%), 500 µg/ml (100%), 600 µg/ml (120%) of standard CET was added in 3 different flasks in triplicate and diluted up to mark giving final concentrations 9, 10, 11 µg/ml of CHD and 90, 100, 110 µg/ml of CET respectively in triplicate. The resulting mixtures were analyzed and the percentage recovery was calculated. (Table 4.20) For determining the limit of detection (LOD) and limit of quantitation (LOQ), the approach based on the standard deviation (S.D.) of the y-intercept and the slope was used and the values were obtained. Recovery greater than 98 % with low SD justified the accuracy of the method. The stability of the working stock solutions was studied at room temperature and in refrigerator (8-25ºC). The summary of validation parameters is as represented in Table 4.20.

Table 4.20 Summary of Validation parameters of RP-HPLC method

Parameter	**CHD**	**CET**
Analytical wavelength(nm)	210	210
Retention time (min)	3.8	3.2
Linearity range (µg/ml)	3-18	30-180
Regression equation	Y = 75,152.4000x – 35884.5333	Y=9132.4200x+452776.0667
Correlation coefficient	0.9991	0.9993
Intraday precision (%RSD)	0.04	0.89

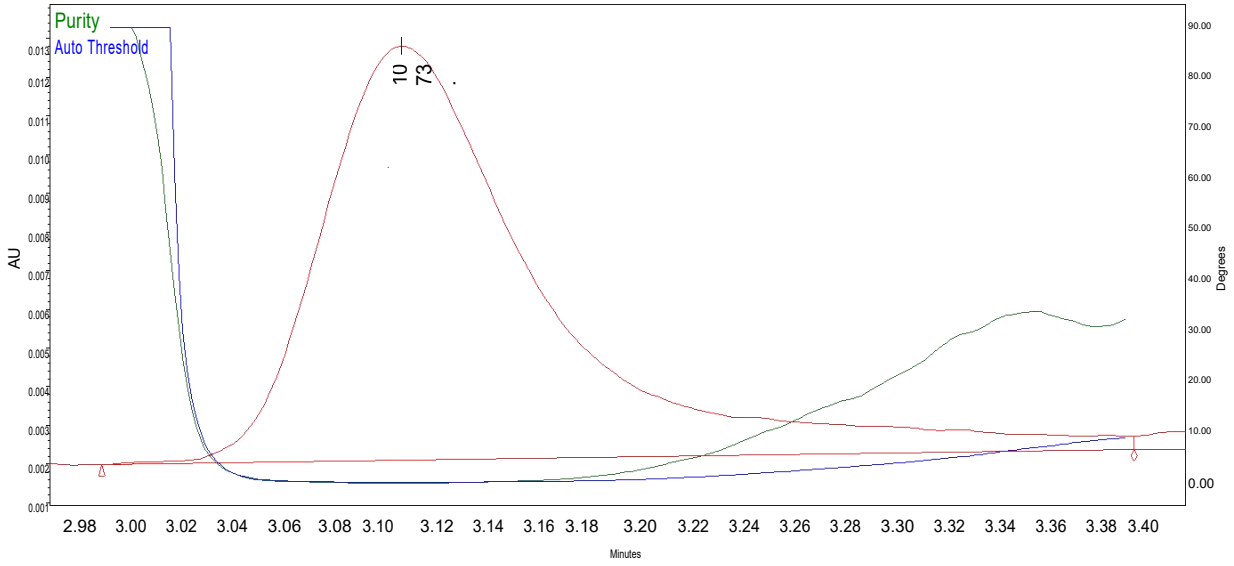
Inter day precision (%RSD)	1.06	0.57
LOD (µg/ml)	0.26	1.47
LOQ (µg/ml)	0.77	4.43
Accuracy (% Mean Recovery)	98-102	98- 102

4.5.2.4 Peak purity studies

Peak purity studies were undertaken for confirming the presence of only one analyte at the retention time of peaks obtained by HPLC-PDA method. The extracted peak purity plots of both standard drugs are presented in Figure 4.29 and further the values of purity threshold higher than the values of purity angles for each peak signify the peaks to be pure. (Table 4.21)

Figure 4.29 Peak purity studies

a) Peak purity plot of CET



b) Peak purity plot of CHD

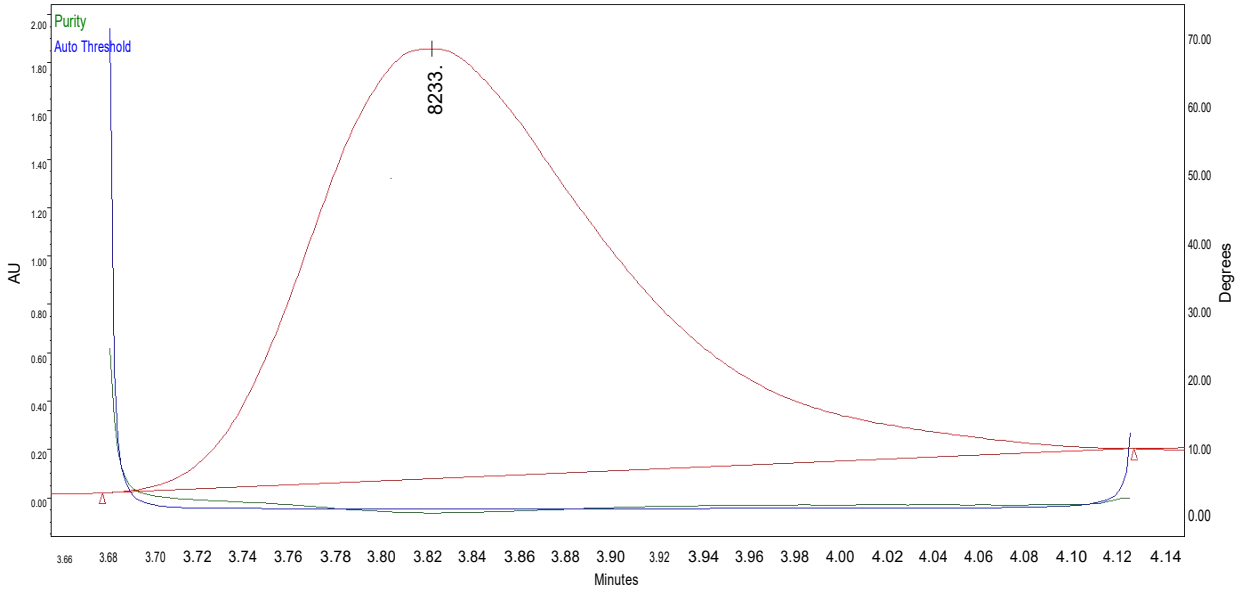


Table 4.21 Peak purity studies

Sr. No	Peak	Peak RT	Peak purity angle	Purity threshold
1	CET	3.1	0.403	0.821
2	CHD	3.8	0.559	0.682

