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Development of an eco-friendly capillary electrophoresis method for the simultaneous determination of piperacillin, tazobactam and ibuprofen in plasma samples: application to a pharmacokinetic study in rats

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Piperacillin (PIP) and tazobactam (TAZ) are broad-spectrum beta-lactam antimicrobial agents, which are frequently co-prescribed in intensive care units (ICUs) worldwide. Ibuprofen (IBU) is a potent pain killer which is commonly co-prescribed with PIP and TAZ postoperatively. The combination therapy of PIP, TAZ, and IBU has been administered commonly after surgical procedures to combat aerobic and anaerobic microbes and exert anti-inflammatory and analgesic effects. This study describes, for the first time, the development of a new capillary electrophoresis (CE) method with a photodiode array detector for the simultaneous determination of PIP, TAZ, and IBU in plasma samples. The experimental factors affecting the elution of analytes were carefully optimized. The final analysis was achieved using a fused silica capillary (58 cm effective length and 75 μm ID) and a background electrolyte solution containing a methanol/borax buffer solution (15 mM and pH 9.3) in a ratio of (10 : 90 v/v) with a driving voltage of 30 kV and detection at 210 nm. The relationship between the peak area and concentration was linear from 1 to 200 $\mu\text{g mL}^{-1}$ for both PIP and TAZ and from 3 to 200 $\mu\text{g mL}^{-1}$ for IBU. The method used was thoroughly validated in accordance with the validation requirements set out by the Food and Drug Administration (FDA) for bio-analytical processes. The proposed CE method was employed to conduct pharmacokinetic and bioavailability studies of the drugs in rat models. The pharmacokinetic results revealed that there is a significant impact upon prescribing this combination concurrently when compared to their single administration. To illustrate, the time required to reach their maximum concentrations (T_{max}) was increased by 0.25 h for both PIP and TAZ, whereas it was increased by 0.5 for IBU. When it comes to their maximum concentration (C_{max}), it was increased by 13.7%, 55.5%, and 44% for PIP, TAZ, and IBU, respectively. Furthermore, the bioavailabilities of PIP, TAZ, and IBU were significantly increased by 55.4%, 19.7%, and 35.6%, respectively. These findings require caution when these drugs are co-prescribed as there is a noticeable augmentation in their therapeutic impacts. Additionally, the greenness of the proposed method was assessed by three metric tools. In conclusion, the method is a valuable tool for further studies on drug–drug interaction in humans.

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1. Introduction

Piperacillin (PIP) is a fourth-generation broad-spectrum β -lactam antimicrobial agent, which is frequently prescribed in intensive care units (ICUs) worldwide to manage sepsis,

especially for patients with related organ dysfunction.^{1–3} The combination of a β -lactamase inhibitor, tazobactam (TAZ), with PIP greatly enhances PIP's activity against β -lactamase-producing bacteria [1]. The combination therapy of PIP and TAZ is marketed as an 8 : 1 fixed-dose combination.⁴ Inflammation and pain are the core symptoms in infected patients;^{5,6} therefore, analgesics and anti-inflammatory agents are commonly prescribed concomitantly with PIP/TAZ therapy.⁷ Ibuprofen (IBU) is a nonsteroidal anti-inflammatory drug (NSAID) commonly prescribed for the management of pain and inflammation. The FDA proposed IBU as a better NSAID to manage infected outpatients and prevent complications.⁸ It is a nonselective blocker of cyclooxygenase enzyme, and it is

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frequently prescribed for children with diverse therapeutic indications, including fever, postoperative pain, tumors, and inflammatory disorders, such as juvenile idiopathic arthritis.⁹ The chemical structures of PIP, TAZ and IBU are given in Fig. 1.

PIP and TAZ were determined by several chromatographic methods either individually or in combination with other drugs. These methods include HPLC-UV,^{1,10–12} UPLC-MS/MS,^{13,14} and capillary electrophoresis (CE).¹⁵ Additionally, IBU was determined by numerous analytical techniques either individually or in combination with other drugs, such as HPLC-UV,¹⁶ HPLC-MS/MS,^{17,18} GC-MS,¹⁹ GC,²⁰ LC-MS/MS,^{21,22} TLC,²³ spectrofluorimetry,²⁴ and spectrophotometry.²⁵ All these techniques were developed for the individual determination of PIP, TAZ and IBU; however, there is no published method for their simultaneous determination. Due to the popular use of the combination of PIP-TAZ-IBU for in-patients²⁶ and post operatively,^{27,28} it is of utmost importance to study the influence of each drug when concurrently prescribed with the other one through a full pharmacokinetic study. Given that the proposed CE method will be the first innovative approach to be followed by a comprehensive pharmacokinetic study to demonstrate the therapeutic impact of the three medications on one another. By gathering information for the determination of the three drugs in biological fluids, PIP and TAZ were determined in human plasma by HPLC-UV,^{10,12} human plasma and urine by UPLC-MS/MS,¹³ human plasma and pleural fluid by LC-MS/MS,²⁹ and rat plasma by LC-MS/MS.³⁰ IBU was detected in human plasma by HPLC-MS/MS,^{17,18} human plasma by LC-MS/MS,^{21,22} human plasma by TLC,²³ human plasma and urine by GC-MS,¹⁹ rabbit plasma by GC,²⁰ mouse plasma and tissue by HPLC-FLU,³¹ and rat plasma by HPLC.³² All the above-mentioned methods were applied for the determination of each drug individually.

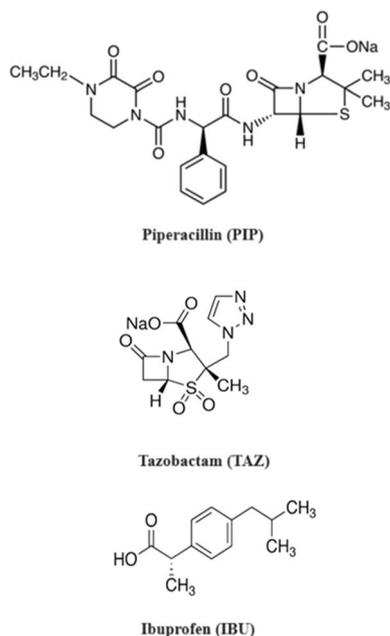


Fig. 1 Chemical structures of piperacillin (PIP), tazobactam (TAZ) and ibuprofen (IBU).

CE is now utilized for the investigation of different aspects of pharmacokinetics because of its unique features including high resolving power, versatility, short analysis time, low consumption of chemicals and samples, small sample volume for cell-based investigations, and fast and simplified sample processing, especially when compared with methods that require tedious sample preparation and sample stacking for increasing the detection sensitivity.³³ This research demonstrates, for the first time, the detailed description for the development and validation of a new ecologically friendly CE method equipped with a diode array detector for the simultaneous quantification of PIP, TAZ and IBU in rat plasma samples followed by a full pharmacokinetic profile. The proposed method combined numerous privileges including a simple and straightforward one-step protein precipitation method for preparing plasma samples, the reduction of sample size, the use of a minimal number of solvents, and a short analysis time (<12 min). The method's straightforward extraction procedure and quick run time contribute to its high throughput, enabling the efficient processing of diverse samples in several clinical laboratories.

