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Validation of a rapid micellar electrokinetic capillary chromatographic method for the simultaneous determination of isoniazid and pyridoxine hydrochloride in pharmaceutical formulation

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Abstract

An efficient and reliable micellar electrokinetic capillary chromatography (MEKC) method has been developed for the simultaneous determination of isoniazid (ISO) and pyridoxine hydrochloride (PYR) in pharmaceutical formulations. A chemometric two level full factorial design approach was used to search for the optimum conditions of separation. Three parameters were selected for this study: the buffer pH, the buffer concentration and sodium dodecyl sulphate (SDS) concentrations. Resolution, peak symmetry and analysis time were established as response. The two analytes were separated within 6 min with the optimized conditions: 50 mM borate buffer, 25 mM SDS pH 7.8, 35 °C, at 50 mbar 4 s injection and 30 kV by using a fused silica capillary (72 cm effective length, 50 μm i.d.). The detection wavelength was set to 205 nm. Meloxicam was used as internal standard. The method was validated with respect to stability, linearity range, limit of quantitation and detection, precision, accuracy, specificity and robustness. The detection limits of the method were 1.0 μg mL^{-1} for ISO and 0.40 μg mL^{-1} for PYR and the method was linear at least in the range of 3.0–100 μg mL^{-1} for ISO and 1.0–100 μg mL^{-1} for PYR with excellent correlation coefficients (0.9995 for ISO and 0.9998 for PYR). Relative standard deviations (R.S.D.s) of the described method ranged between 0.54 and 2.27% for intra-day precision and between 0.65 and 2.69% for inter-day precision. The developed method was applied to the tablet form of ISO and PYR-containing the pharmaceutical preparations and the data were compared with obtained from the standard addition method. No statistically significant difference was found.

Keywords: Experimental design; Validation; Pharmaceutical; Isoniazid; Pyridoxine hydrochloride; Micellar electrokinetic capillary chromatography (MEKC)

1. Introduction

Tuberculosis is still a serious and potentially lethal infection. Isoniazid (ISO) (Fig. 1a) is one of the most effective antitubercular agents available. However, prolonged administration of this drug may be accompanied by side effects with neurotoxic manifestations such as psychotic states, peripheral neuritis, etc. To minimize toxic effects, the drug must be used concurrently with another agent for treatment such as pyridoxine hydrochloride (PYR) (Fig. 1b). If PYR is not administered concurrently in the course of tuberculosis treatment with ISO, peripheral neuritis is the most common reaction of ISO [1,2]. Therefore the drugs should be administered together. To increase patient compliance, the pharmaceutical company (Deva Drug Company) produces combined pharmaceutical forms of ISO with PYR in Turkey.

There are various analytical procedures for the assay of either ISO or PYR in pharmaceutical preparations and biological fluids, only three methods have been reported to be able to analyze both drugs in combination [3–5]. These methods used high-pressure liquid chromatography (HPLC) [3], high performance thin layer chromatography [4] and spectrophotometry [5]. Several capillary electrophoresis methods for quantitation of ISO [6–10] and PYR [11] have been reported. In these studies, ISO was analyzed by UV detection using salicylaldehyde-5-sulfonate as a precolumn derivatizing agent to increase sensitivity [6] and with electrochemical detector [7–10], which is a seldom used detector for capillary electrophoresis.

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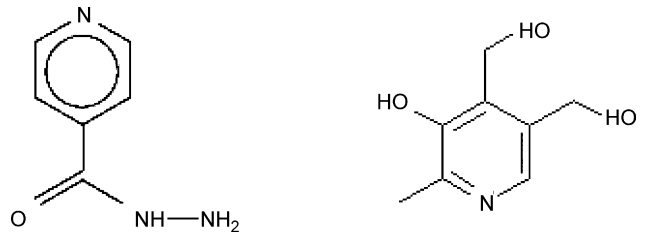

Fig. 1. Chemical structure of (a) ISO and (b) PYR.

Meanwhile, PYR was analyzed in soft drinks with micellar electrokinetic capillary chromatography (MEKC) using UV detector. To our knowledge, there is no reported capillary electrophoresis method for the simultaneous determination of ISO and PYR in pharmaceutical formulations.

