

## EFFECT OF STERILIZATION BY HEATING IN THE PRESENCE OF BACTERICIDE AND BACTERIAL FILTERED MEMBRANE ON THE STABILITY OF EYE DROPS CONTAINING 0.5% CHLORAMPHENICOL AT VARIOUS pH

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### ABSTRACT

**Objective:** The purpose of this study was to determine a sterile 0.5% chloramphenicol eye drop formula with the best potency of antibacterial by determining the appropriate sterilization method and the supporting pH.

**Methods:** 0.5% chloramphenicol was formulated with 0.01% thimerosal, which act as a bactericide and combines with borate buffer to produce eye drop formulas with variations in pH (6.8, 7.0 and 7.4). All formulas were stored at room temperature for 28 d and were evaluated, including: organoleptic of the preparations, sterility, pH stability, and the antibacterial potency of chloramphenicol in eye drops.

**Results:** All dosage formulas did not undergo photodegradation reactions which were marked by no change in color until the end of the storage period. However, the formula with pH 6.8 which was sterilized by heating in a presence of bactericide, showed the presence of more particulate precipitates than in the pH 6.8 formula which was sterilized using membrane filter bacteria. However, both methods of sterilization produced sterile chloramphenicol eye drops. The preparation using a method of heat sterilization with bactericide decreased the pH greater than the preparation using a sterile bacterial filter sterilization method. C2 preparations at pH 7.0 and sterilized using the bacterial filter membrane sterilization method were more stable because they had the smallest pH change of 0.05 and the percentage reduction in antibacterial potential was smaller at 1.15%.

**Conclusion:** The best treatment for the chloramphenicol eye drop was kept the pH formula at pH 7 and sterilized using bacterial filter membrane sterilization method.

**Keywords:** Chloramphenicol, Eye drops, Sterilization, Heating, Membrane filter bacteria

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### INTRODUCTION

Eye infections occur when pathogenic microorganisms contaminated and multiply in the eye. Eye contaminations related to the exposed of the eye to the external environment, because the eyes are very sensitive to external. In addition, the airborne pathogen that contaminated hands can be an alternative source for eye infection [1]. Conjunctivitis, endophthalmitis and bacterial keratitis are some kinds of eye infection caused by various kinds of bacteria [2-4]. The eye is a challenging organ for drug delivery because of its unique anatomy with restriction of drug absorbed into the deep tissues [5]. Chloramphenicol is one of the topical antibiotic occasionally used in the eye infection treatment because of its broad antibacterial spectrum and its capability to penetrate into the aqueous humor and ocular tissues [6]. Chloramphenicol is active against both Gram positive and Gram-negative bacteria. Chloramphenicol acts by binding to the 50S ribosomal subunit of bacteria and inhibiting peptidyl transferase. This enzyme catalyzes the growing polypeptide chain by forming the peptide bonds between adjacent amino acids [7]. The topical application of Chloramphenicol is known to be relatively safe, but its systemic administration may have fatal side effects, such as aplastic anemia and bone marrow suppression [8]. Because of this reason, chloramphenicol use is restricted to ophthalmic applications or other infection treatment when the alternative treatments are not available [9]. However, chloramphenicol has been used as the gold standard of antibiotic in ophthalmic preparation and being text prescribed for 55% patient with red eyes [10, 11]. The chloramphenicol has treated of 91% to 93% in ocular infections [12, 13] and can inhibit more than 94% of eye pathogens [10].

The source of eye infection is not only from the contamination during the preparation used, but it may come from ophthalmic solutions itself [14]. The occurrence of endophthalmitis and bacterial keratitis had been reported to be correlated with contaminated topical eye medications case [3, 4]. The contamination

may lead to the decreasing of efficacious treatment because the contaminants can alter the pH of ophthalmic solution [15]. Therefore, most of ophthalmic preparations always use preservatives to inhibit the growth of microbial contamination. Beside using preservatives or bactericide, the sterility of the ophthalmic solution product also must be ensured. But the determination of the sterilization method used must consider the stability of the active substance in the preparation. The proper sterilization method can optimize the product sterility level and maintain antibacterial stability from eye drops.