Studying the pharmacokinetics of drugs provides critical data on the behavior of molecules within organisms and aids in providing more effective and safer therapies. In the proposed CE approach, the pharmacokinetic findings of the three drugs were compared when administered singly and when administered concurrently. The latter demonstrated the accumulative impact of co-administering the drugs in terms of rapid onset of action with a maximized concentration. This necessitates caution when prescribing the compounds together, as there is a significant change in their bioavailabilities. The entire pharmacokinetic study's data can be employed for therapeutic drug monitoring and as a foundation for future researchers who are interested in conducting additional studies on drug-drug interactions in humans.

In addition, the proposed analytical procedure was evaluated using three green metric tools: Analytical Eco-scale (ESA), Green Analytical Procedure Index (GAPI), and Analytical GREENness Calculator (AGREE). By the ESA tool, the proposed method scored 89 out of 100. The GAPI pictogram demonstrated 7 green parameters, 7 yellow parameters, and only 1 red parameter. By the AGREE tool, the method scored 0.77 out of 1. These results indicated that the CE procedure had a minimal environmental impact while maintaining the analytical parameters.

2. Experimental

2.1. Instrumentation and electrophoretic conditions

This investigation employed a capillary electrophoresis instrument manufactured by Agilent in Germany, which has a PDA detector adjusted at 210 nm. Data processing was performed using the Agilent ChemStation software. A deactivated fused silica capillary (Agilent Technologies, Santa Clara, CA) was employed for electrophoretic analysis. The capillary measured 66 cm in total length, 58 cm in effective length, and 75 μ m in internal diameter. Both the capillary and the sample were maintained at a temperature of 24 °C. A background electrolyte (BGE) solution containing a methanol/borax buffer solution

(15 mM, pH 9.3) in a ratio of 10/90 v/v was used to perform separations. Samples have been injected into the capillary through the anodic side under a pressure of 40 mbar for 30 seconds. In order to conduct electrophoresis, a 30 kV potential was introduced into the capillary, with the cathode positioned at the detector end. To assure the analysis's reproducibility, the capillary was rinsed for two minutes among runs with deionized water and equilibrated for an additional four minutes with a running buffer. The capillary was conditioned with 0.1 M sodium hydroxide for 5 minutes, deionized water for 5 minutes, and a running buffer for 10 minutes prior to sample injection. Prior to analysis, each solution was filtered through 0.2 μm Millipore membrane filters (Millipore, Nihon, Japan).

2.2. Materials used and reagents

Reference standard materials of TAZ and PIP were purchased from Fluka, Sigma Aldrich (GmbH, Germany), with a purity >99%. IBU was obtained from Amgen Inc. (Thousand Oaks, CA, USA). The internal standard (IS) Paracetamol (PAR) was obtained from Jigs Chemical Limited (Ellisbridge, Ahmedabad) with a purity of 99%. Acetonitrile of analytical grade (Sigma-Aldrich, St Louis, MO, USA) was used for protein precipitation. Analytical grade borax was purchased from Winlab (Leicestershire, UK) and sodium hydroxide was obtained from Merck (Darmstadt, Germany). All solvents and other materials used throughout this study were of analytical grade. The investigations were conducted using deionized water and membrane filters (0.2 μm) obtained from Nihon, Millipore (Yonezawa, Japan).

2.3. Standard solutions

Stock standard solutions containing each of TAZ, PIP, IBU and PAR (IS) (1 mg mL⁻¹) were prepared separately in four separate 10 mL calibrated flasks in methanol. These stock solutions were further diluted with the BGE to obtain 10 mL of working standards of 0.5 mg mL⁻¹ of each compound. When stored in a refrigerator, the methanolic stock solutions remained stable for a minimum of three weeks.

2.4. Construction of calibration curves and method validation

Analytes of different concentrations in the range of 1–200 $\mu\text{g mL}^{-1}$ were prepared from the working standard solutions (0.5 mg mL⁻¹) of PIP, TAZ and IBU in three series of test tubes. Each sample solution was mixed with 200 μL of PAR (IS) from 0.5 mg mL⁻¹ standard working solution, and the volume was then adjusted to 1 mL with the BGE and injected into the CE in triplicate under the above-mentioned optimized conditions. Subsequently, calibration curves were generated for the pure samples by using these prepared samples. In the *in vitro* experiments, calibration standards were created by properly transferring different volumes of the medications being tested from their working standard solutions (0.5 mg mL⁻¹) into two separate sets of test tubes. Spiking of each sample was performed with 200 μL blank plasma and 100 μL of working IS solution (0.5 mg mL⁻¹), and then vortexed for 1 min. To each

sample, 1 mL acetonitrile was added for plasma protein precipitation by centrifugation at 5000 rpm for 10 min.

A similar procedure was used for obtaining the quality control samples, which are low-, medium- and high-level quality control samples (50, 100, and 150 $\mu\text{g mL}^{-1}$ for PIP, TAZ and IBU). Until the analysis time, all samples and prepared solutions were kept at $-20\text{ }^{\circ}\text{C}$ in the refrigerator for storage.

For each drug, two calibration curves were generated: one for spiked plasma samples and the other for pure standards. After preparing multiple samples, they were analyzed in triplicate in accordance with the prescribed methodology and the operating conditions of the electrophoresis instruments. The corrected peak area was calculated using the following formula: peak divided of analyte/analyte migration time. To generate the calibration curves, the corrected peak area ratios, calculated as the corrected peak areas of analyte/corrected peak areas of IS, were plotted as a function of analyte concentrations. The data set was analyzed by regression analysis, and the linear fitting equations were derived, along with their parameters (intercepts, slopes, and determination coefficients). For calculations of sample solutions, the automatic integration system (data acquisition and processing software) was used. The percentage of a sample analyte was calculated by determining the peak area as a percentage of the total corrected areas of all peaks, excluding those due to BGE. The quality control samples that had been previously generated were analyzed by the proposed method. To determine the concentrations, regression equations were applied to the injected plasma calibration curves. The results were subsequently utilized to validate the method according to the US-FDA criteria for Industry and Bioanalytical Method Validation.³⁴

2.5. Pharmacokinetic study

For the experiment, 28 Wistar albino rats (250 \pm 30 g) were divided into four groups (7 rats per group), and all rats were housed under the conditions of 25 $^{\circ}\text{C}$ and 50% relative humidity. The rats received the following treatment through intraperitoneal injection: group I received 160/40 mg kg⁻¹ of PIP/TAZ dissolved in dimethyl sulfoxide-saline (5%, v/v),³⁵ group II received 30 mg kg⁻¹ of IBU dissolved in dimethyl sulfoxide-saline (5%, v/v),³⁶ group III received mixed doses of 160/40 mg kg⁻¹ PIP/TAZ and 30 mg per kg IBU, while group IV served as the control. Blood samples (\sim 400 μL) were collected from each rat from the tail vein and transferred into heparinized polythene tubes at predetermined time points of 0.25, 0.5, 0.45, 1, 1.5, 2, 3, 5 and 8 h after drug administration. Plasma was extracted from blood samples by centrifuging them at 5000 rpm for 30 minutes. Plasma samples were obtained and stored in a refrigerated state at $-20\text{ }^{\circ}\text{C}$ until the analysis was performed. Protein precipitation using acetonitrile was used to extract the proposed drugs from the plasma samples. Then, 200 μL of each sample was prepared in a clean centrifuge tube and spiking was performed with 100 μL of the IS's working solution (0.5 mg mL⁻¹). Following a 1 min vortex mixing step, a plasma protein precipitant (1 mL of acetonitrile) was added, followed by precipitation at 5000 rpm for separating the plasma protein.