4.5.2.5 Robustness of the method was determined by using DOE approach.

All runs as per the design domain represented in Table 4.23 were performed independently and the response observed for effect of each variation in factor changes was recorded. The model selected for the response retention time showed that for the retention time of CET, the independent factors flow rate and pH of mobile phase, detection wavelength for RP-HPLC method were not having any significant effect on it. Changing the factors as per the experimental domain did not have any significant impact on the response retention time of CET. All statistical parameters were found to be in range depicting the best fit of the model applied as shown in Table 4.23. Normal plots of residuals were plotted to evaluate the effect of factors on the responses. Normal plots revealed that the factors flow rate and pH of mobile phase as well detection wavelength for method did not have any significant effect on any of the selected responses. 3D contour plots showed that keeping pH of mobile phase constant, when variation of wavelength from 213 to 207 nm was done, decrease in retention time of CHD was observed from 3.862 to 3.800 min and when flow rate of mobile phase was decreased from 0.9 ml/min to 0.7 ml/min again decrease in retention time of CHD was observed from 3.824 to 3.800 min. However, the variation in retention time of CHD observed is not significant and within the USP/NF acceptable limit of ±2.5% for change in retention time of analyte. Thus, this way slight variation embarked on selected factors for RP-HPLC method did not have any impact on any other response signifying robustness of

developed method. ANOVA showed that the F statistic values for factors flow rate and pH of mobile phase as well as detection wavelength of method were higher and the associated p values for them were less than 0.0001 indicating that the model could explain 99.99% of variability. The factors considered for development of design were flow rate, λmax and pH of mobile phase whereas the responses considered for the study were retention time, theoretical plates and resolution between the peaks of CHD and CET as presented in Table 4.22. Figure 4.30, Figure 4.32, Figure 4.34, Figure 4.36 and Figure 4.38 represent 3D contour plots and figure 4.31, figure 4.33, figure 4.35, figure 4.37 and figure 4.39 represent normal plots for residual to check deliberate and small variation in pH, wavelength and flow rate on RT of analytes, theoretical plates of analytes as well resolution between the two analytes in HPLC chromatogram. The plots and the statistical analysis implicate the robustness of the method. The normal plots of residuals having linear relationship suggests that the residuals (error terms) are normally distributed. The alliance of 3D contour plots represents the effect of factors on the responses. Small and deliberate variations by all other factors do not have impact on developed method, signifying the method to be robust and able to tolerate small instrument as well as handling errors. Also the model summary statistics for the experimental design of BBD of RSM in Table 4.24 verifies robustness of method.

Table 4.22: Application of DOE for robustness of method: BBD of RSM [43-45]

Factors	**Responses**
Flow rate	RT of CET
Wavelength	RT of CHD
pH	TP of CET
	TP of CHD
	Resolution

Table 4.23: Experimental runs for the design

Std	**Run**	**Block**	**Factor 1 A: Flow rate ml/min**	**Factor 2 B: Wave length nm**	**Factor 3 C: pH unit**	**Response 1 Rt of CET min**	**Response 2 Rt of CHD min**	**Response 3 TP of CET**	**Response 4 TP of CHD**	**Resolution**
7	1	Block 1	0.7	210	3.2	3.21	3.9	2104	8102	3.25
9	2	Block 1	0.8	207	208	3.2	3.85	2202	8001	3.14
11	3	Block 1	0.8	207	3.2	3.2	3.8	2196	8110	3.09

6	4	Block 1	0.9	210	2.8	3.19	3.81	2099	8053	3.34
3	5	Block 1	0.7	213	3	3.2	3.86	2058	8201	3.01
8	6	Block 1	0.9	210	3.2	3.3	3.79	2150	8103	3.16
4	7	Block 1	0.9	213	3	3.1	3.8	2100	8194	3.12
5	8	Block 1	0.7	210	2.8	3.18	3.8	2083	8120	3.11
12	9	Block 1	0.8	213	3.2	3.2	3.82	2209	8102	3.06
10	10	Block 1	0.8	213	2.8	3.19	3.81	2100	8013	3.30
2	11	Block 1	0.9	207	3	3.21	3.82	2085	8038	3.28
1	12	Block 1	0.7	207	3	3.2	3.8	2108	8024	3.24

Figure 4.30 3D Contour plot representing the effect of pH, Flow rate and Wavelength variation on RT of CET

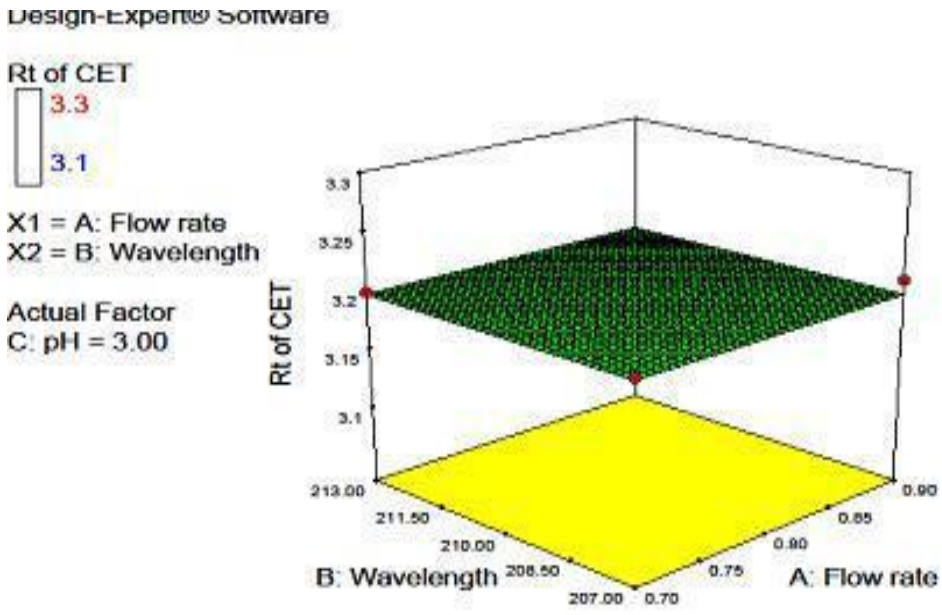


Figure 4.31 Normal plot of residuals representing the effect of pH, Flow rate and Wavelength variation on RT of CET

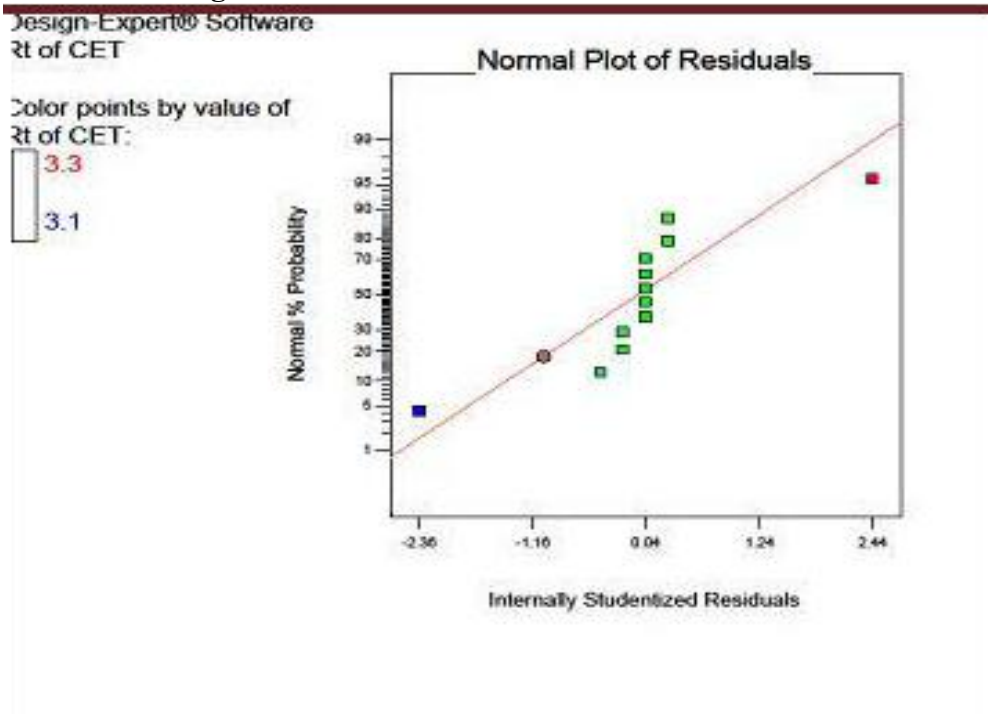


Figure 4.32 3D Contour plot representing the effect of pH, Flow rate and Wavelength variation on RT of CHD