The eye drops can be sterilized using chemical and mechanical method. The common sterilization of eye drops using the chemical method is sterilization by heating with a bactericide at 98-100 °C for 30 min by denaturing the bacterial protein as its mechanism. But chloramphenicol was subjected to forced degradation by chemical oxidation, alkali, acid, and heat [16]. Chloramphenicol heating at a temperature of 100 °C for 30 min resulted in degradation of 4% and by heating 115 °C with the same time, degradation of 10% was produced. This degradation can reduce the stability and efficacy of chloramphenicol in eye drops [17, 18]. Another study compared that 15% hydrolysis of chloramphenicol may occurred after autoclaving the eye drops, and 3 to 4% after heating with a bactericide. This combination of methods was also considered on the fact that spore contaminants in the preparation cannot be killed only by using bactericides but also require heating. It has been reported that bacterial spores can survive after exposed by gamma irradiation, then can be inactivated by heat treatment [19-22]. Others study also observed that spores of bacteria showed higher sensitivity to inactivated by treatment using bactericides at normal temperatures after surviving at gamma irradiation sublethal doses [23]. For this reason, the sterilization process using heat with a bactericide is investigated in this study and compared its efficacy against the mechanical method of sterilization without heat. Meanwhile, for the mechanical method, it can be employed by sieving the bacterial cell

using membrane filter. This sterilization method is used to sterilize a solution that is not heat resistant. The benefits of filter sterilization include the accuracy of filtering the solution in small amounts, the ability to effectively sterilize compounds that are not heat resistant, the equipment used is relatively cheap and can remove living and dead microbes and particles from solution [24]. Besides the heating effect, the stability of eye drops can also be affected by pH. Changes in pH can affect the rate of a chloramphenicol decomposition reaction. But the pH changes can be prevented by adding buffers and adjusting pH through the addition of acids or bases. In addition to maintaining chemical stability, the addition of buffers and pH regulation in eye drops can also reduce the discomfort feeling and increase the solubility of medicinal ingredients [25]. The stability of chloramphenicol eye drops can be improved by adding borate buffer to the pH stability of 7.0-7.5 [18, 25]. Therefore, the effect of different sterilization method was evaluated by measuring the antibacterial potency of 0.5% chloramphenicol in eye drop formula with optimizing the pH formula to determine the most stable eye drops formula.

## MATERIALS AND METHODS

### Materials

Components of eye drops formula were consisted of Chloramphenicol (PT. Cendo Indonesia®), thimerosal (PT. Cendo Indonesia®), boric acid (Merck®), sodium tetraborate (Merck®), and redistillata water (PT. Ikapharmindo Indonesia®). The growth media

used in this study were Tryptone Soya Agar (TSA-Oxoid®), Fluid Tetrathionate Medium (FTM-Oxoid®), Trypticase Soy Broth (TSB-Pronadisa®), Sabouraud Dextrose Agar (SDA-Oxoid®), Pepton (Oxoid®), and Mueller Hinton Agar (MHA-Oxoid®). The tested bacteria used for sterility test were *Candida albicans* ATCC®10231 and *Bacillus subtilis* ATCC® 6633, meanwhile in the antibiotic potency test was *Escherichia coli* ATCC 25922, obtained from the Laboratory of Microbiology, Padjadjaran University, Indonesia. Indonesian Pharmacopoeia reference standard of chloramphenicol was used in a standard solution of 0.5% chloramphenicol in eyes drop preparation for the potency test.

### Eye drops formulation

Chloramphenicol eye drops with different sterilization methods (formula B = heating with bactericidal and formula C = Sterile membrane filter bacteria) and each formula made in three pH variations using borate buffer (pH = 6.8; pH = 7.0 and pH = 7.4). The details formula was performed in table 1. The mixing ingredients of 0.5% chloramphenicol eye drops were conducted in the Laminar Air Flow (LAF) room. After all the raw materials have been dissolved and mixed in a 150 ml beaker glass that has been calibrated for 100 ml, the initial pH check of the preparation was then carried out and then filtered with filter paper. The filtered solution was taken 5 ml of each using a syringe and then sterilized with sterile membrane filter bacteria and heating at a temperature of 98-100 °C for 30 min. After that, the sterilized eye drops were stored at room temperature for 28 d.

Table 1: 0.5% Chloramphenicol eye drop formula

Composition	Formula (%)					
	B1	B2	B3	C1	C2	C3
Chloramphenicol	0.50	0.50	0.50	0.50	0.50	0.50
1.9% Boric acid	0.1805	0.171	0.149	0.1805	0.171	0.149
2.65% Sodium tetraborate	0.01325	0.0265	0.0556	0.01325	0.0265	0.0556
Thimerosal	0.01	0.01	0.01	0.01	0.01	0.01
redistillata water	Ad 100 ml	Ad 100 ml	Ad 100 ml	Ad 100 ml	Ad 100 ml	Ad 100 ml
pH	6.8	7.0	7.4	6.8	7.0	7.4

Notes: B= sterilized using heating with a bactericide; C= sterilized using membrane filter; 1,2 and3= different pH

### Medium preparation

Preparation of all agar media used in this study was done in the same preparation procedure of weighing an amount of media according to the appendix which listed in the medium bottle. The weighed media, then dissolved in 500 ml aquadest in 1000 beaker glass, heated and stirred until it dissolved. The media solution was then sterilized using the autoclave [24].