After conducting three distinct analyses of the samples according to the methodology specified, plasma concentration–time curves were constructed, and subsequently, all pharmacokinetic parameters were calculated.

3. Results and discussion

3.1. Optimization of the electrophoretic parameters

An investigation was conducted to examine the parameters that influence the electrophoretic separation of the analytes. The aim of the study was to obtain sharp and symmetrical peaks of PIP, TAZ, and IBU, with a higher signal-to-noise ratio. To assess the purity of the peaks, the absorption spectra of the analyte peaks were compared to those of standard solutions of each analyte using the diode array detector. The analysis was performed using a detection wavelength of 210 nm as it resulted in the highest response for all components.

3.1.1. Effect of buffer pH and concentration. In CE, the buffer type, pH, and concentration are key parameters for optimizing the separation of ionizable analytes, as they determine the degree of the analyte ionization, the electrophoretic mobility of the analyte, and the magnitude of electroosmotic flow (EOF).³⁷ The pKa values of PIP, TAZ, and IBU were 3.4,³⁷ 2.86,¹¹ and 5.2,³⁸ respectively. Therefore, the three analytes are negatively charged in basic media and can be analyzed under these conditions.

For CE analysis, a variety of circulating buffers at various molarities and pH levels, including phosphate, acetate, and borate, were evaluated. The overlapping of PIP and TAZ could not be resolved by employing phosphate and acetate buffers (pH ranges from 7 to 9.3). The investigation involved using a borate buffer, which exhibited a pH range of 7 to 10. It was observed that complete separation between the medications did not occur for values below 9.3; nevertheless, peaks that were not completely separated at values of pH 10, and the TAZ peak was eluted after a duration of 20 minutes, as demonstrated in Fig. 2(A). It was noticed that a borate buffer with a pH value of 9.3 exhibited sharp and symmetric peaks, indicating optimal separation, and consequently, it was selected as the final background electrolyte considering the additional parameters to be optimized (resolution, migration time, peak shape and height, baseline noise, and electric current generated).

The impact of borate concentration on the separation was examined by varying the amounts from 5 to 50 mM at a pH of 9.3. Buffer concentrations below 15 mM led to a notable overlap between PIP and IBU. Borate buffers at a concentration of 15 mM or more exhibited enhanced separation efficiency. The analysis was conducted with a 15 mM borate buffer with a pH of 9.3 due to its ability to generate analyte peaks that are both well-resolved and symmetrical within an acceptable time of analysis.

3.1.2. Effect of organic modifiers. The potential impact of organic modifiers in the background electrolyte on the separation process can be attributed to alterations in the analytes' partitioning equilibria and electroosmotic flow. Furthermore, the incorporation of organic modifiers may cause a modification in the viscosity of the running buffer.^{39,40} Typically, organic modifiers are introduced into the background electrolyte in

order to enhance peak shape and resolution. The organic modifier methanol was evaluated in concentrations ranging from 0% to 20%. The introduction of methanol resulted in the elimination of distorted TAZ and PIP peaks, as well as enhancements to the analytes' symmetry and peak shape, all while maintaining acceptable migration times. To improve the resolution, a background electrolyte solution comprising 10% methanol was selected as the optimal solution to ensure acceptable peak shapes in a short analysis time.

3.1.3. Effect of applied voltage. The effective separation of capillary electrophoresis is significantly influenced by the applied voltage.³⁵ In general, higher resolution can be attained by raising the applied voltage. A series of experiments were conducted, gradually increasing the applied voltage from 15 to 30 kV, as illustrated in Fig. 2(B). When the voltage increased, it led to a noticeable reduction in the migration time accompanied by an improved peak shape. Nevertheless, reducing the applied voltage to a value below 30 kV led to a decline in the separation efficiency. When a voltage of 30 kV was applied, it led to effective separations with reduced migration time (less than ten minutes). Therefore, a voltage of 30 kV was used as it provided the most favorable balance between peak response, peak purity, and analysis duration (less than 12 minutes) for the analytes.

3.1.4. Effect of injection time and pressure. To determine the optimal sample injection period, a range of durations (30 to 90 seconds) were examined, as demonstrated in Fig. 2(C). It was noticed that the duration of injection did not have a major impact on the migration time of the medications, but it had a noticeable impact on the intensity of response, and hence, the resolution of the studied components.^{40,41} There was no discernible disparity in the resolution when the injection durations ranged from 30 to 60 seconds. Increasing the injection time resulted in a significant decrease in resolution. Therefore, a 30 seconds injection period was selected for the analysis.

The applied pressure was systematically adjusted within the range of 20 to 40 mbar in order to assess its impact on the separation process, as depicted in Fig. 2(D). Raising the pressure led to improved efficiency and reduced migration time. For subsequent analysis, a pressure of 40 mbar and an injection period of 30 seconds were chosen. These parameters were picked because they offered a balance between achieving the best separation, obtaining well-defined peak shapes, and keeping the analysis time within ten minutes.

3.1.5. Effect of capillary temperature. Capillary temperature regulation is critical for ensuring the assay's reproducibility. A portion of the electrical energy that flows through a capillary is transformed into Joule heating. As a result of alterations in buffer viscosity, the temperature influences the migration durations and velocities of the analytes, and thus, the resolution. Temperature regulation is extremely important for regulating the effect of Joule heating. It was observed that as the temperature increased from 15 to 50 °C, the migration duration decreased, resulting in overlap at temperatures above 25 °C. The reason for this is the reduction in the viscosity of BGE as the