The aim of this study was to develop a simple, efficient and reliable MEKC method to analyze ISO and PYR in pharmaceutical preparation simultaneously. Due to number of variables involved, the univariate optimization procedure is too tedious and does not take into account possible interactions between factors [12–15]. A chemometric approach, two level full factorial design was employed to systematically optimize the relevant operating parameters. Resolution, analysis times and peak symmetries were all evaluated to make the developed method more efficient and accurate.

In the present study, a two level full factorial experimental design was used to evaluate the effects of selected independent variables on the responses, to optimize the method. This design is a set of experimental runs where every level of a factor is investigated at both levels of all the other factors [16].

After selecting the optimum conditions, validation studies were performed and the method was applied to the analysis of a commercial tablet containing 100 mg of ISO and 25 mg of PYR.

2. Experimental

2.1. Apparatus

The MEKC analysis was performed on an Agilent 3D-CE (Waldbornn, Germany) apparatus equipped with a fused silica capillary 80.5 cm in total length (72 cm to the detector, 50 (m i.d.), diode array detector (DAD) (190–600 nm), automatic pressure or electrokinetic sample injector, peltier temperature controller (15–60 °C) and 30 kV high voltage power supply.

2.2. Reagents

ISO, PYR and meloxicam (IS) were kindly supplied by Deva (Turkey), Santa Farma (Turkey) and Nobel (Turkey), respectively. Isovit® tablets containing 100 mg of ISO and 25 mg of PYR were obtained from local pharmacies in Turkey. Boric acid and sodium dodecyl sulphate (SDS) were purchased from Sigma. 0.1 N NaOH was purchased from Agilent. All other chemicals were analytical grade. Deionized water was made in laboratory using Milli-Q system (Barnstead, USA).

2.3. Standard solutions

Standard stock solutions of ISO and PYR (1000 μg mL^{-1}) were prepared in water and IS (100 μg mL^{-1}) in methanol. Various aliquots of the standard solution were taken, the IS was added each solution, and then diluted to 5 mL with background electrolyte (BGE) to give a final appropriate analyte concentration.

2.4. Sample Solutions

Ten tablets (each tablet was containing 100 mg of ISO and 25 mg of PYR) were crushed to obtain a fine powder and mixed well. Powder, equivalent to the average weight of one tablet was accurately weighed and dissolved in 100 mL water. The mixture was ultrasonicated for 30 min, then centrifuged for 15 min, 1 mL of supernatant and 1.25 mL of IS (100 μg mL^{-1}) was added in 5 mL volumetric flask and diluted with BGE.

2.5. Synthetic tablet solutions

They were prepared by mixing the solid excipients of commercial tablet form (sodium citrate, lactose monohidrate, polyvinylpyrrolidine, magnesium citrate) and standard substances of ISO and PYR in known amounts and dissolved in deionized water as described in Section 2.4.

Standard, sample and synthetic tablet solutions were kept protected from light with aluminum foil covering and they were stored at 4 °C.

2.6. Background electrolyte (BGE)

154.57 mg boric acid and 360.5 mg SDS were transferred into 50 mL volumetric flask, diluted to a constant volume with water; the pH was adjusted by addition of 0.1 N NaOH then diluted to the mark with distilled water. The final optimal BGE was consisted of 50 mM borate buffer (pH 7.8) and 25 mM SDS.

2.7. Capillary electrophoresis procedure

Prior to each run the capillary was rinsed with 0.1 N NaOH solution for 3 min, distilled water for 2 min and background electrolyte for 3 min. The capillary was filled with BGE, 50 mM borate buffer, pH 7.8 and 25 mM SDS unless specified. The samples were introduced by hydrodynamic injection (50 mbar for 4 s) and run with the applying voltage of 30 kV at 35 °C. Samples were detected using a diode array detector at 205 nm (band with 10 nm). Spectra were also collected during the runs for peak evaluation. All solutions were filtered through a 0.45 μm filter prior to injection the capillary electrophoresis system.

