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RESEARCH ARTICLE

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Stepwise optimization and sensitivity improvement of green micellar electrokinetic chromatography method to simultaneously determine some fluoroquinolones and glucocorticoids present in various binary ophthalmic formulations

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Abstract

A sensitive micellar electrokinetic chromatography method is presented to simultaneously quantify ofloxacin, gatifloxacin, dexamethasone sodium phosphate and prednisolone acetate. The method has the advantages of being rapid, accurate, reproducible, ecologically acceptable and sensitive. The electrophoretic separation utilized 20 mM borate buffer as background electrolyte with pH 10.0 ± 0.1 and 50 mM sodium dodecyl sulfate as a micelle forming molecule. A capillary tube (50 μm i.d., 33 cm) of fused silica was used and on-column diode array detection at 243 nm for dexamethasone sodium phosphate and prednisolone acetate, and 290 nm for ofloxacin and gatifloxacin. Various factors were optimized such as the background electrolyte (type, concentration and pH), addition of sodium dodecyl sulfate and its concentration, detection wavelength, applied voltage and injection parameters. The studied drugs were efficiently separated in 6.2 min, at 20 kV with high resolution. The greenness of the method was estimated using an eco-scale tool and the presented method was found to have excellent green characteristics. The method was validated in conformance with International Conference on Harmonization guidelines, with acceptable accuracy, precision and selectivity. The suggested method can be employed for the economic analysis of the four drugs in dissimilar binary combinations of eye drops saving solvents and chemicals.

KEYWORDS

dexamethasone, electrophoresis, gatifloxacin, green, ofloxacin, prednisolone

1 | INTRODUCTION

Ofloxacin (OFX) [9-fluoro-3-methyl-10-(4-methyl piperazine-1-yl)-7-oxo-2,3-dihydro-7H pyrido[1,2, 3-de]-1,4-benzoxazine-6-carboxylic acid] (British Pharmacopoeia, 2013) (Figure 1a) and gatifloxacin (GFN), [1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methyl piperazine-l-yl)-4-oxo-quinoline carboxylic acid] (Indian Pharmacopoeia, 2010) (Figure 1b) are fluoroquinolone antibiotics with wide range of activity. Both drugs are effective in fighting Gram-positive and Gram-negative bacteria (Wagner, Abelson, Shapiro, & Torkildsen, 2005). Dexamethasone sodium phosphate (DSP) [9-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl disodium phosphate] (British Pharmacopoeia, 2013) (Figure 1c) and prednisolone acetate (PA) [11β,17-dihydroxy-3,20-dioxopregna-1,4-dien-21-yl acetate] (Figure 1d) are effective anti-inflammatory corticosteroids (Sweetman, 2005). The three investigated binary ophthalmic

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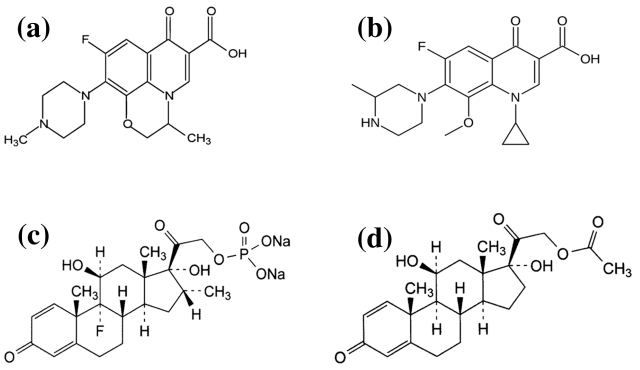

FIGURE 1 Chemical structure of (a) gatifloxacin (GFN), (b) ofloxacin (OFX), (c) dexamethasone sodium phosphate (DSP) and (d) prednisolone acetate (PA)

combinations (GFN–DSP)—combination A, (GFN–PA) combination B and (OFX–PA) combination C—are used to help in the management of acute and sub-acute conjunctivitis and keratitis (Singh, 2001; Yasueda & Inada, 2001). Various methods have been reported describing the assay of the fluoroquinolones and corticosteroids either singly or in combination. These methods include spectrophotometry (Abdel-Razeq, Darwish, Zaazaa, Nasr, & Zeinab, 2015; Patel & Patel, 2013; Pradhan, Raiyani, Shah, Patel, & Upadhyay, 2015; Sversut et al., 2017), HPLC (El Gammal, El-Wasseef, El-Ashry, & Saadia, 2018; Gandhi, Rao, & Rao, 2016; Saad, Essam, Elzanfaly, & Amer, 2020; Sher, Fatima, Perveen, & Siddiqui, 2019; Sversut et al., 2014), thin-layer chromatography (TLC) (Dorofeev, Konovalov, Kochin, & Arzamastsev, 2004; Musharraf, Fatima, & Sultana, 2012; Saad et al., 2020; Seid, Hymete, & Bekhit, 2012), capillary electrophoresis (Al Azzam, Saad, Adnan, & Aboul-Enein, 2010; Alnajjar, 2013; Bourdon et al., 2013; Gallego & Arroyo, 2003; Horstkötter & Blaschke, 2001; Maher et al., 2013; Olędzka, Kowalski, Plenis, & Bączek, 2017; British Pharmacopoeia, 2013; Song, Bai, Jia, & Zhou, 2008; Suliman, Elbashir, & Schmitz, 2015; Wang et al., 2009; Zhu, Jiang, & Hu, 2002) and electrochemical methods (El-Rahman, Lotfy, Hegazy, Rezk, & Rostom, 2015; Smajdor, Piech, & Paczosa-Bator, 2016; Zhang, Gu, Ding, Li, & Liu, 2013; Zhang, Gu, Ding, Zhang, & Li, 2013). After a thorough investigation of literature, it was observed that no capillary electrophoretic method has been published for the simultaneous assay of the four chosen drugs. Moreover, the reported electrophoretic methods have some drawbacks such as long runtime (Al Azzam et al., 2010; Bourdon et al., 2013; Horstkötter & Blaschke, 2001; Suliman et al., 2015; Wang et al., 2009), high energy consumption (Gallego & Arroyo, 2003; Song, Bai, Jia, & Zhou, 2008), high buffer concentration that causes current increase and joule heating (Maher et al., 2013; Olędzka et al., 2017) and low detection sensitivity (Alnajjar, 2013; Song, Bai, Jia, & Zhou, 2008; Zhu et al., 2002). None of the reported methods has evaluated the greenness of the applied technique.

