RESEARCH PAPER

Development and Validation of Reverse Phase High-Performance Liquid Chromatography Based Bioanalytical Method for Isoniazide and Moxifloxacin

R. D. Vyavahare[1*], U. N. Soni[2]

[1*] Research Scholar, Oriental University, Indore, Madhya Pradesh, India, Pin Code - 453555.

Email: vyavahare.ritesh@gmail.com (Corresponding Author)

[2] Research Guide, Professor, Oriental University, Indore, Madhya Pradesh, India, Pin Code - 453555

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ABSTRACT

A straightforward and accurate bioanalytical technique based on RP-HPLC was created and verified for the measurement of isoniazid and moxifloxacin in human plasma. An i.d. 5 µm Phenomenex OOG 2 C18 column measuring 250 × 4.6 mm was used to elute Isoniazide in isocratic mode. At a flow rate of 1.0 mL/min, the mobile phase was made up of an 85:15 v/v combination of water and acetonitrile. Moxifloxacin was examined using a quick and easy HPLC-UV technique. A 250 × 4.6 mm i.d. 5 µm Phenomenex OOG 2 C18 column was used for the separation. At a flow rate of 1 mL/min, the mobile phase consisted of an 80:20 combination of water and acetonitrile. The limits of detection and quantification for isoniazid and moxifloxacin was done at 0.1 and 10 µg/mL each.

Keywords: Bioanalytical methods, Isoniazide, Moxifloxacin, antitubercular drugs.

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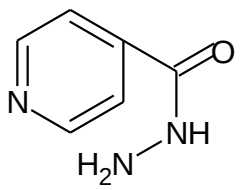
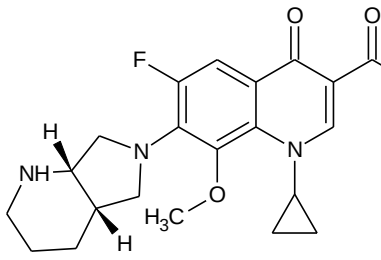
Introduction: Currently, the treatment of tuberculosis is complicated and time-consuming. It involves administering instant-release solid dosage forms (tablets or capsules) of isoniazid (INH) and moxifloxacin in a fixed-dose combination (FDC) for six months [1]. Isoniazid has a plasma half-life of 1–6 hours (3.1 ± 1.1 hours) and is quickly and fully absorbed from the gastrointestinal system. One to two hours after consuming 300 mg of isoniazid, blood concentrations reach their peak at 3–8 mg/mL [2]. The best treatment dosage for each patient may need to be determined by measuring the plasma concentrations of anti-tuberculosis medications, particularly in patients with multidrug resistance [3]. One member of the fourth generation of fluoroquinolones is moxifloxacin (MOXI). It is frequently used to treat multidrug-resistant and sensitive TB. Moxifloxacin was added to standard therapy because of its bactericidal impact on slow-replicating bacteria, which is a significant element that shortens the course of treatment [4-6]. Despite the fact that these medications have been around for decades, there are still concerns regarding the suitability of dosage schedules for particular patient populations, such as young children and those who are co-infected with HIV [7]. A sensitive and appropriately selective analytical technique that can measure the pharmaceuticals in biological fluids is required to answer these issues.



