Estimation of pitavastatin and ezetimibe using UPLC by a combined approach of analytical quality by design with green analytical technique

HEMANTH KUMAR CHANDULURU and ABIMANYU SUGUMARAN

SRM College of Pharmacy, SRM Institute of Science and Technology, Kattankulathur, 603203, India

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ABSTRACT

The current study explores a design and development of the simple, fast, green and selective novel method of UPLC to quantify pitavastatin and ezetimibe simultaneously. The combined approach of Green Analytical Method with Quality by Design-based risk assessment was done using the Ishikawa fishbone diagram followed by a rotatable central composite design used for the optimization. The optimal chromatographic separation was attained through a mobile phase of 72: 28% v/v ethanol and 0.1% orthophosphoric acid (pH 3.5), with a 0.31 mL min$^{-1}$ flow rate. The developed UPLC-PDA method was sensitive and specific for pitavastatin and ezetimibe, with linearity ranging from 2 to 10$^{-150}$ mg L$^{-1}$ with an R$^2$ of 0.9999 and 0.9997, respectively. The forced degradation study of stability-indicating assay results shows the degradation in respective stress conditions. The developed UPLC method was validated and found to have sensible results with good linearity, accuracy and precision. Further, the greenness was evaluated using five states of art metrics like NEMI, GAPI, AES, AMGS, and AGREE metrics and found the greenest results. Based on the results we concluded that the developed UPLC method could be efficient for the simultaneous determination of pitavastatin and ezetimibe in bulk and tablet dosage.

KEYWORDS

analytical quality by design, ezetimibe, green analytical technique, pitavastatin, UPLC

INTRODUCTION

Pitavastatin (PST) also known as nisvastatin, itabavastin, or itavastatin. PST is a dihydroxy monocarboxylic acid chemically, (3R,5S,6E)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoic acid (Fig. 1) [1–3]. A statin class potentially lowers the cholesterols by inhibiting 3-hydroxy-3-methyl glutaryl-coenzyme A (HMG-CoA) reductase. In the De novo synthesis of cholesterol, conversion of HMG-CoA to Mevalonate using HMG-CoA reductase is a committed step. However, PST acts on this enzyme and lowers cholesterol synthesis.

Ezetimibe (EZB) is chemically (3R,4S)-1-(p-fluorophenyl)-3-[(3S)-3-(p-fluorophenyl)-3-hydroxypropyl]-4-(p-hydroxyphenyl) azetidinone (Fig. 1) [4]. Used to treat the diseases like homozygous familial hypercholesterolemia, homozygous sitosterolaemia and primary hyperlipidaemia. EZB lowers cholesterol by averting absorption from dietary sources and blocking cholesterol transport via the intestinal wall.

PST and EZB co-administered due to their synergistic effect for treating primary hypercholesterolemia and homozygous familial hypercholesterolemia if a single statin failed to control the level of cholesterol [5].

There is a snowballing alarm over the death-defying effect of various solvents on the environment. Analytical methods for drug analysis also comprise toxic and hazardous
solvents with an immense volume of generated waste. Pharma industries are now initiating their nods to adopt an eco-friendly analytical method for drug development and analysis. A faster chromatographic approach like ultra-performance liquid chromatography (UPLC) can enhance greener performance by decreasing solvent consumption and waste generation per single analysis. Using miniaturized techniques like UPLC covers 11 out of 12 green analytical principles [6, 7] (except solvent-free analysis).

Further, applying Green Analytical Chemistry (GAC) concepts without disturbing process performance needs a closer inspection of multiple parameters is essential, and the correlation between them needs to be well understood. Implementing Analytical Quality by Design (AQbD) can resolve this problem [8–13]. Adoption of AQbD provides a good advantage in developing a method (i) It saves time, chemicals and waste generated (ii) Method can transfer directly (iii) Re-validation is not required.

