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Comparative Multivariant Spectrophotometric Models for Contemporaneous Determination of Three Antiglaucoma Drugs in Different Matrices

Eman A Bahgat
Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Zagazig University, Zagazig, 44519, Egypt

Esraa S Ahmed (Corresponding Author)
Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Zagazig University, Zagazig, 44519, Egypt
Email: es2526940@gmail.com

Hanaa Saleh
Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Zagazig University, Zagazig, 44519, Egypt

Fathalla Belal
Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Mansoura University, Mansoura,35516, Egypt

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Abstract

The present research conveys an innovative, affordable, simple, and environmentally sustainable multivariate spectrophotometric method for the contemporaneous determination of timolol, brimonidine, and brinzolamide in their co-formulated pharmaceutical formulations and artificial aqueous humor. The studied drugs are widely prescribed either alone or in combination for the effective treatment of glaucoma to prevent the development of progressive glaucoma and visual neuropathy. The five multivariate models that were employed in the analysis were CLS, PCR, PLS, GA-PLS, and ANN. A five-level, three-factor experimental design was implemented to create an assortment of mixtures in the range between 4.0-16.0 µg/mL for timolol, 3.0-5.0 µg/mL for brimonidine, and 4.0-36.0 µg/mL for brinzolamide. The validity of the proposed methodology was evaluated through a set of diagnostic tools, including percentage recoveries, standard deviation, correlation coefficient, the root mean square error of prediction, and the root mean square error of calibration. For timolol, the study revealed that the root mean square errors of prediction for the CLS, PCR, PLS, GA-PLS, and ANN models were, respectively 0.086, 0.072, 0.066, 0.051, and 0.161. The equivalent brimonidine readings were 0.017, 0.031, 0.018, 0.016, and 0.063, respectively. While for brinzolamide, the corresponding findings for the five models were 0.188, 0.191, 0.0106, 0.123, and 0.307, respectively. These outcomes reveal how precise and accurate the generated models were. The reasonable sensitivity of the adopted method enables the analysis of studied medications in artificial aqueous humor. The environmental sustainability of the proposed methodology was assessed through diverse metrics including analytical eco-scale, AGREE, and GAPI.

Keywords: Multivariant spectrophotometry; Comparative study; Timolol, Brimonidine; Brinzolamide; Aqueous humor.

1. Introduction

Glaucoma is a prevalent eye disease that can cause permanent blindness if left untreated. It develops when the eye's natural fluid doesn't drain correctly, causing it to accumulate and raise the intraocular pressure (IOP). The optic nerve is harmed by this pressure and progresses to irreversible sight loss [1]. Lowering IOP with eye drops that work through a variety of mechanisms is the fundamental treatment for preventing progressive glaucoma visual neuropathy. Non-selective beta-blockers are a class of anti-hypertensive medications that are considered the conventional first-line treatment of glaucoma [2].

Timolol **(TIM) (Fig. 1a)** is the most common non-selective beta blocker that is prescribed for managing glaucoma. Additionally, it can be used orally in the management of hypertension, myocardial infraction, and angina pectoris [3]. The efficacy of timolol in lowering intraocular pressure (IOP) through the reduction of aqueous humor production and lowering blood pressure by blocking adrenergic receptors and decreasing sympathetic outflow makes it widely used as a monotherapy eye drop or in combination with other antiglaucoma agents, mainly brimonidine and brinzolamide, for the proper management of glaucoma [4, 5].

Brimonidine **(BRIM) (Fig. 1b)** is a highly selective alpha adrenergic agonist, which is used ophthalmically to lower IOP by reducing aqueous humor production and increasing uveoscleral outflow [6]. While brinzolamide **(BRIZ) (Fig. 1c)** is a selective reversible carbonic anhydrase II inhibitor, which effectively reduces IOP by inhibiting the formation of aqueous humor in the eye [7].

Numerous literatures have been reported for contemporaneous determination of TIM and BRIM in their pharmaceutical formulation including RP-HPLC methods [8-10] and spectrophotometric methods [11-13]. Other

literatures have been reported the assessment of TIM and BRIZ including RP-HPLC methods [14, 15] and spectroscopic methods [16, 17]. On research, it was noticed that there was only one reported RP-HPLC approach for the contemporaneous determination of the three studied drugs [18]. Despite the high sensitivity of the chromatographic method, it has several limitations, including the high cost, the need for multiple procedures, and time consuming [19]. Consequently, this research represents an innovative, simple, alternative, and cost-effective spectrophotometric method for simultaneous determination of TIM, BRIM, and BRIZ in their pharmaceutical formulations and artificial aqueous humor.

The use of direct UV-spectrophotometry in the assessment of multicomponent medications is challenged by the spectrum overlap, the scarcity of specificity, and being unable to utilize the complete spectral data. On the contrary, the utilization of chemometrics enables the incorporation of the entire spectra, resulting in more accurate and thorough information. Chemometrics involves the implementation of mathematical and statistical techniques to analyze data in an attempt to maximize the amount of valuable information that may be extracted. This technique requires the utilization of advanced instruments and the input of data into software for data analysis and resolution of strongly overlapping spectra, that make them accessible for quantitative analysis of active constituents in complex mixtures [20-22]. To carry out the quantitative chemometric analysis, a calibration set of known concentrations is analyzed, and the absorption data from 230 to 330 nm is gathered to create the mathematical models [CLS, PCR, PLS, GA-PLS, ANN]. Mathematical models are then employed to contemplate the concentration of the validation sample. In CLS, the absorptivity matrices [K] are figured through implementing multiple linear regression equations for each of the studied drugs. PCR and PLS have been regarded as the most widely used chemometric techniques, as they are capable of identifying spectra that overlap significantly. Additionally, they are impervious to noise and interference issues because of the decomposition steps. GA-PLS operates by limiting the initial spectra points to those that hold significant information with the goal to reduce the error in the built model [23-25]. For ANN, it is an information processing paradigm that is inspired by the way biological nervous systems such as the brain, process information. ANN consists of multiple layers of simple processing elements called neurons. The neuron performs two functions , namely, collection of input (spectral data at the selected wavelength range) and generation of an output (predicted concentration of the studied drugs for calibration and validation sets) [26].

The ultimate objective of this work is to establish an innovative, simple, green, and cost-effective multivariant chemometric technique for the contemporaneous assessment of TIM, BRIM, and BRIZ in their pure form, combined pharmaceutical formulation, and artificial aqueous humor.

2. Experimental

2.1. Materials and Reagents

- Timolol and brimonidine raw materials were gratefully donated by Egyptian International Pharmaceutical Industries Co. EIPICO (10th Ramadan City, Egypt), with a labeled purity of 99.29%, whereas brinzolamide raw material with a purity of 99.92% was kindly supplied by Novartis Pharma (Cairo, Egypt).
- Alphanova plus® a sterile ophthalmic solution containing 0.5 % TIM and 0.2 % BRIM with a batch number (0724205) manufactured by Orchidia Pharmaceutical Industries (Al-Obour City, Egypt), and AZARGA® an eye drops suspension containing 5 mg/mL TIM and 10 mg/mL BRIZ with a batch number (VFP81A) manufactured by Novartis Pharma (Cairo, Egypt) were utilized in the application study. All pharmaceutical formulations were purchased from the local pharmacy.
- Analytical grade methanol was obtained from Sigma Aldrich (Germany)
- Artificial aqueous humor was prepared by blending two portions: the first portion is a sterile solution (96 mL) containing 7.44 mg of sodium chloride, 0.395 mg of potassium chloride, 0.433 mg of dibasic sodium phosphate, 2.19 mg of sodium bicarbonate, and hydrochloric acid (to modify pH to approximately 7.4). The second portion is a sterile solution (4 mL) containing 4.6 mg of glutathione, 3.85 mg of calcium chloride dihydrate, 5 mg of magnesium chloride hexahydrate, and 23 mg of dextrose.

