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Simultaneous analysis of first-line anti-tuberculosis drugs in tablets by UV spectrophotometry compared to capillary zone electrophoresis

Research Article

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Abstract: The development and optimization of a novel UV spectrophotometric methodology was proposed for simultaneous analysis of ethambutol (ETB), isoniazid (ISO), rifampicin (RIF) and pyrazinamide (PYR), using multivariate calibration based on the partial least squares method (PLS). The methodology was successfully applied for analysis of four-drug fixed dose combination (4-FDC) tablets used for tuberculosis treatment. A 3^4 Box-Behnken design, with triplicate in central point, was used for sample preparation in the calibration step. In the present case, nine latent variables were chosen for the model development that presented the smallest RMSECV and explain 98.76% of data variance in Y block (concentrations of ETB ISO, RIF and PYR) and 99.93% of data variance in X block (spectral data). PLS models for ETB, ISO, RIF and PYR presented RMSEP and R^2 values of 0.23 mg L^{-1} and 0.971; 0.14 mg L^{-1} and 0.731; 0.11 mg L^{-1} and 0.990 and 0.57 mg L^{-1} and 0.972, respectively. A validation step was performed based on the comparison between the UV spectrophotometric proposed methodology and capillary zone electrophoresis (CZE) in 4-FDC real samples and no significant difference was found between two methodologies at 95% of confidence level.

Keywords: *4-FDC • UV spectrophotometry • Chemometrics • Capillary electrophoresis*

1. Introduction

The human tuberculosis (TB) is an infectious disease with register since pre historic times receiving this name only at 1839 by Johann Lucas Schonlein. Its etiologic agent, the Mycobacterium tuberculosis was isolated and discovery by Robert Koch at 1882. For centuries, there were several difficulties to discover the cause and cure of TB, which caused the death of billions of people. The first drug to be used in treating of this disease, discovery in 1943 by North American Selman Waksman, the antibiotic streptomycin, opened perspectives to search for new drugs [1-3]. After the Waksman discovery, other drugs were identified such as isoniazid (ISO) in 1952, rifampicin (RIF) in 1965, ethambutol (ETB) 1968 and pyrazinamide (PYR) in 1970, which are until now used as first choice in the treatment of TB (Fig. 1).

In the early 80's TB reappeared, with non-adherence of patients to treatment, increasing poverty, aging populations, the lack of information, migration,

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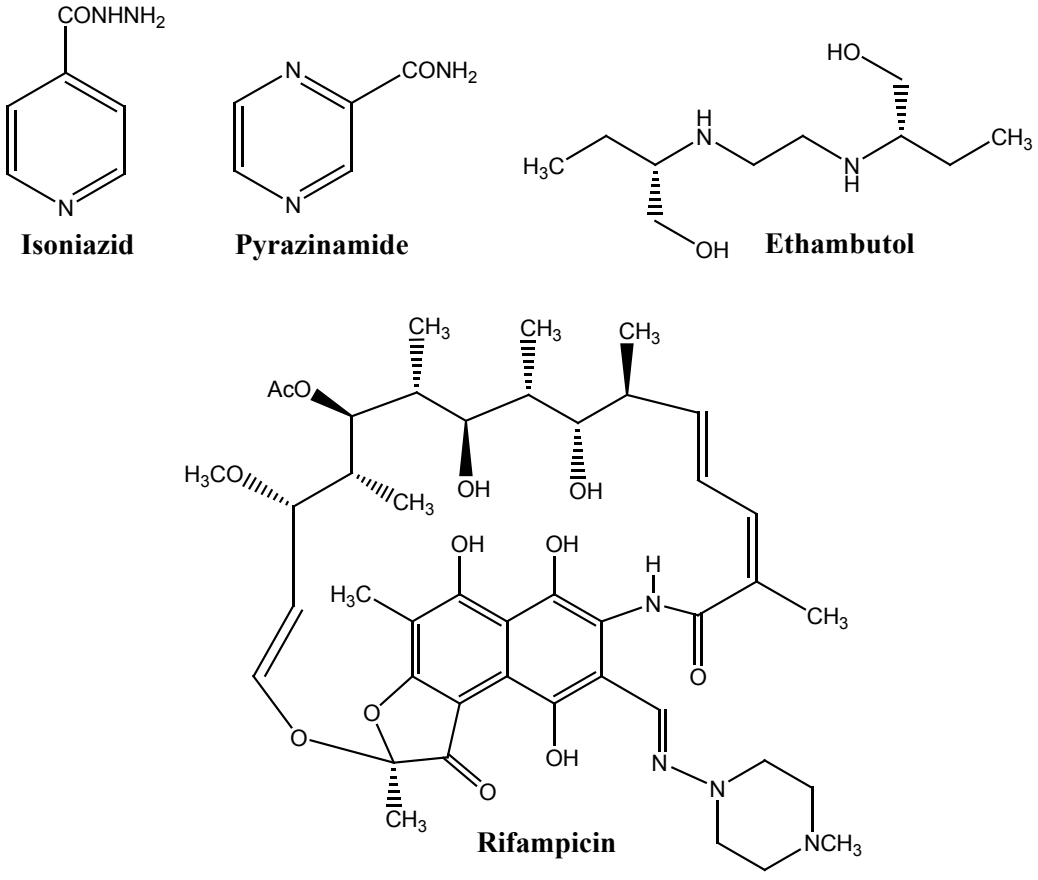

