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Assay Method Validation Of 4-Hydroxy Phenoxymethylpenicillin And Phenoxy Methyl Penicillin In Phenoxymethylpenicillin Tablets By RP-HPLC

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ABSTRACT

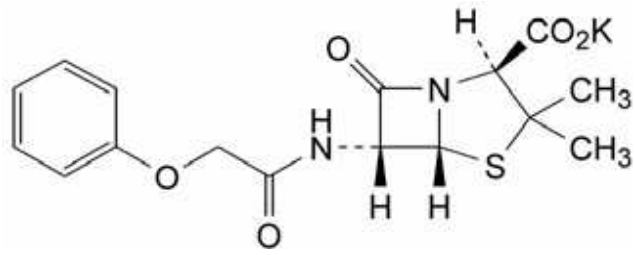
A simple reverse phase HPLC method was developed for the determination of the phenoxymethylpenicillin in pharmaceutical dosage. Efficient chromatographic separation was achieved on Lichrospher 100 RP-18e, 250 x 4.0mm 10µ column from waters-2998 isocratic ode, with simple mobile phase combination of phosphate buffer : methanol : water (8:42:50). Adjust pH of the mixture to 3.5 ± 0.05 with ortho phosphor ic acid used. The flow rate was 1.2ml/ min and effluent was monitored at 254 nm. The retention time of phenoxymethyl penicillin was 16.529 minutes. The proposed method is simple, selective, reproducible, sensitive and accurate with good precision. Some of the methods were proved to be superior to most of the reported methods. All these proposed methods for estimation of selected drug phenoxymethylpenicillin was successfully applied in pharmaceutical formulations.

INTRODUCTION

It is necessary to find the content of each drug either in pure or single, combined dosage forms for purity testing[1] It is also essential to know the concentration of the drug and it's metabolites in biological fluids after taking the dosage form for treatment. The scope of developing and

validating analytical methods is to ensure a suitable method for a particular analyst more specific, accurate and precise. The main objective for that is to improve the conditions and parameters, which should be followed in the development and validation.

Penicillin V is a broad-spectrum antibiotic that kills a wide variety of bacteria that cause a wide variety of commonly occurring infections. Penicillin V may be used to treat infections of the lungs and airways, mouth and throat, skin or soft tissue, or ears[2]. It may also be used to continue treatment for infections that have been treated initially with injections of benzyl penicillin. Penicillin V has *in vitro* activity against gram-positive and gram-negative aerobic and anaerobic bacteria. The bactericidal activity of Penicillin V results from the inhibition of cell wall synthesis and is mediated through Penicillin V binding to penicillin binding proteins (PBPs). Penicillin V is stable against hydrolysis by a variety of beta-lactamases, including penicillinases, and cephalosporinases and extended spectrum beta-lactamases [3].



Chemical Name : (2S,5R,6R)-3,3-dimethyl-7-oxo-6-[[2- (phenoxy)acetyl]amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid.

A survey of literature reveals that HPLC method is not available for simultaneous estimation of the drugs like phenoxy methyl penicillin and 4 hydroxy phenoxy methyl penicillin in combined tablet dosage form Even though very few methods such as UV and HPTLC are available for estimation of above drugs either in single or combination with other drugs, many of them suffer from one disadvantage or the other, such as low sensitivity, lack of selectivity and simplicity etc. [4]

EXPERIMENTAL SECTION

Phenoxymethylpenicillin was obtained from Glen mark (Mumbai, India). Potassium dihydrogen phosphate was of HPLC grade and obtained from E.merck (Mumbai, India) and all other chemicals used were of analytical grade. Purified water from Milli-Q-system (Millipore, Bangalore, India) was used throughout the analysis. Chromatographic measurements were performed on an isocratic HPLC of waters-2998 with Lichrospher 100 RP-18e, 250 x 4.0mm column.

