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Development and Validation of RP–HPLC Method for Simultaneous Determination of Pyridoxine Hydrochloride, Isoniazid, Pyrazinamide and Rifampicin in pharmaceutical Formulation

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Keywords: Reversed phase high-performance liquid chromatography; Validation; Pyridoxine hydrochloride; Isoniazid; Pyrazinamide; Rifampicin

A simple, rapid and precise liquid chromatographic method for simultaneous determination of pyridoxine hydrochloride, isoniazid, pyrazinamide and rifampicin in a tablet dosage form has been developed. Chromatographic analysis was performed on a 250 × 4.6 mm I.D. C_{18} column packed with 5 μm-in-size particles applying gradient elution with a mobile phase composed of acetonitrile (A) and 15 mmol L^{-1} potassium dihydrogen phosphate buffer of pH adjusted to 4.0 ± 0.1 with o-phosphoric acid (B). A:B ratio was 11:89 v/v for the initial 4.5 min, and then it was maintained at 50:50 v/v; the flow rate was 1 mL min^{-1}. UV detection was performed at 235 nm. The total run time was 20 min. Retention times for pyridoxine hydrochloride, isoniazid, pyrazinamide and rifampicin were 3.687, 4.113, 5.041 and 12.829 min, respectively. The method was validated with respect to linearity, accuracy, precision, specificity and sensitivity in accordance with ICH guidelines. Limits of detection were 0.043, 0.063, 0.036 and 0.059 μg mL^{-1} and limits of quantification were 0.13, 0.19, 0.11 and 0.18 μg mL^{-1} for pyridoxine hydrochloride, isoniazid, pyrazinamide and rifampicin, respectively. High recovery and low coefficients of variance confirmed the suitability of the method for the simultaneous analysis of the four considered drugs.

Opracowano prostą, szybką i precyzyjną procedurę jednoczesnego oznaczania chlorowodorku pirydoksyny, izoniazydu, pirazynamidu i rifampicyny w tabletkach za pomocą chromatografii cieczowej. Analizę chromatograficzną prowadzono przy użyciu kolumny 250 × 4,6 mm, napełnionej fazą C_{18} o wielkości cząstek wynoszącej 5 μm. Zastosowano gradient fazy ruchomej składającej się z acetonitrylu (A) i buforu w postaci fosforanu dihydropotasowego o stężeniu 15 mmol L^{-1} i pH = 4 ± 0,1 ustalonego za pomocą kwasu

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o-fosforowego (B). Stosunek A i B wynosił 11:89 v/v przez 45 min, następnie był utrzymywany stosunek 50:50 v/v. Przepływ fazy ruchomej wynosił 1 mL min^{-1}. Całkowity czas rozdzielania składników mieszaniny wynosił 20 min. Detekcję UV prowadzono przy 235 nm. Czasy retencji chlorowodorku pirydoksyny, izoniazydu, pirazynamidu i rifampicyny wynosiły odpowiednio 3,687; 4,113; 5,041 i 12,829 min. Opracowaną procedurę walidowano uwzględniając liniowość, dokładność, precyzję, specyficzność i czułość, zgodnie z zaleceniami Międzynarodowej Konferencji Harmonizacyjnej (ICH). Wykrywalności wynosiły 0,043; 0,063; 0,036 i 0,059 μg mL^{-1} a oznaczalności 0,13; 0,19; 0,11 i 0,18 μg mL^{-1} odpowiednio dla chlorowodorku pirydoksyny, izoniazydu, pirazynamidu i rifampicyny. Wysoki odzysk i małe współczynniki wariancji potwierdziły przydatność metody do jednoczesnej analizy czterech leków.

