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Analytical Method Development and Validation of Cephalexin and Carbocisteine by RP-HPLC

K. Pooja[1], S. Marakatham[2], B. Raj Kamal[3]

[1,2,3] Pharmaceutical Analysis Department, Malla Reddy Institute of Pharmaceutical Sciences, Maisammaguda, Dulapally Post, Medchal Mandal, T.S, India

Corresponding Author: S. Marakatham

ABSTARCT

Carbocisteineand Cephalexin were measured and quantified using a quick, sensitive, and precise RP-HPLC approach that was designed and validated using the Waters HPLC System with PDA detection. Chromatography was performed using a mobile step of filtered, mixed, degassed Methanol: Water (30:70) on a column of Inertsil-ODS C18 (250 x 4.6 mm, 5) at a flow rate of 1.0 ml/min. In addition to being tested for quantification and detection limits, the system was also examined for linearity, precision, accuracy, and specificity.

Main words: Cephalexin, Carbocisteine, Process development and validation.

INTRODUCTION

Cefalexin can treat certain bacterial infections, including those of the middle ear, bone and joint, skin, and urinary tract.[4] It may also be used for certain types of pneumonia and strep throat and to prevent bacterial endocarditis.[4] Cefalexin is not effective against infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), most *Enterococcus*, or *Pseudomonas*.[4] Like other antibiotics, cefalexin cannot treat viral infections, such as the flu, common cold or acute bronchitis.[4] Cefalexin can be used in those who have mild or moderate allergies to penicillin.[4] However, it is not recommended in those with severe penicillin allergies.[4]
Common side effects include stomach upset and diarrhea.[4] Allergic reactions or infections with *Clostridium difficile*, a cause of diarrhea, are also possible.[4] Use during pregnancy or breast feeding does not appear to be harmful to the fetus.[4][6][7] It can be used in children and those over 65 years of age.[4] Those with kidney problems may require a decrease in dose.[4]

Cefalexin was developed in 1967.[8][9][10] It was first marketed in 1969 and 1970 under the names **Keflex** and **Ceporex**, among others.[1][11] Generic drug versions are available under other trade names and are inexpensive.[4][12] It is on the World Health Organization's List of Essential Medicines.[13][14] In 2020, **Carbocisteine**, also called **carbocysteine**, is a mucolytic that reduces the viscosity of sputum and so can be used to help relieve the symptoms of chronic obstructive pulmonary disorder (COPD) and bronchiectasis by allowing the sufferer to bring up sputum more easily.[15][16] Carbocisteine should not be used with antitussives (cough suppressants) or medicines that dry up bronchial secretions.

It was first described in 1951 and came into medical use in 1960.[17] Carbocisteine is produced by alkylation of cysteine with chloroacetic acid.[18]

The chemical structures of both drugs are as shown in (Figures 1, 2).

Figure 1: Chemical structure of Cephalexin.

Figure 2: Chemical structure of Carbocisteine.

MATERIALS AND METHODS

Chemicals

Samples of Cephalexin and Carbocisteinewas gifted by Sun Pharmaceutical Industries Ltd, Goregaon (E), Mumbai. Merck, Schuchardt OHG, Germany provided HPLC grade Acetonitrile, KH2PO4, and phosphoric acid. Millipore Milli Q plus filtration technology was used to create ultra-pure water.

Instruments

We used a Waters 2695 HPLC system with a PAD detector (Waters Corporation, Milford, USA) that could detect wavelengths from 200nm to 400 nm. We also used it to test the method. Empower software was used to look at and process the outgoing signal on a Pentium computer that had a hard drive (Digital Equipment Co). Hydrolysis experiments were conducted using a Cintex computerized water bath. In a photo stability chamber, tests were conducted to determine the shelf life of various items (Sanyo, Leicestershire, UK).

Selection of wave length (λ max)

A solution of 100 µg/ml of Cephalexin and Carbocisteinewere prepared in Mobile phase. The resulting solutions were scanned individually on HPLC PDA detector from 200nm to 400 nm. The optimal response for both of them were obtained at 270nm. Hence the complete method was processed at the wave length of 270nm.