### Microbial preparation for fertility, effectiveness and sterility test

A loopful of each microbial colony was suspended in TSB (for *B. subtilis*) and SDB (For *C. albicans*), then incubated at 37 °C for 18 h (for *B. subtilis*) and 20-25 °C for 3 d (for *C. albicans*). The final microbial inoculum concentration was adjusted to 0.5 Mc Farland ( $1.5 \times 10^8$  cfu/ml) then diluted by each broth media to achieve  $1 \times 10^7$  cfu/ml. 0.5 McFarland solution was made by mixing a total of 0.05 ml of 1% BaCl<sub>2</sub> solution and 9.95 ml of 1% H<sub>2</sub>SO<sub>4</sub> solution, then shaken to homogenize the solution. The solution turbidity was measured spectroscopically at a wavelength of 530 nm by using distilled water as a blank.

### Media fertility test

The fertility test is purposed to ensure that the used media free from inhibitor substances that may inhibit the microorganism growth. *B. subtilis* was used as a bacterial indicator and *C. albicans* as fungal indicator. The *B. subtilis* inoculating media was incubated at 30-35 °C for 7 d, and the fungal medium was incubated at 20-25 °C for 3 d. This fertility test was performed after the standard dilution of microbial colonies for fertility testing. A volume of 1 ml cell suspension was poured into a sterile petri disk, then a total of 5 ml

tested medium was added. The media was allowed to solidify, then incubated at each incubation condition, as previously mentioned [26].

### Media sterility test

Sterile Tioglycolate and Soybean Casein media were incubated at 30-35 °C for Tioglycolate and 20-25 °C for Soybean Casein for no less than 7d. The growth of bacteria or fungi was characterized by the presence of turbidity in the medium.

### Effectiveness test

A total of 5 drops of eye drops preparation was put into two test tubes containing sterile tioglycolate media which had been inoculated with *B. subtilis*, then incubated at 30-35 °C for no less than 7 d. In two tubes containing Soybean Casein medium, *C. albicans* was inoculated and then added 5 drops of eyes drops preparations into the tubes and incubated at a temperature of 20-25 °C for no less than 7 d. The turbidity of the tested media was observed [26].

### Sterility test of eye drops

Sterility testing of each eye drop formula was conducted by direct inoculation method and carried out in the LAF room. A volume of 2 ml test preparation was dropped into each of 3 test tubes containing Tioglycolic media, then incubated at a temperature of 30-35 °C for no less than 14 d and the turbidity observed every day. The same procedure was also carried out in soybean casein media, but this medium was incubated at a temperature of 20-25 °C. In addition, positive, negative and sterility control of the media were made, which were incubated with the test media.

### Evaluation of eye drops

All eye drops formulas were evaluated for the clarity, pH values and antibiotic potency test, with an observation time on days of 1,3,7,14,21 and 28 during storage.

#### Clarity observation

The clarity and the color change of all eye drops were observed visually during storage at room temperature.

#### pH

pH of the prepared eye drops solution was measured by a pH meter.

#### Preparation of microbial suspension for potency test

One Ose of *E. coli* colony was taken and suspended in sterile MHB then incubated at 37 °C for 18 h. The final cell suspension concentration was adjusted to 0.5 Mc Farland ( $1.5 \times 10^8$  cfu/ml). 0.5 McFarland solution was made by mixing a total of 0.05 ml of 1% BaCl<sub>2</sub> solution and 9.95 ml of 1% H<sub>2</sub>SO<sub>4</sub> solution, then shaken to homogenize the solution. The solution turbidity was measured spectroscopically at a wavelength of 530 nm by using distilled water as a blank.

#### Preparation of standard solution

The potency test of chloramphenicol reference standard was done by agar diffusion method using 3+3 pattern. Reference standard of Chloramphenicol was accurately weighed of 25.0 mg and dissolved in ethanol and aquadest till the volume was 25 ml to achieve 1000 mg/ml of chloramphenicol. The solution, then diluted to obtain 3

standard solution with concentration of S1(25 µg/ml), S2 (50µg/ml) and S3 (100 µg/ml).

#### Preparation of sample solution

Each formula of eye drops with different sterilization method was evaluated to determine the percentage of potency value during the storage time at room temperature. From each formula, a 20 ml of eyes drop was pipetted and diluted with aquadest into 100 ml volumetric flask, to obtain a concentration of 1000 mg/ml. The solution, then diluted to obtain 3 sample solution with concentration of U1(25 µg/ml), U2 (50µg/ml) and U3 (100 µg/ml).

