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Simultaneous determination of first-line anti-tuberculosis drugs by capillary zone electrophoresis using direct UV detection

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ABSTRACT

An alternative methodology for simultaneous analysis of ethambutol, isoniazid, rifampicin and pyrazinamide in pharmaceutical formulations by capillary zone electrophoresis under UV direct detection with an analysis time of 8.0 min is proposed. Background running was based on the effective mobility curve of the analytes and an optimum separation condition was achieved using a 3^3 Box-Behnken design, with Brij 35, Cu^{2+} and acetic acid/sodium acetate buffer as factors. An electrolyte consisting of 50.0 mmol L^{-1} of acetic acid/sodium acetate buffer, 12.5 mmol L^{-1} of $CuSO_4$, and standard and sample solutions prepared in 2.00 mmol L^{-1} of Brij 35 and 12.5 mmol L^{-1} of $CuSO_4$ were optimized. After evaluating validation parameters, the method was successfully applied to the analysis of samples in the form of tablets and sachets.

Human tuberculosis (TB) is a communicable disease caused by *Mycobacterium tuberculosis*. Nowadays, TB is transmitted by a single agent that operates worldwide. Its resurgence is due to epidemic HIV infection that dramatically increases transmission, morbidity and mortality of the disease. Tuberculosis is treated with a combination of ethambutol (ETB), isoniazid (ISO), rifampicin (RIF) and pyrazinamide (PYR) for periods of 6–9 months (Fig. 1). These four drugs are considered the best choice, since they combine maximum efficacy with an acceptable degree of toxicity [1,2].

The World Health Organization (WHO) recommends the combined use of multiple drugs to minimize the emergence of resistant strains. In order to follow this recommendation, fixed dose combinations (FDC) has been implemented, which take into account the association of two or more tuberculostatics in a single pharmaceutical formulation. FDC utilization increases treatment adherence, reduces the risk of resistance, lowers treatment costs and reduces errors in drug administration and distribution [3,4]. The combination of drugs has therapeutic advantages, but it brings about new research challenges for the pharmaceutical industry involving stability studies of combined drugs and their simultaneous analysis [5].

Several analytical methodologies have been developed in the last decade to analyze different FDCs. Among the methods most frequently used are UV–vis spectrophotometry combined with multivariate regression methods [6–9] and separation techniques. High performance liquid chromatography (HPLC) [10–13] and ultra performance liquid chromatography (UPLC) [14] with UV detection have been used for ISO, RIF and PYR quantification. Liquid chromatography coupled with mass spectrometry (LC/MS) was applied to the simultaneous determination of ETB, ISO, RIF, PYR and two metabolites, acetilisoniazide and 25-desacetilrifampicine [15]. Micellar electrokinetic chromatography (MEKC) using UV detection was used as an alternative method for ISO, RIF and PYR determinations [16].

In fact, HPLC is the first option in pharmaceutical analysis. However, to the best of our knowledge, all HPLC–UV methods reported in the literature have not considered the simultaneous determination of ETB, ISO, RIF and PYR due to the low ETB molar absorptivity, making their simultaneous analysis very challenging. Furthermore, a new drug association containing ETB, ISO, RIF and PYR in a single tablet (4-FDC) was recently made available by WHO for TB treatment, necessitating the development and optimization of quality control methods that are simple, efficient, robust, inexpensive, fast and suitable for routine analysis [17]. Within this context, this work proposes the development, optimization and validation of an alternative capillary electrophoresis (CE) methodology using direct UV detection at 262 nm for single or simultaneous deter-