2.2. Instruments and Software

- A sonicator (model WUC-A06H, Witeg, Germany) was utilized to properly mix the standard solutions.
- Double-beam Shimadzu Spectrophotometer (model UV-1201, Kyoto, Japan) with 1 cm quartz cells was utilized for UV -spectrophotometric analysis.
- UV probe software version 2.43 was employed to get the spectral data of the studied drugs. MATLAB software version 8.2.0.701 (R2013b) was employed in the creation of models. PLS-toolbox software version 2.1. for PLS and GA-PLS, and MATLAB's Neural Network ToolboxTM were applied to the computation and analysis of data.
- Jenway pH-Meter (model 3510, UK) was used to modify the pH of artificial aqueous humor.

2.3. General Procedures

2.3.1. Standard solutions of TIM, BRIM, and BRIZ

In three distinct 50-mL volumetric flasks, three standard solutions (200.0 µg/mL) of each of TIM, BRIM, and BRIZ were made separately by sonicating 10.0 mg of each studied drug in 30 mL of methanol for five minutes. Volumetric flasks were then completed up to the mark using the same solvent.

2.3.2. Development of Chemometric Models

A five-level, three-factor experimental design [27] was implemented to create an assortment of twenty-five laboratory- prepared mixtures. These mixtures were made by filling a set of 10-mL volumetric flasks with the proper aliquots of TIM, BRIM, and BRIZ standard solutions, then adding methanol to complete the volume up to the mark. The final TIM, BRIM, and BRIZ concentrations were determined to be 4.0-16.0, 3.0-5.0, and 4.0-36.0 µg/mL, respectively. The central level of the design was composed of 10.0 µg/mL TIM, 4.0 µg/mL BRIM, and 20.0 µg/mL BRIZ, which correspond to their concentrations in their combined dosage forms. The prepared mixtures were displayed in **Table. 1**, where thirteen mixtures were utilized for calibration and twelve mixtures for validation. The absorption spectrum of the prepared mixtures was examined over the range of 230-300 nm with 1 nm intervals. UV-probe software was further utilized for data saving. The data saved were then employed for models' creation using MATLAB software.

2.3.3. Analysis of Alphanova Plus® and AZARGA® eye drops

Separately, 1.0 mL aliquots of each of Alphanova Plus® ophthalmic solution and AZARGA® ophthalmic suspension were transferred into 100-mL volumetric flasks and sonicated with 80 mL methanol. The volume was completed up to 100.0 mL with the same solvent to get a stock solution of 50 µg/mL and 20 µg/mL for each of TIM and BRIM, respectively, when regarding Alphanova Plus® ophthalmic dosage form. For AZARGA® eye drops, a stock solution of 50 µg/mL and 100.0 µg/mL for TIM and BRIZ, respectively, was attained. Serial dilutions were carried out by transferring aliquots of the stock solutions into 10-mL volumetric flasks and completing the volume with methanol to get mixtures of the required concentration. The process outlined under **Section 2.3.2** was implemented for chemometric analysis.

2.3.4 Analysis of the Studied Drugs in Artificial Aqueous Humor

Aliquots of TIM, BRIM, and BRIZ standard stock solutions were transferred into an assortment of 10-mL volumetric flasks containing 1 mL of the prepared aqueous humor. Once the mixtures were thoroughly mixed, methanol was added to attain the appropriate concentrations. The process outlined under **Section 2.3.2** was implemented for computation and data analysis.

3. Results and Discussion

Timolol is a non-selective beta blocker that is mostly co-administered with brimonidine or brinzolamide for the proper management of glaucoma and preventing the progressive glaucoma visual neuropathy [4, 5]. It was found that, there was only one reported RP-HPLC approach for the contemporaneous determination of the three studied drugs [18] while there was no spectrophotometric methods reporting their simultaneous determination. While chromatographic measurement is highly sensitive, the UV-spectrophotometric method is considered simpler and less time- consuming. The significantly overlapped spectra of the studied drugs prohibit their direct UV-spectrophotometric measurement **(Fig. 2)**. On the contrary, chemometric measurement enables the incorporation of the entire spectra of the studied medications to represent more accurate and thorough information [20]. This research represents an innovative, alternative, green, simple, economical multivariant chemometric measurement of TIM, BRIM, and BRIZ in their combined pharmaceutical formulations and artificial aqueous humor through the study of the five models, namely CLS, PCR, PLS, GA-PLS, and ANN.

3.1. Identification of Spectrum Zone and Development of Multivariant Chemometric Models

The caliber of multivariate analysis is dependent on the wavelength range and spectral mode employed. The spectrum region between 230 and 300 nm with a 1 nm interval was chosen, giving 71 spectral points. The spectral points before 230 nm were impacted by noise, and points above 300 nm showed poor absorbance readings, as shown in **(Fig. 2)**. Among the twenty-five mixtures that were made, thirteen mixtures were utilized to build the calibration models, and the other twelve were utilized for validation. The five models (CLS, PCR, PLS, GA-PLS, and ANN) were constructed using MATLAB software after the spectral data was introduced.

3.1.1. CLS Model Construction

Utilizing the absorbance matrices of the thirteen mixtures in the calibration set (13 × 71) and the associated concentration matrices (13 × 3), the absorptivity matrix (K matrix) for the CLS model was produced.

3.1.2 PCR and PLS Models Creation

Thorough understanding of the sample component being studied serves as crucial for the CLS model. On the contrary, only a limited comprehension of the component being studied is necessary for PCR and PLS models. This feature made PCR and PLS superior to CLS [28]. The most widely used inverse least squares methods for creating multivariate calibration models are PCR and PLS. It is presumed that the concentration matrix must be error-free, and that the spectrum matrix contains errors for PCR to function. Whereas in PLS, it is presumed that the distribution of error in the spectral and concentration matrices is identical. Consequently, PLS is considered more reliable than PCR since it can eliminate noise from both absorption and concentration matrices [29, 30].

Selecting the ideal number of latent variables was a crucial preconstruction stage in PCR and PLS. A thirteen calibration spectra were utilized to find the optimal number of variables using the cross-validation method of excluding one sample at a time [31]. The ideal was found to be three based on which latent variables in PCR and PLS models had the lowest prediction error values as shown in **Fig. 3**.

3.1.3. GA-PLS Model Creation

PLS is a chemometric model characterized by using full spectrum. Variable selection prior to multivariate calibration can enhance the PLS method's predictive power. Various techniques are available for selecting wavelength [32]. The GA model is the most intriguing of them. PLS regression can effectively be used in conjunction with GA to optimize the selection of wavelength across a wide range of spectral points [33]. Accordingly, GA is a great tool for resolving optimization issues based on the notions of genetic and natural selection. GA-PLS was applied on 71 variables, which declined the initial absorbance matrix to 40.84 %, 54.82 %, and 60.56 % for TIM, BRIM, and BRIZ, respectively. The GA settings that yielded the lowest mean square error are optimized and displayed in **Table. 2**. For the three medications under study, **(Fig. 4)** showed how GA was applied to the PLS model.

3.1.4. ANN Model Creation

There are various types of ANN, and all have their own specific strength. In this research, the feed-forward neural network model is applied, which incorporates three layers. The first layer represented the 71 spectral points and served as an input layer for independent variables. The second represented the hidden layer, where input was corrected and modified based on their weights. while the final one represented the output layer for dependent variables which reflected the predicted concentrations of the studied drugs. The ANN settings were modified till the output met the goal and had the fewest possible errors. The selected parameters were TRAINR for training function, LEARNGD for adaptation learning function, MSE for performance function, PURELIN for the transfer function. Additionally, each of the three drugs has four neurons in the hidden layers. The ANN performance and regression plots were displayed in **Figures 1S- 3S,** for TIM, BRIM, and BRIZ, respectively.

