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Research Article

Development and Validation of Reverse Phase High Performance Liquid Chromatography Method for the Assay of Fosfomycin Trometamol in Granule Dosage Form

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A simple and sensitive reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for the assay of Fosfomycin trometamol in granules dosage form. The potassium dihydrogen phosphate in water pH 4 buffer was used as a mobile phase with isocratic elution at a flow rate of 1 ml/min. Avantor Aminopropyl silyl silica gel column (250mm x 4.6mm x 5µ) was used for chromatographic separation. The effluent was detected by using differential refractometer at 35°C. The retention time was found to be 10 minutes for Fosfomycin trometamol. The method showed linearity over the range of 50-150 µg/ml with correlation coefficient r2 = 0.9996. The % RSD for system precision, method precision and intermediate precision was 0.7%, 0.63% and 0.48% respectively. The system suitability parameters for Fosfomycin trometamol, such as theoretical plates and symmetry factor, were found to be 13634 and 1.0 respectively. The % recoveries were in the range of 98 to 102 (RSD < 2%). There was no interference observed in the blank injections at the retention time of Fosfomycin trometamol and the peaks due to impurities and the active ingredient were well resolved. Hence the method proved to be specific. The drug shows stability in solution upto 20 hrs. The assay of fosfomycin was shown to be stability-indicating. Overall, a new RP-HPLC method was developed and successfully validated for the assay of Fosfomycin trometamol in granule dosage form.

Keywords: Fosfomycin, RP-HPLC, Assay, Forced degradation, Validation.

INTRODUCTION

Fosfomycin is a broad-spectrum antibiotic with activity against gram-positive bacteria, including multi-drug resistant (MDR) pathogens associated with life threatening infections. It is indicated for the treatment of acute uncomplicated urinary tract infections (UTIs) [1-2]. High Performance Liquid Chromatography (HPLC) is a technique in analytical chemistry used to separate, identify and quantify each component in a mixture. One of the most used analytical tools for quantifying diverse analytes in analytical chemistry is HPLC. The reported ultraviolet (UV) detection methods are unsuitable because Fosfomycin exhibits poor UV absorbance due to the presence of an epoxide ring and the absence of a strong chromophore. Hence normal phase liquid chromatography or ultraviolet (UV) detector methods are inadequate and ineffective. The chemical structure of Fosfomycin trometamol is shown as figure 01.

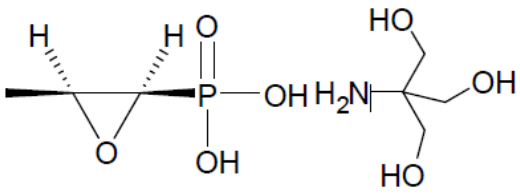

Figure 01: The Chemical Structure of Fosfomycin Trometamol [2]

Further there is no pharmacopeial monograph exists for the finished dosage form such as fosfomycin granules for oral solution in United States Pharmacopoeia (USP), British Pharmacopoeia (BP), Japanese Pharmacopoeia (JP), or World Health Organization (WHO), and existing compendial methods are only available for the drug substance Fosfomycin. There are very few published articles which emphasizes determination of fosfomycin trometamol and its related substances in the bulk drug only. [3-11] Based on literature review, an appropriate RP-HPLC method is required for routine analysis of fosfomycin trometamol in granule dosage form. The present method utilizes readily available, cost-effective laboratory reagents which are suitable for routine analysis and validation of fosfomycin trometamol in the final dosage form. Hence our aim is to develop a method which is more precise, stability-indicating and robust.

MATERIAL AND METHODS:

Reagents and chemicals: Working standard and commercial granule of Fosfomycin trometamol (MONUROL - NDC0456-4300-08 - Zambon/ Forest Pharma) were used as sample containing 5.631g Fosfomycin trometamol equivalent to 3g fosfomycin, Potassium dihydrogen phosphate analytical grade was purchased from Rankem which was used to prepare mobile phase in water, Nylon filter 0.22 µm (PALL Lifesciences P/No. NR047100I) was used for the mobile phase filtration.

Instrumentation:

The chromatographic separation was carried out by utilizing HPLC system Shimadzu (model: 2050 CI Series). An Avantor (Cat No.: EXL-1214-2546U) 250mm x 4.6mm x 5µ, aminopropyl silyl silica gel column with differential refractometer at 35°C was used for chromatographic separation and quantification.

