Simultaneous determination of Bimatoprost and Timolol Maleate by Chemometric assisted Ultra-Violet Visible spectrophotometric methods in its pure drugs and it's combined dosage form

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Abstract:

Chemometric methods including Classical Least Square (CLS), Principal Component Regression (PCR) and Partial Least Square (PLS) were developed for simultaneous determination of Bimatoprost and Timolol Maleate in ophthalmic solution using UV-spectrophotometry. A set of 25 standard mixtures containing both drugs were prepared in range of 1-3 µgmL-1 for Bimatoprost and 16.67-50 µgmL-1 for Timolol Maleate. The developed methods were validated as per IUPAC guidelines. Analytical figure of merit (FOM), such as sensitivity, selectivity, limit of detection and limit of quantitation were determined for Chemometric methods. The proposed methods were applied for determination of two components from combined dosage form.

Keywords: Bimatoprost, Timolol Maleate, Partial Least Square, Principal Component Regression, Classical Least Square.

INTRODUCTION

Bimatoprost (BIM) (Figure 1) {5Z)-7-[(1R, 2R, 3R, 5S)-3,5-dihydroxy-2-[(1E,3S)-3-hydroxy-5-phenylpent-1-en-1-yl]cyclopentyl]-N-ethylhept-5-enamide}[1] having chemical formula $C_{25}H_{37}NO_4$ is a prostamide or synthetic prostaglandin analog, thought to lower intraocular pressure (IOP) by increasing the outflow of aqueous humor through both the trabecular meshwork and uveoscleral drainage systems. BIM is not official in any of the pharmacopoeias.

Timolol Maleate (TM) (Fig. 2) {tert-butyl (2-hydroxy-3-{[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy}propyl)amine}[2] having chemical formula $C_{13}H_{24}N_4O_3S$, is an Anti glucomatic agent, causes a reduction in the production of the liquid (aqueous humor) within the eye, thus decreasing intraocular pressure (IOP) Timolol is official in Indian Pharmacopoeia, 2010 [3].

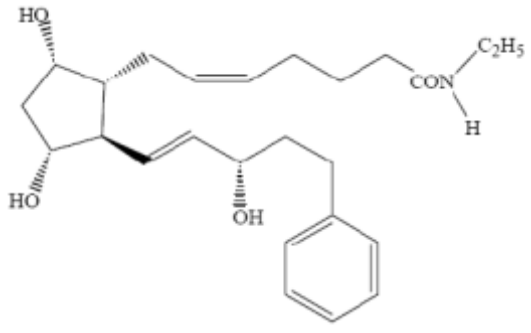


Figure 1 Structure of Bimatoprost

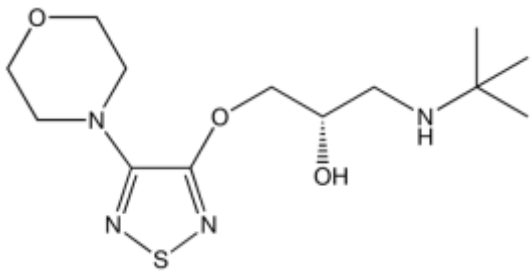


Figure 2 Structuure of Timolol Maleate

The marketed formulation CAREPROST PLUS containing BIM and TM 0.03 and 0.5% respectively (Sun pharmaceuticals Pvt. Ltd., Vadodara) was used throughout the study. Based on literature review it was found that number of methods are available for the estimation of BIM and TM individually as well as in combination with other drugs. Chemometric [4,5], spectrophotometric [6-8]; Near IR and Raman Spectroscopy [9], HPTLC [10] Capillary Zone Electrophoresis [11], UPLC [12] and RP-HPLC [13-18] methods have been used for the estimation of TM and RP-HPLC(by using ELSD Detector) [19,20] methods have been used for the estimation of BIM. As per the literatire non e of the method is available to estimate both the drug simalteniously, hence our thought of interest to develop and validate an analytical method to identified both the drug simalteniopusly.

UV/VIS spectroscopy is a preferred analytical technique as it is easy to operate and interpret the results easily. The various methods UV-spectrophotometry available are:

- Simultaneous Equation estimation Method
- Derivative Spectrophotometry Method
- Absorbance ratio method
- Absorbance Correction method.

