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DETERMINATION OF PRODUCT SHELF LIFE AND EVALUATION OF KINETIC PARAMETERS OF THERMAL DECOMPOSITION OF PYRAZINAMIDE IN SOLID STATE BY HPLC.

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ABSTRACT:
A new high-performance liquid chromatography (HPLC) method for the quantitation of Pyrazinamide has been developed and validated. Pyrazinamide was subjected to thermal decomposition at 130, 140, 150, 160, 170,180°C. Samples were withdrawn at intervals of 7, 14, 21 and 28 days and analyzed for remaining drug content by HPLC assay method. The samples were eluted on a Cosmosil C18 column (250 mm x 4.6 mm, 5.0 µm) at 30°C, with a mobile phase of Methanol : Water :: 95 : 5, (v/v) . The flow rate was 1.0 mL/min, Injection volume : 20µL and detection by PDA detector with detection wavelength at 269 nm.. Pyrazinamide shows thermal-degradation after exposure to high temperature. The shelf life of Pyrazinamide was determined by accelerated stability studies on the basis of first order degradation kinetics. The reaction rate constants, activation energy and half life were also calculated using Arrhenius equation and plots. The shelf life of Pyrazinamide was found to be approximately (t_{90}) 4.95 years. Amounts of Pyrazinamide in all samples were determined by HPLC and the results were compared with those from Infra red spectroscopy and X-ray diffraction technology for qualitative comparison.

Key words: Pyrazinamide, Arrhenius equation, accelerated stability testing, shelf-life, HPLC.

I .INTRODUCTION:

Pyrazinamide **(Pyrazine-2-carboxamide)** (Figure 1) the pyrazine analogue of nicotinamide, is an antituberculous agent. Pyrazinamide (PZA) is an antimicrobial agent that is most commonly used for treatment of active tuberculosis (TB) during the initial phase of therapy (generally the first two months of treatment), in combination with other agents. The spectrum of PZA is relatively narrow; it demonstrates clinically significant antibacterial activity only against *Mycobacterium tuberculosis* and *M. africanum*.

The drug is largely bacteriostatic, but can be bactericidal on actively replicating tuberculosis bacteria. It is soluble in cold water, methanol, partially soluble in diethyl ether. Pyrazinamide is odourless and occurs as a colourless or white crystalline powder or as white crystals. It is stable under recommended conditions of temperature and pressure. Pyrazinamide is slowly affected by exposure to light and air. (27) Pyrazinamide is a generic drug and is available in a wide variety of presentations. Pyrazinamide tablets are usually 500 mg and form the bulkiest part of the standard tuberculosis treatment regimen. Pyrazinamide tablets are so large that some patients find them impossible to swallow; Pyrazinamide syrup is an option for these patients. Pyrazinamide is also available as part of fixed dose combinations with other TB drugs such as Isoniazid and Rifampicin (Rifater is an example). Many HPLC , LC-MS methods have been cited in the research papers for the qualitataive and quantitative analysis of Pyrazinamide as bulk drugs, various formulations and its degradation products (2,6,7,9,16,17,19,20,21,22,23,25).

This paper describes a stability indicating High Performance Liquid Chromatographic (HPLC) method for analysis of Pyrazinamide and Pyrazinamide high temperature degradation samples were developed, followed by validation of the method in accordance with ICH guidelines. With the help of Arrhenius equation the reaction rate constants, activation energy were calculated. Shelf life and half life of Pyrazinamide were also predicted using Arrhenius plot. Qualitative comparison of HPLC results with those obtained by IR and XRD analysis was also carried out.

To ensure the quality, efficacy and safety of pharmaceutical products, stability studies form an essential part of the pharmaceutical pre- and post-formulation studies (Loyd et al., 2003; Watterman et al., 2007; Yunqi and Reza, 2005). Instabilities in modern

formulations are often detectable only after considerable storage under normal conditions. To assess the stability of the formulation, it is usual to expose it to high stress conditions to enhance its deterioration and therefore reduce considerably the time required for testing (Olaniyi, 2000; Tson et al., 2003; Djira et al., 2008). Accelerated stability studies have proved an effective alternative to the time consuming and uneconomical practice of storing the product at room temperature for the time the products would be likely to remain in stock and use. A comprehensive stability plan is an essential and pertinent extension of the quality assurance programme. The effect of temperature on the rate of decomposition as described by the Arrhenius equation (Alfonso et al.,1985) states that:

$$k=Ae^{-Ea/RT}$$

Where k = specific rate constant, T= absolute temperature, Ea = Energy of activation, R =Gas Constant (8.314J/K.Mol), A = Frequency factor. The constants A and Ea may be evaluated by determining k at several temperatures and plotting log k against 1/T. The slope of the graph is –Ea/2.303R and the intercept on the y-axis is log A from which the values of Ea and A may be calculated. Stability of a pharmaceutical product may be defined as the capability of the particular formulation in a specific container/closure system to remain within its physical, chemical, microbiological, therapeutic and toxicological specifications. It is the time from the manufacture and packaging of the formulation until its chemical and biological activity is not less than a predetermined level of labeled potency while its physical characteristics remain unchanged (Wolfgang, 1987; Repeto, 2000; Sprandel et al., 2005).

The physical stability of a suspension is normally assessed by the measurement of its rate of sedimentation, the final volume or height of the sediment and the ease of re-dispersion of the product. An assessment of these three parameters at elevated temperatures would give speedier indication of a rank of order of instability but it is essential to correlate these results with those taken from suspensions stored at ambient temperatures (Sethi, 1997; Jiben, 2002).There have been several reports on accelerated Stability studies, evaluation of kinetic parameters of decomposition

Alibrandi Giuseppe et al. in 2003 carried out Fast drug stability by LC variable-parameter kinetic experiments. Al Omari Mahmoud M. et al. in 2007 observed effect of light and heat on the stability of montelukast in solution and in its solid state. Anderson Geoffrey et al. in 1991 determined Product Shelf Life and Activation Energy for Five Drugs of Abuse. Chena Gang et al. in 2002 determined the rate constants and activation energy of acetaminophen hydrolysis by capillary electrophoresis. Sungthongjee Srisagul in 2004 used "Application of Arrhenius Equation and Plackett-Burman Design to Ascorbic Acid Syrup Development. Syed Akheel A. et al. in 2001 developed LC method for finasteride and applied it to storage stability studies.

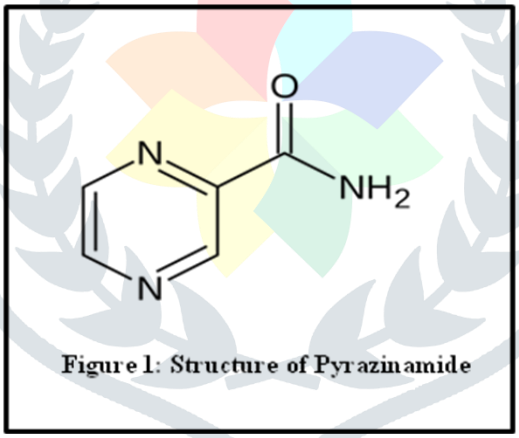

Figure 1: Structure of Pyrazinamide

I .MATERIALS AND METHODS:

Pyrazinamide API, received as a gift sample from Calyx chemicals and pharmaceuticals LTD., Mumbai.
Methanol HPLC grade, water HPLC grade.
Pyrazinamide Sample Pyzina 500mg, Lupin LTD.; (obtained from market).

Instrumentation and chromatographic conditions employed:

The Agilent HPLC (model 1100) system consisted of quaternary pump (model G1311 A), with an auto sampler (model G1313A) having injection capacity of 100 µl. PDA detector was used (model G1515B), data was integrated using Agilent Chemstation software (Ver.10.02) and Cosmosil C18 column (250 mm x 4.6 mm, 5.0µm) and Phenomenex HPLC guard column cartridge (4.0 x 3.0mm, 5.0µm) was also used during analysis.

