Optimization of a Stability-Indicating HPLC Method for the Simultaneous Determination of Rifampicin, Isoniazid, and Pyrazinamide in a Fixed-Dose Combination using Artificial Neural Networks

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Abstract

The aim of this study is to develop and optimize a simple and reliable high-performance liquid chromatography (HPLC) method for the simultaneous determination of rifampicin (RIF), isoniazid (INH), and pyrazinamide (PZA) in a fixed-dose combination. The method is developed and optimized using an artificial neural network (ANN) for data modeling. Retention times under different experimental conditions (solvent, buffer type, and pH) and using four different column types (referred to as the input and testing data) are used to train, validate, and test the ANN model. The developed model is then used to maximize HPLC performance by optimizing separation. The sensitivity of the separation (retention time) to the changes in column type, concentration, and type of solvent and buffer in the mobile phase are investigated. Acetonitrile (ACN) as a solvent and tetrabutylammonium hydroxide (tBAH), used to adjust pH, have the greatest influence on the chromatographic separation of PZA and INH and are used for the final optimization. The best separation and reasonably short retention times are produced on the µ-bondapak C18, 4.6 × 250-mm column, 10 µm/125 Å using ACN–tBAH (42.5:57.5, v/v) (0.0002M) as the mobile phase, and optimized at a final pH of 3.10.

Introduction

Tuberculosis (TB) is a disease that may have first been identified over 15,000 years ago. Even a century after the discovery of its cause, *Mycobacterium tuberculosis*, and decades after the discovery of powerful antituberculosis drugs, TB is still the leading cause of death from an infectious disease in developing countries. Its incidence is increasing particularly among children, the elderly, and among HIV-infected patients. Approximately one-third of the world's population harbors *Mycobacterium tuberculosis* and is at risk for developing the disease (1). In 1993, the World Health Organization (WHO) declared TB to be a global emergency, an epidemic that claims the lives of 2 to 3 million people annually, of whom at least 100,000 are children (2). To control the spread of this global epidemic, fixed-dose combinations (FDCs), containing essential antitubercular agents are now widely recommended by the WHO (3), the International Union against Tuberculosis and Lung Disease (IUATLD), and their partners for better patient concordance and lessening the development of drug resistance. A combination of 150 mg/15 mL of rifampicin (RIF), 100 mg/15 mL of isoniazid (INH), and 500 mg/15 mL of pyrazinamide is recommended [Directly Observed Treatment Short-course (DOTS) program] for the treatment of children with progressive primary or cavitating TB or nonpulmonary TB (4). However, in FDCs the bioavailability of RIF is at risk if poor quality raw materials are used or strict manufacturing procedures are not followed (5). Also, the antituberculosis activity of RIF is dose-dependent (6). Within this context, the WHO and the IUATLD, in their joint statement in 1994, advised that only FDCs of good quality and proven bioavailability of RIF should be used (7).

Most of the developed analytical high-performance liquid chromatography (HPLC) methods that are used to analyze these drugs individually or in combinations require the use of specialized columns, gradient elution, and/or complex mobile phases, resulting in them being cost-ineffective for routine analysis (8–26). Although research is still directed toward further improvements in columns, the improvement of separations may be achieved only by adjusting the composition of the mobile phase. Chromatographic systems are quite complex, and the intentional variation of a system condition can often result in the change of elution order as a function of the system condition. The aim of this study was to optimize the column and the mobile phase composition in order to maximize HPLC performance, optimize RIF, INH, and PZA separation, and to validate the method. The feasibility of a multilayer feed-forward neural network to correlate the experimental conditions with

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separation was examined. The developed model was then used to evaluate the different columns and mobile phases and to identify suitable column and experimental conditions.

Experimental

Apparatus

HPLC separations were performed on a Millipore-Waters liquid chromatograph (Milford, MA) equipped with a WISP Model 710B autosampler, a lambda max UV detector Model 481, a Model 6000A solvent delivery system, and a data module. The separation of the investigated drugs was carried out using four different columns and four different mobile phase compositions. Measurements were made with a 20-µL injection volume at ambient temperature (25 ± 2°C), with a flow rate of 1.0 mL/min and a detection wavelength of 260 nm.