Figure 1. Chemical structure of the first choice pharmaceuticals for TB treatment.

lack of investment and the emergence of Acquired Immunodeficiency Syndrome (AIDS, English : Acquired Immune Deficiency Syndrome). Currently, in addition to AIDS, the discovery of MDR-TB – multidrug-resistant tuberculosis, which occurs when the bacillus becomes resistant to at least RIF and ISO, and super-resistant bacteria to drugs, *i.e.*, extremely drug-resistant tuberculosis (XDR-TB) strains that are resistant to drugs in the first and second choice, has caused serious problems in TB treatment [4,5]. In this context, emerged the fixed-dose combination (FDC) concept, which can be defined as a formulation of two or more substances biologically active, combined in a single drug, available in certain fixed doses. Several advantages can be mentioned in favor of FDC such as reduced risk of emergence of drug resistant strains, better patient compliance, reduced cost of treatment, fewer risks of medication errors, dosages adjustment according to patient's needs and simplified drug supply management, shipping and distribution [6]. This new concept generates also new challenges to the pharmaceutical industry such as drugs stability in the association and the quality control difficulty where simultaneous quantification is required.

For simultaneous analysis of the FDC containing as association ISO, RIF and PYR, different analytical approaches based on multivariate calibration had been considered. In 1998, Benetton et al. developed methods using ultraviolet-visible (UV-Vis) spectrophotometry and first derivative for RIF and ISO analysis in commercial capsules [7]. In 1999, Goicoechea and Olivieri developed a methodology for simultaneous determination of ISO, RIF and PYR in tablets by UV-Vis spectrophotometry using partial least squares (PLS1) regression with higher precision [8]. In 2001, Espinosa-Mansilla *et al*. performed comparative study between hybrid linear analysis (HLA/SX) and PLS1 to simultaneous determination of ISO, RIF and PYR in pharmaceutical formulations. The methodology by HLA/SX yielded similar results to PLS1 for RIF and PYR analysis and slightly worse to ISO [9]. In 2005, Madan *et al*. successfully performed simultaneous determination of ISO, RIF and PYR by UV spectrophotometry in pharmaceutical formulations using principal components regression (PCR) and PLS1 [10]. Two works have demonstrated simultaneous analysis of ETB, ISO, RIF and PYR: one using high performance liquid chromatography tandem mass spectrometry (HPLC-MS) and other using capillary

zone electrophoresis under UV detection [11,12]. Thus, to our knowledge, a methodology for simultaneous analysis of these four drugs by UV spectrophotometry has not been yet developed, mainly due to the low ETB molar absorptivity. However, de Oliveira group have demonstrated in a detailed study that ETB complexation with copper is instantaneous in aqueous solution of acetate buffer at pH 4.6 and present relevant molar absorptivity [13,14]. In this context, the present work has as aim the development and optimization of a novel UV spectrophotometry methodology able to perform simultaneous analysis of ETB, ISO, RIF and PYR in 4-FDC association using multivariate calibration. It is important to stress that 4-FDC tablets for tuberculosis treatment scheme have been distributed in Brazil by Health Unique System (SUS) from august of 2009. Moreover, 4-FDC tablets as tuberculosis treatment scheme also have been used by India and China where disease incidence is very high [15]. Therefore, the use of an efficient alternative analytical methodology that is versatile, simple, without use of organic solvents (which suits the green chemistry principle), low cost and fast can be useful to health regulatory agencies such as National Health Surveillance Agency (Anvisa) for quality control of this drug [16].