AQbD works on statistical and experimental designs follows five different steps (i) Defined analytical target profile (ATP) – outcome of the method, (ii) Determination of critical quality attributes (CQAs) – method attributes and method parameters. (iii) Risk assessment – increases the system’s effectiveness and compliance with the ATP. (iv) Identification of a method operable design region or design space (DS) – the relationship between factors affecting a process and its output (v) control strategy and management of product lifecycle – guarantee the robustness of the method. Combining the GAC concepts with an AQbD approach serves as a synergistic forum for designing environmentally sound, efficient and flexible approaches.

Literature survey for PST and EZB revealed several methods based on varied techniques like spectrophotometry [14, 15], HPLC [16, 17], LCMS [18, 19], are available for individual drugs, and very few methods are available in combinations which showed a tedious effect on the environment. Upon further considerations, the goal of this work was to conduct a new framework for implementing GAC principles in tandem with AQbD principles. This combined framework was used for the first time to develop a green and robust UPLC study of the two drugs in their bulk and marketed formulation.

**EXPERIMENT**

**Chemicals and reagents**

Ethanol (HPLC grade - Union Drug & Chemical Company-China), HPLC grade water -Milli Q, orthophosphoric acid (HPLC grade -MERCK), pure PST and EZB (Glenmark Pharmaceuticals Ltd., Mumbai-India). Marketed formulations available as Ezenon-P4 tablets (2 mg PST and 10 mg EZB), manufactured by Indinon Pharma and purchased from the Indian resident pharmacy.

**Chromatographic system and conditions**

Isocratic separation was executed on Agilent UPLC-PDA system (1290 infinity II LC system) equipped by a binary Infinity II High-Speed pumping system, online degasser, autosampler LC injector and photodiode array (PDA) detector. Kinetex phenyl hexyl (50 × 4.6 mm, 2.6 µm) column was used. The optimized mobile phase contains ethanol and orthophosphoric acid (3.5 pH) with a proportion of 72: 28 (v/v). The mobile phase was degassed and sonicated for 15 min before use. Filtration was done using 0.45-µm Nylon filter membrane and 0.22-µm one-time use syringe filters for mobile phase and samples. Quantification was performed at 0.31 mL min⁻¹ flowrate and 240 nm wavelength.

**Software-aided method development and optimization**

Peak analysis was operated with Empower-2 software; Method optimization was done using Design-Expert® (Stat-Ease Inc., Minneapolis-USA).

**Preparation of orthophosphoric acid solution (OPA)**

1.0 mL orthophosphoric acid (OPA) was accurately placed into a 1000 mL volumetric flask containing 900 mL of milli-Q water. Further the pH was adjusted to 3.0, 3.5 and 4.0 using water. Finally, the solutions were filtered and degassed by sonication.

**Preparation of stock and calibration standards**

The standard stock solutions of PST and EZB (1 mg mL⁻¹) were prepared separately in ethanol. Then, using the respective mobile phase as a diluent, several dilutions for constructing calibration curves were obtained (5–30 and

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**Fig. 1. Structure of pitavastatin (a) and ezetimibe (b)**
20–150 µg mL⁻¹) from their respective standard stock solution. A mixture of PST and EZB were prepared by transferring various aliquot part through the standard stock solution, and the volumes were made up with the mobile phases to get a required concentration.

Pharmaceutical formulation analysis

Ten Ezenon-P4 tablets weighed precisely and grind finely. A weighed quantity of powdered tablets, equivalent to 10 mg PST and 50 mg EZB, transferred into a 50 mL volumetric flask and solubilized in 25 mL ethanol and sonicated for 15 min, make up to mark with ethanol and filtered over Whatman filter paper. An aliquot part from the sample solution was transferred into a 25 mL volumetric flask and diluted using a mobile phase. The final concentrations of the solutions obtained were 20 and 100 µg mL⁻¹ for PST and EZB. Triplicate injection of aliquot 5 µL, followed by an estimation of the concentration of the drug in its dosages with the above-described procedures.

Solution stability

PST and EZB standard sample solutions were checked for stability over three days at 35 ± 2°C. The percentage assay value for the analyzed standard samples was compared to that of freshly prepared samples.

