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Development of thermosensitive and mucoadhesive gels of cabotegravir for enhanced permeation and retention profiles in vaginal tissue: A proof of concept study

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ABSTRACT

As an effective anti-HIV drug, cabotegravir (CAB) is currently administered *via* oral and injection routes, leading to several drawbacks, such as poor oral bioavailability and problems in the injection application process, as well as low drug concentration in vaginal tissue of woman patients. To overcome these issues, for the first time, we formulated CAB into three types of vaginal gels, considering the benefits of vaginal tissue as a delivery route. Thermosensitive gel, mucoadhesive gel, and the combination of these gels were developed as suitable carriers for CAB. Pluronics®, hydroxy propyl methyl cellulose (HPMC), Carbomer and poly(ethylene glycol) (PEG) 400 were used as thermosensitive, mucoadhesive and permeation enhancer agents, respectively. The gels were evaluated for their thermosensitive and mucoadhesive properties, as well as their pH values, viscosities, gel erosions, drug content recovery, *in vitro* drug release, *ex vivo* permeation, *ex vivo* retention, hemolytic activities, *Lactobacillus* inhibition activities and *in vivo* irritation properties. The results showed that all formulations showed desired characteristics for vaginal administration. Importantly, all formulations did not show hemolytic activities and inhibitions to *Lactobacillus* as normal bacteria in the vagina. Furthermore, no irritation in the vaginal tissues of the rats was observed by histopathological studies. Considering the thermosensitive and mucoadhesive properties, the combination of Pluronic® F127, Pluronic F68, and HPMC in thermosensitive-mucoadhesive vaginal gels was selected as the optimum dosage form for CAB as this formulation was able to provide ease administration due to its liquid form at room temperature. The use of PEG in this formulation was able to increase the penetrability of CAB through vaginal tissue with 0.61 ± 0.05 mg and 17.28 ± 0.95 mg of CAB being able to penetrate and localize in the vagina, respectively. Essentially, the optimum formulation was retained in the vaginal mucosa for>8 h. To conclude, further extensive *in vivo* studies should now be conducted to evaluate the efficacy of this approach.

1. Introduction

Human immunodeficiency virus (HIV) is a virus that attacks the immune system of humans by infecting vital cells such as T helper cells (CD4 +) and macrophages (Février et al., 2011). Since its first discovery in 1981, HIV is still a global health problem causing a high mortality rate (Schwetz and Fauci, 2018). Based on data from the United Nations on HIV/AIDS (UNAIDS) in 2020, it is estimated that around 81% or 38 million confirmed cases of HIV have occurred worldwide (UNAIDS, 2020). The main route for HIV transmission is through sexual intercourse. Females have a greater risk of being infected through this route because in the female genital tract mucosa, there are CD4 + T cells and CCR5, acting as HIV receptors and coreceptors (Iyer et al., 2017).

The current available HIV treatment is the use of the antiretroviral drug. One of the antiretroviral drugs used is cabotegravir (CAB) (Kovač and Časar, 2020). CAB has a long half-life and good activity against HIV

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at low concentrations. In addition, it has a low risk of drug interactions (Whitfield and Halsema, 2016). CAB is available in tablet and injection forms. The tablet form is intended for daily medication/intake. However, many HIV patients develop oral candidiasis, which causes swallowing difficulty. This causes the oral route of drug administration to be quite difficult (Williams and Lewis, 2011). CAB is also included in the biopharmaceutics classification system (BCS) II, which has low solubility and high permeability, leading to low bioavailability of CAB (Patel et al., 2019). Furthermore, CAB is also available in long-acting cabotegravir injection, which has a long half-life, thereby reducing the frequency of its administration (Trezza et al., 2015). However, the injection needs to be administered in a health facility by trained healthcare professionals. Additionally, injection often results in hazardous waste and is painful for some patients. This subsequently can decrease patient compliance, leading to discontinuing treatment (Mc Crudden et al., 2019).

Vaginal drug delivery systems (VDDS) is an alternative route for drug delivery, offering various advantages, such as a large surface area, rich blood supply, high permeability, increase of drug bioavailability, avoidance of first-pass metabolism, minimizing side effects, and easy to use when compared to injections (Tuğcu-Demiröz et al., 2013). In addition, the drug administration *via* the vaginal route does not cause pain, tissue damage, and the possibility of infection, which are commonly associated with the injection. Essentially, vaginal administration can increase drug bioavailability in the vaginal area compared to oral administration. With respect to vaginal delivery of anti-HIV drugs, a previous study has shown the effectiveness of vaginal delivery of anti-HIV-1 microbicide, the miniCD4 M48U1, from thermosensitive and mucoadhesive Pluronic® hydrogels (Bouchemal et al., 2013). Considering this benefit, vaginal delivery could be essential to provide a higher concentration of CAB in the vagina. Research conducted by Radzio-basu et al., 2019 showed that CAB concentration in the vaginal fluid after the administration of long acting injection were below four times the protein-adjusted inhibitory concentration (4xPA-IC90) value (0.664 μg/mL) during the entire treatment period. 4xPA-IC90 is a well-established threshold of CAB concentration for rectal and vaginal protection against repeated exposures to HIV (Dobard et al., 2020; Id et al., 2018). By delivering drugs directly to the vaginal area, it was anticipated that the systemic side effects in oral and parenteral administration could be reduced (Ferguson and Rohan, 2011), while improving the drug localized in the vaginal tissue.

Thermosensitive in situ gel is one of the vaginal delivery systems developed to both increase the vaginal localization and the systemic bioavailability of drugs. Thermosensitive gel is an in situ gel system that is sensitive to temperature changes. *In situ* gel systems, especially thermosensitive gels, appear as a solution at room temperature (25 °C) and immediately turn into a gel when they reach body temperature (37 °C) (Chang et al., 2002; Sajid et al., 2014). Several studies have shown the advantages of this system in vaginal delivery (Taurin et al., 2018; Yang et al., 2017). The gel's low viscosity at room temperature allows the easy vaginal administration as well as optimal spread within the mucosa (Vigani et al., 2019). In the formulation, Pluronic® can be used as a thermosensitive polymer. Pluronic® provides the advantages of its sensitivity to temperature changes and its ability to increase the drug's retention time (Mohanty et al., 2018). In addition, Pluronic has low toxicity, good biocompatibility and good miscibility with hydrophobic drugs (Soliman and Fetih, 2016), like CAB. In addition, Pluronic® has also been shown to be non-irritant and compatible with various cell types and biological fluids (Sajid et al., 2014). However, vagina has a self-cleaning mechanism causing poor retention time of drugs in the vaginal mucosa. This leads to the need for repeated dose in therapy to ensure the desired concentration of drug (Regina et al., 2012). This problem can be solved by the formulation of mucoadhesive delivery system. This system has advantages when compared to conventional dosage forms. It is readily localized, improving the bioavailability of drugs. Interaction between the mucoadhesive polymer used in the formulation and the vaginal mucosa could potentially increase the retention time of drugs (Choi et al., 2014; Tuğcu-Demiröz et al., 2013). Carbopol and hydroxy methyl propyl cellulose (HPMC) are the most common gelling agents possessing excellent mucoadhesive properties (Russo and Villa, 2019).

The combination of thermosensitive and mucoadhesive approaches has also been used in the vaginal delivery system. Several drugs had been applied into this system, as itraconazole (Permana et al., 2021a), clotrimazole (Rençber et al., 2017), sildenafil citrate (Soliman and Fetih, 2016), and paclitaxel (Choi et al., 2014). This type of dosage form offers ease of administration, controlled release of drugs and provide prolonged retention time. In the vaginal delivery system, especially in HIV therapy, the drugs are intended to be localized in the tissue and delivered in the systemic circulation. Accordingly, it is crucial to consider the use of permeation enhancers. Polyethylene glycol (PEG) is a chemical enhancer that is commonly used to enhance the permeation of several drugs (Shah et al., 2013). The addition of this compound in the vaginal was considered to be an excellent favor in this case.

To the best of our knowledge, there have been no studies reported on vaginal delivery of CAB. Hence, in this paper, for the first time, we formulated CAB in form of vaginal gels. This study aimed to compare three types of cabotegravir vaginal gels, namely vaginal thermosensitive gel, mucoadhesive gel, and combination of these gels. The thermosensitive gel was prepared utilizing Pluronic® F127- Pluronic® F68, the mucoadhesive gel was prepared using Carbopol 940, and the combination gel was prepared utilizing Pluronic® F127- Pluronic® F68 – HPMC. Specifically, the effect of PEG in the vaginal formulations was also investigated. These gels were evaluated for their physical characteristics, thermosensitive and mucoadhesive properties, *in vitro* and *ex vivo* delivery behaviors, and *in vivo* irritation studies.