Chromatographic conditions:

The solution of potassium dihydrogen phosphate (10.89 g/L) in water was used as mobile phase with isocratic elution. The said solution was sonicated to dissolve and filtered through 0.22µm Nylon 6,6 membrane filter. The mobile phase was pumped into the column at a 1.0 mL/min for a total run period of 25 minutes and 40 minutes for standard and sample respectively. The injection volume was 10µL, the detection of effluent was performed at 25°C as column temperature.

HPLC system cleaning procedure before analysis:

The pressure was given to wash the system by 0.22 micron capillary column, (Part No.228-38994 Make: Shimadzu or equivalent). First washing was carried out with warm water about 30 minutes, followed by methanol for 30 minutes and final washing was done with water for 30 minutes.

Mobile phase and diluent preparation:

Mobile phase was prepared by taking 10.89 g/L solution of Potassium dihydrogen phosphate in water, said mixture was placed it in an ultrasonic bath and degassed for 15-20 minutes. The said mixture is filtered through 0.22 µm Nylon 6, 6 membrane filter. The mobile phase solution was also used as diluent for standard and sample preparation.

Preparation of standard solution:

Carefully weighed quantity of Fosfomycin Trometamol (282 mg) as a working standard was transferred into 10 mL volumetric flask. About 7 mL of diluent was added to it and dissolved by shaking for

about 2 to 3 minutes. With diluent made volume up to the mark and mix well. The standard solution was freshly prepared, before 5 minutes to run on HPLC System.

Preparation of Sample Solution:

Accurately weighed quantity of sample powder (equivalent to about 751 mg of Fosfomycin) 2003 mg was transferred into 50 mL volumetric flask. 35 mL of diluent was added to it and dissolved completely by shaking about 2 to 3 minutes. With diluent made volume up to the mark and mix well. The resulting mixture was filtered through 0.22 µm nylon syringe filter. Open and mix 2 sachets of Fosfomycin trometamol powder. Do not expose it to the environment, do the sample weighing immediately after mixing.

Selection of detector:

Fosfomycin trometamol standard solution was scanned, and spectrum was detected by using differential refractometer at 35°C.

Method Development:

The final method for analyzing Fosfomycin trometamol was described based on the results of the optimization of chromatographic conditions.

Tablet 01: Optimized Chromatographic Conditions

Parameters	Condition
Mode	Isocratic
Column	250mm x 4.6mm x 5µ, Aminopropyl silyl silica gel [Avantor (Cat No.:EXL-1214-2546U)]
Flow Rate	1.0 mL/min
Detection	Differential refractometer at 35°C
Injection Volume	10µL
Column Temperature	25°C
Autosampler Temperature	8°C
Retention Time	About 10.0 Minutes
Run Time	**For Standard:** 25 Minutes **For Sample:** 40 Minutes
Rinse Port	Water (HPLC Grade)

Method Validation:

The developed HPLC methods for assay of Fosfomycin Trometamol in Fosfomycin granules for oral solution (3 g) were validated in accordance with ICH Q2(R1) guidelines. Validation was performed at SRS Pharmaceuticals Pvt. Ltd. (Research Centre), Navi Mumbai. The parameters listed below were used to validate the method.

System suitability:

System suitability parameters were evaluated as per USP criteria. The system suitability parameters like symmetry of the retention time, run time, resolution for close peaks, theoretical plates, symmetry factors were optimized by using different brands of amino column to devise and develop system suitability conditions.

Precision:

The Precision of analytical method is the degree of agreement among individual test results when applied repeatedly to multiple samples of a homogenous sample. Relative standard deviation (RSD) in percent is generally used to present the precision of the method. The precision is determined in three different ways – system repeatability (System precision), method repeatability for a homogenous sample (method precision), and intermediate precision by repeatability of method for samples between two sets of experiments (different days/ different analyst/ different instruments or a combination of any of them). System precision, method precision, and intermediate precision were performed as per methodology.

System Precision: Five injections of the standard solution & two injections of the sample solution were chromatographed as per the methodology. The standard solution was prepared and analyzed in accordance with the methodology. The percentage relative standard deviation was determined of area of Fosfomycin in standard solution. The relative standard deviation for area of five replicate injection of standard solution should NMT 2.0%.

