Validated RP-HPLC Method for Quantitative Analysis of Polymyxin B Sulphate in Fermentation Broth and Pharmaceutical Formulations

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ABSTRACT

Background: Polymyxin B is one of the most valued antimicrobial peptides and plays an indispensable role in the treatment of serious infections caused by Gram-negative bacteria resistant to a variety of antibiotics. Because of its complex structure and the presence of only one chromophore, it is difficult to test Polymyxin B accurately.

Objective: The objective of this research work is to develop and validate an efficient HPLC method for the quantitative analysis of Polymyxin B in fermentation broth and pharmaceutical formulations.

Methods: In order to optimize the reversed-phase HPLC procedure, a C-18 column with a gradient mobile phase system was used. The developed method has been validated based on ICH guidelines with respect to specificity, linearity, accuracy, precision, and robustness.

Results: The developed method proved to be highly accurate because the relative standard deviation is less than 2.0%. All criteria of appropriateness of the system were also satisfied. We were able to measure, with good precision and accuracy, the levels of components B1, B2, B3, and B1-I in Polymyxin B.

Conclusion: The HPLC technique was validated as efficient and reliable in Polymyxin B testing in the complex mixture; it has, therefore, been qualified for use in both research and pharmaceutical industries.

KEY WORDS: Polymyxin B, HPLC, method validation, fermentation broth, dry powder formulation, and quality control.

1. INTRODUCTION

Polymyxin B is still an important antimicrobial peptide needed for the treatment of serious infections caused by Gram-negative bacteria that do not respond to many drugs[1]. This antibiotic will be very useful at a time when others are not effective due to its peculiar mode of action:

attacking bacterial cell membranes[2]. However, it is exceedingly difficult to measure Polymyxin B properly since it possesses a complicated structure and a small chromophore, and various commercial brands are not always the same[3].

Conventional analytical methods usually require complex sample preparation and are time-consuming; most of these procedures have a lack of sufficient sensitivity or specificity for accurate quantitation of Polymyxin B[4]. HPLC is one good way of considering drugs because it separates and counts them much better. However, all current HPLC methods concerning Polymyxin B are tedious, time-consuming, and do not correspond to the need for a simpler and more convenient method[5].

In general, fast and easy-to-execute analytical procedures have considerable importance in pharmaceutical quality control, clinical monitoring, and research. The simplification of the analytical technique would make the gathering of essential analytical data[6] easier on the grounds of cost and time consumption. Consequently, there is a pressing need for a new HPLC method that provides convenience and ease in analysing polymyxin B.

This study will overcome the deficiencies of the current analytical techniques by developing and testing an effective HPLC methodology for assaying Polymyxin B. The objectives are:

(1) to determine the optimal chromatographic conditions that would reduce the overall time required for sample preparation and analysis;

(2) to demonstrate the novelty and usefulness of the technique; and

(3) to perform full validation in compliance with International Conference on Harmonization (ICH) requirements for the method to be accurate, precise, specific, and robust.

This study describes a validated and accessible HPLC technique that enhances the reliability and efficiency in undertaking Polymyxin B analysis in various fields.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

HPLC-grade acetonitrile, water, AR-grade anhydrous sodium sulfate, and orthophosphoric acid were procured from renowned suppliers. A certified reference standard of Polymyxin B sulfate was obtained for use with regard to analytical calibration[6–8].

2.2. Sample Preparation

- **Samples of Fermentation Broth**

The pH of the 10.0 grams of fermentation broth was adjusted to pH 3.20 using orthophosphoric acid. Then, the sample was spun for 10 minutes at 4000 rpm and filtered through a 0.45 µm membrane filter before analysis[9].

➢ **Dry Powder Formulations**

Accurately weigh 100 mg of the dry powder sample and mix with 100 ml of water and acetonitrile (80:20, v/v). Sonicate for 10 minutes, cool, and then filter through a 0.45 µm filter before being introduced into the HPLC system[8–10].

2.3. Chromatographic Conditions

➢ **HPLC System**: Equipped with a UV-Visible detector.

➢ **Columns**:

- Broth samples: C18 (250 mm × 4.6 mm, 5 µm).
- Final product: C18 (250 mm × 4.6 mm, 5 µm).

➢ **Mobile Phase**:

- **Buffer**: 4.5 g/L Anhydrous sodium sulphate in water, pH adjusted to 2.3 with orthophosphoric acid.
- **Mobile Phase A**: 100% Buffer (80%)
- **Mobile Phase B**: 100% Acetonitrile (20%)

➢ **Gradient Program**:

- **Broth samples**: 75% A / 25% B (0-30 min)
- **Final product**: 80% A / 20% B (0-60 min)

➢ **Flow Rate**: 1.0 mL/min (broth), 1.80 mL/min (final product)

➢ **Detection**: 215 nm

➢ **Injection Volume**: 20 µL

➢ **Temperature**: Column at 30°C, Sampler at 23-25°C

All the chromatographic conditions were optimized based on previous publications as well as internal studies related to method development [7-10].