2.8. Software

ChemStation (Rev A.08.03 847) software was used to operate CE system and to evaluate data. Maple 8.0 software (Waterloo

Maple, Canada) was used to calculate matrixes. Response surface graphics were plotted with Microsoft Excel 2003 (U.S.A.).

3. Results and discussion

3.1. Optimization

The optimization procedure was comprised of two steps: a series of initial experiments and two full factorial experimental designs.

3.1.1. Step 1: Initial experiments

The aim of the initial experiments was to establish the basic analytical requirements (the type of buffer, pH range of the BGE, SDS concentration range, temperature, voltage and injection time) of the method.

The pH value of the BGE is an important variable since it affects the charge of the compounds under investigation. In our study, the separation of molecules was achieved at a pH above 9 using capillary zone electrophoresis. In order to achieve a suitable separation for ISO, the experiment had to be performed at a pH of 11 to prevent poor selectivity associated with closer retention times of the peaks of interest and due to the electroosmotic flow (EOF). ISO degrades as pH increases and therefore to overcome the degradation a MEKC technique was selected to separate the molecules from each other with enhanced selectivity and without degradation in the pH range of 7–9.

Phosphate, citrate and borate buffer were tested for BGE. The borate buffer was selected because it yielded the lowest current and the most stable baseline, therefore it was used in all subsequent experiments.

The high voltage and the capillary temperature had significant and positive effects on the analysis time. This was expected since the magnitude of the high voltage influences movement rate of the determinants and the temperature affects their mobility through changes in the viscosity of the BGE [17]. When operating at high voltage and high temperature levels, a satisfactory baseline separation was still achieved. Thus, in further experiments, the high voltage and capillary temperature were held constant at 30 kV and 35 °C, respectively.

Table 1
Factors and factor levels investigated in full factorial design

Factor	Level		
	−1	0	1
x_1: pH	7	8	9
x_2: Buffer concentration (mM)	20	35	50
x_3: SDS concentration (mM)	10	25	40

The effects of injection times of 1–8 s on peak characteristics were studied. An increase in the time over which the injection was made resulted in increasing peak heights up to a time of 4 s. The peak height remained stable when the injection time was longer than 4 s but the shape of peaks broadened. Therefore, 4 s was chosen as the optimum injection time in all further experiments.

3.1.2. Step 2: Experimental design

Initially, experiments were carried out to determine the appropriate parameters and their ranges. From these experiments, three relevant electrophoretic factors investigated: the buffer pH (x_1), the buffer concentration (x_2) and the SDS concentration (x_3). The values of experimental factors are summarized in Table 1. The goal was to locate optimum electrophoretic conditions allowing the separation of the ISO and PYR as well as short analysis time.

A full factorial design (11 experiments) containing the three selected factors was chosen as a 2^3 full factorial design with three trials at the center. The selected experiments were performed randomly as shown in Table 2.

Multiple regression enables the mathematical relationship between the responses and the independent variables. The full factorial design provided sufficient data for the fitting of a quadratic model given below:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

Table 2
Full factorial design of three factors with selected responses

Trial	Random order	Experimental factors			Measured responses				
		x_1	x_2	x_3	Rs	Time (min)		Peak symmetry	
						ISO	PYR	ISO	PYR
1	1	−1	−1	−1	11.3	3.1	3.52	0.95	0.57
2	5	1	−1	−1	40.66	4.07	5.45	1.02	1.34
3	6	−1	1	−1	10.08	3.48	3.79	0.8	0.42
4	8	1	1	−1	45.31	4.09	5.7	0.98	2.09
5	9	−1	−1	1	7.68	4.08	4.49	1.06	0.41
6	7	1	−1	1	39.46	4.53	6.81	0.97	2.14
7	10	−1	1	1	7.99	4.26	4.64	0.94	0.39
8	3	1	1	1	39.66	4.45	6.17	1.03	1.6
9	2	0	0	0	24.79	4.03	5.22	1.02	0.94
10	11	0	0	0	24.31	4.23	5.22	0.95	0.98
11	4	0	0	0	25.16	4.3	5.45	0.98	1.04