Forced degradation studies

Test solutions (10 and 50 µg mL⁻¹) of PST and EZB were exposed to different conditions like 0.1 M HCl, 0.1 M NaOH, 3% H₂O₂, and UV light, and the extent of degradation was analyzed with respect to time of exposure. The percentage of drug degradation was calculated by using the formula as:

\[
\% \text{ degradation} = \frac{\text{Area of unstressed} - \text{area of stressed}}{\text{area of unstressed}} \times 100
\]  

RESULTS AND DISCUSSION

Numerous approaches for analyzing drugs in different matrices have been developed in pharmaceutical analysis; however, most such procedures suffer from difficulties in method transfer, requiring constant re-validation, health and ecological issues. Previous methods developed for the estimation of PST and EZB in the combination and with other drug combinations were utilized the toxic chemicals for the estimation without employing the AQbD technique, the details of the mobile phase used, and the greenness calculated for those methods were depicted in Table 1. As a result, new movements aim at applying a trio combination of UPLC, AQbD and GAC concepts in a single system have emerged to improve long-term viability and robustness. The five steps in the AQbD framework helps understand method variables and their interactions, identify factors that significantly impact process performance, and assign the acceptable limits of their variance. This article provides a detailed description of AQbD based on GAC principles for the application of analytical methods. Instead of using traditional approaches, a combined trio technique was used on a drug combination that enlightens its applicability in other drug analysis.

Analytical target profile and CQA’s

Entire analytical methods comprise ideal quality properties demarcated in ATP. ATP aimed to design simple, fast, green, and cost-effective method to analyze PST and EZB in quality control (QC) laboratories. The need for extremely stable approaches through a high level of consistency in process transfer was also considered. The UPLC-PDA analytical technique was used to accomplish these goals due to its numerous advantages, such as reliability, robustness, and simplicity. CQAs are quantifiable variables that replicate method performance. Extended retention time (RT) was found in early studies with the second compound, imposing close attention on these peaks’ retention. As a result, the most significant Critical Analytical Attributes (CAA) were chosen with a resolution (Rs), retention factor (K’), and retention time of the second peak (RT2) as factors to consider.

\[
\text{Retention factor} = \frac{(\text{Tr} - \text{To})}{\text{To}} \quad (2)
\]

- \(\text{Tr} = \) Retention time of first analyte eluted
- \(\text{To} = \) First baseline disturbance by the mobile phase
- If \(\text{Tr} = \) To specify retention factor is zero (0), it shows co-elution of the analyte and solvent which shows there is no interaction with the stationary phase, which generates inaccurate results.

Risk assessment or scouting phase

Risk Identification was made by using Ishikawa Fishbone Diagram. A risk assessment is considered a general ideology of different parameters on different responses without performing any experiments. This method explores the cause-effect of the experimental conditions; thus, much less effort and time are consumed, while further optimization processes can accelerate. Ethanol was used instead of other toxic solvents as an organic modifier to comply with the GAC principles. GAC principles prompted the need for UPLC, rather than other instruments, to resolve the high solvent consumption and allow for fast and greener analysis. Wavelength at 240 nm providing the highest response for both the compounds and selected for the detection. Isocratic elution was chosen due to its ease and reduced time-consuming procedures rather than using the gradient elution technique.

pH scouting trials have carried to determine the drug’s activity within the permissible pH range. Therefore, the mobile phase was made via combining ethanol and OPA at
various pH levels (3.0–4.0) in various organic phase ratio of ethanol (65–75) and pumping it at a flow rate at a range of 0.25–0.35 mL min⁻¹. Further, this factor was then used in optimization to know more about their effects.

**Experimental design**

Method optimization using rotatable central composite design. The three critical method parameters (CMP) were subjected to experimental study using rotatable central composite design (rCCD) to determine the maximum optimal state of the combination while defining its DS for robust analysis. Based on preliminary research, levels of each parameter were allocated and attain the optimal values with the chosen CAAs. The high ethanol percentage (75%), low pH at 3, and high flow rate (0.4 mL min⁻¹) values improved peak sharpness but negatively impacted peak resolution. Moreover, increasing peak broadening with run time and a reasonable resolution was reached on low ethanol (60%), high pH 4 and flow rate (0.3 mL min⁻¹).

