BUKTI SUBMIT MANUSKRIP

Universitas Hasanuddin

Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

A manuscript number has been assigned: SAA-D-21-02359

1 message

Spectrochimica Acta Part A <em@editorialmanager.com> Mon, Aug 16, 2021 at 11:20 PM
Reply-To: Spectrochimica Acta Part A <j.saa@elsevier.com>
To: Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Ms. Ref. No.: SAA-D-21-02359
Title: Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue for ex vivo vaginal delivery of novel thermosensitive and mucoadhesive vaginal gels
Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

Dear Dr. Permana,

Your submission, referenced above, has been assigned the manuscript number SAA-D-21-02359 and has been assigned to an Editor who will handle peer review.

Please note that in most cases at least two reviews may be required before a decision on a manuscript is made. You will be notified by e-mail each time a reviewer agrees to review your manuscript.

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Thank you for submitting your manuscript to
Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy.

Kind regards,

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

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BUKTI REVIEW DARI REVIEWERS (1)

Universitas Hasanuddin

Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Your Submission SAA-D-21-02359

1 message

Spectrochimica Acta Part A <em@editorialmanager.com> Fri, Oct 1, 2021 at 2:30 PM
Reply-To: Spectrochimica Acta Part A <support@elsevier.com>
To: Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Ms. Ref. No.: SAA-D-21-02359
Title: Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue for ex vivo vaginal delivery of novel thermosensitive and mucoadhesive vaginal gels
Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

Dear Dr. Andi Dian Permana,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript.

To submit a revision, please go to https://www.editorialmanager.com/saa/ and login as an Author.
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When submitting your revised paper, we ask that you include the following items:

Manuscript and Figure Source Files (mandatory)

We cannot accommodate PDF manuscript files for production purposes. We also ask that when submitting your revision you follow the journal formatting guidelines. Figures and tables may be embedded within the source file for the submission as long as they are of sufficient resolution for Production.For any figure that cannot be embedded within the source file (such as *.PSD Photoshop files), the original figure needs to be uploaded separately. Refer to the Guide for Authors for additional information.
http://www.elsevier.com/journals/spectrochimica-acta-part- a:-molecular-and-biomolecular-spectroscopy/1386-1425/guide-for-authors

Highlights (mandatory)

Highlights consist of a short collection of bullet points that convey the core findings of the article and should be submitted in a separate file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point). See the following website for more information
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Graphical Abstract (mandatory)

Graphical Abstracts should summarize the contents of the article in a concise, pictorial form designed to capture the attention of a wide readership online. Refer to the following website for more information: http://www.elsevier.com/graphicalabstracts

On your Main Menu page is a folder entitled "Submissions Needing Revision". You will find your submission record there.

Finally, I would appreciate if you could submit your revised paper by Nov 30, 2021.

Note: While submitting the revised manuscript, please double check the author names provided in the submission so that authorship related changes are made in the revision stage. If your manuscript is accepted, any authorship change will involve approval from co-authors and respective editor handling the submission and this may cause a significant delay in publishing your manuscript.

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We invite you to submit a method article alongside your research article. This is an opportunity to get full credit for the time and money spent on developing research methods, and to increase the visibility and impact of your work. If your research article is accepted, we will contact you with instructions on the submission process for your method article to MethodsX. On receipt at MethodsX it will be editorially reviewed and, upon acceptance, published as a separate method article. Your articles will be linked on ScienceDirect.

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Yours sincerely,

Christian Huck
Editor
Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

Reviewers' comments:

Reviewer #1: This paper describes development and validation of a UV-visible spectroscopic method that is intended to support a development of an alternative approach to administering cabotegravir or CAB (an important anti-HIV drug) to female patients. As the methods based on UV-VIS spectroscopy are generally less expensive and less time-consuming than the traditional chromatography and mass spectrometry based methods, these developments can likely accelerate research progress in the area of new drug delivery systems and, therefore, result in a positive impact on public health.

This paper, in general, is well organized, clearly written and easy to follow. The importance of the study, its fit into the drug delivery system research, and next steps to continue this research are explained well. The proposed UV-VIS spectroscopic method is validated adequately for the purpose. As a result, my recommendation is to accept this paper for publication after minor revisions suggested below:

1) The calibration curves presented in Figure 3 visually appear to be "bilinear" instead of linear. So, a sentence or two explaining this behavior should be added. In addition, the implications of this "bilinear" behavior on the calculations of low limit of detection (LOD) and low limit of quantitation (LOQ) using the eq. (1) and eq. (2) should be discussed.

2) Lines 73-75: Adding a reference to support the statement that CAB is able to prevent further infection to the circulatory system would be helpful. Otherwise, softening the statement is recommended.

3) Line 90: "matrix" should be changed to "matrices".

4) Line 166: Abbreviation "TEA" may not be obvious to every reader.

5) Line 186: "eq. (2)" should be replaced with "eq. (1)"

6) Line 191: "eq. (1) should be replaced with "eq. (2)"

7) Lines 398-401: text in these lines contains a few typos. For example, "released" is used twice in line 398. The punctuation is also to be reviewed.

8) Lines 188, 303, 306 - commas that occasionally separate the decimal figures throughout the paper should be replaced with points.

