



RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR
SIMULTANEOUS ESTIMATION OF ISONIAZID AND PYRAZINAMIDE

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RESEARCH ARTICLE

Abstract

A sensitive and validated RP-HPLC method has been developed for simultaneous estimation of Isoniazid and Pyrazinamide. In RP-HPLC method, methanol:buffer in the ratio of 55:45 v/v was selected as a mobile phase which gives good resolution and good peak shapes for Isoniazid and Pyrazinamide. The flow rate was set at 1.0 ml/min, and the detection was carried out with UV detector at 263 nm. Inertsil-ODS C₁₈ (250 x 4.6 mm, 5 μ) column was used for the separation. The total run time required was 10 mins. The linearity and range was established over the range of 20-80 μg/ml concentration range for Isoniazid and Pyrazinamide respectively. The correlation coefficient of Isoniazid and Pyrazinamide was found to be 0.999. The method validation data showed excellent results for accuracy, precision, linearity, specificity, limit of detection, limit of quantification and robustness. The present method can be successfully used for routine quality control analysis.

Key words: Isoniazid, pyrazinamide, RP-HPLC and validation

INTRODUCTION

Chemically, Isoniazid (Fig.1) is pyridine-4-carbohydrazide. It is a hydrazide of isonicotinic acid ¹. It is the first line antitubercular medication never used

on its own to treat active tuberculosis because resistance quickly develops ². Isoniazid is still considered the primary drug for the chemotherapy of tuberculosis. Isoniazid is bacteriostatic for "resting"

bacilli, but is bactericidal for rapidly dividing microorganisms. Isoniazid is a prodrug; mycobacterial catalase-peroxidase converts Isoniazid into an active metabolite. A primary action of Isoniazid is to inhibit the biosynthesis of mycolic acids³.

Chemically, Pyrazinamide (Fig.1) is pyrazine-2-carboxamide. Pyrazinamide has been elevated to first-line status in short-term tuberculosis treatment regimens because of its tuberculocidal activity and comparatively low short-term toxicity⁴⁻⁶. Pyrazinamide is a mysterious, unconventional and paradoxical drug. Pyrazinamide is primarily active against growing bacteria and no or little activity for non-growing persisters. Pyrazinamide is only used in combination with other drugs and Isoniazid is a prodrug and must be activated by a bacterial catalase-peroxidase enzyme, which has the serious problem of drug resistance. Thus, the two drugs can be used to improvise the activity and nullifies the drug resistance. Literature survey reveals that there is only one HPLC method was reported for simultaneous estimation of Pyridoxine hydrochloride, isoniazid, pyrazinamide and rifampicin in pharmaceutical formulations⁷. Therefore, an attempt has been made to develop a novel, rapid, accurate and precise RP-HPLC method for simultaneous estimation of Isoniazid and Pyrazinamide in tablet dosage form and validated in accordance with ICH guidelines⁸.

MATERIALS AND METHODS

Instrumentation:

To develop a high performance liquid chromatographic method for simultaneous estimation of Isoniazid and Pyrazinamide using Waters 2695 on Inertsil ODS C18 (250 mm ×4.6 mm ID, 5 μ particle

size) column was used. The instrument is equipped with an auto sampler and UV-Visible detector. A 20 μ rheodyne injector port was used for injecting the samples. Data was analyzed by using Spinchrome software. A Global digital pH meter was used for pH measurements.

Chemicals and solvents:

The working standards of Isoniazid and Pyrazinamide were provided as gift samples from Spectrum Labs, Hyderabad, India. HPLC grade water and acetonitrile were purchased from E.Merck (India) Ltd., Mumbai, India. Methanol and potassium dihydrogen phosphate of AR grade was obtained from S.D. Fine Chemicals Ltd., Mumbai, India.

Analytical method development:

Chromatographic conditions:

An Inertsil ODS C18 (250 mm ×4.6 mm ID, 5 μ particle size) was used for chromatographic separation. The mobile phase composed methanol:buffer in the ratio of 55:45 v/v; pH adjusted to 4 with triethylamine. Finally the detection is at a flow rate of 1 ml/min with run time of 10 minutes. Mobile phase and sample solutions were filtered through a 0.45 μ membrane filter and degassed. The detection of both drugs was carried out at 263 nm.

Mobile phase preparation:

Mix methanol:buffer in the ratio of 55:45 v/v; pH adjusted to 4 with triethylamine and sonicates the resulting solution and degasses it using vacuum filtration through 0.45 μ membrane filter.

Preparation of standard stock solution:

The solution was prepared by dissolving 20.0 mg of accurately weighed Isoniazid and 25.0 mg Pyrazinamide in mobile phase, in two 100.0 ml

volumetric flasks separately and sonicate for 20 min. From the above solutions take 10.0 ml from each solution into a 50.0 ml volumetric flask and then makeup with mobile phase and sonicate for 10 min.

