

Antibacterial Activity of Beyond Use Date 0.3% Ofloxacin Eye Drops against *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is a Gram-negative bacterium and a major cause of healthcare-associated ocular infections. The beyond-use date (BUD) of ophthalmic preparations is intended to ensure drug safety and efficacy. This study aimed to analyze the effect of the beyond-use date (BUD) of 0.3% ofloxacin eye drops on the inhibition of the growth of *P. aeruginosa*. This was an *in vitro* laboratory experimental study with a repeated-measures design. Samples were obtained by purposive sampling using *P. aeruginosa* colonies, with five samples in each time group. Antibacterial activity was evaluated by measuring inhibition zone diameters and analyzed using the Friedman test followed by the Wilcoxon test. The mean inhibition zone diameters at 0, 7, 14, 21, and 28 day were 37.4; 34.2; 31.6; 30.0; 29.4 mm. The Friedman test showed a statistically significant difference among the groups ($p < 0.001$). Post-hoc analysis using the Wilcoxon test revealed significant differences between most groups ($p < 0.05$), except between day 21 and day 28 ($p > 0.05$). The findings indicate that prolonged storage time may lead to a decrease in the antibacterial efficacy of the formulation.

Keywords: beyond-use date, ofloxacin, eye drop, *Pseudomonas aeruginosa*

1. INTRODUCTION

Ophthalmic infections represent a significant clinical challenge and may lead to severe complications if not managed appropriately (Lin *et al.*, 2022). *P. aeruginosa*, a Gram-negative bacterium characterized by high virulence and resistance to numerous antimicrobial agents, is among the most prevalent causative organisms of ocular infections (Riedel *et al.*, 2019). Fluoroquinolone antibiotics are widely used in the treatment of bacterial eye infections due to their broad spectrum potency and effective ocular penetration (Ilyas and Yulianti, 2018).

Ofloxacin 0.3% eye drops are widely utilized in clinical practice (Winarto, 2017). Similar to other sterile pharmaceutical preparations, ophthalmic solutions have a limited shelf life after opening, referred to as the beyond-use date (BUD) (Kadek *et al.*, 2024). The BUD is established to maintain the product's quality, stability, and sterility throughout its period of use (Hooper & Jacoby, 2016). Manufacturer labeling for ophthalmic antibiotic preparations

recommends a maximum use period of approximately 28–30 days after opening under appropriate storage conditions, while pharmacopeial guidelines emphasize sterility and stability considerations during this period. Inadequate storage conditions or exceeding the recommended BUD can diminish the medication's efficacy and elevate the risk of microbial contamination (Cokro *et al.*, 2022). Earlier studies have reported that chloramphenicol preparations should not be used beyond 7 days after opening due to reduced effectiveness, as indicated by a decrease in inhibition zone diameter (Tijani, 2021; Oktaviani, Alifiar, & Yuliana, 2022). A decrease in antibacterial activity may lead to inadequate treatment, prolonged infection, and the development of bacterial resistance (Gobetti *et al.*, 2021). However, evidence remains limited regarding the changes in antibacterial activity of ofloxacin 0.3% eye drops over time post-opening, particularly against *P. aeruginosa*.

Therefore, this study aimed to examine the influence of the beyond-use date on the antibacterial activity of 0.3% ofloxacin eye drops against *P. aeruginosa* utilizing an in vitro experimental technique. *P. aeruginosa* is not only a common causative agent of ocular infections but has also shown increasing resistance to fluoroquinolones in recent years, which may compromise treatment effectiveness. In addition, this pathogen is frequently associated with severe ocular conditions such as keratitis, particularly in contact lens users, highlighting its epidemiological significance in ophthalmic infections. The findings of this study are expected to provide scientific data about the significance of adhering to the prescribed BUD for ocular antibiotic preparations. (Abdullah, Hasan and Rajab, 2025).

