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ARTICLES

# Development and Validation of RP-HPLC Method for Simultaneous Estimation of Rifampicin, Isoniazid and Pyrazinamide in Human Plasma[1]

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**Abstract**—A rapid, simple, sensitive and cost effective stability indicating high performance liquid chromatographic method for the simultaneous determination of rifampicin, isoniazid and pyrazinamide in human plasma was developed and validated in accordance to Food and Drug Administration (**FDA**) guidelines. The three drugs were eluted under isocratic mode using a 250 × 4.0 mm i.d., 5 μm Phenomenex ODS 2 C18 column. The mobile phase was composed of a mixture of acetonitrile, methanol and water in the ratio of 30 : 5 : 65 (v/v, pH adjusted to 5.2) at a flow rate of 1.0 mL/min. The limits of detection and quantification for rifampicin were 0.13 and 0.4 μg/mL, for isoniazid—0.6 and 1.8 μg/mL; and for pyrazinamide—0.5 and 1.6 μg/mL, respectively. The method can be successfully applied for pharmacokinetic, bioavailability or bioequivalence studies of rifampicin, isoniazid and pyrazinamide combination in human subjects.

*Keywords*: HPLC, isoniazid, rifampicin, pyrazinamide, anti-tuberculosis drugs

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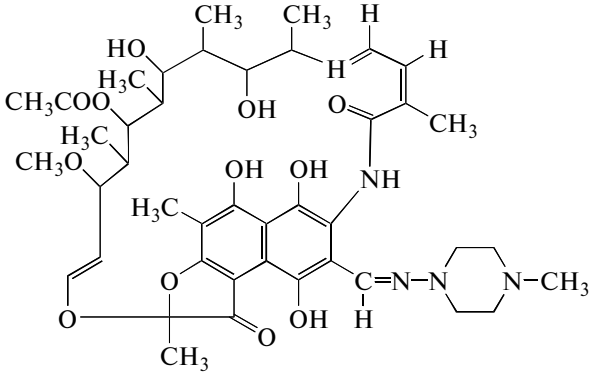
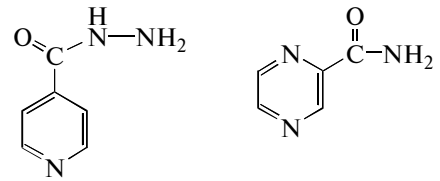
Treatment of tuberculosis is now very complex because of the emergence of multi drug resistant bacteria, which are resistant to first-line anti-tuberculosis drugs, pyrazinamide, isoniazid and rifampicin [1]. These compounds (Scheme) are extensively used as fixed-dose combination in the treatment of tuberculosis [2].

Rifampicin is well absorbed from the gut having bioavailability of 90–95% and has a half-life of 2 to 5 h (3.4 ± 0.7 h). Peak plasma concentrations of 7–10 mg/L appear in blood between 1–4 h after ingestion of 600 mg of rifampicin [3]. Isoniazid is rapidly and completely absorbed from the gastrointestinal tract and has a plasma half-life of 1–6 h (3.1 ± 1.1 h). Peak concentrations of 3–8 mg/mL appear in blood between 1–2 h after ingestion of 300 mg of isoniazid [4]. Pyrazinamide when given orally is readily absorbed from the gastrointestinal tract showing bioavailability of 90% and has a half-life of 9–10 h. Serum concentrations reach a peak level of about 66 mg/L after 2 h of administration of a dose of 3 g [5].

Measurement of plasma concentrations of anti-tuberculosis drugs may be required to determine the optimum drug dose for individuals, especially in patients suffering from multi-drug resistance [6]. Although these drugs have been in use for many decades there are still questions about the appropriateness of dosage regimes in specific patient groups such as those co-infected with HIV and young children [7]. In order to address these questions there is a need for a suitably selective and sensitive analytical method that is capable of measuring the drug in biological fluids.



Rifampicin



Isoniazid Pyrazinamide

The literature survey revealed that colorimetric [8], spectrophotometric [9–13], liquid chromatographic

[1] The article is published in the original.