Isoniazide



HCl

Moxifloxacin hydrochloride

Colorimetric [8], spectrophotometric [9–12], liquid chromatographic (LC) [13–14], and high performance liquid chromatography [15–18] techniques have all been employed to determine isoniazid in pharmaceuticals, according to the literature review. Several spectrophotometric and HPLC techniques that have been previously reported for the detection of isoniazid and moxifloxacin in biological fluids and plasma need a laborious extraction procedure, a long elution time, and a time-consuming mobile phase preparation. With readily available, reasonably priced laboratory chemicals, the current research outlines a straightforward, sensitive reversed phase HPLC technique with a low limit of quantification (LOQ) for UV detection of isoniazid and moxifloxacin in isocratic mode. In order to guarantee the accuracy, precision, robustness, and other analytical technique validation factors specified in the FDA recommendations, considerable effort has been undertaken to develop and validate the reversed phase HPLC method [19].In the subject of developing analytical methods, bioanalytical methods are a relatively new and expanding field. In 1990, the bioanalytical validation workshop was held with the goal of investing in and standardizing the methods needed for method validation for the created method. The study of drugs in bodily fluids is the focus of the bioanalysis area. The qualitative identification of drugs and their metabolites in biological fluid is the focus of the bioanalytical study. It is crucial for assessing and interpreting results from pharmacokinetic, toxokinetic, bioavailability, and bioequivalence studies. The bioanalysis is self-evident. The study of drugs in the bioanalytical matrix is called bioanalysis. Pharmaceutical laboratories, pharmaceutical contract research organizations, the pharmaceutical business, preclinical research, clinical investigations, and the identification of pesticides in the body all employ bioanalytical methods. Because of their accuracy and quick study analysis, bioanalysis's reach is expanding daily. The bioanalysis process begins in the clinical domain and concludes in the regulatory domain, where ethical considerations are taken into account when submitting a drug study report. Non-structural trials and error are the foundation of the creation of a bioanalytical procedure. The investigation was characterized after the different parameters employed for analysis produced suitable results. The developer's theoretical expertise, experience, and preferences all influence the bioanalytical method's quality and performance.

The bioanalysis includes different steps according to sequence as given below.

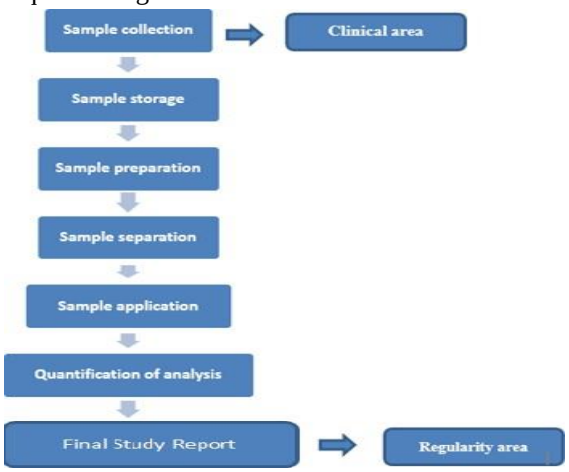


Figure 1: Flow chart of steps in bioanalytical method development

Protein precipitation, liquid extraction, and solid-liquid extraction (also known as solid-phase extraction) are some of the sample preparation methods used in the development of bioanalytical methods. Supercritical fluid extraction, electrophoresis, distillation, evaporation, and dialysis are further, albeit less often employed methods. Alongside extraction techniques, chromatographic parameters must be carefully considered in the development of bioanalytical systems. These chromatographic characteristics, which are necessary for efficient drug analysis, include the choice of solvents, mobile phases, pH-adjusting buffers, and columns. The intricacy of the biological matrix in which the analyte is found is a major obstacle to the development of bioanalytical methods. Based on the drug's pharmacokinetic characteristics, the matrix may consist of blood, plasma, serum, urine, or, less frequently, oral fluid, pus, saliva, or synovial fluid. To guarantee precise and trustworthy assessment of the medication and its metabolites, the existence of these intricate matrices necessitates thorough process optimization.

MATERIALS AND METHODS

Chemicals and Materials

INH and MOXI of pharmaceutical grade were acquired from Dhamtech Pharma Pvt. Ltd., Mumbai (India). Human plasma was kindly donated by Pandharpur Blood

bank, Pandharpur, Solapur, Maharashtra, India. Isonicotinamide and Ciprofloxacin, of pharmaceutical grade, were purchased from Dhamtech Pharma Pvt Ltd, Mumbai (India) and used as internal standards. For the sample processing and HPLC quantification, acetonitrile (HPLC grade, MERK ®, India), Water (HPLC grade, LOBA Chemie ®, India) were used. All other reagents were of pro-analysis quality**.**