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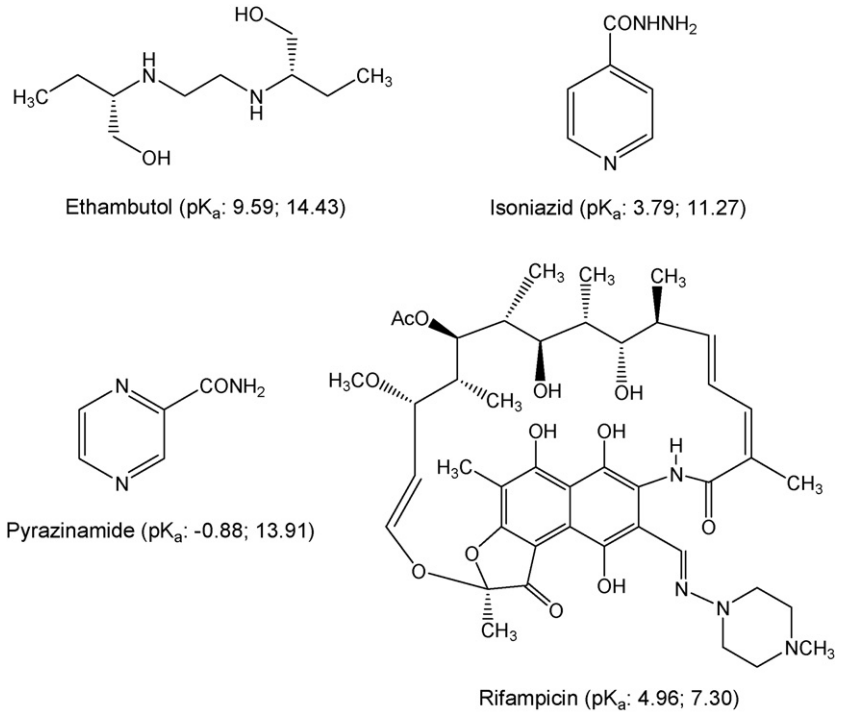

Fig. 1. ETB, ISO, RIF and PYR chemical structures.

mination of ETB, ISO, RIF and PYR in different pharmaceutical formulations.

1. Experimental

1.1. Reagents and solutions

All reagents were of analytical grade. Acetic acid (HAc), sodium hydroxide (NaOH), copper II sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), sodium lauryl sulfate (SDS) and methanol (MeOH) were purchased from Vetec (Rio de Janeiro, Brazil); polyoxyethylene 23 lauryl ether (Brij 35®) was purchased from Sigma–Aldrich (St. Louis, USA); sparfloxacina (SPFLX) was purchased from Xiamen Mchem Pharma Group (Xiamen, China); ethambutol dihydrochloride (ETB·2HCl) was purchased from Genix Pharmaceutical Industry (Goiás, Brazil); isoniazid was purchased from Taizhou Jiangbei Chemical Factory (Taizhou, China), rifampicin was purchased from Xiamem Mchem Laboratories Ltd (Xiangyang, China) and pyrazinamide was purchased from AB Farm Química Ltda (Goias, Brazil). The 4-FDC sachet was manufactured by Svizera Labs (Mumbai, India) and distributed by Svizera Europe BV (Almere, The Netherlands).

The 100 mmol L^{-1} aqueous acetate buffer (HAc/NaAc) (pH 4.6) stock solution used in the present work was prepared as follows: mass corresponding to 100.0 mmol L^{-1} of acetic acid and 50.0 mmol L^{-1} of NaOH were weighed and dissolved in a volumetric flask of 100 mL. An aqueous stock solution of 50.0 mmol L^{-1} of $CuSO_4$ was used to prepare the background electrolyte (BGE) and dilute standards and samples. Also an aqueous stock solution of 50.0 mmol L^{-1} of Brij 35 was used to prepare and dilute standard solutions and samples. The BGE solution was prepared by appropriate dilution of stock solutions of HAc/NaAc buffer (pH 4.6) and $CuSO_4$.

SPFLX stock solution (internal standard—IS) containing 1000 mg L^{-1} was prepared in MeOH. Aqueous stock solutions containing 1000 mg L^{-1} of ETB and ISO, and 2000 mg L^{-1} of PYR were independently prepared and stored in a refrigerator.

A stock aqueous solution containing 60.0 mg L^{-1} of RIF and 2.00 mmol L^{-1} of Brij 35 (maximum concentration capable of dissolving RIF without decomposition) was prepared daily. The compound was dissolved in an aqueous solution containing Brij 35 in an ultrasound bath for about 60 min.

1.2. Samples

1.2.1. 4-FDC sample

For the 4-FDC sample, the solid contents of the sachet were weighed and then two procedures were performed:

(a) Minimum level of the standard addition curve (solution 1): 13.8 mg of the sachet sample corresponding to 2.00 mg RIF was weighed and transferred to a 100 mL volumetric flask. Then aliquots of Brij 35, $CuSO_4$ and IS were added so that the final concentrations were 2.00, 12.5 and 20.0 mmol L^{-1} (fixed additive composition—FAC), respectively.
(b) Maximum level of the standard addition curve (solution 6): 13.8 mg of the sachet sample corresponding to 2.00 mg RIF and 4.00 mg of RIF standard were weighed and transferred to a 100 mL volumetric flask together with FAC. Aliquots of ETB, ISO and PYR were added to this volumetric flask so that the final concentrations were 64.0, 32.0 and 95.0 mg L^{-1}, respectively. The solutions were maintained under sonication in order to complete RIF dissolution and the final volume was adjusted with deionized water.