The columns used were: Nova-pak C18, 3.9 × 150-mm column, 4 µm/60Å, (Waters), µ-bondapak C18, 4.6 × 250-mm column with a C18 guard column, 10 µm/125Å (Waters), Bondex C18, 3.9 × 300-mm column, 10 µm/60Å (Phenomenex, Torrance, CA), and a Nova-pak CN-HP C18, 3.9 × 150 column, 4 µm/60Å (Waters). A F32 pH meter (Beckman, Minnesota), HPLC filter system with 0.5-mm filter paper (Millipore, Ireland), and a Soniclean (Ultrasonic Engineering, RSA, London, UK) were used for the preparation of various mobile phases. The photodiode-array experiments were conducted with a Spectra Gold Photodiode-array system (Beckman, High Wycombe, UK).

Reagents

All reagents employed were of analytical grade. Methanol (MeOH), acetonitrile (ACN), both HPLC grade, and orthophosphoric acid (H_3PO_4) were purchased from BDH (Poole, Dorset, UK). Disodium orthophosphate (Na_2HPO_4) and tetrabutylammonium hydroxide (tBAH) were supplied by Merck (Darmstadt, Germany).

Procedures

Method development and validation

Mobile phases with various combinations of organic–aqueous ratios (v/v or in % ratio) were used. All of the mobile phases consisted of MeOH or ACN as organic solvents and 0.02M Na_2HPO_4 or

Table I. Retention Times Collected Under Different Experimental Conditions (Solvent, Buffer Type, and pH) and Using Four Different Column Types That Were Used to Develop the ANN Model

	Inputs					Outputs		
Data	MeOH	Na_2HPO_4	ACN	tBAH	Type	RIF	INH	PZA
Training and cross validation								
	80	20	0	0	A*	1.73	0.83	0.83
	75	25	0	0	A	2.4	0.91	0.92
	70	30	0	0	A	2.9	1.03	1.04
	65	35	0	0	A	3.32	1.06	1.08
	60	40	0	0	A	6.05	1.11	1.12
	0	40	60	0	A	2.14	0.95	1.04
	0	40	60	0	B†	6.2	2.8	3.15
	0	40	60	0	C‡	4.93	2.61	2.75
	0	40	60	0	D§	1.78	1.3	1.27
	0	50	50	0	A	3.92	1.02	1.06
	0	50	50	0	B	11.59	2.77	3.28
	0	50	50	0	C	7.44	2.53	2.78
	0	50	50	0	D	2.12	1.32	1.32
	0	60	40	0	B	13.27	2.89	3.37
	0	60	40	0	C	> 20	2.66	2.93
	0	60	40	0	D	2.87	1.34	1.35
	75	0	0	25	A	3.34	1.2	1.39
	75	0	0	25	B	8.12	3.75	3.68
	75	0	0	25	C	7.89	3.44	3.45
	75	0	0	25	D	3.46	1.29	1.26
	70	0	0	30	A	2.1	0.99	0.99
	70	0	0	30	B	10.56	3.42	3.46
	70	0	0	30	C	10.22	3.33	3.36
	70	0	0	30	D	2.88	1.17	1.18
	65	0	0	35	A	4.11	1.51	1.7
	65	0	0	35	B	14.45	3.46	3.58
	65	0	0	35	C	14.22	3.15	3.26
	65	0	0	35	D	3.45	2.1	2.78
	0	0	40	60	A	1.71	1.3	1.34
	0	0	40	60	B	13.49	3.15	3.71
	0	0	40	60	C	9.87	2.94	3.18
	0	0	40	60	D	2.51	1.6	1.65
	0	0	45	55	A	1.68	1.09	1.12
	0	0	45	55	B	8.15	3.08	3.63
	0	0	45	55	C	6.99	3.01	3.1
Testing								
	0	0	45	55	D	2.22	1.56	1.58
	0	0	50	50	A	1.6	1.05	1.1
	0	0	50	50	B	6.14	2.72	3.3
	0	0	50	50	C	5.15	2.55	2.81
	0	0	50	50	D	1.77	1.35	1.35
	0	0	60	40	A	1.24	1.05	1.06
	0	0	60	40	B	4.28	2.61	3.42
	0	0	60	40	C	4.02	2.12	2.18
	0	0	60	40	D	1.53	1.16	1.17

* A = Nova-pak C18, 3.9 × 150-mm column, 4 µm/60Å.
† B = µ-bondapak C18, 4.6 × 250-mm column a C18 guard column, 10 µm/125Å.
‡ C = Bondex C18, 3.9 × 300-mm column, 10 µm/60Å.
§ D = Nova-pak CN-HP C18, 3.9 × 150 column, 4 µm/60Å.

