Characterization of Polymyxin B Bio-distribution and Disposition in an Animal Model

Running title: Bio-distribution of Polymyxin B

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Keywords: Polymyxin B, bio-distribution, nephrotoxicity,

This study was presented in part at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, September 5-9, 2014 (Abstract A-030) as well as in part at the 55th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, September 17-21, 2015 (Abstract A-930).
Despite dose-limiting nephrotoxicity concerns, polymyxin B has resurfaced as the last
treatment resort for multidrug-resistant Gram-negative bacterial infections. However, the
pharmacokinetic, pharmacodynamic and nephrotoxic properties of polymyxin B are still not
thoroughly understood. The objective of this study was to provide additional insights to the
overall bio-distribution and disposition of polymyxin B in an animal model. Sprague-Dawley
rats were dosed with intravenous polymyxin B (3 mg/kg). Drug concentrations in the serum,
urine, bile and tissue (brain, heart, lungs, liver, spleen, kidneys and skeletal muscle) samples over
time were assayed by a validated methodology. Among all the organs evaluated, polymyxin B
distribution was the highest in the kidneys. The mean renal tissue/serum polymyxin B
concentration ratios were 7.45 (95% CI, 4.63-10.27) at 3 h and 19.62 (95% CI, 5.02-34.22) at 6 h
post-dose, respectively. Intrarenal drug distribution was examined by immunostaining. Using a
ratiometric analysis, proximal tubular cells showed the highest accumulation of polymyxin B
(Mander's overlap coefficient 0.998), among all other cell types evaluated. Less than 5% of the
administered dose was recovered in urine over 48 h, but all 4 major polymyxin B components
were detected in the bile over 4 h. These findings corroborate previous results that polymyxin B
is highly accumulated in the kidneys, but the elimination is likely via a non-renal route. Biliary
excretion could be one of the possible routes of polymyxin B elimination, which should be
further explored. Elucidation of mechanism(s) of drug uptake in proximal tubular cells is
ongoing.
INTRODUCTION

The emergence of multidrug-resistant bacterial infections has become a medical crisis worldwide (1, 2). Infections caused by Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter* spp., are extremely challenging to treat (3-6). These infections are also associated with high rates of mortality and morbidity (7, 8).

Moreover, there are few new antibacterial agents available in the clinical drug development pipeline for these life-threatening infections. Consequently, this has led to the revival of old antibiotics, such as the polymyxins, as the last treatment resort for infections caused by multidrug-resistant Gram-negative pathogens (9-13).

Polymyxins [primarily polymyxin B and polymyxin E (colistin)] are cyclic polypeptide antibiotics isolated from *Bacillus polymyxa* (14). Commercially available polymyxin B is a mixture of several related analogs: primarily polymyxin B1, B2, B3 and isoleucine B1 (15, 16).

Polymyxin B first became available for clinical use in the 1950s, but the clinical use has largely been limited due to its nephrotoxic potential.

Despite being available for clinical use over 50 years, there is still a paucity of published reports correlating the pharmacokinetics of polymyxin B with its toxicity profile. Furthermore, we lack a thorough understanding about the bio-distribution pattern, cellular disposition, elimination pathways and transport characteristics of polymyxin B *in vivo*. Therefore, studies to delineate the pharmacokinetic, pharmacodynamic and toxicodynamic profile of polymyxin B are warranted. Undoubtedly, such information will play a pivotal role in designing the optimal polymyxin B dosing strategies, which will maximize the clinical efficacy as well as safety of the drug.
The objective of this study is to provide additional insights to the overall bio-distribution and disposition characteristics of polymyxin B using an animal model. It is anticipated that the outcomes from this investigation would unravel the intricacies in our current understanding of pharmacokinetic properties of polymyxin B. The results obtained from this research could guide future studies in designing the optimal dosing strategies of polymyxin B.

MATERIALS AND METHODS

Chemicals and reagents. Polymyxin B sulfate (USP) powder, trichloroacetic acid, phosphate buffer saline with 0.01% Tween 80 (PBST), 4% paraformaldehyde (PFA), 30% sucrose solution in phosphate buffer saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO). Carbutamide was purchased from Aldrich (Milwaukee, WI). LC-MS grade acetonitrile and water were obtained from Mallinckrodt Baker (Philipsburg, NJ). LC-MS grade formic acid was purchased from Fluka Analytical (St. Louis, MO). Murine anti-polymyxin B antibody was purchased from Thermo Scientific (Rockford, IL). Normal goat serum, streptavidin DyLight 488 (green) and 649 (red), fluorogel-II with DAPI (blue) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-mouse IgG secondary antibody, avidin-biotin blocking kits and biotinylated lectins were purchased from Vector Laboratories (Burlingame, CA). The natural sources, targeted nephron regions and optimal dilutions of various lectins are listed in Table 1.