As a result, the combination was tested using three separate ethanol % ranges (a, a, a), pH (a, a, a) and flow rates (a, a, a). A series of twenty experiments were conducted in chronological order, including five centre points for successful experimental error prediction, and the corresponding Retention factor (k'), Resolution (Rs), and retention of second peak (RT2) values were reported. The generated frameworks were then analyzed, and it was

| S.No | Developed by | Chromatographic Conditions | NEMI | GAPI | AES | AGREE |
|------|--------------|---------------------------|------|------|-----|-------|
| 1    | Hiral Panchal et al. | HPLC, 0.1% orthophosphoric acid: acetonitrile: triethylamine | ![Diagram](image1) | ![Diagram](image2) | 8 + 1 + 3 | 0.71 |
| 2    | Ramzia I. El-Bagary et al. | LCMS, ATR, EZB 0.2% formic acid in water–acetonitrile (30:70, v/v) | ![Diagram](image3) | ![Diagram](image4) | 8 + 3 + 3 | ![Diagram](image5) |
| 3    | Hiral J. Panchal et al. | HPLC, 0.1% orthophosphoric acid–acetonitrile–triethylamine (19.8 + 80 + 0.2, v/v/v) | ![Diagram](image6) | ![Diagram](image7) | 8 + 1 + 3 | 0.71 |
| 4    | Ramadevi et al. | LC-MS/MS, 0.1% formic acid and 60:40 acetonitrile | ![Diagram](image8) | ![Diagram](image9) | 8 + 3 + 3 | 0.67 |
| 5    | Proposed method | UPLC Ethanol and orthophosphoric acid (3.5 pH) (72:28) | ![Diagram](image10) | ![Diagram](image11) | 3+ 0 + 0 | 0.89 |

AES = PP for pictograms + Instrument energy + occupational hazard + Wastage.
found that the method follows a second-order polynomial equation.

**Statistical models validation.** ANOVA results, Lack of fit non-significance, reasonable standards of R², adjusted and predicted R² was then used to ensure the statistical viability of the regression models for the three responses. Furthermore, residual diagnostic plot analysis confirms the random scattering of residuals and natural distribution.

**Interpretation and derringers desirability function.** The effects of CMPs on each CAA were studied using graphical data interpretation with Perturbation, contour and 3D surface plots.

Effect of ethanol, flow rate and pH on the Retention factor: The model’s F-value of 142.7 with a P-value of <0.0001 indicates that the model is significant. The 3D (Fig. 2), Perturbation and contour plots and second-order polynomial equation for K’ demonstrate that increase in ethanol concentration and flow rate negatively affects the K’, indicating that PST elutes faster as the ethanol and flow rate increases. The pH as the third factor positively impacts the K’ as the increase in pH increases the K’ value. The polynomial equation for the quadratic model of K’ was represented below

\[
\text{retention factor } K’ = -65.4098 + 1.1489*\text{Ethanol Ratio} + 151.535*\text{Flow Rate} + 8.6675*\text{pH} + -1.9563*\text{Ethanol Ratio}*\text{Flow Rate} + -0.063639*\text{Ethanol Ratio}*\text{pH} + -12.9636*\text{Flow Rate}*\text{pH} + -0.00320309*\text{Ethanol Ratio}^2 + 38.8029*\text{Flow Rate}^2 + -0.0203094*\text{pH}^2
\]

Effect of ethanol, flow rate and pH on the retention of the second peak: The model for RT2 was significant with an F-value of 360.23 and P-value <0.0001. The 3D, Perturbation and contour plots (Fig. 2) and second-order polynomial equation showed an increase in ethanol and pH concentration, a steep increase in Rs. The flow rate has shown significantly less effect on the Rs. The increase in flow rate decreases the RT2 is a minimal effect. The polynomial equation for the quadratic model of retention time was represented below.

\[
\text{Resolution} = 4.37455 + 0.0116034*\text{Ethanol Ratio} + -18.3786*\text{Flow Rate} + 1.5911*\text{pH} + 0.0683443*\text{Ethanol Ratio}*\text{Flow Rate} + -0.0174746*\text{Ethanol Ratio}*\text{pH} + 0.797584*\text{Flow Rate}*\text{pH} + 0.00040561*\text{Ethanol Ratio}^2 + 16.7456*\text{Flow Rate}^2 + -0.0414842*\text{pH}^2
\]