Preparation of stock and sample solutions

In order to make a stock solution of Cephalexin and Carbocisteine(1000 mg/ml), 20 mg of the Cephalexin and 25 mg of the Carbocisteine of the drug ingredient were dissolved in a 100ml volumetric flask of the diluent (mobile phase) for 30 minutes and sonicated for 30 minutes. All of these solutions were pipetted into a 100-ml volumetric flask and mixed with the correct amount of diluent. Then, for 10 minutes, they were sonicated at a high speed. Then prepare 20, 30, 40, 50, 60,70 and 80 ppm solutions were prepared by utilizing the above stock solution.

METHOD OF VALIDATION

Method validation can be defined as International Council for Harmonisation (ICH) "establishing documented evidence which provides a high degree of assurance that specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics. Method validation is an integral part of the method development; it is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, an and drug products. Simply, method validation is the process of proving that and potency of the drug substances analytical method is acceptable for its intended purpose. For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters.

Recovery:

The absolute recovery of analytical method is measured as the response of a processed spiked matrix standard expressed as a percentage of the response of pure standard which has not been subjected to sample pre treatment and indicates whether the method provides a response for the entire amount of analyte that is present in the sample.

Sensitivity

The method is said to be sensitive if small changes in concentration cause large changes in response function. The sensitivity of an analytical method is determined from the slope of the calibration line. The limits of quantification (LOQ) or working dynamic range of bio analytical method are defined as the highest and lowest concentrations, which can determined with acceptable accuracy. It is suggested that, this be set at 15% for both the upper and lower limit of quantitation respectively. Any sample concentration that falls outside the calibration range cannot be interpolated from the calibration line and extrapolation of the calibration curve is discouraged. If the concentration is over range, the sample should be diluted in drug-free matrix and re-assayed.

Precision

The purpose of carrying out a determination is to obtain a valid estimate of a 'true' value. When one considers the criteria according to which an analytical procedure is selected, precision and accuracy are usually the first time to

come to mind. Precision and accuracy together determine the error of an individual determination. They are among the most important criteria for judging analytical procedures by their results.

Accuracy

Accuracy normally refers to the difference between the mean x****, of the set of results and the true or correct value for the quantity measured. According to IUPAC accuracy relates to the difference between results (or mean) and the true value. For analytical methods, there are two possible ways of determining the accuracy, absolute method and comparative method.

Accuracy is best reported as percentage bias, which is calculated from the expression

$$\%Recovery = \frac{\text{Amount found}}{\text{Amount added}} \times 100$$

The accuracy of analytical method is then determined at each concentration by assessing the agreement between the measured and nominal concentrations of the analytes in the spiked drug – free matrix sampler.

Limit of detection (LOD)

The limit of detection (LOD) of an analytical method may be defined as the concentration, which gives rise to an instrument signal that is significantly different from the blank. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (σ), which may be related to LOD and the slope of the calibration curve, S, by

$$LOD = 3\sigma / S$$

Limit of quantification (LOQ)

The LOQ is the concentration that can be quantitate reliably with a specified level of accuracy and precision. The LOQ represent the concentration of analyte that would yield a signal-to-noise ratio of 10.

$$LOQ = 10\sigma / S$$

Where, σ - the estimate is the standard deviation of the peak area ratio of analyte to IS (5 injections) of the drugs. S -is slope of the corresponding calibration curve.

Ruggedness

Method Ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method.

Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small but deliberate variations in method parameters". The robustness of a method is the ability to remain unaffected by small changes in parameters such as Robustness studies are performed by introducing deliberately small changes in the flow rate (±0.2 mL/min).

System suitability

System suitability experiments can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validation have been completed. (or) The USP (2000) defines parameters that can be used to determine system suitability prior to analysis.

The criteria selected will be based on the actual performance of the method as determined during its validation. For example, if sample retention times form part of the system suitability criteria, their variation (SD) during validation can be determined system suitability might then require that retention times fall within a 3 SD range during routine performance of the method.

RESULTS AND DISCUSSION

Validation of the Developed Method

The process of ensuring that the HPLC strategy utilized to perform a given test is appropriate for its intended use is known as HPLC method validation. The planned and refined HPLC technology was put to the test. The validation is carried out according to ICH guidelines

Table No 1: Optimized Chromatographic Condation:

Parameters	Method
Stationary phase (column)	Inertsil -ODS C_{18}(250 x 4.6 mm, 5 µ)
Mobile Phase	Methanol:Water (30:70)
Flow rate (ml/min)	1.0 ml/min
Run time (minutes)	12 min
Column temperature (°C)	Ambient
Volume of injection loop (µl)	20
Detection wavelength (nm)	223nm
Drug RT (min)	4.714min for Cephalexin and 6.691 for Carbocisteine.