(**LC**) [14–16], HPLC [17–23] and HPTLC [24] methods have been used for the simultaneous determination of rifampicin, isoniazid and pyrazinamide in pharmaceuticals. Various spectrophotometric [25] and HPLC methods [26] reported earlier for the simultaneous determination of rifampicin, isoniazid and pyrazinamide in plasma and biological fluids involve a time-consuming mobile phase preparation (involving buffers) along with tedious extraction process and a very lengthy elution time.

Mass spectroscopy (**MS**) methods for the determination of anti-tuberculosis drugs in human plasma have been described using electro spray ionization. Ever since rifampicin being a macromolecule with unusual molecular structure, the electro spray ionization interface leads to the formation of an unstable product ion. In addition, rifampicin, isoniazid and pyrazinamide exhibit a strong chromophore at wavelengths of 337, 263 and 268 nm, respectively. This chromophore not only allows for successful determination in human plasma by UV detection but also offers approximately the same sensitivity offered by LC–MS/MS detection. In the current study we achieved acceptable LODs of 0.13, 0.6 and 0.5 μg/mL for rifampicin, isoniazid and pyrazinamide, respectively, using HPLC–UV interface. This limit of quantification achieved by using HPLC–UV interface can be directly applied to the successful simultaneous estimation of rifampicin, isoniazid and pyrazinamide in a bioequivalence study. Although LC–MS/MS is a versatile tool, the development of HPLC based separation method makes it more economical and simpler both in terms of maintenance and data interpretation.

The present article describes a simple and sensitive reversed phase HPLC method with low LOQ for UV detection of rifampicin, isoniazid and pyrazinamide under isocratic mode using easily available inexpensive laboratory reagents. An attempt has been made to develop and validate the reversed phase HPLC method to ensure the accuracy, precision, robustness and other analytical method validation parameters, as mentioned in the FDA guidelines [27].

## EXPERIMENTAL

**Chemicals and reagents.** Rifampicin, isoniazid and pyrazinamide (purity 98.00%, w/w) were received as gift sample from Lupin Laboratories Ltd. HPLC grade acetonitrile, methanol (HPLC grade), ethyl acetate (AR grade) and ascorbic acid (as stabilizing agent) were purchased from Merck Ltd (Mumbai, India). Deionized water was processed through a Milli-Q water purification system (Millipore, USA). All other chemicals and reagents were of analytical grade.

**Instrumentation.** The chromatographic system consisted of a gradient HPLC Waters system with 515 pump, Rheodyne manual injector, CTO-10Avp column temperature oven, variable wavelength programmable dual UV absorbance detector (Waters 2487) and a Tech comp UV-2301 double beam UV-Vis spectrophotometer (Waters system solvent module, iEEE 488 pump) to carry out spectral analysis. All the components of the system were controlled using SCL-10A VP system controller. Data acquisition and processing were done using Hitachi software (version 3.05.01). The detector was set at a wavelength of 242 nm. Chromatographic separations were accomplished using a reversed phase C-18 column (25 mm × 4.0 mm i.d.; particle size 5 μm). The mobile phase was prepared with acetonitrile, methanol and water in the ratio of 30 : 5 : 65, v/v, with a final pH of 5.2. The components of the mobile phase were filtered before use through 0.45 μm membrane filter, degassed for 15 min and the respective solvent reservoir was pumped to the column at the flow rate of 1 mL/min. The column temperature was maintained at ambient conditions and the volume of the injection loop was 20 μL. The column was equilibrated for at least 30 min with the mobile phase.

**Standard solutions.** The primary stock solution of rifampicin, isoniazid and pyrazinamide was prepared by taking 10, 15 and 50 mg in a 10 mL volumetric flask containing 5 ml methanol, sonicated for about 15 min and made up to final volume with methanol to get a free base concentration of 1, 1.5 and 5 mg/mL for rifampicin, isoniazid and pyrazinamide, respectively. This stock solution of the drug was stored in a refrigerator below 10°C.