The main objective was to develop an AQBD based method without compromising the green analytical principles, mainly waste reduction using UPLC, which should not affect validation guidelines. With a limit of K’ value 2 to 10 for a reasonable chromatogram, ethanol ratio, flow rate and pH were maintained that had not caused any significant impact on the K’ as per guidelines. Following RT2, although there are no limits, considering the principles of GAC, the delayed RT2 was taken into consideration. Finally, the Resolution has a limit, and it should be >1.5. The rationale behind the selection of a higher resolution was to distinguish the degraded peaks with the main PST and EZB peaks. These three responses were interlinked and showed a need for a sensible method that does not alter the other responses. Derringer’s desirability was used to find the best set of conditions based on the importance and constraints of each response. The desirability method illustrated the attainment of specific objectives within limits imposed, and a specific experimental region was explored for compositions whereby constraints set were reached to the limit, i.e., cohesion. The optimum RP-UPLC chromatographic conditions were chosen finally as mobile phase ethanol and OPA 72.28 v/v, flow rate 0.318 mL min⁻¹, and pH 3.5, resulting in retention factor (Y1) 7.62 ± 0.032, EZB retention period (Y2) 1.919 ± 0.001, and resolution (Y3) 6.186 ± 0.11 min. Control Strategy was evaluated with the predicted experimental conditions and studied experimentally from the DS. Then the parameters for the system suitability were calculated, and their values found to be in the optimal range and less than 2%.

**Validation of the proposed method**

**System suitability parameters.** After six repeat injections, the system suitability results indicated a lack of significant difference in the CAAs like K’, RT2, and Rs of PST and EZB. The % RSD values were found to be less than 2%, indicating that the chromatographic instrument has a high degree of accuracy.

**Solution stability.** PST and EZB standard sample solutions were checked for stability over three days at 35 ± 2°C. The percentage assay value for the analyzed standard samples was compared to freshly prepared samples shows within limits with an acceptable % RSD of <2.
**Linearity and sensitivity study.** The calibration curve was observed to have a linear value of 0.9999 and 0.9997 (Fig. 4) for PST and EZB, respectively, with a good regression coefficient ($R^2$), which is less than the specified limits (2%). The method’s LOD and LOQ were obtained theoretically as 0.0192, 0.0584, and 0.0076, 0.023 μg mL$^{-1}$ and practically executed for PST and EZB. Further, the method was analyzed by Mandel’s, IUPAC, and the Lack of fit test by applying the obtained linearity results [20]. The Lack of fit results showed that the calculated F value was less than the critical F value, indicating that the method was significant with the null hypothesis. The Mendel’s H, K, and IUPAC tests show the results were within the boundary, and the interpretation suggests the F critical was less than the F

![Fig. 2. Contour, perturbation and 3D plots for $K'$ (a), RT2 (b), and Rs (c)](image-url)
calculated in the linear regression and indicates the model fits for quadratic with no bias. The overall obtained results are depicted in Table 2.

**Table 2. Application of Mandel’s and Lack of fit for the proposed method**

| Parameters | PST | EZB |
|------------|-----|-----|
| sy^2       | 12732482 | 8800493 |
| F          | 10512 | 13077.71 |
| **Quadratic** sy^2 | 6758704 | 1.13E+08 |
| F          | 4.535457 | 0.125781 |
| F exp IUPAC | 0.883864 | −0.21855 |
| F exp Mandel | 4.535457 | 0.125781 |

F exp = Expected F value; SSLF = Sum of Square Lack of Fit; SSPE = Sum of Square Pure error, F critical value = F table value.

**Precision.** The precision has calculated at three different concentrations, and the data are displayed in Table 3. The % RSD for inter-day and Intra-day precision was within limits for PST and EZB.

