International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.3, No.3, pp 1275-1280, July-Sept 2011

Development and Validation of RP-HPLC Method for Quantitative estimation of Pyrazinamide in Bulk and Pharmaceutical dosage forms

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Abstract: A simple, precise, specific and accurate Reverse phase HPLC method has been developed for the determination of Pyrazinamide in bulk and pharmaceutical dosage forms. Chromatography was performed on a Hypersil C_8 (4.6 x 250mm, 3.5 µm) column with Phosphate buffer (pH 4.4): Methanol 80:20 (v/v) as a mobile phase at a flow rate of 1 ml/ min. Detection was performed at 269 nm. The retention time of Pyrazinamide was found to be 3.62min. By adoption of this procedure Pyrazinamide (PYZ) is eluted completely. Linear calibration plots were obtained between 20-120µg/ml.The method of analysis was used for quantification of PYZ in pharmaceutical preparations with a coefficient of variation <2%. Results of analysis were validated statistically and by recovery studies. The method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision, ruggedness and robustness.
Keywords: Pyrazinamide, Phosphate buffer, Methanol, Coefficient variation.

INTRODUCTION

Pyrazinamide is chemically pyrazine-2-carboxamide, the molecular formula is $C_5H_5N_3O$ and molecular weight is 123.1g/mol. The structural formula is shown in **Fig 1.** It is soluble in water and in chloroform; slightly soluble in ethanol (95 percent) and very slightly soluble in ether.[1-3] Pyrazinamide, the pyrazine analogue of nicotinamide, is an anti-tuberculous agent, an orally administered and First-line drug. Pyrazinamide exhibits bactericidal activity *in vitro* only at a slightly acidic pH. Activity at acid pH is ideal, since *M. tuberculosis* resides in an acidic phagosome within the macrophage.[4,5] It is official drug in Indian Pharmacopoeia 2007 [6], British Pharmacopoeia 2000[7], and United States Pharmacopoeia 2007.[8] From the literature survey, it was found that Pyrazinamide was estimated by analytical methods such as reversed-phase high-performance liquid chromatographic (RP-HPLC) method [9-13],gas chromatography[14] ,UPLC method[15] and some UV-Visible methods[16,17].

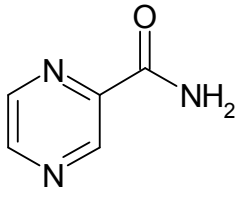

Fig: 1. Chemical Structure of Pyrazinamide

The present developed method was simple, precise, specific and accurate. The statistical analysis proved that method is reproducible and selective for the analysis of Pyrazinamide in bulk drug and tablet formulations.

EXPERIMENTAL

MATERIALS AND METHODS

Shimadzu HPLC prominence model equipped with Auto sample, UV detector with Empower 2 software was employed for the investigation. All the chemicals used in the investigation were of HPLC grade. The chromatographic analysis was performed on a Hypersil C8 (4.6 x 250mm, 3.5 µm) column. The mobile phase consists of buffer (pH 4.4) and methanol in the ratio of 80:20 (v/v). The optimized chromatographic conditions are summarized in **Table 1.**

Preparation of mobile phase

100ml of HPLC grade methanol was mixed with 400ml of sodium dihydrogen orthophosphate buffer prepared in double distilled water and its pH was adjusted to 4.4 using o-phosphoric acid. Then it was ultrasonicated for 20 minutes and then filtered through 0.45µm Nylon 66 (N_{66}) 47mm membrane filter paper.

Preparation of standard stock solution

10 mg of standard drug was weighed accurately and transferred to 100 ml volumetric flasks. The drug was dissolved in 50 ml of mobile phase with shaking and then volume was made up to the mark with mobile phase to get 100 µg/ml of standard stock solution of each drug. This stock solution was filtered through 0.2 µm Nylon 66 (N66) 47mm membrane filter paper.

Preparation of marketed formulations

Twenty tablets of Pyrazinamide were weighed and the average weight was determined. The tablets were then crushed to fine powder and powder equivalent to 100 mg was weighed and transferred to 100 ml volumetric flask and dissolved in sufficient quantity of mobile phase. The contents were ultrasonicated for 20 minutes and the final volume was made up to the mark with mobile phase. The above prepared solution was then filtered through 0.2 µm Nylon 66 (N66) 47 mm membrane filter paper and was used as standard stock solution.

Chromatographic condition

The mobile phase containing methanol and sodium dihydrogen orthophosphate in the ratio of 20:80 was selected as the optimum composition of mobile phase with 4.4 pH, as this solvent system resolved for the component ideally. The flow rate was set to 1.0 ml/min and UV detection was carried out at 269 nm. The mobile phase and sample was degassed by sonication for 20 min and filtered through 0.45 µm Nylon 66 (N66) 47 mm membrane filter paper. All determinations were performed at constant column temperature (25^0C).