For the intermediate levels of the standard addition calibration curve, the following mixtures were made to a final volume of 10.0 mL: Level 2: 8.75 and 1.25 mL; Level 3: 7.50 and 2.50 mL; Level 4: 5.00 and 5.00 mL and level 5: 2.50 and 7.50 mL for solutions 1 and 6, respectively. Each procedure described above was performed six times, and the solutions were filtered through 0.45 μm Millipore filter (São Paulo, Brazil) to obtain clear solutions.

1.2.2. 2-FDC sample

For the combination of 150 mg RIF and 100 mg ISO, four capsules were opened and weighed. The weight corresponding to 20.0 mg of RIF was transferred to a 100 mL volumetric flask together with FAC. After 60 min of sonication, the volume was completed with deionized water and the solutions were filtered through a 0.45 μm Millipore filter to obtain clear solutions.

1.2.3. ETB and PYR samples

For the formulations containing 400 mg of ETB.2HCl or 500 mg of PYR, four tablets were weighed and homogeneously grounded to fine powders. The weight corresponding to 400 mg ETB or 500 mg of PYR was transferred to two different volumetric flasks of 25.0 mL and dissolved in deionized water. After 10 min of sonication the solutions were filtered through a 0.45 μm Millipore filter to obtain clear solutions. Then, a 750 or 800 μL aliquot of the sample solution of ETB and PYR was transferred to two different volumetric flasks of 10.0 mL together with FAC and the final volume adjusted with deionized water.

For the 2-FDC, PYR and ETB samples, quantification by external calibration was performed in quadruplicate.

1.3. Calibration curves

External calibration curves were prepared for the four drugs in triplicate ($n = 3$) from the dilution of standard solutions and the addition of aliquots of Brij 35, Cu^{2+} and IS so that the concentration was 2.00, 12.5 and 20.0 mmol L^{-1} respectively. The concentration ranges were: ETB from 8.00 to 64.0 mg L^{-1}, ISO from 4.00 to 32.0 mg L^{-1}, RIF from 5.00 to 40.0 mg L^{-1} and PYR from 12.0 to 95.0 mg L^{-1}. These curves were used to quantify the ETB, ISO and 2-FDC samples.

Calibration curves for standard addition were prepared, six replicates for the sample of 4-FDC as shown in sample preparation.

1.4. Instrumentation

The experiments involving separation optimization were conducted in a CE system (HP3d CE, Agilent Technologies, Palo Alto, USA) equipped with a DAD set at 262 nm, a temperature control device, maintained at 25 °C, and data acquisition and treatment software (HP ChemStation, rev A.06.01). Samples were injected hydrodynamically (30 mbar 5 s) and the electrophoretic system was operated under normal polarity and constant voltage conditions of +25.0 and +22.0 kV. For all the experiments, a 48.5 cm (40.0 cm effective length) × 75 μm ID × 375 μm OD fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was used.

1.5. Analytical procedures

Conditioning of new capillaries was carried out by a pressure flush of 1.00 mol L^{-1} NaOH solution (30 min), deionized water (5 min) and electrolyte solution (10 min). In between runs, the capillary was replenished with 0.200 mol L^{-1} NaOH solutions (2 min), deionized water (2 min) and fresh electrolyte solution (3 min, pressure flush).

2. Results and discussion

2.1. Background electrolyte optimization

By analyzing the effective mobility (μ_{eff}) curve [18] in Fig. 2, RIF and ISO are found to present a cationic feature within the 0–5.5 pH and the 0–8 ETB ranges, while PYR presents neutral behavior within virtually all intervals. Initially, a possible analysis by CE would be to consider the analytes as a cationic or neutral species.

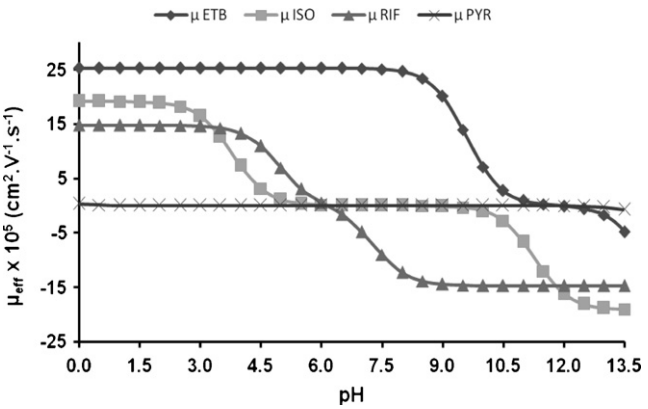

Fig. 2. Effective mobility curve for ETB, ISO, RIF and PYR.