Instrumentation

The chromatographic analysis was performed on an isocratic Waters HPLC system equipped with a Rheodyne manual injector (G1328C 1260, six-port loop) and an Agilent 1260 Infinity G1315D Diode Array Detector (DAD VL) for spectral measurements. Instrument operation was managed using Agilent ChemStation software**,** while data acquisition and processing were carried out with EZChrom Elite software**.** The detection wavelength was set at 277 nm for isoniazid and 296 nm for Moxifloxacin**.** Separation was achieved on a reversed-phase C18 column (25 × 4.6 mm, 5 µm particle size). The mobile phase consisted of water: acetonitrile (85:15, v/v) for isoniazid and water: acetonitrile (80:20, v/v) for Moxifloxacin, both delivered at a flow rate of 1.0 mL/min**.** Before use, the mobile phase components were filtered through a 0.22 µm membrane filter and degassed for 15 minutes. The column was kept at ambient temperature**,** with a 10 µl injection volume**,** and was equilibrated for 30 minutes with the mobile phase prior to analysis.

Preparation of solutions

Standard solutions- Standard stock solutions of each analyte were prepared separately in double distilled water at a final concentration of 1.0 mg/mL**.** The internal standard (IS) was also prepared in the same solvent at a concentration of 1.0 mg/mL. All stock solutions were stored at –20 °C until further use.

Calibration and quality control samples- Aqueous stock dilutions were prepared initially, and an aliquot of 1 µl was transferred into a 10 mL volumetric flask, vortex-mixed for 10 seconds, and the volume was adjusted with double distilled water. The solution was further vortexed for 5 minutes to obtain the desired concentrations of calibration standards. Final calibration standards of isoniazid and Moxifloxacin were prepared in the range of 0.1–10 µg/mL, as shown in Table 1. These solutions were aliquoted into 1.5 mL polypropylene micro centrifuge tubes (Eppendorf) in volumes of 30 µl each and stored at –20 °C until analysis. For quality control (QC) samples, plasma was spiked to achieve final concentrations of 0.3 µg/mL (LQC), 4.5 µg/mL (MQC), and 9.0 µg/mL (HQC), which were used for method validation.

Plasma Sample Extraction

Plasma samples were processed using the protein precipitation method. Frozen, spiked samples were thawed at room temperature, and an aliquot of 50 µl was transferred into pre-labeled 1.5 mL polypropylene centrifuge tubes. To each tube, 200 µl of acetonitrile was added as the extraction solvent, followed by mixing on a vibramax unit with vortexing for 15 minutes. The samples were centrifuged at 10,000 rpm for 10 minutes in a refrigerated centrifuge (4 °C). The resulting supernatant (1 mL) was carefully transferred into shell vials containing vial inserts for HPLC analysis. An injection volume of 10 µl was used, with the column maintained at ambient temperature during separation.

Method validation

The developed HPLC–UV method was validated as per the ICH M10 guidelines. Validation parameters included specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and carry-over assessment, among others.

Selectivity:

Method selectivity was assessed by analyzing eight independent blank human plasma samples to check for possible interference from endogenous substances or environmental contaminants.

Linearity:

Calibration curves were established to confirm the correlation between peak area ratios and drug concentrations in standard samples. Standard calibrators were extracted and analyzed as described in the method section, performed in duplicate on three separate days. Regression analysis was applied to generate the best-fit calibration equation. The concentrations of QC and recovery samples were determined using the regression equation obtained from the calibration curve.

LOD and LOQ:

The LOD and LOQ were estimated based on the signal-to-noise (S/N) ratio. LOD was defined as the lowest concentration that produced a detectable peak, with a minimum S/N ratio of 3:1. LOQ was accepted as the lowest concentration on the calibration curve with an RSD ≤ 20% and an S/N ratio of at least 5:1.

Accuracy-The accuracy of the method was assessed by comparing the peak areas of extracted QC samples

(LQC, MQC, and HQC) with those obtained from blank plasma samples spiked post-extraction at the corresponding nominal concentrations. The percentage accuracy was calculated by relating the measured concentrations to the nominal values.

$$\text{Accuracy}=\frac{\text{Mean observed concentration}}{\text{Nominal Concentration}}\times 100$$

Precision- Intra-day precision was determined by analyzing QC samples at three concentration levels (LQC: 0.3 µg/mL, MQC: 4.5 µg/mL, and HQC: 9.0 µg/mL) with six replicates (n = 6) at each level on the same day. Inter-day precision was evaluated by analyzing QC samples at the same three levels on three consecutive days, with six replicates per level and corresponding calibration curves prepared in duplicate each day. Precision was expressed as the coefficient of variation (CV, %RSD) of the measured concentrations.

$$\text{Coefficient of variation (CV)}=\frac{\text{Standard Deviation}}{\text{Mean Observed Concentration}}\times 100$$

Carryover test- A major concern in the analysis of several drugs is their tendency to adhere to octadecyl (C18) reversed-phase stationary phases, which can lead to undesirable carry-over effects during chromatographic runs.