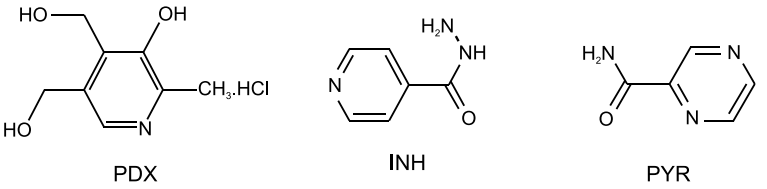
Chemical name of pyridoxine hydrochloride (PDX) is 5-hydroxy-6-methyl-3,4--pyridinedimethanol hydrochloride. It is a water soluble vitamin administrated at a dose of 10–50 mg/day to the patients accepting isoniazid in order to prevent peripheral neuropathy and CNS effects that are associated with the therapy with isoniazid. Isoniazid (INH), isonicotinic acid hydrazide, is the first-line anti-tubercular drug. INH inhibits the synthesis of mycolic acid in the mycobacterial cell wall and is used for the treatment of tuberculosis. Pyrazinamide (PY; pyrazine-2-carboxamide) is used to treat tuberculosis as well; it causes disruption of *Mycobacterium tuberculosis* membrane transport and energetics [1, 2].

Rifampicin ((3-[4-methyl-1-piperazinylimino methyl]-rifamycin – RIF) is a bactericidal semi-synthetic antibiotic derived from *Amycolatopsis rifamycinica*. RIF inhibits DNA-dependent RNA polymerase in bacterial cells by binding to its beta-subunit. It is typically used to treat *Mycobacterium*-related infections.

A combination of the above drugs: PDX(2.5 mg), INH(75 mg), PYR(250 mg) and RIF(100 mg) is commercially available and used in the treatment of childhood tuberculosis. The structures of these four drugs are shown in Figure 1.

PDX, INH, PYR and RIF are officially approved drugs in the Indian, British and US Pharmacopoeias [3–5]. A literature review has revealed that several methods for individual determination of the above compounds are available; they include spectrophotometry [6–14] and chromatography [15–18]. INH and RIF have been determined simultaneously by high-performance liquid chromatography [19], classical least squares analysis of the absorption spectra [20], spectrophotometry [21, 22], and zero- and first-derivative UV spectrophotometry [23]. PDX and INH have been determined simultaneously by spectrophotometry [24]. PDX in combination with other drugs has been analyzed by RP–HPLC [25] and spectrophotometry [26–28]. INH, PYR and RIF have been determined by high-performance thin-layer chromatography [29], high-performance liquid chromatography [30, 31], multivariate spectrophotometric calibration [32], partial least squares method and modified hybrid linear analysis calibration [33]. However, many of these methods suffer from limitations such as

lengthy and tedious procedures, very complex mobile phase composition, large quantities of solvents used, lack of full validation. So far, no method for the simultaneous determination of PDX, INH, PYR and RIF in a combined dosage form has been reported. Therefore, the authors felt a need to develop a simple, economical, fast, precise, and accurate method for the simultaneous analysis of these drugs in a tablet dosage form Rifacept Kid-3.



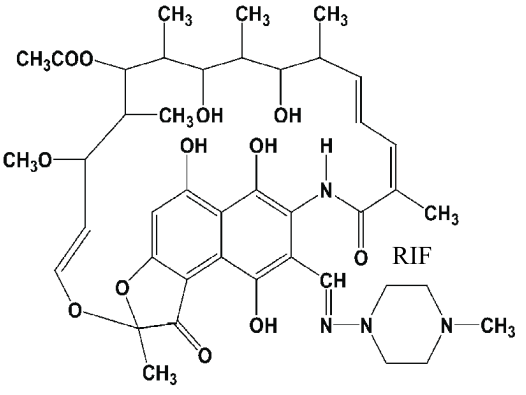


Figure 1. Chemical structures of PDX, INH, PYR and RIF

EXPERIMENTAL

Material and reagents

INH, PYR and RIF were obtained as gift samples from Lupin Limited, SIDCO Industrial Complex, Jammu, India. PDX was obtained as a gift sample from Plethico Pharmaceuticals, Indore, M.P., India. A tablet dosage form, Rifacept Kid–3, manufactured by Concept Pharma Ltd., A–28/3, MIDC, Chikalthana, Aurangabad–431210, (Label claim: 2.5 mg PDX, 75 mg INH, 250 mg PYR and 100 mg RIF) was purchased in the local pharmacy. All chemicals and reagents used were of HPLC grade and were purchased from Spectrochem, Mumbai, India.

