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A Sensitive Capillary Zone Electrophoresis Separation and Determination of Trovafloxacin and Azithromycin in Their Compliance Pak: Applications Stability –Indicating Studies

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A simple and novel capillary zone electrophoresis (CZE) method for simultaneous separation and determination of trovafloxacin mesylate (TRX) and azithromycin (AZ) was developed. The electrophoretic separation was carried out using fused silica capillary (57 cm total length and 50 cm effective length, 75 µm i.d.) along with an applied electric field of 25 kV and a running buffer containing 25 mmol L^{-1} sodium tetraborate decahydrate at pH 9.2 and methanol (80:20%) with diode array detection (DAD) at 260 nm. The sample was hydrodynamically anodic injected for 8 s at 90 mbar and the temperature of the capillary cartridge was kept at 30˚C. Levofloxacin (LVX) was used as internal standard (IS). A Linear concentration response was recorded over the concentration range of 20-140 µg mL^{-1} with limit of detection 12.0 µg mL^{-1} and limit of quantification 20.0 µg mL^{-1} for both drugs, the correlation coefficient was found to be more than 0.999. Precision study showed that the % RSD was within the range of acceptable limits. The electrophoretic method was successfully validated with respect to sensitivity, linearity, accuracy, precision, ruggedness and robustness. Stability indicating studies for the investigated drugs was examined using the proposed method.

Keywords: Capillary zone electrophoresis; Trovafloxacin mesylate; Azithromycin; Pharmaceutical formulations; Stability indicating studies

1. INTRODUCTION

Trovafloxacin (TRX), Figure 1a, is chemically known as (1α, 5α, 6α)-7-(6-amino-3-azabicyclo [3.1.0] hex-3-yl)-1-(2, 4-difluorophenyl)-6-fluoro-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid, monomethanesulfonate. It is a synthetic broad-spectrum antibacterial agent used to treat serious

infections including pneumonia, complicated abdominal infections, gynecologic and pelvic infections. Also, used in the treatment of skin infections [1]. Few analytical techniques were reported for determination of TRX in its pharmaceutical formulations and biological fluids. These include high performance liquid chromatography [2-4], luminescence [5], voltammetry [6], spectrophotometry [7], spectrofluorimetry [8] and capillary zone electrophoresis [9].

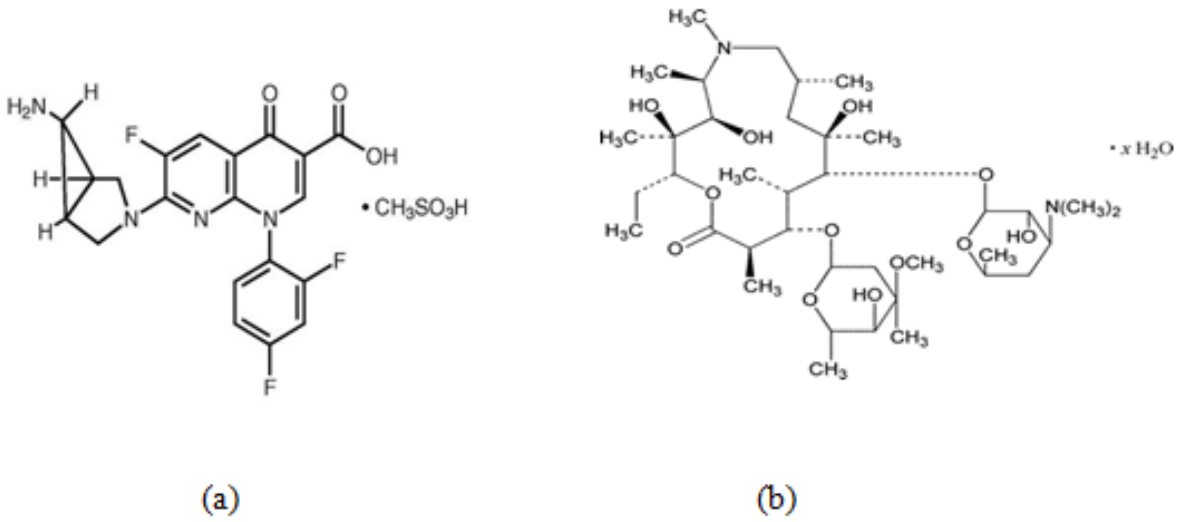

Figure 1. (1a) Chemical structure of trovafloxacin mesylate and (1b) azithromycin hydrate

Azithromycin (AZ), Figure 1b is a member of macrolide antibiotics class called azalide. It is derived from erythromycin; however, it differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring. Azithromycin is used to treat certain infections caused by bacteria, such as bronchitis; pneumonia and infections of the ears, lungs, skin, and throat [10]. Several methods have been reported for determination of azithromycin including high performance liquid chromatography [11-14], liquid chromatography coupled with mass spectrometry [15], thin Layer chromatography [16], spectrophotometry [17, 18], voltammetry [19], capillary zone electrophoresis [20] and chemiluminescence [21].

Capillary zone electrophoresis (CZE) is an establishing separation technique of choice effective for a wide spectrum of analytes, ranging from small inorganic ions to DNA macromolecules as it provides reliable data, requires minimal sample preparation and offers a high degree of automation [22]. In recent years, CZE has gained popularity as a separation technique for routine analysis, and it has widespread applications in many fields of analytical chemistry [23-25].