#### Potency test

The potency test of chloramphenicol was conducted by the agar diffusion method using the perforator technique with a 3+3 dosage arrangement. A total of 20 µl *E. coli* suspension was suspended in MHA medium with a temperature of 45 °C, then the medium was homogenized and let the medium solidify at room temperature. The solid media were then perforated using perforator, thus 6 holes were present in the agar plate. Those holes were provided for sample and standard solution of chloramphenicol, each of 3 holes. Each hole was transferred with 50 µl of sample and standard solution of chloramphenicol and incubated for 18 h at 37 °C. The diameters of the inhibition zone were measured using a caliper.

#### Statistical analysis

The effect of different sterilization method was analyzed to determine its effect to the potency value in different pH solution using 2x3x5 factorial experimental design. The calculation can be seen in table 2.

Table 2: Statistical analysis calculation

Source of varians	dk	jk	Rjk	F cal	F table
mean	1	Ry	Ry		$F_{\alpha}(dk,60)$
M	m-1	My	My/m-1=M	M/MP	$F_{0,05}(dk,60)$
P	p-1	Py	Py/p-1=P	P/E	
H	h-1	Hy	Hy/h-1=H	H/PH	
MP	(m-1)(p-1)	MPy	Mpy/(m-1)(p-1)=MP	MP/E	
MH	(m-1)(h-1)	MHy	Mhy/(m-1)(h-1)=MH	MH/MPH	
PH	(p-1)(h-1)	PHy	Phy/(p-1)(h-1)=PH	PH/E	
MPH	(m-1)(p-1)(h-1)	MPHy	MPHy/(m-1)(p-1)(h-1)=MPH	MPH/E	
mistake		Ey	Ey/mph(n-1)=E		
sum	mphn	$\Sigma y^2$			

**Notes:** M= The effect of the sterilization method used in eye drops; P= Effect of pH variation; H= Effect of observation day; MP= Interaction effects between sterilization methods and pH variations; MH= Interaction effects between sterilization methods and observation days; PH= Interaction effects between variations in pH and observation days; MPH = Interaction effects between sterilization methods, variations in pH and observation days.

## RESULTS AND DISCUSSION

### Results of sterility, fertility and effectiveness test

These tests were a series of steps with different purposes to evaluate the growth media to be used before the method of eyes drop sterilization was fully validated. The fertility test is important to ensure the ability of the media in supporting microbial growth [1]. The accurate and reproducible sterility

test results will obtain by the using of growth media with high quality [27]. The sterility test is purposed to exhibit that the eye drop product is sterile. Meanwhile, the effectiveness test was aimed to demonstrate that the active ingredients in an eye drops did not provide antimicrobial activity against contaminants, so it can be ascertained that the sterility of the media was the result of the mechanism of the used sterilization methods.

The results of these tests were shown in table 3.

Table 3: The results of media sterility, fertility and effectiveness test

Day of observation	Sterility test		Fertility test		Effectiveness test	
	B	C	B	C	B	C
1	-	-	+	+	+	+
2	-	-	+	+	+	+
3	-	-	+	+	+	+
4	-	-	+	+	+	+
5	-	-	+	+	+	+
6	-	-	+	+	+	+
7	-	-	+	+	+	+

Notes: B= *B. subtilis*; C= *C. albicans*; (+) = turbid; (-) = clear

Based on the results of the fertility test, all media to be used for the sterility test of 0.5% chloramphenicol eye drop were defined as high-quality media, because it can support the growth of the tested microbial colonies. From the media sterility test, no microbial colony was a presence in all the tested media. This implied that the tested media were sterile. The sterility of the media was also supported by the result of effectiveness test that resulted in the growth of *B. subtilis* and *C. albicans*, even they were inoculated in the media containing eye drops with chloramphenicol as its active agent. Thus, it could be concluded that all the media were appropriate for the sterility test of eye drops.