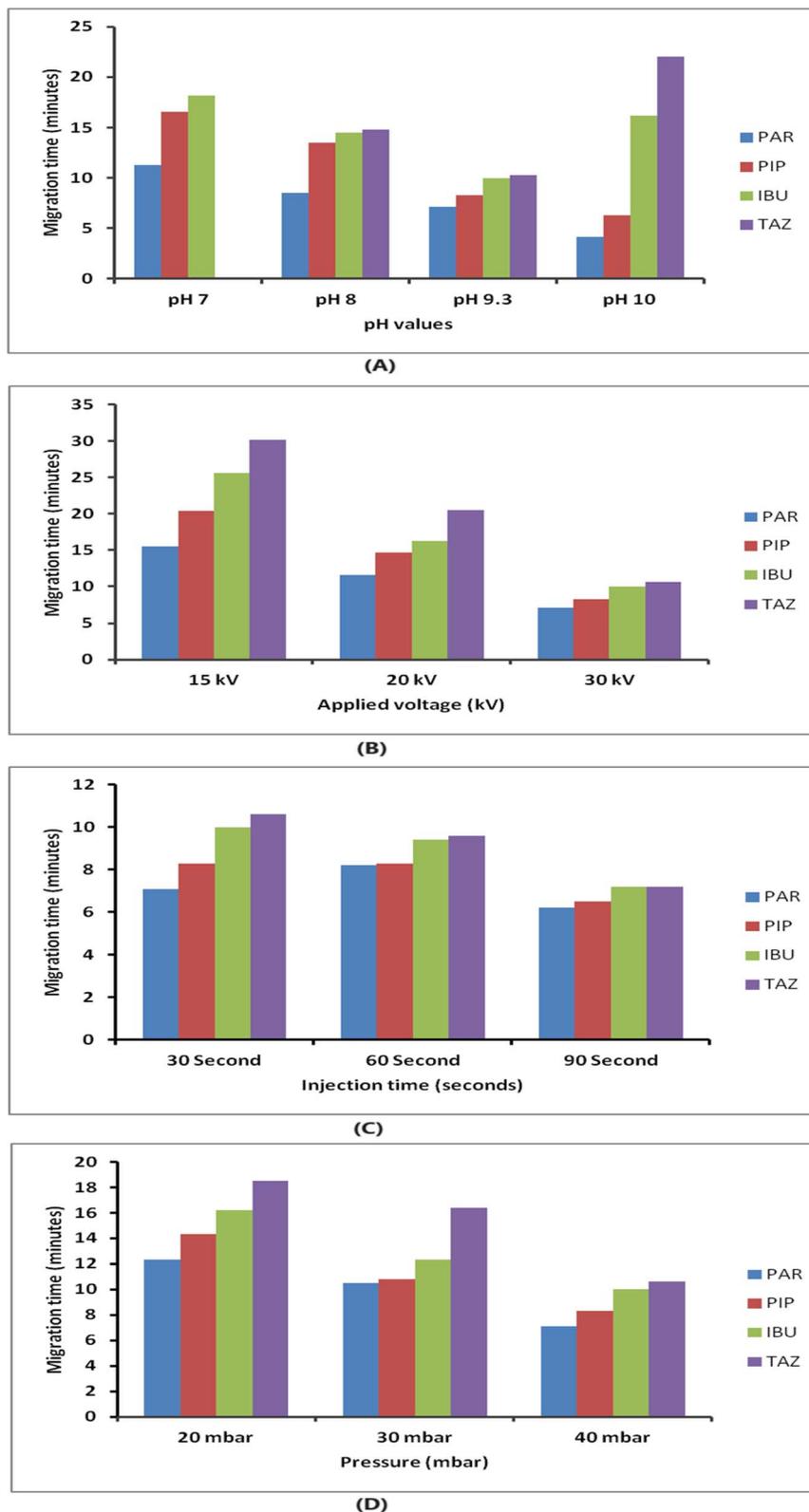


Fig. 2 (A) Effect of borate buffer at different pH values on the compounds' migration time. (B) Effect of applied voltage (kV) on the compounds' migration time. (C) Effect of injection time (seconds) on the compounds' migration time. (D) Effect of pressure (mbar) on the compounds' migration time.



Table 1 Optimization of the experimental conditions for the CE method for the simultaneous determination of PIP, TAZ and IBU

Parameter investigated	Range	Optimum value
Buffer pH	7 to 10	9.3
Buffer concentration (mM)	5 to 50 mM	15 mM
Organic modifier	5 to 20%	10%
Applied voltage (kV)	15 to 30 kV	30 kV
Injection time (seconds)	30 to 90 seconds	30 seconds
Pressure (mbar)	20 to 40 mbar	40 mbar
Temperature (°C)	15 to 50 °C	25 °C

temperature increased. An optimal temperature of 25 °C was determined for the analysis.

In summary, the separation of the analytes was successfully accomplished by employing a fused silica capillary with an effective length of 58 cm and an ID of 75 μm. The background electrolyte employed was a mixture of borax buffer (15 mM, pH 9.3) and methanol (90 : 10, v/v) at a temperature of 25 °C. This information is presented in Table 1. The ideal separation was achieved by employing a voltage of 30 kV, a pressure of 40 mbar, and an injection duration of 30 seconds.

Fig. 3 displays the standard electropherogram of a mixture of PIP, TAZ, and IBU, which was obtained using the optimized approach. The resulting peaks exhibited optimum sharpness and demonstrated complete baseline separation. The employed methodology achieved sufficient resolution of the components within an acceptable time for analysis. The PIP, IBU, and TAZ elution times were 8.3 ± 0.551 , 10 ± 0.451 , and 10.6 ± 0.843 min, respectively. Table 2 displays the system parameters. The use of the CE approach enabled sufficient resolution within appropriate capacity factors. Furthermore, the presence of a significant number of theoretical plates indicates the great efficiency of CE. The degree of peak asymmetry was assessed by calculating the tailing factor, and it was not exceeding the crucial value (1.12), which verified the permitted level of peak asymmetry.

Under these conditions, the system suitability parameters are summarized and presented in Table 2.

3.2. Method validation

The CE technique underwent validation in adherence to the US-FDA guidelines for Industry-Bioanalytical technique Validation.³⁴ Based on the FDA requirements, the method validation was performed on three quality control solutions (QCs) covering the method's range: low QC (LOQ, $50 \mu\text{g mL}^{-1}$), middle QC (MOQ, $100 \mu\text{g mL}^{-1}$), and high QC (HOQ, $150 \mu\text{g mL}^{-1}$) for the three drugs, using these suggested parameters in terms of linearity, selectivity, accuracy, precision, stability, extraction recovery, and LOD and LOQ.

3.2.1. Linear range. For evaluating the linearity of the technique, ten non-zero samples were utilized, which could be spiked with plasma standards or purified standards. A linear regression analysis was employed to assess the linearity, while the concentrations of the analytes were derived from calibration curves. Linear regression analysis was conducted to derive the

linear fitting equations for measuring the analytes, along with their intercepts, slopes, and determination coefficients. Linearity was attained in the range of 1–200, 3–200, and 1–200 $\mu\text{g mL}^{-1}$ for PIP, TAZ and IBU, respectively. The following linear regression equations were derived:

$$\text{For PIP: } P = 0.0290C - 0.0512 \quad (r^2 = 0.9998)$$

$$\text{For TAZ: } P = 0.0314C + 0.1198 \quad (r^2 = 0.9994)$$

$$\text{For IBU: } P = 0.0270C - 0.02465 \quad (r^2 = 0.9990)$$

where P is the peak area of the analyte/the peak area of the IS, C is the concentration of the drug in $\mu\text{g mL}^{-1}$, and r is the correlation coefficient.