But the limitation of UV spectrophotometric methods is its Sensitivity. This limitation could be overcome by use of Chemometric techniques [21-24] that have been reported to be more sensitive than Chromatographic techniques. It was thought of interest to combine the advantage of statistics and UV spectroscopy to develop a simple yet sensitive method for analysis of these drugs. In recent years, multivariate calibrations such as classical least square (CLS), Principal Component Regression (PCR) and Partial lest square (PLS) have been employed extensively in quantitative spectral analysis to get selective information from the unselective data. These methods can be applied for the simultaneous spectrophotometric estimation of drugs in pharmaceutical formulation containing two or more drug compounds. CLS is one of the simplest methods having multivariate least square procedure based directly on Beer's law. PCR and PLS [25-27] are factor analysis methods which are used to establish a relationship between matrices of the chemical data. PLS is related to PCR in that spectral decomposition is performed. PCR decomposition is significantly influenced by variations, which have no relevance to analyte concentration, where as PLS spectral decomposition is weighted to concentration.

METHODS/MATERIALS AND METHODS

Material and Methods

Standards for drugs and reagents

Pure standard sample of BIM and TM were procured as a gratis sample from the Sun pharmaceuticals Pvt. Ltd., Vadodara. Methanol AR grade were purchased from Loba Chemie (Laboratory Chemicals and Fine Reagents).

Instrument and Software

A double beam UV-spectrophotometer, UV-1800 (Shimadzu, Japan) equipped with 1 cm quartz cells and 2 nm fixed slit width connected to a computer loaded with Shimadzu UVPC software was used. An analytical balance New Classic MF (Model No. ML204/A01 METLET TOLEDO Made in Switzerland) was used to weigh accurately the standard and test samples. The additional SOLO software (EIGENVECTOR) was used for various Chemometric methods. The zero order spectra was recorded over 200–400 nm wavelength with one data point per nanometer for Chemometric method.

Pharmaceutical Formulation

Eye drop Careprost Plus, containing 0.03% Bimatoprost and 0.5% TM was taken for the study.

CHEMOMETRIC METHOD[27,28,29,30,31]

Preparation of Stock Solutions

BIM powder (10 mg) was accurately weighed and transferred to a 100 mL volumetric flask. It was dissolved and diluted up to the mark of 100 mL with methanol to obtain a stock solution of BIM with final concentration of 0.1 mg/mol (100µg/ml). TM powder (100 mg) was accurately weighed and transferred to a 100 mL volumetric flask. It was dissolved and diluted up to the mark of 100 mL with methanol to obtain a stock solution of TM with final concentration of 1 mg/mol (1000µg/ml).

Preperation of Working Standard Solution:

Stock solution of BIM (1 mL) was transferred to 10 mL volumetric flask and diluted up to the mark of 10 mL with Methanol to obtain working standard solution of BIM with final concentration of 10 µg/mL. Stock solution of TM (1 mL) was transferred to 10 mL volumetric flask and diluted up to the mark of 10 mL with Methanol to obtain working standard solution of TM with final concentration of 100 µg/mL.

Preparation of Calibration set:

Working standard solutions having concentration range of 1-3 µg mL^{-1} and 16.67-50 µg mL^{-1} for BIM and

TM were prepared by withdrawing 0.1-0.3mL of BIM and 0.16-0.50mL of TM from the above prepared standard stock solutions by using (Tarsons Accupipet, INDIA) micropipette having capacity of 10-100 µL.

Experimental design for CLS, PLS and PCR calibration

A dataset of standard mixture solutions containing 1-3 µg mL^{-1} and 16.67-50 µg mL^{-1} for BIM and TM were prepared from standard stock solution. A set design of concentration data corresponding to the BIM and TM mixture was organized statistically to maximize the information content in the spectra and to minimize the error of multivariate calibrations. The set of 25 standard mixture solutions, with different concentration ratios as mentioned above were systematically prepared (Table 1). From the data set, 9 mixtures have been randomly selected for the validation purpose. The UV absorption spectra were recorded over selected wavelength points. The computations were made using EIGENVECTOR SOLO software for PLS, PCR, CLS methods.

Data Processing

Classical Least Square Method

The Beer–Lambert's law of UV–Vis spectroscopy applied to multiple linear regressions lead to CLS.