Apparatus

HPLC analysis was performed using a Shimadzu LC–10 AT VP solvent delivery system, a Shimadzu SPD–10 AVP UV–VIS photodiode-array detector, and a Rheodyne 7725i universal loop injector of injection capacity 20 μL. The equipment was purchased from Shimadzu (Asia Pasific) PTE Ltd., 16, Science Park Drive #01-01, The Pasteur, Singapore Science Park, Singapore-118227. The analyzed compounds were separated on a 250 × 4.6 mm I.D., 5 μm particle, Phenomenex Luna C_{18} column (Spinco Biotech Pvt. Ltd., P. Box No. 6114, No.4, Vidyaram Street, T. Nagar, Chennai–600 017, India) under the reversed-phase partition chromatography conditions. The measurements were carried out in an air-conditioned room at temperature 25 ± 2°C. The analytes were monitored at 235 nm. The run time was 20 min.

Mobile phase

The selected mobile phase consisted of acetonitrile (A) and 15 mmol L^{-1} potassium dihydrogen phosphate buffer of pH adjusted to 4.0 ± 0.1 with orthophosphoric acid (B). The mobile phase was applied in a gradient elution mode; A:B ratio was 11:89 v/v for the initial 4.5 min and then was maintained at 50:50 v/v. The flow rate was 1 mL min^{-1}. Before the analysis, both mobile phase and sample solutions were degassed by sonication and filtered through a 0.2 μm filter paper.

Preparation of solutions

Standard stock solution and construction of calibration plots. PDX, INH, PYR and RIF (10 mg each) were weighed accurately and separately transferred to 100 mL volumetric flasks. All the drugs were dissolved in HPLC-grade water to prepare 100 μg mL^{-1} standard stock solutions. The obtained standard stock solutions were appropriately diluted to prepare working solutions of different concentrations of the drugs. They were injected into the chromatographic system and for each dilution the areas under the recorded peaks were measured. Calibration curve for each drug was constructed by plotting the peak area *vs* the corresponding concentration. The unknown contents of the drugs in the samples were calculated using the calibration plots as the reference.

Mixed standard solution. The analysis of mixed standard solutions was performed in order to validate the procedure. From 100 μg mL^{-1} standard stock solutions of the drugs different mixed standard solutions of the known concentrations were prepared and analyzed. The results are reported in Table 1.

Table 1. Results of the analysis of mixed standards; S.D. – Standard deviation; CV – Coefficient of riance.

S. No.	Amount present, μg mL^{-1}				Amount found, %			
	PDX	INH	PYR	RIF	PDX	INH	PYR	RIF
1	0.5	35	65	5	100.6	100.3	98.75	98.60
2	1	30	55	10	101.3	99.56	100.2	99.10
3	2.5	25	45	15	99.60	100.3	98.77	100.7

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Table 1. (Continuation)

S. No.	Amount present, μg mL^{-1}				Amount found, %			
	PDX	INH	PYR	RIF	PDX	INH	PYR	RIF
4	5	20	35	20	101.4	99.70	99.45	100.4
5	10	15	25	25	99.80	101.5	101.2	99.32
6	15	10	15	30	100.8	101.2	99.40	99.03
			Mean		100.6	100.4	99.62	99.53
			S.D.		0.753	0.787	0.936	0.852
			%CV		0.748	0.783	0.939	0.856

Sample solutions. For the analysis of a tablet dosage form, 20 Rifacept Kid–3 tablets were weighed individually and their average mass was determined. Then, the tablets were crushed to a fine powder. The powder amount equivalent to the mass of 0.625 mg of PDX (corresponding masses of INH, PYR and RIF were 18.75 mg, 62.5 mg and 25 mg, respectively) was transferred to a 100 mL volumetric flask and dissolved in 50 mL of HPLC-grade water. The solution was shaken vigorously for 25 min and filtered through a Whatman filter paper (#41) into another 100 mL volumetric flask; the residue was washed with a solvent. The solution was diluted up to 100 mL with the same solvent.