**Accuracy.** The PST and EZB drug substances was added to the placebo at 80, 100, and 120% of the sample concentration, respectively, and evaluated using the proposed UPLC-PDA technique. PST and EZB recovery were found to be 99.1%–100.94% and 99.9% to 101.52%, respectively. Each of the individual outcomes for PST and EZB was within 98.0–102.0% criterion. Table 3 shows the accuracy results.

**Robustness.** The method’s robustness was tested by making deliberate adjustments to critical parameters such as ethanol composition, flow rate, and pH (within the established DS). There was no significant variance observed. Such results ensured the methods’ robustness and versatility as they were transferred to other laboratories and used on other UPLC systems.

**Specificity.** Two sets of laboratory-prepared mixtures with various ratios were analyzed for specificity. PDA peak-purity

**Table 3. Validation results for PST and EZB**

| Parameters                  | PST          | EZB          |
|-----------------------------|--------------|--------------|
| **Linearity**               |              |              |
| Range (µg mL⁻¹)             | 5–30         | 25–150       |
| Slope**                     | 3057.62 ± 0.27 | 10579.13 ± 0.29 |
| Intercept**                 | 37232.34 ± 17.87 | 123850.75 ± 24.65 |
| Correlation coefficient     | 0.9999       | 0.9997       |
| STEYX                       | 16391.5      | 46485.73     |
| LOD (µg mL⁻¹)               | 0.0192       | 0.0076       |
| LOQ (µg mL⁻¹)               | 0.0584       | 0.0233       |
| **Accuracy**                |              |              |
| Mean ± SD***                | 100.02 ± 0.92 | 100.71 ± 0.81 |
| Alpha                       | 0.05         | 0.05         |
| n                           | 9            | 9            |
| CI                          | 0.6042       | 0.5323       |
| **Precision**               |              |              |
| Mean ± SD*                  | 99.18 ± 0.57 | 99.79 ± 0.847 |
| % RSD                       | 0.580        | 0.849        |
| Alpha                       | 0.05         | 0.05         |
| n (observations)            | 18           | 18           |
| CI for intraday             | 0.1803       | 0.7964       |
| CI for inter-day            | 0.266        | 0.392        |
| **System suitability parameters** |          |              |
| No. of theoretical plates   | Mean ± SD**  | 3957.8 ± 23.96 | 4036.8 ± 18.63 |
| % RSD                       | 0.61         | 0.46         |
| Retention Time              | Mean ± SD**  | 0.8556 ± 0.01 | 1.919 ± 0.01 |
| % RSD                       | 0.59         | 0.42         |
| Peak area                   | Mean ± SD**  | 340426 ± 188.54 | 1173493.8 ± 793.04 |
| % RSD                       | 0.06         | 0.07         |
| Retention factor            | Mean ± SD**  | 7.624 ± 0.03 | -          |
| % RSD                       | 0.35         | -            |
| Resolution                  | Mean ± SD**  | 1.08 ± 0.01 | 1.04 ± 0.02 |
| % RSD                       | 1.37         | 1.85         |

*Mean of six determinations, **Set of five determinations, *** set of three determinations.
evaluation (Fig. 4) determined that the peaks were well resolved with no intervention, and successful recoveries were achieved. Table 3 summarises various validation parameters.

**Forced degradation studies.** Hydrolysis at different pH: The acid-degraded sample chromatogram showed peaks at Rt of 0.667, 1.364 for acid degradation at 3 and 6 h for PST. Approximate 10.75 and 29.75% of degradation were recorded when subjected to 0.1 M HCl conditions. PST has been susceptible to acid hydrolysis, while EZB has no acid degradation. An extra peak was found in the chromatogram of the base-degraded sample at an Rt of 0.386, 1.267 in EZB. Basic stressed samples have shown that PST was stable in basic, but the EZB was hydrolyzed in basic condition with degradation of 5.06 and 43.40% in 5–60 min exposure to 0.1 M NaOH.

Peroxide and photolytic degradation: PST has been prone to degrade in both conditions, but the degradation rate was considered very slow, were as the degradation of EZB was not observed in these conditions. On exposing to 3% peroxide solution for 1 h, PST has undergone a degradation of 0.21% and shown a tiny spike at 0.499 min. On exposing to UV light for 6 h and having degradation of 4.05% of PST. The complete overlay of all degradations was portrayed in Fig. 3 and resulted in Table 4.

