




AKADÉMIAI KIADÓ

Acta Chromatographica

33 (2021) 3, 216-227

DOI:
10.1556/1326.2020.00783
© 2020 The Authors

Optimization and validation of Eco-friendly RP-HPLC and univariate spectrophotometric methods for the simultaneous determination of Fluorometholone and Tetrahydrozoline hydrochloride

HEBATALLAH M. ESSAM, MARTIN N. SAAD* ,
EMAN S. ELZANFALY and SAWSAN M. AMER

Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, El-Kasr El-Aini Street, 11562, Cairo, Egypt

Received: April 9, 2020 • Accepted: May 28, 2020
Published online: July 20, 2020

ORIGINAL RESEARCH
PAPER



ABSTRACT

A sensitive RP-HPLC method is presented for the simultaneous quantification of Fluorometholone (FLM) and Tetrahydrozoline hydrochloride (THZ). The method has the advantages of being rapid, accurate, reproducible, ecologically acceptable and sensitive. The separation utilized C₈ Xbridge[®] column and mobile phase mixture of Acetonitrile/phosphate buffer pH 3 ± 0.1 (70:30, v/v) with UV detection at 230 nm. Stepwise optimization and factors affecting separation are properly discussed. Different factors were optimized such as stationary phase, selection of organic solvent and its content, buffer pH and concentration, flow rate, elution type and detection wavelength. The studied drugs were efficiently separated in 3.4 min with high resolution. Also, two univariate spectrophotometric methods have been optimized for the quantification of the studied drugs. Method 1: dual wavelength for THZ and iso-absorptive point for FLM, Method 2: ratio difference (RD) for THZ and first derivative FLM utilizing methanol as a solvent. These methods are accurate, precise with minimal data manipulation. Greenness of the methods was estimated using eco-scale tool where the presented methods were found to be excellent green with eco-score of 83 for HPLC and 80 for spectrophotometry. The methods are validated in conformance with ICH guidelines, with acceptable accuracy, precision, and selectivity. The suggested methods can be employed for the economic analysis of THZ and FLM in their pure form and binary ophthalmic formulation, that can be employed by quality control laboratories.

KEYWORDS

Tetrahydrozoline hydrochloride, Fluorometholone, HPLC, spectrophotometry, Eco-friendly

1. INTRODUCTION

Fluorometholone (FLM); [9a-fluoro-11b,17a-dihydroxy-6a-methylpregna-1,4-diene-3,20-dione] [1] (Fig. 1A) is a corticosteroid used for its glucocorticoid activity, usually as eye drops containing 0.1%, in the treatment of allergic and inflammatory conditions of the eye. Tetrahydrozoline HCl (THZ); [1H-Imidazole, 4,5-dihydro-2-(1,2,3,4-tetrahydro-1-naphthalenyl) onhydrochloride] [1] (Fig. 1B) is a sympathomimetic with effects similar to those of naphazoline. It is used as hydrochloride salt for its vasoconstrictor effect in the symptomatic relief of nasal congestion. When present together in an ophthalmic formulation they are used to treat conjunctival irritation [2]. Few methods were reported for the assay of FLM/THZ mixture, these methods include one spectrophotometric method [3] and three HPLC methods [3–5]. Thorough investigation of literature revealed that no pharmacopeial methods have been described for the simultaneous assay of FLM/THZ. Also, the reported methods

*Corresponding author.
Analytical Chemistry Department, Faculty
of Pharmacy, Cairo University, El-Kasr El-
Aini Street, 11562, Cairo, Egypt,
E-mail: martin.nady@pharma.cu.edu.eg

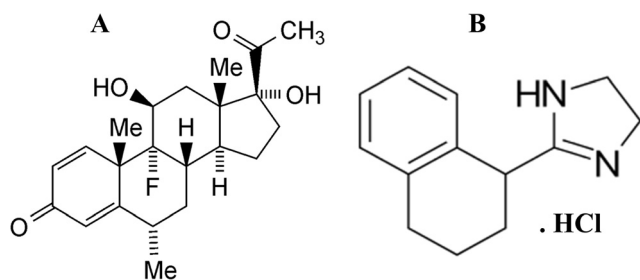


Fig. 1. Chemical structure of (A) Fluorometholone and (B) Tetrahydrozoline HCl

suffer from lack of sensitivity and consume large amounts of organic solvents that harm the environment and increase the cost.

HPLC is the most frequently used and well-developed method for both qualitative and quantitative analysis. HPLC method development can be implemented for various goals including improving selectivity, sensitivity, minimizing runtime, improving efficiency of separation, improving method robustness and transferability and improvement of greenness. However, most of the chromatographic applications apply hazardous solvents and produce large amounts of toxic waste that damage the environment. Recently the Society of Analytical Chemistry has been growingly seeking implementation of eco-friendly methods that eliminate or decrease toxic and corrosive waste [6]. Evaluation of analytical methods greenness is profoundly important and attention grasping for most method developers. One of the latest used greenness valuation tools for analytical techniques is the analytical eco-scale [7], which is a semi-quantitative tool to evaluate and compare analytical methods based on their conformity with green chemistry principles [8]. The eco-scale tool reflects many factors that can have adverse impacts on the environment, such as the class and amount of any chemical used in the procedure, the mass of generated waste, occupational exposure, and energy consumption [9].

UV spectrophotometry has wide applications in the field of pharmaceutical analysis either for identification or for quantitative analysis of different drugs. Many drugs are formulated as mixtures, and the ability to resolve these combination mixtures without prior separation is an extremely important issue in pharmaceutical analysis. Therefore, many univariate spectrophotometric methods were introduced for the analysis of different drugs containing mixtures. The commonly reported dual wavelength technique (DW) [10–13], Iso-absorptive point (ISO) [14], Derivative spectrophotometry (DS) [15–18] and Ratio difference (RD) [19, 20] are well established techniques with minimal data manipulations. These methods can be utilized for their simplicity to improve the sensitivity and allow the application of the drugs in a wide linearity range which are the main drawbacks of the only developed spectrophotometric method for the cited drugs [3].

The aim of this work is to develop a sensitive, rapid and eco-friendly HPLC method as well as two simple univariate

spectrophotometric methods for the simultaneous quantification of FLM/THZ combination in pure form and their binary ophthalmic formulation. In this manuscript the greenness assessment was implemented using the ecoscale tool.

2. EXPERIMENTAL

2.1. Materials

– Pure samples

Pure fluorometholone micronized (FLM) and tetrahydrozoline HCl (THZ); were kindly supplied by Orchidia Pharmaceutical Company, Cairo, Egypt, their purity was certified and found to be $99.81 \pm 0.521\%$ [1] and $99.91 \pm 0.112\%$ [1], respectively.

– Market samples

Efemyo[®] eye drops (1 mg mL^{-1} FLM and 0.25 mg mL^{-1} THZ, batch number 1018201) was manufactured by Orchidia Pharmaceutical Company (Egypt). Efemyo[®] was purchased from the Egyptian market.

– Chemicals and reagents

Acetonitrile, methanol, Ortho-phosphoric acid 85% and sodium dihydrogen phosphate (all of HPLC grade), were purchased from Sigma-Aldrich (Germany). A water purification system (New Human Power I, Korea) was used to obtain ultra-pure water for buffer preparation.