Reviewer #2: The submitted manuscript by Sulistiawati et al. deals with validation of the spectrophotometric method for vaginal gel formulations containing categravir (CAB). The topic is interesting and fits under the scope of the Journal; however, there are deficiencies that should be adequately addressed to strengthen the article.

Since the gels based on pluronic (thermosensitive) and Carbopol (mucoadhesive) are already well known, word "novel" should be removed from the title of the manuscript.

Physico-chemical properties of the drug, particularly solubility data, should be indicated in Introduction section, as these features significantly affect the properties of the formulation.

Regarding the preparation of the gels, why was hydantoin DMDM added at the end, after incorporation of the active ingredient, i.e. CAB?

The authors performed in vitro and ex vivo studies using fresh porcine vaginal mucosa. Preparation of the vaginal tissue is not indicated as well as the age of the animal(s). What was the thickness of the vaginal mucosa used in experiments? Moreover, what was the mass of the gel in donor compartment, and quantity of the drug? Which medium was used as receptor medium in in vitro release studies and ex vivo permeation studies? What was the volume of receptor media? The surface area (Franz cell)? These data must be indicated in the manuscript.

When discussing the results on ex vivo permeation of the drug formulated in the gels, the authors should be more careful with the terms used. Please check and rewrite the sentences on page 17 (first paragraph), i.e. ex vivo permeation studies give information on the permeated drug and the accumulated drug in vaginal tissue, not released drug.

The whole manuscript should be improved/edited for the language, grammar and fluidity.

Data in Brief (optional):
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BUKTI SUBMIT HASIL REVIEW (1)

Universitas Hasanuddin

Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Submission Confirmation for SAA-D-21-02359R1

1 message

Spectrochimica Acta Part A <em@editorialmanager.com> Mon, Oct 4, 2021 at 4:05 AM
Reply-To: Spectrochimica Acta Part A <support@elsevier.com>
To: Andi Dian Permana <andi.dian.permana@farmasi.unhas.ac.id>

Ms. Ref. No.: SAA-D-21-02359R1
Title: Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue for ex vivo vaginal delivery of thermosensitive and mucoadhesive vaginal gels
Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

Dear Dr. Andi Dian Permana,

Your revised manuscript was received for reconsideration for publication in Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy.

You may check the status of your manuscript by logging onto the Editorial Manager as an Author at https://www.editorialmanager.com/saa/.
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Kind regards,

Editorial Manager
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Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue for ex vivo vaginal delivery of thermosensitive and mucoadhesive vaginal gels

--Manuscript Draft--

Manuscript Number:	SAA-D-21-02359R1
Article Type:	Full Length Article
Section/Category:	Analytical Spectroscopy and New Methods
Keywords:	Cabotegravir; UV-Vis spectrophotometry; mucoadhesive; thermosensitive; validation
Corresponding Author:	Andi Dian Permana Universitas Hasanuddin Fakultas Farmasi Makassar, INDONESIA
First Author:	Sulistiawati Sulistiawati
Order of Authors:	Sulistiawati Sulistiawati
	Cindy Kristina Enggi
	Hansel Tridatmojo Isa
	Stevens Wijaya
	Komang Agus Rai Ardika
	Rangga Meidianto Asri
	Ryan F. Donnelly
	Andi Dian Permana
Abstract:	Cabotegravir (CAB) is an antiretroviral therapy (ARV) used for Human Immunodeficiency Virus (HIV) treatment. CAB has low solubility, which affects its bioavailability in oral therapy. Moreover, the injection form of CAB has difficulty in the administration process. Therefore, it is essential to develop a new drug delivery system for CAB. Vaginal drug delivery system offers many advantages such as a large surface area, increased drug bioavailability, and improved drug delivery. CAB was developed in thermosensitive and mucoadhesive vaginal gel preparations that provided optimal distribution in the vaginal mucosa. To support the process of formulation development, in this study, UV-visible spectrophotometry method was validated in methanol, simulated vaginal fluid (SVF) and vaginal tissue to quantify the amount of CAB in the gel preparations, in vitro, and ex vivo studies, respectively. The developed analytical method was subsequently validated according to ICH guidelines. The calibration curves in these matrices were found to be linear with correlation coefficient values (R2) ≥ 0.998. The LLOQ values in methanol, SVF and vaginal tissue were 2.15 µg/mL,2.22 µg/mL,and 5.13 µg/mL, respectively. The developed method was found to be accurate and precise without being affected by dilution integrity. These methods were successfully applied to quantify the amount of CAB in gel preparations, in vitro, and ex vivo studies, showing uniformity of drug content and controlled release manner in the permeationprofile for 24 hours for both thermosensitive and mucoadhesive vaginal gels. Further analytical method is required to be developed for the quantification of CAB in in vivo studies.

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The Editor
Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

September 28, 2021

Dear Sir/Madam,

I wish you to consider our manuscript for publication in **International Journal of Pharmaceutis**. Following the reviewer comments, we have changed our title from from "**Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue for *ex vivo* vaginal delivery of novel thermosensitive and mucoadhesive vaginal gels**" to "**Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue for *ex vivo* vaginal delivery of thermosensitive and mucoadhesive vaginal gels**". Importantly, we have addressed all comments from all reviewers as shown in "Response to Reviewer" file. Also, we have re-checked the manuscript thoroughly and made significant changes to the grammatical and English errors.