2. Experimental procedure

2.1. Chemicals and materials

All reagents were of analytical grade. Acetic acid (HAc), sodium hydroxide (NaOH), copper II sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) 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 MchemPharma Group (Xiamen, China); ethambutol (ETB) dihydrochloride was purchased from Genix Pharmaceutical Industry (Goiás, Brazil); isoniazid (ISO) was purchased from TaizhouJiangbei Chemical Factory (Taizhou, China), rifampicin (RIF) was purchased from Xiamen Mchem Laboratories Ltd (Xiangyang, China) and pyrazinamide (PYR) was purchased from AB Farm Química Ltda (Goias, Brazil). The 4-FDC tablet was purchased from CIPLA LTD, India.

Stock solutions of 50 mmol L^{-1} of buffer acetic acid / sodium acetate (HAc / NaAC), 10 mmol L^{-1} of $CuSO_4$ and 50 mmol L^{-1} of Brij 35 were prepared independently and stored under refrigeration. Standards aqueous stock solutions containing 150 mg L^{-1} of ETB, 200 mg L^{-1} of PYR and 50 mg L^{-1} of ISO were independently prepared and stored under refrigeration. A stock aqueous solution containing 60.0 mg L^{-1} of RIF and 2.00 mmol L^{-1} of Brij 35 (maximum concentration able to dissolve 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. These stock solutions were used to analytical procedures involving UV spectrophotometry and capillary electrophoresis.

2.2. 4-DFC sample preparation

Seven tablets of 4-DFC were weighed and macerated to fine powder in a mortar. A portion of 0.021 g of this powder macerate was transferred to a 50.0 mL volumetric flask, to which was added 2.0 mmol L^{-1} of Brij 35 and kept in ultrasonic bath for 1 h. Then the volume was completed with deionized water and the solution was filtered using a 0.45-µm Millipore filter. An aliquot of 1.2 mL of this solution was transferred into a 10.0 mL volumetric flask, where it was added the stock solutions of Brij 35, $CuSO_4$ and HAc / NaAC, such that the final concentration was equal to 1.76 mmol L^{-1}, 0.5 mmol L^{-1} and 5.0 mmol L^{-1}, respectively. Then UV spectra were obtained in the wavelength range from 200 to 400 nm. These solutions contained concentrations close to 11.0 mg L^{-1} ETB, 3.0 mg L^{-1} ISO, 16.0 mg L^{-1} PYR and 6.0 mg L^{-1} RIF. For the capillary electrophoresis analysis, an aliquot of stock solution of 5.0 mL sample was transferred to a 10.0 mL volumetric flask, which were added stock solutions of Brij 35, $CuSO_4$ and SPFLX (internal standard – IS), such that the final concentration was 2.0 mmol L^{-1}, 12.5 mmol L^{-1} and 30.0 mg L^{-1}, respectively. The procedures were performed seven times.

2.3. External calibration curve for CZE quantification

External calibration curves were prepared in triplicate from dilutions of stock solutions standards and the addition of aliquots of Brij 35, $CuSO_4$ and IS, to a final concentration equal to 2.0 mmol L^{-1}, 12.5 mmol L^{-1} and 30.0 mg L^{-1}, respectively. The concentration ranges were: from 15 to 90 mg L^{-1} for ETB; from 5 to 20 mg L^{-1} for ISO; from 10 to 55 mg L^{-1} for RIF and from 20 to 120 mg L^{-1} for PYR.

2.4. Instrumentation

The measurements of absorption spectra were performed on a double-beam UV-Vis spectrophotometer system (model UV-1800, Shimadzu, Kyoto, Japan) using quartz regular cell of 1.0 cm optical path. Each UV spectrum was obtained with resolution of 0.5 nm from 200 to 400 nm.

The experiments involving separation were carried out on a CE system (HP3d CE, Agilent Technologies,

Palo Alto, CA, USA) equipped with a DAD set at 262 nm, a temperature control device, maintained at 25ºC, 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 kV). For all experiments, a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) 48.5 cm (40.0 cm effective length) × 75 µm ID × 375 µm OD was used.

