DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR THE DETERMINATION OF PHENAZONE IN BULK AND DOSAGE FORM

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ABSTRACT

The objective of the present work is to develop a simple, precise, accurate, validated stability indicating RP-HPLC method for the determination of Phenazone in bulk and tablet dosage form. The HPLC separation was achieved on Agilent TC C18 (2) 250 x 4.6 mm, 5 µ column using mobile phase composition of phosphate buffer pH 2.5, acetonitrile, Methanol (80:10:10V/V/V). Flow rate was maintained at 1.5 ml/min at an ambient temperature. Quantification was achieved with ultraviolet detection at 230 nm. The retention time obtained for Phenazone was at 7.2 min. The result obtained with the detector response was found to be linear in the concentration range of 50-150 µg/ml. The reliability and analytical performance of the proposed methods, including linearity, range, precision, accuracy, detection and quantitation limits, were statistically validated. When Phenazone was subjected to different stress conditions; the proposed methods could effectively separate the drug from its degradation products, and were thus considered as good stability-indicating procedures. It is concluded that this method can be applied for routine quality control of Phenazone in dosage forms as well as in bulk drug.

KEY WORDS: Phenazone, Reverse phase high performance liquid chromatography, Tropex tablet , Method development and validation, Stability indicating.
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INTRODUCTION

Phenazone (INN; also known as phenazon, antipyrine (USAN), or analgesine) is an analgesic, a non-steroidal anti-inflammatory drug and an antipyretic[01] . It is formed by reducing diortho- dinitrodiphenyl with sodium amalgam and methyl alcohol, or by heating diphenylene-ortho-dihydrazine with hydrochloric acid to 150 °C. Phenazone contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of 1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one **(Figure1)**[02] .

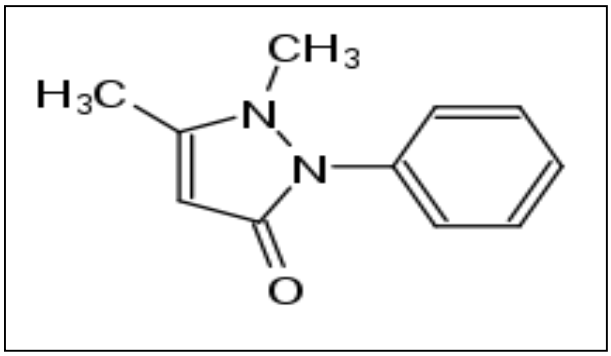

Figure 1: Chemical structure of Phenazone

Antipyrine is an analgesic and antipyretic that has been given by mouth and as ear drops. Antipyrine is often used in testing the effects of other drugs or diseases on drug-metabolizing enzymes in the liver[03].

Literature survey revealed, few analytical methods which include UV-spectrophotometric methods[04-07] liquid chromatographic methods [08-11] have been reported for estimation of PNZ. However, to our knowledge, no information related to the stability-indicating HPLC determination of PNZ in pharmaceutical dosage forms has ever been mentioned in literature. According to the stability test guidelines issued by ICH [12-14],in the present study the stress induced stability studies were carried out for PNZ to establish its stability characteristics. Hence, an attempt has been made to develop an accurate, specific and reproducible method for the determination of PNZ in presence of degradation product for the content analysis during stability studies from pharmaceutical dosage form.

EXPERIMENTAL WORK

Materials and Reagents

Pharmaceutical grade of Phenazone kindly supplied as a gift sample by Glenmark Pharmaceutical Ltd.,Nashik, India. All chemicals and reagents used were of HPLC of analytical grade and were purchased from Merck Chemical. India. Analytical grade sodium hydroxide, hydrochloric acid and 30% hydrogen peroxide were used.

Instrumentation and chromatographic conditions

Agilent technologies 1260 LC system with gradient pump connected to DAD UV detector, LC-GC AGN204PO balance was used for all weighing An Agilent zorbax eclipse C18 column (150 mm × 4.6 mm i.d., 5 µm) was maintained at 30°C. The mobile phase was composed of a mixture of phosphate buffer pH 2.5, acetonitrile, Methanol 80:10:10 (V/V/V). The flow rate of the mobile phase was set at 1.5 mL min^{-1}. Measurements were made with 20 µL of injection volume. The retention time obtained for PNZ was at 7.2 min. For the analysis of forced degradation samples, the photodiode array detector was used in scan mode with a scan range of 200–400 nm.