Capillary electrophoresis (CE) is an instrumental evolution of traditional electrophoretic techniques that has emerged as an excellent separation technique offering various possibilities for pharmaceutical analysis. In CE separation occurs in fused-silica capillaries and involves the application of high voltages across buffer-filled capillaries in order to achieve separation. Several electrophoresis techniques have been utilized for the separation of active pharmaceutical ingredients, proteins, polynucleotides and other biopolymers (Silva, 2013; Terabe, 2010). Capillary electrophoresis has gained momentum in pharmaceutical analysis, being regarded as an alternative and also a complementary technique to the more frequently used high-performance liquid chromatography (HPLC). This is attributed to its speed of analysis, high efficiency, automated analytical equipment, low reagent and sample consumption and rapid method development (Hancu, Simon, Rusu, Mircia, & Gyéresi, 2013). The two most common techniques in CE are capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC).

In pharmaceutical analysis, separation of substances with very similar structural and physico-chemical properties is often required. Being based on differences between the electrophoretic mobilities of the analytes, the classic CZE method is not suitable for the separation of neutral substances, which migrate towards the detector with the same velocity as the electro-osmotic flow (EOF). MEKC extends the applicability of CE to neutral analytes, which cannot be separated using simple free solution CE. The same instrumentation that is used for CZE is used for MEKC, which demonstrates the versatility and adaptability of the method (Silva, 2007). MEKC differs from CZE because it uses an ionic micellar solution instead of the simple buffer salt solution. MEKC can be used for the separation of both ionic and neutral substances while CZE typically separates only ionic substances (Miękus et al., 2016). Thereby MEKC has a great advantage over CZE in the separation of mixtures containing both ionic and neutral analytes (Viglio, Fumagalli, Ferrari, & Iadarola, 2010). The separation principle of MEKC is based on the differential partition of the analytes between micelles and water while CZE is based on the differences between the electrophoretic mobility of the analytes (Sun, Park, Ha, & Kang, 2020). Unfortunately, MEKC still suffers from low detection sensitivity, as does CZE, owing to the small volume of injected sample and short path length which faces the detector. However, this drawback can be overcome by online sample preconcentration techniques such as stacking and sweeping (Olędzka et al., 2018). MEKC on microchips has received much attention recently, with systems having been developed that can perform analytical procedures involving chemical reactions, separation and detection on a single microchip (Breadmore et al., 2019; Deeb, Dawwas, & Gust, 2013; Rezende, Martins, Talhavini, & Coltro, 2020; Štěpánová & Kašička, 2016).

Although HPLC is the most frequently used and well-developed method for both qualitative and quantitative analysis, most of the chromatographic applications use hazardous solvents and produce large amounts of toxic waste that damage the environment. Recently the Society of Analytical Chemistry has been seeking implementation of eco-friendly methods that eliminate or decrease toxic and corrosive waste (Armenta, de la Guardia, & M., 2008). Evaluation of the greenness of analytical methods is

profoundly important for most method developers. One of the most recent greenness valuation tools for analytical techniques is the analytical eco-scale (Gałuszka, Migaszewski, Konieczka, & Namieśnik, 2012), which is a semi-quantitative tool to evaluate and compare analytical methods based on their conformity with green chemistry principles (Al-Alamein, El-Rahman, Abdel-Moety, & Fawaz, 2019). 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 (Tobiszewski, Marc, Galuszka, & Namiesnik, 2015). In this manuscript the greenness assessment was implemented using the eco-scale tool.

Analysis in pharmaceutical quality-control laboratories faces challenges in time, chemicals, solvents, manpower and cost. Therefore, the development of analytical methods that save cost, time and the environment should be encouraged. A very specific situation is the assessment of eye drops, where few pharmaceutical factories have the facilities to produce sterile products for many vendors. This situation forces these facilities to provide production and quality control analysis for various products in a short time. The aim of this work was to help such factories and authoritative quality control laboratories in the evaluation of various eyedrop products in the same run with the advantages of saving time, cost and the environment. One rapid MEKC method can be utilized for the analysis of three eyedrop combinations instead of applying three different techniques, one for each dosage form. Accordingly, the purpose of this study was to unravel the problem of separating a neutral analyte in the presence of charged molecules by MEKC with comparable sensitivity with HPLC, offering advantages over HPLC in being more eco-friendly and with minimal solvent consumption as represented by the greenness assessment. The development and validation of a MEKC method, utilizing one-factor-at-a-time approach, for the concurrent quantification OFX, GFN, DSP and PA in bulk powders and many pharmaceutical dosage forms including the three investigated binary combinations are reported. The analytical figures of merits of the described methods have been checked as well.

