

Research Article



Development and Validation of RP-HPLC Method for the Simultaneous Determination of Ampicillin and Cloxacillin in Oral Suspension Dosage Form

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ABSTRACT

A simple, precise, accurate and rapid reverse phase high performance liquid chromatographic isocratic method has been developed for the quantification of Ampicillin and Cloxacillin in Oral suspension dosage forms. The chromatographic separation was attained on P. Hypersorb ODS (250 x 4.6) mm with precolumn as the stationary phase at ambient temperature. The mobile phase was composed of Acetonitrile and 0.02 M Phosphate buffer pH 3.0, 35:65 with a flow rate of 1.8 mL/min, and UV detection at 225 nm. The run time was 9 minutes. The injection volume was 20 μ L. The retention times of Ampicillin and Cloxacillin were 1.943 ± 0.001 minutes and 6.058 ± 0.002 minutes respectively. Flucloxacillin was used as the internal standard and its retention time was 7.057 ± 0.004 minutes. The linearity of Ampicillin and Cloxacillin were investigated with the concentration ranges of 0.0185 %w/v – 0.0277 %w/v and 0.0175 %w/v – 0.0263 % w/v respectively. The correlation coefficients were greater than 0.99. The mean percentage recovery of Ampicillin at 80 %, 100 % and 120 % concentration levels were 101.5 ± 0.5 , 99.6 ± 0.9 and 99.4 ± 0.3 respectively. That of Cloxacillin were 99.7 ± 0.6 , 100.2 ± 0.1 and 101.4 ± 0.3 for 80 %, 100 % and 120 % concentration levels respectively. The results obtained show a method that is simple, rapid and precise. It can be used for the estimation of Ampicillin, Cloxacillin or Flucloxacillin in single as well as combination pharmaceutical dosage forms.

Keywords: Ampicillin, Cloxacillin, Flucloxacillin, RP-HPLC, Validation.

INTRODUCTION

Ampicillin is a beta-lactam antibiotic. It is bactericidal and has a similar mode of action to that of benzylpenicillin, but as an aminopenicillin with an amino group side-chain attached to the basic penicillin structure, Ampicillin is better able to penetrate the outer membrane of some gram-negative bacteria and has a broader spectrum of activity. The chemical formula is $C_{16}H_{19}N_3O_4S \cdot 3H_2O$ and its molecular weight is 403.45 g/mol.

Cloxacillin is isoxazoly penicillin used similarly to flucloxacillin in the treatment of infections due to staphylococci resistant to benzylpenicillin. It may be used with other antibacterials, including ampicillin, to produce a wider spectrum of activity. The molecular formula is $C_{19}H_{18}ClN_3NaO_5S \cdot H_2O$ and the molecular weight is 475.55 g/mol.¹

A number of methods for the simultaneous determination of Ampicillin and Cloxacillin have been reported.²⁻⁴ The aim of this research is to develop a cost-effective method that can be used to analyze pharmaceutical dosage forms containing either single or combinations of Ampicillin, Cloxacillin and / or Flucloxacillin. This study was done in accordance with the International Conference on Harmonization (ICH) guidelines.⁵

MATERIALS AND METHODS

Materials and Reagents

Knauer HPLC system with autosampler and UV detection, Measuring cylinder, P. Hypersorb ODS (250 x 4.6) mm column; particle size 5 μ m (Make: Knauer (Germany)), Analytical balance (Make: Kern and Sohn GmbH, Model: ABJ 120-4M), pH Meter (Make: Labindia Model: SAB 5000), Potassium dihydrogen orthophosphate, Phosphoric acid, Acetonitrile (HPLC Grade) (Make: Merck, India). Distilled water, volumetric pipettes (100 mL, 50 mL), Column oven (Make: Knauer (Germany)), Syringe-driven filter unit (Make: Millipore Millex, Lot Number: ROAAB2861), Ampicillin trihydrate working standard (potency: 98.95 % on dried basis), Cloxacillin sodium working standard; Potency: 100.79 on dried basis; Flucloxacillin working standard; Potency: 98.79 % on dried basis, Millipore vacuum pump (Make: Millipore, Model: WP6122050), 0.2 μ m Mobile phase filter (Make: Millipore Model: GNWP).

Instrumentation and Chromatographic conditions

A Reverse phase - High Performance Liquid Chromatographic (HPLC) system, controlled by Knauer Advanced Scientific Instrument data handling system⁶ and coupled with an auto sampler was used for the analysis. The data was recorded using Euro Chrome software. A stainless steel column 250 mm long, 4.6 mm internal



diameter filled with Octadecyl silane chemically bonded to porous silica particles of 5 µm diameter (Prontosil Hypersorb ODS, 5 µm, (250 x 4.6) mm, with precolumn, (Make: Knauer Advanced Scientific, Germany)) was used with the mobile phase composed of Acetonitrile and 0.02 M Phosphate buffer pH 3.0 in the ratio, 35:65 at ambient temperature. The flow rate was 1.8 mL/minute. The sample injection volume was 20 µL. The UV detector was set at a wavelength of 225 nm for the detection. The run time was 9 minutes.

