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METHOD DEVELOPMENT AND VALIDATION OF AN ACTIVE METABOLITE OF PYRAZINAMIDE USING ULTRA VIOLET SPECTROPHOTOMETRY

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ABSTRACT

The ultra violet (UV) spectrophotometry measures a particular wavelength absorbed by the sample from the UV spectral region. This method of analysis is very rapid and simple, however, lacks sensitivity and precision while analyzing samples of low concentrations. Despite lack of sensitivity of this method it is very useful when drug concentrations to be estimated are ample and need rapid detection. This method will be useful to estimate released drug from various solid dosage forms containing higher drug concentrations. Here in, we aim to develop a rapid method of analyzing an active metabolite of pyrazinamide viz., pyrazinoic acid (POA) using UV spectrophotometry. 4 µg/ml solution of POA in deionized water was scanned in the spectral range between 200-400 nm and the maximum absorbance was observed at wavelength of 268 nm. Samples of different concentrations analyzed for determining linearity demonstrated a linear response for a quantitation in the range of 2µg/ml to 10µg/ml in the deionized water. A regression equation; y=0.0585+0.019 and r^2 =0.9997 was obtained after plotting the concentration vs. absorbance calibration curve. The method was validated for inter-day and intra-day accuracy, precision, detection limit, robustness and ruggedness. Furthermore, the method validated according to the ICH [Q2 (R1)] guidelines was found to be in acceptance limit. This quantification method is suitable for the estimation or regular quality check of POA in the bulk formulation like tablets, pharmaceutical powders and granules.

KEYWORDS: UV-Spectrophotometry; Pyrazinoic acid; Method development; Validation; Accuracy.

1. INTRODUCTION

Spectroscopy is an analytical technique which deals with the interaction of electromagnetic radiation and the matter. It is the most powerful technique available for the study of atomic and molecular structure which are used for the analysis of wide range of samples. The UV spectroscopy has the region on electromagnetic spectrum between 200 to 400 nm.[1] The region of electromagnetic spectrum is shown in the following table 1.[2]

Table 1: Different regions of the electromagnetic spectrum and corresponding range of wavelength absorption.

Region	Wavelength
Far (or vacuum) ultraviolet	10-200 nm
Near ultraviolet	200-400 nm
Visible	400-750 nm
Near infrared	700-1400 nm
Mid infrared	1400-3000 nm
Far infrared	3000-10000 nm

UV-Visible spectrophotometry is one of the most frequently and powerful used technique in the pharmaceutical analysis. This method of analysis measures the quantum of ultraviolet or visible radiation transmitted by a solute in solution and converts it into the amount of radiation absorbed. A UV-spectrophotometer measures this function as the ratio of the intensity of two beams of light transmitted from standard and unknown samples. Spectrophotometric is a very simple and rapid, method of estimating samples of moderately lower concentrations. Besides quantitative analysis this method of analysis can be used to do qualitative analysis of various organic and inorganic samples. The working principle of this technique is based on Beer and Lambert law.

Beer's law: This law states that the concentration of a solute is directly proportional to the absorbance of light by the solute molecules. In other words, on absorption of light energy by the absorbing molecules, the intensity of monochromatic radiation decreases exponentially. It relates the attenuation of the light to properties of a material. This law is not valid at high concentration.

Lambert's law: This law states that the loss in intensity when a beam of monochromatic radiation propagates in the medium is directly proportional to the intensity of incoming radiation and path length of propagation.

Beer-Lambert law: When a monochromatic beam of light passes across a transparent cell containing a solution of an absorbing compound then there is reduction in the intensity of light. This law has a linear relationship between absorbance and absorbing compound. Mathematically, Beer- Lambert law can be expressed as

$$A= \varepsilon b c$$

Where,

A= absorbance

ε= Molar absorptivity calculated as L mol^{-1} cm^{-1}

b= Sample path length (usually constant according to cuvette size)

c= analyte concentration (mol L^{-1})

Where c is in gm./100 ml, then the constant is called A (1%, 1 cm)[3]

The assay of single component in the sample which contains other absorbing substances is calculated from the measured absorbance by using one of three principal procedures. They are as follow.