Method precision: Six different sample solutions and standard solutions were prepared and analyzed in accordance with the methodology. The average assay of six sample preparations for the known component was calculated and reported. Similarity factors between standard 01 and standard 02 were calculated and it should be between 0.98 to 1.02. The percentage relative standard deviation of area of five replicates of standard solution and bracketing standard solution should be less than 2.0%. The average assay of six sample preparation should be NLT 90 % and NMT 110 %. The percentage relative standard deviation for assay of six sample preparation should be NMT 5.0%.

Intermediate precision: Six different sample and standard solutions were prepared for method precision were used and analyzed on a different day, using different instruments and by a different analyst, as per the methodology. The average assay of six sample preparations for the known component was calculated and reported. The similarity factors between standard 01 and standard 02 was calculated and it should be between 0.98 to 1.02. The percentage relative standard deviation of area of five replicates of standard solution and bracketing standard solution should be less than 2.0%. The average assay of six sample preparation should be NLT 90 % and NMT 110 %. The relative standard deviation for Assay of six sample preparation should be NMT 5.0%. The absolute difference in test result between normal condition and changed condition should be no more than 3.0 %.

Specificity (Selectivity):

For an assay, specificity implies that the signal recorded is from the substance of interest, without any interference from excipients, degradation products and/or impurities, or contamination or the analyte with each other. After each injection, standard solution and sample solution chromatograms should have similar retention times. At the retention time of the analyte peak, the blank chromatogram should show no signal and there is no interference from blank. As a result, the procedure is specific. Each of the blank, placebo solution, standard solution, individual impurities, and sample solution was injected into the HPLC system equipped with a differential refractometer.

Linearity and range:

It was performed by preparing different concentrations of the drug solution. A series of standard preparations were prepared utilizing known components in the standard solution across a range of 50 % to 150 % of the working concentration of Fosfomycin (in mg/mL of API) using diluent. This range corresponded to the concentration used at the 100% level. A minimum of five levels was used for the linearity study.

Accuracy:

Recovery studies were conducted using the standard addition approach to ensure the method's reliability and accuracy. The accuracy studies were conducted by adding fosfomycin trometamol sample drug in the range of 50% to 150% in triplicate. Data from nine measurements at three concentration levels, covering the whole concentration range was obtained. The similarity factors between standard 01 and standard 02 was calculated and it should be between 0.98 to 1.02. The percentage relative standard deviation of five replicates of standard solution and bracketing standard solution should be less than 2.0%. Individual recovery should be within 98% to 102% and mean recovery at each level (n=3) should be within 98.0% to 102.0%. Relative standard deviation of % recovery and overall RSD for % recovery at each respective level should not be more than 2.0%.

Robustness:

Changes in buffer pH, wavelength, flow rate, etc. were used to calculate robustness, and the percentage relative standard deviation was obtained. Small alterations in the above parameter can be used to determine robustness, and the method's resistivity was developed as well. The modification in parameters

could result in considerable variations in peak area, retention time, and RSD. Three sample solutions were analyzed for robustness. The change in flow rate (±0.2ml/min) and change in the column oven temperature (±5°C) were performed. The average assay of three sample preparations for the known component was calculated and reported. The similarity factors were calculated between standard 01 and standard 02 and it should be between 0.98 to 1.02. The percentage relative standard deviation of area of five replicates of standard solution and bracketing standard solution should be less than 2.0%. The average assay of three sample preparation should be between 90 % and 110 %. The relative standard deviation for assay of three sample preparation should be NMT 5.0%. The difference in the mean results of normal and changed condition should be within ± 10.0%.

Solution stability:

Sample and standard solutions were prepared and evaluated for solution stability by analyzing at different time intervals. Solution stability studies were performed for 45 hrs and %RSD of area was calculated. The similarity factors were calculated between standard 01 and standard 02 and it should be between 0.98 to 1.02. % RSD of area of five replicates of standard solution and bracketing standard solution should be less than 2.0%. Sample solutions will be considered stable if the results obtained are within 98.0% to 102 .0% of initial result. The response of standard solution should not differ by more than 2.0% from the initial response.

Forced degradation studies:

Forced degradation studies help to identify chemical behavior of the drug substance and provide data to support identification of possible degradants (impurities) and degradation pathway. Further forced degradation studies are crucial for the development of stability-indicating methods. Forced degradation studies were carried out by exposing samples to acid degradation, base degradation, oxidative degradation, and thermal degradation.