Chromatographic parameters selected:

Column : Cosmosil C18 column (250 mm x 4.6 mm, 5.0 µm),HPLC guard column cartridge (4.0 x 3.0mm, 5.0µm),Mobile phase:- Methanol : Water :: 95 : 5, (v/v) , Flow rate: 1.0 ml/min. ,Detection wavelength: 269 nm , Injection volume: 20.0 µl, Column compartment temperature: 30°C, Diluent:- Methanol : Water :: 94 : 6 , (v/v)

Preparation of Pyrazinamide standard stock solution:

60.0 mg of Pyrazinamide standard was accurately weighed and transferred to a 100.0 ml volumetric flask, 70.0 ml of diluent was added and sonicate till dissolved. Volume make up to 100.0 ml with diluent. Further diluted 5.0 ml from stock solution to 100.0 ml with diluent.

Preparation of Pyrazinamide sample (Pyzina 500mg) solution for assay:
As per label claim, each tablet of Pyzina 500mg (manufactured by Macleods pharmaceuticals LTD, Mumbai) contains 500.0 mg of Pyrazinamide. Hence, 5 tablets (weighing about 3.304 grams) were crushed in mortar and pestle. From that powder 0.1322 grams of powder equivalent to 100 mg of Pyrazinamide was weighed and transferred to a 100.0 ml of volumetric flask, 70.0 ml of diluent was added and sonicated for 10 minutes. Volume was adjusted to 100.0 ml with diluents. Further diluted 3.0 ml from stock solution to 100.0 ml with diluent. The sample solution thus prepared was filtered through a 0.22 µm membrane filter paper and finally chromatographed.

Preparation of Pyrazinamide degradation sample solution for kinetic and accelerated stability study:
60.0 mg of Pyrazinamide sample was accurately weighed and transferred to a 100.0 ml volumetric flask, 70.0 ml of diluent was added and sonicate till dissolved. Volume make up to 100.0 ml with diluent. Further diluted 5.0 ml from stock solution to 100.0 ml with diluent.

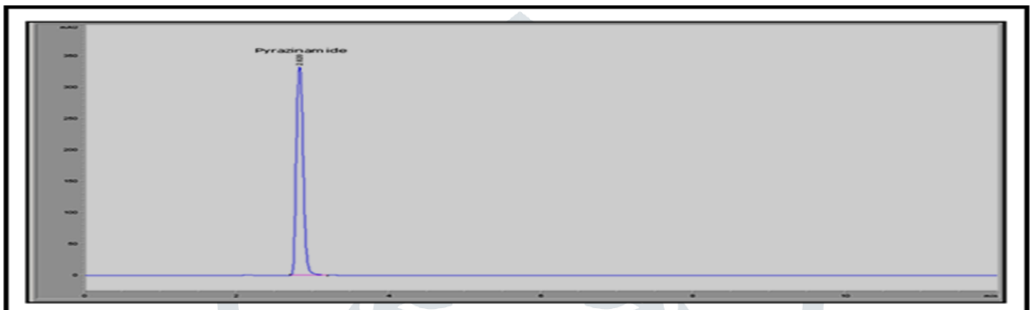

Figure 2: Standard Pyrazinamide chromatogram

II .METHOD VALIDATION:
The objective of the method is to demonstrate that the method is suitable for its intended purpose as it is stated in ICH guidelines. The above method was validated according to ICH guidelines to establish the performance characteristic of a method (expressed in terms of analytical parameters) to meet the requirements for the intended application of the method. They were tested using the optimized chromatographic conditions and instruments.

Specificity:
The peak purity of drug was assessed by comparing the respective spectra of standard drug and sample at peak apex and on the flanks of both sides of the peak. The peak purity of Pyrazinamide was found satisfactory and within the threshold limit, even for the high temperature forced degradation study samples. There was no interference observed from excipients of Pyrazinamide sample, mobile phase, diluent and degradation products with Pyrazinamide peak.

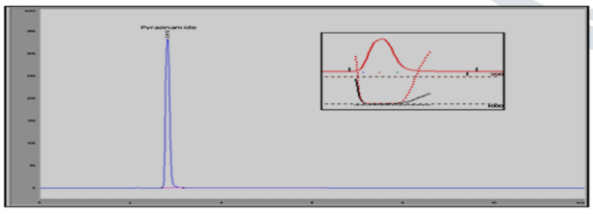
Figure 3: Standard Pyrazinamide chromatogram (Specificity).