2. METHOD

2.1 Research Materials

This was a laboratory experimental study evaluating antibacterial activity across different storage time points to assess the effect of the beyond-use date (BUD) of 0.3% ofloxacin eye drops on *P. aeruginosa* growth. Samples were selected based on inclusion and exclusion criteria. Inclusion criteria included clinical isolates of *P. aeruginosa*, newly opened 0.3% ofloxacin eye drops stored under standard room temperature and sterile conditions, and sterile, non-expired Mueller–Hinton agar and MacConkey media. Exclusion criteria included contaminated cultures, improperly stored eye drops, and damaged, expired, or non-sterile media. The sample size was determined using the Federer formula, resulting in five samples per group, with a total of 25 samples across five storage time points (day 0, 7, 14, 21, and 28).

The eye drop samples were stored at a controlled room temperature of $25 \pm 2^{\circ}\text{C}$, protected from direct light exposure by keeping the bottles in their original packaging. The bottles were opened once daily under aseptic conditions to simulate routine use. All handling procedures, including opening, dispensing, and disc impregnation, were performed inside a biosafety cabinet to minimize contamination. This investigation utilized a biosafety cabinet, autoclave, incubator set at 37°C , micropipettes with sterile tips, sterile Petri plates, and a densitometer. Clinical isolates of *P. aeruginosa* were employed, as well as MacConkey agar and Mueller-Hinton agar (MHA), 0.3% ofloxacin eye drops, sterile blank paper discs, 0.5 McFarland standard, and 0.9% NaCl solution.

2.2 Preparation of Bacterial Suspension and Disc Impregnation

Clinical isolates of *P. aeruginosa* were first cultured on MacConkey agar. Three colonies were suspended in 5 mL of 0.9% NaCl solution until the turbidity reached the 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL). The 0.3% ofloxacin eye drops were stored and evaluated at particular intervals, namely days 0, 7, 14, 21, and 28 after opening. Each time point represented a distinct beyond-use date condition. To prepare the sample, 20 mL of 0.3% ofloxacin eye drops was poured to a sterile Petri dish, and five sterile blank discs were immersed in it for 15 minutes. Negative control discs were created by immersing sterile blank discs in 0.9% NaCl solution for fifteen minutes.

2.3 Antibacterial Activity Test

A sterile cotton swab was dipped in the bacterial suspension, pushed against the tube wall to remove any surplus fluid, and then evenly streaked across the surface of Mueller-Hinton agar in both vertical and horizontal directions. The plates were left to stand for 3–5 minutes. The impregnated discs containing 0.3% ofloxacin eye drops, as well as the negative control discs, were then placed on the inoculated Mueller-Hinton agar with sterile forceps, allowing at least 2.5 cm between them. The plates were incubated inverted at 37 degrees Celsius for 18-24 hours. After incubation, the diameter of the inhibitory zones was measured in millimeters using a caliper. The inhibition zone was defined as a clean area surrounding the disc with no visible bacterial growth.

2.4 Data Analysis

Data analysis was performed using SPSS software. Normality was assessed using the Shapiro–Wilk test. As the data were not normally distributed, nonparametric analysis was applied using the Friedman test to compare differences across multiple groups. The Friedman

test showed a statistically significant difference among the groups $p < 0.001$. Post-hoc pairwise comparisons were conducted using the Wilcoxon signed-rank test. A significance level of $p < 0.05$ was considered statistically significant

3. RESULTS AND DISCUSSION

The antibacterial activity of 0.3% ofloxacin eye drops against *P. aeruginosa* was evaluated at five storage time points after opening, as follows: day 0, 7, 14, 21, and 28. Each time point was evaluated using independently opened bottles of 0.3% ofloxacin eye drops to ensure biological replication. The inhibition zones were measured using the Kirby–Bauer disc diffusion method and expressed as diameters in millimeters.

