RESEARCH ARTICLE

Improved method for isolation of Polymyxin B Sulphate

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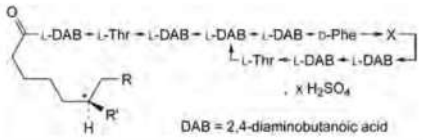
ABSTRACT

This study explores the purification of Polymyxin B sulphate through resin and elution solvent optimization. Diaion WA30 was found to be the most suitable for adsorption, impurity removal, and simple desorption. It had an 85% output, 60.00% total batch yield, and 92.92% content of polymyxin B. Ethanol was the best solvent for the Sephadex SP700 Resin column, with a pH 3.0 solution of ethanol resulting in 98.68% resin stage yeild, 60.00% overall yield, and 92.92% polymyxin B content. The pirification was pharmaceutical compliant, eliminating toxic polymyxins B1-I and B3, and surpassing the microbial assay requirement (9157.58 u/mg). The established method proves to be industrially applicable for attaining high yield and pharmacopeial compliance, Polymyxin B Sulphate.

Keywords:

Polymyxin B Sulphate, downstream processing, HPLC, purification technology, cation exchange resin, adsorption resin.

Introduction



Polymyxin	R	R'	X	Molecular formula	M_r
B1	CH_3	CH_3	L-Leu	$C_{56}H_{98}N_{16}O_{13}$	1204
B2	H	CH_3	L-Leu	$C_{55}H_{96}N_{16}O_{13}$	1190
B3	CH_3	H	L-Leu	$C_{55}H_{96}N_{16}O_{13}$	1190
B1-I	CH_3	CH_3	L-Ile	$C_{56}H_{98}N_{16}O_{13}$	1204

POLYMYXIN B SULPHATE

Polymyxin B Sulphate is an antibiotic used to treat meningitis, pneumonia, sepsis, and urinary tract infections. While it is useful for many Gram-negative infections, it is not useful for Gram-positive infections. It can be injected into a vein, muscle, or cerebrospinal fluid, or inhaled.

Polymyxin B has re-emerged as an ultimate therapy of last resort against multidrug-resistant (MDR) and highly drug-resistant (XDR) Gram-negative infections (1,2). It is a cyclic lipodecapeptide antibiotic with quick bactericidal activity against many Gram-negative bacteria, such as Enterobacteriaceae, Acinetobacter baumannii, and Pseudomonas aeruginosa (3). Polymyxin B's main mechanism of action is against the bacterial membrane, but it also affects crucial respiratory enzymes in the bacterial inner membrane, including type II NADH-quinone oxidoreductases (NDH-2) (4,5). Remarkably, although polymyxin B is structurally related to colistin (polymyxin E), pharmacokinetic differences between the two are substantial and could result in dissimilar clinical and microbiological results (6). Moreover, polymyxin B derivatives, e.g., polymyxin B nonapeptide (PMBN), are not directly bactericidal but permeabilize the outer bacterial membrane, promoting the action of other antibiotics (2,7). New research has also examined the synthesis of new polymyxin analogues possessing greater antimicrobial activity and lower toxicity (8). In summary, although polymyxin B is now an important last-resort therapy for MDR and XDR Gram-negative infections, several areas of understanding its optimal utilization remain incomplete. Additional studies will be necessary to standardize the susceptibility test, define serum and tissue levels, investigate combination therapies, and explain resistance mechanisms (9). In addition, population pharmacokinetic model development and limited sampling strategies can aid therapeutic drug monitoring and optimize polymyxin B dosing in clinical practice.

The isolation and purification of polymyxin B sulphate is the most technically demanding step in its production process because of the molecule's intricate structure, amphiphilic character, and

the occurrence of closely related analogs and impurities. Here's an in-depth look at the major challenges:

- **Separation of Structural Analogue**s: Polymyxin B isn't one compound—it's a mixture, predominantly - Polymyxin B1, Polymyxin B2, and minor variants such as B3 and B1-I. They vary only marginally in fatty acid side chains or amino acid substitutions, which makes them virtually impossible to separate and inseparable by crude chromatographic means. One of the most problematic stages of the polymyxin B production is its purification from nonspecific admixtures and the most toxic components, polymyxin B3 and polymyxin B1-I, which are contained in a pharmaceutical substance, according to international standards, should not exceed 6% and 15%, respectively [10,11].
- **High Surface Affinity:** Polymyxins are cationic cyclic lipopeptides with hydrophilic and lipophilic moieties. As a result, they strongly adsorb to chromatography columns, filters, and glassware. Hence, recovery losses during purification steps are high.
- **Amphiphilic Character:** Polymyxin B contains both hydrophobic (fatty acid tail) and hydrophilic (charged peptide ring) parts, making solvent extraction inefficient (poor partitioning) and leading to solubility issues in some purification operations.
- **Removal of By-products and Impurities:** Fermentation broth has proteins, nucleic acids, cell debris, and other peptides and antimicrobial substances. Owing to the same charge and size, most of these impurities co-elute with polymyxin B on ion-exchange chromatography and size-exclusion or reversed-phase chromatography.
- **pH Sensitivity and Stability Problems:** Polymyxins degrade in acidic or basic environments & high heat, so drastic purification procedures are out; also, prolonged exposure to specific solvents or high temperature must be avoided.
- **Endotoxin Removal:** Since it is applied in injectable products, polymyxin B sulfate should be free of endotoxins (LPS) from gram-negative bacteria. It's especially challenging because Polymyxin itself binds LPS (that's how it kills things), so normal endotoxin removal methods won't work.
- **Polymyxin B Content:** The total content of polymyxins in a pharmaceutical substance should be at least 80%, and the total content of admixtures should not exceed 17% [10,11]. The microbial assay should be more than 6000 u/mg [10].

The purpose of this study is to overcome the above challenges and to develop an efficient and simplified method of industrial isolation and purification of polymyxin B sulphate to the pharmacopeial standard.

Materials and methods

Microorganism and cultivation conditions. A high-yield mutant *Paenibacillus polymyxa* strain obtained by a multi-step selection from *P. Polymyxa* RIA 827 (ATCC 10401) was used as a polymyxin B producer. The strain was maintained at 39ºC on a modified King's medium of the following composition (g/L): sucrose, 40; tryptone type-1, 40; $K_2HPO_4 \times 3H_2O$, 0.5; $MgSO_4 \times 3H_2O$, 1.6; agar, 40 (pH 7.0). The fermentation was carried out for 82 ± 12 h at 39ºC in a liquid medium of the following composition (g/L): wheat flour, 1000; $CaCO_3$, 30; CSM, 0.8; $(NH_4)_2SO_4$, 2.54; $K_2HPO_4 \times 3H_2O$, 1.15.

Polymyxin B isolation and purification. After the completion of fermentation, the culture broth was acidified to pH 4.0±0.2 by using sulphuric acid, the acidified broth was subjected to microfiltration to separate out the cell mass from the product. The product solution is loaded into a weak cation (Indion 225 Na, D-152, or Diaion WA30) resin column. The product was eluted with a 2M sulphuric acid solution. The eluted product solution was treated with a 10% NaOH solution by adjusting pH to 8.0-9.0 and stirred for an hour, then adjusted pH to 4.5 by using 10% sulphuric acid. The solution was filtered through hyflow to remove the undissolved impurities. The filtrate was loaded over an adsorption resin (Sephadex SP700). Product was eluted with 40% Ethanol solution, 40% Acetone Solution, or 40% IPA solution at pH 3.0 or 4.0. The product solution was concentrated by nanofiltration. The concentrated mass was treated with a 10% NaOH solution to isolate the base. The base was filtered & washed with hot water. The wet base was dissolved in water by adjusting the pH to 5.5±0.5. Charcolized & filtered through a 0.45µ filter. Again, pH adjusted to 7.0±0.5 by using 5% NaOH solution & filtered. The product was crystallized by adding the solution to chilled acetone. Filtered, wet cake washed with acetone. The wet cake was dried under vacuum at 55±5°C for 72 hrs.

Identification. Identification of the components of the obtained substance and their quantification was performed by HPLC. The analysis was performed using an Agilent 1200 chromatographic system (Agilent Technologies, USA) with a YMC Pro Pack C18 column (5 µm, 250×4.6 mm). The mobile phase was 20:80, acetonitrile: buffer solution (Na_2SO_4), the flow rate was 1.8 mL/min at 30°C. The absorbance was measured at 215 nm; the sample volume was 20 µl. A standard preparation of polymyxin B sulphate (CRS) dissolved in the acetonitrile: water mix (20:80) up to a concentration of 0.5 mg/mL was used as a reference sample. The retention time for polymyxin B1 (tR_{B1}) was 35 min. The retention times for polymyxin B2, B3, and B1-1

were 0.4, 0.5, and 0.8 of that of polymyxin B1, respectively. The time of registration was $1.7tR_{B1}$.[10,11]

The samples of chromatograms obtained for the standard of polymyxin B preparation are shown in Figure 1, and chromatograms obtained for purified polymyxin B preparations are shown in Figure 2 below.