0.0005M tBAH as buffers. The pH was further adjusted with H_3PO_4 to the apparent pH, and the resulting solution was then filtered and degassed. Optimization of the stationary phase and composition of the mobile phase were carried out using NeuroSolution for Excel, version 4.31 (NeuroDimension, Gainiesville, FL). Retention times collected from four sets of conditions and four different types of column, referred to as the input and testing data (Table I), were used to train, cross validate, and test the artificial neural network (ANN) model. The ANN was used to develop a model that would suggest which variables to change in order to optimize separation. After the ANN was used to evaluate conditions for the optimum separation, based on the sensitivity of the inputs, those conditions were implemented on the HPLC and further optimized. The method was validated for the following parameters: precision, accuracy, linearity, reproducibility, ruggedness, and specificity.

Standard solution preparation

INH (0.05 g) was accurately weighed and dissolved in 100 mL of water to obtain a 0.5 mg/mL stock solution (the same procedure was applied to PZA), while 0.02 g of RIF was transferred into a 100-mL volumetric flask and combined with water to obtain a 0.2 mg/mL stock solution. These stock solutions were further diluted to various relevant concentrations.

ANNs

ANNs are non-linear statistical data modeling tools that can be used to model complex relationships between inputs and outputs. In most cases, an ANN is an adaptive system that changes its structure based on information that is processed through the network. They are composed of a large number of highly interconnected artificial neurons, organized in layers, that use a mathematical model to process the information by adjusting the interconnected weights. The behavior of a neural network is determined by the transfer functions of its neurons, the learning rule, and by the network architecture.

A supervised network with a back-propagation learning rule, sigmoidal transfer function, and multilayer perceptron architecture was used. Detailed descriptions of this type of the ANN model have been published (27–29).

The advantage that ANNs offered over statistical techniques is that the model did not have to be explicitly defined before the experiments began. There were no preconceived ideas about the model. While the ANNs could grasp the relevant data to develop the model and where to derive a statistical model, prior knowledge of the relationships between the factors under investigation was required. However, one of the most important criticisms of neural networks was the difficulty in understanding the nature of the internal representations generated by the network. ANNs are usually presented as "black boxes" with extremely complex work that somehow "magically" transforms inputs into predetermined outputs. Unlike classic statistical models, the importance that each input variable has on the output in a neural network was not easy to find. With the aim of overcoming this limitation, two different approaches were used: sensitivity analysis of the trained network and analysis by means of graphs to reveal the importance of experimental conditions (input variable) on the chromatographic performance (output). A sensitivity analysis was used to measure the effect of varying inputs on the output and to rank inputs in order of importance. Even though neural networks cannot produce explicit rules, sensitivity analysis enabled them to explain which inputs were more important than others. It can give important insights into the usefulness of individual variables and rate variables according to the deterioration in modeling performance that occurs if that variable is no longer available to the model. Neural networks can also assign a single rating value to each variable and visualize how the variation affects the results. Direction of the change in the output variable was important as well. Positive sensitivity indicates the same change of the output as the input change.

Results and Discussion

Method development and ANN

Currently, a wide variety of chromatographic stationary phases, providing significantly different retention and selectivity, are commercially available and principally offer the opportunity to perform any separation. However, many columns present similar characteristics, which made the selection of a proper chromatographic system difficult and problem dependent. The sharpness of a chromatographic peak was an indication of the quality of the chromatographic column and its efficacy. Small particle size and long column dimensions generally increase column efficiency, which in turn increases sensitivity, analytical resolution, and speed, though other characteristics had a greater effect on separation.