Due to the increase in clinical need for the combination drugs or fixed dose combinations, the need for analytical method for qualifying this formulation was increased. The objective of the present study is the development of a CZE method for separation and simultaneous determination of TRX and AZ in their combination formulations. Also stability indicating studies for the investigated drugs was carried out using the proposed method to measure the drugs in their degradation products.

2. EXPERIMENTAL

2.1. Apparatus and software

The CZE separation was performed using (PrinCE 770-Technology) instrument with fused silica capillary (57 cm total length and 50 cm effective length, 75 μm i.d.) and a diode-array detector (DAD). CZE system was equipped with a thermostatted column cartridge, a high voltage built in power supply and autosampler. The system was PC automated controlled and WinPrinCE-770, DAx3D software was used for data acquisition and subsequent treatment. The pH of the electrolyte was measured by HANNA 211-pH meter equipped with glass-combined electrode.

2.2. Material and reagents

All reagents were of analytical grade and used without further purification. Pure grade of TRX, AZ and their Trovan®/Zithromax® compliance pak were supplied from Pfizer Co. Egypt. The compliance pak contains a single 100 mg Trovan (trovafloxacin mesylate) tablet and one Zithromax (azithromycin for oral suspension) Single-Dose Packet 1 gram). Deionized water was used throughout the experiments. The running buffer sodium tetraborate decahydrate pH 9.2 and methanol (80:20%) was prepared daily using sodium tetraborate decahydrate and hydrochloric acid 0.1 mol L^{-1} (BDH) laboratory supplies (England). Methanol was purchased from (BDH) laboratory supplies (England). Sodium hydroxide was purchased from WinLab (UK).

2.3. Preparation of analytical samples

2.3.1. Standard trovafloxacin, azithromycin and levofloxacin solutions

Stock standard TRX and AZ solutions 200 μg mL^{-1} were prepared by dissolving 20 mg of each pure drug in 100 mL methanol: water (20:80 v/v). Serial solutions were prepared daily by appropriate dilution using deionized water. Internal standard (IS) levofloxacin (LVX) solution was prepared in methanolic water to give working solution 200 μg mL^{-1}.

2.3.2. Preparation of Trovan® and Zithromax® compliance pak solution

Ten tablets for Trovan® 100 mg/tablet and one packet Zithromax ® 1000 mg/packet were finally powdered, mixed well and weighed. An accurately weighed portion of the homogenized powder equivalent to 20 mg was transferred into 100-mL volumetric flasks containing 20 mL methanol and dissolved using ultrasonic bath for 15 min, then centrifuged at 2500 rpm for 10 min, filtered through millipore membrane filter paper and diluted to 100 mL with deionized water to obtain 200 μg mL^{-1}. The working solutions were prepared by serial dilution in the range of 20-140 μg mL^{-1} for each drug. The proposed method was employed to determine the investigated drugs in each concentration in the presence of 2.0 mL IS 200 μg mL^{-1}.

2.3.3. Preparation of forced degradation solutions of trovafloxacin and azithromycin

The investigated drugs were subjected to stress conditions to introduce forced degradation. Stress decomposition studies were performed initially with each TRX and AZ working solution of 200 μg mL^{-1} in methanol.

2.3.4. Acid conditions

Acid hydrolysis was performed for each drug separately. 5.0 mL working solution of TRX or AZ 200 μg mL^{-1} was transferred into 10-mL volumetric flask and 5.0 mL of 0.1 mol L^{-1} hydrochloric acid was added, mixed well and refluxed at 60°C for 30 min.

2.3.5. Alkaline conditions

Alkaline decomposition studies were carried out using 200 μg mL^{-1} for both TRX or AZ. 5.0 mL working solution of the investigated drugs were transferred into 10-mL volumetric flask and 5.0 mL 0.1 mol L^{-1} sodium hydroxide was added, mixed well and refluxed at 60°C for 30 min.

2.3.6. Oxidative conditions

Oxidative studies were carried out by mixing 5.0 mL working solution of each investigated drugs with 5.0 mL 3%, 15%, 30% (v/v) hydrogen peroxide in three separate 10-mL volumetric flasks, the resultant solutions were measured after 2, 4, 6, 8 and 10 h at 30°C.

2.3.7. Photolytic and thermal conditions

Photolytic and thermal degradation studies were carried out in bulk drug powder (1 mm thick layer on a petri plate) and in methanol solution (5.0 mL of 200 μg mL^{-1} working solution of TRX or AZ were exposed to sunlight for one week and to 105°C for 4 h, respectively.

2.3.8. Electrophoresis conditions

The electrophoretic analysis for both investigated drugs was carried out using running buffer electrolyte sodium borate decahydrate pH 9.2 and methanol (80:20%). Samples were hydrodynamically anodic injected at 90 mbar for 8 s. The applied voltage was 25 kV and the temperature was kept at 30°C. When using a new capillary, it was washed in a sequence of rinse 0.1 mol L^{-1} sodium hydroxide (2 min), deionized water (2 min) and equilibrated with running buffer electrolyte for (5 min). To ensure the reproducibility of the assay, the capillary was replenished with 0.1 mol L^{-1} sodium hydroxide (5 min), deionized water (5 min) and running buffer electrolyte (10 min).