2.2. Instruments

For HPLC

Chromatographic separations were carried out using an Agilent 1,260 Infinity Series liquid chromatograph consisting of a quaternary gradient pumping system and diode array detector with Rheodyne manual injector (model 7725 I) equipped with a $20\text{-}\mu\text{L}$ injector loop (Agilent, USA). Data analysis and system monitoring was accomplished using Chemstation software (Agilent, USA). Membrane filters with pore size $0.45 \mu\text{m}$ and syringe filters $0.22 \mu\text{m}$ diameter (Alltech Associated, USA). Sonicator (Mettler, Germany) and Jenway 3,505 pH-meter (Jenway, UK), were employed for dissolution and pH adjustment.

For spectrophotometry

A SHIMADZU dual beam (Kyoto, Japan) UV- visible spectrophotometer model UV -1650 PC with two matched 10-mm quartz cells was used. The spectrophotometer was connected to IBM compatible PC and an HP 1020 laser jet printer. UV Probe software version 2.21 was utilized. The spectral band width was 0.2 nm and scanning speed was 200 nm/min .

2.3. Chromatographic conditions

Chromatographic separations were carried out using Xbridge[®] C₈ column ($5 \mu\text{m}$, $250 \text{ mm} \times 4.6 \text{ mm I.D}$) using a mobile phase of acetonitrile/sodium dihydrogen phosphate buffer pH 3.0 ± 0.1 (70:30, v/v) through isocratic elution



with flow rate 1 mL min^{-1} . Phosphate buffer 0.02 M was prepared according to British Pharmacopoeia [1], pH was adjusted to 3.0 ± 0.1 using orthophosphoric acid. Separation was implemented in an air-conditioned room kept at $22 \pm 2 \text{ }^\circ\text{C}$ and UV detection at 230 nm .

2.4. Stock and working solutions

Standard stock solutions of THZ and FLM 1 mg mL^{-1} were prepared in separate flasks using methanol as solvent. Working standard solutions $100 \text{ } \mu\text{g mL}^{-1}$ of THZ and FLM were prepared from their respective stock solutions by appropriate dilution with the mobile phase for the HPLC method while dilution with methanol was done for the spectrophotometric methods.

2.5. Procedures

2.5.1. Spectral characteristics of THZ and FLM. Aliquots equivalent to $5 \text{ } \mu\text{g}$ of THZ and $20 \text{ } \mu\text{g}$ FLM were accurately transferred from their respective working standard solutions into two separate 10-mL volumetric flasks and the volumes were completed to the mark with methanol. Zero-order absorption spectrum of each solution was then recorded over a wavelength range of $200\text{--}400 \text{ nm}$ using methanol as a blank. The recorded spectra were further employed to determine the optimum parameters for each spectrophotometric procedure.

2.5.2. Construction of calibration curves.

For HPLC

Aliquots of THZ and FLM were accurately pipetted from their corresponding standard solutions into two different groups of 10-mL measuring flasks. The mobile phase was used to complete the volume to prepare different concentrations covering the range of $2\text{--}100 \text{ } \mu\text{g mL}^{-1}$ for THZ and FLM. Solutions were then filtered through a $0.22 \text{ } \mu\text{m}$ syringe membrane filter, injected in volumes of $20 \text{ } \mu\text{L}$ in triplicate and chromatographed using the above-mentioned chromatographic conditions. The average peak area attained for each concentration of THZ and FLM was plotted versus the corresponding concentration. The regression equations were calculated. Validation was fulfilled in agreement with ICH guidelines [21].

For spectrophotometry

1 DW for THZ and ISO for FLM

The absorbance difference (ΔA) of THZ and FLM spectra at 234.8 nm and 242.8 nm was calculated to determine THZ where the difference in FLM spectrum is zero. A calibration curve was constructed between this absorbance difference (ΔA $234.8\text{--}242.8$) against the corresponding concentration of THZ and the regression equation was computed (**Method 1.1. DW**), while the absorbance at isoabsorptive point, 224.4 nm is equivalent to the concentration of both THZ and FLM. A calibration curve was constructed by relating the absorbance at 224.4 nm (iso-absorptive point) to the corresponding concentration of FLM, and the regression equation was computed (**Method 1.2. ISO**).

2 RD for THZ and 1D for FLM

The stored zero order spectra of THZ were divided by the standard spectrum of FLM ($50 \text{ } \mu\text{g mL}^{-1}$) and the amplitudes of the ratio spectra at 210 and 230 nm were recorded. A linear relationship was obtained by plotting the amplitude difference of the ratio spectra ($\Delta P_{210\text{--}230 \text{ nm}}$) versus the corresponding concentrations ($3\text{--}30 \text{ } \mu\text{g mL}^{-1}$) of THZ and the regression equation was computed, (**Method 2.1. RD**). For FLM, the first derivative of THZ and FLM spectra was obtained at $\Delta \lambda = 4$ and scaling factor = 10 and the amplitude of the first derivative peak was measured at 271.1 nm where peak amplitude of THZ is zero. Linear relationship between peak amplitude at 271.1 nm of first derivative spectrum of FLM against its corresponding concentration was built and regression equation was computed, (**Method 2.2.A 1D**). Another method for FLM determination, where the amplitudes of the ratio spectra (obtained through dividing the stored spectra of FLM by a standard spectrum of $30 \text{ } \mu\text{g mL}^{-1}$ THZ) at 235 nm and 254 nm were recorded.

2.5.3. Analysis of Laboratory Prepared Mixtures. To assess the specificity of the proposed methods, different aliquots of THZ and FLM were transferred from their corresponding standard working solutions into a series of 10-mL volumetric flasks. The volumes were completed with the mobile phase for HPLC and with methanol for spectrophotometry to prepare different ratios of the two drugs including the ratio of the dosage form.

For HPLC

The prepared flasks were analyzed, where the recovery percentages and %RSD were calculated.

For spectrophotometry

The absorption spectra of laboratory prepared mixtures were scanned and stored then the proposed procedures were applied to determine each drug as follows:

Method 1

The absorbance was recorded for each mixture at the selected wavelength pair ($234.8\text{--}242.8$) where the absorbance difference corresponds to the concentration of THZ and zero contribution of FLM, therefore, THZ can be determined from its corresponding regression equation, (**Method 1.1. DW**) while the absorbance at isoabsorptive point (224.4 nm) was measured and the total concentration of both drugs was obtained from the corresponding regression equation. Then, the calculated concentration of THZ from DW method, was subtracted from the total one to get the concentration that corresponds to FLM (**Method 1.2. ISO**).

Method 2

Zero order spectra of the mixtures were divided by a standard spectrum of $50 \text{ } \mu\text{g mL}^{-1}$ FLM. Then, the peak amplitude difference between 210 nm and 230 nm wavelength was calculated and applied in the corresponding regression equation to determine the concentration of THZ, (**Method 2.1. RD**). Also, the first derivative of the spectra of THZ and FLM mixtures was obtained using $\Delta \lambda = 4$ and scaling factor = 10 and the amplitude of the



first derivative peak was measured at 271.1 nm where the concentration of FLM can be calculated from its corresponding regression equation, (Method 2.2.A¹D).

2.5.4. Application of the suggested methods on pharmaceutical formulations.

For HPLC

Four milliliters of Efemyo[®] eye drops were pipetted into a 10-mL volumetric flask and volume completed to the mark with mobile phase to get 400 µg mL⁻¹ of FLM and 100 µg mL⁻¹ of THZ. A suitable dilution with mobile phase was done to prepare the working solution of 40/10 µg mL⁻¹ of FLM/THZ, respectively.