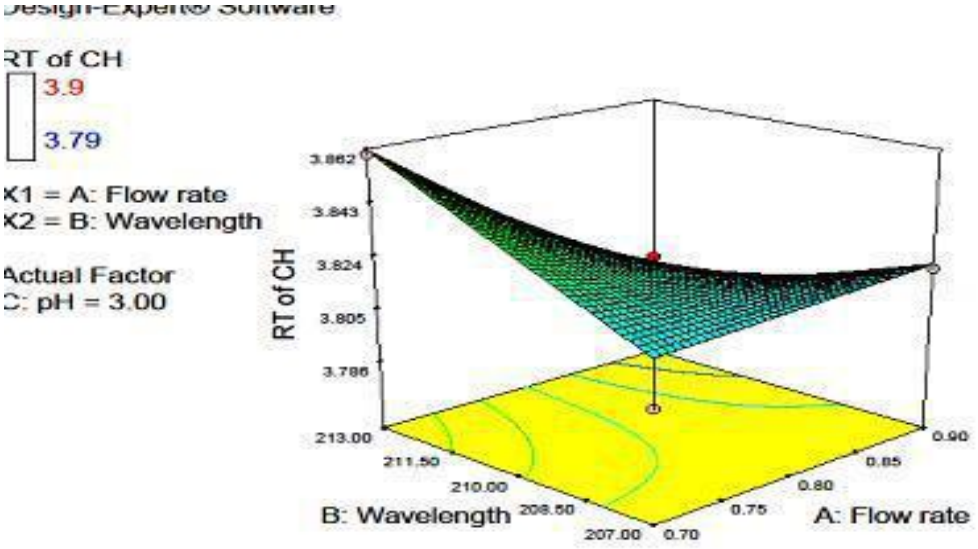


Figure 4.33 Normal plot of residuals representing the effect of pH, Flow rate and Wavelength variation on RT of CHD

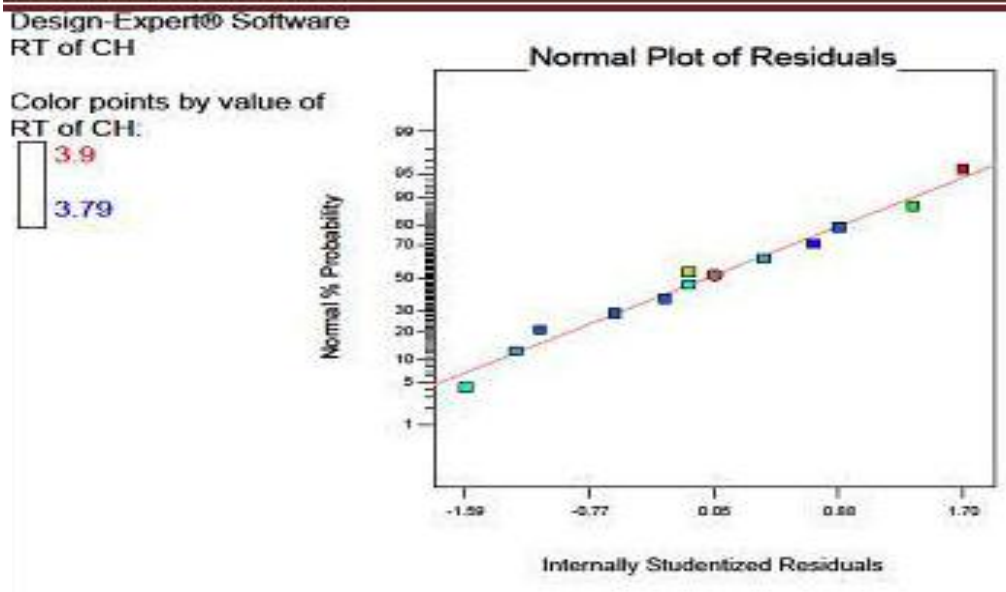


Figure 4.34 3D Contour plot representing the effect of pH, Flow rate and Wavelength variation on TP of CET

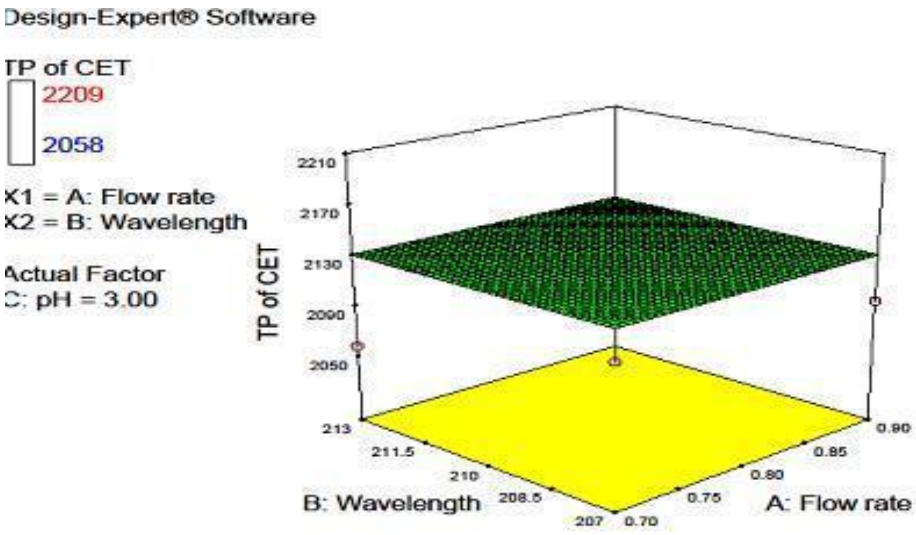


Figure 4.35 Normal plot of residuals representing the effect of pH, Flow rate and Wavelength variation on TP of CET

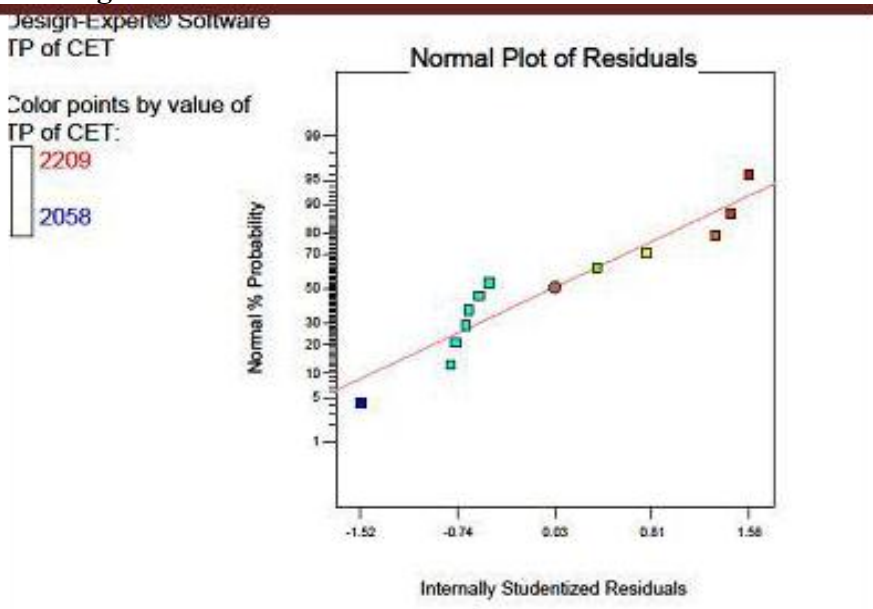


Figure 4.36 3D Contour plot representing the effect of pH, Flow rate and Wavelength variation on TP of CHD