Table 3
Estimated regression coefficients for selected responses

Coefficients	Rs	Time (min)		Peak symmetry	
		ISO	PYR	ISO	PYR
B0	24.796	4.187	5.296	0.983	0.985
B1	14.121	0.247	0.862	0.028	0.610
B2	−0.256	0.039	0.064	−0.028	0.014
B3	−0.904	0.297	0.380	0.028	0.001
B12	1.029	−0.062	−0.070	0.029	0.013
B13	0.765	−0.075	−0.064	−0.025	0.034
B23	−1.287	−0.034	−0.104	0.013	−0.068
B11	0.121	−0.048	−0.060	−0.004	0.036
B22	0.121	−0.048	−0.060	−0.004	0.036
B33	0.121	−0.048	−0.060	−0.004	0.036

where y represents the experimental response, x the independently evaluated factors (in coded variables), b_0 the intercept and b_n the parametric coefficients of the model obtained by multiple regression. Table 3 shows the regression parameters calculated for each response.

The effects after normalization of each factor were listed in Table 3. From this table, the importance of the parameters could be assessed. Buffer concentration showed the least impact on the three responses. But a positive effect of a high buffer concentration was that it promotes stacking due to differences in conductivity between the sample and the BGE, which allowed good peak symmetry. This variable was therefore set to the high level to obtain good peak symmetry. The pH of BGE and SDS concentration had a significant effect on the three responses. To determine optimum value of pH and SDS concentration response surface graphics were plotted.

It was possible to visualize the surface responses as a three-dimensional plot of two factors, while keeping the other constant at its optimal value (Fig. 2). For the sake of simplicity, other surface responses were not reported. These response surfaces allowed the determination of an optimal zone, where a good quality of the separation could be established for further validation.

Response surfaces were drawn for the two response functions as shown in Fig. 2. Because buffer concentration had ranked the least significant among the three factors, their concentration was fixed at an optimum value 50 mM and the response surfaces were traced through variation of pH and SDS concentration. Optimum conditions were chosen by peak symmetry as near 1.00 while keeping migration time as low as possible. At pH 7.8 and 25 mM SDS concentration was achieve good peak symmetry (0.98 for ISO and 0.97 for PYR) with lowest migration time (3.89 for ISO and 5.08 for PYR). Fig. 3 shows the electropherogram obtained with the optimized conditions mentioned above, combined with other appropriate conditions including 50 mM borate buffer, 30 kV, hydrodynamic injection (50 mbar, 4 s) and 35 °C. ISO and PYR were separated within 6 min with good peak symmetry.

A comparison was made between predicted and observed responses (Table 4). The residual error value of each experiment was contained within a range of ±2 S.D., where S.D. is

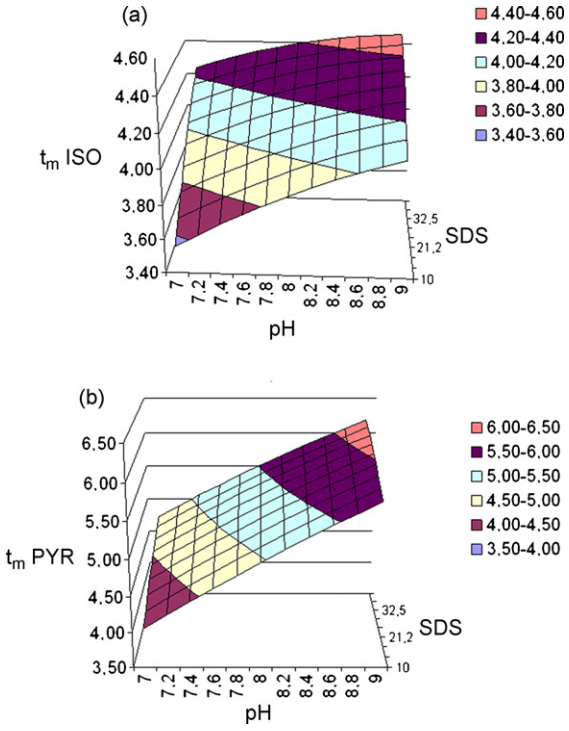

Fig. 2. Surface response plot for migration time of (a) ISO and (b) PYR as a function of pH and SDS concentration. Other factors were set at their optimum value.

the experimental standard deviation obtained through the experiments at the center ($n = 3$), and it can be concluded that each response was sufficiently explained by the regression models. Moreover, the good prediction quality of the model was experimentally verified by means of the good agreement observed between the experimental and the predicted response using the optimized conditions (Table 4).