3.2. Model Validation and Comparative Study

The model prediction capabilities were assessed using a variety of techniques. Two diagnostic tools for the assessment are the root mean square error of prediction (RMSEP) and the root mean square error of calibration (RMSEC). **Tables 3** and **4** display RMSEP and RMSEC figures for each model, which demonstrate the high precision and accuracy of the created models. The validity of models was further assessed through the estimated predicted concentration, percentage recovery, and standard deviation, as shown in **Table. 3**. Furthermore, assessing the correlation coefficient (r) through graphing the actual concentration of the twelve-validation sample versus the predicted concentration was an additional diagnostic tool. The model's correlation coefficient figures were displayed in **Table. 4**, They were higher than 0.9934, indicating the good linear relationship and agreement between the actual and predicted concentration.

The five multivariant models were compared to ascertain the most reliable. RMSEC and RMSEP **(Figure 5)**, correlation coefficients (Table**. 4**,) and percentage recoveries (**Table. 3)** were all compared. The findings demonstrate that GA-PLS has the best percentage recoveries, the lowest RMSEC and RMSEP, and the highest correlation coefficients. As a consequence, using GA-PLS model improved the outcomes and can be regarded as the most effective model for the assay of studied drugs.

3.3 Analysis of the Studied Drugs in Their Co-formulated Dosage Forms

The five multivariant models were employed for the analysis of the studied drugs in their co-formulated dosage forms (Alphanova Plus® and AZARGA® eye drops). The low percentage RSD (less than 2), along with reasonable percentage recoveries in the range between 98.97 and 101.03 % "as indicated in **Table 5**. It shows that, the suggested methodology is reliable to assess the studied drugs in their corresponding co-formulated pharmaceutical formulations. Additionally, these results have been compared to those gathered using comparative methods [11, 17]. The Student *t*-test and the Variance Ratio F-test were utilized to statistically compare the findings of the proposed and comparative approaches [34]. The findings shown in Table 5 validate the appropriate accuracy and precision by demonstrating that, there are no disparities between the proposed and comparative approaches.

3.4. Analysis of the Studied Drugs in Artificial Aqueous Humor

The maximum concentration of each TIM and BRIZ in artificial aqueous humor is about 1 µg/mL, whereas for BRIM is about 0.5 µg/mL [35, 36]. The sensitivity of the adopted multivariant models was down to 0.46, 0.87, and 0.37 µg/mL for TIM, BRIZ, and BRIM, respectively. The results displayed in Table. 6 prove that the proposed methods could be successfully applied for analyzing the aforementioned drugs in artificial aqueous humor.

3.5. Assessment of the Proposed Methods Environmental Impact

Green chemistry has emerged as a key component of contemporary scientific research, that encourages the creation of environmentally friendly technologies for reducing harm to the environment and increasing ecosystem and human safety. Twelve sustainability-focused concepts, including waste minimization, the use of safer solvents and reaction conditions, energy- efficient equipment, and the automation level, serve as a framework for the green chemistry field. These guidelines serve as a basis for creating techniques that are efficient and sustainable. Diverse

greenness assessment metrics were utilized to evaluate the ecological acceptability of the proposed chemometric method. The analytical eco-scale [37] is a semi-quantitative greenness assessment tool that offers a framework to gauge how environmentally friendly analytical techniques are. It aims to mitigate the adverse effect of the thorough analytical process from sample preparation to analysis and waste disposal. The assessment is performed by allocating penalty points based on several factors such as instrument energy, waste amount and potential treatment, amount of reagent used and its hazard. The evaluation result is subsequently subtracted from the original value of 100.0. The proposed chemometric method provides an excellent green analysis with an entire score of 88.0 [Table. 7]. The analytical greenness metric approach (AGREE) [38] is an additional greenness evaluation metric whose primary virtue lies in its thorough examination of the twelve principles of Green Analytical Chemistry (GAC). The output is displayed as a circular pictogram with twelve rectangles, the color of each transition from dark green to red as the output score declines. The middle of the pictogram shows the overall score, which ranges from 0 to 1, demonstrating the degree of sustainability of the analytical approach. AGREE assessment score of the proposed analytical technique is about 0.68 with solely one red rectangle due to the use of non-bio-based reagent, demonstrating how environmentally friendly the proposed approach is **(Fig. 6a)**. The green analytical procedure index (GAPI) is a further assessment tool that offers a rapid and efficient summary of how ecologically sound each step of the analytical methodology is. It consists of a pictogram segmented into five pentagrams. Each pentagram evaluates a certain aspect of the analytical method, including method type, sample preparation, reagent utilized, and energy consumption. A three -level color system “green, yellow, and red” is utilized to reflect the degree of environmental impact: low, medium or high, respectively. **(Fig. 6b)** displays the proposed method GAPI chart, where prevalent colors are green and yellow with only three red sections (1-7-15) due to off-line collection, the usage of an amount of organic solvent, and untreated waste, confirming the minimal environmental impact.

4. Conclusion

The present research reports an innovative, affordable, and ecologically sustainable multivariate spectrophotometric method for simultaneous determination of TIM, BRIM, and BRIZ in their co-formulated pharmaceutical formulation, and artificial aqueous humor, confirming the proposed method sufficient sensitivity and selectivity. A comparative study was conducted among the five employed multivariant models. GA-PLS was found to be the most suitable model for the quantitative analysis of the cited drugs.

References

[1] Weinreb, R. N., Aung, T., and Medeiros, F. A., 2014. "The pathophysiology and treatment of glaucoma: a review." *Jama*, vol. 311, pp. 1901-1911.

[2] Lee, D. A. and Higginbotham, E. J., 2005. "Glaucoma and its treatment: a review." *American journal of health-system pharmacy*, vol. 62, pp. 691-699.

[3] 2009. "Brayfield A, Martindale. The complete drug reference. 36th ed. London: The Pharmaceutical Press; ."

[4] Frampton, J. E., 2006. "Topical brimonidine 0.2%/timolol 0.5% ophthalmic solution: in glaucoma and ocular hypertension." *Drugs & aging*, vol. 23, pp. 753-761.

[5] Syed, M. F. and Loucks, E. K., 2011. "Update and optimal use of a brinzolamide-timolol fixed combination in open-angle glaucoma and ocular hypertension." *Clinical Ophthalmology*, pp. 1291-1296.

[6] Adkins, J. C. and Balfour, J. A., 1998. "Brimonidine: a review of its pharmacological properties and clinical potential in the management of open-angle glaucoma and ocular hypertension." *Drugs & aging*, vol. 12, pp. 225-241.

[7] Iester, M., 2008. "Brinzolamide ophthalmic suspension: a review of its pharmacology and use in the treatment of open angle glaucoma and ocular hypertension." *Clinical Ophthalmology*, vol. 2, pp. 517-523.

[8] Elshanawane, A. A., Abdelaziz, L. M., Mohram, M. S., and Hafez, H. M., 2014. "Development and validation of HPLC method for simultaneous estimation of brimonidine tartrate and timolol maleate in bulk and pharmaceutical dosage form." *Journal of Chromatography & Separation Techniques*, vol. 5, p. 1.

[9] Desai, H. H. and Captain, A. D. "Validated RP-HPLC Method for the Simultaneous Estimation of Brimonidine Tartrate and Timolol Maleate."

[10] Mandan, S., Nerpagar, A., and Laddha, U., 2017. "DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR ESTIMATION OF BRIMONIDINE TARTRATE AS BULK DRUG AND IN OPHTHALMIC FORMULATION." *Journal of Drug Delivery & Therapeutics*, vol. 7, pp. 146-149.