2 | EXPERIMENTAL

2.1 | MATERIALS

Pure samples

Pure GFN was kindly supplied by Global Napi pharmaceuticals, Cairo, Egypt. Its purity was certified to be 99.89 ± 0.69. Pure OFX was kindly supplied by Sanofi Pharmaceutical Company, Cairo, Egypt. Its purity was certified to be 100.15 ± 0.52. Pure DSP was kindly supplied by Orchidia Pharmaceutical Company, Cairo, Egypt. Its purity was certified to be 99.69 ± 0.23. Pure PA was kindly supplied by EIPICO Pharmaceutical Company, Cairo, Egypt. Its purity was certified to be 100.38 ± 0.42.

Market samples

Gatilox DM® eyedrops were from Akums Drugs & Pharmaceuticals Ltd (India), and labeled to contain GFN and DSP in concentrations of 3 and 1 mg ml^{-1}, respectively (batch number FTP 3033). Gatiquin-P® eyedrops by Aditi Pharmaceuticals Pvt. Ltd (India) were labeled to consist of GFN and PA in concentrations of 3 and 10 mg ml^{-1} (batch number DZ5052). Ocepred® eye drops from Sun Pharma Laboratories Ltd (India) were labeled as having OFX and PA in concentrations of 3 and 10 mg ml^{-1}, respectively (batch number HKP0403). All pharmaceutical dosage forms were bought from the Indian market.

Solvents and chemicals

Sodium hydroxide pellets, methanol, phosphoric acid sodium borate decahydrate and sodium dodecyl sulfate were purchased from Sigma-Aldrich (Germany). All chemicals and solvents used were of HPLC grade A water purification system (New Human Power I, Korea) was used to obtain ultra-pure water.

2.2 | Instruments

Electrophoretic separations were carried out using an Agilent HP CE 7100 equipped with an automatic injector and an autosampler, coupled with a diode array detector. ChemStation software (Agilent Technologies, Germany) was utilized for managing the CE system, data acquisition and data inspection. A bare fused silica capillary tube (Agilent Technologies, Germany) 50 μm id, 325 μm o.d. with a total/effective length of 33.5/25.0 cm was used in all investigations.

2.3 | Electrophoretic conditions

Before the initial use the capillary was cleaned with the following procedure: 1 M NaOH (20 min), water (5 min), 1 M phosphoric acid (20 min), water (5 min), 0.1 M NaOH (20 min), and water (5 min). Daily preconditioning was implemented by flushing with 0.1 M NaOH (15 min) and water (15 min) then washing with BGE for 15 min. In order to guarantee the iteration of migration times, pre-run conditioning was employed with 0.1 M NaOH (1 min) and BGE (5 min), while water flushing for 1 min after each run was crucial. Buffer replenishment after every five runs was also necessary. These washing procedures were conducted in accordance with the manufacturer's instructions and literature (Nigović, Sertić, & Mornar, 2013). Separations were accomplished using adjusted BGE that consisted of borate buffer 20 mM as BGE at pH 10.0 ± 0.1 and 50 mM sodium dodecyl sulfate (SDS). Analyses were conducted at 20 kV applied voltage and a stable temperature of 25°C. Samples were introduced into the capillary hydrodynamically using a pressure of 100 mbar for 10 s. Prior to use all solutions were filtered through 0.22 μm pore size disposable polyester filters (Chromafil, Macherey–Nagel, Germany). Detection was performed at 243 nm for DSP and PA, and 290 nm for OFX and GFN.

2.4 | Stock solutions

Standard stock solutions of GFN, OFX, DSP and PA 1 mg ml^{-1} were prepared separately using methanol as a solvent. Working standard solutions of 100 μg ml^{-1} of GFN, OFX, DSP and PA were prepared from stock solutions by appropriate dilution with 50 mM SDS dissolved in water.

2.5 | Background electrolyte

Borate buffer 20 mM was prepared by appropriate dilution of 0.5 M stock solution of sodium borate decahydrate, adjusting the pH with 0.1 M NaOH and completing the volume to 25 ml with 50 mM SDS. The solutions were prepared daily and filtered through 0.22 μm syringe filters before use.

2.6 | Procedures

2.6.1 | Construction of calibration curves

Aliquots of OFX, GFN, DSP and PA were accurately pipetted from their corresponding standard working solutions (100 μg ml^{-1}) into four distinct groups of 10 ml measuring flasks. A 50 mM SDS solution was used to complete the volume to prepare different concentrations covering the ranges 1.5–80 μg ml^{-1} for OFX, 2–80 μg ml^{-1} for GFN and 3–100 μg ml^{-1} for DSP and PA. Solutions were then filtered with a 0.22 μm syringe membrane filter, injected in triplicate using the optimized conditions. The adjusted peak areas acquired for each concentration of OFX, GFN, DSP and PA were plotted vs. the corresponding concentrations. The regression equations were deducted. Validation was fulfilled in agreement with International Conference on Harmonization (ICH) guidelines and the following parameters were investigated: linearity, range, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision.

2.6.2 | Application of the suggested methods on pharmaceutical formulations

One milliliter of Gatilox® eye drops was pipetted into a 10 ml volumetric flask, then filled to the mark with 50 mM SDS to obtain 300 μg ml^{-1} of GFN and 100 μg ml^{-1} of DSP. An appropriate dilution was made with 50 mM SDS to obtain a working solution of 15 μg ml^{-1} of GFN and 5 μg ml^{-1} of DSP.

One milliliter of Gatiquin-P® eye drops was transferred into a 10 ml volumetric flask, then brought up to the mark with 50 mM SDS to obtain 300 μg ml^{-1} of GFN and 1,000 μg ml^{-1} of PA. An appropriate dilution with the 50 mM SDS was made to prepare a working solution of 6.0 μg ml^{-1} of GFN and 20.0 μg ml^{-1} of PA.