Standard preparation:

The stock solutions equivalent to 20-80 µg/ml with respect to both drugs were prepared in combination of Isoniazid and Pyrazinamide above, sonicated and filtered through 0.45 µ membrane.

Procedure:

The column was maintained at an ambient temperature. The run time was set at 10 minutes. The column was equilibrated by pumping the mobile phase through the column for at least 30 minutes prior to the injection of the drug solutions. Inject 20 µl of blank solution, placebo solution, standard solution, disregard peaks due to blank and placebo if any. The optimum chromatogram of the drugs is presented in Fig. 2.

VALIDATION OF METHOD

The HPLC method was validated in accordance with ICH guidelines.

Linearity:

Several aliquots of standard solutions of Isoniazid and Pyrazinamide were taken in six different 10 ml volumetric flasks and diluted up to the mark with diluents such that the final concentrations were in the range of 20-80 µg/ml for Isoniazid and Pyrazinamide. The above solutions were injected into the HPLC system keeping the injection volume constant. The drugs were eluted with UV detector at 263 nm, peak areas was recorded for all the peaks. The linearity curves were constructed by plotting concentration of

drugs against peak areas. The regression equation of this curve was computed. This regression equation was later used to estimate the amount of drugs in tablet dosage forms.

Precision:

The standard solution of Isoniazid and Pyrazinamide was injected for six times and the area for all six injections was measured in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:

To study the accuracy of the method, recovery studies were carried out, by adding a known quantity of the standard to the pre analyzed sample and recovery study were done. The recovery was carried out at 50%, 100%, 150% level and the contents were determined from respective chromatogram.

System suitability:

The system suitability parameters like retention time, theoretical plates and tailing factor were evaluated by six replicate analyses of Isoniazid and Pyrazinamide and compared with standard values. The acceptance criteria are %RSD of peak areas not more than 2%, theoretical plates numbers (N) at least 2000 per each peak and tailing factors not more than 2.0 for Isoniazid and Pyrazinamide.

Specificity and Selectivity:

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix. There should not be any peak in the chromatogram at the retention time of main analyte in the blank and placebo sample injection.

Limit of detection and Limit of quantitation:

Sensitivity of the proposed method was estimated in terms of limit of detection (LOD) and limit of quantitation (LOQ). $LOD = 3.3 \times ASD/S$ and $LOQ = 10 \times ASD/S$, Where, 'ASD' is the average standard deviation and 'S' is the slope of the line.

Robustness:

For demonstrating the robustness of the developed method experimental conditions were purposely altered and evaluated. As part of the robustness, deliberate change in the flow rate, mobile phase composition was made to evaluate the impact on the method.

Assay:

Standard preparations are made from the bulk drug and sample preparations are made from commercial formulation. Both standard and sample solutions were injected in six homogeneous samples. 20 μ l of sample solution was injected and from the peak areas of Isoniazid and Pyrazinamide, amount of each drug in the sample were computed. The results were compared with the label claim of Isoniazid and Pyrazinamide in tablet dosage forms. From the results the average %Assay was calculated.

RESULTS AND DISCUSSION

Mobile phase was optimized to separate Isoniazid and Pyrazinamide using Inertsil ODS C18 column (250 mm x 4.6 mm ID, 2.5 μ m). Initially, mix methanol:buffer in the ratio of 55:45 v/v were tried as mobile phase but the splitting of the peaks for both these drugs was observed. Therefore, after

adjustment of pH of mixed phosphate buffer to 4 with triethyle amine, mobile phase composition (methanol:buffer in the ratio of 55:45 v/v) was tried for resolution of both drugs. Good resolution and symmetric peaks were obtained. The flow rate of the mobile phase was 1 ml/min. Under optimum chromatographic conditions, the retention time for Isoniazid and Pyrazinamide were found to be 2.869 and 3.943 minutes, respectively when the detection was carried out at 263 nm.

The Linear detector response for Isoniazid and Pyrazinamide is demonstrated by concentration versus area. Linearity range is obtained over the range of 20-80 μ g/ml concentration range for Isoniazid and Pyrazinamide. The correlation coefficient (r^2) was found to be 1 for both Isoniazid and Pyrazinamide respectively. The calibration curve was constructed by plotting concentration of standard solutions against mean peak areas and the regression equation was computed. The results show that an excellent correlation exists between peak area and concentration of drugs within the concentration range indicated. The summary of the parameters is shown in Table 1.

The %RSD for 6 replicates for Isoniazid and Pyrazinamide were found to be 0.935% and 0.436% respectively (limit %RSD<2.0%) and hence the method is precise. The precision data of Isoniazid and Pyrazinamide were furnished in Table 2.