2.3.9. Calibration curve

Aliquots of standard stock solutions of TRX and AZ were transferred into 20 mL volumetric flasks, 2.0 mL of IS solution (200 μg mL^{-1}) was added to each flask, then competed to mark with deionized water to yield final concentrations of 20, 40, 60, 80, 100, 120, 140 μg mL^{-1} for TRX while maintaining AZ concentration at a constant level of 70 μg mL^{-1} and concentrations of 20, 40, 60, 80, 100, 120, 140 μg mL^{-1} for AZ while maintaining TRX concentration at a constant level 70 μg mL^{-1}. Triplicate injections of each concentration were performed. The peak-area ratio of each concentration to the IS against the corresponding standard concentration were plotted, to obtain the calibration graphs. Then, the corresponding regression equations were derived.

3. RESULTS AND DISCUSSION

3.1. Optimization of the electrophoretic conditions

3.1.1. Effect of running buffer

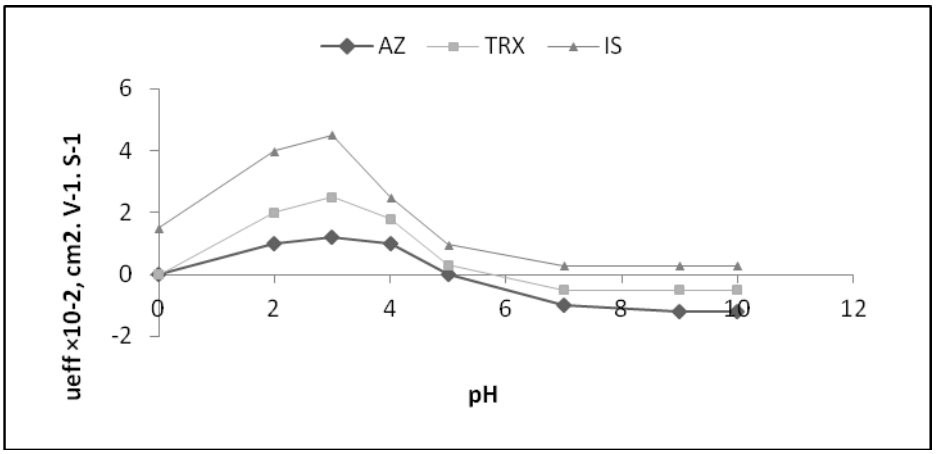

Figure 2. Effective mobility curve in function of the pH for TRX, AZ and IS

The pH value of the running buffer was considered as the most important parameter which influences the sensitivity of the separation and should be carefully studied. The mechanism of separation was affected by the difference between the solute size and the charge at a given pH. Owing to the influence of pH on the resolution and analysis time, we can notice that the latter two parameters were increased by increasing the pH. This can be attributed to the increase in negative charge with increasing the pH, which resulted in a great affinity and a higher complexation between the analytes and the buffer used. The initial study for running buffer optimization was carried out with respect to the mobility (u_{eff}) curve of the two investigated drugs and IS *vs*. pH. To study the influence of pH on the separation of the investigated drugs, different running buffer of pH in the range of 2-10 were used. Figure 2, showed that it was impossible to separate the investigated drugs at pH less than 3.5.

While at pH interval 3.5-6 the investigated drugs were found to present interaction with the internal capillary wall. Therefore, the pH interval of 7-10 was selected for the preliminary study.

To select the suitable running buffer electrolyte, three different buffers namely phosphate buffer, sodium tetraborate decahydrate, acetate were tested to separate mixtures of the investigated drugs containing TRX and AZ in the presence of LVX as IS. The investigation was carried out using five selected concentrations (5, 10, 15, 20, 25 and 30 mmol L^{-1}) for each buffer under constant instrumentation conditions (voltage, injection time, temperature and wavelength, etc.). It was found that sodium tetraborate buffer pH 9.2 was the most reasonable resolution, signal intensity and analysis time in comparison to other electrolyte system. Therefore, it was selected for further investigations.