* indicates Validation data set

Table 1: Concentration matrix of mixtures containing both drugs BIM and TM (Calibration data set)

S.No.	Concentration (µg mL^{-1})	S.No.	Concentration (µg mL^{-1})		
	BIM	TM		BIM	TM
1	1	16.67	14	2	41.67
2	1	24.99	15	2	50
3*	1	33.34	16	2.5	16.67
4	1	41.67	17*	2.5	24.99
5*	1	50	18	2.5	33.34
6*	1.5	16.67	19	2.5	41.67
7*	1.5	24.99	20*	2.5	50
8	1.5	33.34	21	3	16.67
9*	1.5	41.67	22	3	24.99
10	1.5	50	23	3	33.34
11*	2	16.67	24*	3	41.67
12	2	24.99	25	3	50
13	2	33.34			

* indicates Validation data set

The mathematical formulation of this method, in the matrix compact form can be written as

$$A = K \times C \text{----------(1)}$$

for CLS. Here A is the zero order absorbance, C is the concentration matrix and K is the calibration coefficients.

Partial Least Square and Principal Component Regression Method

An appropriate choice of number of principal components or factors is necessary for PCR and PLS calibrations. Various criteria have been developed to select the optimum parameters. Cross validation methods leaving out one sample at a time was employed with a calibration set of 25 calibration spectra. Then PLS and PCR calibration on 24 calibration spectra were performed, and using this calibration, the concentration of the sample left out during the calibration set was determined. The process was repeated 25 times until each calibration sample had been left once.

ppropriate selection of number of factors to be used to construct model is the key to achieving correct quantitation in PLS and PCR calibrations.

Analysis of Marketed Formulation

0.1 mL of formulation were withdrawn 5 times and diluted upto 10 mL with methanol to get a concentration of 3 µg mL^{-1} of BIM and 50 µg mL^{-1} of TM. The absorbance of the test solution was taken at the selected wavelength and concentration was determined using Chemometric method without using any maximum excitation wavelength and absorbtivity.

Validation of Chemometric method[28,29,30,32]

Developed analytical method was ferther validated by IUPAC guideline. For the validation of mathematical models of CLS, PCR and PLS, statistical analysis were applied. Various Parameters like Root mean standard error of calibration (RMSEC), Root mean standard error of cross validation (RMSECV), Root mean standard error of prediction (RMSEP), Predicted Residual Error Sum of Square (PRESS) and correlation coefficient (r^2) were determined. Figure of merits (FOM) is necessary for the validation of Chemometric methods. FOM such as Sensitivity (SEN), Limit of Detection (LOD) and Limit of Quantitation (LOQ) can be estimated and used to compare analytical methods.

RESULTS AND DISCUSSION

Selection of Solvent

The solvent mentioned in the literatures for the analysis of BIM and TM individually or with other drug combinations are methanol, acetonitrile, and phosphate buffers of different pH. TM is very soluble

in water and BIM is sparingly soluble in water, but mixture of both drugs are not stable in water when kept overnight whereas it is stable in methanol and both drugs are freely soluble in methanol. So, methanol was used in the analysis of BIM and TM in eye drops.

Development and Validation of Chemometric assisted UV Spectrophotometric Method

The calibration data set and validation data set were prepared as shown in Table 1. Zero order spectra were scanned in the whole range of 200-400nm.(Figure 3) The spectra range of 200-220 nm was omitted because of the noise and the region from 341 to 400 nm was also omitted because of the zero absorbance in this range and showed high RMSEC, RMSECV value when model was developed. So, wavelength region selected for the analysis was 220-341nm with minimum values of RMSEC, RMSECV for model developed.

CLS, PLS and PCR method

Absorbance matrix (A) was constructed using recording zero order spectra between 220 nm and 341 nm at 10 nm intervals. Thus, 12 wavelength points were selected. CLS, PLS, PCR models was created by introducing absorbance (A) and concentration matrix (C) data in EIGENVECTOR SOLO software. Absorbance values of samples at 12 different wavelength points have been incorporated in Figure 3 Selection of Spectral wavelength region for Chemometric assisted UV spectroscopic method.