The %Recovery of the drugs Isoniazid and Pyrazinamide were found to be 99.19 to 100.05% and 99.46 to 100% respectively and the high percentage of recovery of Isoniazid and Pyrazinamide indicates that the proposed method is highly accurate. The

results of accuracy studies of Isoniazid and Pyrazinamide were shown in Table 3.

The retention times for the drugs Isoniazid and Pyrazinamide was 2.869 and 3.943 minutes respectively. The number of theoretical plates calculated for Isoniazid and Pyrazinamide was 8730.398 and 5934.251 respectively. The tailing factor for Isoniazid and Pyrazinamide was 1.110 and 1.236 respectively, which indicates efficient performance of the column. The limit of detection (LOD) and limit of quantification (LOQ) for Isoniazid were found to be 0.34 and 1.05 µg/ml; 0.25 and 0.77 µg/ml for Pyrazinamide respectively, which indicate the sensitivity of the method. The summary of system suitability parameters and validation parameters were shown in Table 4.

The robustness studies indicated that no considerable effect on the determination of the drugs. Therefore the test method is robust for the quantification of the drugs. In all deliberately varied conditions, the % RSD for replicate injections of Isoniazid and Pyrazinamide were found to be within the acceptable limits. The robustness results are shown in Table 5.

Validated method was applied for the simultaneous estimation of Isoniazid and Pyrazinamide in commercial tablet dosage forms. The %Assay of Isoniazid and Pyrazinamide were found to be 100.52% and 99.68% respectively. The results for the drugs assay showed good agreement with label claims. No interfering peaks were found in the chromatogram of the tablet formulation within the run time indicating that excipients used in tablet formulation did not interfere with the simultaneous estimation of the drugs Isoniazid and Pyrazinamide

by the proposed HPLC method. The assay results are shown in Table 6.

The chromatograms were checked for appearance of any extra peaks under optimized conditions, showing no interference from common tablet excipients and impurities. Also the peak areas were compared with standard and were found to be within limits. As shown in chromatogram, two analytes are eluted by forming symmetrical peaks.

CONCLUSION

A new RP-HPLC method for the simultaneous analysis of Isoniazid and Pyrazinamide 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. 10 min, what enables rapid quantitation of many samples in the routine and quality control analysis of tablet formulation. 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 two drugs in combined dosage forms in quality-control laboratories.

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RERERENCES

1. Florey K. Analytical profiles of drug Substances, Vol. 5, Academic Press, New York, 2005; 183 - 190.
2. <https://www.drugbank.ca/drugs/DB00951>.
3. Indian Pharmacopoeia, Vol. II, the Controller Publication, Govt. of India, New Delhi, 2010; 1515-1517.
4. Zhang Y, Wanliang S, Zhang W and Mitchison D. Mechanisms of Pyrazinamide Action and Resistance. Microbiology Spectrum 2013; 2(4): 1-12.
5. Indian Pharmacopoeia, Vol. II, the Controller Publication, Govt. of India, New Delhi, 1996; 644.
6. Florey K. Analytical profiles of drug Substances, Vol. 5, Academic Press, New York, 2005; 433.
7. Dhal SK and Sharma R. Chemia Analityczna 2009; 54(6): 1487-1499.
8. ICH Harmonised Tripartite Guideline, Validation of analytical procedures: Text and methodology, Q2(R1), International Conference on Harmonization, Geneva. 2005; 1-13.

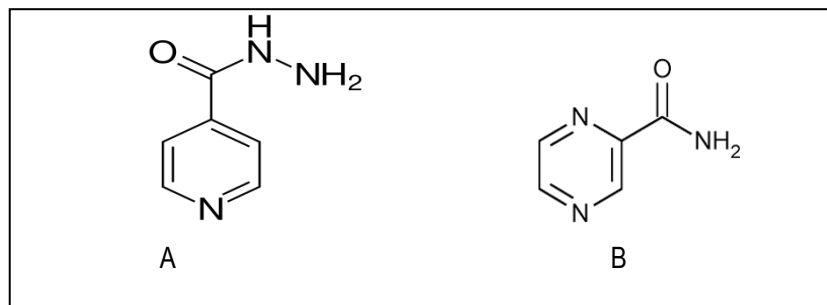


Fig. 1: Chemical structures of analytes
Chemical structure of A. Isoniazid and B. Pyrazinamide