2.4. System Suitability and Method Validation

We checked the appropriateness of the system by infusing the reference standard solution and observing peak symmetry, theoretical plates, and retention time. Then, according to the ICH Q2(R1) recommendations[6], we carried out method validation and observed the following:

➢ Accuracy: Recovery studies at different levels of concentration

➢ Precision: repeatability (intra-day) and intermediate precision (inter-day)

➢ Specificity: No interference from blank or excipients.

➢ Robustness: small, deliberate changes in flow rate and mobile phase composition

2.5. Calculation of Polymyxin B Concentration

For broth samples:

$$\text{Concentration (mg/g)} = \frac{Area\ of\ test}{Area\ of\ STD} \times \frac{Weight\ of\ STD}{Wt.of\ sample} \times \frac{Volume\ (20mL)}{Volume\ (10mL)} \times STD\ Purity\ \times 100$$

For dry powder samples:

$$\text{Concentration} = \frac{\boldsymbol{R_u \times C_s \times P \times 100}}{\boldsymbol{R_s \times C_u}}$$

Where:

Ru: Peak response of Polymyxin B1 from the sample solution

Rs: Peak response of Polymyxin B1 from the standard solution

Cs: Concentration of Polymyxin B Sulphate reference standard (mg/mL)

Cu: Concentration of Polymyxin B Sulphate in the sample solution (mg/g)

P: Potency of Polymyxin B Sulphate reference standard (mg/mg)[7-9]

2.6. Loss on Drying

The test sample was assessed for loss on drying by using both the United States Pharmacopoeia and European Pharmacopeia

United States Pharmacopoeia (USP) Method: As per USP Method[11].

European Pharmacopeia (Ph.Eur.) Method: The sample was weighed out to about 100 mg in an LOD bottle. The sample was dried under vacuum at 60°C for 3 hours.

The weight loss was calculated by the difference between original weight and final weight of the sample.

$$\textbf{LOD \%} = \frac{\{(W2 - W3)\}}{\{(W2 - W1)\}} \times 100$$

Where:

W1 = weight of the empty LOD bottle

W2 = weight of the sample and LOD bottle before drying

W3 = weight of the sample and LOD bottle after drying

3. RESULTS AND DISCUSSIONS

3.1. HPLC Testing of Standard Solutions

Accuracy and repeatability of the HPLC test of standard solutions were quite good. Table 1 illustrates mean peak areas and RSD for Polymyxin B2, B3, B1-I, and B1 over three injections of standard solution. Low values of RSD mean that the method is reliable.

Table 01: Peak areas, retention times (RT), and relative standard deviation (RSD) for Polymyxin B components in standard solution.

Component	Average Area	Standard Deviation	RSD (%)	RT (min)	RRT
B2	4035408	2363.34	0.06	7.60	0.50
B3	1021793	7147.77	0.70	8.40	0.60
B1-I	3283949	25660.21	0.78	11.50	0.80
B1	35512241	73291.46	0.21	13.70	1.00

***** RT = Retention Time; RSD = Relative Standard Deviation

The potency of the standards in USP units (B1). The standard solution was prepared using 0.5 mg/ml of the reference standard and diluent. The sample solution was prepared using 0.5 mg/ml of the crude material and diluted with a solvent mixture.

3.2. HPLC Analysis of Broth Samples

Samples of fermenter broth were analysed by the standard HPLC method. The major peak component B1 eluted from the system at approximately 12 to 15 minutes, as specified in the system suitability criteria. We determined the amount of present Polymyxin B and other associated components based on comparisons of the peak areas to those of the reference standard. Table 02 presents the results of the HPLC analysis of broth sample and Figure 01 shows the graph that represents the variation in Polymyxin B concentration (mg/ml) in fermentation samples at different time intervals (19, 48, 65, and 72 hours). The concentration increases over time, stabilizing at 1.95 mg/ml after 65 hours. obtained through HPLC analysis.

Table 02: Analysis of Polymyxin B in Fermentation Samples

Age (Hrs.)	Area of Sample	Area Of Std	STD Weight	Dilution	Sample Volume	Sample Weight	Potency	Result (mg/ml)
LOG 19	80755102	151590116	10.60	10	20	10.0312	94.50	1.06
LOG 48	127906701	145853331	10.60	10	20	10.0146	94.50	1.75
LOG 65	142292252	145853331	10.60	10	20	10.0117	94.50	1.95
LOG 72	142308902	145853331	10.60	10	20	10.0180	94.50	1.95

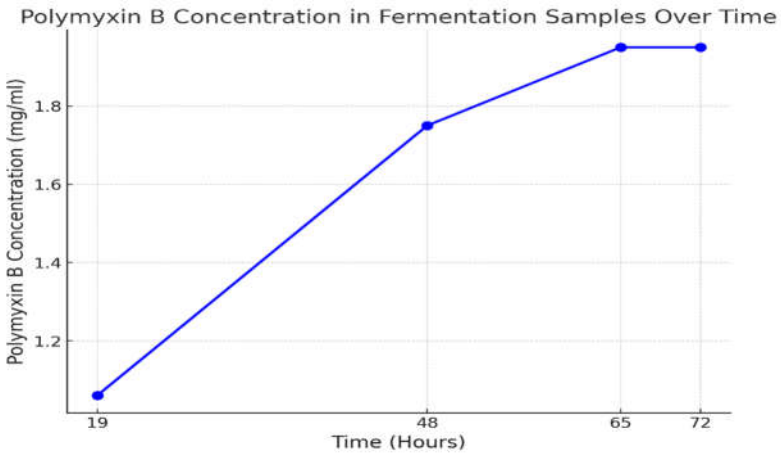

Figure 01. This graph represents the variation in Polymyxin B concentration (mg/ml) in fermentation samples at different time intervals (19, 48, 65, and 72 hours).