For spectrophotometry

Two mL of Efemyo[®] eye drops were pipetted into a 10-mL volumetric flask, made-up to the mark with methanol to get 200 µg mL⁻¹ of FLM and 50 µg mL⁻¹ of THZ. A suitable dilution with methanol was prepared to obtain a working solution of 20/5 µg mL⁻¹ of FLM/THZ, respectively. The procedure previously described under analysis of laboratory prepared mixtures was followed to determine the concentration of each drug in the dosage form.

3. RESULTS AND DISCUSSION

3.1. Method optimization

For HPLC

The main criteria for development of a successful HPLC method is its ability to determine the analyzed drugs in the studied matrix with sufficient resolution in short analysis time. In addition, it should be accurate, reproducible, robust, cost-effective and simple enough for routine use in the quality control laboratory as well [22, 23].

Selection of organic solvent

The chromatographic performance was tested using mobile phase mixtures of various polarities. These initial trials for mobile phase optimization were achieved using C₈ Xbridge[®] column. Xbridge[®] C₈ column (Waters) is categorized as fully porous stationary phase. It offers superior pH stability over wide range (pH 2–12) which is enormously relevant in HPLC method development for pharmaceutical compounds, especially for weak acidic or basic analytes. Also, its

mechanical stability increases the column efficiency and improves column reliability and reproducibility. It shows excellent performance, lifetime and separation while changing instruments and vendors [24]. Initially, green solvents like aqueous isopropyl alcohol and aqueous ethanol were tried but very poor resolution and selectivity were obtained. Then, a preliminary study using different mobile phase ratios of methanol/water and acetonitrile/water were employed. The mobile phase resolved the analytes into broad peaks with long retention time. However, the trials employing acetonitrile showed better resolution and shorter retention time than the mobile phase mixtures containing methanol as shown in Table 1. So, acetonitrile was chosen as the organic solvent for this study.

Organic solvent percentage

Trials of mobile phase mixtures of acetonitrile/water was not the best choice, as mentioned earlier, due to poor resolution and long run time. So, in the next trials water was replaced with buffer. Sodium dihydrogen phosphate buffer was utilized and maintained at 0.05 M (pH 3). Percentage of acetonitrile were varied between 10 and 70 % and investigated. It was observed that increasing buffer up to 70 % increases the retention time especially for eluting FLM. While increasing the acetonitrile up to 70 % decreases the retention time of both components and provides a good resolution between THZ and FLM. The limiting factor was base line resolution of two sharp peaks within minimal analysis time to decrease mobile phase and chemicals consumption. Based on these trials the ratio of acetonitrile/buffer (70:30, v/v) was chosen as optimum mobile phase ratio.

Selection of buffer pH

In order to choose the optimum pH for the buffer, we had to take into consideration the physico-chemical properties of the studied drugs. It is worthy to mention that THZ has pKa of 10.51 [25] and FLM pka of 12.65 [26]. Theoretically, pH range 2–8 would be suitable for the separation in hands.

At this pH range, FLM exist in its unionized form in contrast to THZ, which has fixed positive charge (ionic molecule) allowing considerable resolution, and hence we can expect that THZ elutes first upon using higher ratio of organic solvent. Optimization of pH was achieved by applying the mobile phase in the optimized ratio (acetonitrile/buffer 70:30, v/v), at different pH values in the range

Table 1. System suitability parameters of the proposed HPLC method for THZ and FLM

Parameters	Tetrahydrozoline HCl	Fluorometholone	Reference values [41]
Retention time	2.59 ± 0.1	3.44 ± 0.1	
Retention factor (K')	2.15	2.86	≥2
Selectivity factor (α)	1.61		
Asymmetric factor (T)	0.95	1.05	≤2
Resolution (Rs)	4.29		≥1.5
Number of theoretical plates (N)	2,369	6,607	≥2,000
Height equivalent theoretical plates (HETP) (cm)	0.018	0.004	



of 2–8. It was observed that $\text{pH } 3 \pm 0.1$ showed better peak sharpness, symmetry and better resolution as shown in Table 1, while at higher pH values resolution was slightly decreased. So, the optimum pH for the study in hands is $\text{pH } 3 \pm 0.1$. An effective pH for the mobile phase depends on the fact that the optimum buffer capacity equals pKa of the used buffer \pm one pH unit [27, 28]. According to literature [28] the pK1 of phosphoric acid is 2.15 so the choice of pH 3 assures maximum buffer capacity.

Buffer concentration

Five different concentrations of phosphate buffer were tested (0.02, 0.03, 0.04, 0.05 and 0.06 M) to identify the optimal buffer concentration with acetonitrile content fixed at 70 % (v/v) and pH maintained at 3.0. When the buffer concentration increased from 0.02 to 0.06 M, no significant difference in chromatographic parameters was observed. Low concentration of buffer (0.02 M) is adequate for chromatographic applications. This concentration is low enough to avoid problems with precipitation when significant amounts of organic modifiers are used in the mobile phase [28].

Selection of stationary phase

HPLC columns are the heart of the chromatographic method. The continuous advances in packing technology should help analysts to find more selective, durable, cost-saving, and efficient stationary phase to be used in a certain application [29–31]. As mentioned earlier the Xbridge[®] column was chosen for all the trials in this study due to its high mechanical and pH stability over a wide pH range (2–12). In order to optimize the stationary phase type, C₈ and C₁₈ Xbridge columns, with the same dimensions, were investigated using the optimized mobile phase. It is noteworthy to mention that the FLM and THZ are relatively hydrophilic due to their relatively low Log P values (0.9 for THZ [32] and 2.93 for FLM [33]). This would suggest the choice of normal phase chromatography rather than reversed phase. The trials showed that C₁₈ column shows very short retention time especially for THZ, but with poor resolution and peak shape. C₈ column showed reasonable retention time (2.5 min. for THZ and 3.4 min for FLM), peak shape and resolution for both drugs. Short runtime offers advances in saving solvent and operating cost and most importantly saving the environment. The column length was chosen to be 250 mm to allow satisfying resolution between the two drugs as they

have a considerable difference in their hydrophobicity which goes in agreement with most literatures investigating the same drugs [3, 4, 34–36]. However, the study in hands shows superiority over the reported methods in minimizing run time [3], better peak shape [35], smooth baseline [5], higher efficiency [3] and better sensitivity [4] as shown in Fig. 2. It is noteworthy to mention that using shorter columns (150 or 100 mm) is expected to show shorter runtime and hence saving more solvents and time, but they may affect the system suitability parameter regarding symmetric and retention factors.

Selection of detection wavelength

Zero order spectra presented in Fig. 3A shows that λ_{max} of THZ is 216 nm while λ_{max} of FLM is 243 nm. To optimize detection wavelength, different values were investigated (216, 243, 220 and 230). Maximum sensitivity is obtained at wavelength 230 nm which is considered a center point between λ_{max} of the two drugs. Potassium dihydrogen phosphate (pH 3, 0.02 M) has no effect on absorption at wavelength more than 200 nm [28] The apparent UV cutoff of acetonitrile is less than 200 nm [37], so it has no impact on the measured values.

