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EXPLORATION OF VARIOUS CLASSICAL AND CHEMOMETRIC ASSISTED UV SPECTROPHOTOMETRIC METHODS FOR ESTIMATION OF CHLORHEXIDINE GLUCONATE AND CETRIMIDE IN BULK AND ITS FORMULATION

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ABSTRACT

The present work inculcates with the approach for development of various classical and chemometrics assisted UV spectrophotometric methods for estimation of Chlorhexidine gluconate (CH) and Cetrimide (CET) in bulk and its formulation. The methods developed herein include simple Simultaneous equation method (Vieordt's method), First Derivative spectroscopy method, Multicomponent analysis method, Classical least squares, Inverse least square, Partial least squares, Absorption ratio spectra method and Mean centering of ratio spectra method. The developed methods were successfully validated according to ICH Q2 (R1) guidelines. All methods showed a good linear response. The mean percentage assay values for all UV methods were found to be in the range of 98-102%. Statistical analysis was also applied for assay results of the developed methods which included One-way ANOVA and post hoc analysis like Tukey Honest significant test and Scheffe multiple comparison test. The major outcome of research imbibes to be developed analytical methods were found to be specific, selective, and robust and can be applied for routine analysis of marketed formulation in laboratory premises. Thus, various novel and simple UV analytical methods were explored and available for analysis of the selected drugs.

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INTRODUCTION

Chlorhexidine gluconate is an antiseptic and antibacterial drug with molecular weight of 897.75716 g/mol and pKa value of 10.3. [1] It was approved by USFDA on 19 December 2003. A RP- HPLC analytical method along with its impurity Para chloroaniline is available in literature for its estimation**.** [2] Cetrimide (Cetrimonium bromide) is a local infective agent with molecular weight 364.44 g/mol.[3] It was approved by USFDA on 30 June 2006. Due to absence of any significant chromophoric group in its structure till date no analytical method is available for its estimation. Also no analytical method is available for estimation of Chlorhexidine gluconate and Cetrimide in combination which is found in various marketed formulations like Savlon antiseptic solution [4] and Aceptic Lotion to best of our knowledge. Thus, the major objective of present research is development of analytical methods for estimation of Chlorhexidine gluconate and Cetrimide in combination and its applicability for analysis of its marketed formulations.

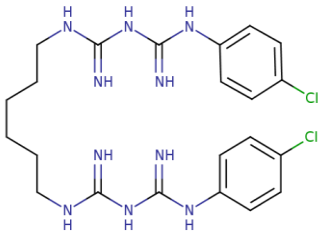

Figure 1 A: Structure of Chlorhexidine gluconate [1]

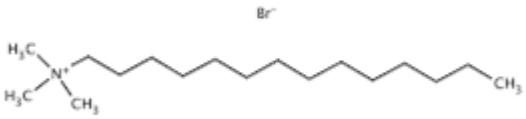

Figure 1 B: Structure of Cetrimide [3]

The Vierordt's method includes simultaneous analysis of two drugs in a sample, applied when both drugs have some absorbance at λmax of other respective drug. [5] First derivative spectroscopy method includes derivatizing zero order spectra of both drugs into its first derivative by differentiating absorbance with respect to wavelength. Thus new derivative λmax for both drugs is available. In this method such wavelength maxima points are considered where there is Zero crossing point (ZCP) for other respective drug. [6] In Multicomponent analysis method, in UV 1700 model, the multicomponent mode of inbuilt software is selected and then manually first standard calibration curve values are fit into the software and a file is saved and then whenever unknown sample simultaneous sample analysis to be done its done using by opening the saved data file. Classical least square(CLS) method is based on mathematical model of 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. Calibration coefficient value K is calculated as K= pin v(C)*A and unknown is computed as C= A* pinv (K). Inverse least square (ILS) method is based on mathematical model of C=AP where P is l x n matrix of unknown calibration, C and A are same as defined for CLS method above at unit concentration and unit path length. Calibration coefficient value P is calculated as P= pin v(A)*C and unknown is computed as C= A* P. [7] Partial least squares (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. 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.[8] Absorption ratio spectra method is applicable for a sample containing x and y analytes in a mixture. 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 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. 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 derivatising the sample and this S/N ratio is improved in it. In this method first the analysis of entire calibration range is done at a λmax of any 1 analyte (say x).This values are then divided by absorptivity of other analyte in the mixture(say y). This Am/αy values for entire calibration range are the mean centered using software packages and thus they can act as a predictor for future analysis of unknown sample. [9] Statistical analysis of developed methods for assay analysis was done by using ANOVA test. [10]

MATERIALS AND METHODS

Apparatus and software:

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 chemo metric models were developed by using the software packages like MATLAB from Math works, Design expert 7.0 and SAS JMP 13.