One milliliter of Ocepred® eye drops was transferred into a 10 ml volumetric flask, then the volume was completed with 50 mM SDS to obtain 300 μg ml^{-1} of OFX and 1,000 μg ml^{-1} of PA. An appropriate dilution was made with the 50 mM SDS to prepare a working solution of 6.0 μg ml^{-1} of OFX and 20.0 μg ml^{-1} of PA.

3 | RESULTS AND DISCUSSION

HPLC is an analytical practice universally used in the pharmaceutical industry for the assessment of drug potency and purity. However, HPLC analysis can be disadvantageous in terms of time consumption and its use of significant amounts of organic solvents, making it costly and not eco-friendly. In this work, the authors describe an alternative to HPLC that can be utilized for pharmaceutical analysis. Capillary electrophoresis is being more widely utilized for biochemical and analytical applications with advances in autosamplers and advancements in injection precision, better resolution and number of theoretical plates. The capability for this instrumentation to be used in conjunction with or as a replacement for HPLC is being assessed in analytical laboratories (Li et al., 2016).

Upon trying to separate the studied drugs, two challenges were faced: (a) the structural similarity between the two studied fluoroquinolones, requiring high resolution power to be separated; and (b) within the pH range PA is present in its unionized form (pKa of PA is 12.61) (Silva, 2013). Therefore it is eluted with organic solvents, making it challenging to quantify it within the calibration range. These two problems are present in the HPLC separation method for the drugs studied by our group (Saad et al., 2020). Although gradient elution HPLC was successful in achieving good separation, it has certain drawbacks. The major weakness is that the compositions of the stationary and mobile phases are altered during analysis and column regeneration is required before the subsequent analysis, consuming time, solvents and column lifetime. Also the well-established problem of baseline drift may require corrections (Dolan & Snyder, 2017; Uchiho, Goto, Kamahori, & Koda, 2017). MEKC is increasingly regarded as a promising alternative method to allow the separation of neutral molecules from charged ones, offering higher resolving power, a requirement for smaller volumes of analytes and shorter analysis times than HPLC (Pappas, Gayton-Ely, & Holland, 2005). The presented method has advantages over the reported electrophoresis methods. It can be utilized for the analytical investigation of more than one dosage form with the same conditions, saving time and capillary usage. The proposed method has a lower detection limit than the reported methods (Alnajjar, 2013; Song, Bai, Jia, & Zhou, 2008; Zhu et al., 2002), shorter runtime (Al Azzam et al., 2010; Bourdon et al., 2013; Horstkötter & Blaschke, 2001; Suliman et al., 2015; Wang et al., 2009), lower buffer salt concentration, limiting the increase in current and joule heating (Maher et al., 2013; Olędzka et al., 2017) and lower voltage consumption, saving power and cost and limiting environmental hazard (Gallego & Arroyo, 2003; Song, Bai, Jia, & Zhou, 2008) with the added value of good greenness assessment in comparison with HPLC. After preliminary screening of experimental conditions, we utilized one-factor-at-a-time approach for fine optimization of the presented method with a limited number of experiments.

3.1 | Method development and optimization

3.1.1 | Optimization of MEKC separation conditions

The method was developed in two steps. The first step was the simple separation of two components of combination A (GFN-DSP) by a simple CZE method using methanol as EOF marker (Figure 2) with full optimization and validation according to ICH as shown in Figures S1 and S2 (Tables S1 and S2). Upon progression of the optimized conditions of CZE for the simultaneous separation of the four studied drugs, a problem was faced of the elution of PA with methanol even with changing the pH, buffer salt, strength and applied voltage as shown in Figure 3. Therefore, to solve the problem, the method was shifted to MEKC, with which PA could be solubilized inside the micelle of SDS (Figure 4) and eluted last owing to the large weight and negative charges on the micelle.

3.1.2 | pH

In CE, the pH, buffer type and applied voltage aree key for the optimization of analyte separation. We had to take into consideration the physico-chemical properties of the studied drugs. It should be stated that DSP is acidic in nature with pK_a 1.89 (Kalam, 2016), the pK_a of GFN is about 6.18 (Ocaña, Barragán, & Callejón, 2005), the average pK_a of OFX is 7.14 (Okeri & Arhewoh, 2008) and that of PA is 12.61 (Yadav, Chandra, Goyal, & Shim, 2013). Therefore, an alkaline pH would be a good choice to apply different electric charges to the compounds. Because OFX and GFN would have a negative charge, DSP would have two negative charges while PA would be included inside the micelle of SDS, thus the whole particle has multiple negative charges. Therefore, the expected order of elution is OFX, GFN, DSP and finally PA inside the SDS micelle. Methanol serves as an EOF marker to monitor the changes in migration time, indicating variations in the driving power of EOF. Also, the choice of alkaline pH rather than neutral or acidic was based on the fact that the alkaline medium kept the inner silica of the capillary tube ionized (Goyon et al., 2018), thus minimizing the run time. The observed increase of apparent mobilities with increasing the buffer pH was attributed to a predominant effect of pH on EOF. Different pH values (7.0–12) were tried; pH 7 showed poor peak shape for DSP and pH 8 and 9 showed poor resolution between OFX and GFN, while pH 11 and 12 showed broad peak shapes especially PA. pH 10 was the optimum in achieving good resolution of all of the components to be separated in a short time.