Table 1: Optimized Chromatographic conditions for the proposed method

Parameters	Optimized condition
Linear range (µg/ml)	20-120
Detection wavelength (nm)	269
Temperature	25^oC
Retention Time (t) (min)	3.62
Run time (min)	10
Limit of Detection (µg/ml)	0.0045
Limit of Quantification (µg/ml)	0.013

Preparation of Calibration Curve and Analysis of Pyrazinamide

Appropriate aliquots were pipetted out from the standard stock solution (100µg/ml) in to a series of 10 ml volumetric flasks. The volume was made up to the mark with the mobile phase to get a set of solutions having the concentration range, ranging from 20-120µg/ml of the drug. The solutions were injected using a 20 µl fixed loop in to the chromatographic system at the flow rate of 1.0 ml / min and the effluents were monitored at 269 nm, chromatograms were recorded. The Pyrazinamide was eluted at 3.62 min as shown in **Fig: 2.**

The calibration curve was constructed by plotting average peak area versus concentration and was presented in **Fig: 3**.The method was extended for determination of Pyrazinamide in pharmaceutical dosage form containing 500 mg.

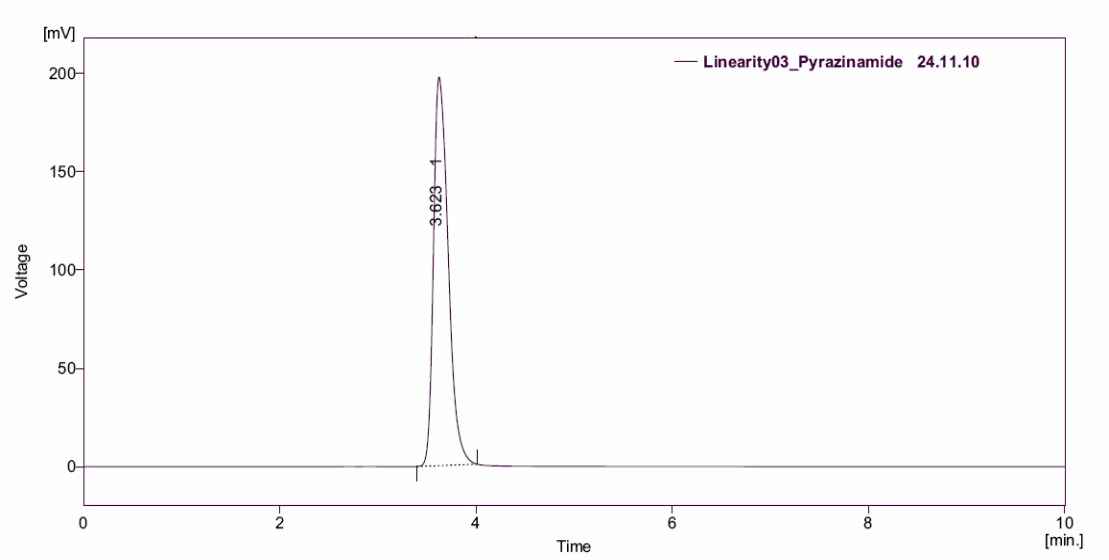

Fig: 2. Chromatogram of Pyrazinamide by RP-HPLC method

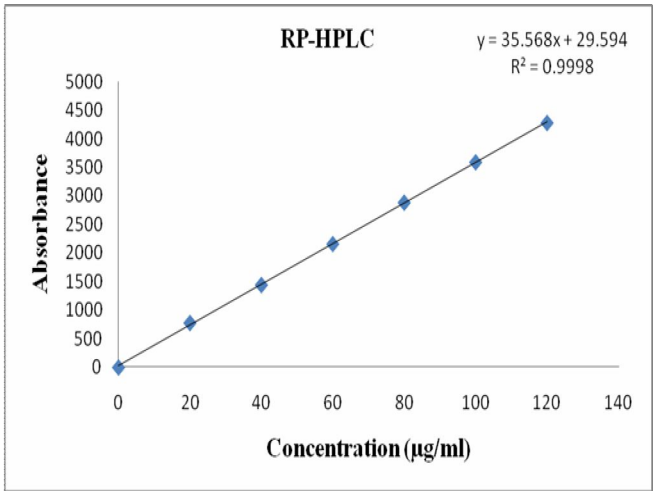

Fig: 3. Calibration curve of Pyrazinamide at 269 nm by RP–HPLC method.