Materials

Gift samples of standard Chlorhexidine gluconate (CH) and Cetrimide (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 CH and 3 gm CET per 100 ml)

Reagents and Chemicals

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.

Preparation of Stock Solution

For all UV spectrophotometric methods, 10mg each of CH and CET were 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 CH and CET respectively.

Preparation of working standards and calibration curve solutions

For all UV spectrophotometric methods, 2.5 ml each of CH 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 CH and CET respectively. Considering the ratio of CH and CET in commercial formulation to be 1:10 appropriate aliquots of CH 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.

Method I: Vierordt's method [5]

In this method for selection of analytical wavelengths, standard solutions of CH and CET were scanned between 200-400 nm wavelength ranges and as shown in figure 3 which implicated a λmax of 217nm for Cetrimide and 260 nm for Chlorhexidine gluconate. The calculations were done by using the formula stated below for simultaneous analysis of both analytes in the mixture.

$$C_{CH} = \frac{A_1\beta_2 - A_2\beta_1}{\alpha_1\beta_2 - \alpha_2\beta_1} \quad (1)$$

$$C_{CET} = \frac{A_2\alpha_1 - A_1\alpha_2}{\alpha_1\beta_2 - \alpha_2\beta_1} \quad (2)$$

Method II: First Derivative spectroscopy method [6]

In this method zero order spectra were derivatized to first order and then on basis of zero crossing points (ZCP) of the corresponding drugs the wavelength for analysis was chosen. As per the study, CET was analyzed at 222 nm which was ZCP of CH whereas CH was analyzed at 275 nm which was ZCP of CET. The parameters delta λ and scaling factor were set to be 5.

Method III: Multicomponent analysis method

This method is developed using the UV 1700 instrument without PC control with UV probe. In this method first λ max determination of single drug is done and then the entire range of mixture solutions is scanned in the multicomponent mode where number of components that is two in our analysis and the λ max of both drugs needs to be selected that is 260 nm and 217 nm for CH and CET respectively. This analytical data are then saved in the inbuilt software of the instrument for analysis of unknown samples.

Method IV: Classical least squares method [7]

This method is developed using the software packages like Design expert 7.0 and Mat lab from Math works. In this method 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. The mathematical model for which 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).

Method V: Inverse least squares method [7]

This method is developed using the software packages like Design expert 7.0 and Mat lab from Math works. In this method 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 Calibration coefficient matrix P, calibration set consisting of thirty sets was utilized. Other 6 sets were considered for validation of the model. The mathematical model for which 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.

Method VI: Partial least squares method [8]

This method is developed using the software packages like Design expert 7.0 and SAS JMP 13. In this method also 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 Absorbance matrix A, calibration set consisting of thirty sets was utilized. Other six sets were considered for validation of the model. 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.

Method VII: Absorption ratio spectra method [9]

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

Method VIII: Mean centering of ratio spectra method [9]

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 derivatising the sample and this 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 (α_{CH}=505446 1/mol/cm). This Am/α_{CH} 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 CH also Am/ α_{CET} is done (where λmax of CH is 260 nm and αCET= 364450 1/mol/cm) and then mean centered and fed for analysis of CH. These values can now act as a predictor for future analysis of unknown sample.

Applicability of the method

The developed UV methods were applied for analysis of their 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 and DDW to produce a clear solution. The resulting solution was again diluted by withdrawing 1 ml and making upto 10 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.

Method Validation [11]

Linearity and range: The proposed spectrophotometric method showed good linearity in the concentration range of 3-18 µg/ml for CH 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 CH 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

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 CH and CET less than 2% revealed the robustness and ruggedness of the method.