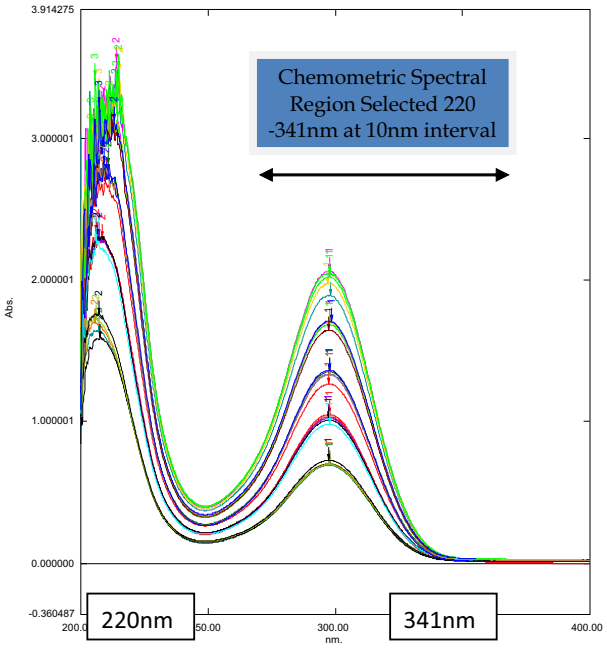


Figure 3 Selection of Spectral wavelength region for Chemometric assisted UV spectroscopic method.

Constructed model and quantities of BIM and TM in validation data set as well as in formulation were predicted as per Table 2.

Table 2: Composition and results of the prediction set by CLS, PLS, PCRPredicted set Concentration

Predicted set Concentration (µg mL^{-1})			Amount of drugs (µg mL^{-1})					
			CLS		PLS		PCR	
Sr. No.	BIM	TM	BIM	TM	BIM	TM	BIM	TM
1	1	33.34	0.9	33.1	0.91	33.1	0.9	33.14
2	1	50	0.94	49.65	0.95	49.6	0.95	49.64
3	1.5	16.67	1.53	16.94	1.53	17.0	1.55	17.01
4	1.5	24.99	1.46	24.97	1.47	25.0	1.47	25.02
5	1.5	41.67	1.49	41.42	1.5	41.4	1.5	41.42
6	2	16.67	2	16.92	2	16.9	2	16.98
7	2.5	24.99	2.59	25.15	2.59	25.16	2.6	25.16
8	2.5	50	2.55	50	2.55	49.93	2.58	49.91
9	3	41.67	3	41.53	2.99	41.47	2.99	41.47

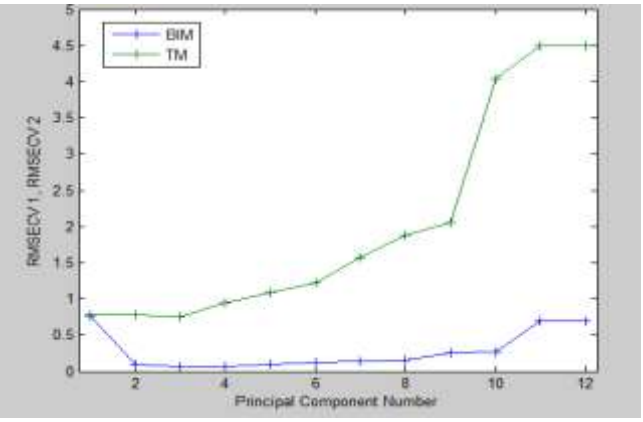
Statistical Analysis in selecting the number of principle components and factors[19]. Various criteria like RMSEC, RMSECV and PRESS have been used to select the optimum parameters. CLS, PCR and PLS calibration on 25 mixtures were performed using this calibration model. The predicted concentrations were calculated and compared with the known concentration of compounds in each calibration sample.(Figure 4). To validate the model, both PRESS and RMSEP values were considered. Both values must be as low as possible for a particular model. Both the values were calculated as follows [19,20,21]:

$$PRESS = S(Ypre - Ytrue)2 \ldots\ldots\ldots\ldots\ldots(2)$$

Y_{pre} and Y_{true} are predicted and true concentrations in µg/ml respectively.

$$RMSECV = \sqrt{\frac{PRESS}{n}} \quad \ldots\ldots\ldots\ldots\ldots(3)$$

Where n is the number of training samples.



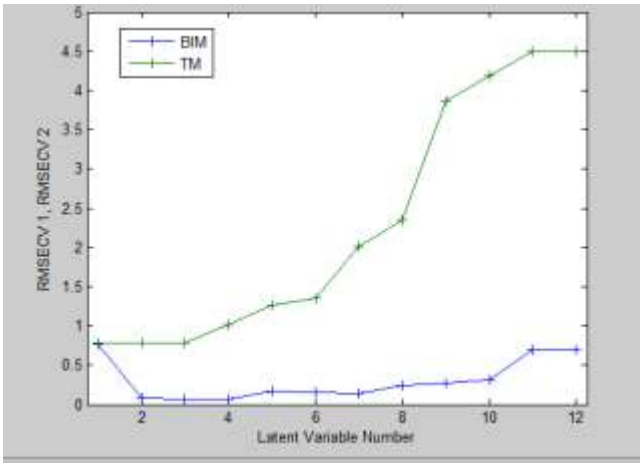


Figure 4 Plot of RMSECV Vs. Number of Components for calibration set prediction using cross-validation of (A) PCR model and (B) PLS model.