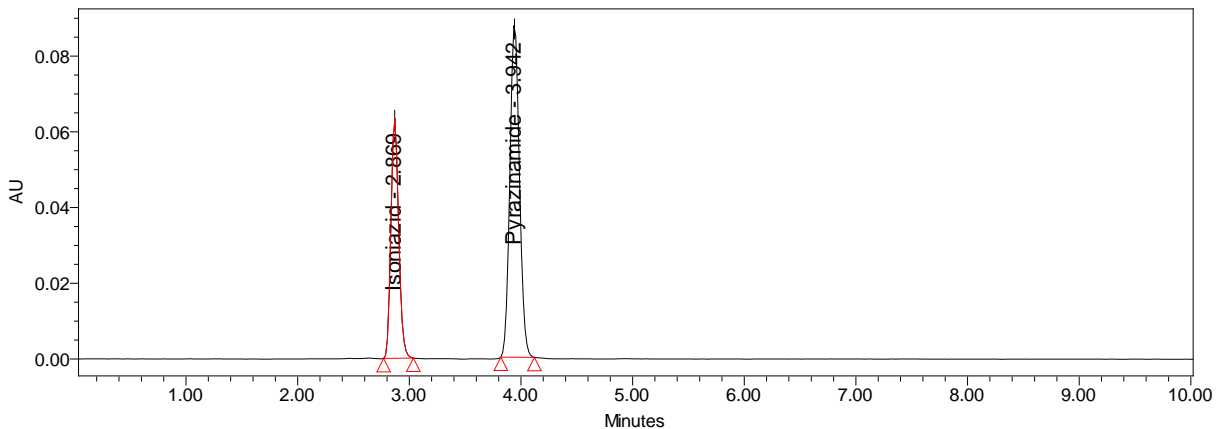


Fig. 2: Optimised Chromatogram

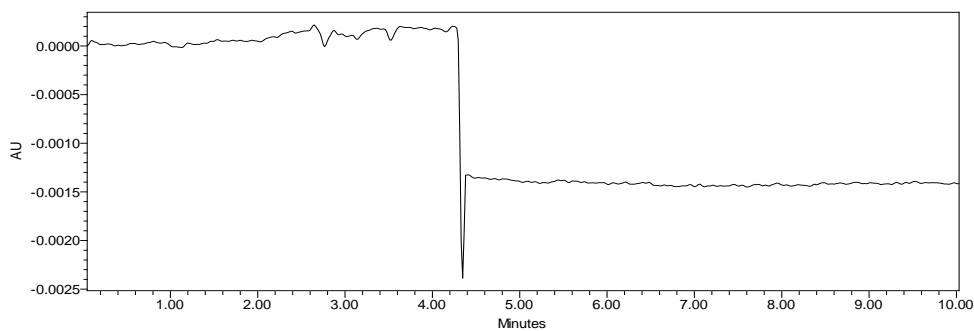


Fig. 3: Placebo chromatogram

Table 1: Linearity-Regression Equation Parameters

Parameter	Isoniazid	Pyrazinamide
Linearity range ($\mu\text{g/ml}$)	20–80	20–80
Correlation co-efficient	0.999	0.999
Slope	20193	31282
Y-intercept	1902	11218

Table 2: Precision Studies

Parameters	Isoniazid	Pyrazinamide
Repeatability		
% RSD of %Assay	0.935987	0.43652
% RSD of Peak Area	0.447297	0.500472
Intermediate Precision		
% RSD of %Assay	0.337299	0.250755
% RSD of Peak Area	0.250755	0.200278

Table 3: Accuracy Data

% Concentration level	Isoniazid			Pyrazinamide		
	Conc. added (µg/ml)	Conc. found (µg/ml)	% Recovery	Conc. added (µg/ml)	Conc. found (µg/ml)	% Recovery
50%	20	19.97	99.88	20	19.89	99.46
100%	40	40.02	100.05	40	39.77	99.76
150%	60	59.51	99.19	60	60.00	100.00

Table 4: System Suitability Results

Parameters	Isoniazid	Pyrazinamide	Range
%RSD of peak area	797201	1138711	<1.0 for n≥6
%RSD of retention time	2.869241	3.943118	<1.0 for n≥6
USP Tailing factor (T)	1.110234	1.236496	T < 2
USP Plate Count (N)	8730.398	5934.251	>2000
USP Resolution (R)		0.98	R > 2

Table 5: Robustness Study

Parameter	Variation	Chromatographic Conditions			
		Std Area		Tailing Factor	
		Isoniazid	Pyrazinamide	Isoniazid	Pyrazinamide
Flow Change	0.8 ml/min	797201	1138711	1.110234	1.236496
	1 ml/min	802606.4	1149717	1.120899	1.247117
	1.2 ml/min	801593	1148852	1.118282	1.245813

Table 6: Assay Results

Drug	Labeled Amount (mg/tab)	Amount found (mg/tab)	% of Assay
Isoniazid	75	75.39	100.52
Pyrazinamide	250	249.2	99.68