As a cation, the problem would be PYR in view of the results above. As regards the MEKC mode, the alternative analysis would be to use sodium dodecyl sulfate (SDS) as an anionic surfactant in pH of about 9.0, as described by Acedo-Valenzuela et al. [16]. However, the main problem in these approaches is due to ETB presenting low molar absorptivity in the UV range, which would require a boundary condition such as an additive cromophore in the electrolyte system in order to achieve indirect UV detection or a derivatization reaction to form an adduct cromophore. Fig. 3 shows the electronic spectra of ETB, ISO, RIF and PIR in aqueous solution each at the 10.0 mg L^{-1} concentration level. However, an interesting alternative methodology would be a complexation study of ETB with copper (II) performed by Jiang et al. [19] using HPLC and adapted by Faria et al. [20] for ETB and its impurity analysis by CZE. For both approaches it was possible to form ETB complexes with copper and achieve good UV signals at 262 nm.

Taking into account the previous discussion, a study using a 3^3 Box-Behnken design was performed to optimize a background electrolyte in order to achieve simultaneous separation of ETB, ISO, RIF and PYR under UV detection through on-column ETB derivatization. The factors selected were Brij 35, Cu^{2+} and HAc/NaAc buffer. Brij 35 was selected to verify which concentration level would be capable of preventing RIF degradation, because RIF undergoes acid hydrolysis resulting in 3-formylrifampicin, which is two times faster in the presence of ISO [21]. Furthermore, 3-formylrifampicin reacts with ISO to form isonicotinil hydrazone, which is catalyzed by PYR and ETB [5,22]. According to Jindal et al. the use of surfactants in the preparation of RIF solution increases its solubility in water and

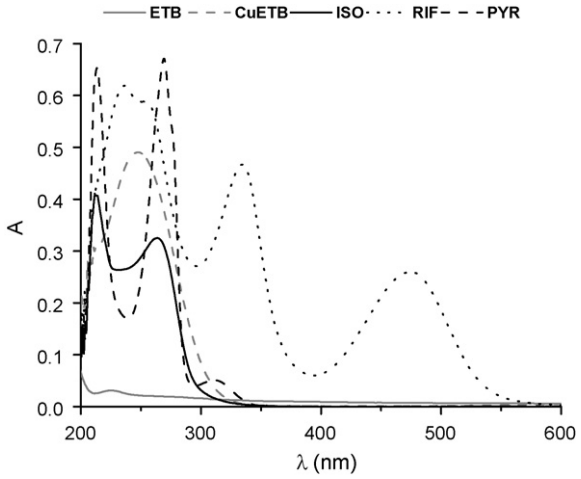

Fig. 3. Electronic spectra for ETB, ISO, RIF, PYR and CuETB.

Table 1
3^3 Box-Behnken design for electrolyte optimization.

Experiment	1	2	3	4	5	6	7	8	9	10	11	12	13
Buffer HAc/NaAc	−1	1	−1	1	−1	1	−1	1	0	0	0	0	0
Cu^{2+}	−1	−1	1	1	0	0	0	0	−1	1	−1	1	0
Brij 35	0	0	0	0	−1	−1	1	1	−1	−1	1	1	0

HAc/NaAc buffer (mmol L^{-1}): (−1) 50.0, (0) 60.0, (1) 70.0; Cu^{2+} (mmol L^{-1}): (−1) 7.50, (0) 10.0, (1) 12.5; Brij 35 (mmol L^{-1}): (−1) 1.50, (0) 2.00, (1) 2.50.

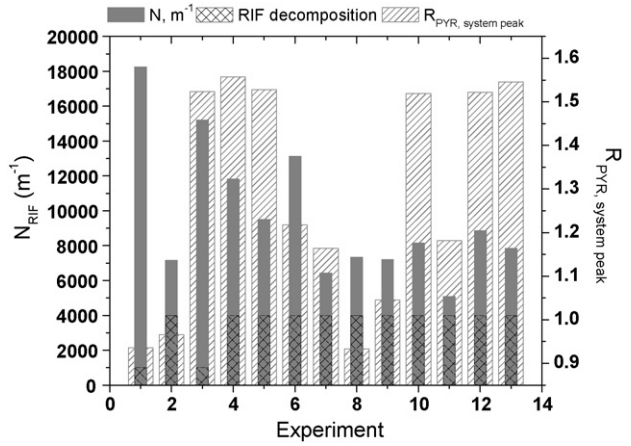

Fig. 4. Plot containing resolution between PYR and system peak, lack (1000) or presence (4000) of RIF decomposition and theoretical plate by meter obtained for 3^3 Box-Behnken design performed for electrolyte optimization.