Before the assay of tablet formulations, 6 replicate aliquots (each 20 μL in volume) of the appropriately diluted tablet stock solution were sonicated for 15 min, then injected into the chromatographic system, and analyzed quantitatively. Chemical identity of all four target compounds was established by comparing the retention time of the components sample solution with those of the mixed standard solution. The contents of PDX, INH, PYR and RIF per tablet were calculated from the calibration curve by extrapolating the peak area (Tab. 2).

Table 2. Results of the assay of tablet formulation; S.D. – standard deviation; CV – coefficient of variance; S.E. – standard error; n – number of replicates

Drug	Label claim, mg/tablet (n = 6)	Amount found, %	S.D.	%CV	S.E.
PDX	2.5	100.36	0.213	0.212	0.087
INH	75	100.52	0.053	0.052	0.022
PYR	250	99.68	0.523	0.525	0.214
RIF	100	99.13	0.647	0.652	0.264

RESULTS AND DISCUSSION

HPLC method development and optimization

Column chemistry, solvent type and strength (volume fraction of an organic solvent(s) in the mobile phase and pH of the buffer solution), detection wavelength and flow rate were varied to determine the chromatographic conditions assuring the best separation. Mobile phase conditions were optimized, so that the tablet components were free from the interference from the solvent and excipients. Other parameters, *e.g.* time required for the analysis, appropriate k range for eluted peaks, assay sensitivity, solvent-related noise, and a use of the same solvent system for extraction of a drug from a drug matrix were also considered.

C_8 and C_{18} columns containing different stationary phases were tested. Satisfactory resolution and run time were achieved on a 250 × 4.6 mm I.D., 5 μm particle Phenomenex Luna C_{18} reversed–phase column. This column was thus selected for further studies.

A series of aqueous mobile phases containing phosphate buffer solutions of different pH in combination with different volume fractions of acetonitrile as a modifier were tested. The best results were obtained applying gradient elution with a mobile phase composed of acetonitrile (A) and 15 mmol L^{-1} potassium dihydrogen phosphate buffer of pH adjusted to 4.0 ± 0.1 with orthophosphoric acid (B). The gradient profile was A:B = 11:89 v/v for the initial 4.5 min; afterwards it was maintained at 50:50 v/v; the flow rate was 1 mL min^{-1}. This optimum flow rate was determined by testing the effect of different flow rates on the peak area and resolution. All experiments were performed at the ambient temperature.

To determine the appropriate wavelength for the simultaneous determination of PDX, INH, PYR and RIF, solutions of these compounds in HPLC-grade water were scanned in the range 200–400 nm using a UV–VIS spectrophotometer (Shimadzu 1700). From the overlain UV spectra (Fig. 2) the suitable wavelength of 235 nm was selected. The solutions of each drug in HPLC-grade water were injected directly for HPLC analysis and the responses (peak areas) were recorded at 235 nm. It was observed that there was no interference from the mobile phase or baseline disturbance, and all the analytes absorbed well at 235 nm. It was therefore concluded that 235 nm was the most appropriate wavelength for the analysis of the drugs with the appropriate sensitivity.