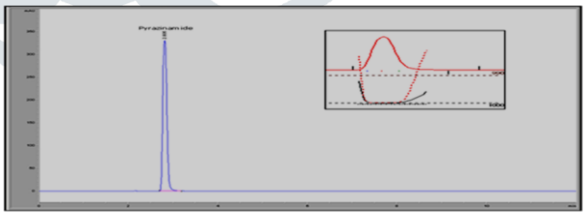
Figure 4: Pyrazinamide sample (Pyzina 500mg) chromatogram (Specificity)

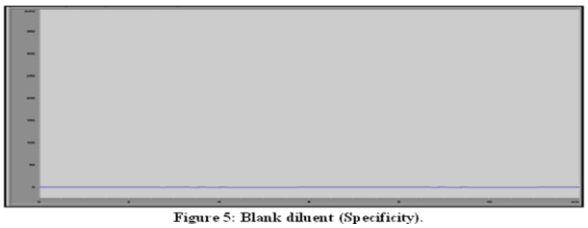
Figure 5: Blank diluent (Specificity).

Linearity:
For the establishment of linearity standard solution of different concentration (ranging from ± 50% and ±4 levels of concentrations of the working range) were prepared from a single stock solution. These solutions were chromatographed and peak areas were obtained subsequently. Peak areas were plotted against corresponding concentrations to obtain the calibration graph.

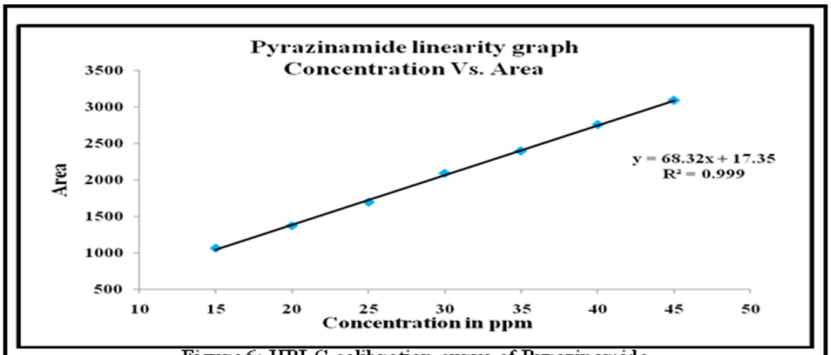

Figure 6: HPLC calibration curve of Pyrazinamide

Solution stability study:
Stability of drug in solution was assessed for the standard preparation of 100 ppm of Pyrazinamide. The solution was kept at room temperature without protection of light and tested after 2, 4, 6, 8, 12, 24 and 48 hrs. The responses for the aged solution were evaluated by comparison with freshly prepared solution.

Precision:
Precision was established by evaluating system precision, method precision and intermediate precision. For system precision six replicate injections of standards and samples were chromatographed. For method precision, six injections of six different standards and samples were chromatographed. Intermediate precision is merely the repetition of method precision but on another day.

Limit of Detection (LOD):
LOD was calculated from the formula: LOD = 3.3 σ / S
Where; σ = Standard deviation of the responses of calibration curve
S = Slope of the calibration curve

Limit of Quantitation (LOQ):
LOQ was calculated from the formula:
LOQ = 10 σ / S
Where; σ = Standard deviation of the responses of calibration curve
S = Slope of the calibration curve

Robustness of the Method:
Robustness of the method was studied at different parameters viz.
Change in the mobile flow rate ± 0.1ml of 1.0 ml
Change in the detection wavelengths ± 2 of 269nm
Change in the column oven compartment temperature ± 2°C of 30°C

Accuracy:
To check the accuracy of the method, recovery studies were carried out by addition of standard drug to sample at three different levels 80 %, 100 % and 120 %. For this 15 ppm of sample solution was prepared. Addition of standard drug was done by spiking from 1000 ppm solution of standard Pyrazinamide to the sample in their respective volumetric flasks. In 100 % recovery study for Pyrazinamide, amount of standard drug added was 20 ppm. In 80 % and 120 % recovery study for Pyrazinamide, the amount of standard drug added was 16 ppm and 24 ppm respectively. Samples were then chromatographed and peak areas were obtained. At each level of the amount, six determinations were performed. Amount of drug recovered was calculated.