**Calibration and quality control samples.** Aqueous stock dilutions were prepared initially. Aqueous stock dilution, 1 mL each, was transferred into a 10 mL volumetric flask, to which 1 mL of 1 M ascorbic acid solution was added and vortexed for 10 s. The final volume was made up with screened drug-free $K_2$EDTA human plasma and vortexed for 5 min to achieve the desired concentration of calibration curve standards. The final calibration standard concentrations were prepared in the range of 40–100, 60–150 and 200–500 μg/mL, as per Table 1 for rifampicin, isoniazid and pyrazinamide, respectively. Each of these standard solutions was distributed into disposable polypropylene micro centrifuge tubes (2.0 mL, Eppendorf) in volume of 0.7 mL and the tubes were stored at −70°C until analysis. Similarly quality control (**QC**) samples were prepared in plasma such that the final concentrations were 0.6, 0.9 and 15.0 μg/mL, respectively, and labeled as low quality control (**LQC**), median quality control (**MQC**) and high quality control (**HQC**), respectively.

The extraction of the plasma samples involved liquid-liquid extraction process. For processing, the stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature. An aliquot of 500 μL was then transferred to prelabeled 2.0 mL polypropylene centrifuge tubes. Extraction solvent, 20 mL of ethyl acetate, was then added to extract the drug. The samples were then kept on a vibramax unit and vortexed for 15 min. Samples were then centri-

**Table 1.** Spiked calibration curve plasma standards

| Standard volume, μL, STD-7 | Final concentration, μg/mL | | | CC STD* ID |
|---|---|---|---|---|
| | rifampicin | isoniazid | pyrazinamide | |
| – | 40.5 | 60.0 | 200.0 | STD-7 |
| 9000.0 | 50.0 | 75.0 | 250.0 | STD-6 |
| 8000.0 | 60.0 | 90.0 | 300.0 | STD-5 |
| 7000.0 | 70.0 | 105.0 | 350.0 | STD-4 |
| 6000.0 | 80.0 | 120.0 | 400.0 | STD-3 |
| 5000.0 | 90.0 | 135.0 | 450.0 | STD-2 |
| 4000.0 | 100.0 | 150.0 | 500.0 | STD-1 |

* CC STD—calibration curve plasma standard.

fuged at 5000 rpm for 5 min in a refrigerated centrifuge (4°C). Supernatant solution, 1 mL, was then transferred into prelabeled polypropylene tubes and was allowed to evaporate to dryness under nitrogen at constant temperature of 40°C. The dried residue was then dissolved in 200 μL of mobile phase and transferred into shell vials containing vial inserts for analysis. Samples, 20 μL by volume, were then injected into the column and analyzed by HPLC on the same day to avoid any degradation. The auto sampler temperature was maintained at 4°C throughout analysis. The column temperature oven was maintained at ambient temperature.

**Validation.** The quantitative HPLC–UV method was validated in accordance to FDA guidelines acceptance criteria for specificity, linearity, limit of detection (**LOD**), limit of quantification (**LOQ**), accuracy, precision, carry over effects, robustness, ruggedness, freeze-thaw and auto sampler stability studies.

**Selectivity.** The selectivity of the method was evaluated by analyzing six independent drug-free $K_2$EDTA human plasma samples with reference to potential interferences from endogenous and environmental constituents.

**Linearity.** Calibration curves were generated to confirm the relationship between the peak area ratios and the concentration of drug in the standard samples. Fresh calibration standards were extracted and assayed as described above on three different days and in duplicate. The regression line was generated to best fit the mathematical model. Drug concentrations in QC samples, recovery samples, and stability samples were calculated from the resulting area ratio and the regression equation of the calibration curve.

**LOD and LOQ.** The parameters LOD and LOQ were determined on the basis of signal to noise ratio. LOD was defined as the lowest concentration that produces a peak distinguishable from background noise (minimum ratio of 3 : 1). The LOQ was accepted as the lowest point on the standard curve with an RSD of less than 20% and signal to noise ratio of 5 : 1.