inhibits its degradation [23]; Cu^{2+} was used as an additive to form a chromophoric adduct with ETB and a cationic adduct with PYR; and the HAc/NaAc buffer was used to maintain the pH within the 4.5 and 5.0 pH range so that copper precipitation does not occur [19]. Table 1 shows the contrast matrix for the 3^3 Box-Behnken design. Instrumental parameters such as voltage, cartridge temperature, detection wavelength, injection time and capillary length were maintained constant. Standard mixtures for each experiment were diluted in aqueous solution containing Brij 35 and $CuSO_4$ to obtain the concentration levels described in Table 1. The monitored responses were the resolution between PYR and the system peak (flow electroosmotic peak—EOF) and RIF plate number (N).

By analyzing the 3^3 Box-Behnken design results in Fig. 4, experiment 3 was the only one found to simultaneously exhibit resolution minimum of 1.50, lack of RIF decomposition and higher theoretical plates. Comparing experiment 1 with 3, that differs only in its $CuSO_4$ concentration level, 7.50 mmol L^{-1} instead of 12.5 mmol L^{-1}, co-migration of the PYR peak together with the electrosmotic flow (EOF) peak could be observed. The explanation for this behavior is that the 7.50 mmol L^{-1} of Cu^{2+} is not sufficient to produce an effective positive charge for PYR capable of producing the migrating shift of the EOF peak. Moreover, by comparing experiment 4 with 3, whose single difference is the level of buffer concentration, there was increased RIF peak deformation, which can be attributed to the occurrence of on-column compound degradation due to the antagonistic effect of the concentration level of this buffer. Finally, comparing experiment 2 with 3, for which only the level of surfactant remained constant, increased RIF peak deformation and PYR co-migration together with the EOF peak were observed. Thus, it can be concluded that there is a correlation between the buffer and the Cu^{2+} concentration for a definite level of surfactant that delimits the separation performance in the context of avoiding RIF degradation and PYR co-migration with the EOF peak. Therefore, quantitative and qualitative analysis show that the electrolyte consisting of 50.0 mmol L^{-1} of HAc/NaAc buffer (pH 4.6) and 12.5 mmol L^{-1} of $CuSO_4$, and standard and sample solution prepared in 2.00 mmol L^{-1} of Brij 35 and 12.5 mmol L^{-1} of $CuSO_4$ can be used as an optimum condition for drug determination.

After method optimization, a fast procedure was performed in order to confirm the interaction among RIF and PYR with Cu^{2+}. Initially, an experiment was carried out using the CE optimal conditions, but without the addition of Cu^{2+}. By comparing the two electropherograms in Fig. 5, it was possible to show that RIF presents decomposition in absence of Cu^{2+} while PYR forms a cationic complex in presence of Cu^{2+}.

2.2. Internal standard selection

After electrolyte system optimization and standard preparations, the need for using an internal standard (IS) was verified.