The dissolution of samples and standards were carried out in a Unique ultrasonic cleaner, USC (2850) model, power of 120 W (São Paulo-SP, Brazil).

2.5. Analytical procedures

Spectrophotometric measurements were performed by using aqueous solution of HAc/NaAc buffer and $CuSO_4$ as reference in the same concentrations used to standards and sample dilution.

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).

2.6. Chemometric analysis

The UV spectra obtained were manipulated using a MATLAB environment. For PLS calculations the data were preprocessed using the mean center. For the calibration models using PLS, a 3^4 Box-Behnken design with triplicate in central point (three levels and four factors) was used for mixture concentration design of ETB, ISO, RIF and PYR, and the number of latent variables of the model was selected based on the root mean square error of cross-validation (RMSECV) obtained from the calibration set by leave one out procedure. Anomalous samples were discarded by the leverage and residue analysis; the occurrence of such samples among the calibration samples can produce models with a low capability of precision and, when present in the validation samples, can influence the validation results, leading to results which are indicative that the model is not adequate.The performance of the models was evaluated by the root mean square error of calibration (RMSEC) and the root mean square error of prediction (RMSEP). A comparison was performed with CZE results.

2.7. Basic statistical analysis

Basic statistical analysis such as normality test (Shapiro–Wilk test), variance homogeneity (Levene and Cochran test) and pared sample t-test were performed using SPSS 8.0 for Windows.

3. Results and discussion

3.1. Range selection of ingredient active content

In a first approach, a study was conducted to check the concentration level of the active ingredient to be used in multivariate calibration procedure, using standard mixtures at concentrations 20 and 25 times smaller than label claimed in 4-FDC pack of tablets usually used for treatment of patients, that is 275 mg of ETB, 75 mg of ISO, 150 mg of RIF and 400 mg of PYR. The standard solution mixtures were prepared using a range of ±15%, as indicated in Table 1. By analyzing the UV electronic spectra of standard solutions mixtures obtained from Table 1 (Figs. 2A and 2B), it is possible to observe that drugs content ratio of 25 times smaller provided better separation of the spectra of the complex CuETB levels of -15% and +15%. In addition, for very concentrated solutions which is providing more than 2 absorbance units, the average distance between the ions or molecules absorbing species decreases to the point that each particle affects the charge distribution and thus the extent of absorption of neighboring particles. So the ratio of the drugs content in tablet by 25 was selected. Moreover, according to the American pharmacopeia the range allowed for the drugs is from 90 to 110% of the declared content [17]. An adequate model takes into account the quantification of samples within concentrations limits used during modeling step. Therefore, a range of variation of ± 15% for modeling step was selected for standard mixture in order to ensure the use of descriptive models for the drugs quantification within a safety range. Thus, a 3^4 Box-Behnken design with triplicate in central point (three levels and four factors) was used to multivariate calibration considerations, as shown in Table 2. The factors selected were ETB, ISO, RIF and PYR, while the concentrations levels of standard mixtures were selected took into account tablet content ratio by 25, while maintaining other variables such as $CuSO_4$, buffer HAc/NaAc and Brij 35, according to described in Table 1. UV electronic spectra obtained from the 3^4 Box-Behnken design within range from 200 to 400 nm is shown in Fig. 3.

The absorbance values of the 29 experiments for standard mixture were studied by the method of partial least squares (PLS1 and PLS2). In the present case, nine latent variables (LV) through PLS 2 approach calibration present the smallest RMSEPCV and better values for the total variance of Y block (concentrations of ETB

Table 1. Concentration variation range of the standard mixtures solution.

Drug	Concentration, mg L^{-1}	ETB	ISO	RIF	PYR
Tablet content /20	-15%	11.7	3.2	6.4	17.0
	+15%	15.8	4.3	8.6	23.0
Tablet content /25	-15%	9.4	2.6	5.1	13.6
	+15%	12.7	3.5	6.9	18.4