Preparation of Standard Solution [5]**:**

Weigh accurately about 55 mg of phenoxymethylpenicillin potassium working standard in to 50.0 ml volumetric flask add to it about 35 ml of diluents and sonicate to dissolve, dilute up to the mark with diluents and mix well. (Concentration of phenoxymethylpenicillin is about 992 µg/ml)

Preparation of Reference solution (a):
Weigh accurately 4.0 mg of 4-hydroxyphenoxymethylpenicillin potassium in to 100.0 ml volumetric flask, add to it about 70 ml of diluents and sonicate to dissolve, dilute up to the mark with diluents and mix well. (Concentration of 4-hydroxyphenoxymethylpenicillin potassium is about 40µg/ml)

Preparation of Reference Solution (b):
Weigh accurately 1.0 mg of phenoxymethylpenicillin potassium and 1.0 mg of benzyl penicillin potassium into 5.0 ml volumetric flask, add to it about 3 ml of diluents and sonicate to dissolve[6] dilute up to the mark with diluents and mix well. (Concentration of phenoxymethylpenicillin potassium & benzyl penicillin potassium is about 200µg/ml respectively).

Preparation of Test Solution:
Weigh accurately not less than 20 tablets and determine the average weight. Crush the tablets to fine powder. Weigh accurately the powder equivalent to 50 mg of phenoxy methyl penicillin into a 50.0 ml volumetric flask add to it 30 ml of diluents and sonicate to dissolve for about 5 minutes. Dilute up to the mark with diluents and mix well. (Concentration of phenoxymethylpenicillin is about 1000µg/ml) Prepare the test solutions immediately before use [7].

Chromatographic conditions
Column used Lichrospher 100 RP-18e, 250×4.0mm, 10µ, Flow rate 1.20ml/min, Detector UV Visible Detector, Wavelength 254 nm, Injection 20 µl ,Column oven Temperature Ambient Sample cooler Temperature 50 c , Run Time 30 minutes.

Method development
Working standard of various concentrations was prepared by taking aliquots of standard solution and diluted to get required concentration for calibration plot and which was injected [8,9].

Assay preparation for commercial formulation
Twenty capsules were taken; average weight was determined and mixed well fine powder. Powder equivalent to 250mg of penicillin v was transferred into 100ml volumetric flask and dissolved in sufficient amount of diluents and sonicated to dissolve. Take 5ml of the aliquot in 50ml standard flask and make up the volume with 50ml with the diluents. Solution was filtered through 0.45µ membrane filter and then the filtrate was further diluted to get the required concentrations

Procedure
20µl of the standard preparation and assay preparation were separately injected.

Method Validation
The objective of method validation is to demonstrate that the method is suitable for its intended purpose as it is stated in ICH guidelines [10-13]. The method was validated for linearity, precision (repeatability and intermediate precision), accuracy specificity, short-term stability and system suitability. Phenoxy methyl penicillin Standard plots were constructed with ten

concentrations in the range of 50-150 % (µg/ml) prepared in triplicates to test linearity. The ratio of peak area signal of each drug to that of IS was plotted against the corresponding concentration to obtain the calibration graph [14]. The linearity was evaluated by linear regression analysis that was calculated by the least square regression method. The precision of the assay was studied with respect to both repeatability and intermediate precision. Repeatability was calculated from six replicate injections of each freshly prepared standard solution in the same equipment at a concentration 50 mcg/mL of the intended test concentration value on the same day.[15] The experiment was repeated by assaying freshly prepared solution at the same concentration additionally on two consecutive days to determine intermediate precision. Peak area ratios of each standard to that of IS were determined and precision was reported as % R.S.D. Method accuracy was tested (% recovery and % R.S.D. of individual measurements) by analyzing samples of each drug at three different levels in pure solutions using three preparations for each level. [16] The results were expressed as the percentage of each drug recovered in the samples. Specificity was assessed by comparing the chromatograms obtained from sample of pharmaceutical preparation and standard solution with those obtained from excipients which take part in the commercial tablets and verifying the absence of interferences

RESULTS AND DISCUSSION

System Suitability:

A Standard solution was prepared by using, phenoxymethylpenicillin working standards as per test method and was injected six times into the HPLC system. The system suitability parameters were evaluated from standard chromatograms by calculating the % RSD from six replicate injections for phenoxymethylpenicillin retention times and peak areas. All system suitability parameters meets the predetermined acceptance criteria's as per the test method indicates suitability of the selected system .