3.1.2. Effect of buffer composition and concentration

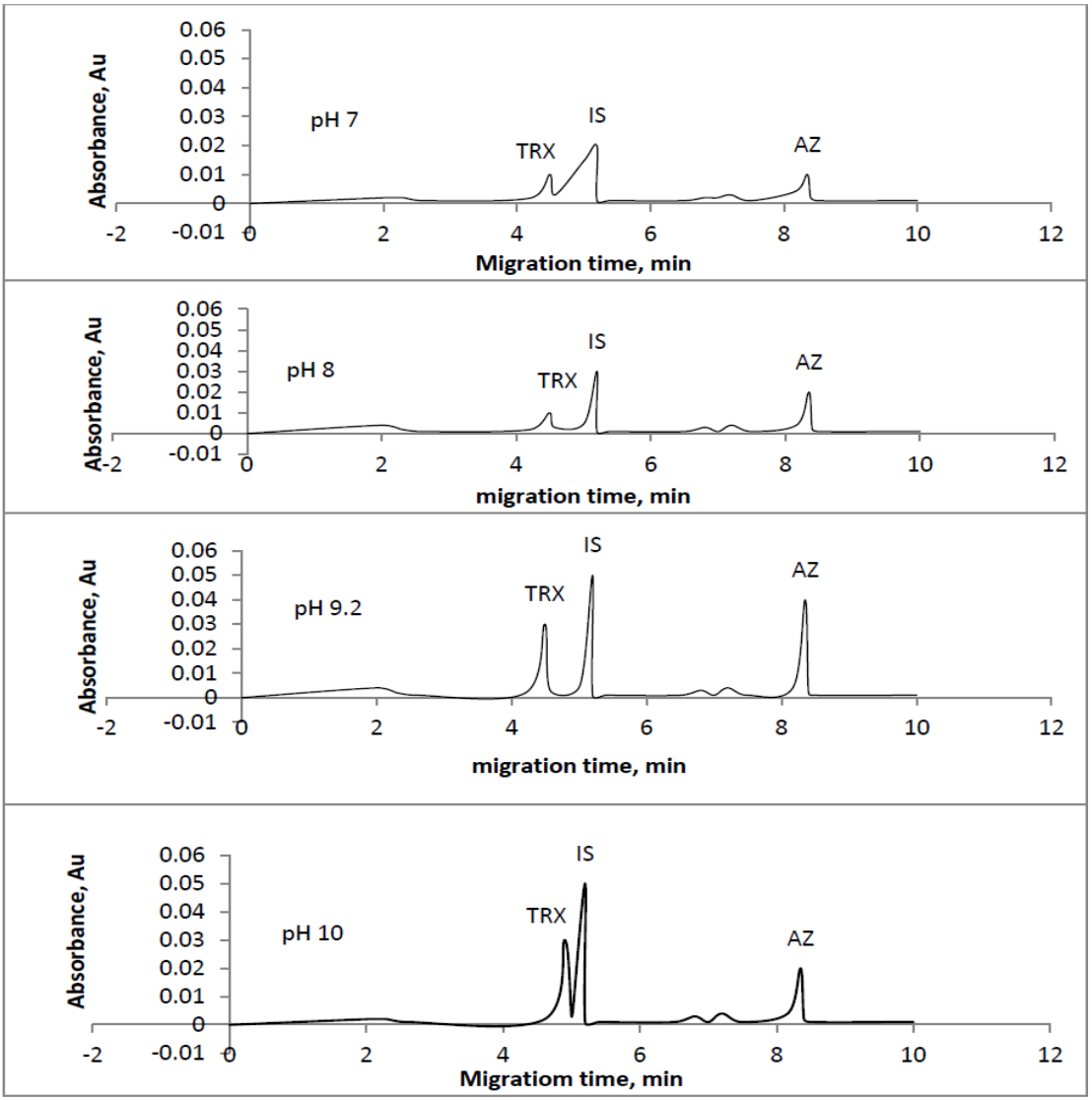

Figure 3. Effect of pH value on separation TRX; (IS) and AZ; Running buffer; 25 mmol L^{-1} sodium tetraborate decahydrate and methanol 80:20% (v/v); injection 90 mbar for 8 s; separation voltage 25 kV; capillary temperature 30°C, DAD detection at 260 nm

Buffer concentration has a considerable effect on the separation performance through its influence on the electroosmotic flow (EOF) and the produced current in the capillary. Different background electrolytes (BGEs) were tested using sodium tetraborate decahydrate buffer pH 7 -10. As shown in Figure 3, the best separation performance, including selectivity, reproducibility and current performance was obtained with sodium tetraborate decahydrate pH 9.2.

To study the suitable concentration of the selected running buffer electrolyte, other parameters were kept constant (pH 9.2, 90 mbar, 25 kV, 30°C). The buffer concentrations were varied in the range of 5-30 mmol L^{-1}. Better sensitivity, migration time as well as produced current was increased with the increase of buffer concentration. No appreciate improvement was observed in buffer concentration more than 25 mmol L^{-1}. Therefore, 25 mmol L^{-1} sodium tetraborate decahydrate at pH 9.2 was selected for further investigations.