3.2. Validation

To obtain reproducible results in capillary electrophoresis, in order to compensate injection errors and minor fluctuations of the migration time it is necessary to use internal standard [18,19]. Meloxicam was selected as internal standard (IS), because of its suitable migration time. The validation assays of ISO and PYR were assessed according to stability, linearity, precision, accuracy, selectivity and robustness [20].

Table 4
Comparison of predicted and measured results ($n = 3$) under optimal conditions

	Measured responses				
	Rs	Time (min)		Peak symmetry	
		ISO	PYR	ISO	PYR
Predicted	21.64	4.14	5.14	0.94	0.91
Measured	22.20	3.92	5.08	0.96	0.97
S.D.[a]	0.43	0.14	0.13	0.04	0.05

[a] S.D. represents the standard deviations obtained by performing three times the point at the center.

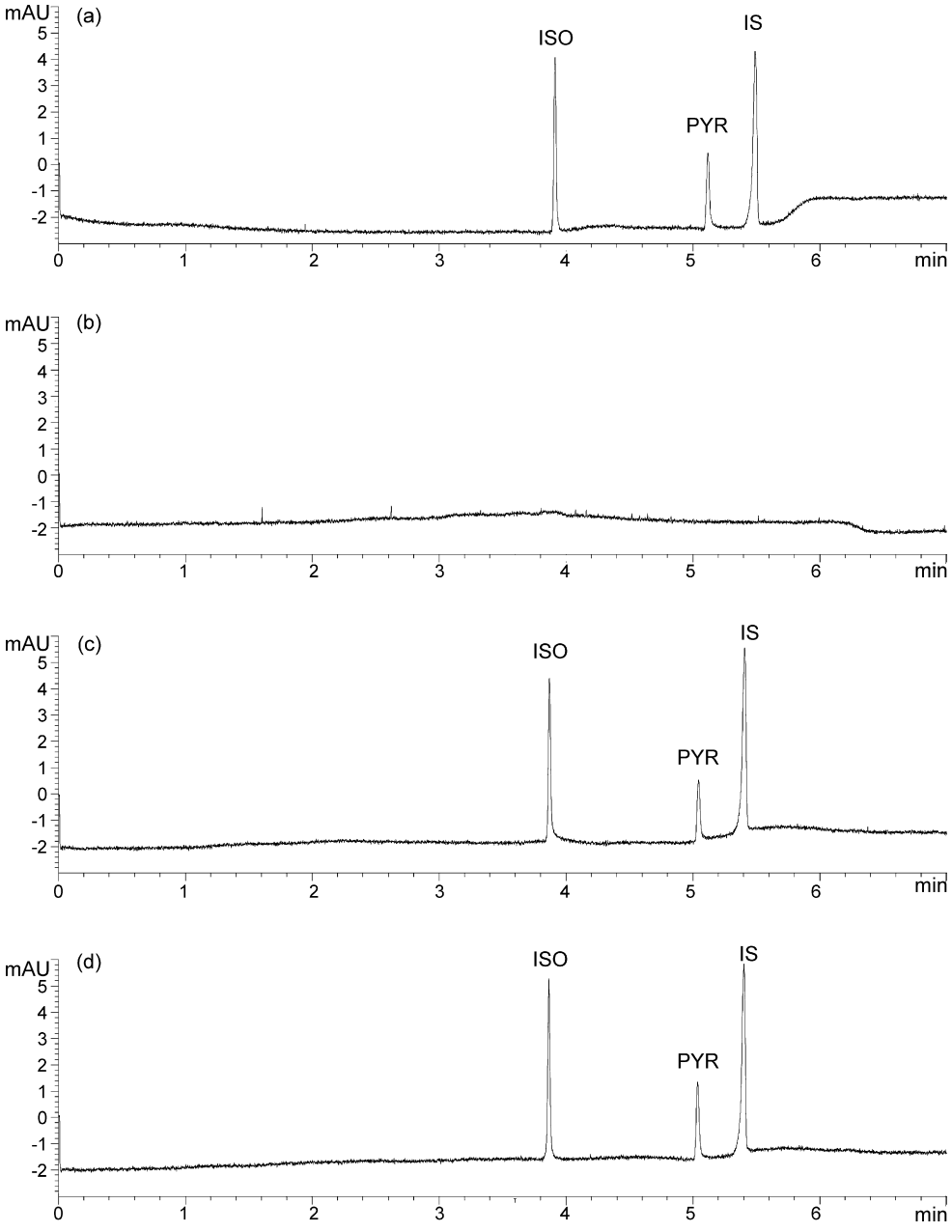

Fig. 3. The electropherograms of (a) 30 μg mL^{-1} ISO and 7.5 μg mL^{-1} PYR standard solution, (b) placebo solutions, (c) synthetic solution including 30 μg mL^{-1} ISO and 7.5 μg mL^{-1} PYR and (d) tablet solution including 30 μg mL^{-1} ISO and 7.5 μg mL^{-1} PYR, in optimum conditions (IS: 25 μg mL^{-1}).

3.2.1. Stability

The standard stock solutions of ISO and PYR, which were protected from daylight, were stored in two different conditions, as 4 °C and room temperature for 3 weeks. During this period, the solutions were analyzed and the peak area ratios were compared with the peak area ratios of daily prepared standard solution and not differences were obtained between the stored and freshly prepared samples (Table 5) ($p > 0.05$). It was determined that ISO and PYR were stable in the mentioned conditions at least 3 weeks.