The manuscript has not been previously published in any language anywhere and it is not under simultaneous consideration by another journal. We have no conflicts of interest.

We appreciate your attention. We hope you will now consider publishing our research in **Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy** and look forward to hearing from you in due course.

Yours Sincerely,

Andi Dian Permana (on behalf of all authors)
Faculty of Pharmacy, Hasanuddin University, Indonesia
Email: andi.dian.permana@farmasi.unhas.ac.id

Ms. Ref. No.: SAA-D-21-02359

Title: Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue for ex vivo vaginal delivery of novel thermosensitive and mucoadhesive vaginal gels

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

Reviewers' comments:

Reviewer #1: This paper describes development and validation of a UV-visible spectroscopic method that is intended to support a development of an alternative approach to administering cabotegravir or CAB (an important anti-HIV drug) to female patients. As the methods based on UV-VIS spectroscopy are generally less expensive and less time-consuming than the traditional chromatography and mass spectrometry based methods, these developments can likely accelerate research progress in the area of new drug delivery systems and, therefore, result in a positive impact on public health.

This paper, in general, is well organized, clearly written and easy to follow. The importance of the study, its fit into the drug delivery system research, and next steps to continue this research are explained well. The proposed UV-VIS spectroscopic method is validated adequately for the purpose. As a result, my recommendation is to accept this paper for publication after minor revisions suggested below:

Response to Reviewer

We are very thankful to the reviewers for taking the time to provide helpful comments for improvements to our manuscript. We are grateful that the reviewer thinks that our manuscript is well organized and easy to follow. We have addressed each of the reviewers' comments in detail below.

1) The calibration curves presented in Figure 3 visually appear to be "bilinear" instead of linear. So, a sentence or two explaining this behavior should be added. In addition, the implications of this "bilinear" behavior on the calculations of low limit of detection (LOD) and low limit of quantitation (LOQ) using the eq. (1) and eq. (2) should be discussed.

Response to Reviewer

We thank the reviewer for this valuable suggestion. Indeed, it was observed that following the construction of the calibration curve, all samples showed bilinear curves. The low concentrations showed a slightly higher slope than the higher concentrations of CAB. The difference in slope values in bilinear could decrease the sensitivity of the methods since the calculation of LOD and LOQ values of the analytical method depends on the slope values of the calibration curves (Equation 1 and Equation 2). Despite this, no significant difference (p > 0.05) was found in the slope values in low and high concentrations of CAB. Importantly, a linear relationship was found between the absorbance and the concentration in the range of 0.5 – 16 µg/mL for CAB-MeOH and CAB-SVF, and 1 – 32 µg/mL for CAB-vaginal tissue. The correlation coefficient values (r2) of three regression equations of CAB in methanol, SVF, and vaginal tissue were 0.9990**,** 0.9989**,** and 0.9985**,** respectively, indicating the acceptable linearity. Furthermore, the LOD and LLOQ values of CAB in methanol were 0.71 and 2.15 µg/mL**,** in SVF were 0.74 and 2.22 µg/mL**,** and in vaginal tissue were 1.69 and 5.13 µg/mL. We have discussed these in the revised manuscript (Section 3.3, Line 311-317).

2) Lines 73-75: Adding a reference to support the statement that CAB is able to prevent further infection to the circulatory system would be helpful. Otherwise, softening the statement is recommended.

Response to Reviewer

We thank the reviewer for the comment and the suggestion. Accordingly, we have rewritten the statement to avoid this ambiguity and improve the clarity. We also have included a reference to support the statement, as follows:

"Importantly, due to the presence of receptors and coreceptors of HIV, such as CD4+ T cells and CCR5, vagina is the main route of HIV infection (Iyer et al., 2017). Therefore, vaginal administration of CAB could be an alternative delivery approach for the treatment and prevention of HIV." (Line 74-77)

3) Line 90: "matrix" should be changed to "matrices".

Response to Reviewer

We have corrected this.

4) Line 166: Abbreviation "TEA" may not be obvious to every reader.

Response to Reviewer

We have included the full name of TEA in the revised manuscript (Line 169).

5) Line 186: "eq. (2)" should be replaced with "eq. (1)"

Response to Reviewer

We have corrected this.

6) Line 191: "eq. (1) should be replaced with "eq. (2)"

Response to Reviewer

We have corrected this.

7) Lines 398-401: text in these lines contains a few typos. For example, "released" is used twice in line 398. The punctuation is also to be reviewed.

Response to Reviewer

We thank the reviewer for pointing this out. Following the suggestions, we have re-checked the manuscript thoroughly and made significant changes to the mistakes.

8) Lines 188, 303, 306 - commas that occasionally separate the decimal figures throughout the paper should be replaced with points.

Response to Reviewer

We thank the reviewer for pointing this out. We have corrected this.

Reviewer #2: The submitted manuscript by Sulistiawati et al. deals with validation of the spectrophotometric method for vaginal gel formulations containing categravir (CAB). The topic is interesting and fits under the scope of the Journal; however, there are deficiencies that should be adequately addressed to strengthen the article.