Results and Discussion

Method development and optimization-

Selection of Detection Wavelength

UV spectra of INH and MOXI were recorded in the range of 200–400 nm using a UV-Visible spectrophotometer to determine the maximum absorbance. INH exhibited λmax at 277 nm, while MOXI showed λmax at 296 nm.

Optimization of Mobile Phase

Multiple trials were performed with various compositions of mobile phases including methanol, acetonitrile, and water with or without buffer systems such as phosphate and acetate buffers at different pH values.

The best resolution, peak symmetry, and baseline stability were observed using a mobile phase consisting of water: acetonitrile in the ratio 85:15 (v/v) for Isoniazide and water: acetonitrile in the ratio 80:20 (v/v) for Moxifloxacin. The mobile phase was filtered through a 0.22 µm membrane filter and degassed before use.

Column Selection

Different reversed-phase columns such as C8 and C18 were evaluated. Ultimately, a C18 column (Phenomenex OOG 2 C18 column, 250 mm × 4.6 mm, 5 µm) was selected due to its ability to provide excellent resolution, peak shape, and reproducibility for both analytes.

Selection of Flow Rate and Run Time

Flow rates ranging from 0.8 to 1.5 mL/min were tested. A flow rate of 1.0 mL/min was selected as optimal since it provided good peak separation with reduced analysis time. The total run time was found to be less than 10 minutes, allowing for high-throughput analysis.

Sample Preparation Method

To extract INH and MOXI from plasma samples, protein precipitation techniques were evaluated. Protein precipitation using acetonitrile: acetone in a 3:1 ratio resulted in better recovery and cleaner chromatograms compared to other methods.

Sample Preparation Steps:

To 50 µL of plasma, 200 µL of cold acetonitrile containing IS was added. The mixture was vortexed for 1 minute and centrifuged at 10,000 rpm for 10 minutes at 4 ^{0} C. The supernatant was transferred and filtered through 0.22 µm membrane filter before injection into the HPLC system.

Injection Volume and System Suitability

An injection volume of 10 µL was chosen to ensure sufficient response without overloading the column. System suitability was evaluated based on parameters such as retention time, peak area, tailing factor, and theoretical plates. The results met the acceptance criteria, confirming method consistency