Auto Sampler stability of the ISO and PYR was performed at 25 ppm. During 48 h, the solution was analyzed at 0, 1, 2, 4, 8, 12, 24, 36 and 48 h and the peak area ratios were compared. Following storage under these conditions it was determined that all test results fell within the calculated confidence interval (95%) (Table 5). It was concluded that PYR and ISO were stable at least 48 h in BGE.

3.2.2. Linearity

The calibration plots were constructed after analysis of eight different concentrations (3, 5, 10, 20, 25, 50, 75 and 100 μg mL^{-1} for ISO and 1, 5, 7.5, 10, 25, 50, 75 and 100 μg mL^{-1} for PYR) and each concentration was measured three times using the ratio of peak area method. This method was easy to use and the Relative standard deviation (R.S.D.) percent values (2.45% for ISO and 2.38% for PYR) were better than peak area (2.94% for ISO and 3.82% for PYR) and peak normalization (2.83% for ISO and 3.97% for PYR).

Table 5
Stability data of ISO and PYR

Standard stock solution stability

Days	ISO[a]			PYR[a]		
	4 °C	Ambient temperature	Daily prepared	4 °C	Ambient temperature	Daily prepared
1	1.116	1.122	1,132	1.508	1.524	1,545
2	1.122	1.100	1,134	1.501	1.572	1,544
5	1.132	1.115	1,116	1.525	1.531	1,545
7	1.133	1.126	1,142	1.538	1.533	1,551
14	1.187	1.125	1,129	1.595	1.598	1,563
21	1.116	1.130	1,127	1.556	1.538	1,574
	$p = 0.726$[b]	$p = 0.115$[c]		$p = 0.137$[b]	$p = 0.357$[c]	

Auto sampler stability

Hours	ISO[a]	PYR[a]
	Ambient temperature	Ambient temperature
0	1.097	1.560
1	1.097	1.574
2	1.100	1.555
4	1.119	1.574
8	1.111	1.538
12	1.121	1.542
24	1.113	1.579
36	1.105	1.568
48	1.121	1.540
Confidence limit	1.091–1.128	1.529–1.589

[a]The concentration of ISO and PYR are 75 and 50 μg mL^{-1}, respectively.
[b,c] *p*-values obtained with ANOVA: two-factor without replication [b] is the comparison of 4 °C and daily prepared and [c] is the comparison of ambient temperature and daily prepared.