**Accuracy.** The accuracy was determined by comparing the area under the curve of extracted QC samples (LQC, MQC and HQC) with direct injection of extracted blank plasma spiked with the same nominal concentration of drug as in the QC samples.

$$\text{Accuarcy}\,(\%) = \frac{\text{Mean observed concentration}}{\text{Nominal concentration}} \times 100.$$

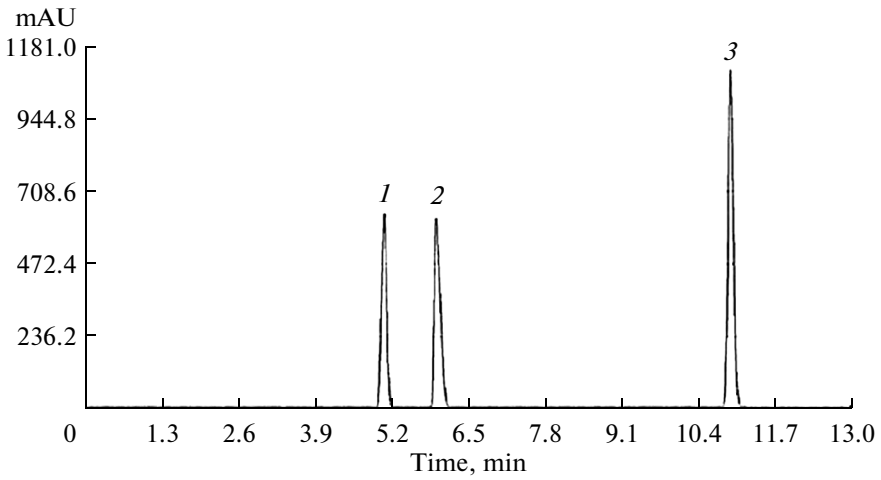
**Precision.** Intra-day precision was evaluated by analysis of QC samples at three levels, μg/mL: LQC—0.60, MQC—0.90 and HQC—15.0, $n = 6$ at each level, on the same day. Inter-day precision was determined by analyzing three QC samples levels on 3 separate days ($n = 6$ at each level) along with three separate standard curves done in duplicate. The precision was expressed by coefficient of variation (**CV**).

$$\text{CV}\,(\%) = \frac{\text{Standard deviation}}{\text{Mean observed concentration}} \times 100.$$

**Carryover test.** A critical issue with the analysis of many drugs is their tendency to get adsorbed by reversed phase octadecyl-based chromatographic packing materials, resulting in the carryover effect.

**Ruggedness and robustness.** The ruggedness was established by comparing the mean peak area obtained by two different analysts. The robustness of the method was assessed by changing the detection of wavelength, mobile phase composition and pH.

**Stability studies.** Auto sampler, and freeze-thaw stability of drug was determined at low, medium and high QC samples concentrations. To determine the impact of freeze-thaw cycles on drug concentration, samples were allowed to undergo 3 freeze-thaw (−50°C) cycles. Following sample treatment/storage conditions, the drug concentrations were determined and compared to the control sample that had been stored at −50°C. Auto sampler stability of extracted samples was determined by comparing drug concentration in freshly prepared samples and samples kept in the auto sampler at 4°C for 24 h.




Typical HPLC chromatogram of plasma spiked with isoniazid (*1*), rifampicin (*2*) and pyrazinamide (*3*).

## RESULTS AND DISCUSSION

**Method development.** Rifampicin, isoniazid and pyrazinamide belong to the basic class of drugs due to their imine, azide and amide functional groups, respectively. Therefore, adjusting the pH of mobile phase to the acidic side obviously ionizes the drug present in plasma, thereby leading to poor recovery. In order to extract the un-ionized form of the drug, it is imperative to adjust the pH to the alkaline side, however, alkaline mobile phase characteristics causes deterioration of the bonded phase in the column due to alkaline hydrolysis of end-capped silica. Compared to acid catalyzed hydrolysis, the hydrolysis of end-capped silica in alkaline conditions is usually very rapid. Therefore, experiments were performed using methanol-water in a limited pH range of 5.0 to 6.0. The response was checked at the detector using a connector (without the column). A pH value of $5.2 \pm 0.1$ gave maximum response for the analytes at 242 nm. The resolution between the peaks was decreased and peaks were not of acceptable shape when the experiment was performed using a shorter column ($150 \times 4.6$ mm i.d.). However better resolution, less tailing and high theoretical plates were obtained with Phenomenex C18 $250 \times 4.0$ mm, 5 μm column.