2. Material and methods

2.1. Chemicals

Cabotegravir (CAB) was kindly gifted by ViiV Healthcare Ltd. (Research Triangle Park, NC, USA). Carbopol 940, DMDM Hydantoin, Hydroxypropyl methylcellulose (HPMC) (viscosity 2,600–5,600 cP, 2 % in H_2O), (Poly(ethylene glycol) (PEG) 400 were obtained from Sigma-Aldrich Pte Ltd. (Singapore, Singapore). Pluronic® F127 and F68 were kindly provided by BASF Indonesia, Jakarta. Other materials were analytical grade

2.2. Simulated vaginal fluid preparation

Simulated vaginal fluid (SVF) was prepared by weighing 5 g of glucose, 3.51 g of NaCl, 2 g of lactic acid, 1.4 g of KOH, 1 g of acetic acid, 0.4 g of urea, 0.222 g of $Ca(OH)_2$, and 0.016 g glycerin. The mixture was then dissolved using deionized water up to 1 L. The pH was evaluated and adjusted with 0.1 N HCl or 0.1 N NaOH to reach a final pH of 4.2 (Das Neves et al., 2012).

Table 1
Composition of thermosensitive gel formulations.

Compositions	%Composition (w/w)				
	F1	F2	F3	F4	F5
CAB	1	1	1	1	1
Pluronic F-127	16,5	16,5	16,5	16,5	16,5
Pluronic F-68	4,5	4,5	4,5	4,5	4,5
PEG 400	–	2,5	5	10	15
DMDM Hydantoin	0,1	0,1	0,1	0,1	0,1
Distilled water	ad 100	ad 100	ad 100	ad 100	ad 100

2.3. Preparation of thermosensitive gel

The thermosensitive gel of CAB was prepared using a modified cold method. The composition of the thermosensitive gels is depicted in Table 1. Briefly, Pluronic® F127 and Pluronic® F68 were slowly added into cold water (4 °C) with continuous stirring. This dispersion was stored in the refrigerator overnight until a clear solution was obtained. After that, CAB was mixed with PEG 400 in mortar and the polymeric solution was added. Finally, DMDM Hydantoin was added and homogenized (Ibrahim et al., 2012; Moreira et al., 2010).

2.4. Preparation of thermosensitive-mucoadhesive gel

Various thermosensitive-mucoadhesive gel formulations of CAB were prepared using Pluronic® F127, Pluronic® F68, and HPMC as polymers, as shown in Table 2**.** The gels were made using a modification of the cold method. Required amount of Pluronic® F127 and Pluronic® F68 were slowly added into cold water (4 °C) with continuous stirring. The gels were then stored in refrigerator until a clear solution was obtained. Furthermore, HPMC was added, and the formulation was kept refrigerated overnight. Then, CAB was mixed with PEG 400 in the mortar, and the polymeric solution was added along with DMDM Hydantoin. The mixture was finally stirred until homogenous (Güven et al., 2010; Permana et al., 2021a).

2.5. Preparation of mucoadhesive gel

Five mucoadhesive gel formulations of CAB were made using Carbopol 940 (0.75% w/w) as the gel base (Aiyalu et al., 2016). Details of composition of gel formulation are exhibited in Table 3**.** An accurately weighed amount of Carbopol 940 was hydrated in distilled water. It was kept for 24 h to form a homogeneous dispersion. Then, the obtained dispersion was neutralized with triethanolamine (TEA), and the mixture was homogenized at 1000 rpm for 15 min. After that, an appropriate amount of glycerin was added into the mixture. Then, CAB was mixed with PEG 400 and added into the gel base. Finally, DMDM Hydantoin was added and mixed at 1000 rpm until homogenous (Gabriela et al., 2016; Jelvehgari et al., 2006).

2.6. pH measurement

The assessment of gel pH was conducted using a digital pH meter (Horiba Scientific, Kyoto, Japan). The measurement was done by soaking the glass electrode entirely into the formulation at room temperature (Aiyalu et al., 2016). The measurement was performed in triplicate.

2.7. Viscosity study

The viscosity of the formulations was examined using a Brookfield viscometer with suitable spindle speed and size (Aiyalu et al., 2016). This evaluation was carried out at 25 °C for the mucoadhesive gels and at various temperatures (4 °C, 25 °C, and 37 °C) for the thermosensitive gels and thermosensitive-mucoadhesive gels (Aiyalu et al., 2016; Permana et al., 2021a). Specifically, the viscosity measurement was carried out using spindle 7, a sample volume of 50 mL, speed of 50 rpm, and time spent of 60 s at each measurement. The measurement was performed in triplicate.

Table 2
Composition of thermosensitive-mucoadhesive gel formulations.

Compositions	%Composition (w/w)				
	F1	F2	F3	F4	F5
CAB	1	1	1	1	1
Pluronic F-127	16	16	16	16	16
Pluronic F-68	6	6	6	6	6
HPMC	0,5	0,5	0,5	0,5	0,5
PEG 400	–	2,5	5	10	15
DMDM Hydantoin	0,1	0,1	0,1	0,1	0,1
distilled water	ad 100	ad 100	ad 100	ad 100	ad 100

Table 3
Composition of mucoadhesive gel formulations.

Compositions	%Composition (w/w)				
	F1	F2	F3	F4	F5
CAB	1	1	1	1	1
Carbopol 940	0,75	0,75	0,75	0,75	0,75
Triethanolamine	2	2	2	2	2
Glycerin	15	15	15	15	15
PEG 400	–	2,5	5	10	15
DMDM Hydantoin	0,1	0,1	0,1	0,1	0,1
Distilled water	ad 100	ad 100	ad 100	ad 100	ad 100

2.8. Rheological properties

The rheological properties of the gel were measured using a Brookfield viscometer (Permana et al., 2021a). In this study, the mucoadhesive gel was evaluated at 25 °C, while the thermosensitive gel and thermosensitive-mucoadhesive gel were examined at 37 °C. Then, the gel was rotated gradually at 5, 10, 20, 50, and 100 rpm with spindle 7. At each speed, the corresponding dial reading was recorded. The measurement was performed in triplicate.

2.9. Gelation temperature test

The measurement of gelation temperature was conducted using a test tube inverting method in 2 different conditions, namely with and without dilution with SVF. An amount of 2 mL of each gel formulation was put into a test tube and placed at 4 °C. Then, the test tube was soaked in water at 20 °C which the temperature was gradually increased by 1 °C until 65 °C. Each gel was then observed visually by inverting the test tube up to 90° for each temperature. The lowest temperature in which the liquid could not flow and turn into a gel was recorded as the gelation temperature. The same method was also applied to determine gelation temperature with dilution, where 0.25 mL of SVF was added prior to evaluation (Permana et al., 2021b). The measurement was performed in triplicate.

2.10. Drug content recovery

This test was conducted by weighing a total of 500 mg of gels and put in 50 mL volumetric flask. The volume was then adjusted using methanol. The absorbance was measured triplicate using UV–Vis Spectrophotometer (Dynamica, HALO XB-10). In this study, the calibration curve of CAB was prepared in methanol with the concentration range between 0.5 and 16 μg/mL. The detection was carried out at the maximum wavelength of CAB in methanol which was 276 nm.

2.11. Gel erosion study

Gel erosion study was carried out in triplicate. Each gel formulation was weighed as much as 5 g and added to a glass vial. Afterwards, 2 mL of SVF was added. After predetermined time (0.5 h, 1 h, 1.5 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, and 24 h), the SVF was removed. The remaining gel left in the glass vial was weighed, and subsequently, 2 mL of fresh SVF was added. Gel erosion rate was determined from the weight loss calculation (Giuliano et al., 2020). The measurement was performed in triplicate.

2.12. Evaluation of mucoadhesive properties

2.12.1. Porcine vaginal mucosa retrieval

The porcine vaginal mucosa was surgically removed, then rinsed slightly with water and cooled at −20 °C. The tissue was stored in a tightly closed container until further testing (Schwarz et al., 2013).

2.12.2. Mucoadhesive strength test

The measurement of mucoadhesive strength was carried out using a modified balance method. Porcine vaginal mucosa was attached to the upper and lower vials. After that, 1 g of each gel formulation was placed between vaginal mucosa (surface area of 4.5 cm^2), which hung on the left arm of the scales. On the right arm of the scale, weight was placed every 30 s on the pan to measure the amount of load required to release the gel from the vaginal mucosa. The addition of the weight was stopped when the surface of the two vials was separated. For thermosensitive and combination of thermosensitive-mucoadhesive gel, the experiment was carried out in triplicate at 37 °C (Galgatte et al., 2014; Manna et al., 2016). Mucoadhesive strength was calculated using equation (1),

$$\text{MucoadhesiveStrength}(N/m^2) = \frac{mg}{A} x0.1 \quad (1)$$

where m is the weight required to remove the gel from the vaginal mucosa (gram), g is gravity acceleration (980 m/s^2), and A is surface area of the exposed mucosa (cm^2).