Application of Proposed Method for the Analysis of Oral Suspension Formulation

Sugar B.P., Sodium C.M.C. B.P. (Prehydrated ticalose CMC 2500), Sodium saccharin, Sodium citrate B.P., Talc B.P., etc. are but a few of the excipients used in the formulation.

The internal standard solution was prepared by weighing and transferring 54.53 mg of Flucloxacillin sodium working standard into a 50 mL volumetric flask. It was dissolved and diluted to volume with the mobile phase.

Samples were analysed by transferring an amount of the reconstituted Ampicillin-Cloxacillin combination oral suspension equivalent to 57.73 mg of Ampicillin trihydrate and 54.71 mg of Cloxacillin sodium into a 50 mL volumetric flask. It was dissolved and diluted to volume with the mobile phase. 5 mL each of the stock solution as well as the internal standard were transferred into a 25 mL volumetric flask and diluted to volume with the mobile phase. Samples were analyzed using the developed RP-HPLC method. The results are reported as means ± SEM (standard error of the mean). Results were statistically analyzed for significant differences.

Validation Parameters

The validation exercise was performed as per ICH guidelines. It is applicable for the routine analysis of Ampicillin and Cloxacillin in combined dosage forms.

Thus, Specificity was performed by injecting 20 µL aliquot of the Mobile phase (diluent), Placebo solution, a solution of Placebo spiked with Ampicillin trihydrate working standard, a solution of Placebo spiked with Cloxacillin sodium working standard, Ampicillin trihydrate working standard solution, Cloxacillin sodium working standard solution and Flucloxacillin working standard solution.

System suitability test was determined by six replicate injections of the standard solution. The respective peak responses and the RSD for six replicate injections were recorded. The retention time, RSD of relative peak areas, tailing factor, number of theoretical plates and mean resolution factors were recorded as represented in Table 1.

The precision of the method was determined by evaluating repeatability and intermediate precision. Intraday precision was determined by preparation and analysis of the same sample in six replicates evaluated on

the same day and by the same analyst. Inter day precision was performed in six replicates at intervals of one day with analysis made by two different analysts. The Percentage contents and relative standard deviation (RSD) were determined in each case as represented in Table 2. The results were subjected to statistical analysis to determine any significant differences.

Accuracy was determined using the method of spiking. Three different amounts corresponding to 80 %, 100 % and 120 % concentrations of Ampicillin trihydrate and Cloxacillin sodium standards were added to the placebo. The nominal concentrations were compared to the actual concentrations and the percentage recoveries were noted. The results were subjected to statistical analysis to determine any significant differences.

The correlation coefficient, slope and intercept were determined after plotting a graph of relative peak area against concentration for Ampicillin and Cloxacillin.

Robustness was determined by making deliberate changes to the following parameters; flow rate, mobile phase composition and the real pH of the mobile phase. Results obtained were subjected to statistical analysis.

The stability of Ampicillin, Cloxacillin and the reconstituted sample in the analytical solution (mobile phase) were evaluated by storing them at ambient temperature (25 °C) and analyzing them at various time points (0 (initial), 3, 6, 9, 24 hours). The responses were compared with those of freshly prepared solutions.

RESULTS AND DISCUSSION

Method Development

The analytes are chromophoric and therefore a UV/Vis detector was chosen. As expected from the UV spectrum of the compounds, far UV detection at 210-230 gave more sensitive detection. Hence, a wavelength of 225 nm was chosen. Ampicillin is acidic and is soluble in dilute acids. Cloxacillin is acidic and freely soluble in water. An acidic mobile phase was chosen as it typically yields better peak shapes. Various mobile phase ratios such as 30:70 and 40:60 (Acetonitrile and 0.02 M Phosphate buffer adjusted to pH 3.0 with orthophosphoric acid) were experimented. The analytes were effectively separated with a ratio of 35:65 (Acetonitrile and 0.02 M Phosphate buffer pH 3.0). With a flow rate of 1.8 mL/min, all the analytes had shorter retention times thereby allowing the analysis to have a relatively short runtime of 9 minutes. A stainless steel column 250 mm long, 4.6 mm internal diameter filled with Octadecyl silane chemically bonded to porous silica particles of 5 µm diameters (Prontosil Hypersorb ODS, 5 µm, (250 x 4.6) mm, with precolumn was used for the entire research. An injection volume of 20 µL was chosen as all the analytes gave detectable peak areas.