The results are given in Table 3. The obtained small intercepts indicated the minimum background signals, and the high determination coefficients indicated the excellent linearity of the calibration lines, and their reliability for the accurate analysis of the three analytes. Every concentration that was computed for the *in vitro* calibration standards remained within 15% of its nominal value.

3.2.2. Selectivity. The determination of specificity involved a comparison of chromatograms obtained from three groups of plasma samples: blank plasma, plasma spiked with IS and each of the investigated pharmaceuticals, and plasma samples obtained from rats that were administered combined doses of the pharmaceuticals under investigation, followed by spiking with IS. Fig. 3 illustrates that the examined components and plasma matrix do not interfere, thereby confirming the selectivity of the method.

3.2.3. Accuracy and precision. An intra-day and inter-day assessment of accuracy and precision was conducted using five replicates for each analyte at three discrete concentration levels: low quality control (LQC), high quality control (HOQ), and medium quality control (MQC). The procedure exhibited a range of intraday accuracies from -5.6 to 1.45% and interday accuracies from -6.25 to 3.02% , as measured by the bias percent. The coefficient of variation was utilized to ascertain precision, which revealed a range of values of 1.06 to 6.31% for interday precision and 0.68 to 5.28% for intraday precision. The technique was demonstrated to be accurate and precise as shown in Table 4.

3.2.4. Stability and extraction recovery. The stability of the prepared samples was assessed using multiple stability methods, including storage at room temperature (25 °C) for 6 hours (Bench-Top stability) and subjecting them to three freeze–thaw cycles, where they were frozen at 20 °C for 12 hours and then returned to room temperature (freeze and thaw stability). The achieved recoveries ranged from 101.88 ± 1.24 to 107.55 ± 8.01 for PIP, from 103.82 ± 7.15 to 101.53 ± 4.83 for TAZ, and from 105.32 ± 5.27 to 99.22 ± 1.21 for IBU, as shown in Table 5. The results have demonstrated that the plasma matrix does not affect the stability of the medications utilized in the generated samples under the evaluated conditions.

The extraction efficiency was taken into account, and the extraction recoveries for TAZ, PIP, and IBU were computed to be



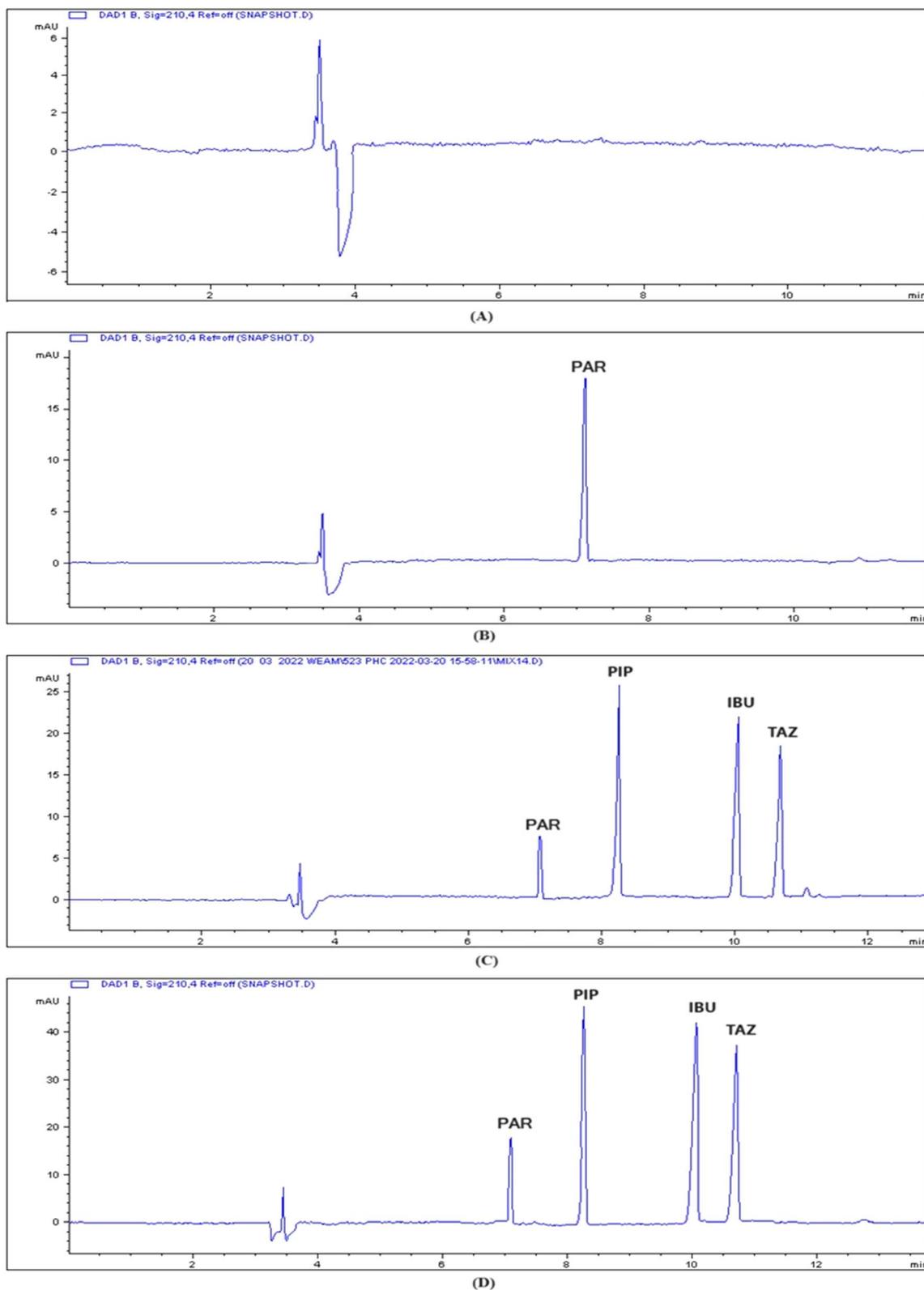


Fig. 3 Capillary electropherogram of (A) blank plasma; (B) plasma sample spiked with 50 µg per mL IS, and scanning at 210 nm; (C) plasma sample spiked with 50 µg per mL IS, 60 µg per mL PIP, 50 µg per mL IBU, 20 µg per mL TAZ, and scanning at 210 nm; (D) real rat plasma sample spiked with 50 µg per mL IS and elution of the studied drugs PIP, IBU, and TAZ, and scanning at 210 nm.