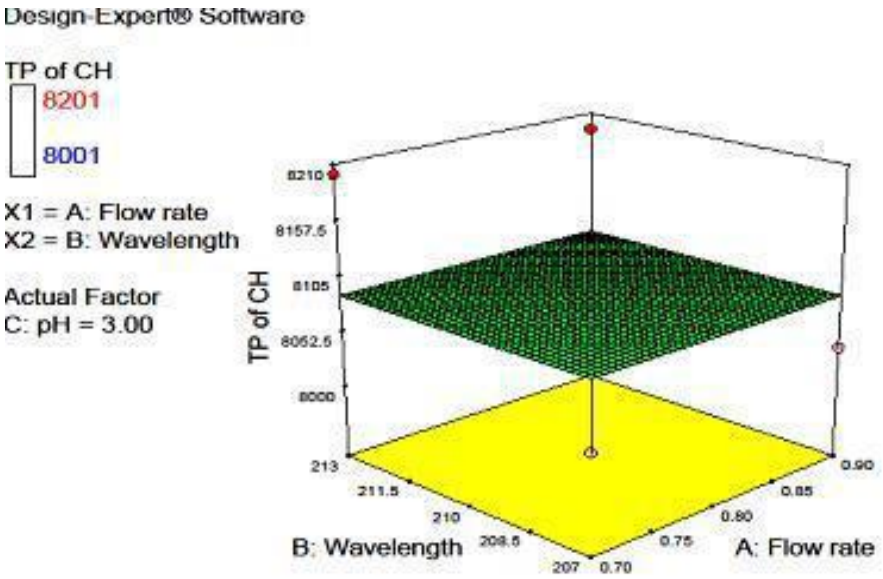


Figure 4.37 Normal plot of residuals representing the effect of pH, Flow rate and Wavelength variation on TP of CHD

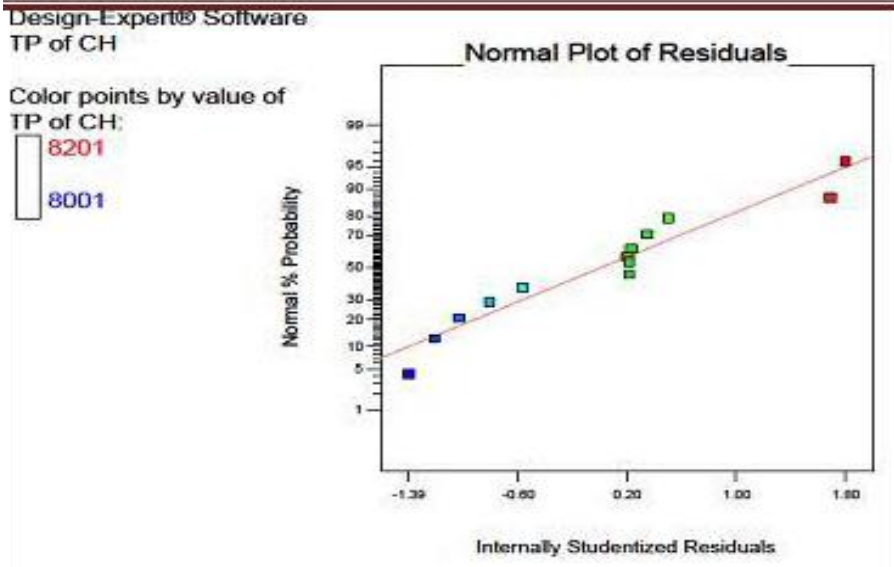


Figure 4.38 3D Contour plot representing the effect of pH, Flow rate and Wavelength variation on Resolution

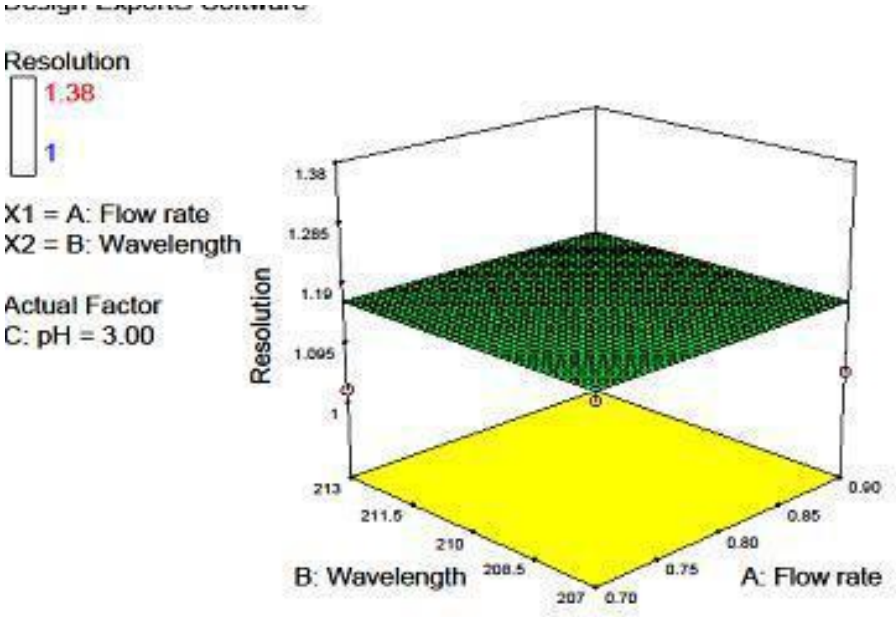


Figure 4.39 Normal plot of residuals representing the effect of pH, Flow rate and Wavelength variation on Resolution

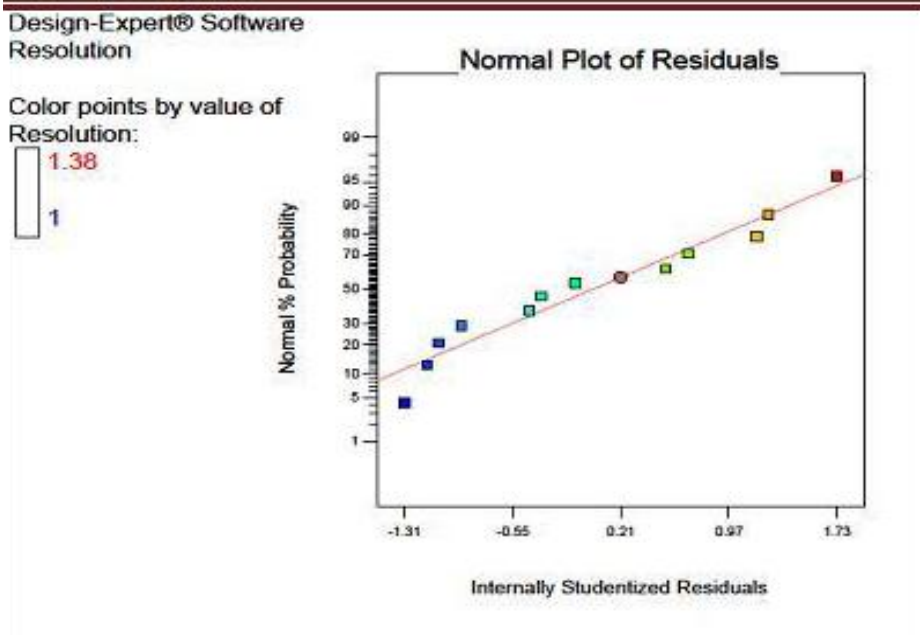


Table 4.24: Model Summary statistics for BBD of RSM

Statistical parameters	R1 = RT of CET	R2= RT of CHD	R3=TP of CET	R4= TP of CHD	R5= Resolution
SD	0.034	0.071	50.86	65.47	0.12
Mean	3.2	3.83	2131.92	8088.42	1.18
CV%	1.06	1.86	2.39	0.81	9.79
R-Squared	0.0000	0.0000	0.0000	0.000	0.0000
Adjusted R^2	0.0000	0.0000	0.0000	0.0000	0.0000
Predicted R^2	-0.1901	-0.1901	-0.1901	-0.1901	-0.1901
F value	1.15	5.06	2586.99	4285.72	0.013
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
PRESS	0.015	0.066	33866	5603.97	0.18

4.5.2.6 Applicability of developed method for analysis of formulation

Applicability of method was checked by analysis of marketed formulation of CHD and CET combination which is Savlon solution comprising of 3% W/V of CHD and 30% W/V of CET in 100ml solution, manufactured by ITC. The sample preparation was done as described in section 4.5.1.8. Table 4.25 consists of the details for the formulation analysis.