Column performance was assessed first using the five sigma

Table II. Predicted and Experimentally Measured Retention Times for the Testing Data Set and Predictive Performance Evaluation

Measured			Predicted		
RIF	INH	PZA	RIF	INH	PZA
2.22	1.56	1.58	2.94	1.29	1.24
1.6	1.05	1.1	4.04	1.18	1.27
6.14	2.72	3.3	13.53	3.60	3.66
5.15	2.55	2.81	6.61	2.97	3.12
1.77	1.35	1.35	2.70	1.27	1.23
1.24	1.05	1.06	1.39	1.03	1.08
4.28	2.61	3.42	8.08	3.51	3.60
4.02	2.12	2.18	5.38	2.88	3.07
1.53	1.16	1.17	2.45	1.23	1.22
Mean-square error			9.03	1.27	0.27
Normalized mean-squared error			3.08	2.89	0.34
Mean absolute error			2.13	0.63	0.34
Minimum absolute error			0.15	0.017	0.02
Maximum absolute error			7.39	0.89	0.88
*r**			0.91	0.97	0.96

*r = correlation coefficient between predicted and experimentally measured retention time for each drug.

efficiency method, by measuring the peak width at 4.4% of the peak height. Sigma efficiency methods measure the peak width at decreasing levels of the peak height. Thus, the three sigma method measures width at 32.4% of the peak height, the four sigma method measures at 13.4%, and the five sigma method (30) measures at 4.4%. The five sigma method was most sensitive to asymmetry because the width was measured at the lowest point. The five sigma method is expressed in the following equation as:

$$N = 25(V/w)^2 \qquad \text{Eq. 1}$$

where N is the number of theoretical plates; V is the distance to the peak (mm); and w is the peak width at 4.4% peak height (mm). All four columns showed good column efficiency because the number of theoretical plates in each column exceeded 12,500. The selection of proper starting conditions for method development was solved by means of a parallel application of the four chromatographic systems. A substantial effort was put into development and subsequent optimization of the chromatographic conditions. The retention data obtained for RIF, INH, and PZA, with MeOH or ACN as organic solvents and Na_2HPO_4 or tBAH buffered solutions on selected columns, were used to train and validate the ANN model (Table I). The predictive performance of the trained network was then tested by predicting the retention times of selected drugs using different mobile phase compositions on the same columns. Table II shows the calculated retention times for the three drugs.

Networks constructed had one hidden layer with four hidden neurons. A sensitivity analysis of the developed and optimized networks was used to evaluate the relative importance of the mobile phase composition, column type, and the effect of their changes on the HPLC separation. This study also aimed to improve the selectivity of the method and achieve separation between INH and PZA. The highest sensitivity, which was associated with column type (Table III), clearly suggests the importance of the column type for the RIF retention time. The reasonably short retention time for RIF and some level of separation between INH and PZA was achieved with the µ-bon-

Table III. Sensitivity of the Chromatographic Separation to the Changes in Column Type, Concentration, and Type of Solvent and Buffer in Mobile Phase

Sensitivity	Rifampicin	Isoniazid	Pyrazinamide
MeOH	0.075	0.001	0.001
Na_2HPO_4	0.189	0.001	0.001
ACN	0.143	0.002	0.002
tBAH	0.245	0.005	0.003
Column type	2.570	0.792	0.796

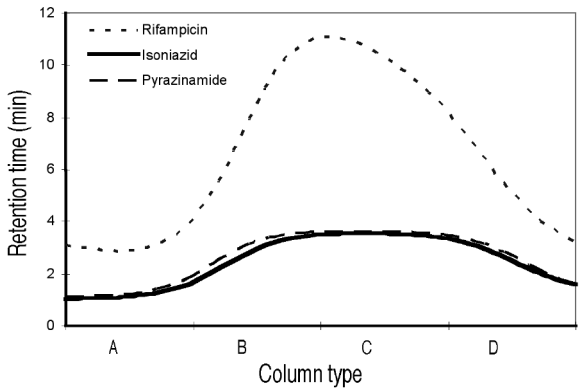

Figure 1. The effect of different column types on HPLC separation (retention times).

Table IV. Mobile Phase Optimization (ACN–tBAH with Column B)

ACN (%)	$(Bu)_4NOH$ (%)	pH	Column type*	Retention time (min) RIF	INH	PZA
50	50	3.7	B	6.14	2.82	3.3
47.5	52.5	3.7	B	7.27	3.02	3.47
45	55	3.7	B	8.15	3.08	3.63
42.5	57.5	3.7	B	9.02	3.19	3.70
40	60	3.7	B	13.49	3.15	3.71

* Column types are the same as in Table I.