The MEKC method developed was linear at least in the range of 3.0–100.0 μg mL^{-1} for ISO and 1.0–100.0 μg mL^{-1} for PYR. The regression equations of ISO and PYR were $y = 0.0145(\pm 0.0010)\ x + 0.0240(\pm 0.0090)$ and $y = 0.030\ (\pm 0.0010)x + 0.050(\pm 0.0021)$, respectively. Where y is the ratio of peak areas, x is the concentrations of ISO or PYR. The correlation coefficients were 0.9995 for ISO and 0.9998 for PYR.

3.2.3. Sensitivity (limit of quantitation and detection)

Limit of quantification (LOQ) is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision [20]. The precisions for ISO and PYR were established by analysing six different standard solutions containing the lowest concentration on the calibration graph (3.00 μg mL^{-1} for ISO and 1.00 μg mL^{-1} for PYR). The relative standard deviations of the response for ISO and PYR were 9.7 and 11.6%, respectively which are well below the limit of the 20% as defines in the ICH guidelines for method validation [20].

Limit of detection (LOD) is the lowest concentration that can be distinguished from the noise level, the concentration of PYR and ISO at a signal-to-noise ratio of 3:1. These were obtained with 1.0 μg mL^{-1} for ISO and 0.40 μg mL^{-1} for PYR.

Table 6
Precision and accuracy of the developed MEKC method for the analysis of ISO and PYR ($n = 6$)

	Added (μg mL^{-1})	Intra day			Inter day		
		Found $\bar{x} \pm$ S.E.	Accuracy bias (%)	Precision R.S.D. (%)	Found $\bar{x} \pm$ S.E.	Accuracy bias (%)	Precision R.S.D. (%)
ISO	5	5.11 ± 0.02	2.20	0.85	5.09 ± 0.04	1.80	2.17
	30	29.81 ± 0.08	−0.63	0.64	30.01 ± 0.30	0.03	2.45
	75	74.61 ± 0.17	−0.52	0.54	74.99 ± 0.20	−0.01	0.65
PYR	3	3.04 ± 0.03	1.33	2.27	3.04 ± 0.03	1.33	2.69
	7.5	7.54 ± 0.05	0.53	1.53	7.50 ± 0.03	0.00	1.03
	50	49.53 ± 0.26	−0.94	1.31	50.12 ± 0.25	0.24	1.24

$\bar{x} \pm$ S.E.: mean (μg mL^{-1}) ± standard error.
Bias (%): [100 × (found − added)/added].

3.2.4. Precision

3.2.4.1. Repeatability of injection and system. The repeatability of the injection and system (while keeping the operating conditions identical) was examined by injecting 25 μg mL^{-1} of ISO, PYR and IS with eight replicate injections of same solution and eight independent series injections, respectively. The electropherograms were evaluated considering migration time, peak area, peak normalization, ratio of peak normalization and ratio of peak area values. The R.S.D. values were between 1.13 and 4.74 for ISO and 1.10 and 3.82 for PYR. The lower R.S.D. values showed that repeatability of the injection and system was good.

3.2.4.2. Intermediate precision. Three different concentrations of ISO and PYR in the linear range were analyzed in six independent series on the same day (intra-day precision) and 6 consecutive days (inter-day precision) within each series every sample was injected three times. The data evaluated by calibration curve are summarized in Table 6. The R.S.D. values varied from 0.54 to 2.17% for ISO and from 1.03 to 2.69% for PYR. The low R.S.D. values of intra-day and inter-day and also the low R.S.D. values of obtained from the analyses of pharmaceutical formulations (1.92% for ISO and 0.59% for PYR) indicated that the intermediate precision of the methods was good.

3.2.5. Accuracy

Accuracy was investigated by analyzing three concentrations of PYR and ISO in the linear range at in six independent replicates on the same day (intra day) and 6 consecutive days (inter day). Accuracy was expressed as bias (%). The results obtained for intra- and inter-day accuracy were shown in Table 6.