Table 2 Performance characteristics of PIP, TAZ and IBU by the CE method

Analyte	Migration time (min)	Number of theoretical plates	Capacity factor (1–10)	Selectivity (>1)	Resolution (>1.5)	Tailing factor (0.9–1.2)
PAR	7.1 ± 0.756	17 203	1.03	1.33	6	1.10
PIP	8.3 ± 0.551	15 876	1.37	1.35	11.3	1.12
IBU	10 ± 0.451	16 796	1.85	1.09	4	1.02
TAZ	10.6 ± 0.843	14 231	2.03			1.11

Table 3 Validation parameters of the CE method for the determination of PIP, TAZ, and IBU

Parameters	PIP	TAZ	IBU
Calibration range ($\mu\text{g mL}^{-1}$)	1–200	3–200	1–200
Slope	0.0290	0.0314	0.0270
Intercept	0.0512	−0.1198	0.02465
Determination coefficient (r^2)	0.9998	0.9994	0.9990
LOD ($\mu\text{g mL}^{-1}$)	0.3	0.7	0.3
LOQ ($\mu\text{g mL}^{-1}$)	1.0	2.3	0.9

104.96 ± 3.35, 99.68 ± 7.63, and 107.85 ± 3.81, respectively, as shown in Table 6. The results validated the efficacy of the used extraction procedure.

3.2.5. LOD and LOQ. The limits of detection and quantitation were detected dependent on the signal-to-noise ratio and the presence of three or ten values of relative standard deviation that were each below 10%.^{40,41} The PIP/TAZ and IBU values are detailed in Table 3. The method's capability to quantify concentrations at low levels renders it suitable for their determination in therapeutic drug monitoring.

3.3. Results of pharmacokinetic study

The assessed pharmacokinetic characteristics of the medications administered singly were utilized as comparison points for evaluating the combined effects of the medications when administered concurrently. The pharmacokinetic characteristics of the administered drugs were calculated by plotting the plasma concentration of the drugs at various time intervals using regression equations. A graph showing the average plasma concentration over time was then plotted, as shown in Fig. 4. PK Solver⁴² was used to conduct a non-compartmental pharmacokinetic study (NCA), and the results of all groups were compared, as shown in Table 7.

From the pharmacokinetic profiles in Fig. 4, it is obvious that all drugs were quickly absorbed almost after 0.25 h in all groups; however, their onset concentrations in group (III) were more than those in groups (I) and (II) by 21.7%, 80% and 14.2% for PIP, TAZ and IBU, respectively. It is noticed that the time required for IBU to record its maximum concentration in group (III) was higher than that in group (II) by 0.5 h, albeit the time attained by PIP and TAZ to reach their upper concentrations in group (III) was raised by 0.25 h when compared to group (I). The half-lives ($t_{1/2}$) of the drugs PIP and TAZ have reduced obviously (from 1.07 to 0.66 and from 2.06 to 1.66 for PIP and TAZ,

Table 4 Intra- and inter-day precision and accuracy of the proposed CE method for the simultaneous determination of PIP, TAZ and IBU in spiked rat plasma samples

Drug concentration ($\mu\text{g mL}^{-1}$)	Intra-day			Inter-day		
	Recovery ^a (%)	RSD ^a (%)	Bias ^b (%)	Recovery ^a (%)	RSD ^a (%)	Bias ^b (%)
PIP						
50	95.81	3.50	−4.19	103.02	6.22	3.02
100	96.90	2.50	−3.13	98.30	2.04	−1.71
150	97.78	3.14	−2.32	99.09	3.81	−0.91
TAZ						
50	93.76	3.31	−3.25	93.75	6.31	−6.25
100	95.54	1.10	−4.46	96.55	2.65	−3.45
150	99.47	1.20	−0.53	99.24	2.01	−0.76
IBU						
50	96.01	5.28	−3.99	99.16	3.25	−0.84
100	94.40	2.17	−5.60	94.40	2.17	−5.60
150	101.45	0.68	1.45	100.58	1.06	0.58

^a Average of 3 determinations. ^b Bias = [(measured concentration - nominal concentration)/nominal concentration] × 100. The values are an average of 3 determinations.



Table 5 Stability results of PIP, TAZ and IBU in spiked rat plasma under different conditions using the proposed CE method

Drug concentration ($\mu\text{g mL}^{-1}$)	Recovery ^a (%)	
	Three freeze thaw cycles ^b	Bench top stability
PIP		
50	110.57	106.10
100	87.23	116.20
150	107.85	100.37
Mean \pm RSD (%)	101.88 \pm 1.24	107.55 \pm 8.01
TAZ		
50	95.94	98.09
100	103.81	107.05
150	111.70	99.43
Mean \pm RSD (%)	103.82 \pm 7.15	101.53 \pm 4.83
IBU		
50	94.82	97.83
100	109.03	100.07
150	112.12	99.76
Mean \pm RSD (%)	105.32 \pm 5.27	99.22 \pm 1.21

^a Average of 3 determinations. ^b Freezing was done at $-20\text{ }^{\circ}\text{C}$.

respectively). The IBU half-life ($t_{1/2}$) was declined somewhat (from 1.20 to 1.09). With regard to the maximum plasma concentration (C_{max}) of all drugs, it was incredibly increased in group (III) compared to groups (I) and (II). PIP and TAZ in group (III) have higher C_{max} than group (I) by about 13.7% and 55.5%, whereas IBU in group (III) have higher C_{max} than group (II) by about 44%. When it comes to bioavailability which is represented by the area under the curve (AUC), the bioavailability of PIP and TAZ was increased incrementally when combined with IBU in group (III) compared to group (I) with 55.4% and 19.7% increase in their plasma levels. Furthermore, the IBU plasma concentration was increased by 35.6% in group (III) than in group (II). Additionally, the distribution volumes (V_d) and clearance rates (Cl) were considerably affected by the concomitant administration of the examined drugs. PIP and TAZ distribution volumes declined by 145.9% and 66.2% and their clearance values were reduced by 57.1% and 20%. For IBU, the distribution volume was minimized by 20%, while its clearance value was reduced by 33.3%. It is now clear that the concurrent administration of the drugs changed their pharmacokinetic

parameters and prescribing them together for in-patients or postoperatively has an accumulative therapeutic impact. This requires dose regimen regulation since there was a noticeable change in their bioavailability. All the pharmacokinetic parameters are given in Table 7.