Based on the system suitability investigations, it was discovered that the optimized standard chromatogram had a peak at 4.714 & 6.691 min and that the mobile phase was Methanol: Water (30:70)v/v at 223nm. For Cephalexin and Carbocisteine, it was discovered that the technique exhibited linearity in the concentration ranges of 20 ppm to 80ppm based on linearity data. A linearity graph was constructed for the peak area of Cephalexin and Carbocisteine versus concentration. The coefficient was found to be within the range of 0.999 for both of these compounds. It was necessary to inject the standard solution five times to determine the compatibility of the system, and the area of each injection was measured using an HPLC. Observations were made to ensure that the % RSD and MEAN remained within the prescribed parameters. Precision was determined to be within the acceptable System Precision, Method Precision, Intermediate Precision and it was computed as percent assay for and percent RSD for assay calculation, respectively. Different concentration levels, such as 50 percent, 100 percent, and 150 percent, were developed in order to improve the accuracy of computations. We discovered that recovery is possible within the parameters. This study calculated robustness for different flow rates of mobile phase, such as 0.8ml/min, 1.0ml/min, and 1.2ml/min, and calculated %RSD We measured ruggedness and found that system to system variability was 100.35, indicating that the approach was accurate. We also measured precision and found that the method was 100.08. Results reveal that RSD of retention time and accuracy, ruggedness, robustness linearity and precision are all within the acceptable ranges of results.

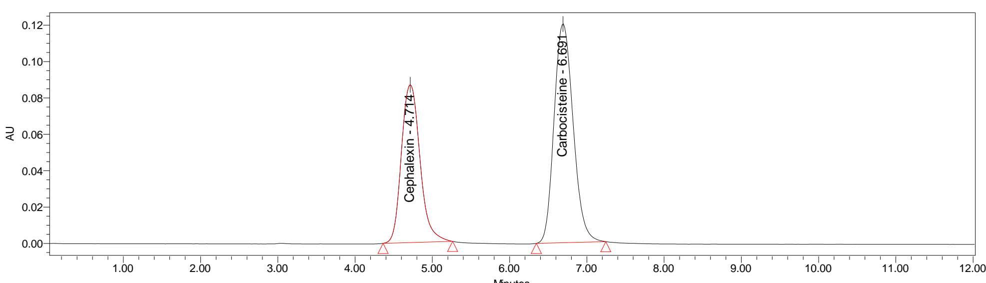

Figure – 1: Standard Chromatography

Inference: The chromatogram was obtained at RTs of 4.714**min for Cephalexin and 6.691min for Carbocisteine.**

Test for System Suitability

Chromatography is a way to make sure that the system is going to work. It is used to check how well the system works for its intended use. System suitability's major purpose is to ensure that the entire testing process, including the instrument and the analyst, is appropriate for the task at hand.

Table - 2: Data of SST for Cephalexin

	RT	Peak Area	USP Plate count	USP Tailing
Mean	4.709948	395567	10036.825471	1.063
SD	0.002754	209.973	-------	-------
% RSD	0.075426	0.053	-------	-------

Table - 3: Data of SST for Carbocisteine

	RT	Peak Area	USP Plate count	USP Tailing
Mean	6.687422	2266430	8358.8754210	1.060
SD	0.0024183	1656.439	-------	-------
% RSD	0.037625	0.073	-------	-------

Precision:

Precision refers to how well a set of measurements that were taken from the same homogenous sample under the same conditions line up. There are three types of parameters system precision, method precision and intermediate precision all of which are important for getting the job done.

Table No 4: Cephalexin Precision Data:

	System Precise %Assay	Precision method %Assay	Intermediate Precision Information %Assay
Mean	100.14	100.15	100.17
SD	0.042	0.054	0.037
% RSD	0.042	0.054	0.037

Table No 4: Carbocisteine Precision Data:

	System Precise %Assay	Precision method %Assay	Intermediate Precision Information%Assay
Mean	100.40	100.36	100.45
SD	0.057	0.052	0.044
% RSD	0.057	0.052	0.044

Linearity

Cephalexin & Carbocisteine assay concentrations 20ppm-80ppm were used to test the method's linearity. The LC system was injected with each solution. Using a correlation coefficients greater than 0.999, the calibration curve between the average peak area and the concentration was found to be linear.