Preparation of Standard solution and calibration graphs

Stock solutions of PNZ (100 µg/mL) was prepared by transferring 100 mg each of PNZ standard in 50 mL volumetric flasks, dissolved in 25 mL of methanol and made up to volume using the same. PNZ were transferred to a series of 10 ml volumetric flasks in the range of 50% to 150% and diluted up to the mark with mobile phase. The prepared dilutions were injected in series, peak area was calculated for each dilution, and concentration was plotted against peak area.

Preparation of sample solution

A quantity of powder equivalent to 100mg PNZ was transfer into 100 mL volumetric flask

containing 25 mL of methanol, volume was made up to the mark with methanol and solution was filtered using 0.45 µm filter (Mill filter, Milford, MA). From filtrate, 10 mL of solution was transferred into 100 mL volumetric flask and the volume was made up to mark with mobile phase to obtain the concentration of 100 µg/mL .From second dilution 7 ml was transferred to 10 ml of volumetric flask and made the volume upto 10 ml with mobile phase to obtain the final concentration 70 µg/mL was subjected to propose method and the amount of PNZ was determined. The assay procedure was repeated for six times.

Method validation[13-15]

The method was validated for its linearity range, accuracy, precision, sensitivity and specificity. Method validation is carried out as per ICH guidelines

Precision

Intra- and inter day precisions of the methods were determined by performing replicate (n=3) analyses of standards and samples. This procedure was replicated on different days (n=3). Recovery studies by standard addition method were performed in view of justifying accuracy of the proposed methods. Previously analysed samples containing PNZ was spiked with standard PNZ, and the mixtures were analysed in triplicate (n=3) by proposed methods. Precision was calculated from percentage relative standard deviation (RSD %) for repeated measurements, whereas accuracy was expressed as % of recovery.

Robustness and ruggedness of the method

Robustness of the method was studied by making deliberate variations in the chromatographic conditions and effects on the peak areas were recovered. Different chromatographic parameters such as variations in flow rate, column oven temperature, mobile phase composition and change in pH of mobile phase were made. It was performed using 70 µg/mL of PNZ and the effects on the peak areas were recorded. The each parameter was repeated for six times.

Ruggedness of the method was studied by analyzing 70 µg/mL of PNZ by two different analyst using same operational and environmental conditions.

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). For study of sensitivity of the method lower part of the linearity curve was selected. From the stock solution, six different concentrations in the range of 10,20,30,40,50µg/mL were prepared and injected into the column. Each concentration was injected into column for three times and peak area was recorded. The LOD was calculated using formula LOD = 3.3 ASD /S and LOQ = 10 ASD/S, where ASD is the average standard deviation and S is the slope of the corresponding calibration curve line.

Specificity

Specificity is a procedure to detect quantitatively the analyte in presence of component that may be expected to be present in the sample matrix, while selectivity is the procedure to detect qualitatively the analyte in presence of components that may be expected to be present in the sample matrix.

The method is quite selective. There was no other interfering peak around the retention time of PNZ; also the base line did not show any significant noise.

Recovery studies

Recovery experiments were performed at three different levels i.e. 80, 100 and 120 %. To the pre-analysed sample solutions, a known amount of standard drug solution of PNZ was added at three different levels.

Forced degradation studies [16-17]

A stock solution containing 10 mg for PNZ in 10 mL methanol was prepared. This solution was used for forced degradation to provide an indication of the stability-indicating property of the method.

Acid degradation

PNZ solution treated with 1 mL of 1 M methanolic HCl. The solutions were kept at room temperature for 8 hr. The solution was

diluted with the mobile phase to reach a final concentration of 50 µg/mL of PNZ and injected to the column.

Base degradation

PNZ solution treated with 1 M methanolic NaOH. The solutions were kept at room temperature for 8 hr. The solution was diluted with the mobile phase to reach a final concentration of 50 µg/mL of PNZ and injected.