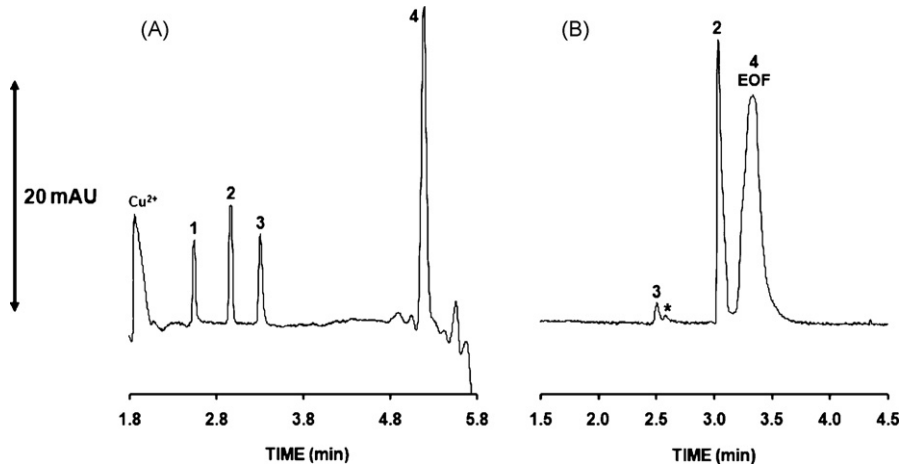

Fig. 5. Standard mixture separations of (1) ETB, (2) ISO, (3) RIF, (4) PYR and (*) RIF degradation product. (A) eletctropherogram obtained at the optimized conditions: electrolyte: 50.0 mmol L^{-1} HAc/NaAc buffer and 12.5 mmol L^{-1} $CuSO_4$, hydrodynamic injection: 30 mbar 5 s, voltage: +22.0 kV, direct detection at 262 nm, capillary: 75 μm × 48.5 cm (40.0 cm effective length); (B) eletctropherogram obtained under the same experimental conditions in letter (A), but without the addition of Cu^{2+} to the electrolyte system and standard dilutions.

Table 2
Statistical results for linearity, selectivity, precision, accuracy and LOQ.

	Linearity and selectivity					Precision (%RSD)		%Recovery	LOQ (mg L^{-1})
	Curve	Slope	Intercept	r	$F_{calculated}$	Area	Time		
ETB	EC	0.477 ± 0.009	− 0.0272 ± 0.0275	0.999	3.00	3.61	0.215	98.7	9.65
	SAC	0.476 ± 0.008	0.618 ± 0.014	0.999		2.53	0.362		
ISO	EC	0.921 ± 0.019	− 0.0671 ± 0.0294	0.999	2.96	3.17	1.61	97.8	2.50
	SAC	0.922 ± 0.018	0.198 ± 0.017	0.999		2.10	0.508		
RIF	EC	0.874 ± 0.007	− 0.0443 ± 0.0130	0.999	3.00	3.29	0.625	100.4	2.09
	SAC	0.865 ± 0.017	1.08 ± 0.02	0.999		1.77	0.609		
PYR	EC	3.18 ± 0.05	−0.179 ± 0.210	0.999	2.99	3.87	2.31	98.4	4.79
	SAC	3.22 ± 0.05	8.80 ± 0.13	0.999		1.78	1.94		

EC: external calibration curve ($n = 3$); SAC: standard addition calibration curve ($n = 6$); $F_{0.05;\ f1=4;\ f2=12} = 3.26$;.

Therefore, some experiments were performed and SPFLX was selected as the IS since it presents chemical behavior and effective mobility similar to the analytes of interest (not shown). In order to optimize peak resolution between ETB and SPFLX (adjacent peak pair), a study applying +20.0, +22.0, +25.0 and +27.0 kV voltages was performed, which presented resolutions of 1.54, 1.86, 1.25 and 1.00 respectively. Therefore, the voltage of + 22.0 kV was selected because it showed an appropriate $R_{ETB,SPFLX}$ and required less analysis time when compared with +20.0 kV.

2.3. Study of the stability of electrolyte solutions and RIF

After optimization of the experimental conditions, a study to evaluate electrolyte system stability was carried out. The mixture of standards containing ETB, ISO, RIF and RIP was subjected to 40 consecutive injections using the same electrolyte system. Then, the electrolyte solutions were exchanged for a fresh solution and the standard mixture resubmitted to 40 consecutive injections. Signs of distortion of the ISO peak were observed that from the 33rd run. Therefore, replacement of the electrolyte at the 30th injection is recommended. Furthermore, the aqueous RIF solution mixed with 2.00 mmol L^{-1} of Brij 35 and 12.5 mmol L^{-1} of $CuSO_4$ was found to be stable since there was no RIF decomposition for a 18-h monitoring.

2.4. Validation parameters

Initially, a system suitability study was performed by twenty-five consecutive injections of the standard mixture using the internal standard. Relative standard deviations (RSD) for repeatability in area and migration time and adjacent peak pair resolutions (RS) were calculated [24,25]. The precision in area and in migration time was below 3.60% and 1.45%, respectively. The adjacent pair resolutions were 1.94 for ETB and SPFLX, 2.60 for SPFLX and ISO, 3.14 for ISO and RIF, and 13.9 for RIF and PYR. Therefore, the system was suitable for carrying out the validation procedure.