Table No 5: Cephalexin Linearity Data:

Statistical Analysis	
Slope	9884
y-Intercept	-380.3
Correlation Coefficient	0.999

Table No 6: Carbocisteine Linearity Data:

Statistical Analysis	
Slope	56481
y-Intercept	-2387
Correlation Coefficient	0.999

Ruggedness:

Method Ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method.

Table No 7: Cephalexin Ruggedness Data:

	Peak area	Assay % of Cephalexin
Mean	395681	100.17
%RSD	0.042	0.042

Table No 7: Carbocisteine Ruggedness Data:

	Peak area	Assay % of Carbocisteine
Mean	2265152	100.36
%RSD	0.039	0.039

Robustness:
The concept of robustness of an analytical procedure has been defined by the ICH as “a measure of its capacity to remain unaffected by small but deliberate variations in method parameters”. The robustness of a method is the ability to remain unaffected by small changes in parameters such as Robustness studies are performed by introducing deliberately small changes in the flow rate (±0.2 mL/min).

Table No 8: Cephalexin Robustness Data:

Flow 0.8 ml	Tailing factor	Flow 1 ml	Tailing factor	Flow 1.2 ml	Tailing factor
Avg	1.067	**Avg**	1.054	**Avg**	1.050
SD	0.024	**SD**	0.020	**SD**	0.025
%RSD	2.262	**%RSD**	1.974	**%RSD**	2.392

Table No 9: Carbocisteine Robustness Data:

Flow 0.8 ml	Tailing factor	Flow 1 ml	Tailing factor	Flow 1.2 ml	Tailing factor
Avg	1.043167	**Avg**	1.060	**Avg**	1.061
SD	0.027622	**SD**	0.021	**SD**	0.034
%RSD	2.647	**%RSD**	2.027	**%RSD**	3.265

Detection and quantification limits (LOD and LOQ)
Cephalexin and Carbocisteine of LOD and LOQ were caluculated by S/N ratio Ex: To determine LOD and LOQ, we must first determine how low a concentration must be and how many samples must be obtained to achieve that concentration and quantity. Both 3:1 and 10:1 signal-to-noise ratios are regarded to be correct. In other method The LOD and LOQ are calculated using the linearity plot. By using the following equation we can calculate LOD value of Cephalexin, and Carbocisteine Here can be calculated by using average area of system suitability data, and slope can be calculated by using linearity data.

Cephalexin:
The LOD and LOQ are computed from the linearity plot:

$$\text{LOD} = \frac{3.3\,\sigma}{S} = \frac{3.3 \times 209.973}{9884} = 0.070$$

$$\text{LOQ} = \frac{10\,\sigma}{S} = \frac{10 \times 209.973}{9884} = 0.212$$

Carbocisteine;

$$\text{LOD} = \frac{3.3\,\sigma}{S} = \frac{3.3 \times 1656.439}{56481} = 0.096$$

$$\text{LOQ} = \frac{10\,\sigma}{S}$$

$$= \frac{10\times1656.439}{56481} = 0.293$$

CONCLUSION

The development of the analytical method involved researching several parameters. First off, it was discovered that the maximal absorbance for Carbocisteine was at 223nm and 254nm, respectively. The peaks purity was outstanding, and the typical wavelength will be 223 nm. The 20 injection volume chosen provided a suitable peak area. Inertsil C18 was the study's chosen column, and ODS selected it for its good peak form. Ambient temperature was found to be adequate for the nature of medication solution. Because of the good peak area, satisfactory retention time, and good resolution, the flow rate was set at 1.0 ml/min. Different mobile phase ratios were investigated, however the ratio of Methanol: Water (30:70) was ultimately chosen because to its good peak symmetry and resolution. Thus, the suggested study made use of this mobile phase.

The precision of the system and the procedure were both confirmed to be precise and within bounds. Curve fitting, correlation coefficient, and a linearity investigation were all successful. For both medicines, it was discovered that the analytical method was linear over the range of 20-80 ppm of the target concentration. The analysis passed the tests for ruggedness and robustness. The relative standard deviation in both circumstances was very acceptable.

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