#### Sterility test results of eye drops

All the eyes drops formulations were found to be sterile in both sterilization methods. The results were displayed in table 4. The sterilization method of heating was done by adding bactericidal substances to the preparation with a certain concentration and heated at a temperature of 98-100 °C for 30 min. Thus, this method can be used as a substitute for the sterilization method using an autoclave that requires a higher heating temperature, which is 121 °C for 15 min. For the preparation of the eye drop solution, bactericides which may be used are benzalkonium chloride 0.1%, chlorhexidine, or thimerosal are used as substitutes for phenyl mercury. In selecting bactericidal substances, toxicity and

compatibility with substances in the preparation must be considered and no interaction with the container [28]. In this study, thimerosal was served as the bactericide to against the contaminants before and after the sterilization process, especially the probable contamination that will occur during the product storage and use. The eye drop sterility was required to be maintained during the period of use [29]. This bactericide is an important substance to be formulated in the eye drops, because the contamination may lead to product degradation thus allowing pathogens to grow and cause eye infections [30]. Therefore, protection of the product, an especially sterile product like an eye drops is essential to improve the product against the opportunistic contamination effects by using appropriate preservatives. Thimerosal is one of common preservative, use in eye drops preparation. Meanwhile, the sterilization method using bacterial filter membrane is used to sterilize a solution that is unstable heat. The benefits of this sterilization method are filtering the solution in small amounts effectively, the used equipment is relatively cheap and can remove particles, living and dead microbes from solution. The disadvantages of this method include certain filters that allow the absorption of several active compounds and give basicity to the solution during the filtration process and the possibility of damage to the filter form which causes the filtering results not to be sterile. In addition, filtering with large volumes will require a longer time than using the other sterilization method [24].

Table 4: Sterility test results of eye drops

Day of observation	CM		NC		PC		B <sub>1</sub>		B <sub>2</sub>		B <sub>3</sub>		C <sub>1</sub>		C <sub>2</sub>		C <sub>3</sub>		
	Bs	F	Bs	F	Bs	F	Bs	F	Bs	F	Bs	F	Bs	F	Bs	F	Bs	F	
1	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-

Notes: CM= control media; NC= negative control; PC= positive control; B= sterilized using heating with a bactericide; C= sterilized using membrane filter; Bs=bacteria; F= fungi; 1,2 and3= different pH; (+) = turbid; (-) = clear

#### Clarity

The appearance of the eyes drops formulas of the both sterilization methods were analyzed. There was no change in color during the storage time. The results were shown in table 5. This implied that chloramphenicol did not undergo photodegradation reactions. The indication of the occurrence of this photodegradation reaction can be seen by the change in color of the solution from colorless to yellow until it formed a yellow-orange precipitate. But preparations

B<sub>1</sub> and C<sub>1</sub> showed the presence of precipitates after 28<sup>th</sup> d of storage time. The precipitations occurred were estimated as hydrolytic degradation compounds from chloramphenicol due to the effect of pH and heating. Another study reported that a lower pH increasing degradation of chloramphenicol and the highest degradation efficiency was achieved at pH 2.96 [31]. The precipitate formed on B<sub>1</sub> preparations was greater than C<sub>1</sub> preparation. This showed in the same pH, the heating process in the sterilization method can accelerate the hydrolytic degradation reaction of chloramphenicol.

Table 5: Clarity observation results

Day of observation	Color and sediment formation of the eyes drop					
	B <sub>1</sub>	B <sub>2</sub>	B <sub>3</sub>	C <sub>1</sub>	C <sub>2</sub>	C <sub>3</sub>
1	-(cl)	-(cl)	-(cl)	-(cl)	-(cl)	-(cl)
3	-(cl)	-(cl)	-(cl)	-(cl)	-(cl)	-(cl)
7	-(cl)	-(cl)	-(cl)	-(cl)	-(cl)	-(cl)
14	-(cl)	-(cl)	-(cl)	-(cl)	-(cl)	-(cl)
28	++(cl)	-(cl)	-(cl)	+(cl)	-(cl)	-(cl)

Notes: B= sterilized using heating with a bactericide; C= sterilized using membrane filter; 1,2 and3= different pH, cl=colorless; (-) = precipitate absence; (+) = precipitate presence.

#### pH stability

The pH of the eyes drops formulation is very important mainly to prevent eye discomfort or pain after the eye drops used. In eyes

drops formulation, the pH value must be considered to rely on the acceptable range of eye pH. The optimum pH for eye drops is 7.4 that equals tear fluid pH range, but other studies mentioned that the pH range of 4–8 was the normal pH of the eyes and the pH of an eye

between pH 5.5-11.4 can still be accepted [32, 33]. The pH incompatibility in eyes may cause irritation and the drug bioavailability decreased because the tearing was increased [32]. Thus, the pH of eye drops must be adjusted to comply with the range of suitable pH for the eyes [34]. In addition, the different pH of eye drops effected the stability of the active substance. A lower pH of

preparations containing chloramphenicol made the increasing of chloramphenicol degradation [31]. Therefore, pH of eye drops containing chloramphenicol was formulated in variations of pH in the range of 6.8-7.4 to determine the compatible pH value of the eyes drop. The pH stability of each formula with different sterilization method was shown in table 6 and fig. 1.