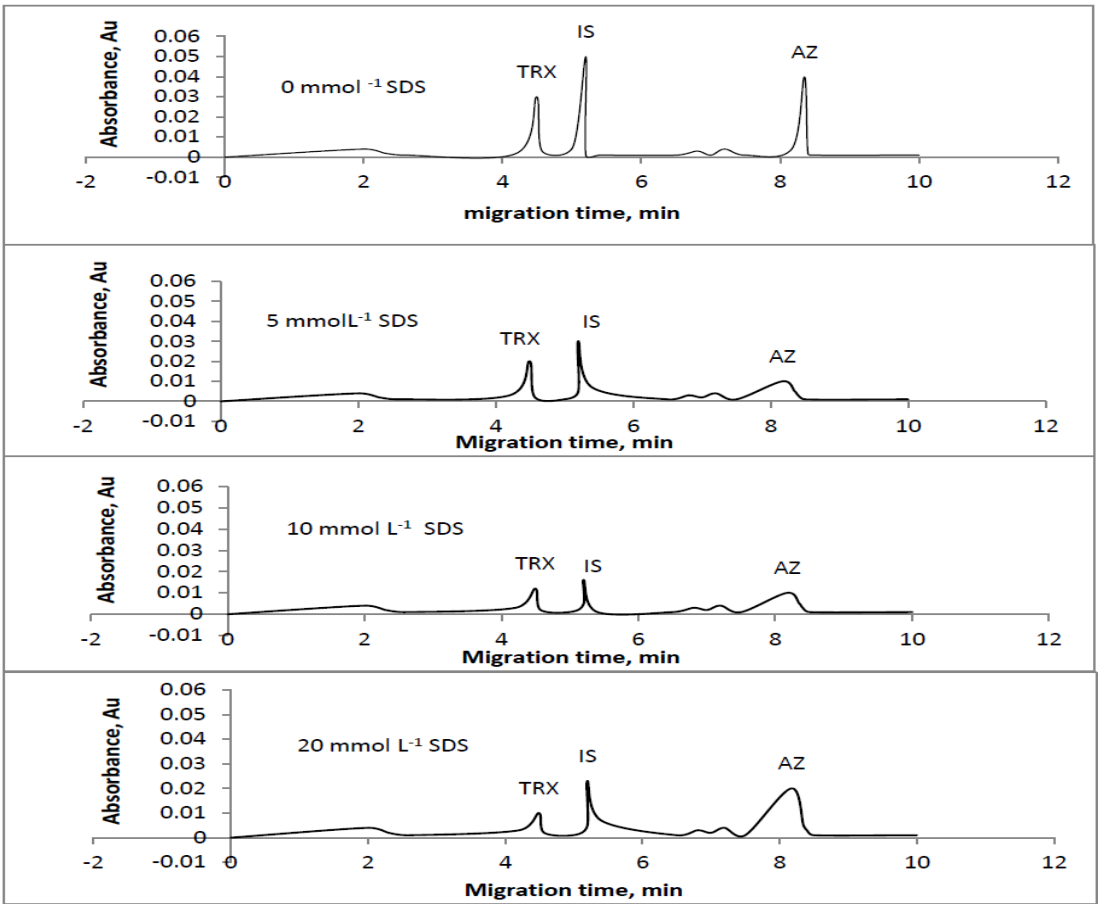

Figure 4. Effect of SDS (5-20 mmol L^{-1}) on separation of TRX; (IS) and AZ; Running buffer; 25 mmol L^{-1} sodium tetraborate decahydrate and methanol 80:20% (v/v); injection 90 mbar for 8 s; separation voltage 25 kV; capillary temperature 30°C, DAD detection at 260 nm

The influence of adding some additives and organic modified to the system electrolyte was tested by adding some additives such as sodium dodecyl sulphate (SDS) and cyclodextrin (CD) in the range of 5-20 mmol L^{-1} or adding some organic modified such as methanol, iso-propanol and acetonitrile in range of 5-30%. It was found that no significant improvement in the separation of the

investigated drugs by adding CD. Moreover, Figures 4 indicated that the addition of SDS in the running buffer over the critical micellar concentration promotes the aggregation of the surfactant molecules forming change in the mobility of the analytes due to hydrophobic interaction [26]. Therefore, the addition of SDS and CD did not exhibit relevant improvement in comparison to experiment carried out in their absence.

On the other hand, the addition of organic modifier such as methanol, ethanol, iso-propanol and acetonitrile may increase the migration window and resolution of separation. It was clear that the use of methanol in the range of 20 % caused a general increase in migration time and resolution factor of the two investigated drugs. Therefore, sodium tetraborate decahydrate pH 9.2 and methanol (80:20%) were used as running buffer for further investigations.

3.1.4. Effect of applied voltage

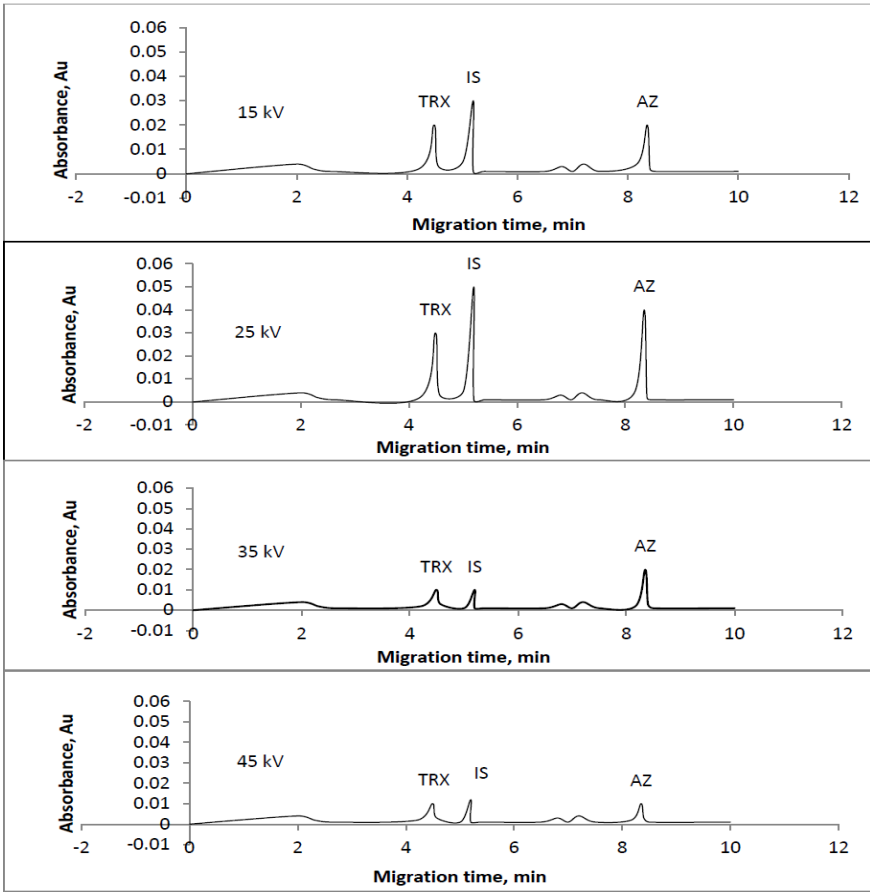

Figure 5. Effect of different voltage on separation of TRX; (IS) and AZ; Running buffer; 25 mmol L^{-1} sodium tetraborate decahydrate and methanol 80:20% (v/v); injection 90 mbar for 8 s; separation voltage (15-45 kV); capillary temperature 30 °C, DAD detection at 260 nm