Y_{pre} and Y_{true} are predicted and true concentrations in µg mL^{-1} respectively. The RMSECV value was used as a diagnostic test for examining the errors in the predicted concentrations. It indicates both of the precision and accuracy of predictions.

The proposed CLS, PCR and PLS calibration models were evaluated by internal validation, recoveries were obtained in between 98.66-100.06% for BIM and 98.79-99.00% for TM. CLS, PCR and PLS methods were evaluated and comparative study of prediction capabilities of mentioned chemometric approaches were carried out.

Two methods were employed for evaluation where the first method was carried out by plotting the known concentration against the predicted concentration. A satisfactory correlation coefficient (r^2) value was obtained for each drug by the mentioned Chemometric approaches, and the second method was carried out by determining Root mean standard error of calibration (RMSEC) and Root mean standard error of prediction (RMSEP) by the following expression:

$$SEC(SEP)=\sqrt{\frac{\sum_{i=1}^{n}(C_i^{Added}-C_i^{Found})^2}{n-1}} \quad \text{.....(4)}$$

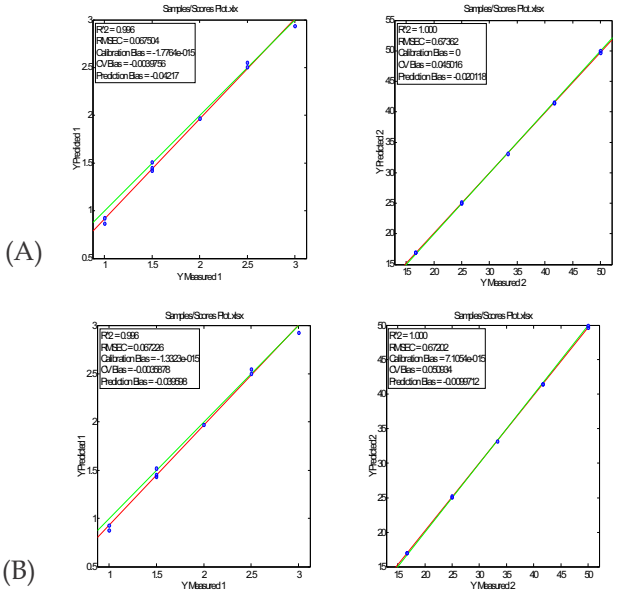
Here, C_i^{Added} represents the added concentration, C_i^{Found} denotes the determined concentration and n is total number of samples. The RMSECV, RMSEC and RMSEP values were obtained by optimizing the calibration matrix of absorption spectra for CLS, PCR and PLS are shown in Table 3 indicating good accuracy and precision. The values of RMSEC, RMSECV, RMSEP and PRESS were found to be minimum for PLS method hence, it could be concluded that PLS is the most suitable method among developed Chemometric methods.

Plots of actual Vs measured values for BIM and TM by (A) CLS (B) PLS and (C) PCR methods called Score plots (Figure 5) and plotting the concentration residuals against the predicted concentrations which were again used to carried out the residual plot (Figure 6). All the residuals distributed in between -2 to +2 value. Chemometric is the technique of separation of necessary informationfrom whole spectra at multiple wavelength, and removal or reducing the noise.

Table 3: Statistical parameters and Figure of Merits for Chemometric methods

Para meter	BIM			TM		
	CLS	PLS	PCR	CLS	PLS	PCR
RMSEC	0.067	0.067	0.06927	0.673	0.672	0.671
RMSECV	0.0850	0.08627	0.090	0.777	0.781	0.779
RMSEP	0.0559	0.0518	0.0584	0.215	0.2428	0.242
PRESS	0.0283	0.027	0.0293	0.4163	0.515	0.51464
r^2	0.996	0.996	0.995	1	1	1
Slope	1.0583	1.0496	1.0567	0.9864	0.9831	0.9829
Noise	0.003745	0.003829	0.003837	0.003745	0.003827	0.003837
Sensitivity	0.944912	0.9527	0.9946345	1.013788	1.01719	1.01739
LOD (µg/mL^{-1})	0.013081	0.0132	0.0133	0.012192	0.01241	0.012445
LOQ (µg/mL^{-1})	0.039638	0.04018	0.04054	0.036945	0.03764	0.03771

The figures in bold indicates that PLS model was found to be the best model amongst all models.