The method selectivity was evaluated by comparing the slopes of the addition calibration curves with the external calibration curves [25,26]. The linearity was evaluated using the correlation coefficient (r) and the application of an *a priori* linearity hypothesis test [23,27] (Eq. (1)). The equations of the curves presented in Table 2 were obtained by regression of ratio analyte area/IS area *versus* ratio analyte concentration/IS concentration. Since the slope values are very similar and all the $F_{calculated}$ values are smaller than the $F_{critical}$ value, which is 3.26, the method presented acceptable selectivity and linearity results (Table 2).

$$F_{calculated} = \frac{{s_{yx}}^2}{{s_y}^2} = \frac{\sum_{i=1}^{p} m_i(\bar{y}_i - \hat{y}_i)^2/(p-2)}{\sum_{i=1}^{p}\sum_{j-h}^{m_i}(y_{ij} - \bar{y}_i)^2/(m-p)} \quad (1)$$

where m_i is the number of measurements of the ith replicate; p is the number of calibration points; m is the pxmi product.

Precision in area and migration time was evaluated for the six levels of the external calibration and standard addition curves. The highest %RSD in area and in migration time for the external calibration and standard addition curves are shown in Table 2.

Accuracy was assessed by calculating recovery and by performing the t test for the levels determined for the different drugs and the contents declared by the manufacturers [25,28]. Recovery was evaluated for the 4-FDC sample in five concentration levels, which is still being tested and may not be consumed. The percentage mean is shown in Table 3. The values were found to be in agreement with the recommended range of 100.0 ± 5.0%. Quantification was performed for the 4-FDC sample and commercially available samples, such as 2-FDC (ISO–RIF) and formulations containing only ETB or PYR (Table 3). The quantification result for the 4-FDC sample presented high discrepancy compared to the declared content, which can be explained by the fact that it is a sample under testing and not recommended for administration. The variations in the drug levels

Table 3
Sample quantification results.

Drug	Sample	Label claim (mg)	Amount found (mg)	$t_{calculated}$
ETB	A[a]	825.0	628.2 ± 4.5	106.4
	B[b]	400.0	400.4 ± 1.8	0.4347
ISO	A	225.0	145.0 ± 0.9	219.6
	C[b]	100.0	99.13 ± 2.36	0.7404
RIF	A	450.0	579.4 ± 5.2	61.01
	C	150.0	152.2 ± 2.7	1.667
PYR	A	1200	1300 ± 14	17.50
	D[b]	500.0	500.1 ± 1.5	0.1283

[a] $n = 6$; [b] $n = 4$; $t_{0.05;\ 3} = 2.353$; $t_{0.05;\ 5} = 2.015$; A: 4-FDC; B: ETB; C: 2-FDC (ISO-RIF); D: PYR.

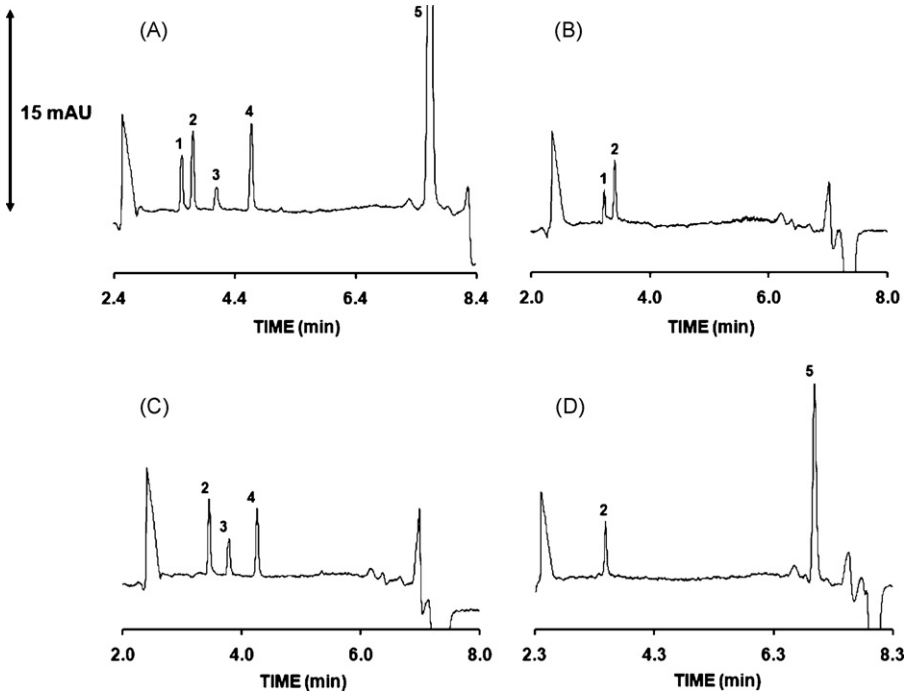

Fig. 6. Sample electropherograms: (A) 4-FDC, (B) ETB (C) 2-FDC (ISO–RIF), (D) PYR, where (1) ETB, (2) SPFLX–PI, (3) ISO, (4) RIF and (5) PYR. Optimum experimental conditions: electrolyte: 50.0 mmol L^{-1} HAc/NaAc buffer and 12.5 mmol L^{-1} $CuSO_4$, hydrodynamic injection: 30 mbar 5 s, voltage: +22.0 kV, direct detection at 262 nm, capillary: 75 μm × 48.5 cm (40.0 cm effective length).