Response to Reviewer

We are very thankful to the reviewers for taking the time to provide helpful comments for improvements to our manuscript. We are grateful that the reviewer thinks that the topic is interesting and fits under the scope of the Journal. We have addressed each of the reviewers' comments in detail below.

Since the gels based on pluronic (thermosensitive) and Carbopol (mucoadhesive) are already well known, word "novel" should be removed from the title of the manuscript.

Response to Reviewer

We thank the reviewer for the suggestion. Accordingly, we have changed the title from "Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue for *ex vivo* vaginal delivery of novel thermosensitive and mucoadhesive vaginal gels" to "Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue for *ex vivo* vaginal delivery of thermosensitive and mucoadhesive vaginal gels"

Physico-chemical properties of the drug, particularly solubility data, should be indicated in Introduction section, as these features significantly affect the properties of the formulation.

Response to Reviewer

We thank the reviewer for the suggestion. Following this, we have included the properties of CAB in the revised manuscript. CAB is categorized as Biopharmaceutics Drug Disposition Classification System (BDDCS) II, which has low solubility and high permeability, affecting the absorption of the drug (Patel et al., 2019; Sareen et al., 2012). It has been reported that the solubility of CAB in the aqueous solution is limited to 4.8 µg/mL (Pons-Faudoa et al., 2019). Furthermore, the log P value of CAB is 1.04 (Rajoli et al., 2018) (Line 59-60).

Regarding the preparation of the gels, why was hydantoin DMDM added at the end, after incorporation of the active ingredient, i.e. CAB?

Response to Reviewer

We thank the reviewer for the question. In the gel preparations, the homogenous mixture of CAB in PEG was initially mixed with the gel base. To ensure that all mixtures were homogenous, DMDM hydantoin was finally added into the formulations. We have included this in the revised manuscript (Section 2.6).

The authors performed in vitro and ex vivo studies using fresh porcine vaginal mucosa. Preparation of the vaginal tissue is not indicated as well as the age of the animal(s). What was the thickness of the vaginal mucosa used in experiments? Moreover, what was the mass of the gel in donor compartment, and quantity of the drug? Which medium was used as receptor medium in in vitro release studies and ex vivo permeation studies? What was the volume of receptor media? The surface area (Franz cell)? These data must be indicated in the manuscript.

Response to Reviewer

We thank the reviewer for the questions and the suggestions. The permeation behavior of CAB from thermosensitive and mucoadhesive gels was evaluated using Franz diffusion cells using a dialysis membrane (Spectra-Por®, 12,000 - 14,000 MWCO dialysis membrane) and porcine vaginal mucosa for *in vitro* and *ex vivo* studies, respectively. The average thickness of the vaginal mucosa was 2.18 ± 0.13 mm. The tissues were obtained from female pigs (three- to four-month-old). The receptor compartment was filled with 24 mL of the receptor media and the temperature was maintained at 37^{o}C. In this study, SVF containing 20% v/v of methanol was used as the receptor media to maintain the sink condition during the experiments. Furthermore, 1 g of formulation (equal to 10 mg of CAB) was placed in the donor compartment with a diffusion area of 4.9 cm^{2}. We have included this information in the revised manuscript (Section 2.8.2).

When discussing the results on ex vivo permeation of the drug formulated in the gels, the authors should be more careful with the terms used. Please check and rewrite the sentences on page 17 (first paragraph), i.e. ex vivo permeation studies give information on the permeated drug and the accumulated drug in vaginal tissue, not released drug.

Response to Reviewer

We thank the reviewer for the comments and the suggestions. We have checked and rewritten this part. We have changed the term “released” to “permeated” in the revised manuscript.

The whole manuscript should be improved/edited for the language, grammar and fluidity.

Response to Editor

We thank the Editor for these suggestions. Following the suggestions, we have re-checked the manuscript thoroughly and made significant changes in the language, grammar and fluidity. We believe that the revised manuscript has now been improved in terms of language, grammar and fluidity.

Rererences:

Iyer, S.S., Sabula, M.J., Mehta, C.C., Haddad, L.B., Brown, L., Amara, R.R., Ofotokun, I., Sheth, A.N., 2017. Characteristics of HIV target CD4 T cells collected using different sampling methods from the genital tract of HIV seronegative women. PLoS One 1–18.

Patel, P., Ford, S.L., Lou, Y., Bakshi, K., Tenorio, A.R., Zhang, Z., Pan, R., Spreen, W., 2019. Effect of a High-Fat Meal on the Pharmacokinetics of the HIV Integrase Inhibitor Cabotegravir. Clin. Pharmacol. Drug Dev. 8, 443–448. https://doi.org/10.1002/cpdd.620

Pons-Faudoa, F.P., Sizovs, A., Di Trani, N., Paez-Mayorga, J., Bruno, G., Rhudy, J., Manohar, M., Gwenden, K., Martini, C., Chua, C.Y.X., Varchi, G., Marzinke, M.A., Grattoni, A., 2019. 2-Hydroxypropyl-β-cyclodextrin-enhanced pharmacokinetics of cabotegravir from a nanofluidic implant for HIV pre-exposure prophylaxis. J. Control. Release 306, 89–96. https://doi.org/10.1016/j.jconrel.2019.05.037