[11] Popaniya, H. S. and Patel, H., 2014. "Simultaneous determination of brimonidine tartrate and timolol maleate in combined pharmaceutical dosage form using two different green spectrophotometric methods." *World J Pharm Pharm Sci*, vol. 3, p. 1330.

[12] Annapurna, M. M., 2018. "Multi component mode and derivative spectrophotometric methods for the simultaneous determination of timolol maleate and brimonidine tartrate." *Asian Journal of Pharmaceutics (AJP)*, vol. 12,

[13] Anwar, M. M., Kormod, L. A., Kessiba, A. M., Abbas, S. S., and El-Gendy, A., 2023. "Development and validation of spectrophotometric methods for the simultaneous determination of Brimonidine Tartrate and Timolol Maleate in bulk and ophthalmic preparations." *Records of Pharmaceutical and Biomedical Sciences*, vol. 7, pp. 97-114.

[14] Khatun, R. and Islam, S. A., 2014. "Development and validation of analytical method for simultaneous estimation of brinzolamide and timolol by HPLC from ophthalmic preparation." *International Journal of Pharmaceutical Sciences and Research*, vol. 5, p. 1001.

[15] El Deeb, S., Abdelsamad, K., and Parr, M. K., 2024. "Whiter and greener RP-HPLC method for simultaneous determination of dorzolamide, brinzolamide, and timolol using isopropanol as a sustainable organic solvent in the mobile phase." *Separations*, vol. 11, p. 83.

[16] Eissa, M., 2016. "Validated spectrophotometric methods for simultaneous determination of brinzolamide and timolol maleate in their pure form and ophthalmic preparation." *Al-Azhar Journal of Pharmaceutical Sciences*, vol. 54, pp. 188-202.

[17] Tambe, S., Das, S. S., Shahane, K., Singh, S. K., Ruokolainen, J., Amin, P., and Kesari, K. K., 2024. "First-order derivative spectrophotometric method for simultaneous determination of brinzolamide and timolol maleate in ophthalmic formulation." *Green Analytical Chemistry*, vol. 8, p. 100098.

[18] Ibrahim, F., Elmansi, H., and El Abass, S., 2019. "A versatile HPLC method with an isocratic single mobile phase system for simultaneous determination of anti-glaucoma formulations containing timolol." In *Annales Pharmaceutiques Françaises*. Elsevier. pp. 302-312.

[19] Dong, M., 2013. "The essence of modern HPLC: advantages, limitations, fundamentals, and opportunities." *LCGC Asia Pacific*, vol. 16, pp. 24–29.

[20] Santos, M. C., Nascimento, P. A. M., Guedes, W. N., Pereira Filho, E. R., Filletti, É. R., and Pereira, F. M. V., 2019. "Chemometrics in analytical chemistry–an overview of applications from 2014 to 2018." *Eclética Química*, vol. 44, pp. 11-25.

[21] Khoshayand, M., Abdollahi, H., Shariatpanahi, M., Saadatfard, A., and Mohammadi, A., 2008. "Simultaneous spectrophotometric determination of paracetamol, ibuprofen and caffeine in pharmaceuticals by chemometric methods." *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 70, pp. 491-499.

[22] Monakhova, Y. B., Mushtakova, S., Kolesnikova, S., and Astakhov, S., 2010. "Chemometrics-assisted spectrophotometric method for simultaneous determination of vitamins in complex mixtures." *Analytical and Bioanalytical Chemistry*, vol. 397, pp. 1297-1306.

[23] Attia, K. A., Serag, A., Eid, S. M., and Abbas, A. E. F., 2022. "A new chemometrically assisted UV spectrophotometric method for simultaneous determination of tamsulosin and dutasteride in their pharmaceutical mixture." *Journal of AOAC International*, vol. 105, pp. 1755-1761.

[24] Sharkawi, M. M., Mohamed, N. R., El-Saadi, M. T., and Amin, N. H., 2023. "Determination of bendamustine, gemcitabine and vinorelbine (BEGEV) regimen in spiked human plasma using multivariate model update chemometric methods." *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 299, p. 122836.

[25] Sayed, R. A., Ibrahim, A. E., and Sharaf, Y. A., 2021. "Chemometry-assisted UV-spectrophotmetric methods for the simultaneous determination of paritaprevir, ritonavir, and ombitasvir in their combined tablet dosage forms: A comparative study." *Journal of Chemometrics*, vol. 35, p. e3339.

[26] Dongare, A., Kharde, R., and Kachare, A. D., 2012. "Introduction to artificial neural network." *International Journal of Engineering and Innovative Technology (IJEIT)*, vol. 2, pp. 189-194.

[27] Brereton, R. G., 1997. "Multilevel multifactor designs for multivariatecalibration." *Analyst*, vol. 122, pp. 1521-1529.

[28] Thomas, E. V. and Haaland, D. M., 1990. "Comparison of multivariate calibration methods for quantitative spectral analysis." *Analytical Chemistry*, vol. 62, pp. 1091-1099.

[29] Hemmateenejad, B., Akhond, M., and Samari, F., 2007. "A comparative study between PCR and PLS in simultaneous spectrophotometric determination of diphenylamine, aniline, and phenol: Effect of wavelength selection." *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, vol. 67, pp. 958-965.

[30] Üstündağ, Ö., Dinc, E., Ozdemir, A., and Tilkan, M., 2015. "Comparative application of PLS and PCR methods to simultaneous quantitative estimation and simultaneous dissolution test of zidovudine-lamivudine tablets." *Acta Chimica Slovenica*, vol. 62,

[31] Kramer, R., 1998. *Chemometric techniques for quantitative analysis*: CRC press.

[32] Sasaki, K., Kawata, S., and Minami, S., 1986. "Optimal wavelength selection for quantitative analysis." *Applied spectroscopy*, vol. 40, pp. 185-190.

[33] Lucasius, C. B., Beckers, M. L., and Kateman, G., 1994. "Genetic algorithms in wavelength selection: a comparative study." *Analytica Chimica Acta*, vol. 286, pp. 135-153.

[34] Mishra, P., Singh, U., Pandey, C. M., Mishra, P., and Pandey, G., 2019. "Application of student's t-test, analysis of variance, and covariance." *Annals of cardiac anaesthesia*, vol. 22, pp. 407-411.

[35] Orii, Y., Kunikane, E., Yamada, Y., Morioka, M., Iwasaki, K., Arimura, S., Mizuno, A., and Inatani, M., 2022. "Brimonidine and timolol concentrations in the human vitreous and aqueous humors after topical instillation of a 0.1% brimonidine tartrate and 0.5% timolol fixed-combination ophthalmic solution: An interventional study." *Plos one*, vol. 17, p. e0277313.

[36] Kadam, R. S., Jadhav, G., Ogidigben, M., and Kompella, U. B., 2011. "Ocular pharmacokinetics of dorzolamide and brinzolamide after single and multiple topical dosing: implications for effects on ocular blood flow." *Drug metabolism and disposition*, vol. 39, pp. 1529-1537.

[37] Gałuszka, A., Migaszewski, Z. M., Konieczka, P., and Namieśnik, J., 2012. "Analytical Eco-Scale for assessing the greenness of analytical procedures." *TrAC Trends in Analytical Chemistry*, vol. 37, pp. 61-72.

[38] Wojnowski, W., Tobiszewski, M., Pena-Pereira, F., and Psillakis, E., 2022. "AGREEprep–analytical greenness metric for sample preparation." *TrAC Trends in Analytical Chemistry*, vol. 149, p. 116553.