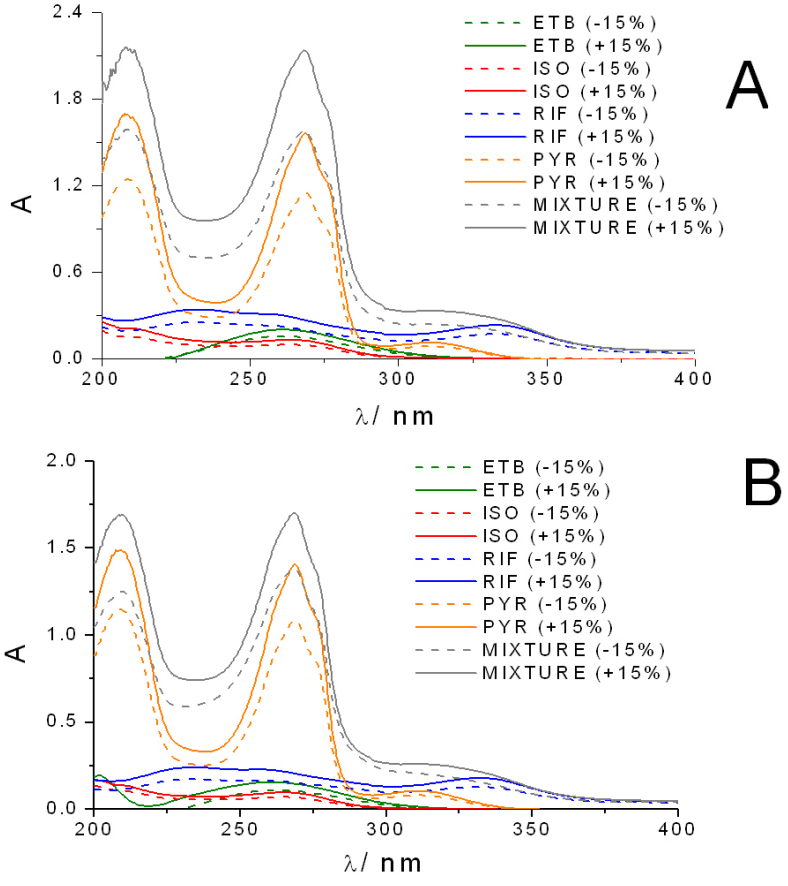

Figure 2. UV spectra of the experiments obtained for the drugs content ratio in tablet by 20 (A) and 25 (B).

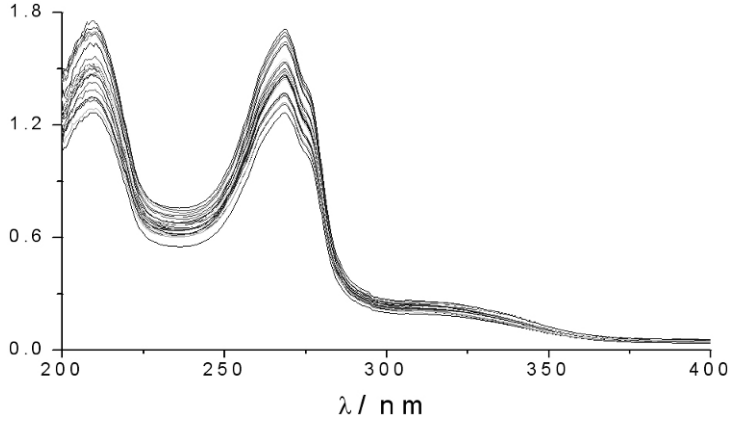

Figure 3. UV spectra obtained from 3^4 Box-Behnken design.

Table 2. 3^4Box-Behnken design matrix.

Experiments	ETB	ISO	RIF	PYR
1	-	-	0	0
2	+	-	0	0
3	-	+	0	0
4	+	+	0	0
5	0	0	-	-
6	0	0	+	-
7	0	0	-	+
8	0	0	+	+
9	-	-	-	-
10	-	0	0	-
11	+	0	0	-
12	-	0	0	+
13	+	0	0	+
14	0	-	-	0
15	0	+	-	0
16	0	-	+	0
17	0	+	+	0
18	0	0	0	0
19	-	0	-	0
20	+	0	-	0
21	-	0	+	0
22	+	0	+	0
23	0	-	0	-
24	0	+	0	-
25	0	-	0	+
26	0	+	0	+
27	+	+	+	+
28	0	0	0	0
29	0	0	0	0

ETB (mg L^{-1}): (-) 9.35; (0) 11.0; (+) 12.65;
ISO (mg L^{-1}): (-) 2.55; (0) 3.0; (+) 3.45;
RIF (mg L^{-1}): (-) 5.1; (0) 6.0; (+) 6.9;
PYR (mg L^{-1}): (-) 13.6; (0) 16.0; (+) 18.4;
$CuSO_4$: 0.5 mmol L^{-1}; Buffer HAc/NaAc: 5.0 mmol L^{-1}; Brij 35: 2.0 mmol L^{-1}.