Statistical analysis [10]

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.

RESULTS AND DISCUSSION

In this study 8 UV spectrophotometric methods were developed in which 3 methods were Chemo metrics assisted methods and 5 were classical spectrophotometric methods. The methods were developed as specified in the introduction section and validated as per ICH guidelines. From the validation data and results it was conferred that the developed methods gave accurate, precise and robust results.

Vierordt's method:

In this method as represented in figure 2, λmax for CH was chosen as 260 nm and λmax for CET was chosen 217nm. The linearity range selected for CH 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 3, Table 5, Table 6 and Table 7.

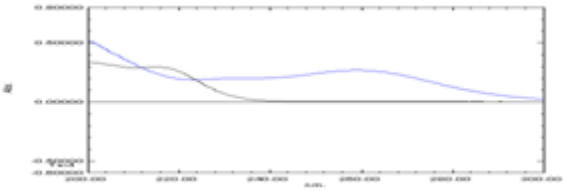

Figure 2: MethodI- Vierordt's method absorption spectra representing λmax of CH=260 nm and λmax of CET= 217nm.

First derivative spectroscopic method:

In this method as represented in figure 3, λmax for CH was chosen as 275 nm as it is the zero crossing point of CET and λmax for CET was chosen 222nm as it is the zero crossing point of CH. The linearity range selected for CH 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 3, Table 5, Table 6 and Table 7.

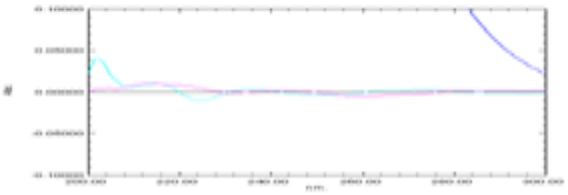

Figure 3: Method II- First derivative spectroscopic method representing λmax of CH=275 nm (ZCP of CET) and λmax of CET=222nm (ZCP of CH).

Multicomponent analysis method:

In this method also the λmax for CH was chosen as 260 nm and λmax for CET was chosen 217nm. The linearity range selected for CH 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 and 5. All validation parameters were within limits as prescribed by ICH guidelines as represented in Table 3, Table 5, Table 6 and Table 7.

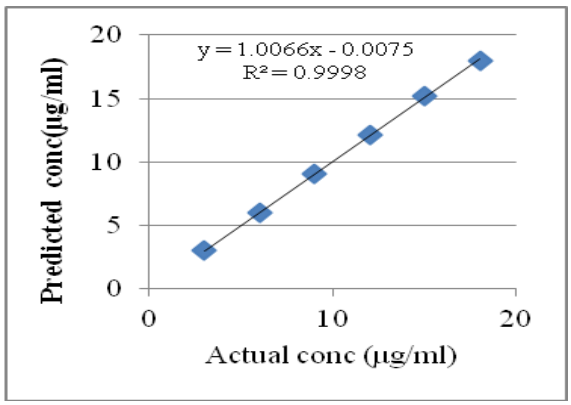

Figure 4: Predicted vs Actual concentration of CH.

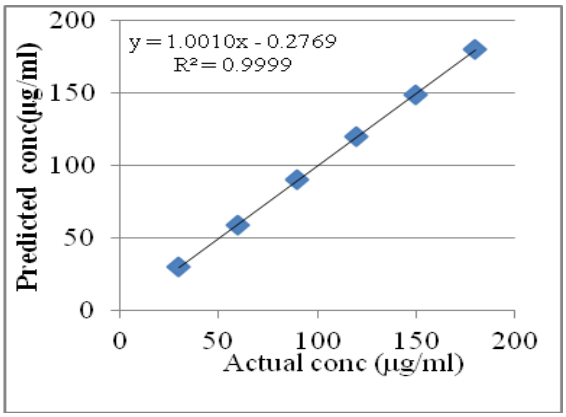

Figure5: Predicted vs Actual concentration of CET.

Classical least square method:

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. 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 I) and thus using it the unknown set can be quantified. The RMSEP values and all other validation parameters represent the validity of the developed method as represented in Table III, Table IV, Table V, Table VI and Table VII.