The mobile phase consists of a mixture of acetonitrile, methanol and water in the ratio of 30 : 5 : 65 (v/v, pH adjusted to 5.2). The flow rate used was 1.0 mL/min. At the reported flow rate peak shape was excellent, however, increasing or decreasing the flow rate increased the tailing factor and resulted in poor peak shape and decreased resolution between the drug and internal standard. The peak shape and symmetry were found to be good when the mobile phase composition of 30 : 5 : 65 (v/v) was used with better resolution of the drug. Increasing the organic portion of the mobile phase caused the drug to elute with high tailing and also merging of the peaks of rifampicin and isoniazid. A mobile phase containing aqueous portion greater than 70% led to very late elution and very poor peak shape. The peaks were also broad and had unacceptable asymmetry factor.

Extraction methods were initially attempted using protein precipitation technique. Organic solvents such as acetonitrile and/or methanol were used as reagents for protein precipitation [28]. Initial experiments of protein precipitation were done using 1 : 3 ratio of plasma-organic solvents. The recovery of the drug was poor as compared with liquid-liquid extraction. Since the noise effects in solid phase extraction (**SPE**) method are similar to that of liquid-liquid extraction (**LLE**), the final analysis was carried out using LLE. SPE methods although render a neat sample for final analysis, polar interferences do enter into the final sample during reconstitution. SPE is further expensive as compared to LLE technique. Various solvents such as ethyl acetate, diethyl ether and dichloromethane were used for extraction. The highest recovery from the plasma samples could be obtained with ethyl acetate alone.

**Detection and chromatography.** The figure shows typical chromatogram of a spiked plasma sample containing rifampicin, isoniazid and pyrazinamide indicating the specificity of the method. The chromatogram of drug-free $K_2$EDTA human blank plasma failed to show any peaks where the relevant peaks were expected with drug spiked plasma. The retention times for rifampicin, isoniazid and pyrazinamide were 5.99, 5.11 and 10.97 min, respectively.

**Method validation.** *Selectivity.* The method was found to have high selectivity for the analytes; since no interfering peaks from endogenous compounds were observed at the retention time for the drug in any of the six independent blank plasma extracts evaluated.

**Calibration curves.** When the drug concentrations and their respective mean peak areas were subjected to regression analysis by least squares method, high correlation coefficients (>0.999) were observed in the

**Table 2.** Characteristics of calibration curves for the estimation of drugs ($y = ax + b$, where $y$ is peak area and $x$ concentration, μg/mL)

| Compound | Slope, $a$ | RSD of $a$, % | Intercept, $b$ | RSD of $b$, % | Correlation coefficient, $R$ |
|---|---|---|---|---|---|
| Rifampicin | $7.92 \times 10^3$ | 1.3 | $3.02 \times 10^3$ | 0.42 | 0.9995 |
| Isoniazid | $4.59 \times 10^3$ | 1.6 | $8.06 \times 10^3$ | 1.5 | 0.9990 |
| Pyrazinamide | $2.23 \times 10^3$ | 0.9 | $3.56 \times 10^3$ | 0.23 | 0.9995 |

**Table 3.** Analytical parameters* of isoniazid, rifampicin and pyrazinamide

| Parameter | Isoniazid | Rifampicin | Pyrazinamide |
|---|---|---|---|
| Retention time, min | 5.10 | 5.99 | 10.96 |
| Area | 424413 | 475680 | 677558 |
| Number of theoretical plates | 12561 | 2886 | 10223 |
| Tailing factor | 1.19 | 1.40 | 0.80 |
| Resolution | – | 4.50 | 25.24 |
| Linear range, μg/mL | 40–100 | 60–150 | 200–500 |
| LOD, μg/mL | 0.13 | 0.6 | 0.5 |
| LOQ, μg/mL | 0.38 | 1.8 | 1.6 |

* Average of 3 values.

ranges of 40 to 100, 50 to 150 and 200 to 500 μg/mL for rifampicin, isoniazid and pyrazinamide, respectively, as shown in Table 2.

**Limits of detection and of quantification.** The approximate LODs of rifampicin, isoniazid and pyrazinamide were 0.13, 0.6 and 0.5 μg/mL. LOQs were found to be 0.4, 1.8 and 1.6 μg/mL for rifampicin, isoniazid and pyrazinamide, as shown in Table 3.

**Accuracy.** The overall recovery of rifampicin, isoniazid and pyrazinamide was 99.79, 99.79 and 99.64% with a coefficient of variation of 0.72, 0.72 and 0.12%, ($n = 3$), respectively, as shown in Table 4.