1. Comparison of Absorptivity of standard vs. unknown
2. Extrapolation of unknown from the standard calibration curve
3. Standardization using single or multipoint analysis

In standard absorptivity value method, there is the use of standard A (1%, 1 cm) or ε values which are used in order to determine its absorptivity. It is advantageous in situations where it is difficult or expensive to obtain a sample of the reference substance. In calibration curve extrapolation method, the absorbance of more than three standard samples is measured as reference substance. A calibration curve is plotted between corresponding values of concentration against absorbance values. The unknown sample is then determined by extrapolating from the standard curve. The single point standardization procedure involves the measurement of the absorbance of a sample solution and of the standard solution of the reference substance. The concentration of the substances in the sample is calculated from the proportional relationship that exists between absorbance and concentration.

$$C_{test}= (A_{test} \times C_{std})/A_{std}$$

Where C_{test} and C_{std} are the concentrations of the sample and standard solutions respectively. Similarly, A_{test} and A_{std} are the absorbance's of the sample and standard solutions respectively.[4]

1.1 Pyrazinoic acid (POA)

POA is a pyrazinamide (PZA) metabolite and the effects of PZA were discovered by *Kushner et aL* in 1952. This was applied to the treatment of human tuberculosis by *Yeager et al*.[5] Hepatic toxicity, due to excessive doses, led to suspension of its use for several years but there has been more recent interest in its role in short-term antituberculous chemotherapy.[6] POA is the active form of its prodrug, PZA. The active metabolite is formed by the action of amidase enzymes produced by Mtb. It primarily accumulates intracellularly in an acidic environment and is active against non-growing persisters as compared to a majority of antibiotics, (failing to kill persisters) that are used for TB. POA is not impermissible through the mammalian cell membrane. It acts on multiple targets such as energy production, translation and maybe a pantothenate/ coenzyme therefore, , its mode of action is different from regular antibiotics.[7]

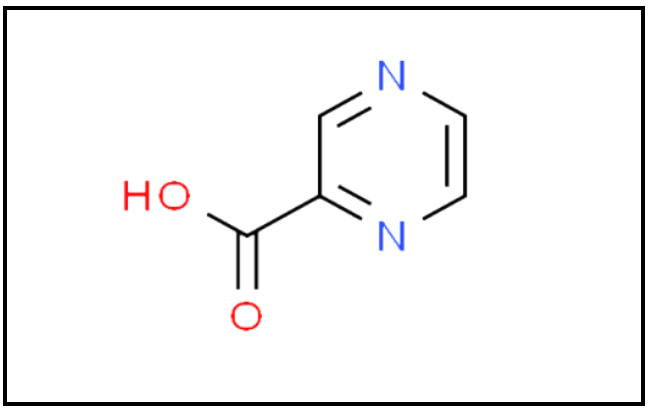

Fig. 1: Chemical structure of pyrazinoic acid (Pyrazine-2-carboxylic acid).

2. EXPERIMENTAL

2.1 Materials

Pyrazinoic acid (POA) (CAS [97-97-5]) were purchased from GLR Innovations, India. All the chemicals and reagent used for the experiments were pure and of analytical grade. A Merck Millipore, Milli Q apparatus (Bedford, MA, USA) was used for the entire experimental water requirements.

2.2 Instruments and apparatus

UV-spectrophotometer from Eppendorf® (Basic Model 6135HM604128) was used for recording absorbance of samples while analysis was done using BioSpectrometer software (SW-version 4.3.6.0).

2.3 Method development and spectrum measurement

The development of UV spectrometric method was started with the selection of the solvent system and determination of the maximum wavelength of absorption of UV light. The solubility of POA was checked in various solvent system by performing practically and also through literature survey and it was found that having maximum solubility in water and methanol. A standard solution containing drug (POA) was scanned between range of 200-400 nm and the UV spectrum was obtained.

2.4 Preparation of standard solution and calibration curve

The stock solution was prepared by dissolving 10mg of POA in 100ml of triple distilled water to obtain concentration of 100µg/ml. Further different concentration of 2, 4, 6, 8, and 10µg/ml of solution prepared by taking 2, 4, 6, 8, and 10 ml respectively from stock solution in a volumetric flask and adjusting volume up to 100ml. The absorbance of each sample was measured at λ max against blank triple distilled water on UV-visible spectrophotometer. The above procedure was repeated for three times for each sample and average of three readings of absorbance was calculated. Taking these readings, a calibration curve was plotted by taking concentration of POA in X-axis and corresponding absorbance in Y-axis.