Chromatographic System Suitability-

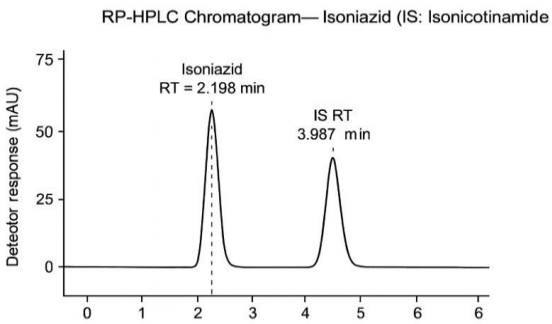


Figure 2. chromatogram of isoniazide with internal standard

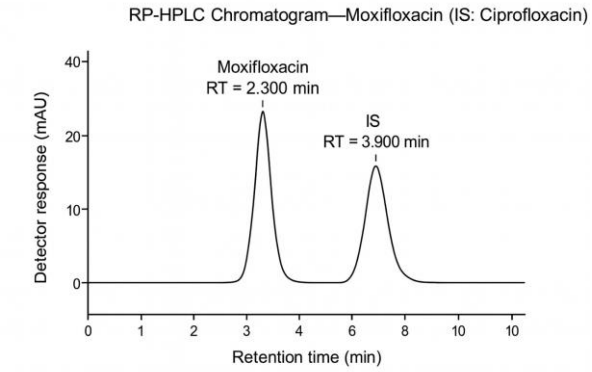


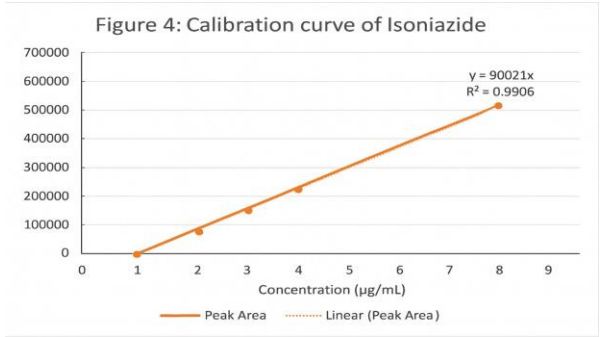
Figure 3. chromatogram of Moxifloxacin with inenal standard

Calibration Curve and Linearity Study

Calibration standards of INH and MOXI were prepared separately in plasma and analysed under optimized chromatographic conditions. Peak area against nominal concentration to generate the calibration curve was shown in table 1.

Table 1: Calibration Data of Isoniazid and Moxifloxacin at Different Concentrations

Particular	Concentration (µg/mL)	Peak Area (INH)	Peak Area (MOXI)
LLOQ	0.1	1,25,343	1,21,237
CC1	0.5	1,90,173	1,35,739
CC2	1	2,56,653	1,62,136
CC3	2.5	3,30,659	1,81,694
CC4	5	4,60,257	2,13,954
CC5	7.5	5,28,937	2,35,991
CC6	8	6,51,037	2,63,043
ULOQ	10	7,16,723	2,87,904



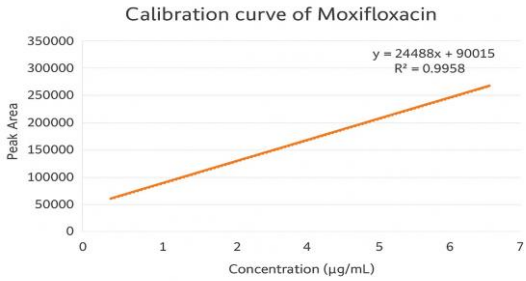


Figure 05 –Calibration curve of Moxifloxacin

The method showed excellent linearity over the concentration range of 0.1-10 µg/mL for INH and 0.1-10 µg/mL for MOXI with regression coefficients (R^2) of 0.9906 and 0.9958 respectively.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection (LOD) and limit of quantification (LOQ) for Isoniazid and Moxifloxacin were calculated based on the standard deviation of the response and the slope of the calibration curve as per ICH guidelines. The following formulas were used:

$LOD = (3.3 \times \sigma) / S$, $LOQ = (10 \times \sigma) / S$

Where:

σ = Standard deviation of the response (typically the y-intercepts of regression lines from multiple calibration curves)

S = Slope of the calibration curve

The values of LOD and LOQ obtained for INH and MOXI are shown in the table 2 as below:

Table 2- The values of LOD and LOQ obtained for INH and MOXI

Drug	Slope (S)	SD of Response (σ)	LOD (µg/mL)	LOQ (µg/mL)
INH	51,184.6	2,121.2	0.136	0.414
MOXI	12,863.9	471.2	0.121	0.366

The results indicate that the developed method is highly sensitive and capable of detecting and quantifying low levels of INH and MOXI in plasma samples.

Accuracy

The accuracy of the developed HPLC method was determined by analyzing quality control samples at three concentration levels (low, medium, and high) in six replicates (n = 6). The percent recovery was calculated by comparing the measured concentrations with the nominal concentrations. The results are summarized in table 3 as below.

Table 3-Accuracy and Precision Data for QC Samples of Isoniazid and Moxifloxacin

Drug	QC Level	Nominal Conc. (µg/mL)	Mean Measured Conc. (µg/mL)	% Recovery	% RSD
INH	LQC	0.3	0.095	95.0	2.4
INH	MQC	4.5	4.77	95.4	1.8
INH	HQC	9	9.76	97.6	1.6
MOXI	LQC	0.3	0.094	94.0	3.1
MOXI	MQC	4.5	4.61	92.2	2.0
MOXI	HQC	9	9.82	98.2	1.7

The accuracy results for INH and MOXI were evaluated at three different concentration levels. For INH, the mean percentage recovery ranged from 95.0% to 97.6%, and for MOXI, it ranged from 94.0% to 98.2%. All values were within the acceptable limits as per regulatory guidelines (±15% of nominal concentration), indicating that the method is accurate for quantitative determination of both drugs in plasma samples.