Flow rate and type of elution

Different flow rates (0.6, 0.8, 1.0, 1.2 and 1.5 mL min⁻¹) were tested. Flow rates of 0.6 and 0.8 mL min⁻¹ increase the peak broadening and run time by 30%, while increasing the flow rate to 1.5 mL min⁻¹ decreases resolution by 50%. The optimum resolution, peak symmetry, and run-time are achieved by fine-tuning of the flow rate to 1 mL min⁻¹ as shown in Table 1. Isocratic elution was chosen rather than gradient elution as an optimum elution type. Isocratic elution is a better choice especially if the number of analytes under investigation is small due to several advancements over gradient elution. These advancements include easier method development, simpler instrument adjustment, less re-equilibration time and hence saving solvents, time and the environment, less baseline disturbances and easier method transferability.

The proposed method shows better HPLC performance with advances over the reported methods investigating THZ and FLM. These advances include chromatographic characters and general separation method improvements. The chromatographic advances involve better peak shape [35], smooth baseline [5] and higher separation efficiency [3]. Other general characters include short run time [3, 4,

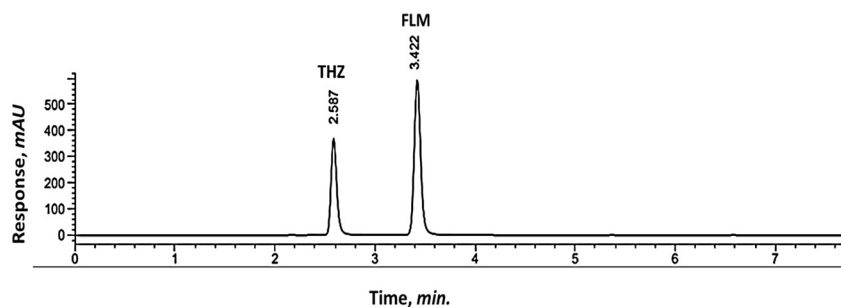


Fig. 2. HPLC chromatogram of: THZ [50 $\mu\text{g mL}^{-1}$], FLM [50 $\mu\text{g mL}^{-1}$] at 230 nm

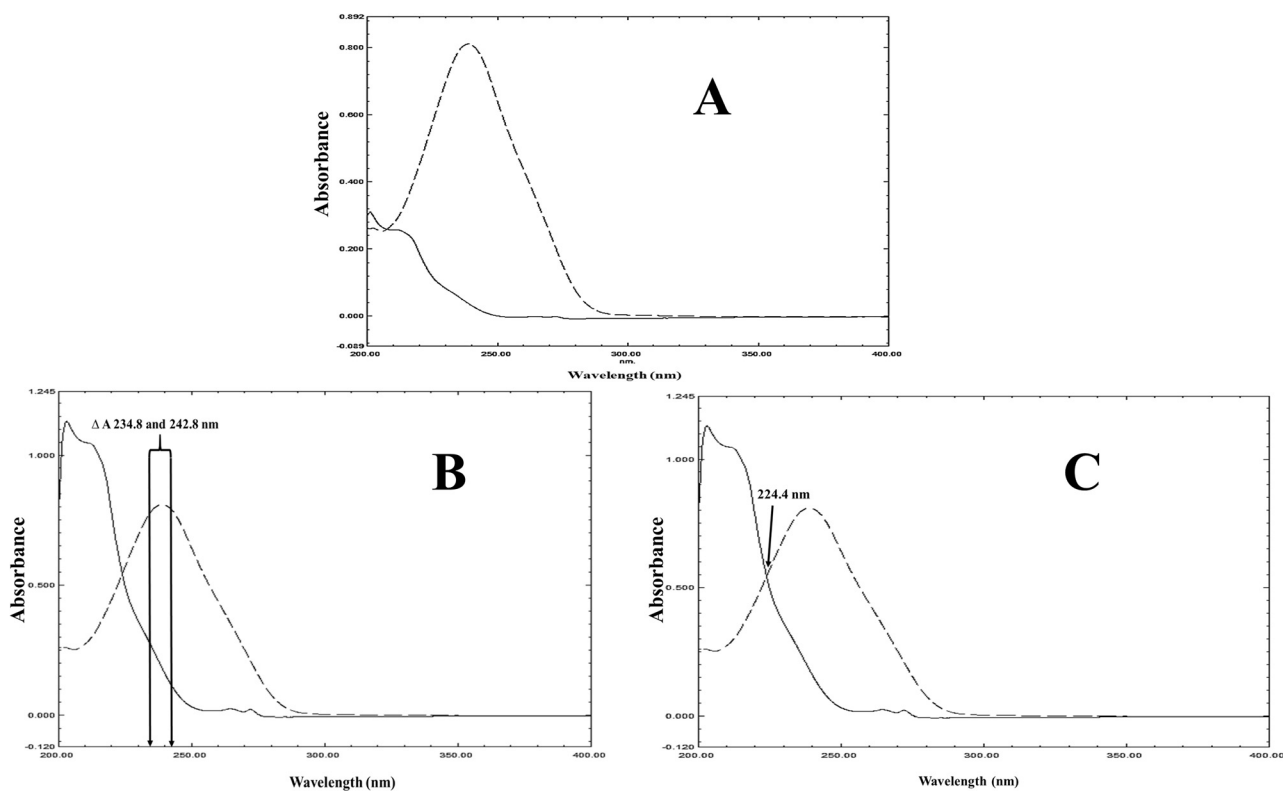


Fig. 3. (A) Zero order absorption spectra of $5 \mu\text{g mL}^{-1}$ THZ (—) and $20 \mu\text{g mL}^{-1}$ FLM (---) in methanol (same ratio as in their dosage form). (B): Zero order absorption spectra of $10 \mu\text{g mL}^{-1}$ THZ (—) and $10 \mu\text{g mL}^{-1}$ FLM (---) showing the same absorbance for FLM and different absorbance for THZ at 234.8 and 242.8 nm. (C): Zero order absorption spectra of $10 \mu\text{g mL}^{-1}$ THZ (—), $10 \mu\text{g mL}^{-1}$ FLM (---) using methanol as blank showing the isoabsorptive at 224.4 nm

34–36] and hence cost-effectiveness, eco-friendly characters, better sensitivity [4] and wider linearity range [35, 36, 38]. Greenness assessment presented in this study was not offered by any of the reported methods for the drugs under investigation. Under optimized conditions, good separation of the two analytes with acceptable system suitability parameters, Table 1, is obtained within 4 min Fig. 2.

For spectrophotometry

Zero order spectra of THZ and FLM (Fig. 3A), show complete overlap between the two drugs along the wavelength range 200–300 nm, which hinders the direct determination of THZ or FLM. This overlap can be resolved by the two proposed methods.

Method 1

It covers the application of dual wavelength (DW) method which is simple, accurate and need minimal data manipulation to determine of THZ, then iso-absorptive point method can be applied to determine FLM.

• Method 1.1. DW for THZ determination

The developed method can determine compounds in their binary or ternary mixtures [10–13], where each component can be selectively determined after completely removing the interference of the other component. The described method has the advantage of minimal data manipulation. THZ could be determined at one step. The most important parameter for successful application of the method was the choice of the

two wavelengths ($\Delta\lambda$) at which the interferent contribution is canceled while the concentration of the analyte is determined. Two different λ_1 and λ_2 were tested for the calculation of the absorbance difference of THZ where the FLM showed the same absorbance, but the selected (234.8–242.8) nm showed better accuracy and better recovery percentages in the analysis of laboratory prepared mixtures. Fig. 3B shows equal absorbance at (234.8–242.8) nm for FLM, but for THZ, the difference in absorbance at wavelengths $\Delta 234.8$ –242.8 nm corresponds to its concentration.