2.5 Analytical method validation

In order to prove the suitability of method, optimized method parameters were validated as per ICH guidelines Q2A; Q2B.[8] The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below:

1. Specificity
2. Linearity
3. Quantitation limit
4. Detection limit
5. Range
6. Accuracy
7. Precision

8. Repeatability
9. Intermediate Precision
10. Reproducibility
11. Robustness
12. System Suitability Testing

1. **Specificity and selectivity:** The analyte solution containing 4µg/ml POA was scanned between the range of 200-400 nm and the spectrum was obtained. A spectrum of blank solvent was obtained and compared for any interference at maximum wavelength of absorbance of analytes.
2. **System suitability:** It involves testing of any analytical system or method to ensure consistency of the system before or after any unknown sample study. It is an important part of all methodology used to check the system's reproducibility and resolution to carry out further analysis.
3. **Linearity:** Linearity for an analytical procedure or method is the ability to achieve the test results within a given range that is directly proportional to the analyte concentration of the sample. It can be documented as the linear regression curve of the measured response (absorbance) with corresponding incremental analyte concentration. To determine UV analytical method for POA, dilutions of 2, 4, 6, 8, and 10µg/ml were prepared from the stock solution of 100µg/ml. Absorbance of these samples was than recorded in triplicates at 268 nm using UV spectrophotometer. Least square regression method was used for determination of regression coefficient, r and the equation y = ax+ b for the best fitting straight line.
4. **Range:** The range of analytical determination comprises of upper and lower analyte levels that has been found analytically evaluated with high degree of precision, consistency and linearity in any system.
5. **Detection and Quantitation limit of analyte**: The limits of detection and quantification are calculated by the using the formula:

   LOD = 3.3 × Standard deviation of y-intercepts / slope of the calibration curve

   LOQ = 10 × Standard deviation of y-intercepts / slope of the calibration curve
6. **Accuracy:** The consistency of the analytical procedure is given by the degree to which the measured value deviates from the true value.[8] The result should be found in the absence of error i.e. "true" value. Accuracy of the test is defined as the percentage of agreement between the value measured and the value of truth.[9]

7. **Precision:** Precision refers to the degree of the variation of a measurement of a group measured under identical experimental conditions.[10] It gives an indicator of random errors and is typically subdivided into two classes: 1. Reproducibility 2. Repeatability. Precision is frequently expressed in imprecision terms. This is reckoned as either Standard deviation, uncertainty or variation coefficient (CV) of the testing results. If there is the Large standard deviation it indicates an inaccuracy. Often the minimum and the maximum precision estimates are important. It causes outcome variation. Measurement accuracy quantitatively is fundamentally dependent on the conditions stipulated, which in turn depends on factors influencing outcome variation from a measuring system. Laboratory, time spent between measurements, operator, equipment calibration etc. are some of the factors that causes variation in the outcome.[11]
8. **Robustness:** It is the ability of any system to remain unaffected by any small changes or intentional variance in parameters of the system. This primarily tests how effective any system is. It must be evaluated in the late development or in the early of any method development validation process. It is used to establish the system suitability parameters.
9. **Ruggedness and Reproducibility:** In order to prove the ruggedness and reproducibility of any method six replicates of solution containing the analytes were prepared and the absorbance of each sample were measured by different analyst and different instruments and %RSD were calculated for the absorbance obtained.

3. RESULTS AND DISCUSSIONS

3.1 Method Development

For the estimation of POA the basic criteria is to found such a solvent in which the analyte shows its maximum solubility. By performing few trials, it was found that the POA shows its maximum solubility in water and methanol hence water is chosen as a solvent system for further experimentation. The detection wavelength of POA was obtained by scanning the analyte solution in the UV-spectrophotometer. The spectrum shows its maximum absorbance at 268nm.