Table-1. Concentrations of twenty-five mixtures of TIM, BRIM, and BRIZ utilized in calibration and validation sets.

Sample number	TIM (µg/mL)	BRIM (µg/mL)	BRIZ (µg/mL)
1[a]	10	4	20
2[a]	4	4	4
3	16	3	4
4	7	3	12
5	16	5	12
6	10	3.5	36
7[a]	7	20	5
8[a]	7	4	12
9	13	3.5	12
10	16	3.5	28
11[a]	13	4.5	36
12	10	5	28
13[a]	16	4.5	20
14[a]	16	4	36
15	4	5	36
16	13	5	4
17[a]	4	3	28
18	10	4.5	4
19[a]	13	3	20
20	13	4	28
21[a]	7	4.5	28
22	4	4.5	12
23[a]	7	3.5	4
24[a]	10	3	12
25	4	3.5	20

[a] concentration of mixtures employed in the validation set.

Table-2. The GA settings yielding the lowest mean square error.

Parameters	TIM	BRIM	BRIZ
Population size	38	38	38
The number of variables in a window (window width)	2	2	2
% Wavelengths used at initiation	50%	50%	50%
Maximum generation	32	32	32
Percent of population the same at convergence	100%	100%	100%
Mutation rate	0.005	0.005	0.005
Maximum number of latent variables	3	3	3
Number of subsets to divide data into for cross-validation	4	4	4
Number of iterations	2	2	2
Cross-validation	Random	Random	Random
Crossover type	Double	Double	Double

Table-3. Prediction recoveries of five multivariant chemometric models generated for the validation set.

Mix No.	Actual conc.(µg/mL)	TIM % recoveries					Actual conc.(µg/mL)	BRIM % recoveries					Actual conc.(µg/mL)	BRIZ % recoveries				
		CLS	PCR	PLS	GA - PLS	ANN		CLS	PCR	PLS	GA - PLS	ANN		CLS	PCR	PLS	GA - PLS	ANN
1	10	100.76	101.16	100.07	99.80	101.04	4	100.26	99.23	99.92	100.05	99.56	20	99.88	100.26	100.03	99.36	97.20
2	4	97.77	99.75	98.78	100.77	99.03	4	100.50	100.27	100.75	99.73	98.16	4	102.25	96.04	98.78	99.01	98.13
7	7	99.00	99.57	99.78	99.60	100.56	5	100.66	99.40	99.76	100.41	101.83	20	99.80	99.20	99.48	99.85	98.64
8	7	98.71	100.67	99.35	99.82	98.52	4	99.51	99.50	99.26	100.75	98.29	12	98.92	100.38	99.62	100.62	97.01
11	13	99.85	100.23	99.82	101.12	100.27	4.5	100.29	100.06	100.23	100.24	100.31	36	99.69	99.31	99.81	99.48	99.90
13	16	100.27	100.75	99.34	99.87	97.36	4.5	99.78	99.33	100.45	99.76	101.30	20	101.00	101.05	99.32	101.63	98.09
14	16	99.13	99.50	99.97	100.30	98.50	4	99.25	99.75	100.52	99.47	100.56	36	98.78	98.83	99.99	99.95	99.22
17	4	102.56	100.48	100.87	100.55	101.76	3	99.91	101.00	98.98	101.01	96.37	28	101.04	100.32	100.48	99.90	99.82
19	13	100.92	100.96	99.91	99.70	100.77	3	99.43	99.77	99.62	99.58	97.00	20	100.50	100.75	99.44	100.27	99.07
21	7	98.43	99.29	102.12	99.94	101.62	4.5	99.56	100.16	99.75	99.95	100.61	28	100.11	100.82	100.53	99.75	97.98
23	7	100.98	99.00	100.64	100.44	98.47	3.5	100.03	100.26	99.97	100.24	101.60	4	101.25	101.75	101.12	101.38	99.53
24	10	99.81	99.63	101.07	100.51	98.82	3	99.67	99.52	100.36	100.46	98.65	12	102.00	100.92	101.72	100.63	98.55
Mean		99.76	100.38	100.27	100.20	99.73		99.82	99.85	99.97	100.14	99.53		100.43	99.79	100.03	100.15	98.60
SD		1.31	0.96	0.84	0.48	1.44		0.55	0.51	0.53	0.47	1.81		1.11	1.44	0.82	0.79	0.95
%RSD		1.31	0.96	0.84	0.48	1.44		0.55	0.51	0.53	0.47	1.82		1.11	1.44	0.82	0.79	0.96
RMSEP		0.08	0.10	0.06	0.05	0.17		0.04	0.05	0.03	0.01	0.06		0.19	0.18	0.11	0.10	0.31

Table-4. Statistical performance data of the five multivariant models created.

Parameters	TIM					BRIM					BRIZ				
	CLS	PCR	PLS	GA - PLS	ANN	CLS	PCR	PLS	GA - PLS	ANN	CLS	PCR	PLS	GA - PLS	ANN
Wavelength* range (nm)	230 – 300 nm														
Linearity range (µg/mL)	4.0 – 16.0					3.0 – 5.0					4.0 – 36.0				
RMSEC	0.06	0.07	0.09	0.04	0.09	0.04	0.03	0.03	0.02	0.03	0.15	0.14	0.13	0.07	0.15
Latent variable number (LV)		3	3	3			3	3	3			3	3	3	
Mean (%)	100.23	100.34	99.54	100.20	100.13	100.49	100.33	100.39	100.11	99.78	100.32	100.16	99.92	99.94	99.89
RSD (%)	0.99	0.79	1.12	0.62	0.96	1.15	0.59	0.43	0.41	0.69	0.87	1.03	0.94	0.63	1.69
R**	0.9995	0.9997	0.9998	0.9999	0.9984	0.9993	0.9994	0.9992	0.9994	0.9934	0.9997	0.9999	0.9999	0.9999	0.9989
Slope**	1.01	1.01	0.99	0.99	0.99	1.01	0.98	1.01	0.99	0.99	0.99	0.99	0.99	0.99	1.00
Intercept**	-0.02	-0.04	0.05	0.001	0.009	-0.03	0.05	-0.02	0.01	0.08	0.17	0.06	0.003	0.09	-0.02

*The selection is based on trial-and-error method based on RMSEC.
**Computed for the validation set actual and predicted concentrations.

Table-5. Analysis of studied drugs in their co-formulated dosage forms using the proposed and comparative methodology.

Method	**Preparation**									
		Mean (%)		**SD**		**N**	**Student *t*- test (2.22)***		**F-test (5.05)***	
		TIM	BRIM	TIM	BRIM		TIM	BRIM	TIM	BRIM
Reported method [11]	Alphanova Plus® TIM + BRIM	100.60	100.30	1.20	0.75	6	-	-	-	-
CLS		99.55	100.35	1.46	1.04	6	1.59	0.10	1.48	1.92
PCR		100.00	100.03	0.47	1.18	6	1.20	0.51	4.71	2.48
PLS		100.26	100.13	0.7	0.95	6	0.64	0.43	2.94	1.11
GA-PLS		100.37	100.22	0.68	0.72	6	0.43	0.21	2.25	1.09
ANN		99.30	100.09	0.79	0.81	6	2.40	0.53	2.31	1.17
Method	Parameter									
		Mean (%)		SD		N	Student *t*- test (2.26)*		F-test (6.26)*	
		TIM	BRIZ	TIM	BRIZ		TIM	BRIZ	TIM	BRIZ
Reported method [34]	AZARGA® TIM + BRIZ	101.26	101.28	0.63	0.48	6	-	-	-	-
CLS		100.31	100.59	1.00	0.49	5	1.76	1.94	2.52	1.04
PCR		100.57	100.53	0.67	0.56	5	1.54	1.99	1.13	1.36
PLS		100.41	100.07	0.48	0.92	5	2.18	2.02	2.05	3.67
GA-PLS		101.78	100.38	0.29	0.49	5	0.26	1.68	1.24	1.04
ANN		100.38	100.25	0.53	0.97	5	2.15	1.98	1.41	4.08

*The figures in the parentheses represent the corresponding theoretical values of Student *t* and F at (p=0.05)

Table-6. Application of the proposed methodology for the measurement of studied drugs in artificial aqueous humor.