Parameter	HPLC Validation report of Pyrazinamide
Linearity range	**15 – 45 ppm**
Limit of detection (LOD)	**0.96 ppm**
Limit of quantitation (LOQ)	**2.92 ppm**
Accuracy (%Recovery)	**Between 99 – 101%**
Solution stability	**Stable up to 48 Hours**
% Assay	**Pyzina 500mg: 100.02%**

Precision (% RSD) **System precision** **Method precision (Inter – day)** **Intermediate precision (Intra – day)**	 **Less than 2.0 %** **Less than 2.0 %** **Less than 2.0 %**
Robustness	**Robust**
Specificity	**Specific**

Table 1: Statistical data for the validation of Pyrazinamide by HPLC method

III .EXPERIMENTAL PROCEDURES:

In accordance with ICH stability testing of drug substances and products, Pyrazinamide standard were stored at 130, 140, 150, 160, 170,180°C. Stress testing of a drug, in this case Pyrazinamide, which can in turn help establish the intrinsic stability of the molecules, and validate the stability indicating power of the analytical procedures used (2,6,7,9,16,17,19,20,21,22,23). The nature of the stress testing depends on the chemical and physical characteristic of drug and the type of formulation (5,15,18,24). Stress testing according to ICH is likely to be carried out on a single batch of the drug substance. It should include the effect of temperature (in 10°C increments; for accelerated testing). In this work, thermostability was determined under dry and dark conditions. At the accelerated storage condition, a minimum of three time points, including the initial and final time points. Samples were taken at 7,14,21 and 28 days after being stored at 130, 140, 150, 160, 170,180°C. and analyzed by HPLC and IR and XRD technique for qualitative purpose. All samples were analyzed simultaneously and comparison was made with time zero Pyrazinamide standard at room temperature on HPLC, to establish the percentage of drug remaining in the high temperature forced degradation samples. The data thus generated from this accelerated high temperature degradation study was used to calculate reaction rate constants, activation energy, shelf life t_{90} and half life $t_{1/2}$.

DATA ANALYSIS:

The temperature dependence of the rate of chemical degradation of Pyrazinamide in bulk was investigated by determining the concentration of active principal (Pyrazinamide) by HPLC as a function of time. A linear correlation was obtained at each temperature, and the observed rate constant (k) was calculated as the slope of these curves. The activation energy (Ea) was derived from the slope of the natural logarithm of the observed rate constants (ln k) plotted against the inverse absolute temperature (1/T) using the Arrhenius equation ln k = ln A – Ea/RT Eq. 1 where the constant (A) is the frequency factor; Ea is the energy of activation; R is the universal gas constant; and T is the absolute temperature. Shelf life t90 and half life t1/2 were also calculated from the apparent rate constant value from the Arrhenius plot.

ASSAY OF PYRAZINAMIDE:

The content of authentic Pyrazinamide was determined in samples as a function of time and temperature (Table II). The changes occurring in the assay values with respect to temperature are listed in the following table. Data of temperature sets from 130 to 180°C was considered for stability calculations since the higher temperature did not have enough data points for calculations.

	Time 0	1week	2week	3week	4week
26°C (RT)	**100.00**	**99.81**	**99.90**	**99.76**	**99.71**
130°C	**99.76**	**100.19**	**98.62**	**95.66**	**92.51**
140°C	**99.86**	**100.00**	**95.33**	**91.94**	**88.32**
150°C	**99.76**	**98.19**	**93.03**	**88.55**	**83.25**
160°C	**99.86**	**96.90**	**90.04**	**84.27**	**75.64**
170°C	**99.95**	**95.33**	**86.90**	**78.57**	**67.62**
180°C	**99.95**	**93.10**	**80.67**	**71.43**	**61.81**

Table 2: Amount of drug remaining in %

	Time 0	1week	2week	3week	4week
26°C (RT)	**4.6052**	**4.6033**	**4.6042**	**4.6028**	**4.6023**
130°C	**4.6028**	**4.6071**	**4.5912**	**4.5608**	**4.5273**
140°C	**4.6037**	**4.6052**	**4.5573**	**4.5212**	**4.4810**
150°C	**4.6028**	**4.5869**	**4.5330**	**4.4836**	**4.4219**
160°C	**4.6037**	**4.5737**	**4.5002**	**4.4340**	**4.3260**
170°C	**4.6047**	**4.5574**	**4.4648**	**4.3640**	**4.2139**
180°C	**4.6047**	**4.5336**	**4.3903**	**4.2687**	**4.1241**