Table 4.25: Applicability of method:

%Label claim ± SD for CHD	%Label claim ± SD for CET
100.11 ±0.68	100.36 ±0.83

4.5.2.7 Statistical analysis using Anderson darling normality test

Statistical analysis was undertaken using Anderson darling normality test as this test is especially effective in detecting departure from normality in low and high values of distribution. It is used in our study to verify the normality of data distribution. We have taken area of HPLC peaks for checking normal distribution of data. The test is conducted using online calculator for Anderson darling test using the following formula.

$$AD = -N - \frac{2i-1}{N}\left(\ln\left(F\left(Y_i\right)\right) + \ln\left(1 - F\left(Y_{N+1-i}\right)\right)\right)$$

$$AD^* = AD\left(1 + \left(\frac{0.75}{N} + \frac{2.25}{N^2}\right)\right)$$

For checking the normal distribution of data for CHD, Anderson darling normality plot was prepared for CHD as shown in Figure 4.40. Data summary in Table 4.26 and Test statistics in Table 4.27 infer p- value greater that alpha value of 0.05 signifying normal distribution of data

For CHD. Similarly, for checking the normal distribution of data for CET, Anderson darling normality plot was prepared for CET as shown in Figure 4.41. Data summary in Table 4.28 and Test statistics in Table 4.29 infer p- value greater that alpha value of 0.05 signifying normal distribution of data for CET.

Figure 4.40: Anderson darling normality plot for CHD

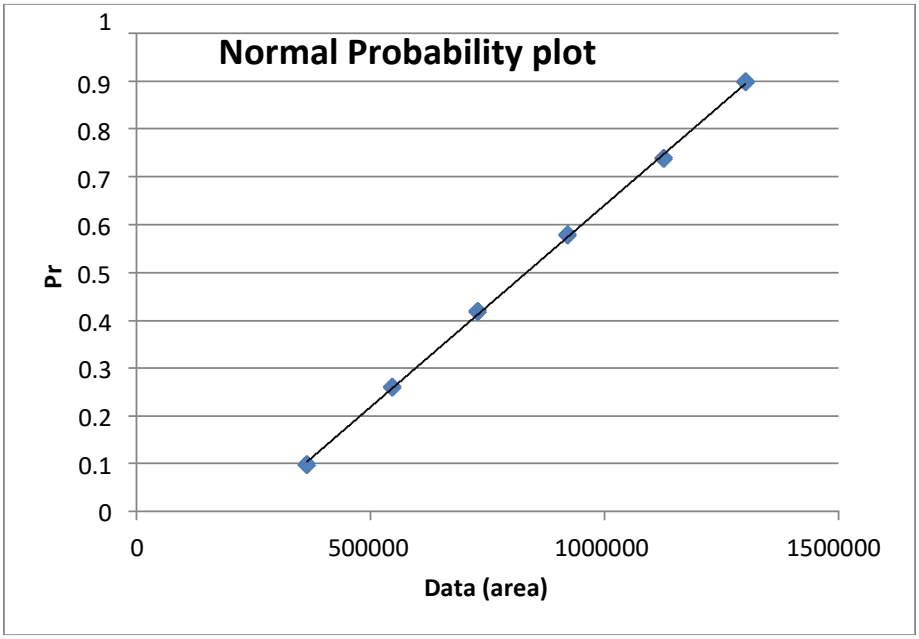


Table 4.26: Anderson Darling normality test for checking normal distribution of data for Chlorhexidine gluconate.

Data	Sorted	Count	F1i	1-F1i	F2i	Si	N Plt Line	NPlt Line
363678	363678	1	0.09323358	0.9067666	0.0922127	-4.7563052	377662.5	0.1
545997	545997	2	0.2102753	0.7897247	0.202243	-9.4728691	603319.8	0.26
728291	728291	3	0.3859460	0.6140539	0.399137	-9.3525411	759408.7	0.42
921163	921163	4	0.6008629	0.3991370	0.614054	-6.9794258	902180	0.58
1125552	1125552	5	0.7977570	0.2022429	0.7897247	-4.158199	1058269	0.74
1300085	1300085	6	0.9077873	0.0922126	0.9067664	-2.1407708	1283926	0.9

Table 4.27: Test statistics for CHD

0.1434	AD test statistic
0.17023	AD* test statistic
0.932945	P-value

Figure 4.41: Anderson darling normality plot for CET

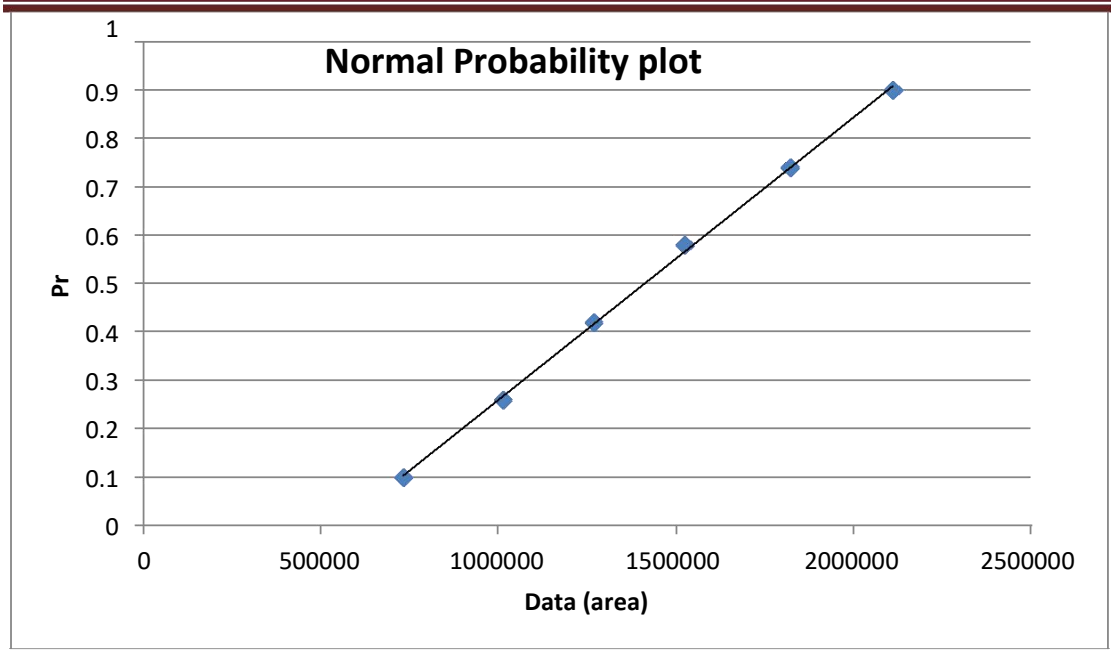


Table 4.28: Anderson Darling normality test for checking normal distribution of data for Cetrimide.

Data	Sorted	Count	F1i	1-F1i	F2i	Si	N Plt Line	N Plt Line
730687	730687	1	0.09206744	0.90793256	0.0860661	-4.8378735	754570.3	0.1
1011844	1011844	2	0.21775591	0.78224409	0.2121202	-9.2249475	1081808	0.26
1269729	1269729	3	0.39094947	0.60905053	0.4128188	-9.1196179	1308160	0.42
1524638	1524638	4	0.58718123	0.41281877	0.6090505	-7.1979306	1515200	0.58
1821408	1821408	5	0.78787976	0.21212024	0.7822441	-4.3559842	1741553	0.74
2111775	2111775	6	0.91393389	0.08606611	0.9079326	-2.0524044	2068790	0.9

Table 4.29: Test statistics for CET

0.1315	AD test statistic
0.156108	AD* test statistic
0.954926	P-value

4.6 SECTION – C

Marketed formulations in form of lotions and creams are available in market having only Cetrimide as active constituent. Examples include brands like merarine, dermacare etc. Till date no HPLC method for analysis of Cetrimide in bulk as well dosage form is available in literature best to our knowledge. Thereby, we decided to develop a simple HPLC method for analysis of Cetrimide.

4.6.1 Experimental

Development and validation of RP-HPLC method for Cetrimide

4.6.1.1 Chemicals and materials

Cetrimide was kindly supplied as a gift sample by Mil Laboratories Pvt Ltd, Vadodara. methanol and acetonitrile used were of HPLC grade and were purchased from Fisher Scientific Pvt. Ltd. Double distilled water was prepared at the laboratory premises. All other reagents and chemicals used were of analytical grade.