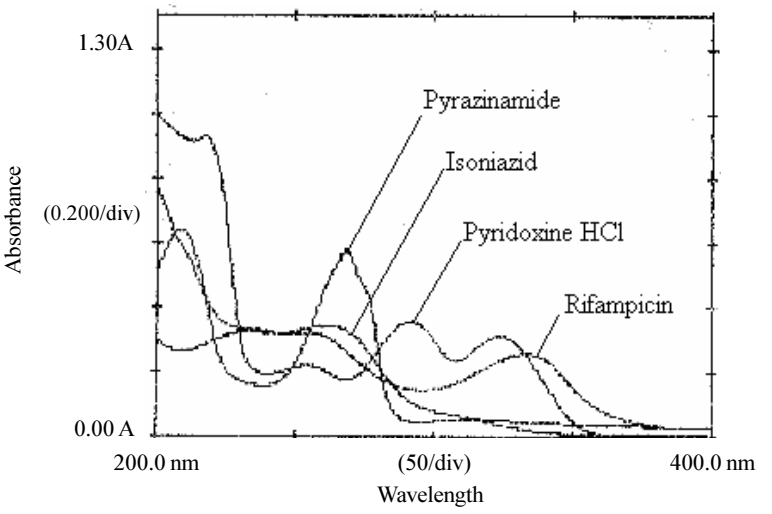

Figure 2. Overlain spectra of PDX, INH, PYR and RIF at 15 μg mL^{-1} concentration

Under the optimum chromatographic conditions, the retention times obtained for PDX, INH, PYR and RIF were 3.687, 4.113, 5.041 and 12.829 min, respectively. Resolution (R_S) between PDX and INH, INH and PYR, and PYR and RIF was 2.15, 3.83 and 5.94, respectively. Retention factors, tailing factors and a number of theoretical plates are given in Table 3. The values of k and R_S ($1 < k < 10$, $R_S > 2$) confirmed that the applied chromatographic conditions were appropriate for the separation and quantification of all the analytes. The number of plates (N) is a measure of column efficiency; the calculated N values indicated high separation efficiency of the column used.

Table 3. System suitability parameters; R_t – retention time; T_f – tailing factor; k – retention factor; N – number of theoretical plates; R_S – resolution

Property	PDX	INH	PYR	RIF
R_t	3.687	4.113	5.041	12.829
T_f	1.07	1.01	1.05	1.13
k’	1.16	1.83	2.87	4.42
N	5018	7271	8671	10271
R_s	–	2.15	3.83	5.94

Validation of the method

The method was validated with respect to linearity, accuracy, precision, repeatability, selectivity and specificity, according to the ICH guidelines. Validation studies were carried out by replicate injections of the sample and standard solutions into the chromatograph.

Linearity

Linearity was determined separately for PDX, INH, PYR and RIF by plotting the peak area against the corresponding concentration. The obtained calibration plots were linear over the following concentration ranges: 0.3–20 μg mL^{-1} for PDX, 2–50 μg mL^{-1} for INH, 1–150 μg mL^{-1} for PYR and 3–65 μg mL^{-1} for RIF. The linear regression equations describing the obtained calibration plots for PDX, INH, PYR and RIF are as follows:

PDX: $y = 48000x - 13536$ ($n = 5$, $r^2 = 0.9995$)
INH: $y = 40316x - 59434$ ($n = 5$, $r^2 = 0.9992$)
PYR: $y = 68111x + 76784$ ($n = 5$, $r^2 = 0.9991$)
RIF: $y = 68866x - 34500$ ($n = 5$, $r^2 = 0.999$)

where y is the response (peak area) and x is the concentration.

Accuracy

The accuracy of the developed method was confirmed by replicate analysis ($n = 6$). For this purpose, a recovery study at three different concentration levels: 80, 100 and 120% was performed in accordance with ICH guidelines. Standard drug solutions were added to the pre-analyzed sample solution and the percentage drugs contents were calculated. The results of the accuracy study are reported in Table 4. All the results were within the acceptable limits, *i.e.* CV < 2.0% and S.D. < 1.0. Therefore it was clear that the method enabled very accurate quantitative determination of PDX, INH, PYR and RIF in the tablet dosage form.