Table 1: Repeatability

Injection number	**Phenoxymethylpenicillin**	**4-hydroxyphenoxymethylpenicillin**
1	1603817	34926
2	1605640	34864
3	1604464	34902
4	1604426	34864
5	1603631	34871
6	1604134	34829
7	1603507	34908
8	1605524	34926
9	1603452	34847
10	1601310	34830
Average	1603991	34877
SD	1219.1	36.8
%RSD	0.1	0.1

Precision Studies:

Repeatability:

Standard solution of phenoxymethylpenicillin working standard at 100% targeted concentration was prepared as per the proposed test procedure for repeatability studies. Ten replicate injections were injected into the HPLC system. %RSD for the peak responses as the peak area was calculated, results are shown in Table No.1 and 2

Table 2 Method Precision

Sample number	Phenoxy methylpencellin		4-Hydroxy phenoxy methyl pencillin		Total	
	Mg/tab	% Assay	Mg/tab	% Assay	Mg/tab	% Assay
1	249.69	99.9	5.20	2.1	254.89	102.0
2	248.75	99.5	5.10	2.0	253.85	101.5
3	249.26	99.7	5.20	2.1	254.46	101.8
4	248.94	99.6	5.06	2.0	254.0	101.6
5	249.33	99.7	5.05	2.0	254.38	101.7
6	249.41	99.8	5.05	2.0	254.46	101.8
Average	249.23	99.7	5.11	2.03	254.34	101.73
SD	0.3	0.1	0.1	0.1	0.4	0.2
% RSD	0.1	0.1	1.4	2.5	0.1	0.2

Method Precision:

Six test preparations were prepared as per the proposed test method by weighing the uniform sample matrix for individual test preparation. All individual test preparations were injected into the HPLC system as per the test method.

Table 3 Linearity

Concentration (µg/ml)	Peak Area Response	
	phenoxymethylpenicillin	4-hydroxyphenoxymethylpenicillin
50%	817731	16260
60%	969460	19567
70%	1124207	22463
80%	1282818	25712
90%	1444186	28881
100%	1587668	31943
110%	1760459	34905
120%	1920452	38566
140%	2236959	44696
150%	2392500	48279
Correlation coefficient	0.9999	0.9998
Slope (m)	1592.4850	895.2565
Intercept (y)	21964.2944	272.1407
Statistical Y intercept	1.4	0.9

Linearity

The linearity studies of detector response for phenoxymethylpenicillin were evaluated in the concentration range from about 50% to 150% of the targeted concentration. The diluted standard solutions were prepared from stock solution in the above range and analyzed using proposed analytical method by injecting each level in duplicate injections. The linearity graph of average area response verses concentration was plotted and the correlation coefficient was calculated. The correlation coefficient meets the acceptance criteria indicates that the peak responses are linear. This concludes that the method is linear throughout the range selected. Results are tabulated in table-3