As per ICH Q1A(R2) and Q2(R1), stress testing was performed under the following conditions:

- Acid degradation: 1M HCl, 15 min at room temperature
- Base degradation: 4M NaOH, 4 hrs at room temperature
- Oxidative degradation: 10% H_2O_2, 5 hrs at room temperature
- Water hydrolysis: 70°C, 15 min
- Thermal degradation: 80°C, 6 hrs (dry heat)
- Untreated samples: As reference control

RESULT AND DISCUSSION:

Method development:

To resolve the Fosfomycin trometamol peak, several mobile phase mixtures were attempted. The optimum mobile phase containing buffer 10.89 g/L potassium dihydrogen phosphate in water was selected because it could resolve the peak of Fosfomycin trometamol. Determination was achieved with differential refractometer at 35°C on the basis of peak area at 1.0 ml/min flow rate. A typical HPLC chromatogram achieved during Fosfomycin trometamol determination is shown in Figure 2 to 4.

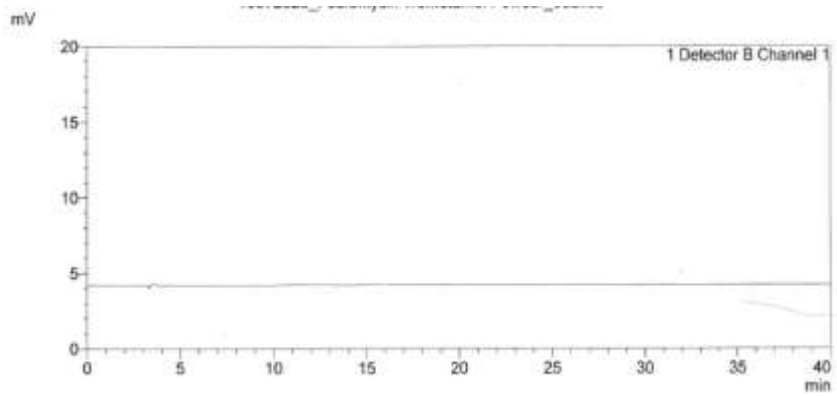

Figure 02: Blank chromatograph

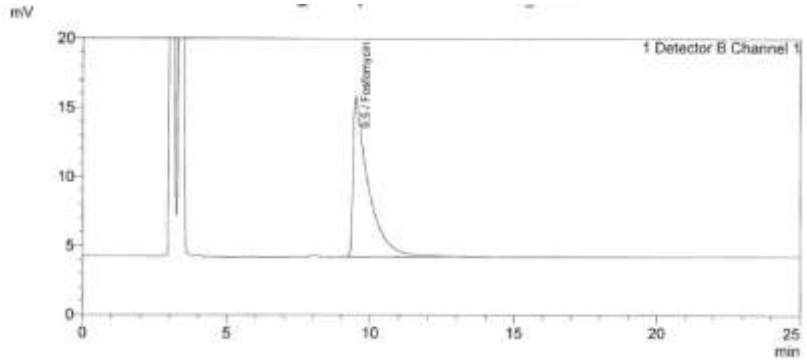

Figure 03: Standard solution chromatograph

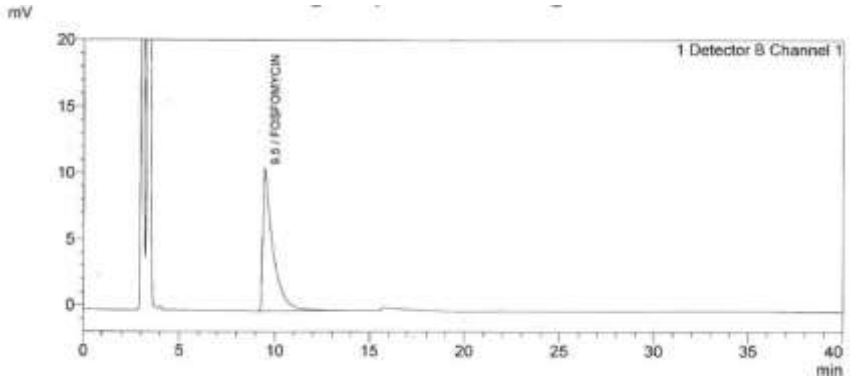

Figure 04: Sample solution chromatograph

A rapid and specific RP - HPLC method has been developed for the assay of Fosfomycin in Fosfomycin Trometamol powders 3 g. The developed method also ensured early elution of active peak thereby significantly reducing chromatographic run times and enabling faster analysis. Methods find specific with respect to their known and unknown impurity. Based on the conducted experimental trials and using optimized method, the Fosfomycin peak was consistently eluted at approximately 10 minutes with improved peak shape, adequate response, and satisfactory system suitability.