**Precision.** A detailed summary of the intra- and inter-day precision data generated for the assay validation is presented in Table 5. The inter- and intra-day precision was <2% for all QC concentrations, which was within the general assay acceptability criteria for QC samples.

**Carryover test.** In this analysis no quantifiable carryover effect was observed when a series of blank (plasma) solutions were injected immediately following the highest calibration standard.

**Ruggedness and robustness.** The ruggedness was established by determining the concentrations of rifampicin, isoniazid and pyrazinamide by two differ-

**Table 4.** Accuracy of the proposed HPLC method

| Drug | Level, % | Concentration, μg/mL | Amount recovered*, μg/mL | Recovery, % | RSD, % |
|---|---|---|---|---|---|
| Rifampicin | 50 | 60.0 | 59.63 | 99.4 | 1.87 |
| | 100 | 80.0 | 79.38 | 99.2 | 0.48 |
| | 150 | 100.0 | 100.74 | 100.7 | 0.86 |
| Isoniazid | 50 | 90.0 | 90.04 | 100.1 | 1.22 |
| | 100 | 120.0 | 120.78 | 100.7 | 0.84 |
| | 150 | 150.0 | 150.57 | 100.4 | 1.07 |
| Pyrazinamide | 50 | 300.0 | 300.9 | 100.3 | 0.70 |
| | 100 | 400.0 | 397.0 | 99.2 | 0.49 |
| | 150 | 500.0 | 496.9 | 99.4 | 0.53 |

* Average of 3 values.

**Table 5.** Inter- and intra-day precision of the proposed HPLC method

| Drug | Concentration, μg/mL* | Intra-day | | Inter-day | |
|---|---|---|---|---|---|
| | | mean | RSD, % | mean | RSD, % |
| Rifampicin | 0.60 | 785534 | 0.26 | 791931 | 0.53 |
| | 0.90 | 453930 | 0.78 | 477152 | 1.06 |
| | 15.1 | 314807 | 1.02 | 330445 | 1.80 |
| Isoniazid | 0.60 | 692183 | 0.15 | 693042 | 0.27 |
| | 0.90 | 444486 | 0.70 | 430631 | 1.64 |
| | 1.51 | 274748 | 1.38 | 285726 | 1.45 |
| Pyrazinamide | 0.60 | 1099603 | 1.91 | 1105728 | 0.42 |
| | 0.90 | 679098 | 1.25 | 675436 | 0.87 |
| | 15.1 | 443987 | 0.68 | 424305 | 1.10 |

* Average of 6 values.

**Table 6.** Ruggedness of the proposed HPLC method

| Parameter | Isoniazid, 90 μg/mL* | | Rifampicin, 60 μg/mL* | | Pyrazinamide, 300 μg/mL* | |
|---|---|---|---|---|---|---|
| | *1* | *2* | *1* | *2* | *1* | *2* |
| Mean peak area | 430630.5 | 414685.2 | 477152.3 | 478650.3 | 475436.2 | 681657.3 |
| RSD, % | 1.64 | 1.14 | 1.06 | 1.39 | 0.87 | 0.84 |

* Average of 6 values.
*1*, *2*—different analysts.

ent analysts having reproducibility with a coefficient of variation less than 1.4, 1.7 and 0.9%, respectively, as shown in Table 6. The robustness was established by determining the change in mobile phase, wavelength and pH with a coefficient of variation less than 0.9, 0.8 and 0.6%, respectively, as shown in Table 7.

**Stability studies.** Determination of drug stability following three freeze–thaw cycles showed that for all QC samples there was a minor change in the drug concentration, as shown in Table 8.