ISO, RIF and PYR), 98.76%, and the total variance of the block X (spectral data), 99 93%. Besides, leverage and residues of Students for concentration presented results within normal range. In the present case nine LV was necessary due to nonlinear behavior of ETB and ISO, as shown in the scatter diagram of latent variables found for RMSEP (Fig. 4). It is important to stress that, curiously, all results obtained using PLS1 modeling (not shown) were worst when compared to PLS2.

Since there was no significant difference in the UV spectra between standard mixture and 4-FDC pills the model developed was used to predict the compounds in real samples. Fig. 5 shows PLS2 fitted models for ETB, ISO, RIF and PYR, which presented RMSEP and R^2 values of 0.23 and 0.971; 0.14 and 0.731; 0.11 and 0.990 and 0.57 and 0.972 respectively. The low R^2 value obtained for ISO can be considered acceptable since ISO concentration value is very low in comparison with other compounds in 4-FDC pills.

3.2. Comparison between UV spectrophotometric methodology and CZE

In order to verify if PLS2 model optimized can be useful for analysis in real sample of 4-FDC tablets, a comparison using CZE methodology recently published by de Oliveira group [14] was carried out. Thus, the quantification of the samples analyzed by CE was achieved by calibration curve using SPFLX as internal standard. The curves were performed using concentration levels for each drug in authentic triplicate through the least-square regression with internal standard approach. The linearity was evaluated by homoscedasticity (Levene and Cochran' test) and lack of fit test by priori hypotheses test (Eq. 1).

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

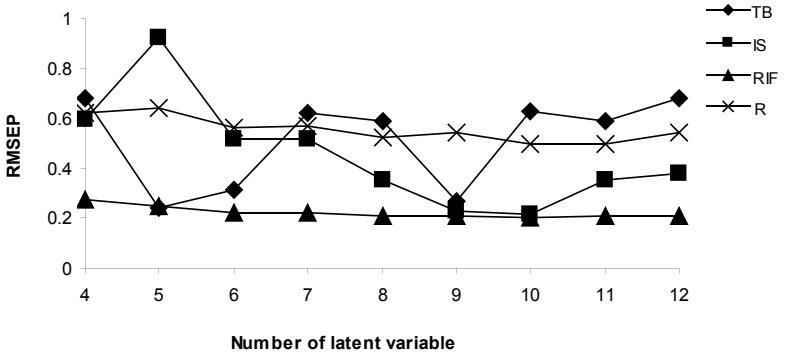

Figure 4. RMSEP scatter diagram of latent variables for ETB, ISO, RIF and PYR.