Table 4. Results of accuracy study; S.D. – standard deviation; CV – coefficient of variance

Drug	Amount taken, μg mL^{-1}	Amount added		Recovery, % ± S.D.	%CV
		%	μg mL^{-1}		
PDX	0.625	80	0.5	98.78 ± 0.416	0.421
INH	18.75		15	101.17 ± 0.213	0.211
PYR	62.5		50	99.81 ± 0.619	0.620
RIF	25		20	99.83 ± 0.716	0.717
PDX	0.625	100	0.625	99.73 ± 0.352	0.352
INH	18.75		18.75	100.57 ± 0.276	0.274
PYR	62.5		62.5	100.93 ± 0.458	0.453
RIF	25		25	98.75 ± 0.571	0.578
PDX	0.625	120	0.75	100.12 ± 0.725	0.724
INH	18.75		22.5	100.73 ± 0.123	0.122
PYR	62.5		75	101.31 ± 0.525	0.518
RIF	25		30	100.38 ± 0.471	0.469

Precision, limit of detection and limit of quantitation

Both intra-day and inter-day precisions were studied. Five replicate sample solutions were prepared from the stock solution. In the study of intra-day precision the concentrations of the four drugs were measured thrice on the same day at time intervals of 1 h. In the inter-day precision study drug concentrations were measured on three different days. The limits of detection and quantitation (LOD and LOQ, respectively) were calculated using the equations: $LOD = 3.3\sigma/S$, $LOQ = 10\sigma/S$, where σ is the mean standard deviation of the peak area and S is the slope of the calibration plot. The obtained results are given in Table 5.

Table 5. Intra-day and inter-day precision, LOD and LOQ values; CV – coefficient of variance; LOD – limit of detection; LOQ – limit of quantitation

Drug	Intra-day precision (n = 6) % CV					Inter-day precision, % CV			LOD, µg mL^{-1}	LOQ, µg mL^{-1}
	1st h	2nd h	3rd h	4th h	5th h	Day 1[a]	Day 2[a]	Day 3[a]		
PDX	0.368	0.117	0.451	0.723	0.589	0.412	0.254	0.615	0.043	0.13
INH	0.091	0.318	0.769	0.428	0.377	0.133	0.189	0.345	0.063	0.19
PYR	0.366	0.282	0.516	0.379	0.836	0.223	0.519	0.256	0.036	0.11
RIF	0.463	0.538	0.813	0.768	0.519	0.716	0.481	0.971	0.059	0.18

[a] Averaged from six determinations.

Selectivity and specificity

Selectivity of the method was checked by injecting the solutions of all four drugs into the chromatograph. Four sharp peaks of PDX, INH, PYR and RIF were obtained at retention times of 3.687, 4.113, 5.041, and 12.829 min, respectively. These peaks were not obtained for the blank solution.

Specificity of the method was assessed by comparing the chromatograms of drug standards (Fig. 3) and mixed standard solutions (Fig. 4) to those obtained for tablet solutions. Retention times of the drugs in standard solutions, in the mixed standard solutions and in the sample solutions were the same. This result indicated specificity of the method. Furthermore, there was no interference from the excipients present in the tablets; thus, the method was considered specific and selective.