3.1.3 | Buffer type and concentrations

Borate and phosphate buffers were tested in the studied pH range. The best results concerning migration time, resolution, peak shape, peak height, baseline noise and the electric current produced were achieved using 20 mM borate (pH 10) adjusted with 0.1 M NaOH. The

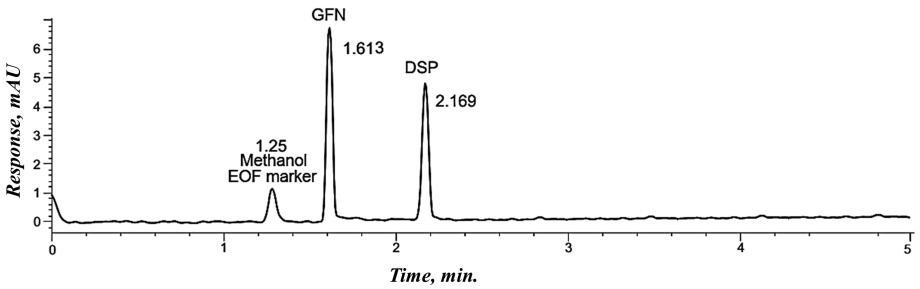

FIGURE 2 Capillary zone electrophoresis (CZE) electropherogram of GFN–DSP using 20 mM borate as BGE and 20 kV. Methanol (MT = 1.25), GFN (50 μg ml^{-1}, MT = 1.613) and DSP (50 μg ml^{-1}, MT = 2.169) at 243 nm

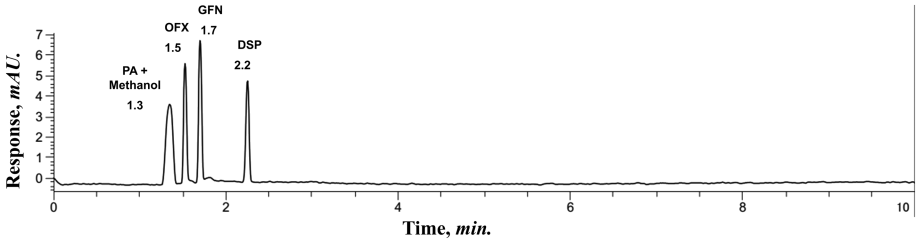

FIGURE 3 CZE electropherogram of using 20 mM borate as BGE and 20 kV. PA and methanol (50 μg ml^{-1}, MT = 1.63), OFX (50 μg ml^{-1}, MT = 1.84), GFN (50 μg ml^{-1}, MT = 2.06) and DSP (50 μg ml^{-1}, MT = 2.169) at 243 nm

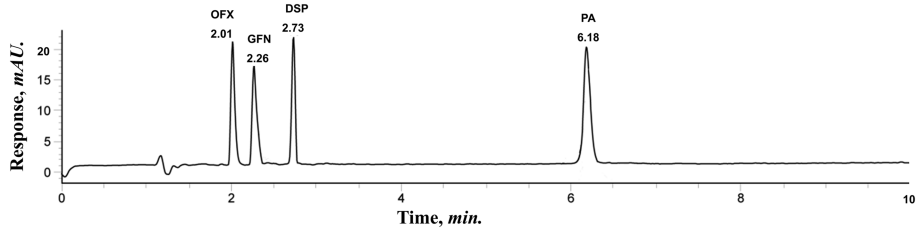

FIGURE 4 MEKC electropherogram showing separation using 20 mM borate as BGE 50 mM and SDS as a solvent and 20 kV. SDS (MT = 1.25), OFX (50 μg ml^{-1}, MT = 2.01), GFN (50 μg ml^{-1}, MT = 2.26), DSP (50 μg ml^{-1}, MT = 2.73) and PA (50 μg ml^{-1}, MT = 6.18) at 243 nm

influence of BGE concentration on the separation was examined in the range from 10 to 50 mM at a stable SDS concentration of 20 mM. The results demonstrated a delay in migration time with the rise of buffer molarity. This effect is correlated with the decline of zeta potential formed at the capillary–BGE interface and subsequently lower EOF. At 50 mM buffer the developed current in the capillary tube was high (>120 A), while the resolution was not significantly improved. These results are in accordance with the literature (Nigović et al., 2013). Therefore, a BGE of 20 mM borate buffer was selected.

3.1.4 | Surfactant

SDS was chosen as surfactant because at concentration higher than the critical micelle concentration (CMC), it would have an external negative charge. That would improve the separation and solve the problem of PA being eluted with the organic solvent in CZE. Separation of PA requires partitioning into charged micelles that migrate at a different rate from the EOF. SDS was selected as a widely used and low-priced surfactant. In order to optimize the SDS concentration, a range of 10–50 mM was studied. Different concentrations of SDS were tried from 10 to 50 mM, all above the CMC (Hancu et al., 2013). Prednisolone is a relatively lipophilic compound PA with reported log*P* of 2.5 (Vogt et al., 2007) and its migration time increased with the rise in SDS concentration. Methanol on the other hand is relatively hydrophilic with reported log*P* of 0.8 (Moffat, David, Widdop, & Watts, 2011), so it is not retained inside the SDS micelle, which makes it the perfect EOF marker in addition to its role in preparing the stock solutions. This is exactly the purpose of using MEKC in this separation instead of CZE. In CZE, methanol and PA are eluted at the same time, while in MEKC, PA is incorporated in the SDS micelle and methanol is not. With SDS, repulsion between negatively charged and the formed micelle was observed owing to dissimilarities in their electrophoretic mobilities and lipophilic properties. The best concentration, chosen based on the effect on resolution runtime and current, was 50 mM SDS, while concentrations <50 showed poor peak shape and resolution, especially between OFX and GFN.