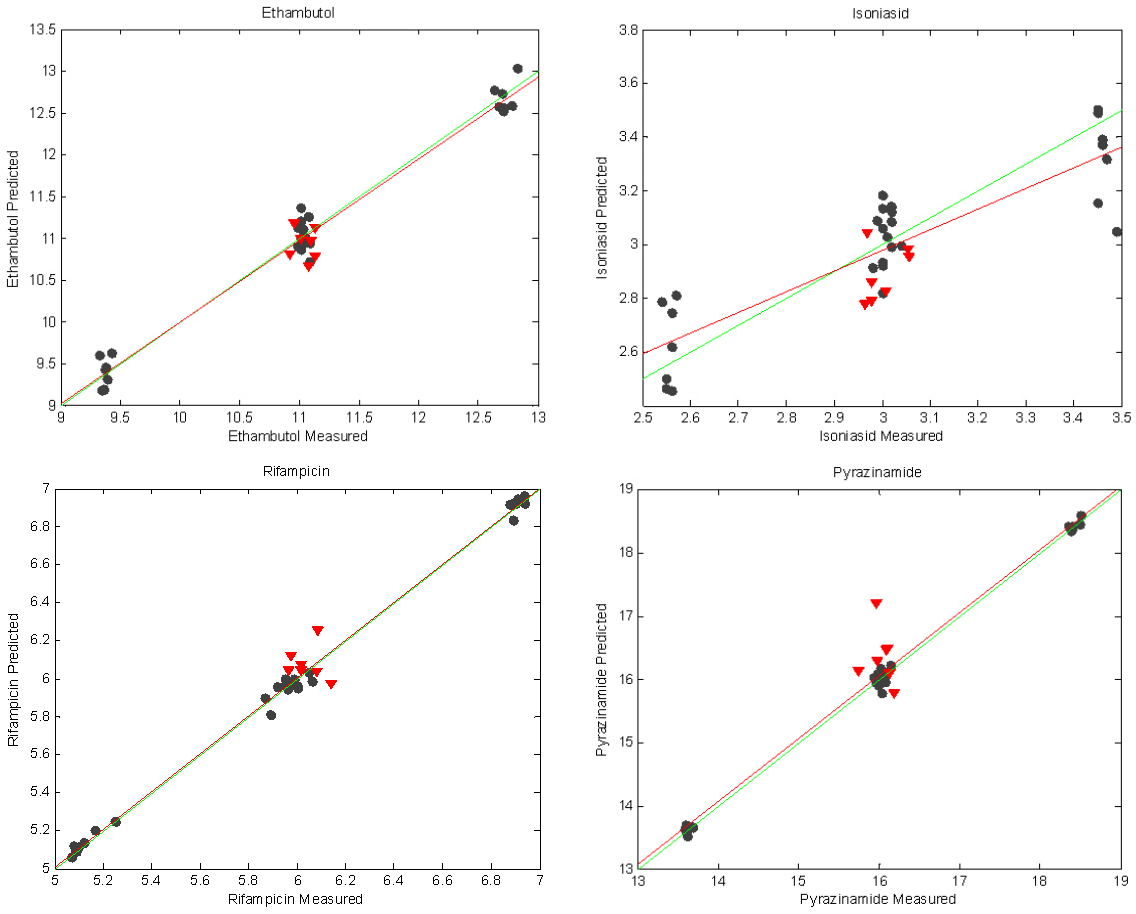

Figure 5. Plots forETB, ISO, RIF and PYR PLS2 modeling (●) standard mixture solutions- calibration; (▼) 4-DFC tablets samples-validation.

Table 3. Quantification values of 4-FDC by CZE.

Drugs	Linearity				*Precision (%CV)	%Recovery
	Slope	Intercept	R^2	F_{calc} Lack of fit		
[a]ETB	0.39 ± 0.01	- 0.04 ± 0.01	0.995	2.76	4.7	101.9
[b]ISO	0.78 ± 0.01	- 0.07 ± 0.01	0.997	3.99	4.3	103.1
[c]RIF	1.16 ± 0.02	- 0.05 ± 0.03	0.999	2.19	4.7	104.5
[d]PYR	2.79 ± 0.05	- 0.34 ± 0.13	0.995	2.32	4.6	101.6

$^{a}F_{Sig.\ (1,\ 16)} = 3292$; $^{b}F_{Sig.\ (1,10)} = 3656$; $^{c}F_{Sig.\ (1,14)} = 3421$; $^{d}F_{Sig.\ (1,16)} = 2894$

** higher variation coefficient obtained for external calibration curve with internal standard;*

$^{a}F_{tab.\ (0.05,\ 4;\ 12)} = 3.26$; $^{b}F_{tab.\ (0.05,\ 2;\ 8)} = 4.46$; $^{c}F_{tab.(0.05;\ 4,\ 10)} = 3.48$; $^{d}F_{tab.\ (0.05,\ 3;\ 12)} = 3.26$

As homoscedasticity was violated by RIF, and then weighted least-square regression was used. As $F_{calculated}$ values obtained for lack of fit analysis were lower than $F_{critical}$, showing no lack of fit in the range of 95% confidence interval according to shown in Table 3. Besides, all F_{sig} and determination coefficient (R^2) values obtained were considered satisfactory, indicating an optimal adjustment of the data [18-20]. Other statistical considerations such as precision and recovery also were considered.

3.3. Pharmaceutical formulation analysis

The optimized multivariate UV spectrophotometry analysis using PLS2 model was applied to analysis of 4-FDC pharmaceutical formulations. In validation procedure, results obtained by CZE were used for comparison with PLS2 method, taking into account seven tablets analyzed in replicates. Table 4 shows the results obtained for CZE and UV spectrophotometry analysis and references values for each compound in pill and Fig. 5 shows the electropherogram obtained

Table 4. Average of values found by CZE and UV spectrophotometry (n=7) for 4-FDC tablets.