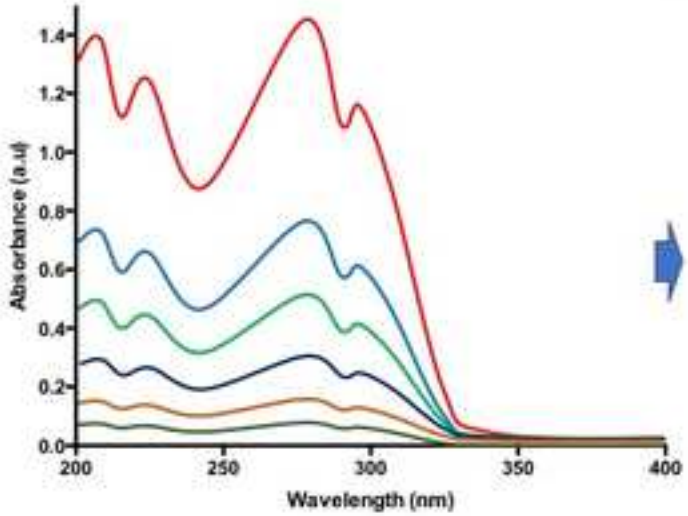
Rajoli, R.K., Curley, P., Chiong, J., Back, D., Flexner, C., Owen, A., Siccardi, M., 2018. Predicting drug–drug interactions between rifampicin and long-acting cabotegravir and rilpivirine using physiologically based pharmacokinetic Mmodeling. J. Infect. Dis. 219, 1735–1742. https://doi.org/https://doi.org/10.1093/infdis/jiy726

Sareen, S., Joseph, L., Mathew, G., 2012. Improvement in solubility of poor water-soluble drugs by solid dispersion. Int. J. Pharm. Investig. 2, 12. https://doi.org/10.4103/2230-973x.96921

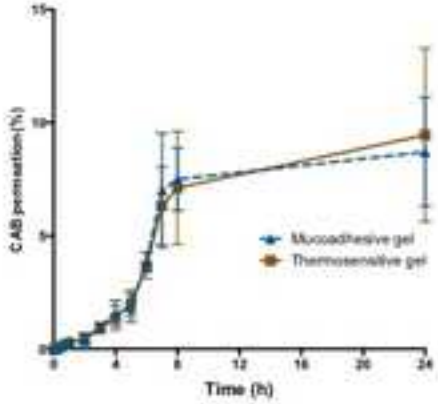
Graphical Abstract

Thermosensitive and mucoadhesive vaginal gels of cabotegravir

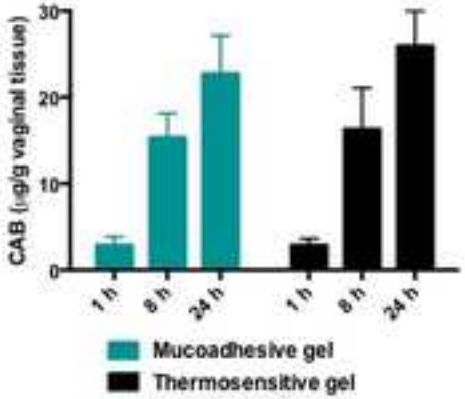
Spectrophotometric validation



CAB spectrum in vaginal tissue



***Ex vivo permeation* study**



***Ex vivo retention* study**

Highlights:

- Spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue was developed
- The analytical method was validated according to ICH guidelines
- The validated method was applied in *in vitro* study and *ex vivo* vaginal delivery of thermosensitive and mucoadhesive vaginal gels

Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue for *ex vivo* vaginal delivery of thermosensitive and mucoadhesive vaginal gels

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Abstract

Cabotegravir (CAB) is an antiretroviral therapy (ARV) used for Human Immunodeficiency Virus (HIV) treatment. CAB has low solubility, which affects its bioavailability in oral therapy. Moreover, the injection form of CAB has difficulty in the administration process. Therefore, it is essential to develop a new drug delivery system for CAB. Vaginal drug delivery system offers many advantages such as a large surface area, increased drug bioavailability, and improved drug delivery. CAB was developed in thermosensitive and mucoadhesive vaginal gel preparations that provided optimal distribution in the vaginal mucosa. To support the process of formulation development, in this study, UV-visible spectrophotometry method was validated in methanol, simulated vaginal fluid (SVF) and vaginal tissue to quantify the amount of CAB in the gel preparations, *in vitro*, and *ex vivo* studies, respectively. The developed analytical method was subsequently validated according to ICH guidelines. The calibration curves in these matrices were found to be linear with correlation coefficient values (R^2) ≥ 0.998. The LLOQ values in methanol, SVF and vaginal tissue were 2.15 µg/mL, 2.22 µg/mL, and 5.13 µg/mL, respectively. The developed method was found to be accurate and precise without being affected by dilution integrity. These methods were successfully applied to quantify the amount of CAB in gel preparations, *in vitro*, and *ex vivo* studies, showing uniformity of drug content and controlled release manner in the permeation profile for 24 hours for both thermosensitive and mucoadhesive vaginal gels. Further analytical method is required to be developed for the quantification of CAB in *in vivo* studies.