3.1.5 | Applied voltage

Voltages of 15–25 kV were applied. Increasing the voltage led to shorter migration time and lower resolution and increased the produced current. Therefore, the optimum separation and migration times with reasonable current (50 μA) were achieved with 20 kV.

3.1.6 | Detection wavelength

The detection was tested at 200.0, 243.0 and 290 nm, and the best sensitivity was obtained with wavelength 290 nm for OFX and GFN and 243 nm for DSP and PA. Peak purity of the examined drugs was assessed according to the manufacturer's specifications as shown in Figure S1. The 3D plot of the separation showing the spectrum of each drug, which serves as proof of purity and identity, is also shown in Figure S2. The average migration times were 2.015 min for OFX, 2.266 for GFN, 2.731 for DSP and 6.182 min for PA.

3.1.7 | Injection parameters

Hydrodynamic injection parameters were optimized to increase the sensitivity without negatively affecting the resolution. Different pressures and timing were tested, and the best sensitivity was obtained with 100 mbar for 10 s. These results were accordance with the literature (Iio, Chinaka, Takayama, & Hayakawa, 2005; Liu, Shi, & Liu, 2010; Lourenço, Aguiar, de Oliveira, & de Gaitani, 2015). Increasing the time to longer than 10 s was found to cause sample overload and changes in peak shape.

3.1.8 | Temperature

The effect of temperature on the migration time and resolution was examined for 15, 20, 25 and 30°C under the separation conditions selected above. Temperature alters the electrolyte viscosity.

by 2–3% for every °C and hence changes the electrophoretic mobility and EOF speed (Damić & Nigović, 2010; Jimidar, Van Nyen, Van Ael, & De Smet, 2008). A temperature of 20°C was optimum in terms of resolution and peak shape. As expected, at higher temperature (30°C) the run time was shorter, but the resolution decreased. At 15 and 20°C poor peak shape was obtained. These results are in accordance with the literature (Damić & Nigović, 2010; Hancu et al., 2013).

3.1.9 | Sample stacking to increase sensitivity

It is well known that detection sensitivity in CE is low in terms of concentration sensitivity when photometric detectors are used, mostly because of the limited amounts of samples injected and the short light pathlength for the photometric detection. To overcome this weakness, numerous online sample preconcentration practices have been utilized (Simpson, Quirino, & Terabe, 2008). When the analyte is prepared in a dilute electrolyte solution or a low-conductivity solution, and when the BGS is a high-concentration electrolyte solution or a high-conductivity solution, the analyte ion migrates rapidly in the sample zone but slowly in BGS under electrophoresis because the electrophoretic velocity is proportional to the field strength. This easily implemented technique yields effective sample preconcentration (Olędzka et al., 2017; Šlampová, Malá, & Gebauer, 2019). In the presented manuscript stacking was achieved by preparing the samples in SDS and pure water, which have low conductivity, while the BGE consisted of buffer and SDS with higher conductivity that allowed refocusing of the sample in narrow zone inside the capillary tube. This technique increased the sensitivity more than 13-fold. For example, the LOD of GFN is 0.65 μg ml^{-1} compared with 9.00 μg ml^{-1} obtained

TABLE 1 System suitability parameters of the proposed MEKC method

Parameter	OFX	GFN	DSP	PA	Reference values (US Pharmacopeia Convention, 2017)
Migration time	2.01	2.26	2.73	6.18	
K′ (retention factor)	0.43	0.61	0.95	3.41	The higher the retention factor, the smaller the retardation factor
α (Relative retention)	1.12		1.21	2.26	>1
Resolution	2.52		4.94	30.04	>1
Symmetry factor	1.05	1.1	0.98	1.01	=1 for typical symmetric peak
N (Number of theoretical plates)	7,895	6,991	19,006	27,117	>2,000

MEKC, Micellar electrokinetic chromatography ; OFX, ofloxacin; GFN, gatifloxacin; DSP, dexamethasone sodium phosphate; PA, prednisolone acetate

by typical injection. These results agree with the literature (Wang, Chen, Chen, Cheng, & Wu, 2012) (Olędzka et al., 2017, Šlampová et al., 2019). System suitability tests were performed to ensure the capability of the MEKC system and the procedure of providing quality data as shown in Table 1.

3.2 | Method validation

The suggested method was validated in conformance with ICH guidelines (ICH, 2005) and the calculated validation parameters are described below and shown in Table 2.

3.2.1 | Range and linearity

Under the specified optimized conditions, a linear correlation was acquired between the relative peak areas at the two selected wavelengths and the corresponding concentrations of the drugs in the concentration ranges of 1.5–80 μg ml^{-1} for OFX, 2–80 μg ml^{-1} for GFN and 3–100 μg ml^{-1} for DSP and PA. Seven concentrations of each drug were utilized for calibration curve construction. These ranges allowed the examination of the dosage form in a single run. Linear relationships for both drugs were obtained and the regression equations were computed to confirm the linearity claims.