4-DFC	Label claim[a,b] (mg)	Found average concentration (mg)	
		CZE	UV[a,b]
ETB	275	276.3 ± 0.8	273.3 ± 1.7
ISO	75	75.0 ± 1.3	72.3 ± 3.5
RIF	150	151.0 ± 1.1	152.0 ± 1.5
PYR	400	400.8 ± 0.9	408.8 ± 2.7

[a]*Normality test p-value: 0.23.*
[b]*Paired samples t-test p-value: 0.58.*

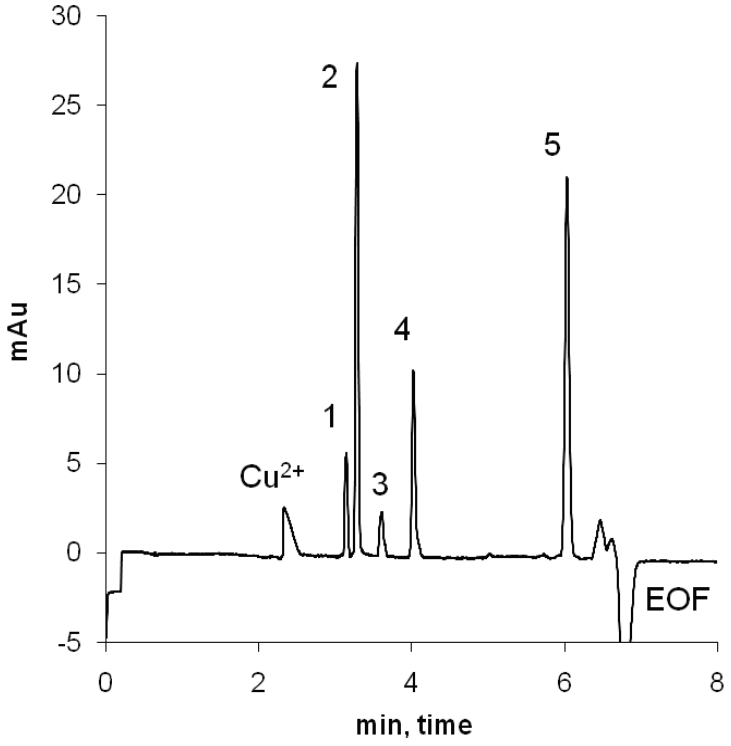

Figure 6. Sample electropherograms of 4-FDC, where (1) ETB, (2) SPFLX–PI, (3) ISO, (4) RIF and (5) PYR. Experimental conditions: electrolyte: 50.0 mmol L^{-1} HAc/NaAc buffer and 12.5 mmol L^{-1} $CuSO_4$, hydrodynamic injection: 30 mbar. 5 s, voltage: +25 kV, 25ºC cartridge temperature, direct detection at 262 nm; capillary:75 μm × 48.5 cm (40.0 cm effective length).

for sample analysis by CZE. The EOF acronym in Fig. 6, means the migration time of electrosmotic flow, a characteristic phenomenon in capillary electrophoresis [21]. In order to show the reliability of the PLS2 method a normality test using Shapiro-Wilk test followed by paired sample t-test was performed between reference and UV spectrophotometry associate to PLS2 method and no significant difference was found for 95% confidence interval, since p-value found were higher than α equal to 0.05.

4. Conclusions

In the present work we have developed and optimized a novel UV spectrophotometric methodology using multivariate approach able to perform simultaneous analysis of ETB, ISO, RIF and PYR using PLS2 model. The Box-Behnken approach associate to standard calibration showed that is not necessary to use the full formulation in calibration step. Besides, the methodology optimized presented acceptable prediction ability for all pharmaceuticals, obeying the American pharmacopeia regulation, which consider adequate range from 90 to 110% of the declared content for the drugs [17]. Finally, in the present case, the UV spectrophotometry and the multivariate calibration associated to the ethambutol copper complexation showed to be promising for use in routine analysis for quality control monitoring. Thus, this compounds analysis in pharmaceutical formulations presents lower cost, versatility and simplicity as its main advantages.

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