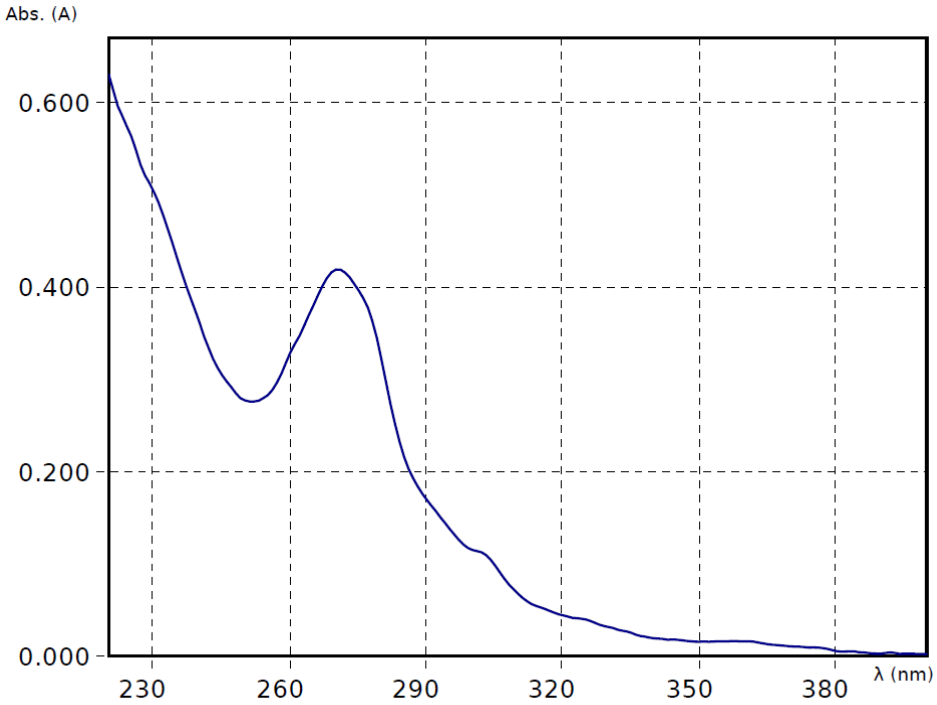

Fig. 2: Maximum wavelength absorption (λ_{max} UV) by 4µg solution of POA in water. The spectrum was taken by scanning the drug solution between 400-200 nm using benchtop UV spectrophotometer (Eppendorf®).

The above spectrum of POA (Figure2) shows maximum absorption at 268nm which comply reported $\lambda_{max.}$ Hence it was selected for further use in the analysis.

3.2 Linearity and Range

For UV-Spectrophotometric method a regression curve was plotted between absorbance and concentration in the range of 2-10µg/ml. The regression coefficient (R^2) was found to be equal to 0.9997. The linear regression equation was found to be y = 0.0585x + 0.019. Results of linearity data is summarized in **Table: 2** and **Fig: 2.**

Table 2: Mean absorbance values against corresponding concentrations (µg/ml) at λ_{max} of 268nm.

Sr. No.	Concentration (µg/ml)	Absorbance (Mean)
1.	2	0.136
2.	4	0.249
3.	6	0.375
4.	8	0.487
5.	10	0.601

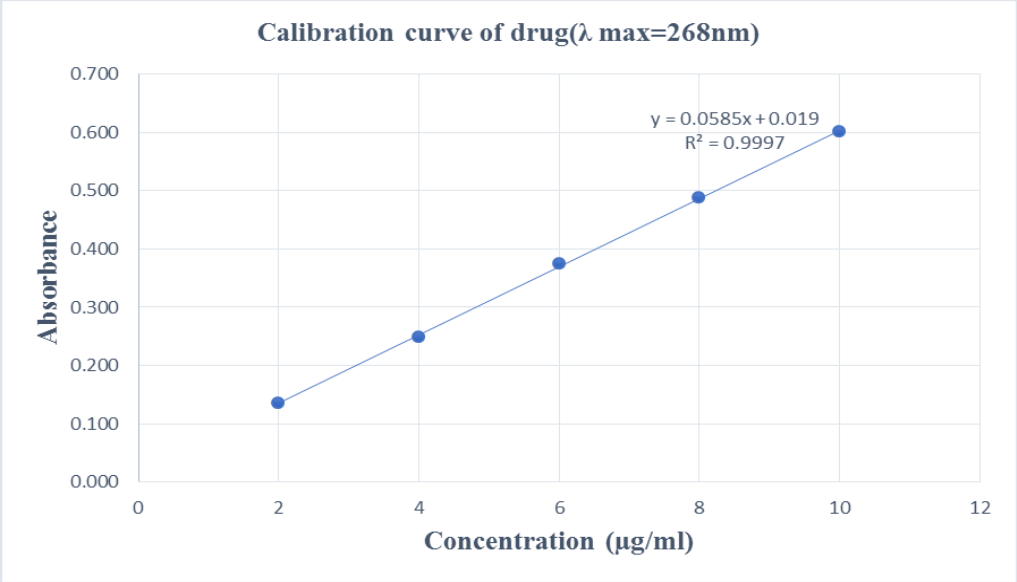

Fig. 3: A calibration curve obtained by plotting different values of absorbance against corresponding standard concentrations of POA at λ_{max} of 268nm.