4.6.1.2 Equipments and analysis conditions

The liquid chromatographic system was of Waters, Ahmedabad and consisting of following components a gradient pump, PDA detector, a manual injection facility with 20 µl fixed loop. The chromatographic analysis was performed using Empower 3 software on a Hypersil BDS C_{18} column (250×4.6 mm, 5 µm particle size).

4.6.1.3 Preparation of mobile phase buffer

20 mM ammonium formate buffer was prepared by dissolving 0.12 g of ammonium formate in sufficient water to produce 100 ml. The pH was adjusted to 3 using formic acid. The buffer was filtered through 0.22 µ membrane filter, stored at ambient temperature.

4.6.1.4 Preparation of mobile phase: Ammonium formate buffer 20 mm (pH=3, pH adjusted with formic acid): ACN: Methanol (20:30:50, v/v/v)

The appropriate volumes of ammonium formate buffer, acetonitrile and methanol were transferred into a reagent bottle, mixed thoroughly, sonicated for 10 min and filtered through 0.22 µm membrane filter and used as mobile phase.

4.6.1.5 Preparation of stock solution (1000 µg/ml):

10mg CET was weighed accurately and transferred into a 10 ml volumetric flask containing 1 ml acetonitrile. DDW was added up to the mark to produce a stock solution containing 1000 µl/ml of CET.

4.6.1.6 Preparation of working standards and calibration curve solutions

For preparation of working standard solution, 2.5 ml of CET transferred into a 25 ml volumetric flask containing 2.5 ml acetonitrile. DDW was added up to the mark to produce a stock solution containing 100 µl/ml of CET. Appropriate aliquots of CET working standard solutions were taken in different 6 ml volumetric flasks each and diluted up to the mark with mobile phase to obtain final concentrations of 30-180 µl/ml respectively.

4.6.1.7 Method Development

For development of liquid chromatographic method, various parameters were considered like

1) Selection of appropriate λmax for detection. For this parameter, whole UV range was scanned by PDA detector
2) Mobile phase selection was based on one factor at a time method (on trial and error basis). The pH of buffer was selected based on pKa values of drugs.
3) For selection of diluents of final samples, various solvents were tried.
4) The flow rate was also selected based on one factor at a time method (on trial and error basis).

4.6.1.8 Applicability of the method

The developed HPLC method was applied for analysis of its formulation available in market. "Cetrilak solution" manufactured by Menarini India Pvt. ltd was procured from local pharmacy. 0.5 ml of the sample formulation was withdrawn in a 50 ml volumetric flask and diluted up to the mark using acetonitrile and DDW to produce a clear solution. The resulting solution was again diluted by withdrawing 1 ml and making up to 10 ml to give the final solution for analysis. The final solution was analyzed and chromatogram was recorded. Concentrations of both analytes were then calculated from the calibration graph. Six replicate samples were used for analysis.

4.6.1.9 Method Validation [39]

Method validation was done as per ICH guidelines encasing parameters like linearity, range, precision, accuracy, LOD, LOQ, robustness, ruggedness, specificity and selectivity.

System suitability parameters were checked for the developed method and we found them to be within the prescribed limits assuring the suitability and effectiveness of chromatographic system.

4.6.1.10 Statistical analysis [41, 42]

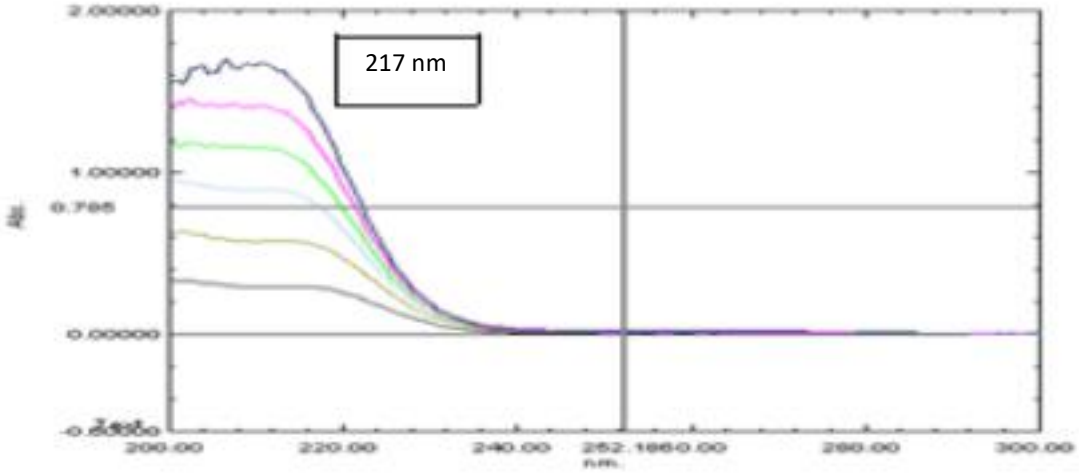
In order to verify whether a statistical procedure follows a normal distribution or not, 3 common types of Normality tests are performed namely 1) Anderson-Darling Test 2) Ryan-Joiner Test 3) Kolmogorov-Smirnov Test. As Anderson-Darling Test is especially effective in detecting departure from normality in low and high values of distribution it is used in our study to verify the normality of data distribution.

4.6.2 Results and discussion

4.6.2.1 Determination of suitable wavelength

The UV spectrum of CET is presented in figure 4.42. The spectrum indicates that detection wavelength 217 nm to be optimal for development of analytical method for CET by RP-HPLC method.

Figure 4.42: Selection of wavelength maxima for combination dosage form =217nm



4.6.2.2 Method optimization and development

For optimization of chromatographic conditions for CET, various combinations of solvents, dilutions solvents, pH were tried as shown in Table 4.30. For analysis of CET by HPLC, method described in section-B can be applied but we were attempting to develop LCMS compatible method, so that it can aid as a helpful method for future scope of study for CET. The final chromatographic conditions selected for analysis of sample are as represented in Table 4.31. Diluent for sample preparation was mobile phase. Figure 4.43 represents the overlay chromatogram of CET. Figure 4.44 represents the calibration curve for CET. The system suitability parameters were within limits as prescribed by ICH guidelines for optimized HPLC method. (Table 4.32)

Table 4.30 Mobile phase optimization trials for RP-HPLC method for CET

Mobile phase	**Ratio (%v/v/v)**	**Column**	**Flow rate (ml/min)**	**Cetrimide**	
				Rt (min)	Peak shape
methanol: water	50:50	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	3.5	Distorted peak shape
methanol: water	60:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	3.6	Distorted peak shape
methanol: water	40:60	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	3.2	Broad peak
ACN: water	50:50	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	2.8	Distorted peak shape
ACN: water	60:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	3.1	Fronting was observed
ACN: water	40:60	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	2.7	Broad peak
0.1% Formic acid: ACN	70:30	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	2.8	Distorted peak
0.1% Formic acid: ACN	60:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	2.3	Distorted peak
0.1% Formic acid: ACN	50:50	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	2.1	Broad peak
ammonium formate buffer 20 mm(pH=3, pH adjusted with formic acid:ACN: methanol	30:30:40	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	2.8	Broad peak
ammonium formate buffer 20 mm(pH=3, pH adjusted with formic acid:ACN:methanol	25:25:50	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)	1.0	3.1	Symmetric peak but not sharp
ammonium formate buffer 20 mm(pH=3, pH adjusted with formic acid:ACN:methanol	**20:30:50**	**BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)**	**1.0**	**3.1**	**Symmetric and sharp peak**

Table 4.31 Optimized chromatographic parameters for RP-HPLC method of CET

Method parameter	Optimized value
Column	BDS Hypersil C-18 (250 mm x 4.6 mm, 5 µm)
Mobile phase	Ammonium formate buffer 20 mM(pH=3, pH adjusted with formic acid): ACN: methanol(20:30:50)
Retention time	3.1 min
Detection wavelength	217 nm
Flow rate	1 ml/min
Temperature	Ambient