Oxidative degradation

PNZ solution was treated with 1 mL of hydrogen peroxide 10%. The solution was kept at room temperature for 8hr. Both solutions were kept protected from light. After the specified time intervals, the solutions were diluted with the mobile phase to reach a final concentration of 50 µg/mL of PNZ. After the previous treatments, the solutions were filtered with a 0.45-µm filtration disc prior to injection to the column.

Photochemical degradation

The drug solution was left in sunlight for 8h. The resultant solution was treated as described for hydrogen peroxide-induced degradation.

Dry heat degradation

The powdered drug was stored for 3h. under dry heat conditions at 55°C. In UV, 10 mg sample was dissolved methanol and volume made up to 10 ml. The solution was diluted with the mobile phase to reach a final concentration of 70 µg/mL PNZ. The chromatograms were run by injecting the sample in the column.

RESULT AND DISCUSSION

Optimization of procedures

Initially, combination of acetonitrile and water (50:50 v/v) was tried as mobile phase but broadening of the peak was observed .Then, pH of the mobile phase was adjusted to 3.0, the chromatogram showed fronting and splitting. To overcome the problem combination of phosphate buffer: acetonitrile: methanol (80:10:10v/v/v) was tried for resolution of PNZ. Good resolution and symmetric peak was obtained for PNZ when the pH of the mobile phase was adjusted to **2.5** and column oven temperature was kept at **30^{0}C.** Under these optimum chromatographic conditions, the retention time for PNZ was found to be **7.2 ± 0.02 min** at a flow rate of 1.5 mL/min. The detection was carried out at **230 nm**.

Linearity

Linearity was studied by injecting eleven concentrations of standard PNZ (50-150 µg/mL) in triplicate. Peak area versus concentration data was performed by least square linear regression analysis, whereby slope, intercept, and correlation coefficient were determined.Calibration curve is shown in **Figure 2**.

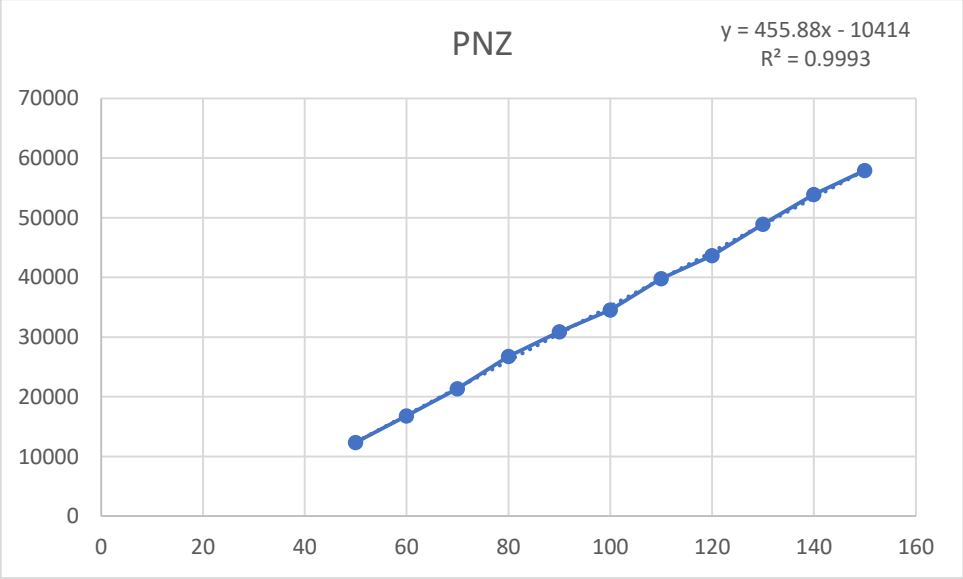

Figure 2: Calibration curve of PNZ

Precision

Precision was determined as intra-day and inter-day variations.

Intra-day variation of the method was performed by analyzing, the three different concentrations 70 µg/mL, 80 µg/mL and 90 µg/mL of PNZ, for three times in the same day. Inter-day variations of the method were performed by analyzing the same concentrations for the period of the three consecutive days over a period of week. %R.S.D. was found to be 0.68-0.79 for intra-day and 0.70-1.45 for inter-day precision.% R.S.D. less than 2 indicates method is more precise.The results are shown in **Table1**.