Table V. Mobile Phase Optimization (pH Adjustment)

ACN (%)	tBAH (%)	pH	Column type	Retention Time (min) RIF	INH	PZA
50	50	3.7	B	6.14	2.82	3.3
42.5	57.5	3.70	B	9.02	3.19	3.7
42.5	57.5	3.50	B	9.39	3.17	3.69
42.5	57.5	3.40	B	9.85	3.11	3.63
42.5	57.5	3.20	B	10.68	2.92	3.56
42.5	57.5	3.10	B	10.97	2.90	3.54
42.5	57.5	3.00	B	11.68	2.95	3.47
42.5	57.5	2.85	B	12.02	2.89	3.45

* Column types are the same as in Table I.

Table VI. Precision Data

								Mean CV
RIF	Conc (mg/mL)	0.02	0.04	0.05	0.08	0.10	0.25	0.19
	CV(%)	0.04	0.42	0.23	0.00	0.26	0.18	
INH	Conc (mg/mL)	0.02	0.04	0.05	0.08	0.10	0.14	0.28
	CV(%)	0.26	0.15	0.43	0.26	0.17	0.38	
PZA	Conc (mg/mL)	0.01	0.02	0.05	0.06	0.10	0.12	0.23
	CV(%)	0.19	0.39	0.09	0.05	0.39	0.25	

dapak C18 column, 4.6 × 250-mm, 10 µm/125Å (Waters) (Figure 1).

Sensitivity of the separation (retention time) to the changes in MeOH and Na_2HPO_4 was the lowest at only 0.001, suggesting that MeOH and Na_2HPO_4 were not suitable because separation between INH and PZA could not be achieved. The use of MeOH and Na_2HPO_4 in various ratios resulted in close retention times for INH and PZA, with a lack of baseline resolution between the two peaks. Although a small degree of separation between INH and PZA was found, adequate baseline resolution could not be achieved. The retention (apparent hydrophobicity) of RIF increased with an increased ratio of buffer solution and decreased with the increased ratio of solvents. RIF and PZA were both soluble in MeOH and an increase in the MeOH ratio would decrease their retention. ACN, and especially tBAH, showed greater influence on the chromatographic separation of PZA and INH, and further investigation was carried out employing ACN as the solvent and tBAH as the buffer (Table IV). The use of tBAH resulted in improved separation between INH and PZA. The adjustment of pH would further optimize the separation. The same chromatographic conditions as for the mobile phase development were applied, except the pH was altered. Table V shows the effect of pH on the the selected mobile phase.

All of the mobile phase compositions listed in Table V produced good separation results, proving that the method showed acceptable ruggedness. As in most reverse-phase systems, retention and selectivity were controlled by the concentration and nature of the organic modifier, pH, and to a lesser extent, the concentration and nature of the buffer. The best separation was produced by using the mobile phase consisting of ACN–tBAH (42.5:57.5, v/v) (0.0002M), with a final pH of 3.10. However, the pH range of 3.10 to 3.40 was also acceptable. After six injections, the retention times were found to be 2.85 ± 0.01, 3.54 + 0.01, and 10.97 + 0.01 min for RIF, INH, and PZA, respectively, when using the described conditions with the pH set at 3.1.

Precision, accuracy, linearity, and reproducibility

The accuracy and precision were determined using six (including blank sample) determinations for each concentration (0.02–0.25 mg/mL). The standard solutions were injected and analyzed six times, and the coefficient of variation (CV) was calculated. In order to produce acceptable reproducibility, the mean CV value should not exceed ± 15% of the actual value

Table VII. Linearity of the HPLC Method for RIF, INH, and PZA

Drug	Gradient	y-intercept	Correlation coefficient (*r*)
RIF	144672497.25 units/mg	–535.57 units	0.9999
INH	171655612.60 units/mg	250.68 units	0.9999
PZA	224671655.34 units/mg	116.75 units	0.9998