**Assay of marketed formulation**

The developed UPLC method’s applicability was demonstrated by analyzing their marketed pharmaceuticals (Fig. 4). The mentioned drugs (PST and EZB) were calculated selectively with good recovery values in their mixture.

**Table 4. Forced degradation studies of PST and EZB**

| S.No | Description  | Drug peak area | % Recovery (Assay) | % Total Degradation | Mass ratio |
|------|--------------|----------------|--------------------|---------------------|------------|
|      |              | PST            | EZB                | PST                 | EZB        |          |
| 1    | Acid 3h      | 303725         | 1168968            | 89.25               | 100.00     | 10.75    | 0.00     | 100.00   |
| 2    | Acid 6h      | 239061         | 1168968            | 70.25               | 100.00     | 29.75    | 0.00     | 100.00   |
| 3    | Alkali 5 min | 340308         | 1109762            | 100.00              | 94.94      | 0.00     | 5.06     | 100.00   |
| 4    | Alkali 1 h   | 340308         | 661665             | 100.00              | 56.60      | 0.00     | 43.40    | 100.00   |
| 5    | Peroxide 0min| 340308         | 1168968            | 100.00              | 100.00     | 0.00     | 0.00     | 100.00   |
| 6    | Peroxide 1 h | 339592         | 1168968            | 99.79               | 100.00     | 0.21     | 0.00     | 100.00   |
| 7    | Photo 6 h    | 326512         | 1168968            | 95.95               | 100.00     | 4.05     | 0.00     | 100.00   |
Furthermore, using the standard addition method, the extraction effectiveness and excipients intervention were investigated. Results show that good recoveries were achieved, and there was no interference from common tablet excipients. Statistical comparison of the suggested method has acquired results method and the reported ones for evaluating each of the studied substances, Student’s $t$-test and F-test were used (Table 5). None of the values obtained was higher than the tabulated values, indicating insignificant variations in the methods’ accuracy and precision.

### Table 5. Statistical comparison of proposed and reported method

|                      | Proposed method | Reported method |
|----------------------|-----------------|-----------------|
| Mean ± SD            | 99.636 ± 0.16   | 99.44 ± 0.19    |
| Variance             | 0.0508333       | 0.037433        |
| Observations         | 3               | 3               |
| Pooled Variance      | 0.0441333       |                 |
| Df                   | 4               |                 |
| $t$ Stat             | 1.127117        |                 |
| P (T ≤ t) one-tail   | 0.1613646       |                 |
| t Critical one-tail  | 2.1318468       |                 |

**F-test two-sample for variances**

| F                    | 1.35796972 |
|----------------------|------------|
| P(F ≤ f) one-tail    | 0.42409366 |
| F Critical one-tail  | 19         |

Eco-friendly method assessment

GAC has proposed many approaches for creating sustainable analytical approaches. Among those strategies, the 3R ideologies [21] (Replace, Reduce, Reuse), toxic solvents were replaced with eco-friendly and more cost-effective ones are diminished if their function is unnecessary. Using shorter columns with high pressure and rapid examination is another viable way of improving UPLC sustainability. By applying such methods, the proposed approach effectively quantified the PST and EZB in their combination. Compared to prior existing techniques for each drug combination, the proposed method effectively used greener ethanol rather than hazardous methanol or acetonitrile. The proposed UPLC method makes negligible use of solvents and energy. All of these characteristics established the proposed technique as an environmentally friendly than the reported one. The greenness profile was assessed using five state-of-the-art metrics, namely NEMI, GAPI, Analytical Eco-Scale, AMGS, and AGREE metrics, which state that a process eco-friendly.

A circular pictogram divided into four quadrant colours coded with green [22]. (1st quadrant) The solvent used in the process was not listed in the persistent, bioaccumulative, and toxic (PBT)-list. (2nd quadrant) substances used in the process was not listed in the Environmental Protection Act [23] (EPA), Toxicity Reactivity Ignitability (TRI) list of Hazardous substances. (3rd quadrant) non-corrosive (pH 2–12). (4th quadrant) for the produced waste is below 50 mL g$^{-1}$.
Figure 5 shows the NEMI pictogram for the proposed method.