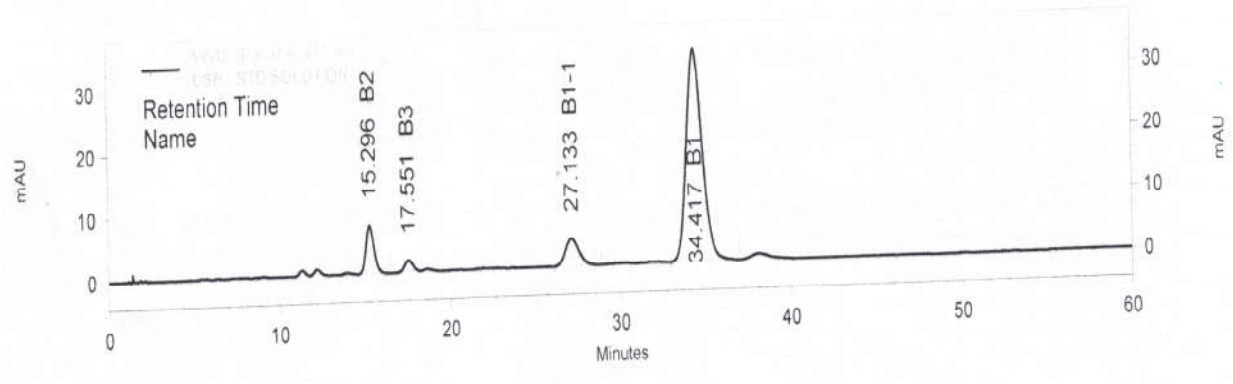


Figure 1: Chromatograms of the standard polymyxin B preparations

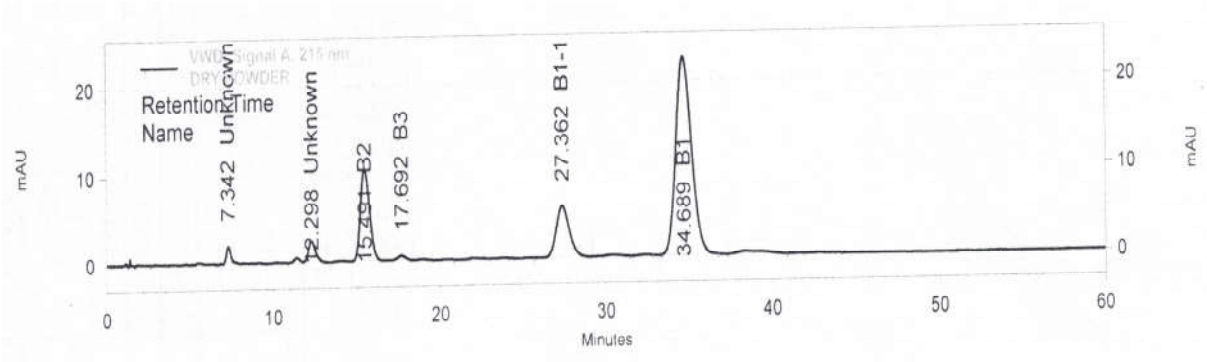


Figure 2: Chromatograms of the purified polymyxin B preparations.

Results and discussion

In this study, we tested various resins to determine the most efficient elimination of accompanying admixtures. Special attention was paid to the search for a new resin type and the purification. For the initial purification stages, we choose super-cross-linked methacrylic weak cation resin for better adsorption, impurity removal & easy desorption abilities. Among these resins (Indion 225 Na, Diaion WA30, or D-152), the final choice was made to be Diaion WA30, having a good sorptive capacity and used for the sorption of high-molecular-weight compounds. Unlike other mentioned sorbents, which provided the output of about 60-70%, Diaion WA30 made it possible to achieve 85% column output. The overall yield of the batch is higher (60.00%) as compared with others. The polymyxin B content is well above the limits in Diaion WA30 as

compared to other resins**.** In addition, complete polymyxin B desorption from this resin requires a small volume of eluent, allowing us to recommend the developed purification method for industrial use. The results of the comparison between different resins are shown in Table 1.

Sr. No.	Resin Name	Output of Resin Stage (%)	Overall Yield of batch	Polymyxin B content
1.	Indion 225 Na	No product	NA	NA
2.	D-152	66.0%	46.21%	60.62%
3.	Diaion WA30	85.0%	60.00%	92.92%

Table 1: Comparison between different resins

During Sephadex SP700 Resin column, different elution solvents (Ethanol, Acetone & IPA) are used for higher output and better quality of eluted product, among these solvents, ethanol made it possible to achieve the highest output and better quality of product. The pH of the solution also plays an important role in output yield. It is observed that the 3.0 pH elution solution observed a higher yield compared with the 4.0 pH elution solution. The comparison of results between the different elution solvents in terms of overall yield, Polymyxin B Content, output of resin stage, and pH of elution solution is shown in Table 2.