Fig 1Phenoxymethylpenicillin

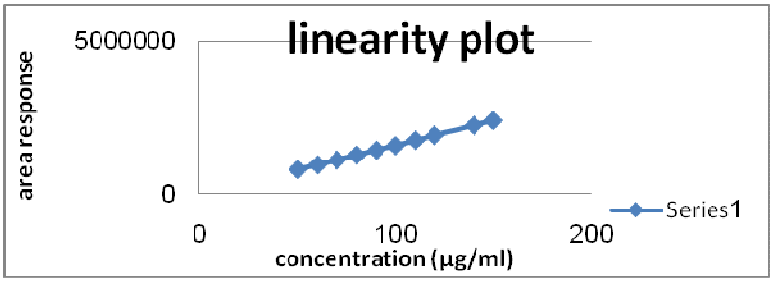


Fig 2 4-Hydroxyphenoxymethylpenicillin

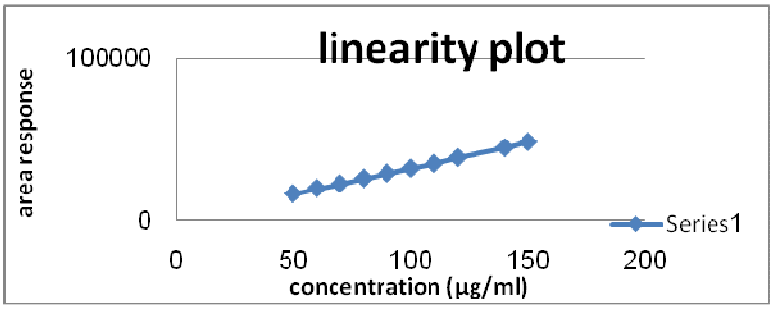


Table-4 Accuracy

Recovery level	Mean peak area response of PMP	mean% Recovery	% RSD recovery	Mean peak area response of 4 hydroxy PMP	mean% Recovery	% RSD recovery
50%	817704	99.5	0.2	15738	99.8	0.0
100%	1582339	99.5	0.2	31716	99.1	0.3
150%	2380278	99.5	0.2	47268	101.5	0.4

Accuracy:

An accuracy study was conducted by spiking the known amount of phenoxymethylpenicillin in the equivalent weight of placebo. Accuracy study was conducted in triplicate at three different levels, (50%, 100%, and 150% of targeted concentration). The samples were analyzed as per the proposed test procedure and the % recovery for each spike level was calculated. The precision at

each spike level was also established. The results found within acceptance criteria, hence the method are accurate throughout the selected range. The results are tabulated in Table-4

Specificity:

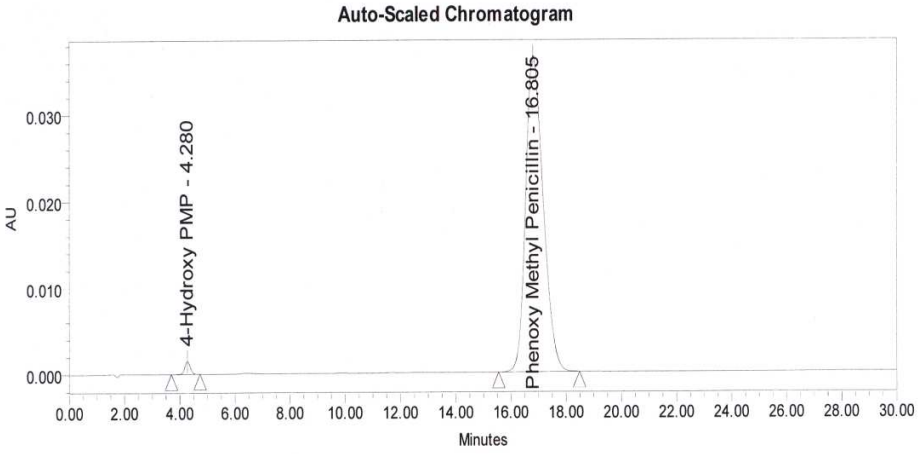
Placebo Interference:

Placebo solution were prepared in triplicate by weighing the equivalent amount present in the finished drug product and analyzed as per proposed method. There was no interference from placebo at retention time of phenoxymethylpenicillin and 4-hydroxyphenoxymethylpenicillin peak. Hence the method is specific.

Sample As Such:

Test preparation was prepared as per the test method and injected into HPLC system and % assay of phenoxymethylpenicillin and 4-hydroxyphenoxy methyl penicillin was calculated. The peak purity result of the sample solution was evaluated. Purity angle of phenoxymethylpenicillin peak and 4-hydroxyphenoxy methyl penicillin in sample chromatogram was lesser than the purity threshold. As per waters, empower software it can be concluded that the peak purity of phenoxymethylpenicillin peak and 4-hydroxyphenoxymethylpenicillin was passed and method is specific.