2.12.3. Mucoadhesive time

The test of mucoadhesive time was carried out using the rotating cylinder method using USP dissolution test apparatus 2 (paddle apparatus). The medium temperature in the test was maintained at 37 °C, and the rotating speed was 100 rpm. In this study, the porcine vaginal mucosa was clamped and attached to the paddle in the dissolution apparatus. After that, 1 g of each gel formulation was applied to the entire vaginal mucosa and subsequently immersed in SVF medium in the dissolution apparatus. Mucoadhesive time was determined based on the time required by the gel formulation to be released from the vaginal mucosa (Sanz et al., 2017). The experiment was performed in triplicate.

2.13. Evaluation of in vitro drug release

The release study was carried out using a dialysis method with SVF containing 20% v/v methanol as the release medium. Our preliminary study showed that the addition of 20% v/v methanol in SVF could maintain sink condition during the experiment. Each gel formulation that was equivalent to 10 mg of CAB was inserted into the dialysis membrane (Spectra-Por®, 12,000–14,000 MWCO dialysis membrane) (Permana et al., 2020, Permana et al., 2019). After that, the dialysis membrane was placed in 100 mL of the release medium at 37 °C. Then, the experiment was carried out in an orbital shaker at 100 rpm. At each predetermined time, 1 mL of the sample was taken and then replaced by 1 mL of fresh release medium. The amount of drug released was calculated by analyzing the sample using UV–Vis spectrophotometry (Dynamica, HALO XB-10). In this study, the calibration curve of CAB was prepared in SVF containing 20% v/v methanol with the concentration range between 0.5 and 16 μg/mL. The detection was performed at the maximum wavelength of CAB in SVF containing 20% v/v methanol which was 278 nm. All measurement was performed in triplicate.

2.14. Ex vivo permeation test

The *ex vivo* permeation study was carried out using the vertical Franz cell. In this experiment, porcine vaginal mucosa was utilized to enable the drug permeation from donor compartments to receptor compartments. The surface area of the donor compartment was 4.9 cm^2. Briefly, the receptor compartment was filled with 24 mL of the medium (SVF with 20% v/v methanol) and then stirred at 100 rpm with the temperature kept at 37 ± 1 °C. Afterwards, the donor compartment was filled with each gel formulation that was equivalent to 10 mg of CAB. At each predetermined time, 1 mL of the sample was taken from receptor compartment and then replaced by 1 mL of fresh release medium to maintain the sink condition. All samples were analyzed using UV–Vis spectrophotometry (Dynamica, HALO XB-10) (Permana et al., 2021b). The study was performed in triplicate.

2.15. Ex vivo retention test

Retention test was performed by measuring the amount of CAB retained in the vaginal mucosa following the *ex vivo* permeation studies. At each predetermined time, vaginal mucosa was taken and rinsed with distilled water. CAB was extracted using methanol in bath sonicator for 1 h. After that, the mixture was centrifuged at 5000 rpm for 30 min. The supernatant then analyzed using UV–Vis spectrophotometry (Dynamica, HALO XB-10) (Permana et al., 2021b). In this study, the calibration curve of CAB was prepared in vaginal tissue with the concentration range between 1 and 32 μg/mL. The detection was performed at the wavelength with no interference of vaginal tissue which was 305 nm. The study was performed in triplicate.

2.16. In vitro hemolytic activity

In vitro hemolytic activity study was carried out to determine the safety and biocompatibility of CAB and CAB vaginal gels. A fresh blood sample was obtained from Wistar rats. Red blood cells (RBC) was separated from the plasma through centrifugation at 2000 rpm for 20 min. The RBC was washed three times using PBS followed by mixing using a vortex mixer and centrifugation at 2000 rpm for 10 min. The washed RBC was resuspended in PBS to obtain concentration of 10% v/v. An aliquot of 100 μL of tested samples was added to 900 μL of RBC and incubated at 37 °C for 1 h. Afterwards, the samples were centrifuged at 7000 rpm for 10 min. The absorbance of the supernatant was measured using UV–Vis Spectrophotometer (Dynamica, HALO XB-10) at 540 nm. Blood sample were added with PBS and water as positive and negative control, respectively (Mir et al., 2020). The experiment was performed in triplicate. Calculation of the hemolysis percentage was done using following equation:

$$\text{Hemolysis}(\%) = \frac{(OD\ \ test\ \ sample) - (OD\ \ negative\ \ control)x100}{(OD\ \ positive\ \ control) - (OD\ \ negative\ \ control)} \quad (7)$$

2.17. In-vitro Lactobacillus inhibition evaluation

The *in vitro* antimicrobial activity test was performed to evaluate any possible microbiota growth inhibitory effect of the formulated vaginal gels to Lactobacilli, the dominant species of bacterial microbiota in a healthy human vagina. Disk diffusion method was utilized for this evaluation. *Lactobacillus acidophilus* (ATCC 53544) cultures were procured from *in vitro* bacterial culture, prepared in a suspension using De Man, Rogosa, and Sharpe Broth (MRSB) with a final concentration of 10^6 colony-forming units (CFU)/mL. There were 6 samples tested namely (1) blank thermosensitive gel, (2) blank mucoadhesive gel, (3) blank thermosensitive-mucoadhesive gel, (4) thermosensitive gel containing CAB, (5) mucoadhesive gel containing CAB, and (6) thermosensitive-mucoadhesive gel containing CAB. The 6 formulated gel samples were applied separately in the paper disk and placed on MRSA surface with *Lactobacillus* culture, then was further incubated for 24 h at 37 °C. Visible clear zone around the paper disc indicated the possible antimicrobial inhibitory zone of the formulated gel samples (Renschler et al., 2020)

2.18. Irritation and histopathological studies

2.18.1. Vaginal mucosa irritation study

The *in vivo* irritation evaluation study was carried out using female Wistar rats. The study was approved by the Ethical committee of the Faculty of Medicine, Hasanuddin University, Indonesia (Protocol number: UH21060385). Prior to the experiments, the rats were acclimated for one week. Five cohorts (n = 5) were prepared and labeled: (1) positive control, (2) negative control, (3) thermosensitive gel, (4) mucoadhesive gel, and (5) thermosensitive-mucoadhesive gel. For the positive control, 0.2 mL of basic gel containing low dose 5% sodium dodecyl sulfate (SDS) as the irritant material was applied to the rats' vaginal mucosa (Ishii et al., 2016), and no treatment was given for the negative control group. All other cohorts received 0.2 mL of each formulation. All samples were applied to the vaginal mucosa of the rats once a day for three days, administered directly to their vaginal mucosa using disposable syringe without needles. At the end of the experiment, the rats were dissected for their vaginal mucosa sample collection and preserved in formaldehyde in five separate sample containers.

2.18.2. Histopathological study

The vaginal mucosa samples were taken for histopathology examination. The histopathology examination was performed to examine the irritating potency of the formulated gel on the vaginal mucosa. All vaginal tissue samples were examined for vaginal irritation, defined by four endpoints: (1) epithelial exfoliation, (2) vascular congestion, (3) leukocyte infiltration, and (4) lamina propria edema. A vaginal irritation categorizing system with grades from 0 (no irritation) to 4 (high irritation) was applied to examine each tested material for the endpoints.

The composite average scores from the four endpoints are graded based on their range:

a) 1–4: Minimal vaginal irritation
b) 5–8: Mild vaginal irritation
c) 9–11: Borderline vaginal irritation
d) 12–16: Unacceptable vaginal irritation

Formulations showing scores in the range of 1–8 were considered safe as vaginal dosage form (Costin et al., 2011).

2.19. Statistical analysis

All data were calculated and processed using ® Excel® 2021 (Microsoft Corporation, Redmond, USA). Data displayed is based on mean ± SD. Analysis was performed statistically using IBM® SPSS® Statistics 25.0 (IBM, Armonk, New York, USA). Where appropriate, one-way ANOVA was used to compare multiple cohorts. Furthermore, *t*-test was used to compare two cohorts.