Sr. No.	Elution Solution	pH of Elution Solution	Output of Resin Stage (%)	Overall Yield of batch	Polymyxin B content
1.	40% Acetone Solution	4.0	78.40%	51.88%	83.20%
2.	40% IPA solution	4.0	80.84%	41.95%	81.84%
3.	40% Ethanol solution	4.0	85.81%	50.61%	81.61%
4.	40% Ethanol solution	3.0	98.68%	60.00%	92.92%

Table 2: Comparison of Results between the different elution solvents in terms of Overall Yield, Polymyxin B Content, Output of Resin Stage, and pH of elution solution

The comparison of results between the different elution solvents in terms of overall yield, Polymyxin B Content, output of resin stage, and pH of elution solution as per Table 2 has been graphically represented in Figure 3 below.

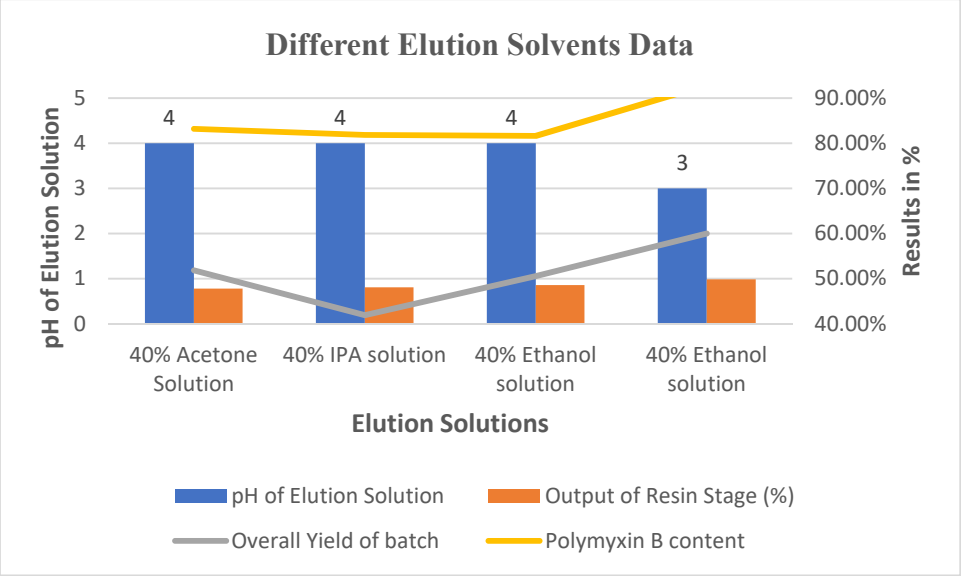


Figure 3: Comparison of Results (Table 2) between the different elution solvents in terms of Overall Yield, Polymyxin B Content, Output of Resin Stage, and pH of elution solution

The results obtained prove an adequate degree of purification of the polymyxin B sulphate material gained by means of the suggested purification technology. Moreover, an adequate degree of removal of toxic polymyxins B1-1 and B3 was proved; it was equated to the adopted pharmaceutical requirements for polymyxin B sulphate.

The optimum results with respect to the output of the final product and the degree of elimination of admixtures associated therewith (polymyxins B1-1 and B3) were achieved by using Diaion WA30 resin as a weak cation resin and using a 60% Ethanol solution of pH 3.0 for elution during a Sephadex SP700 resin column. The microbial assay observed is 9157.58 u/mg well above the limits (more than 6000 u/mg).

Conclusions

The research undertaken resulted in the development of an effective and streamlined process of polymyxin B sulphate purification and separation appropriate for large-scale production. The process involves the following steps: (1) culture broth pretreatment to give a native polymyxin B solution, (2) removal of impurities from the native solution using a weak cation exchange resin, (3) inorganic impurity removal through pH treatment (4) purification of the product solution using an microporous adsorption resin, (5) solution concentration using nanofiltration, (6) precipitation of the base of polymyxin B, (7) crystallization of Polymyxin B Sulphate.

The research indicates an optimized technique for the purification of polymyxin B sulfate through screening resins and eluents. Below is the conclusion in a nutshell:

1. Selection of Resin: Diaion WA30 resin was determined to be the optimum selection because of its greater sorptive power, greater column yield (85%), improved total batch yield (60.00%), and greater content of polymyxin B (92.92%) as compared to other tested resins.