• Method 1.2. ISO for FLM determination

Upon trying to measure the absorbance of the same concentration of THZ and FLM, it was found that 224.4 is an Iso-absorptive point that can be used for the simple determination of FLM after subtracting the corresponding THZ concentration. This method has the advantages of being simple, applied to zero order spectra thus need no data manipulation. The developed method can determine compounds in their binary mixture. where each component can be selectively determined at iso-absorptive point after subtraction of the other component concentration [14]. The described method has the advantage of minimal data manipulation. FLM could be determined at one step. The most important parameter for successful application of the method was the exact determination of the iso-absorptive point, at which the two drugs have the same

absorptivity in zero order spectra. Fig. 3C shows that both drugs have isoabsorptive point at 224.4 nm. Absorbances at wavelength 224.4 nm were recorded where it corresponds to total concentration of THZ and FLM. After subtraction of THZ concentration, we can get the concentration of FLM.

Method 2

THZ is determined by RD method while FLM is determined by first derivative method or RD method.

• Method 2.1. RD For THZ determination

RD method proved to be a simple, accurate and reproducible method for resolving severely overlapped spectra. Unlike the dual wavelength method, the interfering component is completely eliminated in the form of constant and the difference at any two points will be equal to zero, so there is no need for critical measurements which leads to highly reproducible and robust results. The presented work shows that values of accuracy and precision for RD method are better than those obtained by DW method as shown in Table 2. The developed method can determine compounds in their binary mixture, where each component can be selectively determined after removing the interference by the other component [39, 40]. There were two important parameters for successful application of the method. The first one was finding the most suitable divisor. For determination of THZ different divisors of FLM were tried (10, 30, 50 $\mu\text{g mL}^{-1}$), and 50 $\mu\text{g mL}^{-1}$ was found to be optimum in terms of sensitivity and linearity. The second parameter was finding the two wavelengths, where the difference in amplitude is linear relative to concentration for one drug, but for the other

drug is zero. The two selected wavelengths in the ratio spectra for estimation of THZ were 210 nm and 230 nm Fig. 4A.

• Method 2.2. A¹D For FLM determination

The most significant benefit of DS is the ability to determine drugs in binary mixtures simultaneously in one-step manipulation. This resolving power can be confirmed by first order derivative, where the first derivative of FLM has zero crossing of THZ at 271.1 nm, Fig. 4B. The interference of THZ is completely canceled not subtracted as in ISO method and FLM could be determined at 271.1 nm, accurately. There were two important sets of parameters for successful application of the method. The first set was finding $\Delta\lambda$ and scaling factor. Different values were tried, where $\Delta\lambda = 4$ and scaling factor = 10 were found optimum in terms of sensitivity and linearity. The second parameter was finding the wavelength at which FLM has reasonable amplitude, where the amplitude of THZ equal zero. The described method has the advantage of minimal data manipulation. The method shows better sensitivity than ISO method as shown in Table 2. It is noteworthy to mention that by scanning the first order spectra of both drugs, no single wavelength has shown reasonable peak amplitude for THZ that correspond to zero contribution of FLM. That is why THZ could not be determined using 1D method and RD method was suggested as mentioned in method 2.1. RD.

After developing the two above-mentioned methods, we continue to investigate simple, sensitive and selective methods with minimal manipulations. Many more methods were applied such as second derivative method, derivative

Table 2. Validation parameters of the proposed methods for the determination of pure samples of THZ and FLM according to ICH guidelines

Parameters	THZ			FLM		
	HPLC	DW	RD	HPLC	ISO	¹ D
Range ($\mu\text{g mL}^{-1}$)	2–100	3–30	3–30	2–100	5–50	5–50
Slope ^a	50.17	0.0079	0.0644	51.99	0.028	–0.0097
Intercept ^a	6.22	–0.00002	–0.0018	7.22	–0.0051	–0.00007
SE of the slope		0.00002	0.010		0.00009	0.0007
SE of the intercept		0.0004	0.0005		0.0030	0.00002
Correlation coefficient (r)	0.9999	0.9999	0.9997	0.9999	0.9999	0.9999
LOD ^b ($\mu\text{g mL}^{-1}$)	0.31	0.19	0.54	0.54	0.35	0.24
LOQ ^b ($\mu\text{g/mL}$)	0.94	0.57	1.64	1.64	1.07	0.75
Accuracy ^a						
Mean \pm RSD	100.35 \pm 0.84	100.85 \pm 0.40	99.97 \pm 0.30	99.73 \pm 0.60	100.77 \pm 1.23	100.58 \pm 1.27
Precision (RSD)						
Repeatability ^c Intermediate precision ^d	\pm 1.14 \pm 1.38	\pm 1.12 \pm 1.71	\pm 0.45 \pm 1.14	\pm 0.41 \pm 1.15	\pm 0.76 \pm 1.58	\pm 1.13 \pm 1.23

^a Average of three determinations.

^b Determined via signal to noise ration calculations for HPLC and ¹D method and by calculations for the remaining methods, LOD = 3.3 (SD of the response/slope), LOQ = 10 (SD of the response/slope).

^c The intraday ($n = 3$) standard deviation of concentrations (20, 50, 60 $\mu\text{g mL}^{-1}$) both drugs for HPLC, (7, 13, 26 $\mu\text{g mL}^{-1}$) THZ and (6, 26, 34 $\mu\text{g mL}^{-1}$) FLM for spectrophotometry repeated three times within the same day.

^d The interday ($n = 3$) relative standard deviation of concentrations (20, 50, 60 $\mu\text{g mL}^{-1}$) both drugs for HPLC, (7, 13, 26 $\mu\text{g mL}^{-1}$) THZ and (6, 26, 34 $\mu\text{g mL}^{-1}$) FLM for spectrophotometry repeated three times in three successive day.