3. Results and discussion

3.1. Preparation of CAB vaginal gels

In this study, CAB was prepared into thermosensitive, mucoadhesive, and thermosensitive-mucoadhesive vaginal gels using different types of polymers. CAB thermosensitive and thermosensitive-mucoadhesive gels were prepared using combination of Pluronic® F127 and Pluronic® F68 as thermosensitive polymers. The combination of Pluronic® F127 and F68 was advantageous because Pluronic® F68 could increase the low gelation temperature of Pluronic® F127. This combination resulted in more compatible properties for biomedical applications in the human body (Holmgren, 2013). Pluronics® or Poloxamers are water soluble nonionic triblock copolymers containing polar (polyethylene oxide) and non-polar (poly propylene oxide) parts which can undergo sol-to-gel transition with the increase of temperature (Russo and Villa, 2019). HPMC was also added in thermosensitive-mucoadhesive gel in order to increase the residence time of CAB in vaginal mucosa (Güven et al., 2010). While for CAB mucoadhesive gel, Carbopol 940 was used as the gelling agent, and glycerin as humectant. DMDM Hydantoin was added to formulation as preservative. Furthermore, to provide improved permeation of CAB, PEG 400 was also included in formulation. All formulations were evaluated to obtain the optimum formulation. The representative images of vaginal three various types of vaginal gels are shown in Fig. 1.

3.2. pH measurement

The pH value of CAB vaginal gels preparation should be in the appropriate range to prevent irritation on the vagina. The pH value of less than 4 can induce the growth of *Lactobacillus vaginalis* while the pH value of>8 can cause the vaginosis due to *cocci* bacteria (Miller et al., 2016). The results showed that the pH values of the gel preparation were between 4.73 and 5.19 for thermosensitive gel; 6.26–7.20 for mucoadhesive gel; and 4.61–5.10 for combination of thermosensitive-mucoadhesive gel (Table 4). Based on these results, the pH of three types of vaginal gels were acceptable to be used for application in human vagina.

3.3. Viscosity study and rheological properties

The viscosity of the thermosensitive and thermosensitive-mucoadhesive gel were performed in 3 various environment namely cold temperature (4 °C), room temperature (25 °C), and body temperature (37 °C). This is due to the requirement of thermosensitive gel to have a free-flowing viscosity below the body temperature to provide easy administration of the vaginal gel and exhibit gel properties at body temperature to enhance the localization time. For mucoadhesive gel the test was performed at room temperature (25 °C). It was found that the viscosity of mucoadhesive gel in body temperature were not statistically ($p > 0.05$) different compared to the values at room temperature. The results also showed an increase in gel viscosity with higher PEG 400 concentration. It was important to note that the thermosensitive formulations showed low viscosity at cold and room temperature, indicating the liquid form of the formulations. All formulations exhibited high viscosity, which were>30,000 mPa.s at body temperature, implying the successful transformation of our approaches. All tested vaginal gels exhibit pseudoplastic behavior. The viscosity and rheological properties are depicted in Fig. 2.

3.4. Gelation temperature test

A tube inverting method was applied to determine the temperature when the liquid form turned into gel. Fig. 1 **(A&C)** depict the illustrative images of thermosensitive gel formulation at room temperature (liquid) and at body temperature (gel). The test was also carried out with and without dilution using SVF because it was critical to investigate the effect of vaginal fluid on the gelation temperature of the gel (Permana et al., 2021b). The results of gelation temperature test are shown in Table 5. The result showed that F1 for both thermosensitive and thermosensitive-mucoadhesive gel which did not contain PEG 400 turned into gel at ~ 35 °C closest to body temperature (37 °C) for both without and with dilution. It was found that the higher amount of PEG 400 produced the lower $T_{sol\text{-}gel}$ due to strong hydrogen bond between Pluronic and PEG 400 (Bain et al., 2013). In the thermosensitive gel, it was observed that there was no significant difference of $T_{sol\text{-}gel}$ among five formulations without dilution ($p > 0.05$). However, there was a significant difference of $T_{sol\text{-}gel}$ between F1 (not containing PEG 400) and F5 (containing 15% of PEG 400) with SVF dilution (p less than 0.05). Furthermore, in the thermosensitive-mucoadhesive gel, it was found that $T_{sol\text{-}gel}$ values between F1 and F4, also F1 and F5 were significantly different both with and without SVF dilution. In this case, F4 and F5 contained 10% and 15% of PEG 400, respectively. On the other hand,

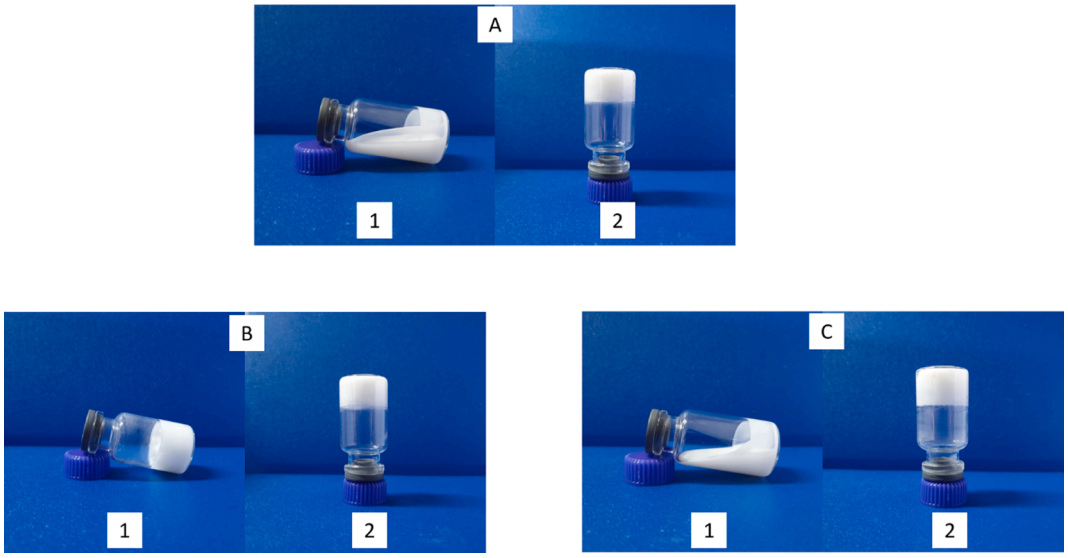

Fig. 1. Representative of (A) thermosensitive gel, (B) mucoadhesive gel, (C) thermosensitive-mucoadhesive gel at (1) room temperature and (2) body temperature.

Table 4
pH of CAB vaginal gels preparation (Means ± SD, n = 3).

Types of gel	pH				
	F1	F2	F3	F4	F5
Thermosensitive gel	4.73 ± 0.11	4.81 ± 0.15	4.96 ± 0.15	5.05 ± 0.14	5.19 ± 0.12
Mucoadhesive gel	6.26 ± 0.02	7.16 ± 0.01	7.19 ± 0.10	7.19 ± 0.06	7.20 ± 0.06
Combination of thermosensitive-mucoadhesive gel	4.61 ± 0.37	4.71 ± 0.17	4.87 ± 0.10	5.03 ± 0.17	5.10 ± 0.19

PEG 400 was not added in F1. Therefore, it was important to consider the concentration of PEG 400 as a permeation enhancer in *in situ* gel formulations. Considering all the results, despite the difference in gelation temperature, it was confirmed that all formulations could turn into gel at the body temperature.

3.5. Drug content recovery

Drug content analysis was carried out to determine the amount of CAB comprised in all three vaginal gel formulations. The results in Table 6 showed that the drug content percentage in the three types of vaginal gel formulations was in the range of 98.61–100.17%. This showed that the method used in the gel fabrication could produce a homogeneous formulation. Furthermore, the drug content recovery also fulfilled the requirements set by ICH regarding the recovery percentage, which was in the range of 95–105% (Walfish, 2006). Therefore, it was confirmed that the amount of CAB in gel was not affected by the vaginal gel preparation process.

3.6. Gel erosion study

It should be noted that conducting *in vitro* studies that are completely similar to *in vivo* environments is unlikely possible. However, the use of suitable media could be effective to evaluate the characteristics of the formulation developed. In this study, SVF was utilized to mimic the real condition after the administration of the gels into the vagina. The ability of the gels to resist from the erosion of vaginal fluid following their administration was then evaluated. As shown in Fig. 3, despite the difference in erosion profiles, all formulations did not show any significant difference (p less than 0.05), showing that>80% of the gels could resist the erosion from SVF. However, to further evaluate the residence time of the gels in the vaginal tissue, the mucoadhesive properties evaluations were then performed.