Precision

The precision of the method was assessed in terms of intra-day (repeatability) and inter-day (intermediate precision) at three concentration levels (LQC, MQC, HQC) for both INH and MOXI. Intra-day and Inter-day Precision Data for QC Samples of Isoniazid and Moxifloxacin are shown in table 4 and the results are presented as %RSD.

Formula used: %RSD = (Standard Deviation / Mean) × 100

Table 4-Intra-day and Inter-day Precision Data for QC Samples of Isoniazid and Moxifloxacin

Drug	QC Level	Nominal Conc. (µg/mL)	Intra-day Mean ± SD (µg/mL)	Intra-day %RSD	Inter-day Mean ± SD (µg/mL)	Inter-day %RSD
INH	LQC	0.3	0.096 ± 0.0023	2.4 %	0.095 ± 0.0027	2.8 %
INH	MQC	4.5	4.87 ± 0.082	1.7 %	4.84 ± 0.091	1.9 %
INH	HQC	9	9.78 ± 0.142	1.45 %	9.74 ± 0.158	1.6 %
MOXI	LQC	0.3	0.094 ± 0.0028	3.0 %	0.093 ± 0.0030	3.2 %
MOXI	MQC	4.5	4.79 ± 0.088	1.8 %	4.76 ± 0.095	2.0 %
MOXI	HQC	9	9.81 ± 0.136	1.38 %	9.77 ± 0.141	1.44 %

The intra-day and inter-day precision results showed %RSD values well within the acceptable limits of ≤15% across all QC levels. For INH, intra-day %RSD ranged from 1.45 % to 2.4% and inter-day %RSD ranged from 1.6 % to 2.8%. Similarly, MOXI showed intra-day precision between 1.38 % and 3.0%, and inter-day precision between 1.44 % and 3.2%. These results indicate that the developed HPLC method is precise and reproducible for the simultaneous estimation of INH and MOXI in plasma.

Carryover Test

Carryover was evaluated to determine whether residual analyte from one injection could influence subsequent analyses. This parameter is essential in bioanalytical method validation to ensure accurate quantification of isoniazid (INH) and moxifloxacin (MOXI). For assessment, a blank plasma sample was injected immediately after the upper limit of quantification (ULOQ) standard. Chromatograms of the blank were examined at the retention times of the analytes and internal standard (IS). According to acceptance criteria, the peak response in the blank should not exceed 20% of the mean LLOQ response for the analyte and 5% of the IS response. The results confirmed that carryover was within these limits, as no detectable peaks corresponding to INH, MOXI, or IS were observed, indicating negligible carryover.

Conclusion

The present study was undertaken to develop and validate a simple, sensitive, and reproducible bioanalytical method for the simultaneous and separate estimation of isoniazid (INH) and Moxifloxacin (MOXI) in human plasma using High-Performance Liquid Chromatography (HPLC).The developed method was optimized by evaluating different chromatographic conditions including mobile phase composition, flow rate, detection wavelength, and extraction procedures. Both drugs exhibited good resolution and peak symmetry under the selected conditions. The extraction procedure ensured high recovery and minimal matrix interference. The validated method was subjected to rigorous validation as per the current USFDA and ICH guidelines, including parameters such as linearity, accuracy, precision, sensitivity, selectivity, recovery, carryover etc. The calibration curves for both INH and MOXI were linear over the tested concentration ranges, with correlation coefficients (R^2) greater than 0.9554. The limits of detection (LOD) and quantification (LOQ) demonstrated the method's high sensitivity. Precision and accuracy results were well within acceptable limits, indicating the method's reliability. Carryover was negligible. The validated method is suitable for routine quantitative analysis of INH and MOXI in plasma samples and can be effectively applied for pharmacokinetic, bioavailability, and therapeutic drug monitoring studies in clinical settings.

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Conflict of Interest

The author(s) declare that there is no conflict of interest regarding the publication of this project work.

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