Table 3: Natural log of % drug remaining

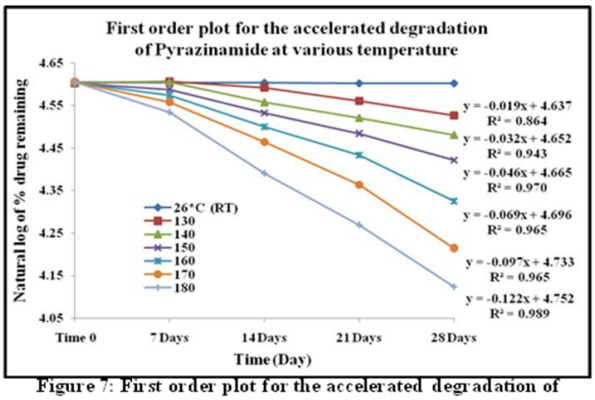

Figure 7: First order plot for the accelerated degradation of Pyrazinamide at various temperatures.

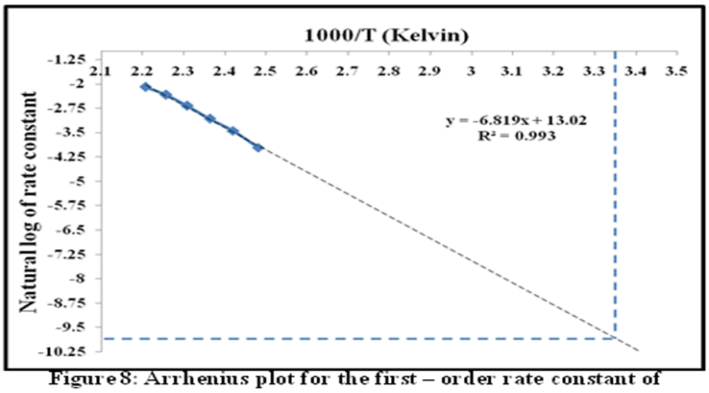

Figure 8: Arrhenius plot for the first – order rate constant of Pyrazinamide degradation over the temperature range of 130 – 180° C

From the first order plot for the accelerated degradation of Pyrazinamide (Fiure 8) at various temperatures, the rate constants were obtained, which were equal to the slope of the lines for the particular degradation temperature.

Temperature (T)	T in Kelvin	1000/T	Rate constant	ln of Rate constant
130°C	403	2.4813	0.019	-3.9633
140°C	413	2.4213	0.032	-3.4420
150°C	423	2.364	0.046	-3.0791
160°C	433	2.3094	0.069	-2.6736
170°C	443	2.2573	0.097	-2.3330
180°C	453	2.2075	0.122	-2.1037

Table 4: Results obtained by first order degradation study for Pyrazinamide

IV . SHELF LIFE AND HALF LIFE CALCULATIONS:

0.1054/ kapp = t_{90}

Where; kapp = Reaction rate constant at particular temperature

t_{90} = Shelf life of the compound

A perpendicular is dropped on X axis from a value 3.35 (Inverse of Room temperature 26°C) (Figure 8) which coincides at a particular point on the extrapolated slope line. From this point again a perpendicular is dropped on Y axis which corresponds to a particular point which suggests the natural log value of rate constant at that temperature. The antilog of this value gives the direct value of rate constant at room temperature. From the above graph (Figure: 8) the rate of reaction at 26°C is found out to be approximately 0.0000583^{-1}. Substituting this value of rate constant at 26°C in the above equation $\mathbf{t_{90}}$ can be calculated.

Therefore,

t_{90} = 0.1054/0.0000583

t_{90} = 1807.89 days

t_{90} = 4.95 years.