3.7. Mucoadhesive strength and time

Vaginal gel preparations must have good mucoadhesive strength because it affects the contact time of the gel with the vaginal mucosa. The tests of mucoadhesive strength and time were conducted to determine the capability of the vaginal gel to adhere to the mucosa. In this study, three types of gels with different polymers, namely thermosensitive gel (Pluronic® F127 & F68), mucoadhesive gel (Carbopol 940), and thermosensitive-mucoadhesive gel (Pluronic® F127 & F68 - HPMC) were tested. Table 7 shows the mucoadhesive properties of each vaginal gel. The results of the three gels showed that regardless of the polymer, the addition of PEG 400 was able to increase the mucoadhesive properties of each gel with F1 < F2 < F3 < F4 < F5 (Wang et al., 2008). PEG has been reported to possess mucoadhesive properties, which may be due to interpenetrating polymer network (IPN) effects between PEG chain and mucosa. There was no significant difference in the mucoadhesive time test of each gel formulation ($p > 0.05$). However, the results of the mucoadhesive strength test showed that F1 and F2 were not significantly different ($p > 0.05$) in the thermosensitive gel formulation. The mucoadhesive gel formulations F1 & F2 and F4 & F5 also showed results that were not significantly different ($p > 0.05$). Furthermore, the thermosensitive-mucoadhesive combination gel showed no significant difference ($p > 0.05$) between F1 & F2, F2 & F3, and F4 & F5. Therefore, due to the less excipients used, in this test the formulation of each vaginal gel showed that F4 was the optimum formulation with desired mucoadhesive strength and time properties.

The properties of F4 formulations of each type of gel were then compared. Although the results obtained showed that the mucoadhesive gel had the highest mucoadhesive value, the statistical analyzed data showed that the mucoadhesive properties of the mucoadhesive gel and the thermosensitive-mucoadhesive combination were not significantly different ($p > 0.05$). Furthermore, the thermosensitive gel without mucoadhesive agent showed the lowest mucoadhesive value compared to other gels. This was because Pluronic®, the main polymer in the thermosensitive gel, has lower mucoadhesive properties than Carbopol, HPMC, and several other adhesive materials (Russo and Villa, 2019). Pluronic® F127/F68 combined with HPMC as a thermosensitive-mucoadhesive gel combination polymer had a stronger hydroxyl interaction with the mucus layer than without HPMC. Therefore, this

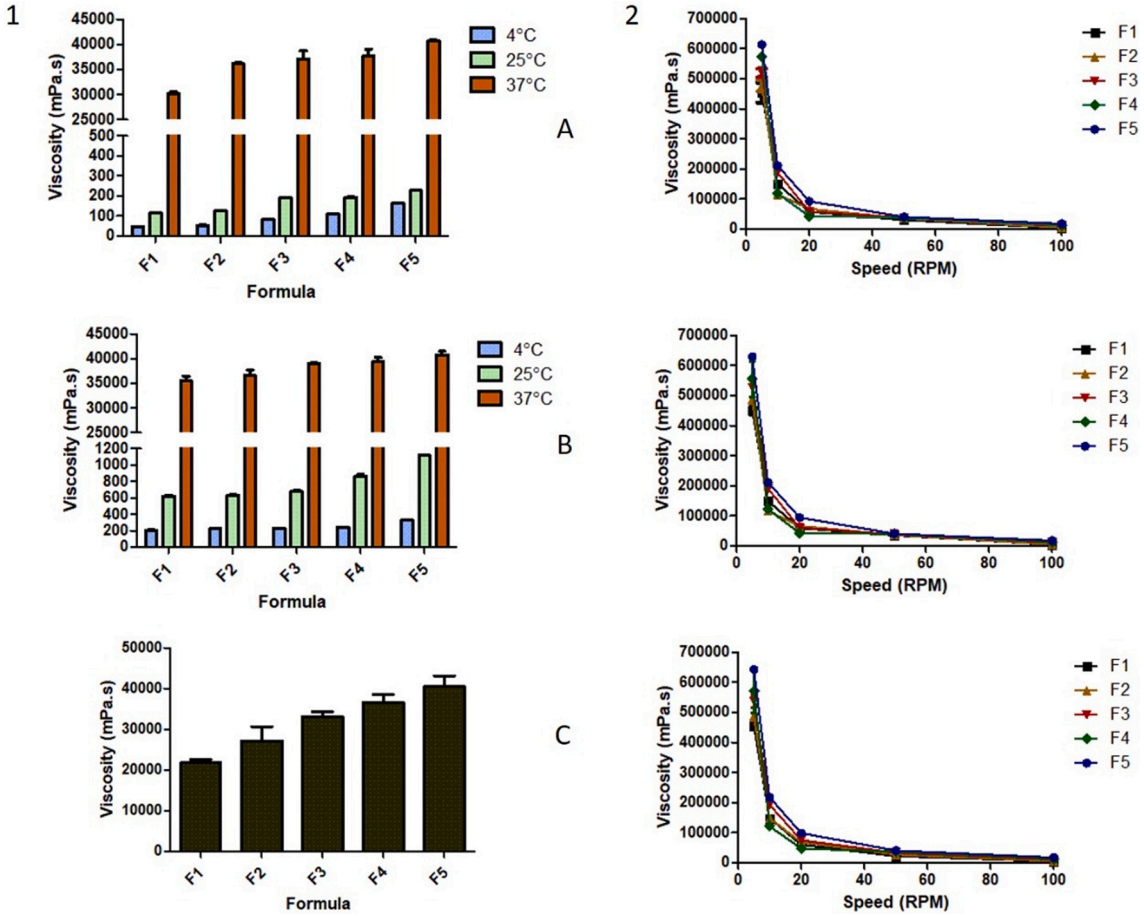

Fig. 2. Viscosity (1) and rheological properties (2) of (A) thermosensitive gel, (B) thermosensitive-mucoadhesive gel, (C) mucoadhesive gel (Means ± SD, n = 3).

Table 5
Gelation Temperature of Vaginal Gel (Means ± SD, n = 3).

	$T_{sol\text{-}gel}$ (Without Dilution)		$T_{sol\text{-}gel}$ (With Dilution)	
Formula	**Thermosensitive**	**Thermosensitive-Mucoadhesive**	**Thermosensitive**	**Thermosensitive-Mucoadhesive**
F1	35.33 ± 2.08	35.00 ± 1.00	35.33 ± 1.53	34.67 ± 2.08
F2	33.33 ± 2.08	33.67 ± 1.15	32.33 ± 2.52	32.00 ± 2.00
F3	32.33 ± 1.53	32.33 ± 1.53	32.00 ± 1.00	31.67 ± 1.15
F4	32.00 ± 2.00	31.33 ± 1.15	31.33 ± 1.53	30.33 ± 0.58
F5	31.67 ± 0.58	31.33 ± 0.58	30.33 ± 0.58	30.00 ± 1.00

Table 6
Drug Content Recovery of Vaginal Gel (Means ± SD, n = 3).

Drug Content Recovery (%)			
Formula	**Thermosensitive**	**Mucoadhesive**	**Thermosensitive-Mucoadhesive**
F1	98.70 ± 2.21	100.07 ± 0.82	98.80 ± 1.83
F2	99.93 ± 0.92	99.14 ± 1.75	99.34 ± 2.19
F3	99.68 ± 1.14	98.66 ± 1.25	100.17 ± 1.77
F4	99.63 ± 0.92	99.44 ± 1.18	99.19 ± 2.44
F5	99.29 ± 1.71	98.80 ± 2.44	98.61 ± 1.84

combination showed better mucoadhesive properties (Aka-any-grah et al., 2010). Then, the mucoadhesive gel polymer, namely Carbopol, showed higher mucoadhesive properties than other gel polymers used in this study. Carbopol has higher mucoadhesive properties than HPMC because HPMC, with its non-ionic nature, has lower hydrogen bonding ability on mucous membranes (Dhawale et al., 2018).

3.8. *In vitro* drug release

The *in vitro* drug release profile of each vaginal gel is illustrated in Fig. 4. In the formulation of each vaginal gel, various concentrations of PEG 400 were used. PEG 400 was selected because of its safety and tolerance when administered to the body, especially through the vaginal route, which has become one of the FDA-approved ingredients for drug delivery systems (Zhang et al., 2020). This study showed that after 24 h, the use of PEG could increase the release of CAB from all formulations. Analyzed statistically, from each of these vaginal gels, the CAB released from F1 and F2 were not significantly different ($p > 0.05$). The similar trend was also observed in F4 and F5. In this case, because F4 and F5 were not significantly different ($p > 0.05$), and therefore, to minimize the use of excipient, F4 with PEG 400 10% was chosen as the optimum formulation. Drug release of the selected formulation (F4) of each vaginal gel was then compared using statistical analysis. The analysis results showed that the release of CAB from the three gels was also not significantly different ($p > 0.05$). Specifically, after 24 h, the drugs released from each vaginal gel were 2.06 ± 0.30 mg, 1.86 ± 0.27 mg,