Method validation:

The developed HPLC methods for assay of Fosfomycin Trometamol in Fosfomycin granules for oral solution (3 g) were validated in accordance with ICH Q2(R1) guidelines.

1. System suitability:

The system suitability parameters like symmetry of the retention time, run time, resolution for close peaks, theoretical plates, symmetry factors were optimized by using different brands of amino column in order to devise and develop system suitability conditions.

Table 02: Result of systems suitability study

System suitability parameters	Column description		
	Princeton amino column (25cm x 4.6 mm,5µ)	**Avantor amino column (25cm x 4.6 mm,5µ)**	**Zorbax amino column (25cm x 4.6 mm,5µ)**
Retention time	About minutes	About minutes	About minutes
Run time	7 minutes	10 minutes	12 minutes
Resolution for close peaks	Not injected due to peak distortion observed	3.7	2.6
Theoretical plates	10858	13634	7656
Symmetry factor	1.2	1.0	1.1

Precision:

System precision:

The obtained %RSD value of system precision was 0.7 which meet the ICH guidelines permitted standards. The result of system precision is given in table 03. The precision studies results reported in %RSD, which meet the ICH guidelines permitted standards and shows strong repeatability, suggesting that the developed method has excellent precision.

Table 03: Results of System precision

S/N	Fosfomycin	
	Retention Time	Area
1	10.3	414738
2	10.2	412079
3	10.2	417093
4	10.2	418441
5	10.2	419281
Mean	10.2	416326
%RSD	0.4	0.7

Method precision and Intermediate precision:

The average assay of six sample preparations by method precision was found to be 96.2% and the % RSD was found to be 4.4%. The average assay of six sample preparations by intermediate precision was found to be 94.2% and the % RSD was found to be 4.4%. The absolute difference in test results between method precision and intermediate precision was found to be 2.0 %. The method and intermediate precision other parameter results are within the acceptance criteria.

Table 04: Results of Method precision and Intermediate precision

Sample No.	Method Precision					Intermediate Precision				
	Sample Weight	Area	%Assay	Avg. Assay(%)	% RSD	Sample Weight	Area	%Assay	Avg. Assay(%)	% RSD
Sample 1	2003.56	409771	98.5	96.2%	4.4%	2008.44	392393	94.5	94.2%	4.4%
Sample 2	2010.30	413991	99.2			2005.10	372499	89.9		
Sample 3	2004.76	384842	92.5			2008.49	377010	90.8		
Sample 4	2003.20	378061	90.9			2014.74	399166	95.8		
Sample 5	2011.15	424586	101.7			2011.24	386944	93.1		
Sample 6	2005.85	391861	94.1			2009.09	420584	101.3		

Specificity:

Standard and sample solutions were prepared and analyzed as per the methodology. Each of the blank, placebo solution, standard solution, individual impurities, and sample solution was injected into the HPLC system equipped with a differential refractometer detector.

Table 05: Results for specificity for Blank interference

S/N	Components	Retention time in min of Individual identification solutions	Peak area of at retention time in blank solution	% Blank interference
01	Fosfomycin	10.3	Not detected	No interference
02	Fosfomycin Trometamol Impurity A	8.4	Not detected	No interference
03	Fosfomycin Trometamol Impurity B	4.0	Not detected	No interference
04	Fosfomycin Trometamol Impurity C	4.8	Not detected	No interference
05	Trometamol	3.0 and 3.4	Not detected	No interference

Table 05: Results for specificity for placebo interference

S/N	Components	Retention time in min of Individual identification solutions	Peak area of at retention time in Placebo solution	% Placebo interference
01	Fosfomycin	10.3	Not detected	No interference
02	Fosfomycin Trometamol Impurity A	8.4	Not detected	No interference
03	Fosfomycin Trometamol Impurity B	4.0	Not detected	No interference
04	Fosfomycin Trometamol Impurity C	4.8	Not detected	No interference
05	Trometamol	3.0 and 3.4Min	Observed*	No interference