Actual conc. (µg/mL)	TIM % recoveries					Actual conc. (µg/mL)	BRIM % recoveries					Actual conc. (µg/mL)	BRIZ % recoveries				
	CLS	PCR	PLS	GA - PLS	ANN		CLS	PCR	PLS	GA - PLS	ANN		CLS	PCR	PLS	GA - PLS	ANN
4	99.50	98.83	99.60	101.37	100.82	3	99.07	99.11	99.07	99.03	100.01	4	100.62	100.56	100.58	102.09	102.68
7	99.34	98.49	99.57	101.32	100.88	3.5	99.43	100.43	101.11	100.23	101.73	12	99.45	101.14	101.84	101.25	99.83
10	101.55	101.42	100.46	99.49	101.61	4	102.28	102.07	100.26	100.25	99.60	20	100.61	99.62	99.51	99.72	99.65
13	100.84	100.71	101.38	99.56	99.35	4.5	99.65	100.69	100.32	100.45	100.28	28	99.83	98.20	98.66	99.32	100.41
16	99.33	99.45	98.18	101.79	99.64	54	100.59	99.27	98.97	99.24	99.33	36	100.40	100.69	100.67	100.63	99.66
Mean (%)	100.11	99.78	99.84	100.71	100.46		100.20	100.32	99.94	99.84	100.19		100.18	100.04	100.26	100.60	100.44
SD	1.02	1.25	1.18	1.09	0.93		1.28	1.20	0.91	0.65	0.93		0.52	1.17	1.21	1.12	1.28
RSD (%)	1.02	1.25	1.18	1.09	0.93		1.28	1.19	0.91	0.65	0.93		0.52	1.17	1.21	1.12	1.27

Table-7. Eco-Scale for assessing the greenness of the proposed methods.

Parameter	**Penalty points (pp_s)**
Methanol	6
Instrument	
Energy (Spectrofluorimetry: ≤0.1 KWh per sample)	0
Occupational hazards (analytical process hermitization)	0
Waste (1-10 mL, no treatment)	6
Total penalty points.	∑18
Analytical eco-scale total score[a] [b], Excellent green analysis	88

[a] Analytical Eco-Scale total score = 100 – total penalty points

[b] If the score >75, it represents excellent green analysis. If the score is >50, it represents acceptable green analysis. If the score is <50, it represents inadequate green analysis.

Figures

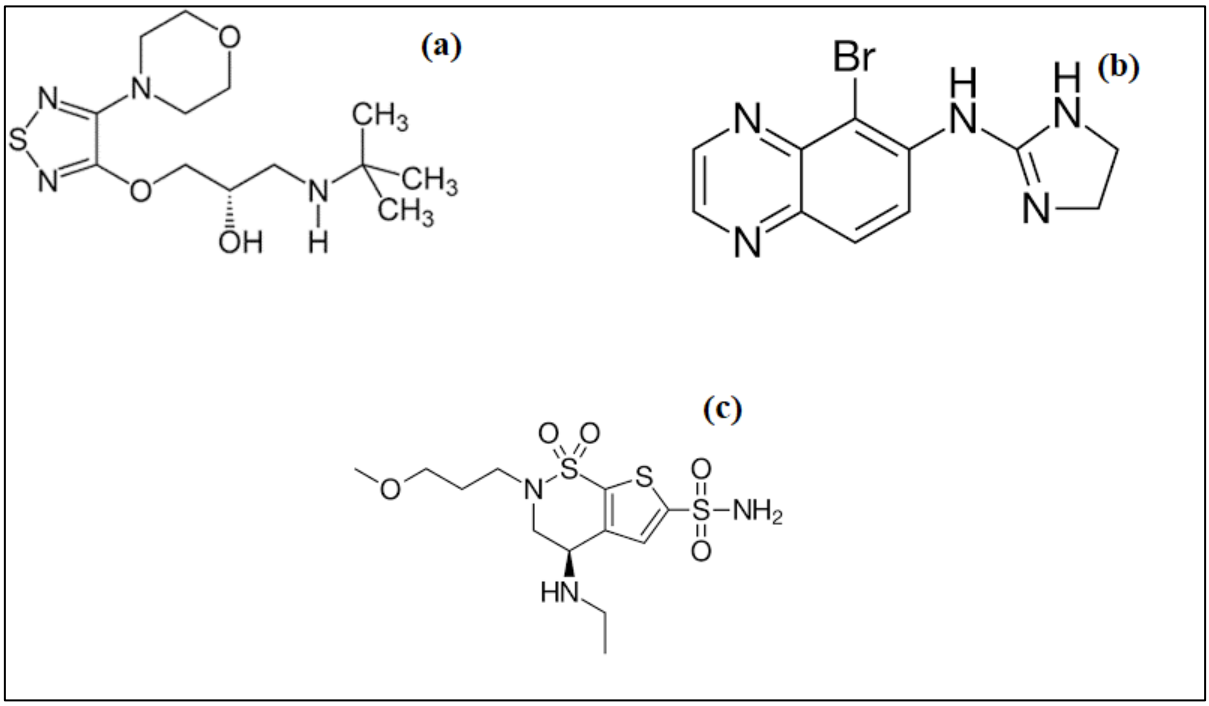

Fig. 1. The chemical formula of (a) TIM, (b) BRIM, and (c) BRIZ.