Table I: Method IV- K matrix for Classical least square method.

Wavelength	CH	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

Inverse square method:

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. 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, ILS model was developed for obtaining the P matrix (Table II) and thus using it the unknown set can be quantified. The RMSEP values and all other validation parameters represent the validity of the developed method as represented in Table III, Table IV, Table V, Table VI and Table VII.

Table II: Method V- P matrix for Inverse square method.

Wavelength	CH	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

Partial least square method:

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. 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, PLS model was developedThe RMSEP values and all other validation parameters represent the validity of the developed method as represented in Table 3, Table 4, Table 5, Table 6 and Table 7.

Absorption ratio spectra method:

In this method as represented in figure 6, λmax for CH was chosen as a difference of 217 nm and 200 nm while for CET it was chosen a difference of 263 and 225 nm. The linearity range selected for CH 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 3, Table 5, and Table 6 and Table 7.

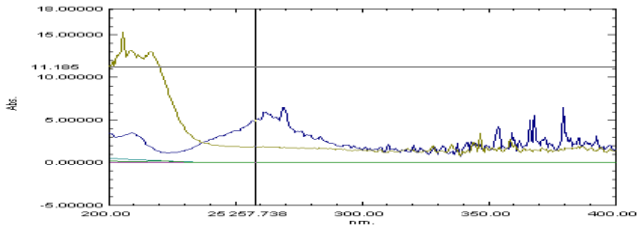

Figure 6: Method VII- Absorption ratio spectra method representing values of 217-200 nm (λ2- λ1) for CH and 263-225 nm (λ2- λ1) for CET.

Table III: 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**		**Absorption ratio spectra method**		**Mean centering of ratio spectra method**	
	CH	CET	CH	CET	CH	CET	CH	CET	CH	CET	CH	CET	CH	CET	CH	CET
Analytical wavelength(nm)	260	217	275	222	260	217	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
Slope	0.0732	0.1721	0.002	0.0071	1.0066	1.0010	0.994	1.005	1.004	0.998	0.991	0.999	0.353	0.343	0.164	0.184
Intercept	0.0175	0.197	0.036	0.002	-0.007	-0.276	0.015	-0.028	0.060	0.008	0.025	0.007	1.267	1.016	2.86	1.584
Correlation coefficient (R^2)	0.998	0.9990	0.9991	0.9990	0.9998	0.9999	0.9996	0.9998	0.9994	0.998	0.999	0.997	0.998	0.997	0.999	0.998
LOD(µg/ml)	0.087	0.154	0.120	0.098	0.178	0.124	0.641	0.125	0.234	0.103	0.564	0.238	0.145	0.251	0.124	0.155
LOQ(µg/ml)	0.261	0.462	0.360	0.294	0.534	0.372	1.923	0.375	0.702	0.309	1.692	0.714	0.435	0.753	0.372	0.465

Table IV: RMSEP values for developed Chemo metrics methods.

Method	Drug	RMSEP
Classical least squares	CH	0.105
	CET	0.114
Inverse least square	CH	0.099
	CET	0.109
Partial least squares	CH	0.114
	CET	0.104

Table V: Results of Intraday and Interday precision.

Methods	Precision	%RSD
Vieordt's method	Intraday	0.245
	Interday	1.354
First Derivative spectroscopy method	Intraday	0.984
	Interday	0.458
Multicomponent analysis method	Intraday	1.559
	Interday	1.021
Absorption ratio spectra method	Intraday	0.151
	Interday	0.786
Mean centering of ratio spectra method	Intraday	0.651
	Interday	1.250
Classical least squares	Intraday	0.564
	Interday	0.654
Inverse least squares	Intraday	1.025
	Interday	0.845
Partial least squares	Intraday	0.458
	Interday	0.785

Table VI: Assay results of UV spectrophotometery methods.