Keywords: Cabotegravir, UV-Vis spectrophotometry, mucoadhesive, thermosensitive, validation

1. Introduction

Cabotegravir (CAB) is known as an HIV-1 integrase inhibitor, one of the antiretroviral medications for HIV patients. It is categorized as Biopharmaceutics Drug Disposition Classification System (BDDCS) II, which has low solubility and high permeability, affecting the absorption of the drug [1,2]. It has been reported that the solubility of CAB in the aqueous solution is limited to 4.8 µg/mL [3]. Furthermore, the log P value of CAB is 1.04 [4]. Currently, CAB is available in tablet and injection forms. However, following oral administration, many HIV patients were found to develop oral candidiasis, causing difficulty in swallowing. [5]. On the other hand, the long-acting injectable form of CAB also possesses several limitations. Despite having less frequency of administration, CAB injection needs to be administered by healthcare professionals. Additionally, the injection form is not comfortable and painful, which could lead to a decrease in the patient compliance to the medications [6].

In order to increase patient compliance and obtain sustained drug delivery, in this study, CAB was separately developed in the form of thermosensitive and mucoadhesive vaginal gels. Thermosensitive dosage forms would enable easy administration due to the liquid form of the formulation, which would transform to semisolid form in the vaginal temperature [7]. Furthermore, the mucoadhesive preparations offer the advantage, mainly due to their ability to prolong the contact of the drugs in the vaginal tissue [8]. The delivery of CAB through vaginal delivery can be an alternative therapy as it provides various advantages, including large surface area and rich blood supply, resulting in higher drug bioavailability. Importantly, due to the presence of receptors and coreceptors of HIV, such as CD4+ T cells and CCR5, vagina is the main route of HIV infection [9]. Therefore, vaginal administration of CAB could be an alternative delivery approach for the treatment and prevention of HIV.

Drug detection and quantification are essential parts of the development of novel drug delivery systems. Accordingly, an appropriate analytical method is crucial to be developed for this purpose. In this study, calculation of drug content in the formulations and measurement of drug concentration in *in vitro* and *ex vivo* studies were critical evaluations during the development of CAB thermosensitive and mucoadhesive vaginal gels. *In vitro* studies are an initial step to evaluate drug release behavior using a suitable release medium. Besides, *ex vivo* studies are performed using tissues from organisms by mimicking natural conditions. *Ex vivo* studies are advantageous for evaluating the drug behavior in specific organs/tissues as they can eliminate other physiological factors, resulting in more controlled conditions compared to living organisms. This indicates the importance of *ex vivo* experiments before proceeding to *in vivo* studies [10]. As the gel was intended for vaginal application, the concentration of CAB

was measured both in the simulated vaginal fluid (SVF) and vaginal mucosa. To the best of our knowledge, analytical methods to quantify CAB in both of these media have not been reported previously. Various analytical methods have been developed to quantify CAB in various matrices, including HPLC-UV [11] and HPLC-MS/MS [12]. However, the reported methods are expensive, time-consuming and require sophisticated equipment and, thus, are difficult to apply in the middle- and low-income countries/laboratories. On the other hand, spectrophotometer UV-visible for CAB detection and quantification in *in vitro* and *in vitro* studies would be promising due to its simplicity, cost-effectiveness and high adaptability in small laboratories. Therefore, in the present study, for the first time, CAB quantification methods were developed in SVF and vaginal mucosa using a spectrophotometer UV-visible. Several previous studies have reported the ability of spectrophotometer UV-visible in quantifying numerous types of drugs in both *in vitro* and *ex vivo* studies [13,14]. Following the development of analytical procedures, method validation must be carried out to ensure reliability, traceability, and comparability of the results.

This study aimed to develop and validate analytical methods of CAB in thermosensitive and mucoadhesive vaginal gels using a spectrophotometer UV-Visible. The developed analytical method was subsequently validated according to the International Conference Harmonization (ICH) guidelines. Linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) were established. The validated method was finally applied to determine the drug content in vaginal preparations, as well as the *in vitro* and *ex vivo* permeation profiles.

2. Materials and methods

2.1 Materials

Cabotegravir (CAB) was kindly provided by ViiV Healthcare Ltd. (Research Triangle Park, NC, USA). Pluronic® F127 and F68 were kindly gifted by BASF Indonesia, Jakarta. Other materials were analytical grade.

2.2 Preparation of simulated vaginal fluid

Simulated vaginal fluid (SVF) was prepared by weighing 5 g of glucose, 0.4 g of urea, 3.51 g of NaCl, 2 g of lactic acid, 1.4 g of KOH, 1 g of acetic acid, 0.22 g of $Ca(OH)_2$, and 0.016 g glycerin. Deionized water (800 mL) was added to dissolve the mixture, and the pH was adjusted to 4.2 [15]**.** Deionized water was then added to obtain 1 L of SVF. In this study, as SVF was used in the *in vitro* and *ex vivo* permeation studies, 20% v/v of methanol was added into SVF to achieve the sink condition during the experiment.

2.3 Preparation of CAB stock solution

An amount of 10 mg of CAB was carefully weighed and placed into a 10 mL volumetric flask. Afterwards, methanol was added to dissolve CAB, obtaining the concentration of 1000 µg/mL.