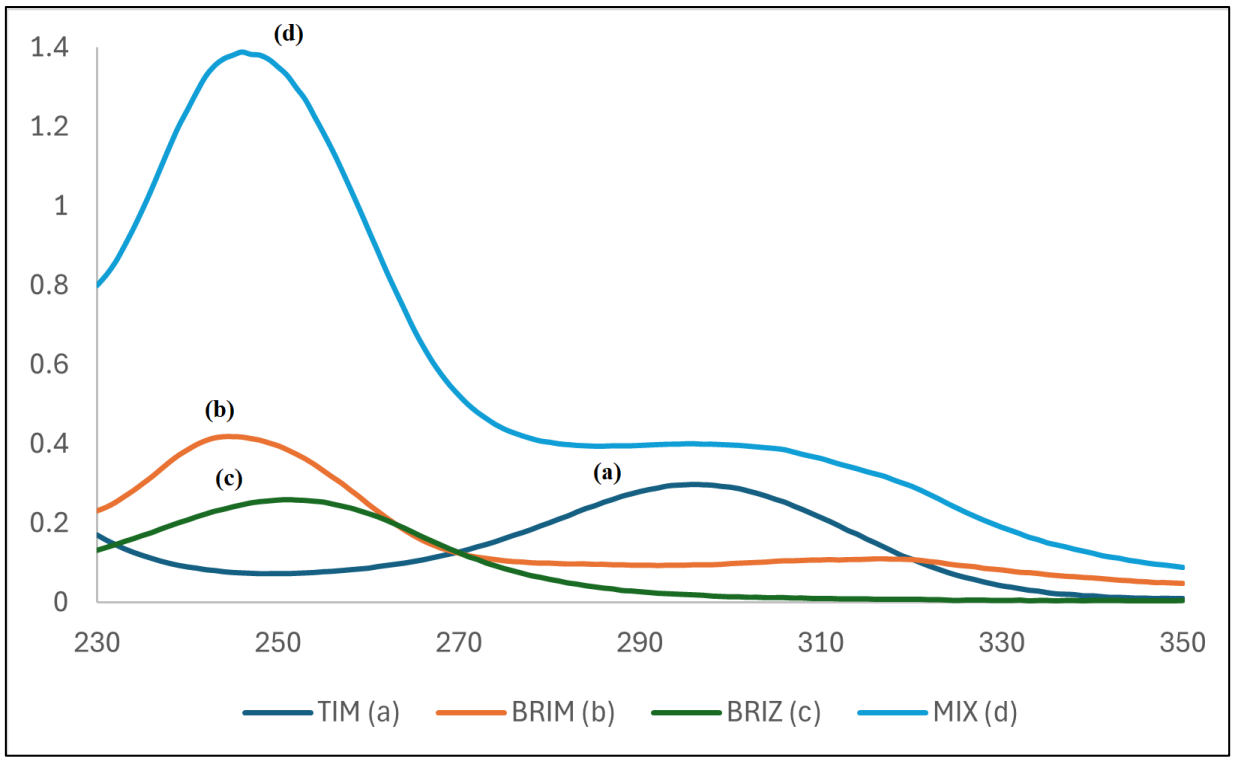

Fig. 2. Zero-order spectra of (a) TIM, (b) BRIM (, (c) BRIZ, and (d) their mixture.

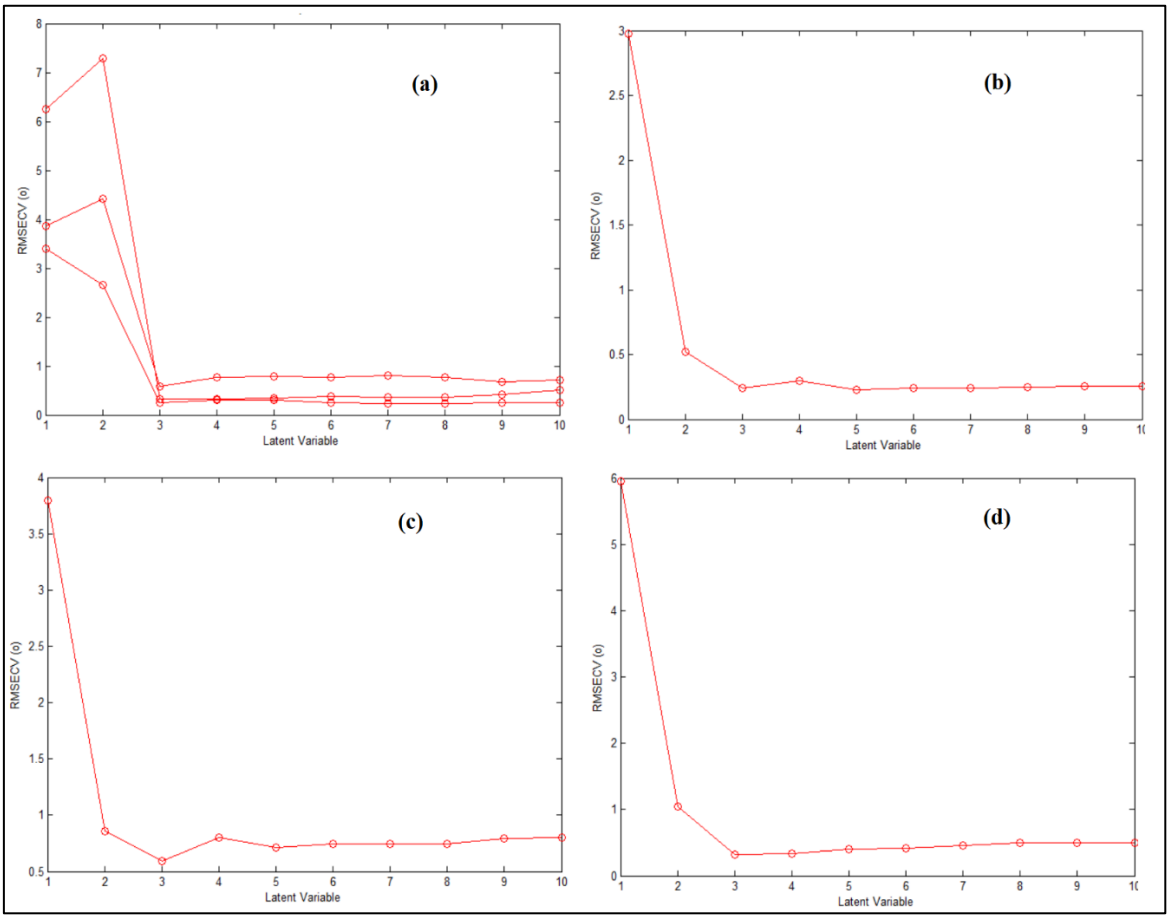

Fig. 3 Plots of cross-validation for (a) PCR, (b) PLS for TIM, (c) PLS for BRIM, (d) PLS for BRIZ.