Fig 3



Robustness:

Robustness of the proposed analytical method was evaluated by making deliberate changes in the chromatographic system method parameters i.e. flow rate and wave length), the standard solution and test solutions were injected for each of the changes made to access the robustness of proposed analytical method.

The effect due to change in flow rate on the system suitability parameters are compared. The system suitability parameters found comply as per acceptance criteria, hence it is concluded that the analytical results remain unaffected even there is change in flow rate by ±10% and wave length by ±5 nm. .

Forced Degradation Studies:

The stress degradation study was carried out on the sample preparations (higher strength) of phenoxymethylpenicillin tablet, and the degradation was evaluated by calculating the %

degradation of 1.0 % -50 % was tried by the stress conditions like acid stress, alkaline ,peroxide, thermal and photolytic degradation to prove the stability indicating characteristics of the method. Purity angle of phenoxymethylpenicillin and 4-hydroxyphenoxymethylpenicillin peak in stressed sample chromatogram was lesser than the purity threshold. As per waters, empower software it can be concluded that the peak purity of 4-hydroxyphenoxymethylpenicillin and phenoxymethylpenicillin peak was passed and method is specific. From forced degradation studies, it is observed that the proposed acceptance criteria meet the requirements. The peak purity results of complete forced degradation studies for the sample solution are summarized in Table 5 & 6 and Fig No 4-8.

Based on the forced degradation studies carried out proposed analytical method can be considered as stability indicating method and can be used for release and stability studies for effective evaluations.

Table No 5 Forced degradation studies

Stress condition	Phenoxymethylpenicillin		4-hydroxy PMP	
	% assay	% degradation	% assay	% degradation
As such (unstressed sample)	101.5	NA	2.5	NA
Acid degradation	85.8	15.5	1.8	28
Alkali degradation	66.7	34.3	1.6	36
Peroxide degradation	66.7	34.3	1.8	28
Thermal degradation	100.2	1.3	2.1	16

Table No 6 Peak Purity Results

Stress condition	phenoxymethylpenicillin				4-hydroxyphenoxymethylpenicillin			
	Purity angle	Purity threshold	Purity flag	remarks	Purity angle	Purity threshold	Purity flag	remarks
acid degradation	0.101	4.009	No	Passes	2.889	4.156	No	Passes
Alkali degradation	0.072	0.252	No	Passes	0.194	0.324	No	Passes
Peroxide degradation	0.111	2.030	No	Passes	0.620	2.241	No	Passes
Thermal degradation	0.090	0.258	No	Passes	0.109	0.273	No	Passes
Photolytic degradation	0.099	0.099	No	passes	0.085	0.264	No	passes