The applied voltage was considered as a critical parameter which should be carefully investigated and optimized. The efficiency of resolution (Rs) of analysis was directly proportional to the applied voltage [27]. Therefore, the effect of applied voltage on separation and resolution of the investigated drugs was tested. Several runs were performed with gradual increase in the applied

voltage from 15-45 kV. It was found that an increase in resolution efficiency (Rs factor) with the increase of applied voltage from 15-30 kV. A further increase in the applied voltage more than 35 kV caused a decrease in capillary Rs efficiency. This can be attributed to the generation of excessive Joule heat, which decreases the separation efficiency. So 25 kV was selected for further investigation (Figure 5).

3.1.5. Effect of separation temperature

The control of capillary temperature is considered as one of the most important parameter which influences the reproducibility of the separation and assay. Electropherograms and resolutions under different capillary temperature It can be seen that, increasing the temperature resulted in a decrease in migration time and resolution. Separation was performed at temperature 30°C this can be attributed to the short migration time and good resolution *Rs*, 2.1 and 1.6 for TRX and AZ, respectively.

3.2. Separation performance

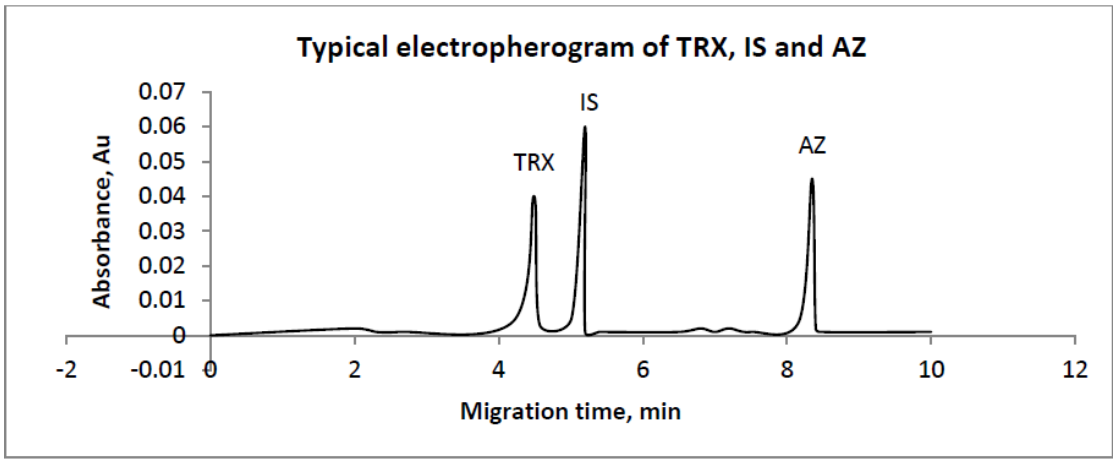

Figure 6. Typical electropherogram corresponding to the separation of 100 µg mL^{-1} TRX; 200 µg mL^{-1} IS and 70 µg mL^{-1} AZ

In order to reduce the injection related imprecision and to ensure better reproducibility and greater control the amount of sample injected, the quantitative analysis was preferred. Levofloxacin (LVX) was used as an internal standard to guarantee a high level of quantitative performance. Figure 6 shows the typical electropherogram obtained by the separation of the two investigated drugs in the presence of IS under the optimum conditions. The migration time for TRX, AZ and LVX were found to be 4.49, 8.35 and 5.22 min, respectively with %RSD less than 1.0 % for the migration time of each peak.

3.3. Method validation

Under optimum conditions the proposed method was validated with respect to linearity, precision, accuracy, lower limit of detection, lower limit of quantification, robustness and ruggedness according to ICH guidelines [28].

3.4. Selectivity

The selectivity of the method is defined as the ability of the proposed method to discriminate the analytes from all potential interfering species. To evaluate the selectivity of the proposed method, peak purity and spiking excipients with pure standard drugs were used. The peak purity was evaluated using a diode array detector and prinCE-770 DAx3D software. There were no peaks detected at the retention time of each individual drug and of internal standard at the level of LOQ.

The possible commonly coformulated excipients such as magnesium stearate, starch, lactose, cellulose, calcium hydrogen phosphate and coloring agents were also tested. No interference was recorded during the determination of TRX and AZ, indicating high selectivity of the proposed method.

3.5. Linearity, lower limit of detection and quantification

The linearity of the detector responses for both TRX and AZ were determined by plotting peak area ratios of the drug to internal standard *vs.* concentrations. The analytical data for the calibration graphs were listed in Table 1.