2.4 Determination of maximum UV light absorption wavelength, preparation of calibration standards and quality control samples

The maximum UV light absorption wavelength was determined using a UV-Visible spectrophotometer (Dynamica, HALO XB-10). CAB solutions, with the concentration of 50 µg/mL, in methanol (CAB-MeOH) and SVF (CAB-SVF) were scanned between 200-400 nm at room temperature. Furthermore, the calibration solutions were prepared in six different concentrations in triplicate in the range concentration between 0.5 µg/mL and 16 µg/mL by spiking the stock solution with methanol and SVF. In addition, quality control (QC) samples were prepared using each solvent in four different levels, namely lower limit of quantification (LLOQ), low quality control (LQC), medium quality control (MQC), and high quality control (HQC). For CAB-MeOH, the QC samples included LLOQ – 2.15 µg/mL, LQC - 4 µg/mL, MQC – 7.5 µg/mL, and HQC - 12 g/mL. For CAB-SVF, the QC samples were LLOQ – 2.2 µg/mL, LQC - 4 µg/mL, MQC – 7.5 µg/mL, and HQC - 12 µg/mL. All samples were prepared and measured in triplicate.

To prepare the calibration standards of CAB in vaginal tissue (CAB-VT) for *ex vivo* studies, fresh vaginal tissue of porcine was used. Vaginal mucosa matrices were initially prepared by mixing vaginal tissue with deionized water (9:1) using UltraTurrax homogenizer for 10 min. The calibration standard solutions were made by mixing 200 mL of drug stock solutions into 1.8 g of blank vaginal matrices to obtain the concentrations in the range of 1 µg/mL – 32 µg/mL. In addition, Quality Control (QC) samples in vaginal tissue include LLOQ – 5.13 µg/mL, LQC – 7.5 µg/mL, MQC – 15 µg/mL, and HQC 24 µg/mL were prepared.

2.5 Sample preparation and CAB extraction from vagina samples

The preparation of the vaginal mucosa sample was carried out to precipitate proteins and other molecules in the organ to avoid any interferences during the measurement. The CAB extraction method was performed using methanol and acetonitrile. The volumes of methanol and acetonitrile used to extract the drug were varied, as shown in Table 1. Initially, 1 g of the matrices-spiked CAB were mixed with the extraction solvent. The mixture was then homogenized for 10 minutes using a vortex mixer and centrifuged for 15 minutes, 14000 x g. The supernatant obtained was then placed at room temperature to allow the evaporation of the organic solvent. Finally, 1 mL of methanol was added to reconstitute the dry extract,

homogenized, and centrifuged as previously mentioned. The supernatant obtained was then measured using spectrophotometry UV-visible.

Table 1. Volume of organic solvent for CAB extraction from vaginal samples

Organic Solvent	Methods	Volume (mL)
Methanol	A	1
	B	3
	C	5
	D	7
Acetonitrile	A	1
	B	3
	C	5
	D	7

2.6 Preparation of thermosensitive and mucoadhesive vaginal gels

CAB thermosensitive gel was prepared by dissolving 16% w/w of Pluronic® F127 and 4% w/w of Pluronic® F68 in cold water using a magnetic stirrer to produce a gel base. Then, 1% w/w of CAB was dispersed in 5% w/w of (poly(ethylene glycol) (PEG) 400 before being added into the gel base. Finally, after all mixtures were homogenous, 0.1% w/w of DMDM hydantoin was added into the mixture and homogenized for 15 minutes at 1000 rpm.

CAB mucoadhesive gel was prepared by hydrating 0.5% w/w of Carbopol 940 in distilled water for 24 hours. Afterwards, 2% w/w of triethanolamine (TEA) was added into Carbopol 940, and the mixture was homogenized for 15 minutes, 1000 rpm to obtain a gel base. The 1% w/w of CAB was first dispersed in 5% w/w of PEG 400 before being added into the gel base. After all mixtures were homogenous, 0.1% w/w of DMDM hydantoin was added into the mixture and homogenized for 15 minutes at 1000 rpm.

2.7 Validation of analytical method

2.7.1 Specificity

Specificity was determined by comparing the UV spectra of blank thermosensitive gel, mucoadhesive gel and vaginal tissue with appropriate CAB standard solution after scanning between 200 – 400 nm. This parameter was assessed to identify any possible interferences between the responses of the analyte and other compounds at the relevant wavelength.

2.7.2 Linearity

Linearity was evaluated using six different concentrations of each CAB-MeOH, CAB-SVF and CAB-VT. The sample solutions were analyzed in triplicate using UV-Vis spectrophotometry at 276 nm for CAB-MeOH, 278 nm for CAB-SVF and 305 nm for CAB-VT. The calibration curve, which consisted of six different concentrations versus absorbance was analyzed to obtain the value of correlation coefficient (r^2), slope, and y-intercept [16].