3.4. Greenness profile of the CE method

Green Analytical Chemistry (GAC) is the design and development of analytical procedures to determine certain compounds in a given sample that generate less hazardous substances without impairing the efficiency even at low concentrations of analytes in complex matrix compositions.⁴³ Three metric assessment tools were used to assess the greenness of the proposed method: the eco-scale assessment, ESA,⁴⁴ green analytical procedure index, GAPI,⁴⁵ and analytical greenness, AGREE.⁴⁶

The ESA tool assesses the environmental influence of the analytical procedure/approach. The procedure/approach encompasses various factors such as the solvents and reagents employed, the energy consumption of the analytical instrument, the occupational dangers involved, and the generation of waste. The negative results of each procedure/approach are assigned as penalty points (PPs), which are then deducted from a perfect score of 100 points (representing an optimum eco-friendly procedure with no penalty points) in order to calculate the final result. The result of the calculation is ranked on a scale, where the score >75 represents excellent green analysis, >50 represents acceptable green analysis, and <50 represents inadequate green analysis.⁴⁴ The CE approach, as proposed, achieved a cumulative ESA score of 89. To illustrate: PP (penalty points) of solvent = subtotal PP \times number of pictograms \times signal word. For methanol = $1 \times 3 \times 2 = 6$, while for Acetonitrile = $1 \times 2 \times 2 = 4$. As the energy used was <0.1 kW h per sample (PP = 0), there was no analytical process hermetization (PP = 0), and the wastes were less than 10 mL (PP = 1), the total penalty points were 11. After deducting 11 from 100, the net ESA score was 89, as indicated in Table 8. Based on the ESA scale, the score designates the suggested CE technique as an optimum environmentally friendly procedure.

The GAPI instrument is responsible for assessing the overall analytical approach and delivering a comprehensive evaluation of 15 parameters, pertaining to the procedures' greenness, as shown in Table 8. The results of GAPI pentagrams revealed that the proposed method had 7 parameters colored green, 7

Table 6 Extraction recovery results of PIP, TAZ and IBU in spiked rat plasma

	PIP		TAZ		IBU	
	Concentration ($\mu\text{g mL}^{-1}$)	Recovery ^a (%)	Concentration ($\mu\text{g mL}^{-1}$)	Recovery ^a (%)	Concentration ($\mu\text{g mL}^{-1}$)	Recovery ^a (%)
	50	108.65	50	90.95	50	109.91
	100	102.10	100	105.05	100	110.19
	150	104.15	150	103.07	150	103.46
Mean \pm RSD (%)		104.97 \pm 3.35		99.69 \pm 7.63		107.85 \pm 3.81

^a Average of 3 determinations.



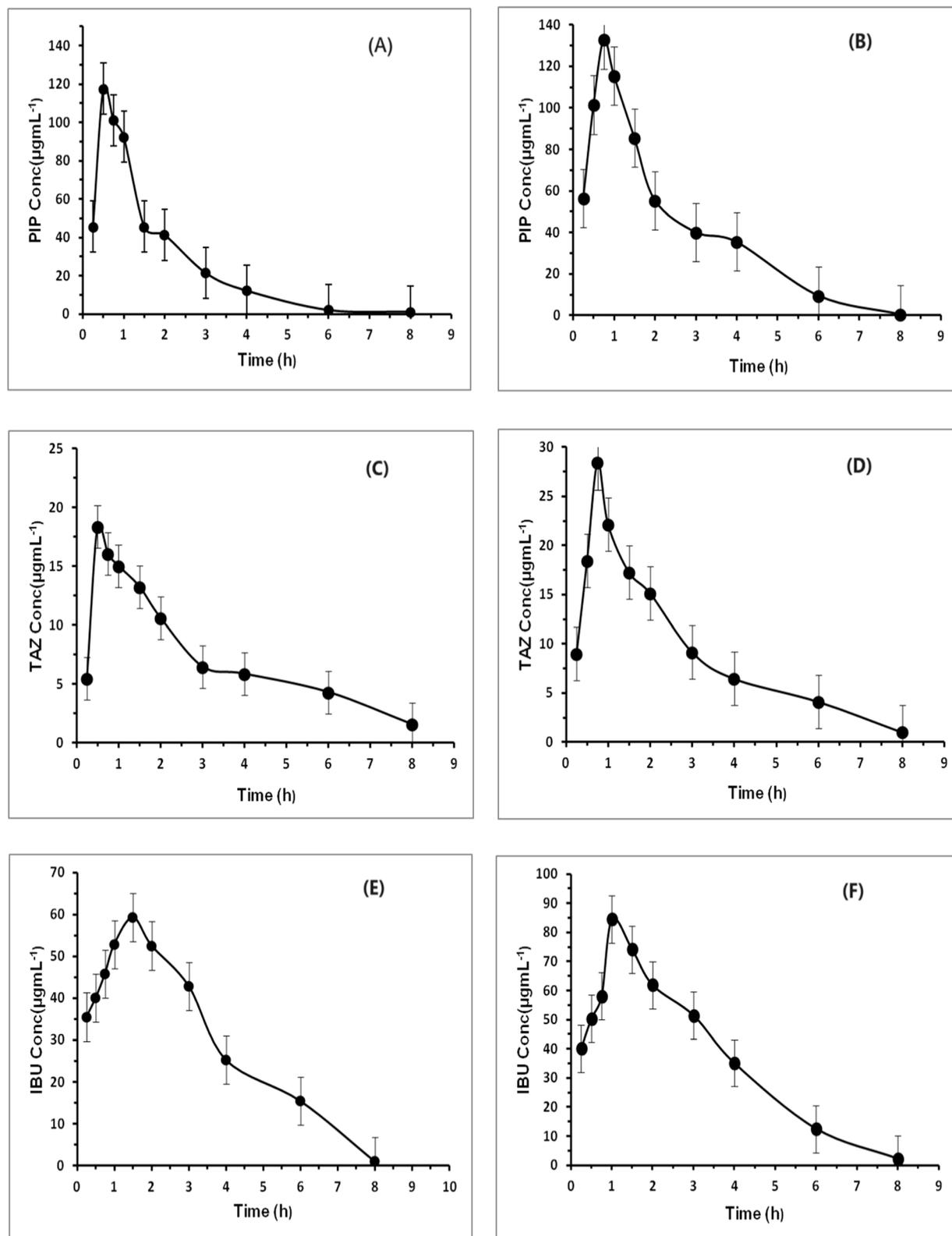


Fig. 4 Mean plasma concentration–time curves of PIP after intraperitoneal administration of mixed doses of 160/40 PIP/TAZ mg kg⁻¹ (A), PIP after intraperitoneal administration of mixed doses of 160/40 mg per kg PIP/TAZ and 30 mg per kg IBU (B), TAZ after intraperitoneal administration of mixed doses of 160/40 mg per kg PIP/TAZ (C), TAZ after intraperitoneal administration of mixed doses of 160/40 mg per kg PIP/TAZ and 30 mg per kg IBU (D), IBU after single intraperitoneal administration of a dose of 30 mg kg⁻¹ (E), and IBU after intraperitoneal administration of mixed doses of 160/40 mg per kg PIP/TAZ and 30 mg per kg IBU (F).