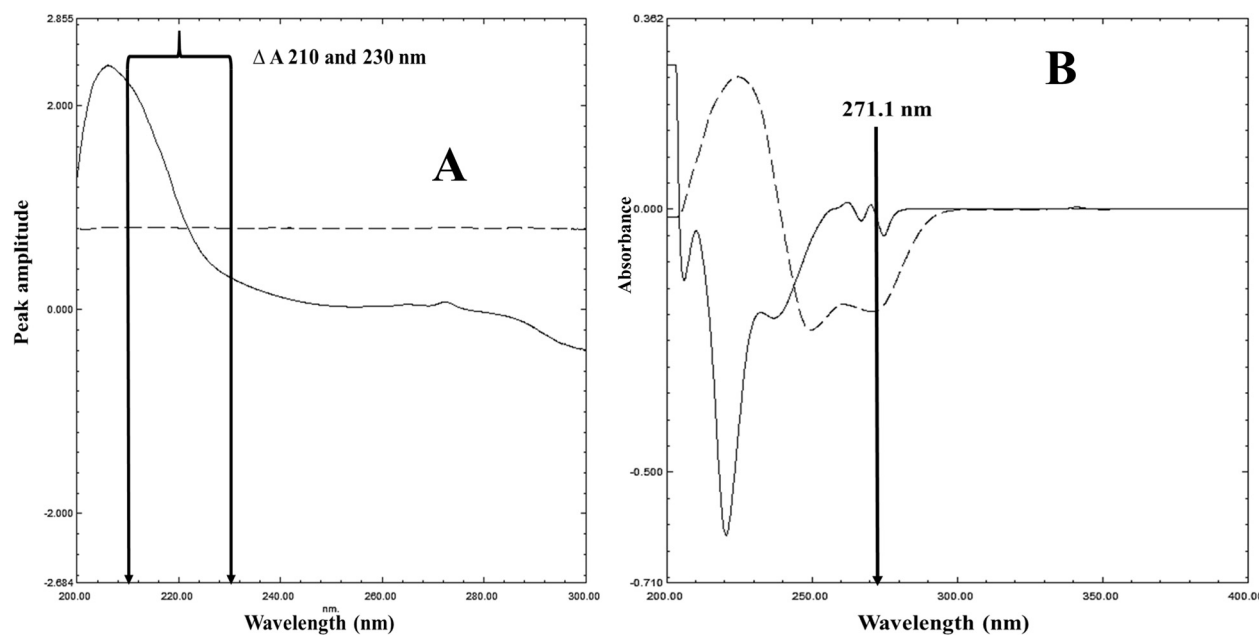


Fig. 4. (A) Ratio spectra of 20 $\mu\text{g mL}^{-1}$ FLM/50 $\mu\text{g mL}^{-1}$ FLM (—) and 20 $\mu\text{g mL}^{-1}$ THZ/50 $\mu\text{g mL}^{-1}$ FLM (---) showing the two selected wavelengths for ratio difference. (B) First order absorption spectra of 20 $\mu\text{g mL}^{-1}$ THZ (---) and 20 $\mu\text{g mL}^{-1}$ FLM (—) for FLM determination using first derivative method

ratio and mean centering but they were inferior in terms of accuracy and simplicity to the presented methods.

3.2. Method validation

Validation of the proposed univariate spectrophotometric and HPLC methods was performed according to ICH guidelines [21] by assessment of linearity, range, accuracy, precision. Obtained results are represented in Table 2. The specificity of the developed methods was assessed by analyzing laboratory prepared mixtures containing different ratios of THZ and FLM. Satisfactory results were obtained and listed in Table 3. The proposed methods were applied for the determination of THZ and FLM in pharmaceutical formulations and good results were obtained where no impurities were detected as represented in Table 4.

– Range and Linearity

The linearity of the presented methods was examined by managing variable concentrations of the different calibration curves on 3 different days. The calibration curves were built within concentration ranges that are related to drugs concentration in the dosage forms.

– Accuracy

The suggested methods were applied for measuring three concentrations of THZ and FLM within their linearity ranges – each repeated three times – and the concentrations were calculated from their corresponding regression equations. The percentage recovery for each drug was calculated Table 2. The accuracy of the proposed methods was further assessed by applying the standard addition technique for the analysis of Efemyo[®] eyedrops.

– Precision

The precision of the methods expressed as %RSD was calculated by the determination of three different

concentrations of pure drugs chosen along the linearity range. The intra-day precision was obtained from the throughout results of three replicate determinations of three pure drugs samples throughout a single day. To determine the inter-day (intermediate) precision, the same samples were analyzed on 3 consecutive days. The % RSD of the results of both inter and intraday precisions were less than 2.00% indicating the good precision [41]. The results are illustrated in Table 2.

– Limits of detection and quantification

The limit of detection and quantification for the HPLC and ¹D method were calculated using signal to noise ratio method [21] while LOD and LOQ for the remaining methods were determined via calculations, LOD = 3.3 (SD of the response/slope), LOQ = 10 (SD of the response/slope).

– Specificity

The specificity of the spectrophotometric methods was assessed by analyzing different laboratory prepared mixtures of THZ and FLM within the linearity range Table 3, where good results were obtained. Moreover, the specificity of the HPLC method is presented by resolution of the investigated drugs, Table 1 [21], also with the peak purity and 3D figures imported from Chemstation software, Figs. S1 and S2.

– Robustness

The robustness was examined by testing the samples under a minor variety of experimental conditions. For RP-HPLC methods, small changes in the pH (± 0.2), small changes in percentage of acetonitrile by up to $\pm 2\%$ were introduced to the mobile phase, small changes in the detection wavelength (± 5 nm) and small changes in flow rate (± 0.1 mL min⁻¹). A slight modification in the elution time and peak symmetry was observed, however, the



Table 3. Determination of pure THZ and FLM in laboratory prepared mixtures by the proposed spectrophotometric methods

Ratio of the studied mixtures		THZ						FLM					
		HPLC		DW		RD		HPLC		ISO		1D	
		Found $\mu\text{g mL}^{-1}$	Recovery %	Found $\mu\text{g mL}^{-1}$	Recovery %	Found $\mu\text{g mL}^{-1}$	Recovery %	Found $\mu\text{g mL}^{-1}$	Recovery %	Found $\mu\text{g mL}^{-1}$	Recovery %	Found $\mu\text{g mL}^{-1}$	Recovery %
5 ^a	20 ^a	5.02	100.40	5.06	101.31	5.04	100.87	20.23	101.15	20.15	100.76	19.79	98.93
5	30	4.96	99.20	4.94	98.78	5.09	101.8	29.77	99.23	30.17	100.57	29.89	99.63
20	10	20.24	101.20	20.00	100.00	20.33	101.69	9.95	99.50	9.89	98.93	9.89	98.89
10	25	10.13	101.30	9.88	98.80	9.96	99.65	25.42	101.68	24.52	98.08	24.84	99.35
8 ^a	32 ^a	7.95	99.38	7.98	99.71	8.02	100.31	32.36	101.13	31.92	99.74	31.95	99.84
15	15	15.11	100.73	15.06	100.43	14.89	99.25	14.88	99.20	14.94	99.58	15.04	100.29
15	25	14.88	99.20	14.94	99.95	15.01	100.08	24.26	97.04	24.67	98.68	24.63	98.52
Mean \pm RSD		100.2 \pm 0.93		99.80 \pm 0.90		100.52 \pm 0.97		99.85 \pm 1.61		99.48 \pm 0.98		99.35 \pm 0.61	

^aRatio of the dosage form.

Table 4. Quantitative determination of THZ and FLM in Efemyo[®] eye drops by the proposed methods and standard addition technique

Pharmaceutical formulation	Efemyo [®] eyedrops (Labeled to contain 250 µg mL ⁻¹ THZ and 1 mg mL ⁻¹ FLM) Batch number 1018201						
	Drug	THZ			FLM		
	Method	HPLC	DW	RD	HPLC	ISO	¹ D
Mean ±%RSD ^a		99.93 ± 0.97	99.37 ± 0.99	99.73 ± 1.25	100.28 ± 0.90	99.67 ± 0.88	99.70 ± 0.66
Recovery of standard added ±%RSD ^a		101.39 ± 0.45	100.05 ± 1.28	100.00 ± 0.23	100.53 ± 1.07	101.20 ± 0.87	98.81 ± 0.41

^aAverage of three determinations.

Table 5. System suitability parameters for robustness of the proposed HPLC method for THZ and FLM

Parameters		Symmetry		Retention factor		Number of theoretical plates		Selectivity	Resolution
		THZ	FLM	THZ	FLM	THZ	FLM	FLM	FLM
		Wavelength nm	230+5	0.97	1.05	2.16	2.85	2,004	7,284
	230–5	1.10	1.05	2.16	2.85	2,217	6,607	1.59	4.25
Flow rate mL min ⁻¹	1.10	0.92	1.05	2.16	2.93	2,217	7,744	1.67	4.89
	0.90	0.94	1.05	2.14	2.79	2,165	7,009	1.58	3.42
Mobile phase ratio v/v	72–28	0.88	1.13	2.40	3.16	2,692	7,093	1.54	3.96
	68–32	1.13	1.20	2.04	2.59	2,704	6,021	1.67	4.49
pH	3.30	0.92	1.10	2.08	3.10	2,212	6,087	1.20	4.27
	2.70	1.00	1.20	2.15	2.86	2,072	9,056	1.61	4.38

system suitability parameters are still within the acceptable values, Table 5.