A HPLC method was developed and validated for the simultaneous determination of rifampicin, isoniazid and pyrazinamide in human plasma. The extraction process involved a single-step LLE procedure employing ethyl acetate as extracting solvent. LLE method is usually devoid of polar interferences thus rendering the sample clean for final analysis. The noise is usually absent or at minimum as compared to precipitation or SPE techniques. This assay requires only a small volume of plasma (500 μL) and uses easily available inexpensive laboratory reagents. The mobile phase consists of a mixture of acetonitrile, methanol and water in the ratio of 30 : 5 : 65 (v/v, pH adjusted to 5.2). It does not use any tedious preparation of buffers which commonly block the column, and no carryover effect was observed. Due to the LLE method of extraction, the baseline noise is minimal. Matrix effects are not observed. In conclusion, method validation following FDA guideline indicated that the developed method has high sensitivity, acceptable recovery, reliability, specificity and excellent efficiency with a total running time of 13.0 min per sample and retention time of 5.99, 5.11 and 10.97 min for rifampicin, isoniazid and pyrazinamide, respectively, which is important for large batches of samples. The method showed good linearity in the ranges of 40–100, 50–150 and 200–500 μg/mL, with high correlation coefficient values ($R^2 > 0.999$) for rifampicin; isoniazid and pyrazinamide, respectively. The LOD and LOQ (μg/mL) for rifampicin were 0.13 and 0.38, for isoniazid 0.6 and 1.8, and for pyrazinamide 0.5 and 1.6, respectively. This HPLC method is simple, sensitive, precise and highly accurate. It requires small quantity of plasma sample and hence could be successfully applied to determine rifampicin, isoniazid and pyrazinamide combination in spiked human plasma for pharmacokinetic analysis. The therapeutic assay developed here requires small sample volume, short chromatographic

**Table 7.** Robustness of the proposed HPLC method

| Condition | Isoniazid | | Rifampicin | | Pyrazinamide | |
|---|---|---|---|---|---|---|
| | mean* | RSD, % | mean* | RSD, % | mean* | RSD, % |
| Unaltered | 424413 | – | 475680 | – | 677558 | – |
| MP change 1: A–M–W (25 : 5 : 70, v/v) | 425717 | 0.31 | 473512 | 0.46 | 680745 | 0.48 |
| MP change 2: A–M–W (35 : 5 : 60, v/v) | 421354 | 0.72 | 480578 | 1.03 | 674754 | 0.41 |
| WL change 1: 240 nm | 429030 | 1.09 | 481876 | 1.30 | 684124 | 0.97 |
| WL change 2: 244 nm | 424027 | 0.10 | 480824 | 1.08 | 677168 | 0.06 |
| pH change 1: 5.0 | 423893 | 0.13 | 469504 | 1.30 | 677937 | 0.04 |
| pH change 2: 5.4 | 417802 | 1.56 | 468735 | 1.47 | 674742 | 0.42 |

* Average of 3 values.
Notations: MP—mobile phase, WL—wavelength, A—acetonitrile, M—methanol, W—water.

**Table 8.** Short-term, long-term and freeze-thaw stability studies*

| Parameter | Rifampicin | | Isoniazid | | Pyrazinamid | |
|---|---|---|---|---|---|---|
| | 0.60 (LQC) | 15.1 (HQC) | 0.60 (LQC) | 15.1 (HQC) | 0.60 (LQC) | 15.1 (HQC) |
| Short-term stability, 4 days | | | | | | |
| Mean | 99.42 | 100.08 | 100.88 | 98.49 | 98.63 | 100.32 |
| CV, % | 0.71 | 1.04 | 0.39 | 0.44 | 1.02 | 0.63 |
| Long-term stability, 12 days | | | | | | |
| Mean | 99.49 | 99.69 | 100.78 | 99.21 | 100.16 | 99.78 |
| CV, % | 2.90 | 1.56 | 0.88 | 1.21 | 1.85 | 0.87 |
| Freeze-thaw, 3 cycles | | | | | | |
| Mean | 99.56 | 99.30 | 100.67 | 99.93 | 100.69 | 99.23 |
| CV, % | 0.43 | 1.34 | 1.07 | 0.10 | 0.79 | 0.46 |

* Nominal concentrations of analyts (μg/mL) are given, average of 3 values.

time, simple sample pre-treatment and mobile phase composition.