Table 7 Results of the pharmacokinetic study using the proposed CE method for the determination of PIP, TAZ and IBU

Parameters	PIP		TAZ		IBU	
	Group administered with PIP and TAZ	Group administered with PIP,TAZ and IBU	Group administered with TAZ and PIP	Group administered with PIP,TAZ and IBU	Group administered with IBU	Group administered with PIP,TAZ and IBU
T_{\max} (h)	0.5	0.75	0.5	0.75	1.5	1
$T_{1/2}$ (h)	1.08	0.67	2.28	1.66	1.30	1.41
C_{\max} ($\mu\text{g mL}^{-1}$)	117.46	132.82	18.35	28.41	59.23	84.56
Elimination constant (k)	0.64	1.03	0.30	0.42	0.53	0.49
V_d^a (mL) ($\mu\text{g}^{-1} \text{mL}^{-1}$)	1.55	0.61	2.17	1.32	0.45	0.36
Cl^b (mL) ($\mu\text{g}^{-1} \text{mL}^{-1} \text{h}^{-1}$)	0.99	0.64	0.66	0.55	0.24	0.18
AUC $0 \rightarrow t$ ($\mu\text{g mL}^{-1} \text{h}^{-1}$)	199.72	313.33	55.62	70.42	202.94	277.78
AUC $0 \rightarrow \infty$ ($\mu\text{g mL}^{-1} \text{h}^{-1}$)	201.59	313.87	60.75	72.84	208.59	282.13

^a V_d = Volume of distribution. ^b Cl = Clearance.

Table 8 ESA, GAPI and AGREE tools for the assessment of greenness values of the proposed CE method

ESA tool (PPs)	GAPI tool	AGREE tool
Methanol	6	
Acetonitrile	4	1- Sample treatment
Distilled water	0	2- Sample amount
Energy used (instrument)	0	3- Device positioning
Waste	1	4- Sample prep. Stages
Occupational hazards	Total	5- Method automation/miniaturization
PPs	11	6- Derivatization
ESA score	89	7- Waste
		8- Analysis throughput
		9- Energy consumption
		10- Source of reagent
		11- Toxicity
		12- Operator's safety

parameters colored yellow, and 1 parameter shaded red. Because the samples were prepared offline and stored at -20 degrees Celsius prior to analysis, parameter 1 (relating to sample preparation) in the GAPI pictogram of the proposed CE method was colored red. Due to the absence of chemical, physical, and physico-chemical preservation, parameter 2 was assigned the color green. Similarly, since there is no transportation, parameter 3 is also colored green. Parameter 4 was

tinted yellow due to storage being conducted under standard conditions. The reason parameter 5 is colored yellow because the CE method involves operations such as filtration and do not require extraction. Parameter 6 was marked as yellow due to the successful drug detection using the micro-scale in the CE method. Parameter 7 was also marked as yellow because the CE method relies heavily on the use of green solvents. Parameter (8) was assigned the color green since the process of preparing the



Table 9 Comparison of the performance and features of the proposed method with the published methods

Method	Analyte	Linearity range	Remarks	Ref.
CE-PDA	PIP, TAZ, and IBU	1–200 $\mu\text{g mL}^{-1}$ for both PIP and TAZ, and 3–200 $\mu\text{g mL}^{-1}$ for IBU	The first method for ternary mixture and applied to pharmacokinetic studies	Proposed study
UHPLC-MS/MS	PIP, TAZ, and IBU	0.5–500 $\mu\text{g mL}^{-1}$ for PIP and 0.625–62.5 $\mu\text{g mL}^{-1}$ for TAZ.	The method was not applied to pharmacokinetic studies	13
HPLC-UV	PIP and TAZ	0.5–400 $\mu\text{g mL}^{-1}$ and 1–100 $\mu\text{g mL}^{-1}$ for PIP and TAZ, respectively	The method was not applied to pharmacokinetic studies	1
RP-HPLC	PIP and TAZ	1.0–200 $\mu\text{g mL}^{-1}$ for PIP and 0.78–50 $\mu\text{g mL}^{-1}$ for TAZ	The method was not applied to pharmacokinetic studies	10
RP-HPLC	PIP and TAZ	50–100 ppm for both PIP and TAZ	The method was not applied to pharmacokinetic studies	11
Capillary zone electrophoresis	PIP and TAZ	20–400 for PIP and 10–100 $\mu\text{g mL}^{-1}$ for TAZ	The method was not applied to pharmacokinetic studies	15

sample does not necessitate any simple or advanced treatment. While the remaining parameters were colored yellow or green, the majority of the parameters were green. Hence, the suggested CE approach may be regarded as a relatively environmentally friendly method.

The AGREE metric, which is widely used, assesses analytical methodologies by considering a set of 12 parameters that are scaled from 0 to 12 on a common scale. The metric provides a visual result in the form of a number and a color. The final evaluation result is obtained by adding up the assessment findings for each concept. The AGREE pictograms indicate the degree to which specific principles are adhered to, as shown in Table 8. The final score, which is generated automatically in the center, is presented alongside the twelve evaluation parameters in the form of a colored circular pictogram representing the results. The AGREE pictogram represents the assumed CE technique. Table 8 presents the level of adherence to the principles, with a value of 0.77 out of 1, and a middle color of light green which ascertains that the performance of the principles is quite excellent, and the CE method is an eco-friendly method.

In summary, the proposed CE method satisfies the GAC's criteria for routine application in the analysis of TAZ, PIP and IBU in plasma samples. The comprehensive assessment of the method's environmental impact, as evidenced by the ESA, GAPI, and AGREE tools, substantiates its ecological friendliness and greenness.

4. Conclusion

For the first time, a sensitive, selective, and green capillary electrophoresis method equipped with diode array detection was employed to determine the co-administered drugs PIP, TAZ, and IBU in the plasma of rats. The proposed technique combined numerous privileges as plasma samples were prepared by simple straightforward one-step protein precipitation with miniaturizing of sample size, high efficiency, few solvents required and a short analysis time. The method's straightforward extraction procedure and short run time contribute to its high throughput, enabling the efficient

processing of diverse samples across several clinical laboratories. The CE method complied with FDA recommendation, and it was used for the full pharmacokinetic study of the drugs on rats. The pharmacokinetics of the drugs when administered singly was compared with that of the drugs when administered concurrently, and it showed the accumulative impact from co-administering the drugs in terms of rapid onset of action with a maximized concentration, which requires caution when prescribing the compounds together as there is a massive change in their bioavailability. The data resulting from the full pharmacokinetic study can be used for therapeutic drug monitoring and as basics for future researchers who are interested in further studies on drug–drug interaction in humans. Furthermore, the proposed analytical method was assessed using three green metric tools, namely ESA, GAPI and AGREE. The resulted outcomes revealed that the method has minimum environmental impacts while maintaining analytical parameters.

The proposed method is the first capillary electrophoretic method for simultaneous analysis of PIP, TAZ, and IBU and applied for pharmacokinetic studies. A comparison of the performance and features of the proposed method with the reported separation-based methods for simultaneous analysis of binary mixtures is summarized in Table 9.

Ethical statement

The Committee of Beni-Suef University, Faculty of Pharmacy approved the study protocol from an ethical point of view. Approval no.: REC-H-PhBSU-022-234.

Data availability

The data that support the findings of this study are included within the article, and any additional data will be available upon reasonable request.

Conflicts of interest

There are no conflicts to declare.



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