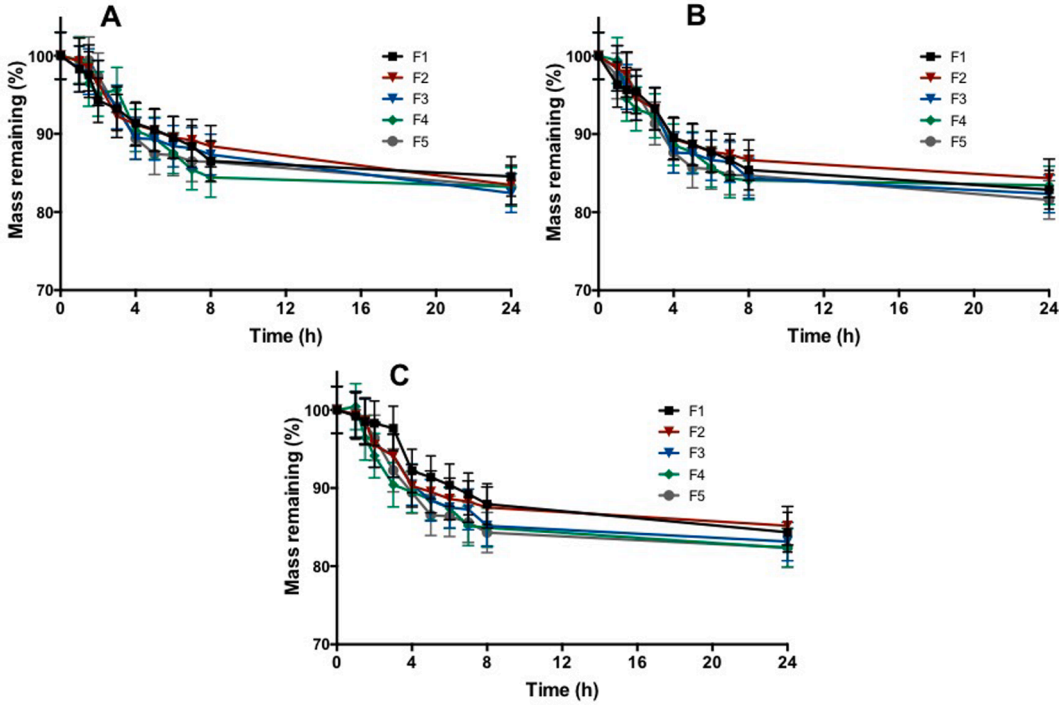

Fig. 3. Result of gel erosion study in (A) Thermosensitive gel, (B) Mucoadhesive gel, (C) Thermosensitive-mucoadhesive gel (Means ± SD, n = 3).

Table 7
Mucoadhesive Strength and Time of Vaginal Gel (Means ± SD, n = 3).

Mucoadhesive Strength (N/m^{-2})0.100				Mucoadhesive Time (h)		
Formula	**Thermosensitive**	**Mucoadhesive**	**Thermosensitive-Mucoadhesive**	**Thermosensitive**	**Mucoadhesive**	**Thermosensitive-Mucoadhesive**
F1	3.1 ± 0.5	14.8 ± 2.6	10.9 ± 1.6	0.99 ± 0.20	8.17 ± 0.15	8.04 ± 0.23
F2	3.5 ± 0.3	15.5 ± 8.8	14.5 ± 1.0	1.22 ± 0.09	8.28 ± 0.10	8.12 ± 0.16
F3	6.1 ± 0.7	20.0 ± 7.1	16.1 ± 1.7	1.54 ± 0.21	8.29 ± 0.09	8.21 ± 0.11
F4	8.8 ± 0.7	23.7 ± 0.4	21.5 ± 2.6	1.92 ± 0.05	8.31 ± 0.10	8.26 ± 0.07
F5	11.1 ± 0.9	25.4 ± 9.7	23.3 ± 1.8	1.95 ± 0.06	8.32 ± 0.13	8.28 ± 0.15

1.69 ± 0.22 mg for the thermosensitive, mucoadhesive, and thermosensitive-mucoadhesive gel, respectively.

3.9. Ex vivo permeation test

Ex vivo permeation profiles of CAB in different types of vaginal gel was carried out using porcine vaginal mucosa over 24 h. The results obtained are depicted in Fig. 5. After 24 h, F4 and F5 from each type of gels showed highest permeation profile compared to other formulations. An amount of 1.18 ± 0.21 mg and 1.43 ± 0.12 mg of CAB were permeated from F4 and F5 thermosensitive gels, respectively. On the other hand, CAB permeated from F4 and F5 mucoadhesive gels were 0.92 ± 0.20 mg and 1.07 ± 0.02 mg, while from F4 and F5 thermosensitive-mucoadhesive gels were 0.88 ± 0.08 mg and 0.94 ± 0.1 mg, respectively. It can be clearly observed that the amount of CAB permeated from F1, F2, and F3 were lower compared to F4 and F5. This was due to F1, F2, and F3 contained lower concentration of PEG 400. As it was reported that PEG 400 was able to enhance dissolution of drug, higher concentration of PEG 400 led to higher dissolution rate of CAB, which culminated in more amount of CAB permeated to the receptor compartment (Dyja and Jankowski, 2017). The data obtained were statistically analyzed, and there was no significant difference between F4 and F5 ($p > 0.05$). Thus, F4 was chosen as the optimum formula in all types of vaginal gels due to the less amount of excipient used while still providing high amount of permeated CAB. Comparison between 3 types of vaginal gels was also carried out, resulting in no significant difference of CAB permeation ($p > 0.05$) after 24 h. Furthermore, it was extremely important to evaluate the CAB permeated based on the mucoadhesive time of the formulations. Considering the mucoadhesive time, CAB permeated from thermosensitive gels (F4) possessing mucoadhesive time of less than 2 h was only 0.12 ± 0.009 mg. On the other hand, with mucoadhesive times of>8 h, CAB permeated from mucoadhesive and thermosensitive-mucoadhesive gels were found to be 6-folds, which were 0.61 ± 0.05 mg and 0.72 ± 0.11 mg, respectively. Accordingly, although all formulations showed non-significant different permeation after 24 h, following the consideration of mucoadhesive time, mucoadhesive and thermosensitive-mucoadhesive gels were more effective to deliver CAB through the vaginal mucosa.

3.10. Ex vivo retention test

Ex vivo retention study was carried out to investigate the amount of CAB localized in the vaginal tissue following the application of the vaginal gels. It can be seen that thermosensitive-mucoadhesive gel retained higher amount of CAB compared to other types of vaginal gels (Fig. 6). This might be due to combination of Pluronic F127-F68 and HPMC, resulting in a stronger hydroxyl interaction with the mucus layer (Aka-any-grah et al., 2010). Carbopol which is presence as polymer in mucoadhesive gel had carboxyl groups that bind strongly through a hydrogen bond with the oligosaccharide chain of mucin (Bera et al., 2015). On the other hand, Pluronic had lower mucoadhesive properties compared to other mucoadhesive polymers (Russo and Villa, 2019).

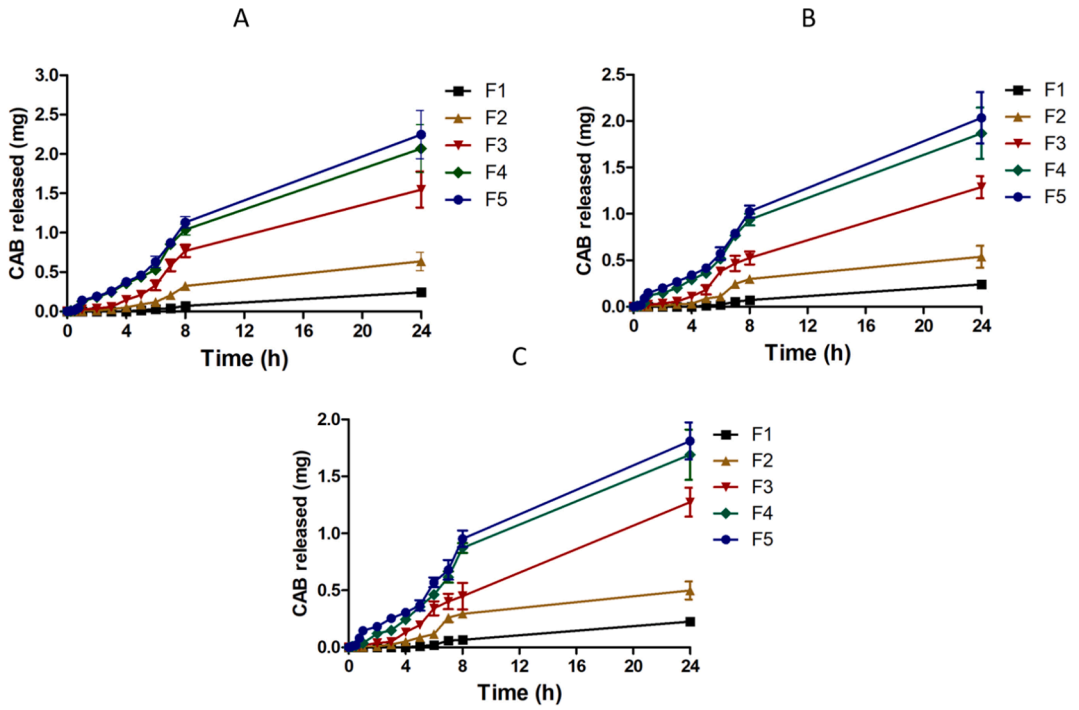

Fig. 4. *In vitro* release profile of (A) Thermosensitive gel, (B) Mucoadhesive gel, (C) Thermosensitive-mucoadhesive gel (Means ± SD, n = 3).