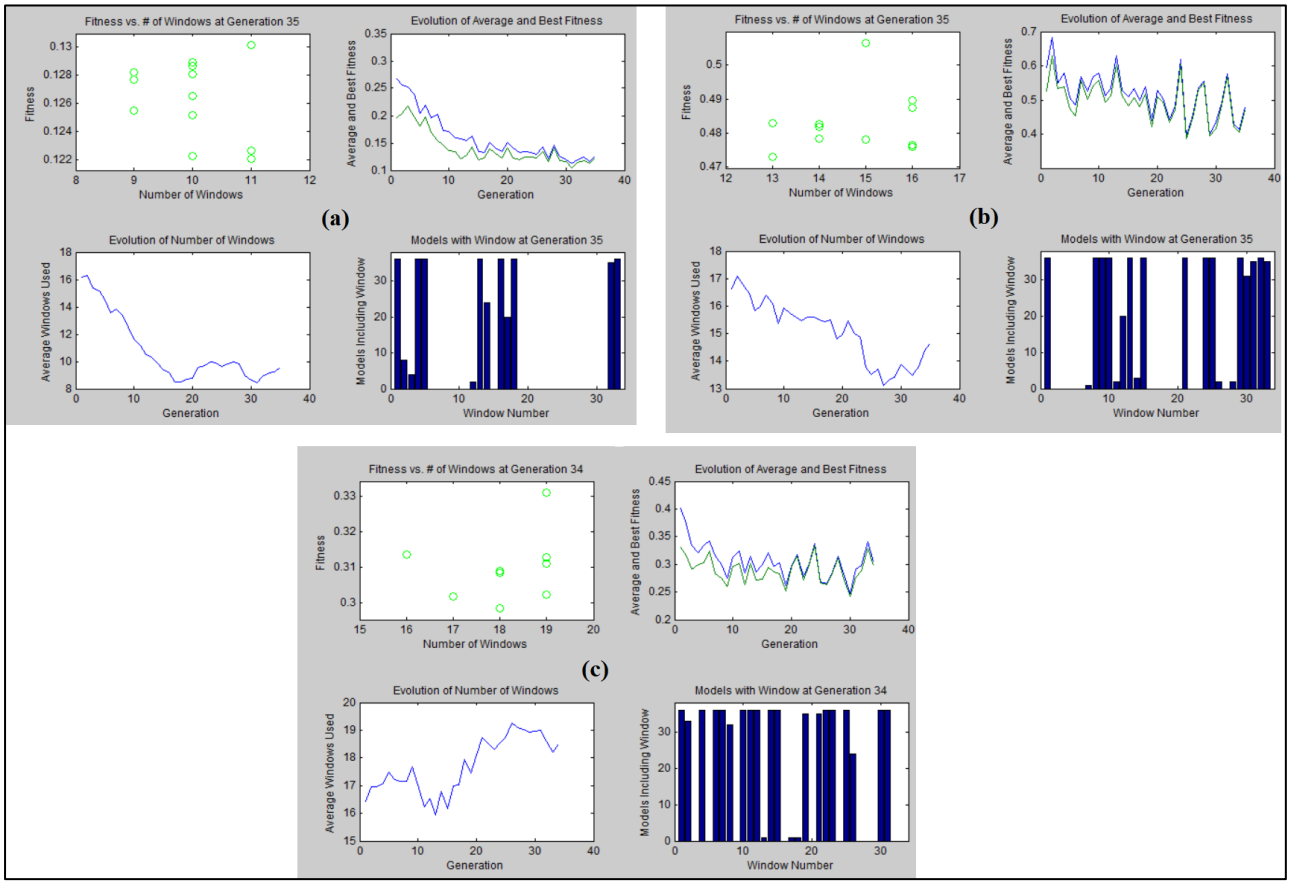

Fig. 4. The entire set of parameters used when applying the genetic algorism (GA) to the partial least square (PLS) model for (a) TIM, (b) BRIM, (c) BRIZ).

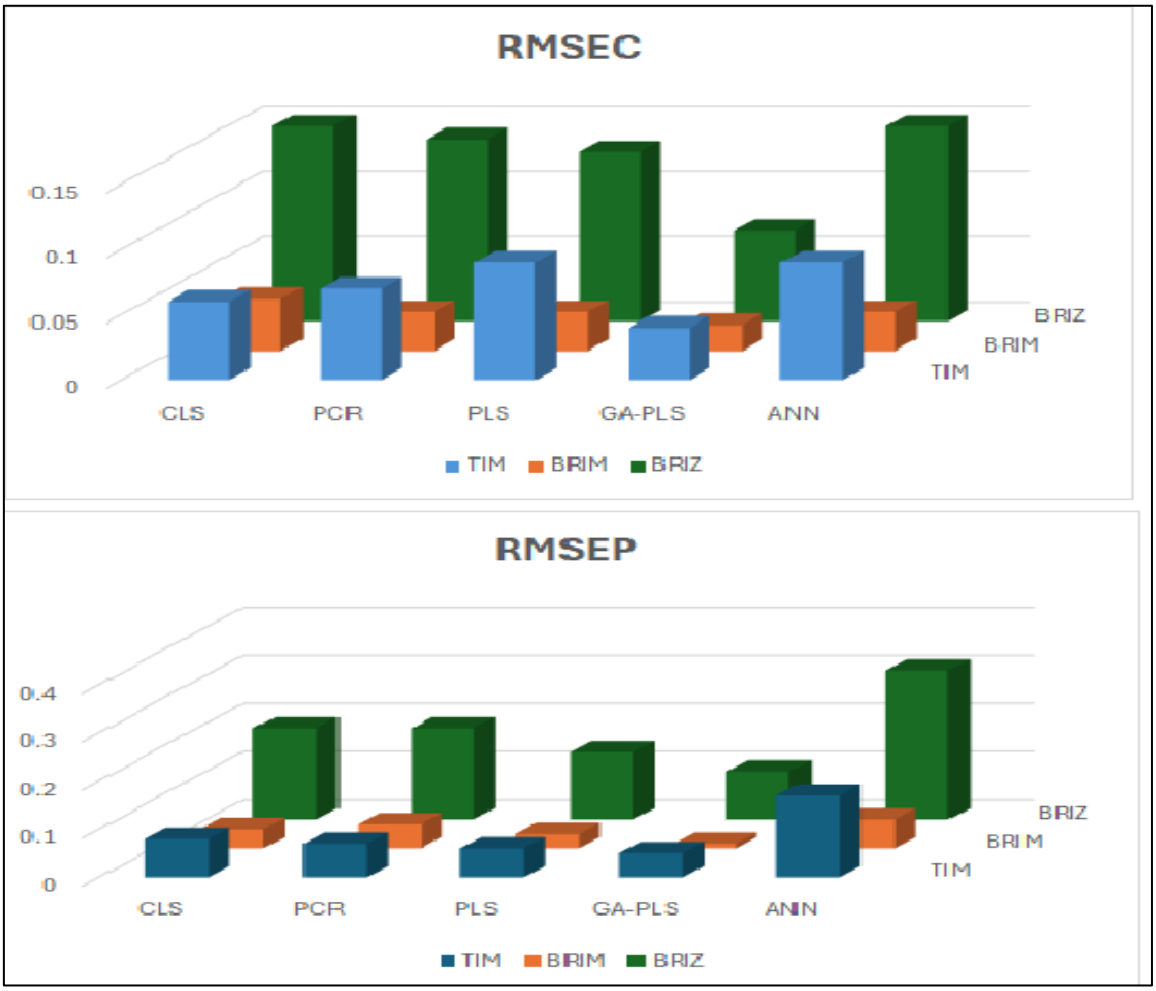

Fig. 5. The Root Mean Square Error of calibration (RMSEC) and Root Mean Square Error of Prediction (RMSEP) calculated for each drug**.**

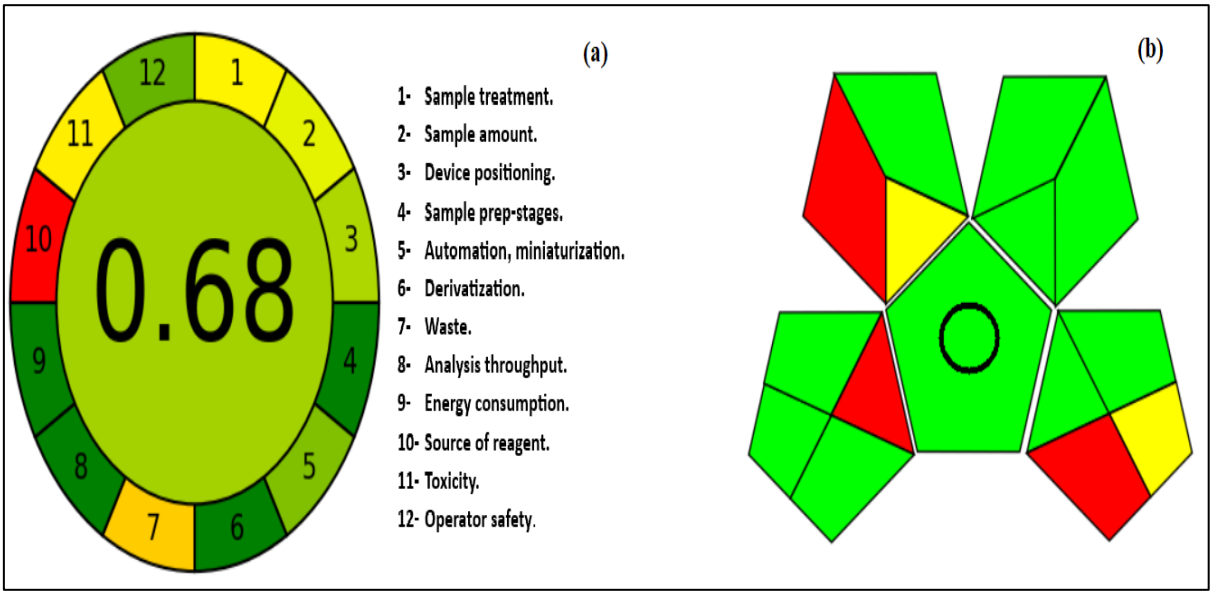

Fig. 6. The analytical greenness metric (AGREE) (a), and the green analytical procedure index (GAPI) (b) for evaluating the ecological suitability and applicability of the adopted multivariant spectrophotometric method.