Table 6: pH stability

Day of observation	pH					
	B <sub>1</sub>	B <sub>2</sub>	B <sub>3</sub>	C <sub>1</sub>	C <sub>2</sub>	C <sub>3</sub>
0	6.80	7.08	7.41	6.80	7.08	7.41
1	6.78	7.07	7.39	6.80	7.08	7.40
3	6.77	7.04	7.37	6.79	7.06	7.37
7	6.74	7.04	7.36	6.77	7.05	7.35
14	6.72	7.03	7.33	6.74	7.04	7.34
28	6.70	7.01	7.32	6.71	7.03	7.33

Notes: B= sterilized using heating with a bactericide; C= sterilized using membrane filter; 1,2 and3= different pH

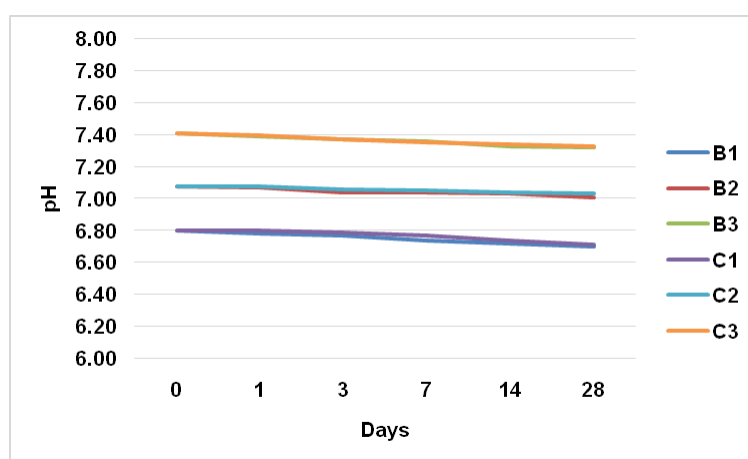


Fig. 1: pH stability results

Based on the data in table 6 and fig. 1, it can be seen that all preparations have decreased in pH value. But, the pH decreasing of the eye drops which sterilized using heating with bactericide were greater than using a sterile bacterial filter membrane sterilization method. The longer the storage time, the price of the pH of the dosage will decrease even though the preparation has been added to borate as a pH stabilizer, but in reality the values of pH decreased after storage for 28 d. These could be caused by the hydrolysis of chloramphenicol during storage as reported in another study that the stability of chloramphenicol was independent of pH between 4 to 6.2, and it was susceptible to hydrolysis in aqueous media [35]. From the data above, it can be seen that C2 preparations at pH 7.0 and sterilized using the bacterial filter sterilization method are more stable because they had the smallest pH change of 0.05. However, all pH of eyes drops formulas from both sterilization methods still complies the acceptable pH of the eye drops.

#### Potency test results

Stability of drug is a great importance factor that must be considered for its efficacy. The antibiotic effectiveness is described in the terms of potency value, thus, the accurate measurement of antibiotic potency is a critical step to ensure the antibiotics quality [36]. A mild change in the concentration of active components in preparations containing antibiotic may have impact in actual efficacy and contributed in the resistance cases [37, 38]. Therefore, quantification of chloramphenicol in these eye drop preparations was very important. Based on the data in table 7 and fig. 2, it can be concluded that the C2 preparations, which were formulated at pH 7.0 and sterilized using the bacterial filter membrane sterilization method, were more stable because the potential reduction percentage was smaller than the other formulas.

Table 7: Potency test results

Day of observation	Potency (%)					
	B <sub>1</sub>	B <sub>2</sub>	B <sub>3</sub>	C <sub>1</sub>	C <sub>2</sub>	C <sub>3</sub>
1	97.72	98.86	98.17	97.50	98.63	98.40
3	96.38	97.72	97.50	96.38	98.62	98.17
7	97.72	97.50	96.82	93.97	98.40	97.28
14	90.57	96.82	94.62	90.57	97.72	95.71
28	88.31	96.61	94.40	89.33	97.50	95.94

Notes: B= sterilized using heating with a bactericide; C= sterilized using membrane filter; 1,2 and3= different pH