Method Validation

In evaluating specificity, there were no peaks in the Diluent. There was a peak however in the placebo and this peak was identified to be Sodium saccharin.

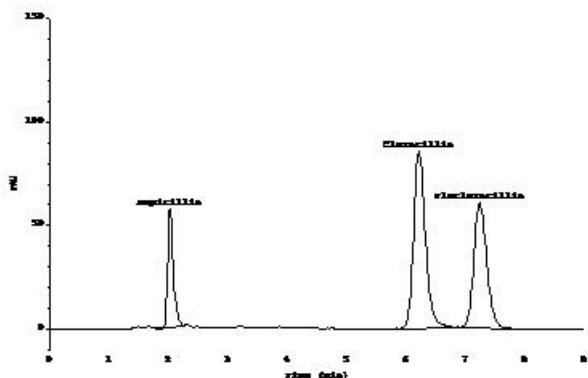


Figure 1: Chromatogram of Ampicillin, Cloxacillin and Flucloxacillin

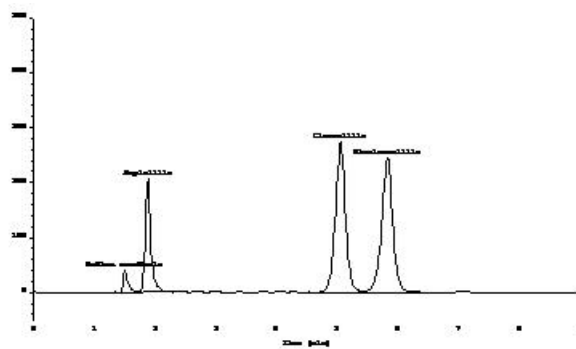


Figure 2: Chromatogram of synthetic blend

The order of elution from synthetic blend is as follows;

1. Sodium saccharin
2. Ampicillin
3. Cloxacillin
4. Flucloxacillin

Table 1: Results of Specificity and System Suitability Test

Specificity		System Suitability Test		
Solution	Retention time (minute)	Parameter	Ampicillin	Cloxacillin
Diluent	No peak	Retention Time (minute)	1.942 ± 0.004	6.056 ± 0.008
Placebo	1.487 ± 0.001 (Peak emanating from Sodium saccharin)	Theoretical plates	3600 ± 79.66	4566 ± 130.04
Ampicillin	1.943 ± 0.001	Tailing Factors	1.037 ± 0.009	1.046 ± 0.007
Cloxacillin	6.058 ± 0.002	RPA	0.3959 ± 0.0016	1.0247 ± 0.0016
Flucloxacillin	7.057 ± 0.004	RSD of RPA (%)	0.97%	0.38%
Placebo spiked with Ampicillin, Cloxacillin and Flucloxacillin	1.485 ± 0.006; 1.945 ± 0.004; 6.057 ± 0.008; 7.096 ± 0.003	Resolution		
		AMPI and CLOX	CLOX and INT	AMPI and INT
		10.717 ± 0.234	1.743 ± 0.049	12.459 ± 0.282

In terms of System suitability, the numbers of theoretical plates were greater than 2000 indicating high efficiency with regards to the column. The tailing factors were less than 2.000 thereby giving symmetrical peaks.

The % RSD obtained from Repeatability test for Ampicillin and Cloxacillin were 1.09 % and 0.57 % respectively for the first day of analysis, and 1.67 % and 1.87 % respectively for the fifth day of analysis. That of intermediate precision were in the range of 0.64 % - 1.87 % for both first and fifth day analysis. Hence, the developed method was highly precise.

Recovery studies ensures the quantification of target compounds. It is therefore very important in determining the accuracy of an analytical method. The percentage recovery were found to be in the range of 99.4 % - 101.5 %. There were no significant differences between the results obtained between the various concentration levels.

The working concentration ranges from 0.0185 %w/v - 0.0277 %w/v for Ampicillin and 0.0175 %w/v - 0.0263 %

w/v for Cloxacillin. Correlation co-efficient of Ampicillin was 0.9990 and that of Ibuprofen was 0.9984. Residual analysis showed values that were randomly dispersed around the horizontal axis indicating the appropriateness of linear regression data.