Two peaks were observed from the standard identification of Trometamol. The Trometamol-1 peak did not interfere in the placebo, whereas the Trometamol-2 peak overlapped with the placebo peak. However, since Trometamol is not an active component and did not interfere with the active ingredient or its related impurities, it was considered non-impacting to the assay evaluation. Injections of the blank and placebo solutions did not exhibit any peak at the retention time of any known component. The peaks due to impurities and the active ingredient were well resolved.

Linearity and range:

The method's linearity was established throughout a concentration range of 50 to 150 %. From the standard stock solution, aliquots of 50%, 75%, 100%, 125%, 150% were prepared. For each concentration the solution was injected, and a calibration curve was obtained by plotting the peak area v/s the drug concentration, which showed a linear relationship over a concentration range of 50 to 150 µg/ml. The regression equation was found to be y = 26.297x+14291. The correlation coefficient was found to be, r^2= 0.9996. The calibration curve and observation were shown in the figure 04 and table 06 respectively. All parameters met their respective acceptance criteria; hence, the method was considered linear.

Table 06: Results of linearity study

Injection No.	Peak Area				
	50 %	75 %	100 %	125 %	150 %
1	208545	314234	411304	512168	601679
2	209328	313975	415757	514252	607867
3	207392	308012	411345	505024	601328

4	213665	--	--	--	608077
5	208133	--	--	--	600714
6	206857	--	--	--	610392
Average Value	**208987**	**312074**	**412802**	**510481**	**605010**
SD	2449.72	3519.89	2559.19	4839.69	4234.22
%RSD	1.17	1.13	0.62	0.95	0.70

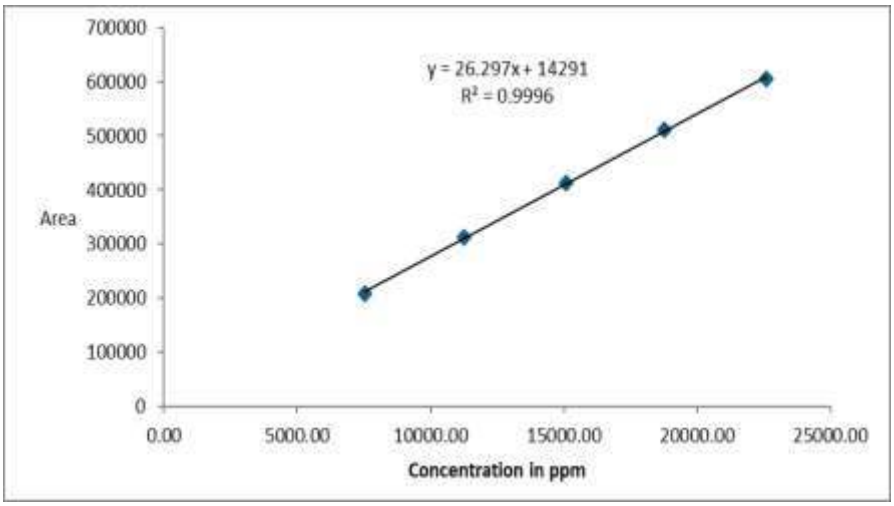

Figure 04: Linearity Plot of Fosfomycin

Accuracy:

All accuracy levels were well established within their acceptance limits; hence, the method was considered accurate.

Table 07: Results for Accuracy studies

Accuracy level	Amount added (mg)	Amount recovered (mg)	% Recovery	% Mean Recovery	%RSD
Level 1 (50%)	751.10	746.52	99.4	100.0	0.6
	751.54	753.53	100.3		
	751.38	754.48	100.4		
Level 2 (100%)	1502.17	1494.62	99.5	99.8	0.3
	1502.30	1502.91	100.0		
	1502.32	1499.75	99.8		
Level 3 (150%)	2253.34	2231.61	99.0	98.9	0.3
	2253.43	2222.12	98.6		
	2253.42	2234.26	99.1		

Robustness:

Three sample solutions were analyzed for robustness. Change in flow rate and column oven temperature were used to calculate robustness, and the %RSD was obtained. Table 08 shows the findings, which were acquired in the same order as the optimised results. The %RSD was within the acceptable range, i.e. less than 2 and average assay of three sample preparation was found to be in between 90 % and 110 %. None of these variables had a considerable impact on the retention time of drug suggesting that the presented method is considered as robust and suitable for the analysis of Fosfomycin when the analytical conditions were slightly changed.