– System suitability

According to the ICH [21] system suitability is a crucial part of many analytical methods, particularly liquid chromatographic methods. They are applied to authenticate that the resolution and reproducibility of the proposed methods are suitable for the real-life quality control analysis to take place. Different parameters, namely retention factor (k'), symmetry factor, selectivity factor (α) and resolution (R_s), were calculated and proved to be within the accepted values Table 1.

3.3. Application to pharmaceutical formulation and standard addition technique

The suggested HPLC method is valid for the determination of THZ and FLM in pure form and in Efemyo[®] eye drops. The validity of the proposed method and interference of added excipients in the pharmaceutical products was further investigated by implementing the standard addition technique, which produced acceptable results, Table 4. Good accuracy confirmed that the excipients in pharmaceutical products did not interfere in the determination of these drugs. The results support the suitability of the suggested method for the regular analysis of these compounds in their combined formulations.

3.4. Greenness Assessment: Analytical eco-scale

Applying the eco-scale metric to determine the proposed methods greenness is based on giving penalty points to any

aspect that doesn't conform with perfect green technique, where the ideal green analysis has its eco-scale value of 100, excellent green analysis should score >75 eco-scale, acceptable green analysis scores >50, while if the method scores <50, it will be considered as inadequate green analysis [7]. Penalty points' calculations for the proposed methods are shown in Table 6, where both methods were found to be excellent green method. HPLC has comparable score to spectrophotometry. The calculation of the penalty point for each used chemical is based on the calculation of (amount penalty points × hazard penalty points). Hazard penalty

Table 6. Penalty points for greenness assessment of the proposed methods

Hazard	Penalty Points	
	HPLC	Spectrophotometry
Reagents		
Acetonitrile	8	
Phosphate buffer	0	
Methanol		12
Instruments energy	1 ^b	0 ^b
Occupational hazard	0	0
Waste	8	8
Total penalty points	17	20
Analytical eco-scale total score	83 ^a	80 ^a

^a>75 represents excellent green analysis, >50 represents acceptable green analysis, <50 represents inadequate green analysis.

^bEnergy consumed by instrument per sample (0 for methods using less than 0.1 kWh per sample, 1 for methods using 0.1–1.5 kWh per sample, 2 for methods using more than 1.5 kWh per sample).



points is the number of pictograms in material safety data sheet of the chemical \times the score for the signal word (safe = 1, danger = 2). Amount penalty points are assigned based on the rule that (less than 10 mL = 1, 10–100 mL = 2, more than 100 mL = 3). So, for acetonitrile (2 pictograms, signal word is danger, amount is between 10 and 100 mL) the penalty point score is $[2 \text{ pictograms} \times 2 \text{ (danger)} \times 2 \text{ (amount 10–100 mL)}] = 8$ penalty points. Methanol penalty points calculated as follows $[3 \text{ pictograms} \times 2 \text{ (danger)} \times 2 \text{ (amount 10–100 mL)}] = 12$ penalty points. Sodium dihydrogen phosphate has no pictograms, so zero penalty points. The instrumental energy consumption also has penalty points as following (0 for methods using less than 0.1 kWh per sample, 1 for methods using 0.1–1.5 kWh per sample, 2 for methods using more than 1.5 kWh per sample). Spectrophotometry is assigned zero while HPLC is assigned one penalty point. Waste penalty points is calculated as follows (None = 0, <1 mL (g) = 1, 1–10 mL (g) = 3, >10 mL (g) = 5) then processing points are added where (recycling 0, degradation = 1, passivation = 2, no treatment = 3). The waste penalty points for both developed methods is $[(1-10 \text{ mL (g)} = 3) + (\text{no treatment} = 3)] = 8$.

4. CONCLUSION

The proposed spectrophotometric and HPLC methods have the advancements of being green, simple with no excessive data manipulation, reproducible, fast and accurate according to the study in hands. Under optimized chromatographic conditions, excellent separation of THZ and FLM was obtained. The chosen buffer and flow rate had shown superior performance and selectivity regarding system suitability parameters, analysis time, analysis cost, conditioning time, backpressure. Spectrophotometric methods have the advantages of minimal data manipulation and being eco-friendly. The developed methods have advantages over the reported method in being greener, with lower cost, lower volumes consumption and most importantly better sensitivity. The proposed approaches can be used for the routine analysis of THZ and FLM, in their binary pharmaceutical preparation or in bulk powder form. The methods are characterized by broad applicability, short analysis time and adequate robustness. The suggested methods are validated utilizing ICH guidelines. they could be implemented in QC laboratories for a cost-effective analysis.

REFERENCES

1. B. P. Commission. British Pharmacopoeia, British Pharmacopoeia Commission. London, TSO, UK, **2013**.
2. Fanta, H.; Kurz, M.; Marx, J.; Zotti, A. Fluorometholon/tetryzoline in the treatment of conjunctival irritation. *Klinische Monatsblätter für Augenheilkunde* **1983**, *183*, 471–6.
3. Altuntas, T.; Korkmaz, F.; Nebioglu, D. Determination of tetrahydrozoline hydrochloride and fluorometholone in pharmaceutical formulations by HPLC and derivative UV spectrophotometry. *Die Pharmazie* **2000**, *55*, 49–52.
4. El-Bagary, R. I.; Fouad, M. A.; El-Shal, M. A.; Tolba, E. H. Stability-indicating RP-HPLC methods for the determination of Fluorometholone in its mixtures with sodium cromoglycate and Tetrahydrozoline hydrochloride. *J. Chromatogr. Sci.* **2016**, *54*, 923–33.
5. Pham, T.-V.; Mai, X.-L.; Song, H.-R.; Ahn, S.-B.; Cha, M.-J.; Kang, J.-S.; Woo, M. H.; Na, D.-H.; Chun, I.-K.; Kim, K. H. Simultaneous determination of fluorometholone and tetrahydrozoline hydrochloride in ophthalmic suspension by HPLC. *Anal. Sci. Technol.* **2018**, *31*, 88–95.
6. Armenta, S. G.; de la, S.; Guardia, M. Green analytical chemistry. *TrAC Trends Anal. Chem.* **2008**, *27*, 497–511.
7. Gatuszka, A.; Migaszewski, Z. M.; Konieczka, P.; Namieśnik, J. Analytical Eco-Scale for assessing the greenness of analytical procedures. *Trends Anal. Chem.* **2012**, *37*, 61–72.
8. Al-Alamein, A. M. A.; El-Rahman, M. K. A.; Abdel-Moety, E. M.; Fawaz, E. M. Green HPTLC-densitometric approach for simultaneous determination and impurity-profiling of ebastine and phenylephrine hydrochloride. *Microchem. J.* **2019**, *147*, 1097–102.
9. Tobiszewski, M.; Marc, M.; Galuszka, A.; Namiesnik, J. Green chemistry metrics with special reference to green analytical chemistry. *Molecules* **2015**, *20*, 10928–46.
10. Fernandes, N.; Nimdeo, M.; Choudhar, V.; Kulkarni, R.; Pande, V.; Nikalje, A. Dual wavelength and simultaneous equation spectrophotometric methods for estimation of atenolol and indapamide in their combined dosage form. *Int. J. Chem. Sci.* **2008**, *6*, 29–35.
11. Bindaiya, S.; Bankey, S.; Jain, D. Simultaneous determination of nitazoxanide and ofloxacin in tablet by ultraviolet spectrophotometry (dual wavelength method). *Int. J. Chem. Tech. Res.* **2010**, *2*, 11–5.
12. Ramadan, N. K.; Mohamed, H. M.; Moustafa, A. A. Simultaneous determination of rabeprazole sodium and domperidone. *J. Appl. Pharm. Sci.* **2011**, *1*, 73.
13. Lotfy, H. M.; Saleh, S. S.; Hassan, N. Y.; Elgizawy, S. M. A comparative study of the novel ratio difference method versus conventional spectrophotometric techniques for the analysis of binary mixture with overlapped spectra. *Am. J. Anal. Chem.* **2012**, *3*, 761–9.
14. Connors, K. A. A Textbook of Pharmaceutical Analysis; John Wiley & Sons, **2007**.
15. Satana, E.; Altinay, S.; Göğür, N.; Ozkan, S. A.; Şentürk, Z. Simultaneous determination of valsartan and hydrochlorothiazide in tablets by first-derivative ultraviolet spectrophotometry and LC. *J. Pharm. Biomed. Anal.* **2001**, *25*, 1009–13.
16. Thomas, O.; Burgess, C. Techniques and instrumentation in analytical chemistry. In *UV Visible Spectrophotometry of Water Wastewater*, vol. 27, **2007**.
17. Hopkala, H.; Kowalczyk, D. Application of derivative UV spectrophotometry for the determination of ciprofloxacin, norfloxacin and ofloxacin in tablets. *Acta. Pol. Pharm.* **2000**, *57*, 3–13.
18. Meras, I. D.; Mansilla, A. E.; López, F. S.; Gómez, M. R. g. Determination of triamterene and leucovorin in biological fluids by UV derivative-spectrophotometry and partial least-squares (PLS-1) calibration. *J. Pharm. Biomed. Anal.* **2002**, *27*, 81–90.