Table1: Intraday and interday precision studies

Drug	Conc. [µg/mL]	Intra- day Amount found [µg/mL]		Inter- day Amount found [µg/mL]	
		SD [n= 3]	% RSD	SD [n= 3]	% RSD
BZP	70	6485.06	0.70	2924.65	077
	80	9883.02	0.40	9662.60	0.68
	90	13332.38	1.45	9353.15	0.79

Robustness and ruggedness of the method

Various parameter such as mobile phase composition, PH of mobile phase, change in flow rate and change in column temperature were studied. The standard deviation of peak areas was calculated for each parameter and %R.S.D. was found to be 0.28-0.87. The low % R.S.D. values indicated robustness of the method.

The ruggedness of the proposed method was evaluated by two different analysts. The results for PNZ were found to be 99.78% and 100.22% respectively

LOD and LOQ

Detection limit and quantification limit was calculated by the method as described in Section **2.5.3.2** LOD and LOQ were found to be **0.65 µg** and **0.89 µg**, respectively.

Recovery studies

To the preanalysed 70µg/mL solutions a known amount of standard drug solutions of PNZ at 80 %,100 % and 120 % levels.% RSD of recovery study was found to be 0.88 - 1.28, which indicated that the method is accurate.

System Suitability

System suitability tests were also carried out to verify reproducibility. The parameters such as capacity factor (K), injection repeatability tailing factor (T), theoretical plate number (N) and resolution (Rs) for the principal peak and its degradation product were tested on a 70 µg/mL sample of PNZ to assist the accuracy and precision of the developed HPLC system .The results are as shown in **Table 2.**

Table 2:System Suitability Parameter

System Suitability Parameters	Proposed Method
Retention time (T_R)	7.2
Capacity factor (K')	0.86
Theoretical plate (N)	12372
Tailing factor (T)	1.13

Summary of validation parameter are as shown in **Table 3.**

Table 3:Summary of Validation Parameter

Parameter	**HPLC**
Linearity range	50-150 µg/mL
Regression equation [Y = mX + C]	Y=455.88X-10414
Correlation coefficient	0.9993
Limit of detection	0.65 µg
Limit of quantitation	0.89 µg
% Recovery [n = 3]	99.47 – 100.30
Ruggedness [%]	
Analyst I [n = 3]	99.78
Analyst II [n = 3]	100.22
Precision [% RSD]	
Repeatability [n = 6]	0.31
Inter-day [n = 3]	0.70-1.45
Intra-day [n = 3]	0.68-0.79
Robustness	Robust
Specificity	Specific

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Analysis of the marketed formulation

Six replicate determinations were performed on the commercially available tablet. For PNZ recovery was found to be 99.86±0.56%. There was No interference was observed from the excipients commonly present in the tablets. It may, therefore, be inferred that degradation of PNZ had not occurred in the marketed formulations analysed by this method. The low RSD indicated that the method is suitable for routine estimation of PNZ in pharmaceutical dosage forms.

Force degradation studies

The chromatogram of samples degraded with acid, base, hydrogen peroxide and light showed well separated peaks of pure PNZ as well as some additional peaks at different Retention time. PNZ was successfully separated from all the degradation products as confirmed by the resolution values calculated for each chromatogram. The number of degradation

product with their retention time values are as shown in **Figure 3**.