Table 1. Performance characteristics of the proposed method for determination of TRX and AZ

Parameter	TRX	AZ
Concentration range, μg mL^{-1}	20-140	20-140
Regression equations	y= 0.9846x + 0.2285	y = 0.9927x −1.1474
Correlation coefficient (r)	0.9998	0.9995
Slope	0.9846	0.9927
Intercept	0.2285	1.1474
LOD, μg mL^{-1}	12	12
LOQ, μg mL^{-1}	20	20

The calibration graphs were found to be linear over the concentration range of 20-140 μg mL^{-1} for both TRX and AZ with correlation coefficient (r) of more than 0.999. The regression equations were y= 0.9846 + 0.2285 for TRX and y = 0.9927-1.1474 for AZ.

The limit of detection (LOD) was determined as the sample concentration that produces a peak with a high three times the level of the baseline noise [29] and the limit of quantification (LOQ) was calculated using signal to noise ration equal ten times. The LOD value for the investigated drugs was found to be 12 μg mL^{-1} and the LOQ was 20 μg mL^{-1}. The obtained results permit the detection of the investigated drugs in their compliance pak.

3.6. Accuracy and precision

The accuracy and precision of the proposed method were evaluated using stock solutions spiked with a standard drug solution containing 0.2%, 0.5% and 1.0% of the labeled amount of both drugs (Trovan® 100 mg/tablet TRX and Zithromax®1000 mg/packet AZ). The accuracy of the investigated drugs was calculated as mean % recoveries (n=6) and the obtained results were found to be 99.45±0.87 and 99.15±0.74 for TRX and AZ, respectively. The intra-day and inter-day were evaluated as % RSD. The obtained results were 0.92% and 1.26% for TRX and AZ, respectively, indicating good precision of the proposed method.

3.7. Robustness of the proposed method

The robustness of the proposed method is the capacity of the method to remain unaffected by small, but deliberately introduced. Variations in the method variables were carried out to provide an assurance of its reliability during normal usage. The robustness were evaluated regarding the migration time, peak area and resolutions in the presence of IS. The variance and standard deviation for 10 replicate were (migration time 4.51±0.14 and 8.72 ±0.27, peak areas 1.4±0.12 and 1.6±0.35 and Rs 1.7±0.21 and 2.4±0.34 for TRX and AZ, respectively. The obtained results showed fair repeatability of the proposed method.

3.8. Analytical applications

3.8.1. Quantification of trovafloxacin and azithromycin in authentic mixture

Table 2. Determination of TRX and AZ in their authentic mixture using capillary zone electrophoresis in the presence of 2.0 mL IS 200 µg mL^{-1}

Ratio TRX/AZ w/w%	Taken µg mL^{-1} TRX:AZ	TRX		AZ	
		Found µg mL^{-1}	% Recovery	Found µg mL^{-1}	% Recovery
1:1	20:20	19.95	99.8	19.91	99.6
1:2	35:70	34.99	99.9	69.45	99.2
1:4	20:80	19.83	99.2	80.01	100.0
1:6	20:120	19.68	98.4	119.42	99.5
Mean±SD		99.33±0.69		99.58±0.33	

The proposed method was employed in the determination of the investigated drugs in their authentic mixture. Working solution 100 µg mL^{-1} of each drug was prepared. Aliquots of standard solutions of TRX and AZ were transferred into 20-mL volumetric flasks, 2.0 mL of IS solution 200 µg mL^{-1} was added to each flask and then completed to volume with deionized water to give a final concentration ratio 1:1, 1:2, 1:4 and 1:6 w/w, respectively. Triplicate injections for each solution were made. The peak area ratio of each concentration to the IS was calculated. The corresponding regression

equations were used to evaluate the concentration of each drug. The obtained results were summarized in Table 2.

3.8.2. Determination of trovafloxacin and azithromycin in Compliance pak

Table 3. Determination of TRX and AZ in their compliance pak using capillary zone electrophoresis in the presence of 2.0 mL IS 200 µg mL^{-1}

Ratio TRX/AZ w/w%	Taken µg mL^{-1} TRX:AZ	TRX		AZ	
		Found µg mL^{-1}	% Recovery	Found µg mL^{-1}	% Recovery
1:1	20:20	19.90	99.5	19.96	99.8
1:2	35:70	34.94	99.8	69.52	99.3
1:4	20:80	19.87	99.4	80.15	100.2
1:6	20:120	19.66	98.3	119.47	99.6
Mean±SD		99.25±0.66		99.72±0.38	

Table 4. Comparative data for determination of TRX and AZ in other reported methods

Method of analysis	AZ Linear conc. range µg mL^{-1}	TRX Linear conc. range µg mL^{-1}	References
Proposed method (CZE)	20-140	20-140	
Spectrophotometry	10-75	----	[16]
RP-HPLC	0.02-0.6	----	[13]
Differential–pulse adsorptive voltammetry	-----	0.005	[8]

The proposed method was employed to determine the investigated drugs TRX and AZ in their compliance pak. The evaluation of both drugs was calculated as mean % recoveries. The obtained results were found to be 99.25±0.66 and 99.72±0.38 for TRX and AZ, respectively as summarized in (Table 3). From the literature survey, it was found that there is no reported method employed for detection of both drugs in their compliance pak. The obtained results for the proposed method was compared to previously reported methods for determination of each TRX and AZ in pharmaceutical dosage forms (Table 4).