The average inhibitory zone diameter reduced gradually as storage duration increased. On day 0, the average inhibition zone was 38 (36-38) mm. The result fell to 34 (33-36) mm on day 7 and 32 (31-32) mm on day 14. On day 21, the mean diameter decreased to 30 (29-31), with the lowest mean inhibition zone detected on day 28 was 29 (29-30) mm. These findings suggest that 0.3% ofloxacin eye drops lose antibacterial action over time after opening. Diameter of inhibition zone of 0.3% ofloxacin eye drops show in table 1.

Table 1. Diameter of Inhibition Zones of 0.3% Ofloxacin Eye

Day	Drops					Median (Min- Max)	<i>p</i> -value
	Sample 1 (mm)	Sample 2 (mm)	Sample 3 (mm)	Sample 4 (mm)	Sample 5 (mm)		
0	36	38	37	38	38	38 (36-38)	<0,001*
7	33	33	34	35	36	34 (33-36)	
14	31	31	32	32	32	32 (31-32)	
21	30	29	31	30	30	30 (29-31)	
28	29	29	30	29	30	29 (29-30)	

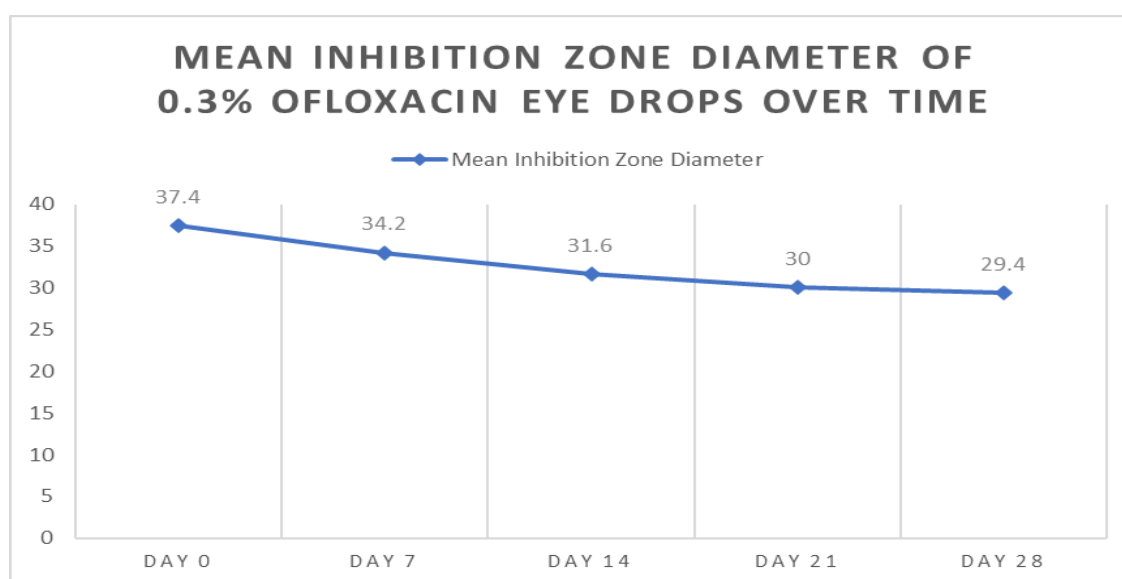
*Friedman Test

Normality testing was performed using the Shapiro–Wilk test due to the small sample size in each group ($n < 50$). The results indicated that data from Day 7, and 21 were normally distributed ($p > 0.05$), whereas data from Day 0, 14, and 28 were not normally distributed ($p < 0.05$). Variance homogeneity testing using Levene’s test showed homogeneous variances among groups ($p=0.710$). Because the overall normality assumption was not met, non-parametric analysis was applied. The Friedman test demonstrated a significant variation in inhibition zone widths across the five storage time periods ($p < 0.001$), showing that storage duration had a substantial impact on the antibacterial activity of 0.3% ofloxacin eye drops