Animals. Female Sprague-Dawley rats (225-250g) (Harlan, Indianapolis, IN) were used. Jugular veins were cannulated to facilitate intravenous drug administration. The rats received food and water ad libitum. All animals were cared for in accordance with the highest humane
and ethical standards, as approved by Institutional Animal Care and Use Committee (IACUC) of the University of Houston.

**Polymyxin B assay.** A validated liquid chromatography tandem mass spectrometry (UPLC/MS/MS) method was modified to determine the concentrations of polymyxin B in rat serum, bile, brain, heart, lungs, liver, spleen, kidneys and skeletal muscle tissues (17). Briefly, serum, bile or tissue homogenate was spiked with 10 µl of an internal standard (carbutamide 5 µg/ml) and extracted with 150 µl of 5% trichloroacetic acid. The samples were vortexed for 1 min, followed by centrifugation as previously described (17). The supernatant was transferred to a new tube and evaporated to dryness under a stream of ambient air. The residue was then reconstituted with 100 µl of a mixture of acetonitrile and 0.1% formic acid (1:1 v/v). The samples were again centrifuged at 18,000 \( \times g \) for 20 min at 4°C, and 10 µl of the supernatant was injected into the UPLC-MS/MS system for quantitative analysis. The bile samples were treated in a similar fashion as serum or tissue homogenate, except for inclusion of blank rat serum (> 2 times the volume of bile) to the bile as an attempt to minimize the matrix effect caused due to bile component.

The calibration curves in serum/bile/tissue samples were constructed using at least eight concentration standards. The linearity of the calibration curves was determined by the best fit of peak-area ratios (analyte/internal standard) versus concentrations, and fitted using a linear regression (1/\(x^2\) weighing) method. The lower limit of quantification (LLOQ) was determined based on the signal to noise ratio of 10:1. The assay was validated based on accuracy, precision, inter and intraday variability, which were well within 15% of the coefficient of variation (CV) and did not exceed 20% of the CV for the LLOQ (17).
A validated microbiological assay method was also modified to determine the concentrations of polymyxin B in the rat urine samples, as previously described (18). *Bordetella bronchiseptica* ATCC 10580, obtained from American Type Culture Collection (Manassas, VA), was used as the reference organism. The calibration curves were made by plotting the inhibition zone diameter (mm) versus the logarithmic standard polymyxin B drug concentration in urine. The assay was previously validated based on accuracy, precision, inter and intraday variability; LLOQ was determined to be 2 µg/ml.

**Bio-distribution of polymyxin B.** Prior to each experiment, polymyxin B powder was dissolved in sterile water for injection and diluted to the desired concentration. The rats were given a single intravenous dose of polymyxin B (3 mg/kg) and sacrificed after 3 h (n=3), 6 h (n=6) and 24 h (n=3), respectively. The harvested tissues were weighed and homogenized in de-ionized water (1:2). The mean tissue/serum drug concentration ratios were estimated for each tissue matrix. The groups were compared using one-way ANOVA, followed by Tukey’s post-hoc test. P-values <0.05 were considered significant.

**Intrarenal distribution of polymyxin B**

*Harvesting and processing of rat kidney.* The rats (n=3) were given a single intravenous dose of polymyxin B (4 mg/kg) and sacrificed at 6 h post-dose. The kidneys were perfusion fixed with 4% paraformaldehyde and stored overnight. Subsequently, the kidneys were cryoprotected in 30% sucrose solution in PBS and finally cryosectioned at 20µm intervals into thin tissue cross-sections.
Identification of renal cell type and immunostaining for polymyxin B. Different anatomical regions of the nephron were identified by staining with specific lectin markers. The fresh frozen kidney tissue sections were placed in PBST maintained at pH 7.4 for 5-10 min before staining. Briefly, the sections were pre-incubated in avidin-biotin blocking solution, followed by incubation with biotinylated lectins diluted in PBST to the concentrations specified in Table 1, each for 30 min at room temperature. After washing 3 times in PBST, the sections were incubated for 30 min at room temperature with streptavidin DyLight 649 (10 µg/ml diluted in PBST). Subsequently, the sections were washed in PBST followed by washing 3 times in distilled water. The sections were then mounted using the fluorogel-II mounting agent with DAPI. Controls were prepared by the same staining procedure, except that the lectins were replaced by PBST.