Analysis of Pyrazinamide in Pharmaceutical formulations

The procedure for the preparation of the sample solution remains same as explained above. From this stock solution, various dilutions of the sample solution were prepared and analysed. A 20 µl volume of each sample solution was injected in to the sample injector of HPLC system and their chromatograms were recorded under the same chromatographic conditions as described above. The area of each peak was determined at 269 nm and the amount of drug present in the sample was determined.

Method validation [18-20]

The developed analytical method was subjected to validation with respect to various parameters such as accuracy, precision, linearity and range, robustness, ruggedness, LOD and LOQ of the proposed method was validated as per the ICH guidelines.

Table 2: System Suitability Test Parameters for the proposed method

Parameters	Optimized condition
Retention Time (t) (min)	3.62
Theoretical plates (N)	2554
Peak asymmetry	1.758

Table 3: Regression analysis of the Calibration curve for the proposed method

Parameters	Optimized condition
Linearity range (µg/ml)	20-120
Regression equation (Y=mx +c)	
Slope (m)	35.56
Standard deviation of slope (S_m)	0.035501
Intercept (c)	29.594
Standard deviation of Intercept (S_c)	1.5715
Correlation coefficient (r^2)	0.9998
Relative standard deviation (%)	0.009095
Retention time	3.62
Range of error	0.147329

Table 4: Summary of Validation Parameters for the proposed method

Parameters	Values
Limit of detection (µg/ml)	0.0045
Limit of quantitation (µg/ml)	0.013
[a]Accuracy (% RSD)	
80%	0.0111
100%	0.0180
120 %	0.2571
[a]Precision (% RSD)	
Intra Day precision	0.227545
Inter Day precision	0.373256
[a]Ruggedness (% RSD)	
Analyst I	0.007071
Analyst II	0.021213
[a]Robustness (% RSD)	
Changed condition I (Flow rate)	
0.9 ml/min	0.7791
1.1 ml/min	0.7813
Changed condition II (Detection nm)	
263nm	0.5788
265nm	0.7685

[a]Mean of six determination s,RSD indicates relative Standard deviation

Table 5: Assay Results of Pyrazinamide using proposed method

Brand name	Labeled amount (mg)	Amount found (mg)	%Recovery ± SD**
Brand I	500	499.86	99.97±0.4546
Brand II	500	500.24	100.04±0.4744

RESULTS AND DISCUSSION

In this method the conditions were optimized to obtain complete elution of Pyrazinamide. Mobile phase and flow rate selection was based on peak parameters (height, tailing factor, theoretical plates, capacity or asymmetry), run time, resolution. The system with methanol: Sodium dihydrogen orthophosphate buffer (0.1M) (20:80 v/v) with pH 4.4.

The run time was set at 10 min and the retention time for Pyrazinamide was found 3.62 min as shown in Fig: 2.The sample solution was injected 6 times and the retention times were found to be same. When the concentrations of Pyrazinamide and its respective peak areas were subjected to regression analysis, a good linear relationship (r^2 = 0.9998) was observed between the concentration of Pyrazinamide and the respective peak areas in the range 20-120 µg/ml. The regression of Pyrazinamide was found to be Y = 35.568X + 29.594, where 'Y' is the peak area and 'X' is the concentration of Pyrazinamide. (Table 3).

The regression equation was used to estimate the amount of Pyrazinamide, either in tablet formulations or in validation study (precision and accuracy).

The proposed RP-HPLC method was validated for intra and inter-day variation. When the solution of Pyrazinamide was repeatedly injected on the same day, the coefficient of variance (%CV) in the peak area for three replicate injections was found to be 0.2279. Also the inter day variation was found to be 0.3750. (Table 4)

A known amount of the drug solution (80%, 100% and 120 %) was added to the powder sample of the tablet formulation and subjected to the estimation of the drug for the recovery studies. There was a high recovery of Pyrazinamide indicating that the proposed procedure for the determination of Pyrazinamide in the tablet formulation is highly accurate. (Table 4)

Robustness and ruggedness of the proposed method was determined by analysis of sample by changes in different parameter like flow rate, detection and analyst using similar operational and environmental conditions, the % R.S.D. reported was found to be less than 2 %.

The proposed method was validated in accordance with ICH parameters and applied for analysis of the same in marketed formulations.

CONCLUSION

Thus, it can be concluded that the method developed in the present investigation was economical, simple, sensitive, accurate, robust, rapid and precise. Hence, the above said method can be successfully applied for the estimation of Pyrazinamide in pharmaceutical dosage forms..

ACKNOWLEDGEMENT

We would like thank to Arch pharmalabs Limited Mumbai, India for providing reference sample of Pyrazinamide to facilitate this work.

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