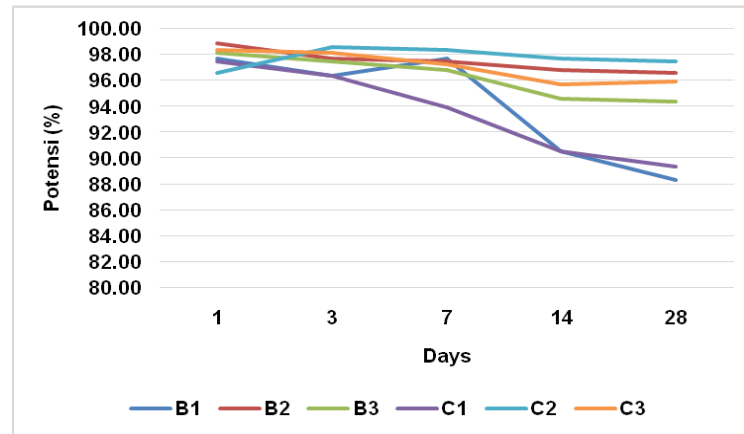


Fig. 2: Potency stability results

### Statistical analysis result

This statistical analysis was aimed to determine the effect of sterilization methods and variations in the pH of the eyes drops preparation on the stability of chloramphenicol potency. Statistical testing using 2x3x5 factorial experimental design with the assumption that the model used is a mixed model with two factor levels of the sterilization method (sterilization method B which is heating with

bactericidal addition at certain temperature and C method sterilization using bacterial filter membrane), three levels of variation pH (pH 6.80; pH 7.0 and pH 7.4) and five observational day factor levels (1, 3, 7, 14 and 28 d). The results of statistical testing using the 2x3x5 factorial experimental design can be seen in table 8. From these results, it can be concluded that there was a difference in the chloramphenicol potency due to variations in pH during storage time, while for other factors did not indicate an effect on potency difference.

Table 8: Statistical analysis result

Source of varians	dk	JK	RJK	F cal	F table
mean	1	833332.600	833332.600		$F_{\alpha}(dk,60)$
M	1	0.013	0.013	0.001	4.00
P	2	220.214	110.107	11.624	3.15
H	4	248.711	62.178	4.245	2.53
MP	2	20.432	10.216	1.079	3.15
MH	4	6.136	1.534	0.416	2.53
PH	8	117.175	14.647	1.546	2.10
MPH	8	29.515	3.689	0.389	2.10
mistakes	60	568.340	9.472		
Sum	90	834543.100			

### CONCLUSION

In conclusion, variations in the sterilization method did not give a significant difference in the efficacy of chloramphenicol. In contrast, the variation in pH gives a significant difference in the chloramphenicol potency during storage. Based on the results, the most stable formula for chloramphenicol eye drops was the preparation at pH 7.0 and sterilized using a bacterial filter membrane.

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### AUTHORS CONTRIBUTIONS

All the author have contributed equally

### CONFLICT OF INTERESTS

Declared none

### REFERENCES

- Kusuma SAF, Hendriani R, Genta A. Antimicrobial spectrum of red piper betel leaf extract (*Piper crocatum* Ruiz and Pav) as natural antiseptics against airborne pathogens. J Pharm Sci Res 2017;9:583-7.
- Jithan AV, Krishna MC, Vimaladevi M. Development and evaluation of a chloramphenicol hypertonic ophthalmic solution. Indian J Pharm Sci 2008;70:66-70.
- Templeton WC, Eiferman RA, Snyder JW, Melo JC, Raff MJ. Serratia keratitis transmitted by contaminated eyedroppers. Am J Ophthalmol 1982;93:723-6.
- Schein OD, Wasson PJ, Boruchoff SA, Kenyon KR. Microbial keratitis associated with contaminated ocular medications. Am J Ophthalmol 1988;105:361-5.
- Saxena P, Kushwaha SKS. Temperature sensitive ophthalmic hidrogels of levofloxacin hemihydrate with enhanced solubility and prolonged retention time. Int J Pharm Pharm Sci 2013;5:877-83.
- De Souza MVN. Promising drugs against tuberculosis. Recent Pat Antiinfect Drug Discovery 2006;1:33-44.
- Schwarz S, Kehrenberg C, Doublet B, Cloeckert A. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. FEMS Microbiol Rev 2004;28:519-42.
- Lam RF, Lai JS, Ng JS, Rao SK, Law RW, Lam DS. Topical chloramphenicol for eye infections. Hong Kong Med J 2002;8:44-7.
- Zuorro A, Fidaleo M, Fidaleo M, Lavecchia R. Degradation and antibiotic activity reduction of chloramphenicol in aqueous solution by UV/H2O2 process. J Environ Manag 2014;133:302-8.
- Buckley SA. Survey of patients taking topical medication at their first presentation to eye casualty. Br Med J 1990;300:1497-8.
- Kirkness CM, Seal DV, Hay J. Topical chloramphenicol: use or abuse? Eye 1995;9:7-8.
- Hall AV, Das SS, Tabaqchali S. Is it time to stop using chloramphenicol on the eye? Risk is low in short courses [Letter]. Br Med J 1995;311:450-1.