The double immunostaining of renal tissue section was carried out using polymyxin B antibody and lectins. The sections were incubated with 5% normal goat serum followed by incubation with avidin-biotin blocking solution for 30 min at room temperature. Subsequently, the sections were treated with polymyxin B antibody (1:20 dilution in 5% normal goat in PBS) for 1 h at 4°C, and later incubated with biotinylated mouse anti-polymyxin B antibody (1:150 dilution in 5% normal goat serum). After washing 3 times in PBST, the sections were incubated with streptavidin DyLight 488 (10 µg/ml, diluted in PBST) for 30 min each at room temperature. Subsequently, staining with a specific lectin marker was performed on the same tissue section, as described above. Controls were prepared by same staining procedure except primary antibody and lectins were replaced by PBST.

Co-localization of polymyxin B antibody with lectin. Images were captured using an Olympus IX61 DSU. Several region of interest (ROI) were randomly selected from individual overlay/co-
localized image for each cell type. Selected ROI(s) from each overlay image were further analyzed using a computer assisted software image J (version 1.47); the Mander's overlap coefficient ($R_{col}$), which signifies the percentage of overlap of two signal intensities, was calculated using Just Another Co-localization Plug-in (JACoP). The coefficient value above 0.7 was deemed to be a good degree of co-localization.

**Elimination of polymyxin B**

*Pre & post-dose urine sampling.* The rats (n=3) were housed individually in separate metabolic cages to facilitate urine collection. Blank urine was collected from each rat, prior to the administration of a single intravenous dose of polymyxin B (3 mg/kg). Subsequently, the cumulative urine of each rat was collected in aliquots for up to 48 h following drug administration. The urine was concentrated 2× by evaporation to reduce the lower limit of detection.

*Bile sampling.* The bile ducts of anesthetized rats were cannulated to facilitate bile sampling (n=5). Prior to drug administration, the blank bile was collected (over 30 min) from each rat. The rats were given a single intravenous dose of polymyxin B (3 mg/kg), and the bile was further collected cumulatively for another 4 hours.

**RESULTS**

**Polymyxin B assay.** The linear concentration ranges for serum, bile, brain, heart, lungs, liver, spleen, kidneys and muscle tissue homogenate were 0.1-12.8 µg/ml, 0.05-12.8 µg/ml, 0.05-12.8 µg/ml, 0.05-12.8 µg/ml, 0.0125-12.8 µg/ml, 0.05-12.8 µg/ml, 0.05-12.8 µg/ml, 0.05-12.8 µg/ml.
µg/ml, and 0.1-6.4 µg/ml, respectively. The linear regression coefficients were ≥ 0.99 in all the calibration curves. For the microbial assay of polymyxin B in urine, the linear concentration range was 2-64 µg/ml respectively.

**Bio-distribution of polymyxin B.** Among all the organs evaluated, polymyxin B distribution was the highest in the kidneys. The mean renal tissue/serum polymyxin B concentration ratios were 7.45 (95 % CI, 4.63-10.27) at 3 h and 19.62 (95 % CI, 5.02-34.22) at 6 h post-dose, respectively as shown in Figure 1. As shown in Table 2, all tissue concentrations at 24 h were lower than those observed at 6 h, but the mean tissue/serum ratio at 24 h could not be calculated because the polymyxin B concentration in the serum was below the limit of detection. The kidney concentration at 24 h was roughly 1.4-5.8 times those observed in organs other than the brain. Polymyxin B levels in the brain remained consistently lower than that observed in serum. All other organs exhibited a similar distribution profile as serum (Figure 1).

**Intrarenal distribution of polymyxin B.** A gross cross-section of the kidney revealed that the intrarenal distribution of polymyxin B was heterogeneous, as shown in Figure 2. Polymyxin B accumulated mainly in the renal cortex and outer stripe of the outer medulla.