Recovery of the method was investigated by preparing synthetic tablet solutions as mentioned previously. The method developed for tablet preparations was applied to this synthetic tablet mixture. Each study ($n = 6$) was performed triplicate. The mean recoveries of ISO and PYR (100 × found/added) was obtained as 100.78 (±1.87) and 99.46 (±1.22)%, respectively.

The low bias values and high recovery indicated that the method has a high degree of accuracy.

3.2.6. Specificity and selectivity

The electropherograms, obtained from pharmaceutical formulations and placebo (excipients) solution, (Fig. 3) were identical with that obtained from standard solution containing an equivalent concentration of ISO (30 μg mL^{-1}) and PYR (7.5 μg mL^{-1}) (Fig. 3a). There was no peak when the analyses of synthetic solution without ISO and PYR (Fig. 3b). In addition, the standard addition technique was applied to the same preparations which were analyzed by calibration curve of methods. The regression equations of standard addition curves of ISO and PYR were found to be $y = 0.0144\ (\pm 0.0003)x + 0.4411(\pm 0.0053)$ ($r^2 = 0.9993$) for ISO and $y = 0.0306\ (\pm 0.0070)x + 0.2282(\pm 0.0049)$ ($r^2 = 0.9991$) for PYR. There was no difference between slopes of calibration curve and standard addition techniques. These results showed that there was no interference from matrix components. Therefore, it could be said that method was highly selective.

3.2.7. Robustness

In robustness testing of an analytical method, the aim was to explore how sensitive the responses were to small changes in the factor settings. Ideally, a robustness test should show that the responses are not sensitive to small fluctuations in the factors, that is, the results are the same for all experiments. In our study, the surface response plots showed small changes around optimum conditions. These proved to be quite stable towards the variations of the experimental conditions. When a parameter was deliberately changed 1% from its optimum condition, the shifting of migration times of ISO and PYR were not more than 0.29% from their migration time at optimum condition. These results showed that the robustness of the developed method was good.

3.3. Analysis of tablets

Tablets containing 100 mg ISO and 25 mg PYR were analyzed through the procedure as given in Section 2.4. Quantitative analyses of ISO and PYR in the tablets were performed using the developed method. Tablets were analyzed in six independent series and sample from each series were measured there times. The results obtained for ISO and PYR from calibration method were compared with the data obtained from standard addition method. The statistical comparison of two methods was done by Wilcoxon paired test ($p_{calculated} > p = 0.050$). The results showed that there was no significant difference between calibration and standard addition methods (Table 7).

Table 7
The results of tablets analyzed by calibration and standard addition methods (Theoretical amount: 100 mg ISO and 25 mg PYR) ($n = 6$)

	ISO		PYR	
	Calibration	Standard addition	Calibration	Standard addition
$\bar{x} \pm$ S.E.	99.65 ± 0.78	100.45 ± 0.68	24.93 ± 0.06	25.35 ± 0.13
S.D.	1.91	1.66	0.15	0.32
R.S.D. (%)	1.92	1.65	0.59	1.25
	$p_c = 0.345 > p = 0.05$		$p_c = 0.116 > p = 0.05$	

$\bar{x} \pm$ S.E.: mean (mg) ± standard error.
S.D.: standard deviation, R.S.D.: relative standard deviation.
p_c: calculated p-value.

4. Conclusion

In this study, a simple and rapid MEKC method was developed and optimized using two level full factorial design with three trials at the center. Three experimental factors were investigated: the pH of the buffer, buffer concentration and the SDS concentration. The migration times, resolution and peak symmetries were evaluated. Response surface graphs were drawn to determine optimum condition. Finally, optimized conditions were selected and the method was validated. The validation assays have concluded that the developed method is linear, sensitive, accurate, precise and robust for the determination of ISO and PYR in pharmaceutical formulations. When comparing the method with reported HPLC methods it is clear that this MEKC method has advantages in that the analysis times are shorted and there is lower reagent consumption. Therefore, the proposed method provides an alternative procedure for the quality control of ISO and PYR in pharmaceutical formulations.

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