Table 4
3^3 Box-Behnken design for robustness evaluation.

Experiments	Brij 35	Cu^{2+}	Voltage	$R_{ETB,\ SPFLX}$	$R_{PYR,\ System\ peak}$	N_{RIF} ($\times 10^4$) m^{-1}
1	−1	−1	0	1.86	2.77	6.69
2	1	−1	0	1.84	2.87	6.43
3	−1	1	0	1.78	2.81	6.82
4	1	1	0	1.75	3.12	6.66
5	−1	0	−1	2.12	4.40	6.71
6	1	0	−1	1.82	3.86	6.96
7	−1	0	1	1.92	3.64	7.19
8	1	0	1	2.01	3.80	6.97
9	0	−1	−1	1.81	3.02	6.96
10	0	1	−1	1.76	3.48	7.23
11	0	−1	1	1.90	3.12	7.00
12	0	1	1	1.88	3.59	6.87
13	0	0	0	1.92	3.34	6.89
14	0	0	0	1.93	3.50	6.79
15	0	0	0	1.96	3.48	6.77

Brij 35 (mmol L^{-1}): (−1) 1.80, (0) 2.00, (1) 2.20; Cu^{2+} (mmol L^{-1}): (−1) 12.0, (0) 12.5, (1) 13.0; voltage (kV): (−1) 21.5, (0) 22.0, (1) 22.5.

can be explained by the difficulty of maintaining formulation quality, owing to problems such as a change in the RIF crystalline form, adsorption of drug ingredients, drug addition order and formulation decomposition [5,22]. However, based on recovery values, the method has been shown to be effective for quantitative monitoring of these combined drugs. The other samples, already commercially available, had levels close to those reported, which was demonstrated by the $t_{calculated}$ values that are smaller than the $t_{critical}$ value, indicating no significant differences between the observed levels. Fig. 6 shows the electropherograms for the analyzed samples.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the 2-FDC (ISO–RIF) electropherograms and for formulations containing only ETB or PYR, taking into account signal-noise ratios equal to 3 and 10, respectively [24,25]. These values were considered suitable for pharmaceutical formulations (Table 2).

Robustness was evaluated executing a 3^3 Box-Behnken design using Brij 35, Cu^{2+} and applied voltage as factors. These factors were selected in order to evaluate method robustness for three critical analytical characteristics: resolution between the PYR and EOF peaks, RIF decomposition and resolution between ETB and SPFLX. Table 4 shows the results and experimental description performed using a 3^3 Box-Behnken design, where the three experiments at central point are in triplicate for calculating experimental error.

Robustness results are presented in Table 4. Small variations seen in the $CuSO_4$ concentration and the applied voltage do not interfere with resolution between the PYR and EOF peaks and resolution between ETB and SPFLX. Additionally, no decomposition was detected in any of the 15 experiments performed, because the N of RIF presented negligible variations ($6.68 \times 10^4 \pm 0.21$). Therefore, the decomposition of RIF does not occur with small variations in the concentration of Brij 35 and Cu^{2+}. The estimated regression models do not present lack of fit at the 95% confidence interval since the $F_{calculated}$ values obtained by ANOVA for $R_{ETB,SPFLX}$, $R_{PYR,\ System\ peak}$ and N_{RIF}, 8.07, 11.48 and 9.14, respectively, were smaller than

the $F_{0.05;3,2}$ value which is 19.33, characterizing robustness for the range investigated.

3. Conclusions

An alternative methodology for the simultaneous analysis of ETB, ISO, RIF and PYR in pharmaceutical formulations using Cu(II) complexation by CZE under direct UV detection was demonstrated. The method presented the following advantages: ability to simultaneously analyze the four drugs using a UV detection system, use of a simple and cheap electrolyte system; sample preparation without off-column derivatization steps and short analysis time. Therefore, CE proved suitable for routine analysis in the quality control of these drugs.

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