(C)

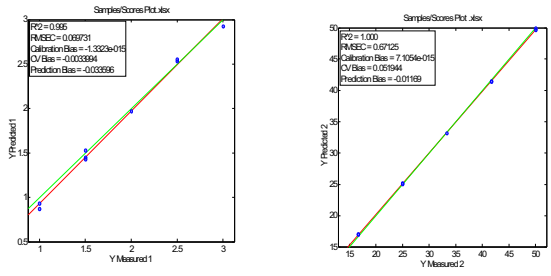


Figure 5 Plots of actual Vs. predicted values for BIM and TM by (A) CLS (B) PLS and (C) PCR methods.

Validation of Chemometric Methods

Analytical figure of merits (FOM) are very important to quantify the quality of a given methodology or for method comparison. In multivariate calibration, several FOM have been reported e.g. sensitivity (SEN), Limit of detection (LOD) and Limit of quantitation (LOQ). Results of FOM are shown in Table 3.

Analysis of Marketed Formulation

The developed methods were applied for analyzing the BIM and TM in marketed formulation and study was repeated five times. The results obtained are shown in Table 4.

Table 4: Assay of Marketed Formulation (Careprost Plus) by proposed methods

BIMATORPOST			
	Label Claim (%)	Amount found (%)	Mean ± RSD(%)
CLS	100	98.8	98.8 ± 0.2317
PLS	100	98.66	98.66 ± 0.33
PCR	100	100.06	100.06 ± 0.36
TIMOLOL MALEATE			
	Label Claim (%)	Amount found (%)	Mean ± RSD(%)
CLS	100	99	99 ± 1.78
PLS	100	98.83	98.83 ± 1.77
PCR	100	98.79	98.79 ± 1.76

The results obtained were complying with the label claim. The statistical comparison of the developed methods were made by using one way ANOVA to determine the variance between the recovery data for the marketed formulation and the method developed. For formulation the F-calculated obtained is less than F-critical. This indicates that there is no significant difference between the above all three developed methods.

(A)

(B)

(C)

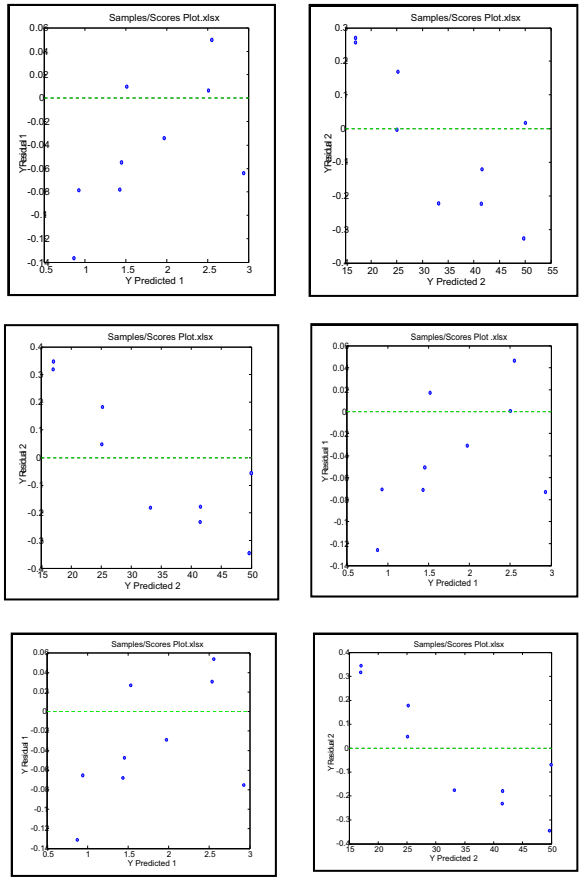


Figure 6 Plots of Concentration Residual vs. Predicted Concentration for BIM and TM by (A) CLS (B) PLS and (C) PCR methods.