The differential staining pattern was satisfactory with each lectin marker (data not shown). Strong, focal staining of the brush border epithelium of proximal tubular cells was seen with Phaseolus vulgaris erythroagglutinin (PHA-E) lectin. Soybean agglutinin (SBA) lectin predominantly stained the distal convoluted tubular cells. The staining pattern of SBA lectin was reasonably distinct; cytoplasmic staining of distal cells was observed. For glomeruli, a strong, global and diffused staining pattern was seen with Wheat germ agglutinin (WGA) lectin.
The double staining with polymyxin B antibody and lectins is shown in Figure 3. Polymyxin B was found to be accumulated primarily in the proximal tubular cells, with Mander’s overlap coefficient ($R_{\text{col}}$) of 0.998 for proximal tubular cells, 0.366 for distal tubular cells and 0.097 for the glomerulus, respectively.

Elimination of polymyxin B

The concentration of polymyxin B in the rat urine was below the limit of detection; less than 5% of administered dose (or pharmacologically active metabolites, if any) could have been recovered in the urine over 48 h. In contrast, all 4 major polymyxin B components (polymyxin B1, B2, B3 and isoleucine-polymyxin B1) were detected in the bile collected over 4 h (data not shown).

DISCUSSIONS

As we are faced with a dearth of viable treatment options for infections caused by multidrug-resistant Gram-negative bacteria, polymyxins have undergone a resurgence as a treatment of last resort. However, the clinical utility is hindered by concerns of dose-limiting nephrotoxicity and a limited understanding of polymyxin pharmacokinetics.

Over the years, several attempts have been made to characterize the pharmacokinetics and nephrotoxicity of polymyxin B. Kunin et al. examined the bio-distribution of polymyxin B in rabbits and observed that the distribution was non-uniform in various organs (19, 20). Our group also reported that polymyxin B was preferentially accumulated in rat kidneys with a prolonged residence time (21). In a mouse model, polymyxin B was found to be substantially accumulated...
in the renal proximal tubular cells (22), which was previously observed to be the primary renal
injury site in a rat model (23). Furthermore, polymyxin B recovery from urine was reported to be
very low in several studies. Abdelraouf et al. reported that less than 1% of polymyxin B was
recovered in an unchanged form in the urine, over 48 h after single intravenous dose (21, 23).
Based on the urinary excretion data of polymyxin B available from 17 critically ill patients,
Sandri et al. also reported the median urinary recovery to be 4.0% (range 1.0% - 17.4%) of the
administered dose (24). In line with previous findings, Zavascki et al. reported that less than 1%
of the dose was recovered unchanged in urine samples following intravenous administration of
polymyxin B in critically ill patients (25).

In this study, we provided additional insights to the following. First, despite using different
animal species, methods for sample preparation and analysis, our bio-distribution results are
concordant with previous results; suggesting that polymyxin B distribution to different organs is
not homogenous and the drug is preferentially accumulated in the kidneys. However, in contrast
to previous studies by Kunin et al (19, 20), we observed consistently low drug concentrations in
the brain. The earlier studies by Kunin et al, used a non-specific microbiological assay to
quantify the polymyxin B in various tissue homogenate rather than a sensitive, specific UPLC-
MS/MS assay. Moreover, in the previous study a liquid-liquid extraction method was used for
sample preparation; the drug was estimated separately in the organic and aqueous extraction
layers in the bound and free form, respectively (19). This procedure might have over-estimated
the drug concentration as the final drug recovery from serum and tissue homogenate was found
to be greater than the dose administered. Our brain tissue bio-distribution results were in
accordance with a recent review of clinical findings (26). We were somewhat surprised to
observe a higher concentration of polymyxin B in lung tissues as compared to serum at 6 h (but
not at 3 h). This finding is in contrast to our previously reported low polymyxin B levels in the epithelial lining fluid (ELF) in mice (17). Since the lungs are consisted of several sub-compartments (lung parenchyma, alveolar epithelium, macrophages, ELF and blood-alveolar barrier etc.); assaying drug concentrations in the whole organ could be subjected to a high degree of variability.

Second, Yun et al. provided circumstantial evidence linking polymyxin B distribution to the site of renal injury (22). We have previously shown histological evidence that the polymyxin B induced injury was mainly confined to the proximal tubular cells of the rat kidneys (23). Using the same model, we evaluated drug accumulation in the multiple cell types in a semi-quantitative fashion, establishing a more concrete association between site of drug accumulation and the site of renal injury. These findings are expected to guide future investigations focusing on intervention(s) to reduce nephrotoxicity. In addition to demonstrating a low urine recovery of polymyxin B, the use of a microbiological assay in this study provided additional evidence that renal excretion of active metabolite(s) are unlikely. Finally, to the best of our knowledge, the present study is the first to examine the biliary excretion of polymyxin B. We detected all 4 major polymyxin B components in the bile over 4 h. Biliary excretion could be one of the possible routes of polymyxin B elimination, which should be further explored.