3.3. HPLC Testing of Anhydrous Powder Samples

HPLC analysis of the dry powder sample is described in Table 03. The analysis showed the presence of Polymyxin B2, B3, B1-I, and B1, plus two unknown impurities. The retention time and relative retention time for each component are given. Area percent and composition of each component and composition on a dried basis are shown.

The summation of all the peaks' area was 41067833. Polymyxin B2, B3, B1-I and B1 totalled 84.76% which is above the NLT 80% mark. The total unknown impurity amount was 3.46%, below the maximum amount of 17%. The sample had an AREA % of 96.54%.

Table 03: HPLC Analysis of Dry Powder Sample

Sr. No.	Name	RT (min)	RRT	Area	Area (%)	Composition	Composition on Dried Basis	Limit (%)
01	Unknown-01	7.779	0.192	679486	1.655	--	--	NMT 3%
02	Unknown-02	13.358	0.331	740977	1.804	--	--	NMT 3%
03	B2	17.085	0.424	9129149	22.229	18.43%	19.21%	--
04	B3	19.699	0.489	279122	0.680	0.56%	0.59%	NMT 6%
05	B1-1	31.030	0.772	2765596	6.734	6.03%	6.29%	NMT 15%
06	B1	40.190	1.000	27473503	66.898	56.26%	58.66%	--

Figure 2 shows the bar chart that represents the composition on a dried basis (%) of various components identified through HPLC analysis of a dry powder sample. The primary component, B1, accounts for 58.66%, while B2, B3 and B1-I contribute 19.21%, 0.59% and 6.29%, respectively.

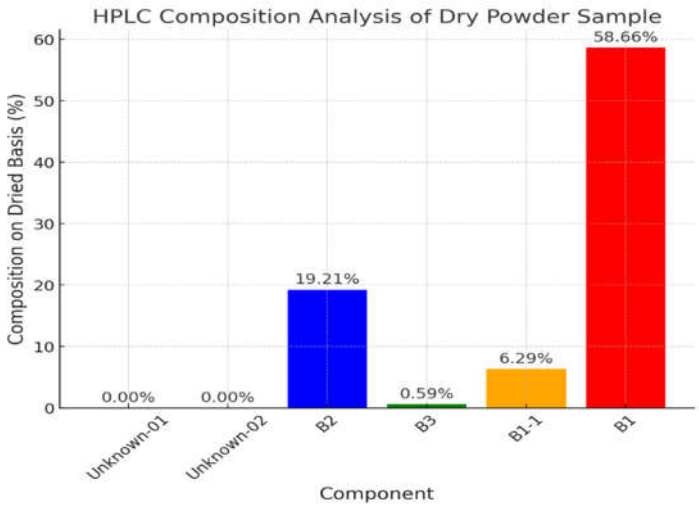

Figure 2. This bar chart represents the composition on a dried basis (%) of various components identified through HPLC analysis of a dry powder sample.

3.4. Calculated concentrations:

We determined the amount of each Polymyxin B component, namely B1, B2, B3, and B1-I, present in a dry powder sample. The calculations were based on maximum responses of the sample and standard solutions, the concentration of the Polymyxin B Sulphate reference standard, and the strength of said standard.

3.5. Loss on Drying (LOD)

We used the formula below to determine that the LOD was 3.37%.

$$\text{LOD } \% = \frac{\{(W2 - W3)\}}{\{(W2 - W1)\}} \times 100$$

Where:

W1 = weight of the empty LOD bottle

W2 = weight of the sample and LOD bottle before drying

W3 = weight of the sample and LOD bottle after drying

The weights used in the calculation were W1 = 44.4826, W2 = 45.6985, and W3 = 45.6576. The calculated LOD was 3.37%.

3.6. Inference

- **The effectiveness of the strategy and the degree to which it fits**

Low RSD and good separation of the parts of Polymyxin B show the correctness and exactitude of the approach. The method measures Polymyxin B correctly in both its dry powder and broth forms.

- **Comparison with Requirements of USP and EP**

The developed approach was found to meet the various USP and EP requirements for the analysis of Polymyxin B including system compatibility and impurity determinations.

- **Limitations and Future Directions**

More studies are needed to ensure that the method works with more sample matrices and detects small levels of contaminants better.

4. CONCLUSION

The validated HPLC method herein represents an effective, rapid, and reliable method for the determination of Polymyxin B from its dry powder and fermentation broth formulations, and this is very helpful in carrying out research studies and quality control processes within the pharmaceutical industry.

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