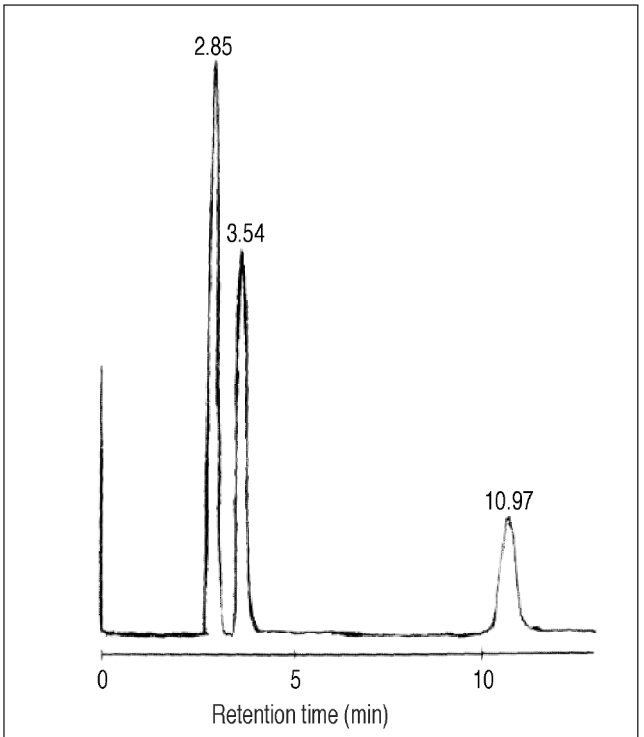

Figure 2. The HPLC chromatograms of RIF, INH, and PZA with retention times at 2.85, 3.54, and 10.97 min represent, respectively, using a mobile phase containing ACN–tBAH (42.5%:57.5%, v/v) (0.0002M), with pH of 3.10.

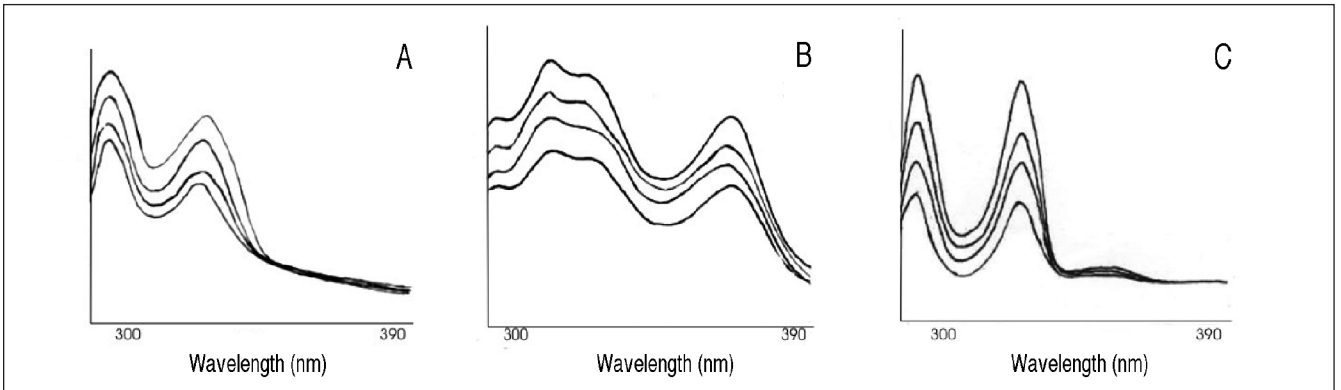

Figure 3. The photodiode-array spectra of RIF (A), INH (B), and PZA (C).

[except during the limit of quantitation (LOQ) determination study, where the mean value must be within 20% of the actual value (31)]. The data are listed in the Table VI. The linearity of the HPLC method over the investigated concentration range and the correlation coefficients were determined (Table IV). Low CV and good correlation coefficients (r) indicated that the levels of precision and accuracy for the method were satisfactory. Furthermore, good linearity with $r \geq 0.9998$ was achieved.