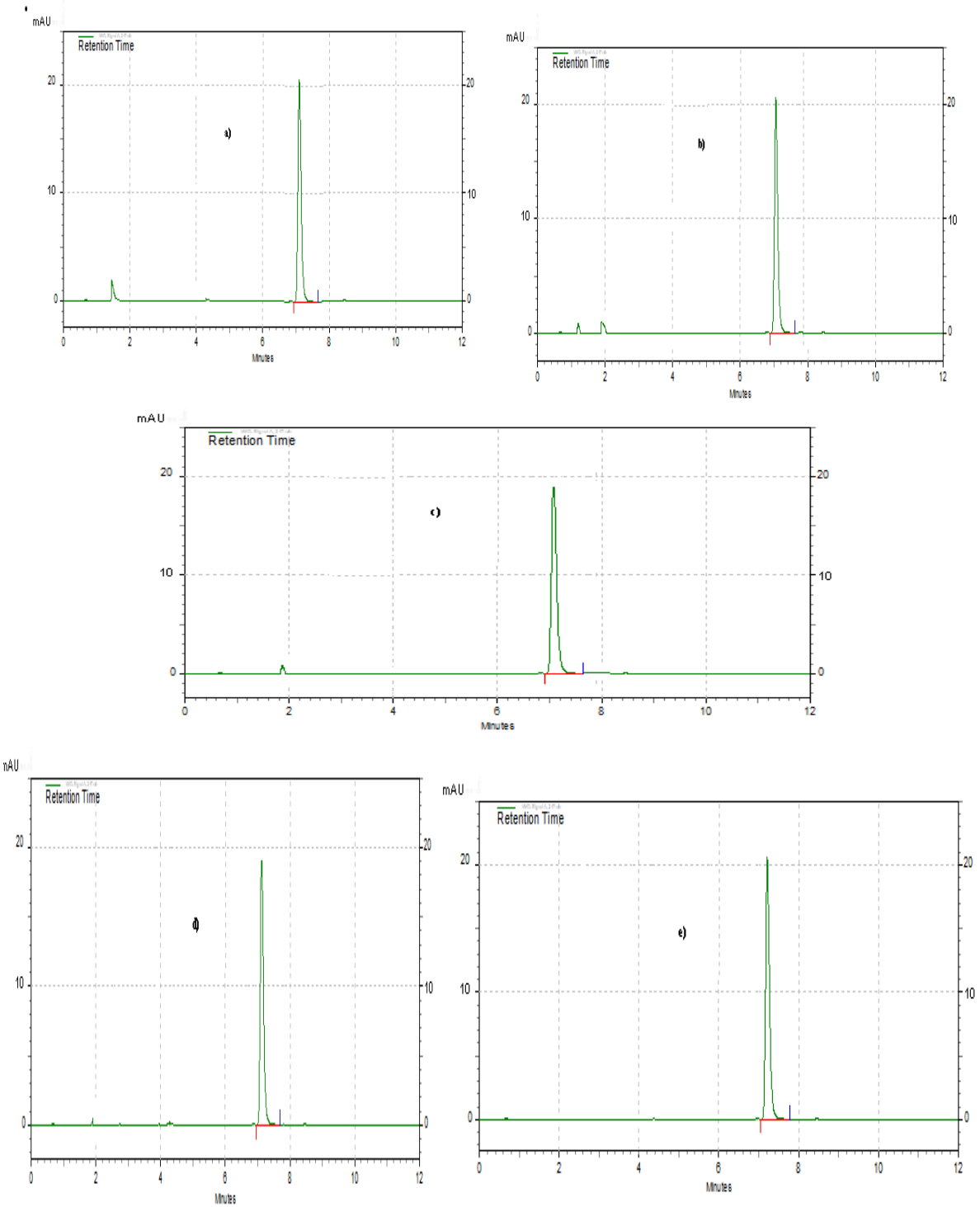

Figure 3: Forced degradation of PNZ by HPLC: A) 1N HCl + PNZ; B) 1N NaOH + PNZ; C) 30% H_2O_2 + PNZ; D) Dry heat PNZ; E) Light heat PNZ

Content of PNZ remained, and percentage recovery were calculated and listed in **Table 4.**

Table 4: Forced Degradation of PNZ

Sample exposure condition	Number of degradation products [Rt values]	PNZ remained [50ug/ml]	SD	Recovery [%]
1 M HCl, 8h,RT[a]	1 (1.5)	48.871	8.36	97.74
1M NaOH,8h, RT[a]	2 (1.2,2.0)	45.513	10.23	91.03
30%H_2O_2,8h,RT[a]	1(1.9)	48.57	7.42	97.14
Heat, 3H, 55^0C	1(1.9)	46.721	2.76	93.44
Photo, 8 h	No degradation	49.98	1.56	99.96

[a]RT = Room Temperature

CONCLUSION

The proposed HPLC method provide simple, accurate and reproducible quantitative analysis for determination of PNZ in tablet. The method was validated as per ICH guidelines. As the method could effectively separate the drugs from their degradation products; therefore, it can be employed as a stability indicating study.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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