CONCLUSIONS

This study is mainly done for check the interference of excipients and additives for two drugs. Three different Chemometric methods (CLS, PLS, PCR) were developed and validated as per IUPAC guideline. The developed method was applied successfully for simultaneous estimation of BIM and TM in an ophthalmic formulation. The values of RMSEC, RMSECV, RMSEP and PRESS were found to be minimum for PLS method hence, it was concluded that PLS is the most suitable method among developed all Chemometric methods. CLS, PLS, PCR were compared statistically by applying ANOVA test shows that there is no observed any significant difference between all developed methods. The developed methods are beneficial in terms of ease of performing and reduced cost of the analysis for routine analysis, quality control of mixture and commercial preparation containing these two drugs.

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References:

1. https://www.drugbank.ca/drugs/DB00905 (07/14/2017)
2. https://www.drugbank.ca/drugs/DB00373 (07/14/2017)
3. Indian Pharmacopoeia, Government of India, Ministry of Health and Family Welfare, the Indian Pharmacopoeial Commission, Ghaziabad 2010, Volume 3, Page No.2229.
4. Azzouz T, Puigdomenech A, Aragay M, Tauler R. (2003) 'Comparison between different data pre-treatment methods in the analysis of forage samples using near-infrared diffuse reflectance spectroscopy and partial least-squares multivariate calibration method.' *Analytica Chemica Acta,* Vol. 484 PP. 121-134.
5. Patel NS, Nandurbarkar VP, Patel AJ, Patel SG. (2014) 'Absorption Correction and Chemometric Method for estimation of Celecoxib and Diacerin in bulk and Capsule dosage form.' *Spectrochimica Acta Part A: Molecular and Bimolecular Spectroscopy.* Vol. 125 pp.46-52.
6. Bebawy L I. (2002) 'Application of TLC-densitometry, first-derivative UV-spectrophotometry and ratio derivative spectrophotometry for the determination of Dorzolamide Hydrochloride and Timolol Maleate.' *Journal of pharmaceutical and biomedical analysis.* Vol. 27(5) pp. 737-746.
7. Olivieri AC, Faber NM, Ferré J, Boqué R, Kalivas JH, Mark H. (2006) 'Uncertainty estimation and figures of merit for multivariate calibration (IUPAC Technical Report).' *Pure and applied chemistry*.Vol. 78(3) pp.633-461.
8. Haaland DM, Thomas EV.(1998) 'Partial least-squares methods for spectral analyses. 1. Relation to other quantitative calibration methods and the extraction of qualitative info'mation.' *Analytical Chememistry.* Vol. 60(11), pp.1193-1202.
9. Steves M. Short, Robert P. Cogdill, and Carl A. Anderson.(2007). 'Determination of Figures of Merit for Near-Infrared and Raman Spectrometry by Net Analyte Signal Analysis for a 4-Component Solid Dosage System.' *AAPS pharmaceutical science and technology.* Vol. 8 (4) Article 96, pp. E1–E11.
10. Kulkarni SP, Amin PD. (2000) 'Stability indicating HPTLC determination of Timolol Maleate as bulk drug and in pharmaceutical preparations. *Journal of pharmaceutical and biomedical analysis.*'vol. 23(6), pp.983-987.
11. Maguregui M, Jimenez R, Alonso R, Akesolo U. (2002) 'Quantitative determination of Oxprenolol and Timolol in urine by capillary zone electrophoresis.' *Journal of Chromatography A.* vol. 949(1), pp.91-97.
12. Sharma N, Rao SS, Reddy AM. (2012) 'A Novel and Rapid Validated Stability-Indicating UPLC Method of Related Substances for Dorzolamide Hydrochloride and Timolol Maleate in Ophthalmic Dosage Form.' *Journal of Chromatographic Science.vol.* 50 (9), pp. 745-755.
13. Kumar SS, Natraj K, Khan A, Kumar BK, Rao JV. (2011) 'Development and Validation of RP-HPLC Method for Estimation of Bimatoprost in Pharmaceutical Dosage Forms.' *Journal of Pharmacy Research* Vol. 4(10) pp. 1-2.
14. Nasir F, Iqbal Z, Khan A, Ahmad L, Shah Y, Khan AZ, et al.(2011) 'Simultaneous determination of Timolol Maleate, Rosuvastatin calcium and Diclofenac sodium in pharmaceuticals and physiological fluids using HPLC-UV.' *Journal of Chromatography B.* vol. 879(30), pp. 3434-343.
15. Gasco M, Gallarate M, Trotta M, Bauchiero L, Gremmo E, Chiappero O. (1989) 'Microemulsions as topical delivery vehicles: ocular administration of Timolol.' *Journal of pharmaceutical and biomedical analysis. Vol.* 7(4), pp. 433-439.
16. El-Laithy HM.(2009) ' Novel Transdermal delivery of Timolol Maleate using sugar esters: preclinical and clinical studies.' *European Journal of Pharmaceutics and Biopharmaceutics.* Vol. 72(1), pp. 239-245.
17. Lee V H, Li S Y, Sasaki H, Saettone M F, Chetoni P.(1994) 'Influence of drug release rate on systemic Timolol absorption from polymeric ocular inserts in the pigmented rabbit.' *Journal of Ocular Pharmacology & Therapeutics. Vol.* 10(2), pp. 421-429.
18. Rakić D, Antunović M. (2006) 'Preparation and testing of buffered eye drops containing pilocarpine chloride with Timolol Maleate.' *Vojnosanitetski pregled. Vol.* 63(10), pp. 873-877.