Ruggedness

A 48-h stability trial was carried out on the standard solutions (1.0 mg/mL) of RIF, INH, and PZA. Two sets of stock solutions were placed either at 25 ± 1°C or in the refrigerator (5 ± 1°C) and protected from any light source. The stability-time analysis indicated that RIF was unstable in the aqueous solution at room temperature. A 40% loss of the total amount of the RIF occurred after 48 h. This effect was less dramatic for both INH and PZA. The standards were deemed to be reliable only if the analysis was carried out within 8–12 h after preparation.

Specificity

The specificity of the method was investigated by conducting a photodiode-array analysis to investigate the integrity of the drug peaks and to clarify the purity of the peaks. These chromatograms were plotted as absorbance of the specific drug peak region against a range of wavelength (190 to 400 nm). The pattern of each UV spectrum of an individual drug was unique; thus, the shape of the UV spectra should maintain almost an identical faction (except differ in their intensities). Therefore, the diode-array scan of a particular elution peak in a different time region (Table VIII) should also reproduce a similar argument. The impurities within the elution peak would cause an alteration of the shape of the peak in the diode-array scan.

The HPLC chromatogram and the photodiode-array spectra are presented in Figures 2 and 3, respectively. In the HPLC chromatogram, the peaks at retention times of 2.85, 3.54, and 10.97 min represent INH, PZA, and RIF, respectively. The photodiode-array spectra were obtained by scanning at four different points of each drug peak. The photodiode array spectra confirmed the integrity of the three drug peaks.

Table VIII. Photodiode-Array Scans Time Region for Figure 3

RIF Retention time (min)	INH Retention time (min)	PZA Retention time (min)
10.91–10.93	2.81–2.82	3.52–3.53
10.93–10.96	2.82–2.84	3.55–3.57
10.97–10.99	2.83–2.86	3.50–3.56
10.99–11.02	2.85–2.87	3.51–3.52

Table IX. LOD and LOQ Estimation

Drug	IUPAC method estimation	Serial dilution method estimation
RIF	0.133 µg/mL	0.200 µg/mL
INH	0.111 µg/mL	0.150 µg/mL
PZA	0.137 µg/mL	0.150 µg/mL

Limits of detection and LOQ

Limit of detection (LOD), defined as the lowest concentration of an analyte that the analytical process can reliably differentiate from background noise level, was determined using the International Union of Pure and Applied Chemistry and the serial dilution method (32). The LOD for RIF, INH, and PZA was estimated by diluting the stock solutions of known concentration until the ratio analyte response signal became three times that of the noise. The LOQ was the lowest concentration of an analyte that could be measured with an acceptable degree of confidence. The LOQ of RIF, INH, and PZA was determined by diluting these solutions until the signal-to-noise ratio was greater than three and the accuracy and precision of the response was less than 10%. The serial dilution method was performed by diluting RIF, INH, and PZA stock solutions until the HPLC system was unable to observe the parent peak. The LOD and LOQ were calculated and are listed in Table IX.

Conclusion

The rapid and simultaneous determination of RIF, INH, and PZA is only possible if these drugs are adequately resolved in a timely manner. The complexity of chromatographic systems lies in the existence of multiple optimal experimental conditions. The intentional variation of system conditions can often cause peaks to "cross" one another and to change their elution order. Conditions for which all peaks are separated from each other represent the maximum chromatographic performance. However, many sets of experimental conditions might provide peak separation. The problem then is to predict the experimental condition with certain constraints, such as analysis time. Usually, optimum separation means that all components of the sample are separated in a reasonable time. The time of analysis is limited by the retention time of the most retentive component. Another important aspect of separation is resolution. Hence, two criteria are important: retention time, which should be as low as possible, and resolution, which should possess the greatest value while reasonable maximum peak retention time is maintained.

Optimization of the chromatographic conditions for RIF, INH, and PZA separation was achieved using the ANN as a modeling tool. The aim of this optimization was to achieve a reasonably short retention time for RIF, INH, and PZA to be adequately resolved because the structural similarity between INH and PZA and the differences between those two drugs and RIF makes that difficult to achieve.

The selectivity and optimization of separation was achieved by controlling the amount of organic modifier to adjust the retention and type of organic modifier, type of buffer, and pH

to control the separation. Once optimized, the method proved to be valid for the simultaneous determination of RIF, INH, and PZA in liquid or solid dosage form used to treat multi-drug resistant TB, especially in developing countries.

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