3.8.3. Forced degradation studies

Forced degradation studies (Figure 7) were carried out under different stress conditions. For photo-degradation, no detectable changes were present in the exposure of the two investigated drugs to sunlight. On the other hand the investigated drugs were relatively stable on heating at 105°C for 4 h.

Acid and base of TRX and AZ solution was started with 0.1 mol L^{-1} hydrochloric acid and sodium hydroxide separately for 30 min. Both drugs showed extensive degradation under these hydrolytic conditions and almost complete degradation of both drugs occurred.

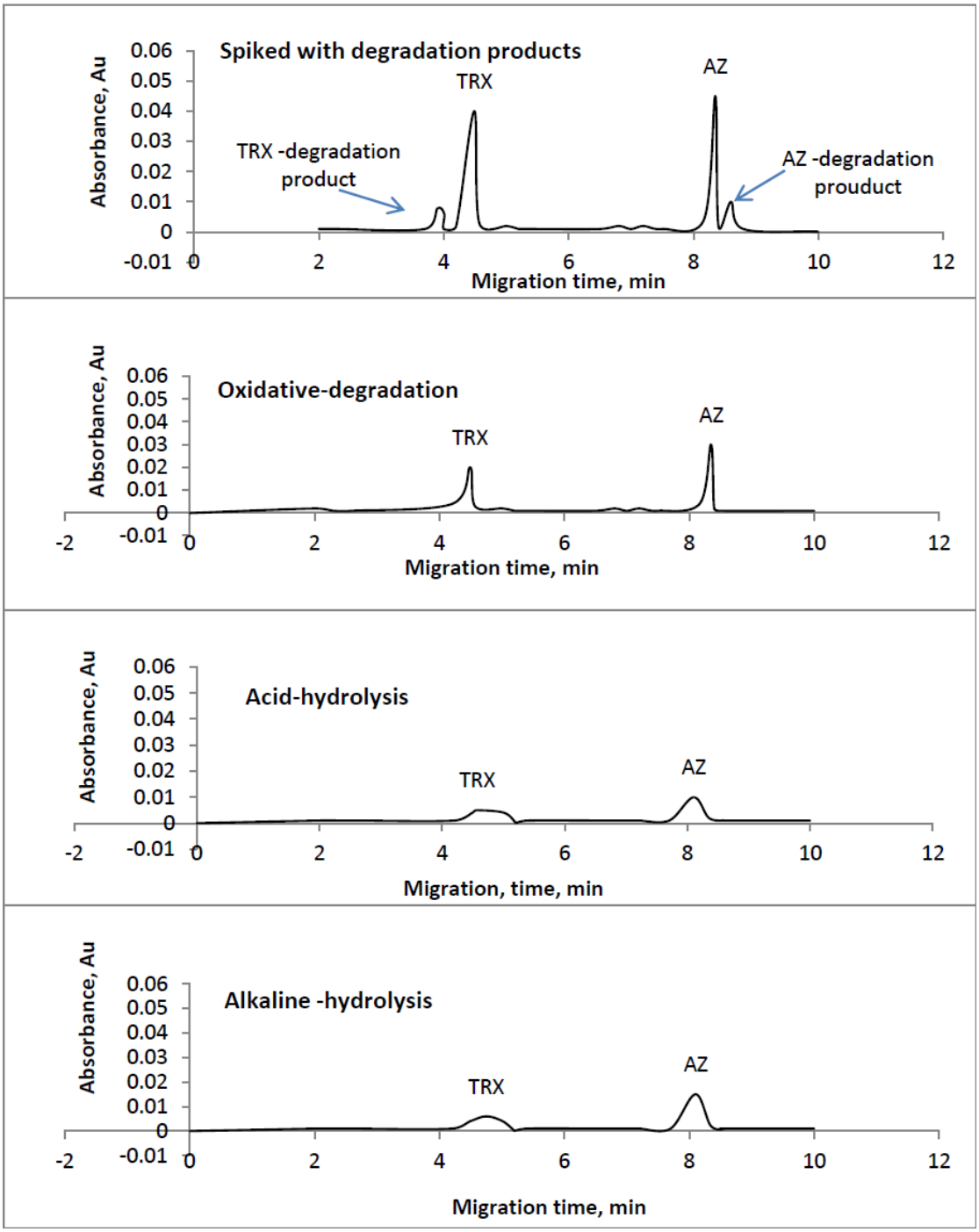

Figure 7. Electropherograms of TRX and AZ forced degradation

In the case of oxidative degradation both TRX and AZ were unstable towards oxidation. No degradation was observed on using 3% H_2O_2, while the degradation was accelerated by increasing

H_2O_2 concentration to 15% (v/v). The concentration of the tested drugs was significantly decreased to 86% and 73% for TRX and AZ, respectively.

4. CONCLUSION

A simple and novel electrophoretic method for simultaneous separation and determination of trovafloxacin and azithromycin in their combination pharmaceutical formulations was developed. The developed method was considered as the first method proved for determination of the investigated drugs in their compliance pak. The optimal condition for the effective separations and determination of the investigated drugs were 25 mmol L^{-1} sodium tetraborate decahydrate and methanol 80:20%, the injection at 90 mbar for 8 s, along with applied voltage 25 kV at 30°C. The proposed method gave satisfactory results relevant to migration time, peak area and resolution. The obtained electropherograms facilitate the determination of the investigate drugs in the presence of their degradation products. The method was simple, accurate and precise might be useful for clinical applications in the further research.

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