GAPI concentrates mainly on two aspects: sample preparation and instrumentation assessment [24]. Depending on the method used, the colour coding like green, yellow and red was represented. The sample preparation should not include any preservation, transport, or storage to create an environmentally friendly process. The process should be inline and avoid the use of any solvents. Extraction steps should avoid, if not either by using green solvents can be accepted without any additional treatments or using low solvent consumption. The reagents used should be less toxic and safe, according to National Fire Protection Act (NFPA) [25]. Instrumental aspects like using low energy consumption and waste-producing instruments to preferred. Applying these steps in the GAPI pictogram (Fig. 5) showed no red zone, and very few yellow zones and rich green zones indicate this method was greenest.

AES [26, 27] represented a score of 100. Penalty point (PP) was calculated and subtracted from the method using five different steps; it was calculated as follows.

- **The amount of reagent** used was less than 10 mL per analysis. So, 1 PP for this step. Then total PP for the amount has calculated as amount PP × Hazard PP.
- **Hazard PP** calculated based on pictograms that represent the nature of reagents. Pictograms were categorized into two based on the severity of the hazard, such as Danger (severe hazard) and Warning (less hazard); In our method, the ethanol and OPA represents a danger symbol with two and one pictograms, respectively. Considering this, the method has three penalty points multiplied by the amount PP (1) gives 3 PP.
- **Energy** consumption for UPLC is ≤0.1 kWh per sample, so no penalty/0 PP.
- **Occupation hazard** carries 0 PP due to usage of ethanol and water.
- **Wastage** in this method was less than 1 mL so no penalty/0 PP.
- Finally, the total PP lost for the method is 3 + 0 + 0 + 0 = 3
- Analytical Eco-score = 100 - PP = 97.
- The method with a score of 75 considered greener; the proposed method got a score of 97, which indicates the impact of the method on future usage with environmental friendliness.

AMGS is a semi-quantitative method for determining environmental sustainability [28]. AMGS is a combination of several metrics, including SHE (safety, health, and environmental assessment), which is used for solvent safety calculation via geometric mean, AMVI [29] (Analytical
Mass Volume Intensity), which is used for calculating solvent volume waste, and CED (Cumulative Energy Demand), which is used for both instrument and solvent selection. When the required information was entered into the system, it produced a score of 49.38 (Fig. 5), which should stand as low as possible to make the method eco-friendly, and a high score in solvent EHS, suggesting that the solvent usage should reduce to make the method slightly greener [30].

The AGREE metrics were calculated using software that operates on 12 parameters, corresponding to the 12 GAC principles [31–33]. Each principle or parameter has a score range of 0–1 determined based on the hazard to a specific principle’s greenness. The AGREE tool portrays greenness as a classic clock shape with numbers 1–12 on the circle’s side, reflecting the theory of 12 principles. The proposed method’s overall score of 0.89, as seen in Fig. 5, shows that it was the greenest in all aspects of green principles.

**CONCLUSION**

A novel green and extremely robust analytical methodology were designed based on a pioneering amalgamation of the AQbD method and GAC principles. We have effectively developed a green QbD method that effectively estimates the two drugs, PST and EZB, in the bulk and marketed formulation. A detailed stepwise description of the AQbD method was implemented to provide an actual image of method variables and create stable and robust methods that can be effectively applied to QC laboratories without the need for further re-verification. For statistical optimization studies, experimental designs such as rotatable central composite were used. The obtained results established the optimal working conditions for a mix of drugs within the DS, which were practically validated with additional runs. Ethanol-based mobile phases were used instead of harmful organic solvents like methanol and acetonitrile. Furthermore, shorter columns with the finest particle-sized columns at high pressure in UPLC enabled a 4-min analysis time and high throughput analysis. Finally, the results obtained by the green assessment tools also proved that the method was most eco-friendly and can easily adaptable for industrial and quality control purposes.

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