There are several limitations for this study. As a preliminary investigation, we examined polymyxin B elimination in the bile only for 4 h. For future investigations, surgically modified rats could be used to evaluate biliary drug excretion for a longer duration. The sites of polymyxin B accumulation within the kidneys were identified at the cellular level. For better resolution, the investigations could be performed at the sub-cellular/organelle level. Finally, the bio-distribution
and disposition characterization of polymyxin B was investigated following a single intravenous
dose. Further investigations to assess the steady state pharmacokinetics of polymyxin B after
multiple doses are warranted.

In conclusion, this study provides valuable insights to the overall *in vivo* distribution properties
of polymyxin B. Drug accumulation within the kidneys is heterogeneous, and is primarily
confined to the proximal tubular cells. Among different renal cell types evaluated, proximal
tubular cells show the highest accumulation of polymyxin B. In conjunction with previous
findings, it is likely that there is a direct link between the site of drug accumulation and injury at
the cellular level within the kidneys. Polymyxin B could be eliminated unchanged via the biliary
route. Elucidation of the mechanism(s) of drug uptake in proximal tubular cells is ongoing.
REFERENCES


Figure 1. Tissue/serum concentration ratio of polymyxin B in various tissues; 3 h post-dose (A), 6 h post-dose (B).

Vertical error bars represent the 95% confidence intervals.
Panel A represents a kidney cross-section from control rat whereas panel B represents a cross-section from polymyxin B treated rat. Blue color represents counterstaining with nuclear stain (DAPI) whereas green color represents staining with anti-polymyxin B antibody.
Figure 3. Double staining with polymyxin B antibody and lectins

Panel A-C represent control section; D-F represent the staining with anti-polymyxin B antibody; G-I represent staining with lectin; J-L represent multichannel images of double staining with polymyxin B antibody and lectin.

Green color represents staining with anti-polymyxin B antibody and red color represents staining with specific lectin marker, respectively. The green-red co-localized signals produced a yellow signal in the overlay images.
Table 1. Lectins used for the staining of rat kidney section

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Source</th>
<th>Target</th>
<th>Final dilution of Lectin in PBST (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phaseolus vulgaris erythroagglutinin (PHA-E)</td>
<td>Red kidney bean</td>
<td>Proximal convoluted tubules (PCT)</td>
<td>10</td>
</tr>
<tr>
<td>Glycine max/ Soybean agglutinin (SBA)</td>
<td>Soybean</td>
<td>Distal convoluted tubules (DCT)</td>
<td>20</td>
</tr>
<tr>
<td>Wheat germ agglutinin (WGA)</td>
<td>Wheat germ</td>
<td>Glomerulus</td>
<td>20</td>
</tr>
</tbody>
</table>
Table 2. Distribution of polymyxin B in rat serum and tissue homogenates

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Serum (µg/ml)</th>
<th>Kidney (µg/g)</th>
<th>Muscle (µg/g)</th>
<th>Liver (µg/g)</th>
<th>Heart (µg/g)</th>
<th>Lung (µg/g)</th>
<th>Spleen (µg/g)</th>
<th>Brain (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.46 ± 0.38</td>
<td>10.57 ± 1.21</td>
<td>1.31 ± 0.41</td>
<td>1.10 ± 0.29</td>
<td>1.50 ± 0.78</td>
<td>4.93 ± 3.71</td>
<td>0.86 ± 0.43</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td>6</td>
<td>0.69 ± 0.29</td>
<td>11.32 ± 5.06</td>
<td>0.77 ± 0.34</td>
<td>0.82 ± 0.24</td>
<td>0.57 ± 0.18</td>
<td>1.33 ± 0.41</td>
<td>1.11 ± 0.50</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>24</td>
<td>&lt; LLOQ</td>
<td>0.76 ± 0.41</td>
<td>0.53 ± 0.18</td>
<td>0.13 ± 0.04</td>
<td>0.28 ± 0.06</td>
<td>0.25 ± 0.04</td>
<td>0.50 ± 0.07</td>
<td>0.01 ± 0.02</td>
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