Similarly $\mathbf{t_{1/2}}$ can also be calculated using following formula,

0.6931/ kapp = $t_{1/2}$

$t_{1/2}$ = 0.6931/0.0000583

$t_{1/2}$ = 11888.50 days

$t_{1/2}$ = 32.57 years

Activation energy:

The - slope of above graph = -Ea/R

Therefore

-6.819 = -Ea/R

Ea = 6.819 × 8.314

Ea = 56.693 kJ/Mol

Or

Ea = 56693 J/mol

Finally from the graph in (figure 8), looking at the straight line nature of the graph it can be stated that Pyrazinamide degradation follows a first order reaction mechanism.

Temperature	Rate constant (day^{-1})	t_{90} (days) at 26°C	t_{90} (years) at 26°C	$t_{1/2}$ (days) at 26°C	$t_{1/2}$ (years) at 26°C	Activation energy (Ea) kJ/mol
130°C	0.019	1807.89	4.95	11888.50	32.57	56.69
140°C	0.032					
150°C	0.046					
160°C	0.069					
170°C	0.097					
180°C	0.122					

Table 5: Summary of Results obtained by first order degradation study for Pyrazinamide

V .XRD ANALYSIS WAS CONDUCTED AS FOLLOWS:

Samples were filled in a glass holder and exposed to Cu Kα radiation (40 kV x 30 mA) in a wide angle X-ray diffractometer at ambient temperature. The instrument was operated in a continuous scan mode, over the angular range of 5 to 60° 2θ.

X -ray powder diffraction patterns of the Pyrazinamide time 0 standard and the degradation samples (140, 160, 180°C) are given below. Based on the results, generated by HPLC technique it was decided to analyze only two, three and four week samples for XRD.

VI . (DRS) IR SPECTROSCOPIC ANALYSIS WAS CONDUCTED AS FOLLOWS:

FTIR spectra of Pyrazinamide degradation samples were obtained using Shimadzu FTIR spectrophotometer using diffuse reflectance technique (KBr disc technique) as a part of qualitative analysis by comparing it with the spectra of Pyrazinamide standard. Samples of Pyrazinamide degradation sample powder and Pyrazinamide standard were previously ground and mixed with KBr, an infrared transparent matrix. The KBr discs were prepared by compressing the powder and the scans were obtained in the mid-infrared regions of the spectrum form 4000 – 400 cm^{-1}. Infra red spectroscopic results (spectrum/pattern) of the Pyrazinamide time 0 standard and the degradation samples (140, 160, 180°C) are given below. Based on the results, generated by HPLC technique it was decided to analyze only two, three and four week samples for IR spectroscopy.

VII .SUMMARY AND CONCLUSIONS:

A stability indicating High Performance Liquid Chromatographic (HPLC) method for routine analysis in a Quality Control (QC) lab was developed for the assay of Pyrazinamide. The developed HPLC method is a stability-indicating. Stability indicating nature of the method was established after subjecting the drug to accelerated stress temperature parameter. The method was properly validated and showed satisfactory data for all the method validation parameters tested. The HPLC method developed and validated for Pyrazinamide is rapid, precise and selective. The developed method was found "specific" to the drug as there were no peaks of the degradation products which interfere with the drug peak. Thus the proposed method can be employed for assessing the stability of Pyrazinamide as a bulk drug.Stability testing is usually performed to determine the product shelf life during the early stages of product development. The results obtained in the study demonstrate that Arrhenius equation could be applied for calculating Pyrazinamide shelf life, half life, rate constant and activation energy.

A secondary objective was to qualitatively compare the high temperature degradation assay results of HPLC with those of Infrared spectroscopy and X – Ray diffraction methods. Infrared spectroscopic and X – Ray diffraction results of degraded samples also showed significant changes in the graphical patterns when compared with fresh samples. Thus the data generated from these two techniques can be used as supporting data with data of HPLC analysis. HPLC analysis shows no changes in retention time of peak of interest with respect to temperature and time. However, differences are seen in x-ray diffraction and diffuse reflectance infrared spectroscopic studies. It may be due to internal morphology changes e.g. particle size, aggregation, loss of moisture, crystallanity etc. In summary, accelerated stability testing and Arrhenius equation available for interpreting the data can provide valuable information for evaluating reagents and control products. By optimizing the analytical precision and other aspects of test protocol design, one can expect both real time and accelerated stability studies to provide more valid information.

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