Table 08: Results for Robustness

Robust Condition→ Sample No.↓	Flow 1.2 mL	Flow 0.8 mL	Column Temp 30°C	Column Temp 20°C
Sample_1	94.4	97.9	97.8	97.3
Sample_2	92.3	102.3	96.3	96.0
Sample_3	93.9	100.7	95.8	95.9
% Average assay Series 2	93.5	100.3	96.7	96.4
%RSD	1.2	2.2	1.1	0.8
Series 1 (Method Precision)	96.2			
DBM	2.7	-4.1	-0.5	-0.2

#Difference between mean (DBM)

Solution Stability:

The standard and sample solution's stability was evaluated by assessing samples at ambient temperature for a specified time interval. The % area difference values at different time intervals were found to be < 2 of the initial zero-time interval solution and the % assay obtained are within 98.0% to 102 .0% of initial result, showing that the solutions were stable.

Table 09: Results for solution stability

Hours	Retention Time	Area	% Assay	% Difference
0 Hr.	10.2	409771	98.53	NA
4 Hr. 30 Min	10.2	410600	98.73	- 0.2
9 Hr. 10 Min	10.2	410572	98.73	- 0.2
15 Hr. 10 Min	10.1	413360	99.40	- 0.9
19 Hr. 50 Min	10.1	405673	97.55	1.0
22 Hr.	10.1	419097	100.78	- 2.2
24 Hr.	10.1	416586	100.17	- 1.6

Forced degradation studies assay results:

Forced degradation studies were carried out by exposing sample to acid degradation, base degradation, peroxide degradation, thermal and photolytic degradation. Percentage assay decreased notably under acidic and hydrolytic conditions, correlating with impurity formation. Forced degradation confirmed acidic and hydrolytic conditions are the most critical for Fosfomycin degradation. The percentage degradations determined by the method are given in table 10.

Table 10: Results for forced degradation study (Assay results)

Conditions	Assay (%)
Untreated	101.7
Acid degradation	89.8
Water hydrolysis	93.4
Base degradation	104.7
Oxidative degradation	103.0
Elevated temperature	100.3

Mass balance remained within acceptable limits (≥90%), confirming method stability-indicating capability.

Table 11: Results of Mass Balance (Fosfomycin Granules):

Condition	Total Impurities (%)	Assay (%)	Mass Balance (%)
Acid degradation	18.75	89.8	106.7
Water hydrolysis	11.84	93.4	103.5
Base degradation	0.34	104.7	103.3
Oxidative degradation	3.38	103.0	104.6
Elevated temperature	1.44	100.3	100.0

The assay percentage was found to be in the specified limit i.e. NLT 90% and NMT 110%. The developed method was therefore shown to be suitable for the routine analysis of fosfomycin trometamol in granule formulation.

CONCLUSION:

The prior art and literature disclose the ion pair HPLC method for determination of fosfomycin trometamol in bulk drug. Hence there is no teaching or guidance for the development of HPLC method for estimation fosfomycin trometamol in pharmaceutical formulation. The present author developed a sensitive, precise, and accurate RP-HPLC method for measuring fosfomycin trometamol in granule dosage form. The validation study confirms that the developed HPLC method is specific, precise, accurate, linear, robust and stability-indicating. The assay is decreased notably under acidic and hydrolytic conditions, correlating with impurity formation. Forced degradation confirmed acidic and hydrolytic conditions as the most critical for Fosfomycin degradation. Mass balance remained within acceptable limits (≥90%), confirming method stability-indicating capability. Mass balance findings (>90%) demonstrate that the method accounts for degradation loss, supporting its use for routine QC and stability studies. For routine quality monitoring of fosfomycin in pharmaceutical formulations, the developed method is therefore easy to use and has quick analytical time. The overall study results demonstrate that the developed HPCL method is appropriate for routine quantitative analysis of fosfomycin in dosage form and for routine use in release testing, stability studies, and regulatory submissions.

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