Methods	%Assay±SD	%Assay±SD
	CH	CET
Vieordt's method	100.35±0.037	100.12±0.25
First Derivative spectroscopy method	100.11±0.545	99.94±0.056
Multicomponent analysis method	99.86±1.042	99.86±1.08
Classical least squares	100.51±0.097	100.34±0.65
Inverse least square	101.08±0.341	100.51±0.089
Partial least squares	100.56±1.002	100.27±0.21
Absorption ratio spectra method	99.72±0.785	99.72±1.01
Mean centering of ratio spectra method	100.67±0.027	100.67±0.45

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

Method	Drug	%Spiking	%Recovery±SD
Vieordt'method	CH	80	100.44±1.021
		100	99.39±0.078
		120	101.56±1.001
	CET	80	99.32±0.034
		100	100.10±0.970
		120	99.29±0.089
First Derivative spectroscopy method	CH	80	101.43±1.031
		100	100.34±0.098
		120	99.49±0.670
	CET	80	101.45±0.089
		100	100.21±1.007
		120	100.89±1.010
Multicomponent analysis method	CH	80	100.49±0.098
		100	101.11±0.790
		120	99.27±0.672
	CET	80	100.02±0.089
		100	99.23±0.098
		120	100.34±1.001
Absorption ratio spectra method	CH	80	101.22±1.082
		100	100.34±1.089
		120	100.56±0.098
	CET	80	101.10±0.560
		100	100.88±0.460
		120	99.70±0.093
Mean centering of ratio spectra method	CH	80	99.44±0.078

		100	101.12±0.374
		120	100.23±1.013
	CET	80	100.56±1.082
		100	100.39±0.098
		120	101.00±1.078
Classical least squares	CH	80	100.75±0.45
		100	101.12±0.865
		120	100.78±0.261
	CET	80	99.76±1.024
		100	100.86±0.484
		120	101.82±0.892
Inverse least squares	CH	80	100.46±0.591
		100	99.55±1.025
		120	100.36±1.001
	CET	80	101.22±0.465
		100	100.79±0.234
		120	100.23±0.211
Partial least squares	CH	80	100.69±0.685
		100	101.04±0.478
		120	99.45±0.251
	CET	80	101.45±0.958
		100	100.63±0.125
		120	100.48±0.045

Statistical analysis

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 CH 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 CH whereas for CET , all methods give results with insignificant difference from each other. (Table VIII and Table IX)For further investigation for CH 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. In Scheffe test no significant difference was obtained in any of the methods. 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 VIII: One way ANOVA for CET.

Group	Count	Sum	Average	Variance
Viordt's method	6	600.72	100.12	0.16
First derivative spectroscopy method	6	599.66	99.94	0.53
Multicomponent analysis method	6	599.19	99.86	0.44
Classical least squares	6	602.08	99.34	0.23
Inverse least squares	6	603.06	100.51	0.16
Partial least squares	6	601.66	100.27	0.023
Absorption ratio spectra methed	6	598.34	99.72	0.073
Mean centering of ratio spectra method	6	604.06	100.67	0.24

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F-statistics	p-value
Treatment	2.90	7	0.41	1.85	0.10
Error	8.95	40	0.22		
Total	11.85	47			

Table VIII: One way ANOVA for CH.

Group	Count	Sum	Average	Variance
Viordt's method	6	602.12	100.35	0.51
First derivative spectroscopy method	6	600.66	100.11	1.12
Multicomponent analysis method	6	599.19	99.86	0.44
Classical least squares	6	603.08	99.34	0.44
Inverse least squares	6	606.48	100.51	0.48
Partial least squares	6	603.40	100.08	0.34
Absorption ratio spectra methed	6	598.34	99.72	0.073
Mean centering of ratio spectra method	6	604.06	100.67	0.24

Source	Sum of squares	Degrees of freedom	Mean sum of squares	F-statistics	p-value
Treatment	6.59	7	0.94	3.41	0.0059
Error	11.03	40	0.27		
Total	17.63	47			

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. The future scope for current research is chromatographic and other hyphenated techniques can be explored for perceiving more information regarding the analytes in the pharmaceutical formulation.

Abbreviations

CH- Chlorhexidine gluconate
CET- Cetrimide
ZCP- Zero crossing point
ICH- International conference of Harmonisation
CLS-Classical least squares
ILS-Inverse least squares
PLS-Partial least squares
NIPALS-Non-iterative Partial least squares
RMSEP-Root mean squares error of prediction

Competing Interests

We hereby declare that the work submitted in this manuscript has not been published or under consideration in any other journal.

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