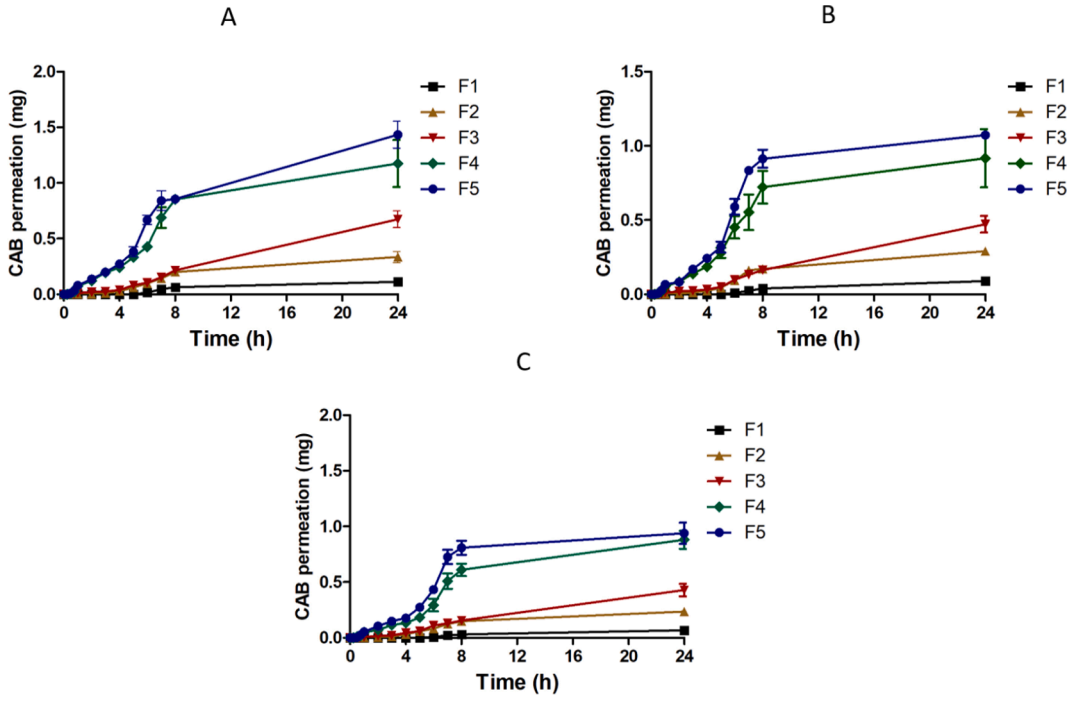

Fig. 5. *Ex vivo* permeation profile of (A) Thermosensitive gel, (B) Mucoadhesive gel, (C) Thermosensitive-mucoadhesive gel (Means ± SD, n = 3).

However, in this study it was shown that thermosensitive gel exhibited higher retention than mucoadhesive gel. After 24 h, the amount of CAB retained in the vaginal mucosa were calculated to be 26.62 ± 1.42, 24.43 ± 3.13, 32.40 ± 1.91 μg/g vaginal tissue for F4 thermosensitive, mucoadhesive, thermosensitive-mucoadhesive gel, respectively. As per *ex vivo* permeation study, the concentrations of CAB retained in the vaginal tissue were also investigated based on their mucoadhesion time. According to their mucoadhesion times in the vaginal tissue, the amount of CAB retained were found to be 1.5 ± 0.08, 17.28 ± 0.95, 15.29 ± 1.34 μg/g vaginal tissue for F4 thermosensitive, mucoadhesive, thermosensitive-mucoadhesive gel, respectively. Therefore, mucoadhesive and thermosensitive-mucoadhesive gels were found to be more effective to increase the concentration of CAB in vaginal mucosa. Recent study investigating the delivery of CAB in long-acting implant formulation for HIV prevention was carried out by Karunakaran *et al* (Karunakaran et al., 2021). Administered subcutaneously, they were able to deliver 350 μg/day of CAB to the systemic circulation. Despite this promising result, following the quantification of CAB in rectal and

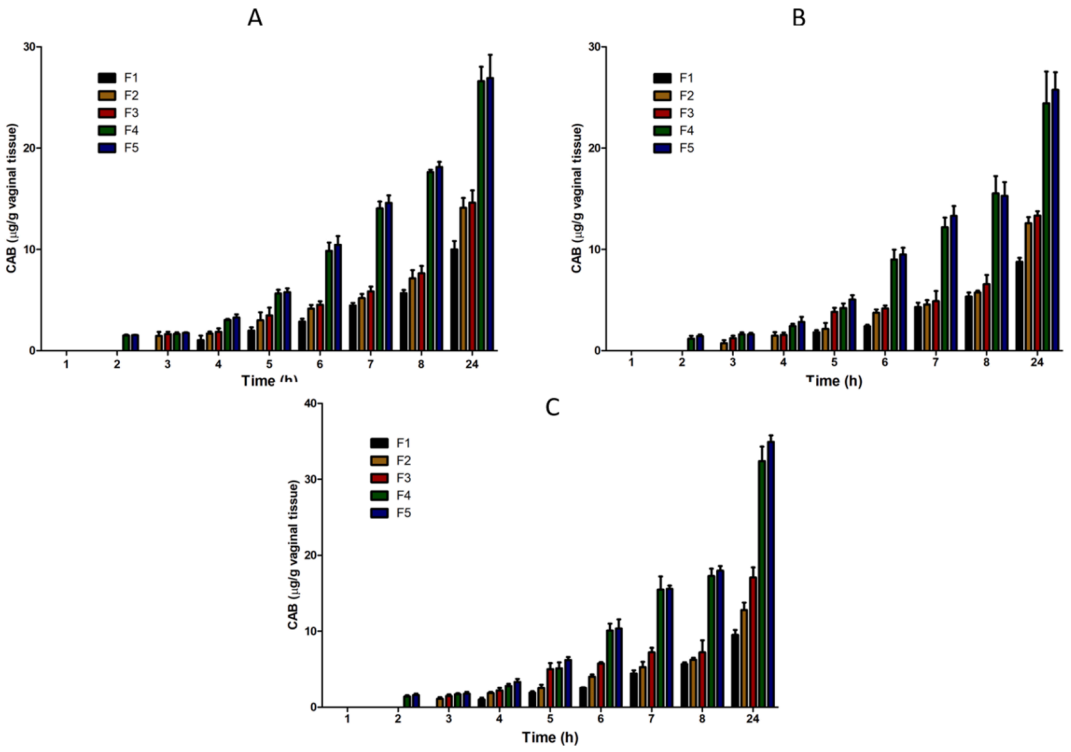

Fig. 6. Ex vivo retention of (A) Thermosensitive gel, (B) Mucoadhesive gel, (C) Thermosensitive-mucoadhesive gel (Means ± SD, n = 3).

vaginal tissues, only less than 0.2 µg/g and no CAB reported were found in rectal and vaginal tissues, respectively. With this in mind, vaginal delivery of CAB could be an alternative approach to improve the concentration of CAB in the vaginal tissue.

3.11. *In vitro hemolytic activity*

Hemolytic activity test was carried out using Wistar rats to study the initial toxicity (Greco et al., 2020) of CAB against erythrocytes. The results obtained are shown in Table 8**, and** Fig. 7b. It can be clearly seen that all types of the prepared vaginal gels exhibited less than 5% of hemolysis values in all concentrations tested. It has been previously reported that the recommended value of the hemolysis index is less than 5%. Within this limit, the compounds tested are considered safe (Zhou et al., 2011). Therefore, based on the results obtained, it clearly indicated that CAB and CAB vaginal gels would be safe to use at the tested concentration.