19. Sharif N, Ke T, Haggard K, Kelly C, Williams G, Graff G, et al.(2002) 'Bimatoprost Hydrolysis to 17-Phenyl PGF2alpha by Human and Rabbit Ocular Tissues and Agonist Activity of Bimatoprost and 17-Phenyl PGF2alpha.' *Investigative Ophtalmology and Visual Science.* Vol. 43(12), pp. 4080.
20. Shafiee A, Bowman LM, Hou E, Hosseini K.(2013),'Ocular pharmacokinetics of Bimatoprost formulated in DuraSite compared to Bimatoprost 0.03% ophthalmic solution in pigmented rabbit eyes.' *Clinical ophthalmology* (Auckland, NZ). Vol. 7, pp. 1549.
21. Marwada KR, Patel JB, Patel NS, Patel BD, Borkhatariya DV, Patel AJ.(2014), 'Ultraviolet spectrophotometry (dual wavelength and chemometric) and high performance liquid chromatography for simultaneous estimation of meropenem and sulbactam sodium in pharmaceutical dosage form.' *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy.* Vol. 124, pp. 292-299.
22. Rajput S J, George R K, Deepti B R. (2008) "Chemometric Simultaneous estimation of Clopidogrel Bisulphate and Aspirin from Combined Dosage Form.' *Indian Journal of Pharmaceutical Sciences,* vol. 70(4),Jul-Aug, pp. 450-454.
23. Damiani PC, Moschetti AC, Rovetto AJ, Benavente F, Olivieri AC.(2005) 'Design and optimization of a Chemometrics-assisted spectrophotometric method for the simultaneous determination of Levodopa and Carbidopa in pharmaceutical products.' *Analytica Chimica Acta.*, Vol. 543(1), pp. 192-198.
24. Ferraro MC, Castellano PM, Kaufman TS. (2004) 'Chemometric determination of amiloride hydrochloride, atenolol, hydrochlorothiazide and timolol maleate in synthetic mixtures and pharmaceutical formulations'. *Journal of Pharmaceutical and Biomedical Anaysis.* Vol. 34(2), pp. 305-314.
25. Martens H, Naes T, Multivariate Calibration plot, Wiley, New York, 1992, 3rd Edition, 116-158.
26. Mc Kay B, Hoogenraad M, Damen E, Smith A.(203) 'Advances in multivariate analysis in pharmaceutical process development.' *Current opinion in drug discovery & development.* Vol. 6(6), pp. 966-77.
27. Faber N.M. (1999), 'Notes on two competitng definitions of multivariate sensitivity.' *Analytica Chimica Acta*, vol . 381, pp. 103-110.
28. Kumar N, Bansal A, Sarma G, Rawal RK.(2014), 'Chemometrics tools used in analytical chemistry: An overview.' *Talanta.*, vol. 123, pp. 186-199.
29. Wise BM, Gallagher NB.(1996), 'The process chemometrics approach to process monitoring and fault detection.' *Journal of Process Control.* Vol. 6(6), pp. 329-48.
30. Kokot S, Grigg M, Panayiotou H, Phuong TD. (1998), Data interpretation by some common chemometrics methods. *Electroanalysis.* Vol. 10(16), pp. 1081-1088.
31. Esbensen KH, Hjelmen KH, Kvaal K. (1996) 'The AMT approach in chemometrics—first forays.' *Journal of Chemometrics*. Vol. 10(5 6), pp. 569-90.
32. Olivieri AC, Faber NM, Ferré J, Boqué R, Kalivas JH, Mark H. Uncertainty estimation and figures of merit for multivariate calibration (IUPAC Technical Report). Pure and applied chemistry. 2006;78(3):633-61.