13. Sinclair NM, Leigh DA. A comparison of fusidic acid viscous eye drops and chloramphenicol eye ointment in acute conjunctivitis. *Ther Res* 1988;44:468-74.
14. Jokl DH, Wormser GP, Nichols NS, Montecalvo MA, Karmen CL. Bacterial contamination of ophthalmic solutions used in an extended care facility. *Br J Ophthalmol* 2007;91:1308-10.
15. Perry HD, Donnenfeld ED. Issues in the use of preservative-free topicals. *Manag Care* 2003;12:39-41.
16. Prakash K, Sireesha KR, Kumari AS. Stability indicating HPLC method for simultaneous determination of dexamethasone sodium phosphate and chloramphenicol in bulk and formulations. *Int J Pharm Pharm Sci* 2012;4:505-10.
17. Connors, Kenneth A. Stabilitas kimiawi sediaan farmasi. Translated by: Drs. Didik Gunawan. Vol II. IKIP Semarang Press: Semarang Indonesia; 1992.
18. Martindale. The complete drug reference. 32<sup>th</sup> ed. The Pharmaceutical Press: London; 1999.
19. Kan B, Goldblith SA, Proctor BE. Complementary effects of heat and ionizing radiation. *Food Res* 1957;22:509-18.
20. Kempe LL. Combined effects of heat and radiation in food sterilization. *Appl Microbiol* 1955;3:346-52.
21. Licciardello JJ, Nickerson JTR. Effect of radiation environment on the thermal resistance of irradiated spores of *Bacillus subtilis*. *Appl Microbiol* 1963;11:216-9.
22. Morgan BH, Reed JM. Resistance of bacterial spores to gamma irradiation. *Food Res* 1954;19:357-66.
23. Pershina ZQ, Vasil'eva IG. The combined effect of irradiation and antibacterial substances on bacteria. *Zh. Mikrobiol Epidemiol Immunobiol* 1961;32:132-7.
24. Lachman L, Herbert AL, Joseph IK. Teori dan praktek farmasi industri. 3<sup>th</sup> ed. UI Press, Jakarta Indonesia; 1994.
25. Voigt R. Buku pelajaran teknologi farmasi. 5<sup>th</sup> ed. Gadjah Mada University Press: Yogyakarta Indonesia; 1995.
26. Kusuma SAF, Yus Hargono, Hendro Winarno. Betalactamase enzyme role in minimizing the false-positive result of cefotaxime injection end-product sterility. *J Pharm Sci Res* 2018;10:1036-40.
27. Sandle T. Selection of microbiological culture media and testing regimes. *Microbiology and Sterility Assurance in Pharmaceuticals and Medical Devices*. Business Horizons, New Delhi; 2010.
28. Aulton ME. *Pharmaceutics: the science of dosage form design*. Churchill Livingstone, New York; 1988.
29. British Pharmacopoeia. Efficacy of antimicrobial preservation. Vol. IV. Appendix XVI C A367-A369. The Stationery Office, London; 2007.
30. Fegghi M, Mahmoudabadis AZ, Mehdinejad M. Evaluation of fungal and bacterial contaminations of patient-used ocular drops. *Med Mycol* 2008;46:17-21.
31. Nie M, Yang Y, Zhang Z, Yan C, Wang X, Li H, *et al.* Degradation of chloramphenicol by thermally activated persulfate in aqueous solution. *Chem Eng J* 2014;246:373-82.
32. Jitendra PK, Banik SA, Dixit S. A new trend: ocular drug delivery system. *Int J Pharm Sci* 2011;2:1-25.
33. Lukas S. *Formulasi steril*. Penerbit ANDI, Yogyakarta; 2006.
34. Abdassah M, Kusuma SAF. Comparison of thimerosal effectiveness in the formulation of eye drops containing neomycin sulfate and chloramphenicol. *Int J Appl Pharm* 2019;11:130-5.
35. Abachi FT, Bander F, Al-Deeb NN, Nasoh N, Gafar ZM. Formulation and stability studies of chloramphenicol as ophthalmic eye drop. *Tikrit J Pharm Sci* 2010;6:70-7.
36. Branson E. Clinical relevance of minimum inhibitory concentration. *Aquaculture* 2001;11:289-96.
37. Dafale NA, Semwal UP, Agarwal PK. Development and validation of microbial bioassay for quantification of levofloxacin in pharmaceutical preparations. *J Pharm Anal* 2015;5:18-26.
38. Hewitt W. *Microbiological assay: an introduction to qualitative principles and evaluation*. 1st ed. Academic Press: New York; 1977.