2. Elution Optimization: For Sephadex SP700 Resin column - Ethanol as elution solvent gave the maximum output and optimum quality product. The use of a pH 3.0 ethanol solution improved results significantly, with resin stage output of 98.68%, overall yield of 60.00%, and polymyxin B content of 92.92%.

3. Pharmaceutical Standards: The process efficiently separated toxic polymyxins B1-1 and B3 in accordance with pharmaceutical standards. Also, the microbial assay (9157.58 u/mg) was many times higher than the acceptable level (6000 u/mg), confirming the efficiency of purification.

This purification technique, leveraging Diaion WA30 resin and optimized elution conditions (40% Ethanol solution with 3.0 pH), is recommended for industrial-scale applications due to its efficiency and compliance with pharmaceutical standards.

Statements & Declaration:

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Ravindra Krishnarao Burde, Mukesh Kumar, Karunasagar K. M., Dr. Amitabh Chaturvedi, and Dr. Umesh Luthra. The first draft of the manuscript was written by Ravindra Krishnarao Burde, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript."

References:

1. Suneel Kumar Garg, Omender Singh, Salman Motlekar, Amit Qamra, Niraj Tyagi, Amandeep Singh Khurana, Hanmant Barkate, Deven Juneja, Resurgence of Polymyxin B for MDR/XDR Gram-Negative Infections: An Overview of Current Evidence, https://doi.org/10.1155/2017/3635609, Critical Care Research & Practice, 2017
2. Martti Vaara, Polymyxin Derivatives that Sensitize Gram-Negative Bacteria to Other Antibiotics, https://doi.org/10.3390/molecules24020249, Molecules, 2019.

3. Maria Helena Rigatto, Diego R Falci, Alexandre P Zavascki, Clinical Use of Polymyxin B., https://doi.org/10.1007/978-3-030-16373-0_14, Advances in experimental medicine and biology, Jan 2019
4. I Ofek, E Rubinstein, R Rahmani, K Kabha, S Cohen, Y Herzig, D. Tamarkin, Antibacterial synergism of polymyxin B nonapeptide and hydrophobic antibiotics in experimental gram-negative infections in mice, https://doi.org/10.1128/aac.38.2.374, Antimicrobial Agents and Chemotherapy, Feb 1994
5. Zakuan Z Deris, Philip E Thompson, Sivashangarie Sivanesan, Jian Li, Kade D Roberts, Tony Velkov, Jesmin Akter, Roger L Nation, A secondary mode of action of polymyxins against Gram-negative bacteria involves the inhibition of NADH-quinone oxidoreductase activity, https://doi.org/10.1038/ja.2013.111, The Journal of Antibiotics, Oct 2013
6. Peile Wang, Zhenfeng Zhu, Xiaojian Zhang, Tongwen Sun, Min Feng, Qiwen Zhang, Jing Yang, Population Pharmacokinetics and Limited Sampling Strategy for Therapeutic Drug Monitoring of Polymyxin B in Chinese Patients With Multidrug-Resistant Gram-Negative Bacterial Infections, https://doi.org/10.3389/fphar.2020.00829, Frontiers in Pharmacology, Jun 2020
7. Alejandra Gallardo-Godoy, Wanida Phetsang, Alysha G Elliott, Soumya Ramu, Johnny X Huang, Ruby Pelingon, Craig Muldoon, Bernd Becker, Matthew A Cooper, Angela M Kavanagh, Lawrence H Lash, Mark S Butler, Mark A T Blaskovich, Activity and Predicted Nephrotoxicity of Synthetic Antibiotics Based on Polymyxin B., https://doi.org/10.1021/acs.jmedchem.5b01593, Journal of Medicinal Chemistry, Jan 2016
8. Zhe Yuan, Vincent H Tam, Polymyxin B: a new strategy for multidrug-resistant Gram-negative organisms, https://doi.org/10.1517/13543784.17.5.661, Expert Opinion on Investigational Drugs, April 2008
9. G Schindler, Michael Teuber, Action of Polymyxin B on Bacterial Membranes: Morphological Changes in the Cytoplasm and in the Outer Membrane of Salmonella typhimurium and Escherichia Coli B, Peter R. https://doi.org/10.1128/aac.8.1.95, Antimicrobial Agents and Chemotherapy, Jul 1975
10. United States Pharmacopeia USP-48, https://doi.org/10.31003/USPNF_M66570_02_01, 2025
11. European Pharmacopoeia EP-11.0, European Directorate for the Quality of Medicines & HealthCare EDQM, 2025, pp. 3747-3748.