Tabel 2. Wilcoxon Test

Pairwise comparisons between periods	<i>p</i> -Value
Day 0 and 7	0,039*
Day 0 and 14	0,041*
Day 0 and 21	0,041*
Day 0 and 28	0,041*
Day 7 and 14	0,039*
Day 7 and 21	0,042*
Day 7 and 28	0,038*
Day 14 and 21	0,038*
Day 14 and 28	0,034*
Day 21 and 28	0,083

The results of the post-hoc analysis using the Wilcoxon test are presented in Table 2. As shown in Table 2, statistically significant differences were observed in most pairwise comparisons between observation days ($p < 0.05$). However, no significant difference was found between day 21 and day 28 ($p > 0.05$), indicating that the reduction in antibacterial activity was less pronounced during the later storage period.

**Figure 1.** Mean Inhibition Zone Diameter Of 0.3% Ofloxacin Eye Drops Over Time

In general, manufacturer labeling for ophthalmic antibiotic preparations recommends a maximum use period of approximately 28–30 days after opening under appropriate storage conditions, while the United States Pharmacopeia (USP) states that ophthalmic preparations should not be used after 30 days of opening to guarantee efficacy and sterility (United States Pharmacopeia [USP], 2019). Ofloxacin, a fluoroquinolone antibiotic, works by blocking DNA gyrase and topoisomerase IV, inhibiting bacterial DNA replication (Yefet *et al.*, 2018).

Fluoroquinolones in liquid formulations may deteriorate over time due to factors such as light exposure and storage conditions after opening (Unnisa *et al.*, 2025). Several studies have reported that topical antibiotics may exhibit reduced antibacterial potency, even when no visible physical changes in the formulation are observed (Abdullah, Hasan and Rajab, 2025).

The clinical relevance of this reduction is enhanced by the fact that *P. aeruginosa* has intrinsic resistance mechanisms such as biofilm development and limited outer membrane permeability. The observed decrease in inhibition zone widths in this investigation lends support to the idea that prolonged storage after opening can reduce the antibacterial activity of ocular antibiotic formulations (Abdullah, Hasan and Rajab, 2025). However, this study did not assess chemical stability or active ingredient degradation; therefore, no definitive conclusions regarding physicochemical instability can be drawn.

The findings of this study demonstrated a progressive reduction in the diameter of inhibition zones of 0.3% ofloxacin eye drops against *P. aeruginosa* from day 0 to day 28 of storage. Statistical analysis confirmed that storage time significantly affected antibacterial activity ($p < 0.001$), with a decreasing trend observed across most time points, although the difference between day 21 and day 28 was not statistically significant, indicating a tendency that the reduction in antibacterial activity was less pronounced during the later storage period.

This study evaluated antibacterial activity solely based on inhibition zone diameters. It did not assess other parameters, such as minimum inhibitory concentration (MIC) or evaluate chemical stability or active ingredient degradation. Thus, no definitive conclusions regarding physicochemical instability can be made. (Balouiri *et al.*, 2016). Furthermore, the experiments were conducted solely *in vitro*, which limits their ability to represent clinical conditions, including ocular drug absorption, tissue distribution, interactions with tear fluid, and host immune responses (Balouiri, Sadiki and Ibnsouda, 2016). Therefore, further *in vivo* studies are required to confirm the clinical relevance of the observed decrease in antibacterial activity (Mofidfar *et al.*, 2021).

4. CONCLUSION

The antibacterial activity of 0.3% ofloxacin eye drops against *P. aeruginosa* decreased significantly with increasing storage time after opening, particularly between day 0 and day 21. Although the reduction appeared less pronounced between day 21 and day 28, as the difference was not statistically significant. The findings emphasize that compliance with the recommended beyond-use date (BUD) is critical for preserving the antibacterial efficacy of the formulation.

As this study was conducted in vitro, the findings are limited to laboratory conditions and require further investigation

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