Figure 4.43: Optimized Peak and calibration curve for Cetrimide

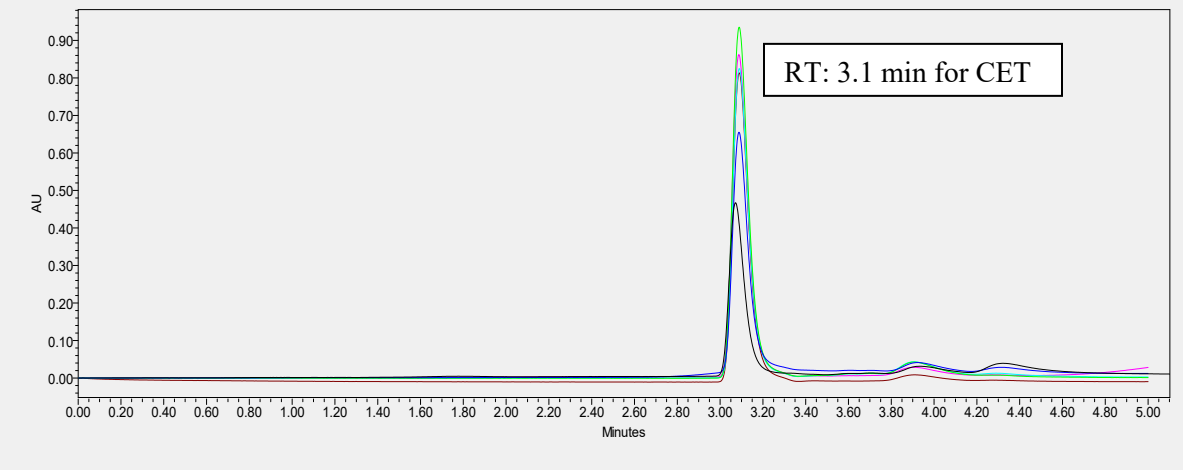


Figure 4.44: Calibration curve for Cetrimide

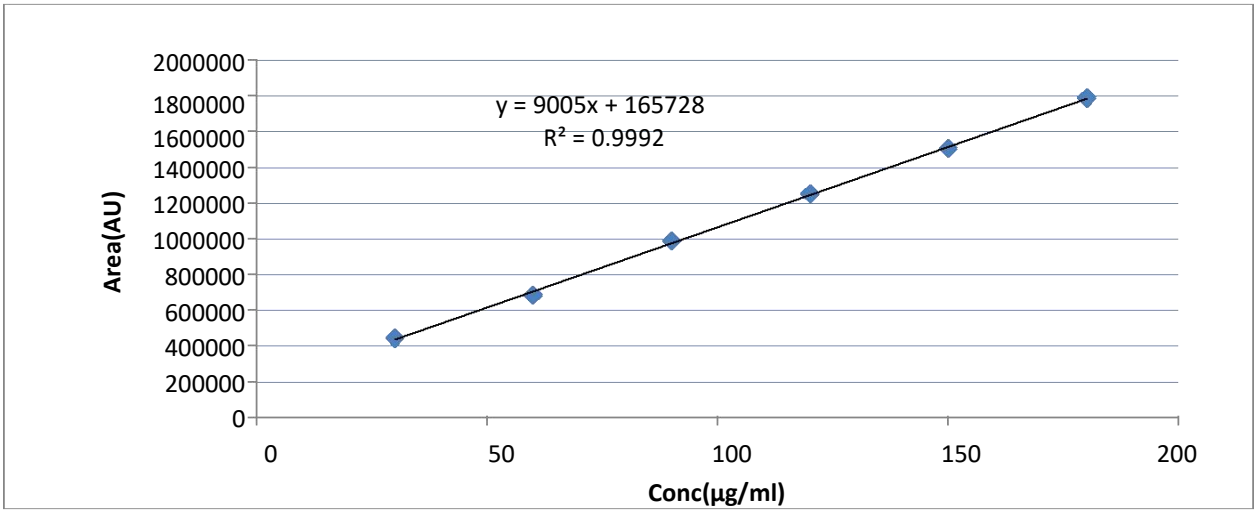


Table 4.32: System suitability test (SST) parameters

Parameters	Data obtained* for Cetrimide
Retention time (min) ± SD	3.1 ± 0.06
Theoretical plate ± SD	7539± 264.58
Tailing factor ± SD	1.12 ± 0.02

*Data obtained from 6 replicate Injections

4.6.2.3 Method validation using ICH Q2 (R1) guideline

Linearity and range: The proposed RP-HPLC method showed good linearity in the concentration range of 30-180 µg/ml for CET.
Precision: Inter-day and intra-day precision for the method were measured in terms of % RSD. The experiment was repeated 3 times in a day (Intraday precision) and the average % RSD values of the results were calculated. Similarly the experiment was repeated on 3 different days (Inter day precision) and the average % RSD value for absorbance of CET was calculated. The low value of SD obtained confirms the precision of the method.
LOD and LOQ: Calibration curve was repeated for 9 times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were measured as follows. LOD=3.3 * SD/slope of calibration curve, LOQ=10 * SD/slope of calibration curve where SD = Standard deviation of intercepts
Accuracy: Accuracy of the method was confirmed by recovery study from marketed formulation at 3 level of standard addition (80%, 100%, and 120%) of label claim. Recovery greater than 98 % with low SD justified the accuracy of the method.
The stability of the working stock solutions was studied at room temperature and in refrigerator (8-25ºC).
Robustness study was performed by making small but deliberate changes in flow rate by ±0.1 (0.9 mL/min, 1.0 mL/min, 1.1 mL/min) and change in the composition of Organic solution (acetonitrile) by ±2% (48%, 50% and 52%) and variation in detection wavelength) by ±2% (215, 217, 219 nm) as represented in Table 4.34. From these results it is concluded that the method has capacity to withstand some extent of human or system errors
The summary of validation parameters is as represented in Table 4.33.

Table 4.33 Summary of Validation parameters of RP-HPLC method

Parameter	Cetrimide
Analytical wavelength(nm)	217 nm
Retention time (min)	3.1min
Linearity range (µg/ml)	30-180
Regression equation	Y=9005x+16572
Correlation coefficient	0.9994
Intraday precision (%RSD)	0.14
Inter day precision (%RSD)	1.08

LOD (µg/ml)	0.36
LOQ (µg/ml)	1.08
Accuracy (% Mean Recovery)	98- 102 %

Table 4.34 Robustness study for developed HPLC method

Factor	Retention time (min) CET	PEAK AREA (AU)
A. Flow rate (mL/min)		
0.9	3.13	443422
1	3.10	443420
1.1	3.00	443421
Mean ±SD	3.07±0.06	443421±548
B. Ratio of ACN:		
28 %	3.14	443421
30%	3.11	443418
32%	3.17	443420
Mean ±SD	3.14±0.03	443420±453
C. Wavelength		
215 nm	3.16	443425
217 nm	3.11	443425
219 nm	3.18	443423
Mean ±SD	3.15±0.04	443424.33±615

4.6.2.4 Peak purity studies

Peak purity studies were undertaken for confirming the presence of only one analyte at the retention time of peaks obtained by HPLC-PDA method. The extracted peak purity plots of standard drug is presented in Figure 4.45 and further the values of purity threshold higher than the values of purity angles for each peak signify the peaks to be pure. (Table 4.35)