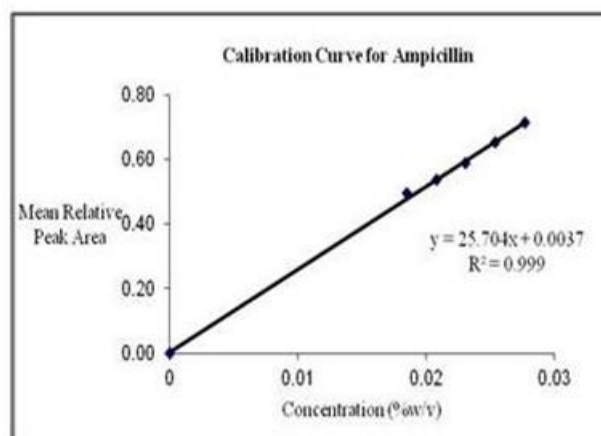


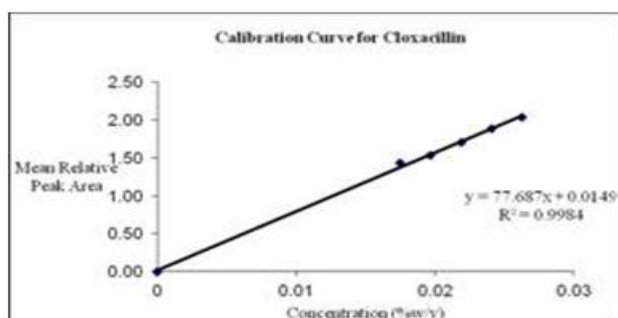
Figure 3: Calibration curve for Ampicillin

Table 2: Results of Intermediate Precision

Intraday Precision								
Ampicillin					Cloxacillin			
Analyst A		Analyst B			Analyst A		Analyst B	
	First day analysis (%)	First day analysis (%)	First day analysis (%)	First day analysis (%)	First day analysis (%)	First day analysis (%)	First day analysis (%)	First day analysis (%)
Mean±SEM	106.5 ± 0.5	102.2 ± 0.7	101.9 ± 0.3	100.3 ± 0.4	117.1 ± 0.3	95.8 ± 0.7	113.6±0.1	94.3±0.7
RSD	1.09%	1.67%	0.64%	0.92%	0.57%	1.87%	0.13%	1.82%
Ampicillin					Cloxacillin			
	First day of analyst A	First day of analyst B	First day of analyst A	First day of analyst B	First day of analyst A	First day of analyst B	First day of analyst A	First day of analyst B
Variance	1.35	0.41	2.93	0.83	3.65	2.67	3.22	2.94
F-test	3.33		3.54		1.37		1.10	
Interday Precision								
Ampicillin					Cloxacillin			
	Sample One		Sample Two		Sample One		Sample Two	
	First day analysis	First day analysis	First day analysis	First day analysis	First day analysis	First day analysis	First day analysis	First day analysis
	% content	% content	% content	% content	% content	% content	% content	% content
Mean±SEM	106.9±0.5	102.2±0.7	101.7±0.8	97.6±0.6	117.1±0.3	95.9±0.7	114.7±0.7	90.9±0.3
RSD	1.09%	1.67%	1.92%	1.40%	0.57%	1.87%	1.45%	0.84%
Ampicillin					Cloxacillin			
	First day of analyst A	First day of analyst B	First day of analyst A	First day of analyst B	First day of analyst A	First day of analyst B	First day of analyst A	First day of analyst B
Variance	1.39	3.77	2.94	1.89	1.39	2.79	3.23	2.96
F-test	2.72		1.55		2.01		1.09	

Table 3: Results from Accuracy Study

Ampicillin						
Level	80%	100%	80%	120%	100%	120%
Mean % Recovery	101.5	99.6	101.5	99.4	99.6	99.4
T-calculated (Absolute values)	1.946		4.172		0.213	
Cloxacillin						
Level	80%	100%	80%	120%	100%	120%
Mean % Recovery	99.7	100.2	99.7	101.4	100.2	101.4
T-calculated (Absolute values)	0.778		2.471		4.243	
T-tabulated	4.303 at 95 % probability level					

**Figure 4:** Calibration curve for Cloxacillin**Table 4:** Linear regression data for calibration curves

API	Ampicillin	Cloxacillin
Concentration range (%w/v)	0.0185 to 0.0277	0.0175 to 0.0263
Slope	25.704	77.687
Intercept	0.0037	0.0149
Correlation coefficient (R ²)	0.9990	0.9984



Irrespective of the deliberate changes made to the chromatographic conditions, all the system suitability parameters remained significantly unaffected.

Stability studies of Ampicillin and Cloxacillin in the analytical solution were studied within a period of 24 hours and they were stable within that period.

CONCLUSION

The proposed RP-HPLC method for the estimation of Ampicillin and Cloxacillin in model oral suspension is precise, rapid, specific, robust, accurate and precise for the estimation of Ampicillin and Cloxacillin in combined dosage form. It can be adopted for routine quality control analysis of raw materials and formulations containing single and / or combined forms of Ampicillin, Cloxacillin and / or Flucloxacillin.

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