3.12. *In vitro Lactobacillus inhibition evaluation*

Lactobacilli are the major source of L-lactic acid and D-lactic acid, which at physiological concentrations acidify vaginal secretions (to pH levels less than 4), enhancing the protective activities of H_2O_2 and bacteriocins, and inhibiting the opportunistic infections (Amabebe and Anumba, 2018). Therefore, it was important to consider that the application of vaginal dosage forms should not disturb the population of this bacteria. The results of the antimicrobial activity test are presented in Fig. 7**.** It was clear that no inhibitory zone was formed in all tested samples, indicating no antimicrobial activity exhibited by the formulated gel against *Lactobacilli*. It can be inferred that the CAB vaginal gels did not inhibit the growth of the vaginal microbiota (*Lactobacilli*). Some studies have reported the successful delivery of several *Lactobacillus* strains in vaginal gels. Vigani *et al* developed vaginal in situ gel containing *Lactobacillus gasseri* (Vigani et al., 2019). Furthermore, Gnaman *et al* formulated vaginal gel of *Lactobacillus crispatus* to treat gonorrhea (N'Guessan Gnaman et al., 2020). Both studies showed that the formulation of *Lactobacillus* strains in vaginal gels was able to preserve the viability of *Lactobacillus* in the gel formulations. Therefore, as our formulations only contact with *Lactobacilli* at the time of application, it could be ensured that the viability of these bacteria would not be affected following the application of our approaches.

3.13. *Irritation and histopathological studies*

Following the promising results obtained from hemolytic assay and *Lactobacillus* inhibition assay, the *in vivo* irritation effect of the gels was further assessed by observing the histology of the vaginal tissues of the rats following the application of the vaginal gels. It was important to consider irritability as a safety factor in the vaginal drug delivery system. The results of histopathological study are depicted in Fig. 8**.** As observed, there was no congestion, infiltration and edema in the vaginal tissue following the application of gel formulations. Similar results were also exhibited by the negative control group. On the other hand, in the positive control group, epithelial thinning, edema, erosion, and

Table 8
Hemolytic Activity of CAB and CAB Vaginal Gels (Means ± SD, n = 3).

Hemolysis (%)				
Concentration (µg/ml)	**CAB**	**Thermosensitive**	**Mucoadhesive**	**Thermosensitive-Mucoadhesive**
500	2.40 ± 0.07	0 ± 0.09	0 ± 0.12	0 ± 0.36
50	0.29 ± 0.09	1.39 ± 0.27	0 ± 0.20	0.08 ± 0.09
5	0.49 ± 0.21	0 ± 0.15	0 ± 0.10	0.41 ± 0.21

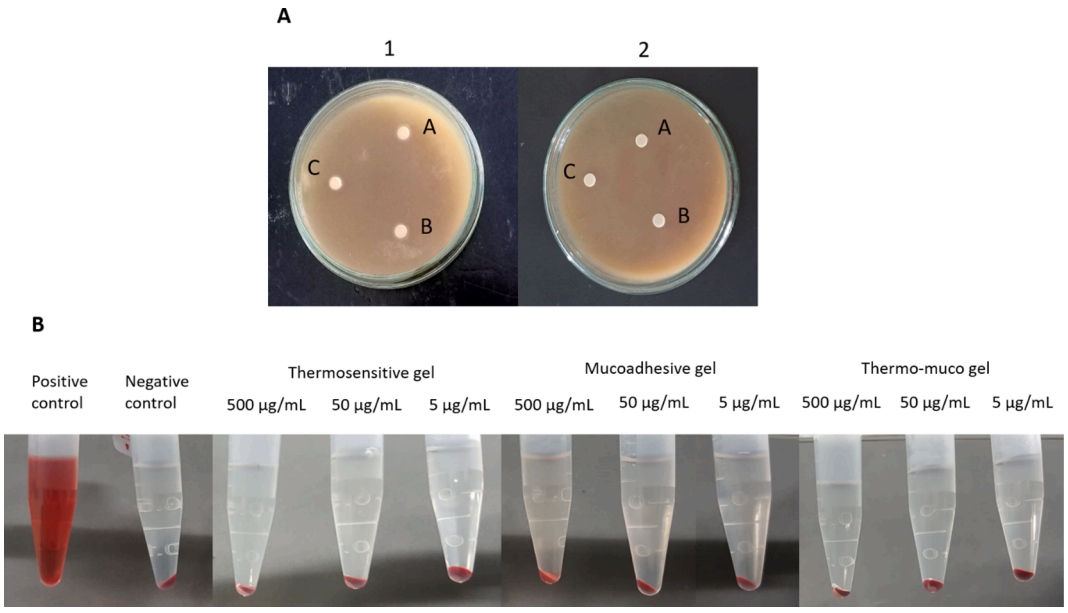

Fig. 7. (A) *In vitro* antimicrobial activity of (1) blank vaginal gel and (2) vaginal gel containing CAB (A), themosensitive gel, (B) mucoadhesive gel, and (C) thermosensitive-mucoadhesive gel. (B) Result of hemolytic activity test.

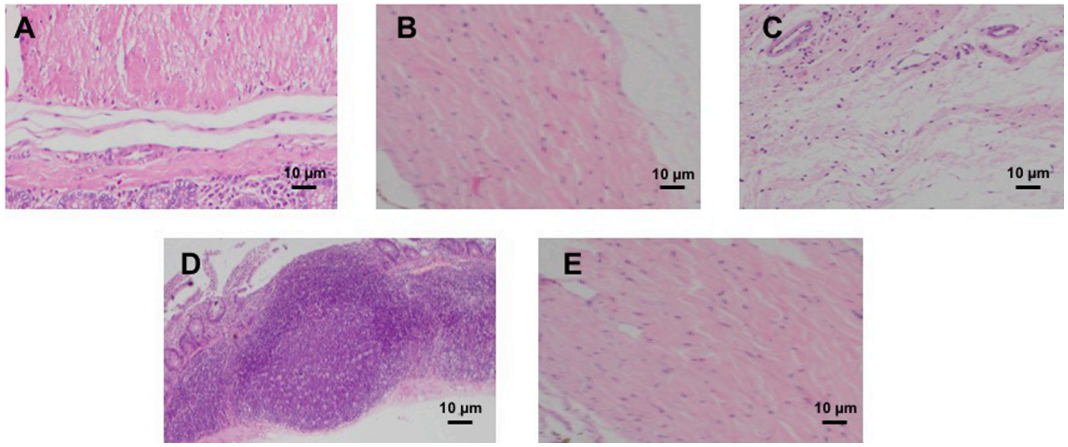

Fig. 8. Histopathological result of (A) thermosensitive gel, (B) mucoadhesive gel, (C) thermosensitive-mucoadhesive gel, (D) positive control, and (E) negative control.

infiltration were observed. Accordingly, the application of the prepared vaginal gel formulations was considered to be safe for vaginal delivery.

Based on the outcomes obtained in this study, it is imperative that the development of CAB into vaginal dosage form could be beneficial for HIV treatment and prevention as this route could potentially deliver CAB through vaginal tissue to reach systemic circulation and localize the concentration of CAB in the vaginal. In this study, as discussed, we developed three types of vaginal gels. Due to their mucoadhesive properties, mucoadhesive gels and the combination of thermosensitive and mucoadhesive gels showed promising delivery profiles compared to thermosensitive gels. Furthermore, considering the easy administration of liquid form to the vaginal cavity, the combination of thermosensitive and mucoadhesive gels possessed desired characteristics to provide convenience administration to the patients. Following this study, in vivo studies must now be carried out to evaluate the pharmacokinetic profiles of CAB following vaginal delivery of this approach. Importantly, the pharmacokinetic profiles of CAB by this approach should also be compared to injection and oral administration as the currently available delivery routes of CAB. In addition, acceptability and usability in humans should be evaluated prior to its use in clinical applications.

4. Conclusion

In this study, CAB was formulated into three different types of vaginal gels, namely thermosensitive gel, mucoadhesive gel, and thermosensitive-mucoadhesive gel. several evaluation parameters were carried out to determine the optimum formulations of CAB vaginal gels. Thermosensitive-mucoadhesive vaginal gel, prepared using PF127-PF 68-HPMC, was found to be the optimum dosage form providing ease administration of CAB due to the pseudoplastic flow of the gel. Additionally, this formulation was also able to exhibit prolonged retention time in vaginal mucosa after 8 h. Furthermore, the use of PEG 400 as permeation enhancer improved the permeation and retention of CAB in the vaginal tissue in *ex vivo* studies. These findings showed that our approach could be potential to deliver CAB through vaginal mucosa for HIV treatment in the future. Moving forward, *in vivo* experiment is essential to be carried out in the future to investigate the pharmacokinetic profiles of CAB using suitable animal models.

CRediT authorship contribution statement

Cindy Kristina Enggi: Conceptualization, Methodology, Funding acquisition, Writing – original draft. **Hansel Tridatmojo Isa:** Methodology, Writing – original draft. **Sulistiawati Sulistiawati:** Methodology, Writing – original draft. **Komang Agus Rai Ardika:** Methodology, Data curation. **Stevens Wijaya:** Data curation, Validation. **Rangga Meidianto Asri:** Validation, Supervision. **Sandra Aulia Mardikasari:** Validation, Supervision. **Ryan F. Donnelly:** Project administration. **Andi Dian Permana:** Conceptualization, Project administration, Funding acquisition, Validation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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