3.3 LOD & LOQ: It was found to be 0.1714µg/ml and 0.5194µg/ml respectively.

3.4 Accuracy: The accuracy of method was ascertained by performing recovery study at the three concentration levels i.e. 80%, 100% and 120%. The percent recovery obtained indicates non-interference from the excipients used in the formulation. The results of recovery study are given in **Table 3** and **Table 4**.

Table 3: Percent recovery of the quality control samples at different levels of addition.

Level of addition	Standard (µg/ml)	Stock added(µg/ml)	Total concentration (µg/ml)	Absorbance (Mean)	Drug recovered	%Recovery
80%	4	3.2	7.2	0.445	7.28	101.14
100%	4	4	8	0.486	7.98	99.72
120%	4	4.8	8.8	0.537	8.86	100.69

Table 4: Validation of the recovery studies using statistical analysis.

Sr.NO	Level of addition	%Recovery (Mean±SD) *	%RSD
1	80%	101.139± (0.002)	0.449
2	100%	99.715± (0.003)	0.518
3	120%	100.686± (0.002)	0.387

*n=3

3.5 Precision

Precision of this method was determined for intra-day and inter-day variations. These are expressed in terms of percent relative standard deviation (%RSD). Results of precision are summarized in **Table 5** and **Table 6.**

Table 5: Determination of intra-day precision.

Intra-day Precision study							
		Absorbance					
Sr.No	Conc.(µg/ml)	Test 1	Test 2	Test 3	Average	SD	%RSD
1	3	0.193	0.195	0.195	0.194	0.001262	0.649018
2	5	0.318	0.316	0.314	0.316	0.001836	0.581376
3	9	0.553	0.543	0.545	0.547	0.005292	0.967368

Table 6: Determination of inter-day precision.

Inter-day Precision study							
		Absorbance					
Sr.No	Conc.(µg/ml)	Test 1	Test 2	Test 3	Average	SD	%RSD
1	3	0.193	0.195	0.195	0.195	0.001347	0.692425
2	5	0.318	0.312	0.316	0.315	0.002874	0.912025
3	9	0.553	0.545	0.553	0.550	0.004426	0.804141

3.6 Robustness

In this study effect of minor, intentional variations of the analytical parameters on drug absorbance was investigated and results are shown in the **Table 7.**

Table 7: Determination of robustness of the method.

Robustness data for POA							
		Absorbance					
S. No	Wavelength(nm)	Test1	Test2	Test3	Average	SD	%RSD
1	267	0.242	0.244	0.239	0.243	0.0025	1.041356
2	268	0.251	0.255	0.253	0.253	0.0020	0.790514
3	269	0.245	0.248	0.249	0.247	0.0021	0.841644

3.7 Ruggedness

The effect of minor, systematic variations of the analytical parameters on product absorbance was explored in the Ruggedness test. Results are shown in **Table 8.**

Table 8: Determination of ruggedness of the method.

Ruggedness Data of POA							
		Absorbance					
S No.	Conc.(µg/ml)	Test 1	Test 2	Test 3	Average	SD	%RSD
1	3	0.189	0.191	0.192	0.190667	0.001528	0.80115
2	5	0.318	0.315	0.313	0.315333	0.002517	0.79808
3	9	0.544	0.541	0.542	0.542333	0.001528	0.281658

4. CONCLUSION

A simple and robust method was developed for active metabolite of PZA using UV spectrophotometry. The precision and accuracy of this method was validated using intra-day and inter-day system suitability test. In addition this method was observed for ruggedness and robustness besides LOD and LOQ. The method was found to be highly reproducible and can be used for estimation of unknown drug samples of POA in solid dosage forms and pharmaceutical powders. However samples of lower concentration found in plasma samples cannot be determined using this method due to low sensitivity and require more advanced methods like High pressure liquid chromatography.

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