19. Lotfy, H. M.; Hagazy, M. A.-M. Comparative study of novel spectrophotometric methods manipulating ratio spectra: an application on pharmaceutical ternary mixture of omeprazole, tinidazole and clarithromycin. *Spectrochim. Acta Part A: Mol. Biomol. Spectrosc.* **2012**, *96*, 259–70.
20. Elzanfaly, E. S.; Saad, A. S.; Elaleem, A. E. B. A. A smart simple spectrophotometric method for simultaneous determination of binary mixtures. *J. Pharm. Anal.* **2012**, *2*, 382–5.
21. ICH. Validation of analytical procedures: text and methodology Q2 (R1). In: International Conference on Harmonization, Geneva, Switzerland, **2005**; pp 11–2.
22. Sahu, P. K.; Ramisetty, N. R.; Cecchi, T.; Swain, S.; Patro, C. S.; Panda, J. An overview of experimental designs in HPLC method development and validation. *J. Pharm. Biomed. Anal.* **2018**, *147*, 590–611.
23. Unger, K. K.; Skudas, R.; Schulte, M. M. Particle packed columns and monolithic columns in high-performance liquid chromatography-comparison and critical appraisal. *J. Chromatogr. A.* **2008**, *1184*, 393–415.
24. Chen, Y.; Zhang, Z.; Zhang, Y.; Zhang, X.; Zhang, Z.; Liao, Y.; Zhang, B. A new method for simultaneous determination of phenolic acids, alkaloids and limonoids in phellodendri amurensis cortex. *Molecules* **2019**, *24*, 709.
25. Florey, K. Profiles of Drug Substances, Excipients and Related Methodology; Academic Press, **1979**.
26. Annapurna, M. M. A new liquid chromatographic method for the simultaneous determination of ketorolac tromethamine and fluorometholone in the presence of hydrochlorothiazide. *Int. J. Green Pharm.* **2018**, *12*.
27. Kromidas, S. HPLC Made to Measure: A Practical Handbook for Optimization, John Wiley & Sons, **2008**, pp. S221–s233.
28. Dolan, J. A guide to HPLC and LC-MS buffer selection. In: ACE HPLC columns, **2009**; pp 1–20.
29. Tanaka, N.; McCalley, D. V. Core-shell, ultrasmall particles, monoliths, and other support materials in high-performance liquid chromatography. *J. Anal. Chem.* **2015**, *88*, 279–98.
30. Dittmann, M. M.; Wang, X. New materials for stationary phases in liquid chromatography/mass spectrometry. In: Handbook of Advanced Chromatography/Mass Spectrometry Techniques; Elsevier, **2017**; pp 179–225.
31. Gama, M. R.; Bottoli, C. B. G. Nanomaterials in liquid chromatography: recent advances in stationary phases. In: Nanomaterials and Chromatography; Elsevier, **2018**; pp 255–97.
32. Timmermans, P. B. M. W. M.; van Zwieten, P. A. Correlations between central hypotensive and peripheral hypertensive effects of structurally dissimilar alpha-adrenoceptor agonists. *Life. Sci.* **1981**, *28*, 653–60.
33. Yoshida, F.; Topliss, J. G. Unified model for the corneal permeability of related and diverse compounds with respect to their physicochemical properties. *J. Pharm. Sci.* **1996**, *85*, 819–23.
34. Pradhan, P. Study of forced degradation behavior of fluorometholone by reversed-phase high-performance liquid chromatography. *Int. J. Green Pharm.* **2018**, *12* 1–8.
35. Hafez, H. M.; Elshanawany, A. A.; Abdelaziz, L. M.; Mohram, M. S. HPLC method for determination of fluorometholone and sodium cromoglycate in bulk and ophthalmic solution. *J. Appl. Pharm.* **2015**, *7*, 451–8.
36. Angirekula, N.; Mukthinuthalapati, M. A. Development and validation of stability indicating HPLC method for the determination of fluorometholone in eye drops formulations. *Acta Sci. Pharm. Sci.* **2018**, *2*, 07–14.
37. Winfield, J. M. Acetonitrile, a convenient solvent for inorganic fluorides. *J. Fluor. Chem.* **1984**, *25*, 91–8.
38. Al-Rimawi, F.; Zareer, W.; Rabie, S.; Quod, M. Development and validation of a reversed-phase HPLC method for analysis of tetrahydrozoline hydrochloride in eye drop formulations. *J. Pharm. Anal.* **2012**, *2*, 67–70.
39. Lamie, N. T. Comparative study of spectrophotometric methods manipulating ratio spectra: An application on pharmaceutical binary mixture of cinnarizine and dimenhydrinate. *Spectrochim. Acta Part A: Mol. Biomol. Spectrosc.* **2015**, *141*, 193–201.
40. Lotfy, H. M.; Hegazy, M. A.; Rezk, M. R.; Omran, Y. R. Novel spectrophotometric methods for simultaneous determination of timolol and dorzolamide in their binary mixture. *Spectrochim. Acta Part A: Mol. Biomol. Spectrosc.* **2014**, *126*, 197–207.
41. U.S.P. Convention. Physical Tests/621 Chromatography in: USP 40-NF 35; United States Pharmacopeia, **2017**; pp 1–12.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1556/1326.2020.00783>.