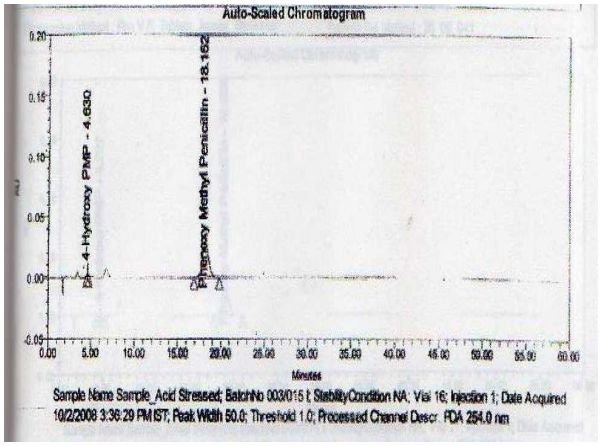


Fig No 4

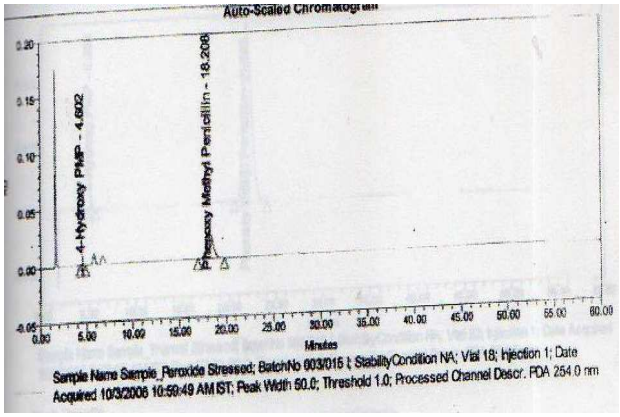


Fig No 5

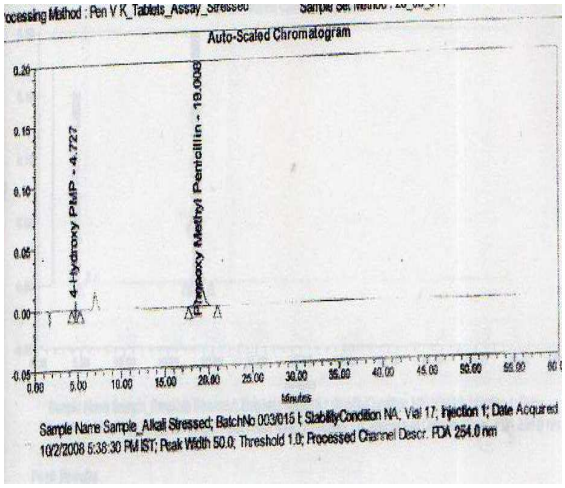


Fig No 6

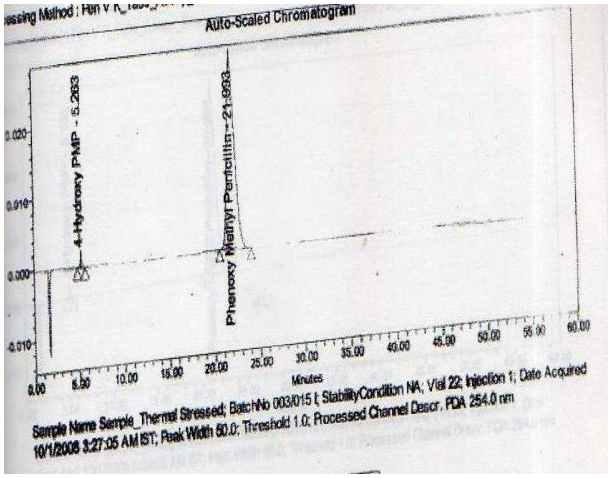


Fig No 7

CHROMATOGRAMS OF FORCED DEGRADATION STUDIES

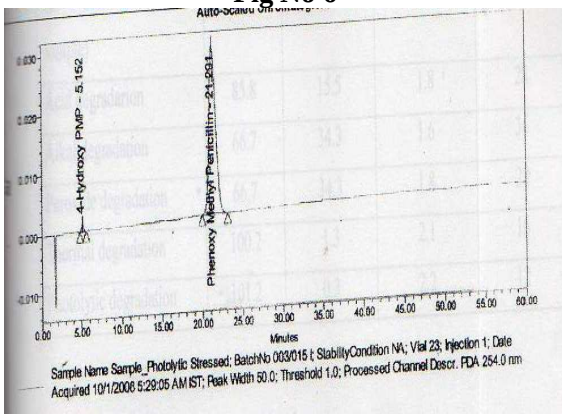


Fig No 8

Fig No 4-8: Acid degradation, Alkali degradation, Peroxide degradation, Thermal degradation, Photolytic degradation respectively

CONCLUSION

Analysis of drugs present in combined pharmaceutical dosage forms is a quite challenging problem and hence attempts were made to develop analytical method for phenoxymethylpenicillin present in dosage forms.

The proposed method is simple, selective, reproducible, sensitive and accurate with good precision. Some of the methods were proved to be superior to most of the reported methods. All these proposed methods for estimation of selected drug phenoxymethylpenicillin was successfully applied in pharmaceutical formulations. The proposed method can be used as alternative methods to the reported ones for the routine determination of selected drug under the study in pharmaceutical dosage forms. Thus the purpose of the present investigation was successfully achieved.

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