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## REFERENCES

1. Fivy, K., Syed, A., Syed, S., and Syed, W.G., *Int. J. Pharm. Pharm. Sci.*, 2012, vol. 4, p. 733.
2. Enoche, F.O., *Int. J. Pharm. Pharm. Sci.*, 2010, vol. 2, p. 55.
3. *Indian Pharmacopoeia*, Delhi: The controller of publications, 1996, vol. 2, p. 665.
4. *Indian Pharmacopoeia*, Delhi: The controller of publications, 1996, vol. 2, p. 408.
5. *Indian Pharmacopoeia,* Delhi: The controller of publications, 1996, vol. 2, p. 644.
6. Prasanthi, B., Santosh Kumar, T., and Vijaya Ratna, J., *Int. J. Pharm. Sci. Rev. Res.,* 2013, vol. 23, p. 108.
7. *Treatment of Tuberculosis,* Guidelines for national programmes, World Health Organization (WHO),

2003.http://www.emro.who.int/stb/media/pdf/Guidelines2003.pdf. Cited June, 2004.

8. Ellard, G.A., *Int. J. Tuberc. Lung. Dis.*, 1999, vol. 3 (11 Suppl. 3), p. S343.
9. Rote, A.R. and Sharma, A.K., *Indian J. Pharm. Sci.,* 1997, vol. 59, p. 119.
10. Hector, C.G. and Alejandro, C.O., *J. Pharm. Biomed. Anal.*, 1999, vol. 20, p. 681.
11. Espinosa, M.A., Acedo, V.M.I., Munoz, D.L.P.A., and Canada, F.S.L., *Anal. Chim. Acta*, 2001, vol. 27, p. 129.
12. Ayla, G., Ece, K., and Sumru, O., *Int. J. Pharm.*, 2004, vol. 271, p. 115.
13. Rasha, M.Y. and Hadir, M.M., *Spectrochim. Acta*, 2008, vol. 70, p. 1152.
14. Calleri, E., De, L.E., Furlanetto, S., Massolini, G., and Caccialanza, G., *J. Pharm. Biomed. Anal.*, 2002, vol. 29, p. 1089.
15. Espinosa, M.A., Acedo, V.M.I., Munoz, D.L.P.A., and Canada, F.S.L., *Talanta*, 2002, vol.8, p. 273.
16. Khuhawar, M.Y. and Rind, F.M.A., *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2002, vol. 766, p. 357.
17. Jindal, K.C., Chaudhary, R.S., Singla, A.K., Gangwal, S.S., and Khanna, S., *J. Pharm. Biomed. Anal.*, 1994, vol. 12, p. 493.
18. Mariappan, T.T., Singh, B., and Singh, S.A., *Pharm. Pharmacol. Commun.*, 2000, vol. 8, p. 345.
19. Gorzata, T., Jolanta, F., and Halina, S., *J. Planar Chromatogr.—Mod. TLC,* 2005, vol. 18, p. 207.
20. Glass, B.D., Agatonovic, K.S., Chen, Y.J., and Wisch, M.H., *J. Chromatogr. Sci.*, 2007, vol. 45, p. 38.
21. Kaushik, G.K.M.P., Hiral, R.T., and Shital, D.F., *Int. Bull. Drug Res.,* 2011, vol. 1, p. 71.
22. Shyam, P.T., Rao, K.N.V., Chaitanya, Y., Raghavendra, P., Surendar, M., and David, B., *Int. J. Pharm. Res. Dev.*, 2012, vol. 4, p. 153.
23. Lai, Y.T., Lik, T.T., and Steven, A.N., *J. Liq. Chromatogr. Relat. Technol.*, 2013, vol. 36, p. 12.
24. Argekar, A.P., Kunji, S.S., and Puranadre, K.S., *J. Pharm. Biomed. Anal.*, 1996, vol. 14, p. 1645.
25. Barsoum, N., Barsoum, M., Kamel, S., and Mohamed, M.A.D., *Res. J. Agric. Biol. Sci.*, 2008, vol. 4, p. 471.
26. Unsalan, S., Sancar, M., Bekce, B., Clark, P.M., Karagoz, T., Izzettin, F.V., and Rollas, S., *Chromatographia*, 2005, vol. 61, nos. 11–12, p. 595.
27. *Guidance for Industry, Bioanalytical Method Validation,* U.S. Department of Health and Human Services: Food and Drug Aadministration, Center for Drug Evaluation and Research, 2001.
28. Cara, P., Pratibha, S., Bev, I., Vanaja, R., and Russell, G., *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2003, vol. 785, p. 263.