Figure 4.45 Peak purity studies

a) Peak purity plot of CET

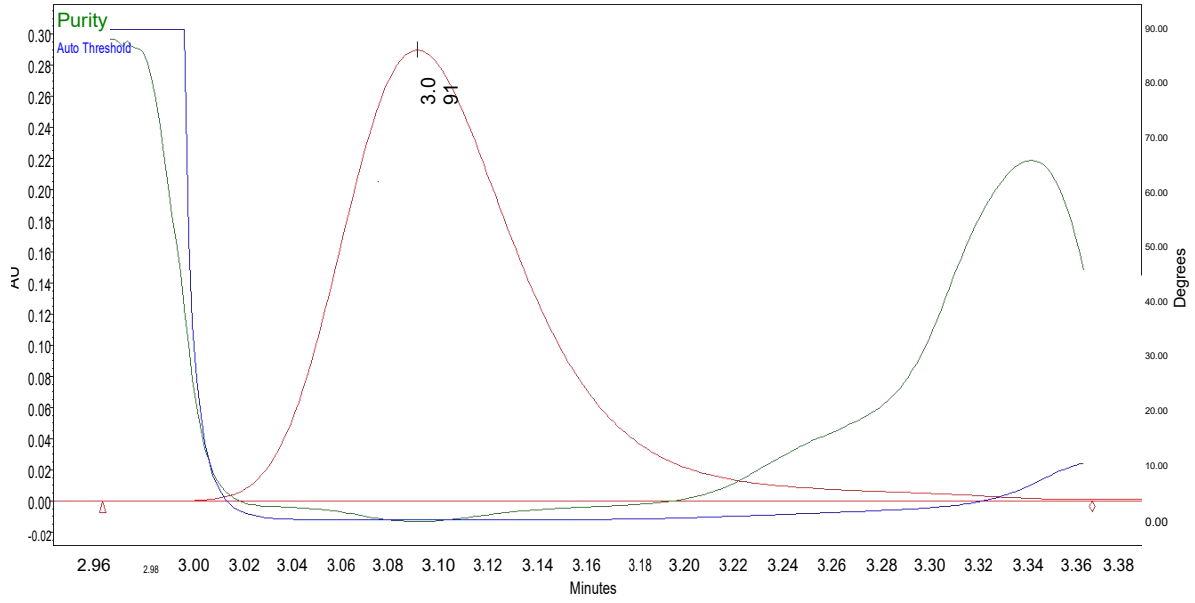


Table 4.35 Peak purity studies

Sr. No	Peak	Peak RT	Peak purity angle	Purity threshold
1	CET	3.1	0.499	3.109

4.6.2.5 Applicability of developed method for analysis of formulation

Applicability of method was checked by analysis of marketed formulation of CET combination which is Cetrilak solution comprising of 3% W/V of CET solution, manufactured by Menarini India Pvt. Ltd. Table 4.36 consists of the details for the formulation analysis.

Table 4.36: Applicability of method

Actual conc. (mg in 10 ml)	Amount of CET found (mg in 10 ml)	%Label claim	Standard Deviation	%RSD
3	3.05	101.866%	0.96	0.95 %
3	3.06	101.900%		
3	3.00	100.066%		
3	3.04	101.300%		
3	2.98	99.630%		
3	3.04	101.500%		

4.6.2.6 Statistical analysis using Anderson darling normality test

Statistical analysis was undertaken using Anderson darling normality test as this test is especially effective in detecting departure from normality in low and high values of distribution. It is used in our study to verify the normality of data distribution. The test is conducted using online calculator for Anderson darling test using the following formula.

$$AD = -N - \frac{2i-1}{N}\left(\ln\left(F\left(Y\right)\right) + \ln\left(1 - F\left(Y\right)\right)\right)$$

$$AD^{*} = AD\left(1 + \frac{0.75}{NN} + \frac{2.25}{NN^{2}}\right)$$

For checking the normal distribution of data for CET, Anderson darling normality plot was prepared for CET as shown in Figure 4.46. Data summary in Table 4.37 and Test statistics in Table 4.38 infer p- value greater that alpha value of 0.05 signifying normal distribution of data for CET.

Table 4.37: Anderson Darling normality test for checking normal distribution of data for Cetrimide.

Data	Sorted	Count	F1i	1-F1i	F2i	Si	N Plt Line	N Plt Line
443112	443112	1	0.0931688 5	0.9068311 5	0.090062	-4.780598 3	463305. 2	0.1
684459	684459	2	0.1992961	0.8007039	0.218418 2	-9.402922 3	785978. 7	0.26
990130	990130	3	0.4053353 6	0.5946646 4	0.386952 7	-9.262467 2	1009174	0.42
125649 3	125649 3	4	0.6130473 4	0.3869526 6	0.594664 6	-7.063495 4	1213327	0.58
150437 4	150437 4	5	0.7815818 2	0.2184181 8	0.800703 9	-4.218295 5	1436523	0.74
178893 6	178893 6	6	0.9099379 5	0.0900620 5	0.906831 1	-2.113956 6	1759196	0.9

Figure 4.46 Anderson darling normality plot for CET

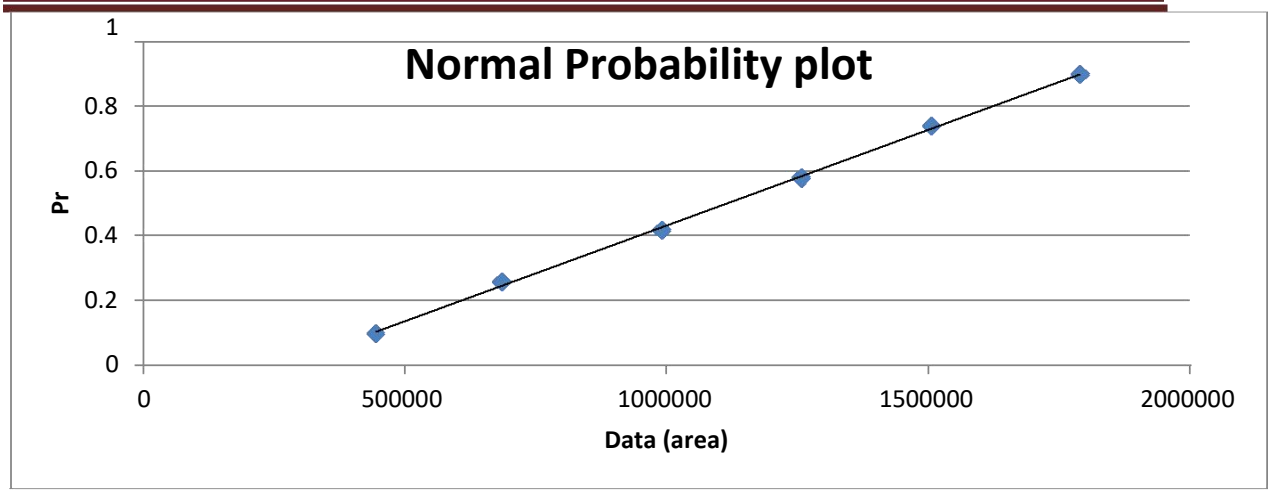


Table 4.38 Test statistics for CET

0.1403	AD test statistic
0.166593	AD* test statistic
0.938946	P-value

4.7 CONCLUSION

The developed UV spectrophotometric methods were found to be valid, simple, rapid, accurate, precise and specific and sensitive for estimation of Chlorhexidine gluconate and Cetrimide. The sample recoveries for all methods were in good agreement with their respective label claims, which suggested non-interference of formulation additives in its estimation. Hence, the developed methods could be successfully applied for estimation of Chlorhexidine gluconate and Cetrimide in bulk and its marketed formulation. All methods were validated as per ICH guidelines. Statistical analysis was undertaken to check if results obtained from all methods are in good agreement with each other. Statistical analysis using ANOVA and post hoc analysis suggested no significant difference between various methods developed except for the assay results significantly varying between Inverse least square methods and Absorption ratio spectra method.

Also, a simple RP-HPLC method was developed for the combined dosage form of Chlorhexidine gluconate and Cetrimide. The retention times were found to be 3.10 mins and 3.9 mins for Cetrimide and Chlorhexidine gluconate respectively. It was also validated as per ICH guidelines and robustness study was done using DOE approach. All parameters were found to be robust as per the limits prescribed in regulatory guidelines. Normal distribution of data was inferred by application of Anderson darling normality test.

Along with it, a simple UV and RP-HPLC method was developed for analysis of Cetrimide. Validation for the developed method was carried out as per ICH guidelines

and Normal distribution of data was inferred by application of Anderson darling normality test.

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