TABLE 2 Validation parameters of the proposed MEKC method for the determination of OFX, GFN, DSP and PA

Parameter	OFX	GFN	DSP	PA
Wavelength (nm)	290 nm	290 nm	243 nm	243 nm
Regression parameters				
Working range (μg ml^{-1})	1.5–80	2–80	3–100	3–100
Intercept	−4.7003	−7.6841	−0.4716	−10.631
Slope	10.778	7.5128	4.2921	7.0968
Correlation coefficient	0.9999	0.9999	0.9999	0.9999
Accuracy				
Mean ± RSD[a]	100.42 ± 1.21	99.07 ± 0.78	100.93 ± 1.13	100.70 ± 0.76
Precision (± RSD, %)				
Repeatability[b]	±1.06	±1.34	±1.07	±1.11
Intermediate precision[c]	±1.27	±1.61	±1.44	±1.45
LOD[d] (μg ml^{-1})	0.48	0.65	0.89	0.65
LOQ[d] (μg ml^{-1})	1.48	1.98	2.70	1.99

[a]Average of three concentrations (5, 20 and 55 μg ml^{-1}) for OFX, GFN, DSP and PA.
[b]Intraday precision: the RSD of three different concentrations (6, 25 and 35 μg ml^{-1} for OFX, GFN, DSP and PA)/three replicates each, within the same day.
[c]Interday precision: the RSD of three different concentrations (6, 25 and 35 for μg ml^{-1} OFX, GFN, DSP and PA)/3 replicates each, repeated on three successive days.
[d]Limits of detection and quantitation. Determined via signal-to-noise ratio calculations.

TABLE 3 System suitability parameters for robustness of the proposed MEKC method

Drug	Robustness parameter		T^a	N^a	Rs^b	Assay (%)[c]
OFX	Buffer concentration	20 + 5 mM	1.05	7,895		100.35
		20–5 mM	1.04	7,884		99.34
	SDS concentration	50 + 5 mM	1.01	7,862		99.87
		50–5 mM	1.03	7,835		100.76
	pH	10 + 0.2	0.99	7,899		99.74
		10–0.2	1.05	7,902		98.69
	Voltage applied	20 + 2 kV	1.05	7,855		100.74
		20–2 kV	1.03	7,890		99.69
	Wavelength	290 + 2 nm	0.97	7,895		101.39
		290–2 nm	1.01	7,893		101.74
	Temperature	25 + 2°C	1.02	7,799		99.39
		25–2°C	0.96	7,850		98.79
GFN	Buffer concentration	20 + 5 mM	1.13	6,992	2.51	98.99
		20–5 mM	1.11	6,990	2.43	101.42
	SDS concentration	50 + 5 mM	1.09	6,950	2.34	99.67
		50–5 mM	1.11	6,980	2.11	98.99
	pH	10 + 0.2	1.16	7,005	1.89	100.25
		10–0.2	1.17	6,987	2.3	100.45
	Voltage applied	20 + 2 kV	0.99	6,959	2.44	101.26
		20–2 kV	1.13	7,010	1.99	99.79
	Wavelength	290 + 2 nm	1.11	6,995	2.36	100.85
		290–2 nm	1.12	6,990	2.55	99.28
	Temperature	25 + 2°C	1.15	7,050	1.88	100.37
		25–2°C	1.11	7,000	2.58	99.3
DSP	Buffer concentration	20 + 5 mM	0.99	19,000	4.94	99.85
		20–5 mM	0.98	19,050	4.99	100.95
	SDS concentration	50 + 5 mM	1	19,081	5.02	99.53
		50–5 mM	0.97	19,100	5.33	98.17
	pH	10 + 0.2	1.01	19,006	5.21	100.67
		10–0.2	0.99	19,087	4.99	99.67
	Voltage applied	20 + 2 kV	0.99	19,105	4.84	99.39
		20–2 kV	1.02	19,008	5.26	100.09
	Wavelength	243 + 2 nm	1.02	19,020	4.98	99.49
		243–2 nm	1.01	19,015	5.31	99.98
	Temperature	25 + 2°C	0.99	19,084	5.52	100.99
		25–2°C	0.98	19,027	5.1	98.24
PA	Buffer concentration	20 + 5 mM	1.01	27,117	30.24	101.42
		20–5 mM	1.03	27,321	29.5	99.39
	SDS concentration	50 + 5 mM	0.99	27,110	28.5	101.85
		50–5 mM	1.05	27,204	30.3	99.27
	pH	10 + 0.2	1.11	27,186	29.5	99.76
		10–0.2	0.97	27,058	29.9	99.45
	Voltage applied	20 + 2 kV	1.15	27,351	30.5	100.44
		20–2 kV	1.11	27,025	28.5	101.69
	Wavelength	243 + 2 nm	0.99	27,111	30.1	99.37
		243–2 nm	0.96	27,116	30.1	100.71

TABLE 3 (Continued)

Drug	Robustness parameter		T^a	N^a	Rs^b	Assay (%)[c]
	Temperature	25 + 2°C	1.05	27,316	29.6	99.39
		25–2°C	0.94	27,014	29.74	100.78

[a]Tailing factor and number of theoretical plates determined for individual peaks.
[b]Resolution factor determined between each drug peak and the previous one.
[c]Assay (%) calculated from the regression equation.

TABLE 4 Results obtained from the analysis Gatilox-DM®, Gatiquin-P® and Ocepred® eyedrops using the proposed MEKC method and application of standard addition technique