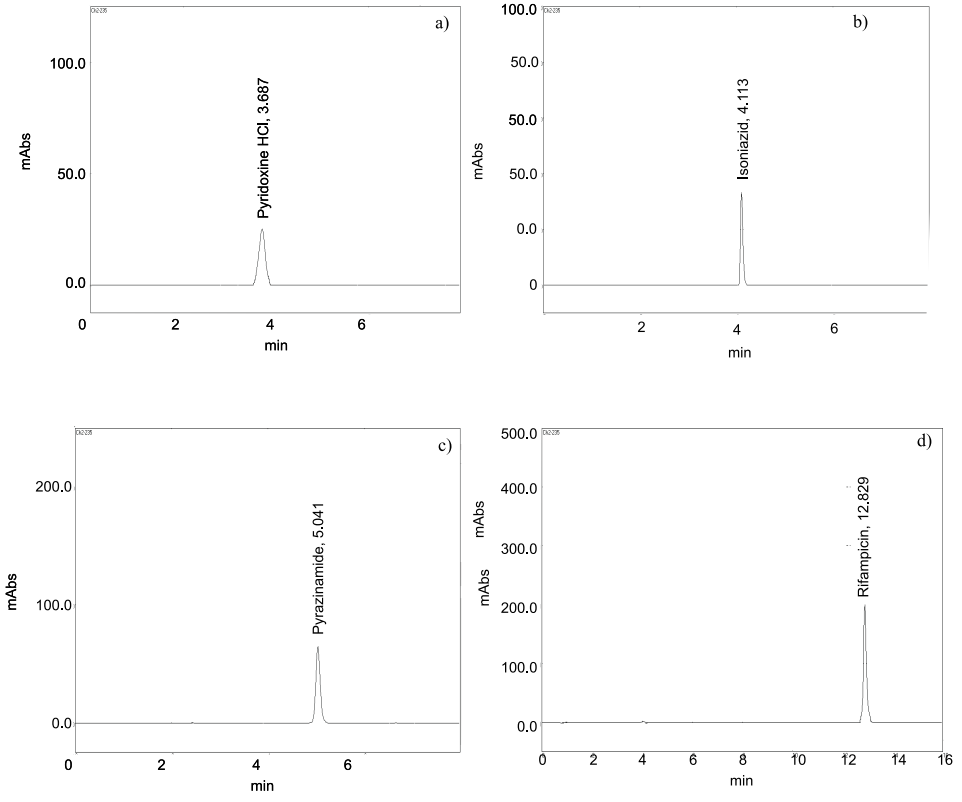

Figure 3. Chromatograms of PDX (a), INH (b), PYR (c) and RIF (d) at 20 μg mL^{-1} concentration

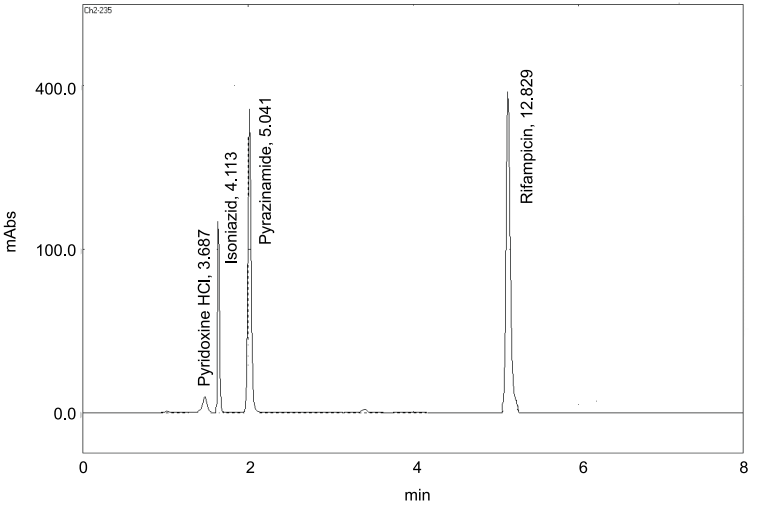

Figure 4. Chromatogram of PDX (0.625 μg mL^{-1}), INH (18.75 μg mL^{-1}), PYR (62.5 μg mL^{-1}) and RIF (25 μg mL^{-1}) in the mixed standard solution

CONCLUSIONS

A new reversed-phase HPLC method for the simultaneous analysis of PDX, INH, PYR and RIF in a tablet formulation has been developed. It has been shown that the method is accurate, reproducible, repeatable, linear, precise, selective, and thus reliable. The run time was relatively short, *i.e.* 20 min, what enables rapid quantitation of many samples in the routine and quality control analyses of tablet formulations. The same solvent was used throughout and no interference from any excipient was observed. These results indicate that the proposed method may find practical applications as a quality-control tool in the simultaneous analysis of the four drugs in combined dosage forms in quality-control laboratories.

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