			Standard addition technique			
			Taken	Pure added	Found	
Pharmaceutical formulation	Drug	Found (%)[a] ± RSD	(μg ml^{-1})	(μg ml^{-1})	(μg ml^{-1})	Recovery (%)[a]
Gatilox-DM® eyedrops (claimed to contain 3 mg GFN and 1 mg DSP per 1 ml eyedrops), batch no. FNP3001	GFN	99.76 ± 0.29	30.00	15.00	14.923	99.49
				30.00	29.844	99.48
				35.00	35.00	100.00
	Mean ± RSD					99.65 ± 0.85
	DSP	100.58 ± 1.06	10.00	5.00	5.056	101.13
				10.00	9.949	99.49
				15.00	15.075	100.50
	Mean ± RSD					100.37 ± 0.911
Gatiquin-P® eyedrops (claimed to contain 3 mg GFN and 10 mg PA per 1 ml eyedrops), batch no. DZ5052	GFN	100.01 ± 1.01	6.00	3.00	3.011	100.35
				6.00	5.965	99.42
				10.00	10.051	100.51
	Mean ± RSD					100.09 ± 10.07
	PA	100.27 ± 0.32	20.00	10.00	10.061	100.60
				20.00	19.881	99.40
				25.00	25.057	100.23
	Mean ± RSD					99.94 ± 1.27
Ocepred® eyedrops (claimed to contain 3 mg OFX and 10 mg PA per 1 ml eyedrops), batch no. HKP0403	OFX	100.04 ± 0.55	6.00	3.00	3.006	100.20
				6.00	6.046	100.77
				10.00	9.983	99.83
	Mean ± SD					100.27 ± 1.13
	PA	100.46 ± 0.49	20.00	10.00	10.032	100.32
				20.00	19.914	99.57
				25.00	25.147	100.58
	Mean ± RSD					100.16 ± 0.89

[a]Average of three determinations.

3.2.2 | Limits of detection and quantification

The newly developed method had shown better sensitivity relative to previously reported methods with an LOD of 0.489 μg ml^{-1} for OFX, 0.654 μg ml^{-1} for GFN, 0.891 μg ml^{-1} for DSP and 0.658 μg ml^{-1} for PA (Agarwal, Dadhich, Tiwari, & Nagariya, 2013; Razzaq et al., 2017; Yu, 2014). The LOD and LOQ were calculated utilizing the signal-to-noise ratio method (ICH, 2005).

TABLE 5 Penalty points for greenness assessment for the proposed MEKC method compared with the reported HPLC method

	Penalty points	
Hazard	**MEKC**	**HPLC**
Reagents		
Acetonitrile		8
Phosphate buffer		0
Sodium dodecyl sulfate	6	
Instruments		
Energy (>1.5 kWh per sample)	2	1
Occupational hazard	0	0
Waste	6	8
Total penalty points	14	17
Analytical eco-scale total score	86[a]	83[a]

[a]>75 represents excellent green analysis;
>50 represents acceptable green analysis;
<50 represents inadequate green analysis.

3.2.3 | Accuracy

To investigate the accuracy of the presented method. Three varying concentrations of pure drugs for three replicates were injected using optimum conditions. The accuracy results are shown in Table 2.

3.2.4 | Precision

Precision of the presented approach expressed as RSD (%) was calculated by the determination of three dissimilar concentrations of pure drugs chosen from the linearity range. The intra-day RSD (%) was obtained from the results of three replicate determinations of three pure drug samples on a single day. To determine the inter-day (intermediate) precision, the same samples were examined on three consecutive days. It was found that the utilized MEKC method is precise with RSD < 2%. The results are illustrated in Table 2.

3.2.5 | Robustness

Robustness of the suggested approach was assessed by making small but deliberate variations in electrophoretic conditions. The robustness was conducted at three different concentrations for each analyte (30, 40 and 50 $\mu g\ ml^{-1}$). The extent of reproducibility and system suitability parameters of the results were within reasonable ranges, indicating that the methods were robust enough, as shown in Table 3.

3.2.6 | System suitability

System suitability was investigated according to the US Pharmacopeia by calculating the potential aspects that affect the separation, such as asymmetric factor, retention factor, relative retention, resolution, column efficiency (N) and the plate height (HETP) (Table 1; US Pharmacopeia Convention, 2017).

3.3 | Application to pharmaceutical formulation and standard addition technique

The suggested MEKC method is valid for the quantification of GFN and DSP in Gatilox® eye drops, GFN and PA in Gatiquin-P® eye drops and OFX and PA in Ocepred® eye drops. The validity of the designed method and interference of excipients in the pharmaceutical products were further investigated by implementing the standard addition technique, which showed acceptable results, as shown in Table 4. Good accuracy confirmed that the excipients present in pharmaceutical products did not disturb the determination of these drugs. The results confirm the suitability of the suggested methods for the regular examination of these active pharmaceutical ingredients in their combined formulations.

3.4 | Greenness assessment: analytical eco-scale

Eco-scale calculation is based on giving penalty points to any aspect that does not conform with perfect green technique, where the perfect green analysis has an eco-scale value of 100, excellent green analysis should score >75 and acceptable green analysis scores >50, while if the method scores <50, it will be considered as inadequately green (Gałuszka et al., 2012). The penalty point calculation for the proposed approach compared with the cited HPLC method (Saad et al., 2020) is shown in Table 5, and it is found to greener than the HPLC method, although both methods are considered green.

4 | CONCLUSION

The proposed MEKC method has solved the problem of separating neutral and ionic drugs without interference from methanol. Under optimized electrophoretic conditions, excellent separation of OFX, GFN, DSP and PA was obtained. The chosen buffer and SDS concentration showed superior system suitability parameters. The developed method has advantages over the cited HPLC method in being greener, with lower cost, lower volume consumption and better peak sharpness. Sample stacking showed significant 13-fold improvement in sensitivity of MEKC in comparison with HPLC. The proposed approach can be used for everyday analysis in QC laboratories for a cost-effective analysis of OFX, GFN, DSP and PA, in different binary, tertiary or ternary pharmaceutical preparations or in bulk powder form. The method is characterized by broad applicability, short analysis time and adequate robustness. The suggested method was validated utilizing ICH guidelines. This technique shows a promising future in lab-on-a-chip research where systems are developed that can perform

analytical procedures involving chemical reactions, separation and detection on a single microchip.

Conflict of interest and authorship conformation form

All authors have participated in (a) conception and design or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version. This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue. The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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