2.7.3 Limit of detection (LOD)

LOD expresses the smallest concentration of analyte which can be identified in a sample [17]. LOD was determined by using eq. (1), where sy is the standard deviation of the blank (without analyte) and b is the slope obtained from the regression equation of the calibration curve

$$LOD = \frac{3.3sy}{b} \quad \text{(Equation 1)}$$

2.7.4 Lower limit of quantification (LOQ)

LLOQ is expressed as the smallest concentration of samples which can be determined accurately with satisfactory accuracy and precision [16]. LLOQ was determined by using eq. (2), where b is the slope obtained from the regression equation of calibration curve and sy is the standard deviation of the blank (without analyte) [18].

$$LLOQ = \frac{10sy}{b} \quad \text{(Equation 2)}$$

2.7.5 Accuracy and precision

Accuracy and precision demonstrate the closeness to the reference value and degree of scattering between a series of measurements attained from numerous testing in an analytical method. These parameters were evaluated by intra-day and inter-day measurements of QC samples (HQC, MQC, LQC, and LLOQ). Percentage of relative error (%RE) and relative standard deviation (%RSD) were calculated to represent the values of accuracy and precision, respectively [19].

2.7.6 Dilution integrity

Dilution integrity was evaluated by preparing 75 µg/mL for both CAB-MeOH and CAB -SVF and 150 µg/mL for CAB-VT. Each of the solutions was diluted 5 and 10 times with appropriate solvents. The experiment was in triplicate and the absorbance of the analyte was observed [17].

2.7.7 Extraction recovery

The extraction recovery determination was conducted by comparing the values obtained from all samples at LLOQ, LQC, MQC, and HQC extracted from vaginal tissue with the measured values of the concentrations of the same samples (LLOQ, LQC, MQC, and HQC) [20].

2.8 Application of the analytical method

2.8.1 Drug content measurement

A total of 0.1 g of gel was dissolved in methanol up to 10 mL in a volumetric flask, obtaining a CAB concentration of 100 µg/mL. The solution was then diluted by taking 1 mL of the gel solution and diluted with methanol up to 10 mL. The absorbance of the final solution was determined using UV-Vis spectrophotometry at 276 nm. Measurements were performed in triplicate.

2.8.2 In vitro permeation, ex vivo permeation and ex vivo retention studies of CAB from thermosensitive and mucoadhesive gels

The permeation behavior of CAB from thermosensitive and mucoadhesive gels was evaluated using Franz diffusion cells using a dialysis membrane (Spectra-Por®, 12,000 - 14,000 MWCO dialysis membrane) and porcine vaginal mucosa for *in vitro* and *ex vivo* studies, respectively. The average thickness of the vaginal mucosa was 2.18 ± 0.13 mm. The tissues were obtained from female pigs (three- to four-month-old). The receptor compartment was filled with 24 mL of the receptor media and the temperature was maintained at 37ºC. In this study, SVF containing 20% v/v of methanol was used as the receptor media to maintain the sink condition during the experiments. Furthermore, 1 g of formulation (equal to 10 mg of CAB) was placed in the donor compartment with a diffusion area of 4.9 cm^2. A sample of 1 ml from the receptor compartment was taken at the predetermined time points (0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, and 24 h) and replaced by the same volume of fresh medium. The absorbances of the samples were then measured using UV-Vis spectrophotometry at 278 nm. The calculated cumulative amount of CAB permeating through the dialysis membrane and the porcine vaginal mucosa, was plotted versus time [21].

Following the *ex vivo* permeation study, the concentration of CAB retained in the vaginal tissue was also determined. After 1 h, 8 h and 24 h, the vaginal tissue was removed from Franz cells and the gel remained on the surface of the tissue was carefully removed. Afterwards, CAB was extracted using the method described previously.

2.8.3 Mathematical Modelling for In Vitro and Ex Vivo Permeation Studies

The data obtained from the *in vitro* and *ex vivo* permeation studies were fitted to five different mathematical models, such as zero-order kinetics (ZO), first-order kinetics (FO), Higuchi, Korsmeyer – Peppas (KP), and Hixson – Crowell (HC) to determine the release kinetics of

CAB from thermosensitive gels and mucoadhesive gels. The equations of each model are described below [22]:

$$Zero\ Order\ Kinetics: C_t = C_0 + k_0 t \quad \text{(Equation 3)}$$

$$First\ Order\ Kinetics: \ln C_t = \ln C_0 + k_1 t \quad \text{(Equation 4)}$$

$$Higuchi\ Model: C_t = k_H \sqrt{t} \quad \text{(Equation 5)}$$

$$Korsmeyer - Peppas\ Model: C_t = k_{KP} t^n \quad \text{(Equation 6)}$$

$$Hixson - Crowell\ Model: C_t^{1/3} = C_0^{1/3} k_{HC} t \quad \text{(Equation 7)}$$

C_t represents CAB concentration at time t, C_0 represents the initial concentration of CAB in the media (t = 0), k_0 denotes the zero-order constant, k_1 denotes the first-order constant, k_H denotes the Higuchi constant, k_{KP} denotes the Korsmeyer - Peppas constant, and k_{HC} denotes the Hixson-Crowell constant. All calculations were carried out using the DD-solver software. The release kinetics were determined from the value of correlation coefficient (r^2) [23].

2.9 Statistical Analysis

All data were expressed as means ± standard deviation (SD). The values of mean, SD, relative standard deviation (RSD) and reduction of error (RE) were calculated utilizing Microsoft Excel® 2019 (Microsoft Corporation, Redmond, USA). To analyze the data statistically, GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA) was applied, where p value < 0.05 indicates a statistical difference.

3. Results and discussion

3.1 Selection of sample preparation method and drug extraction

In this work, CAB extraction from vaginal tissue was performed using organic solvents, which were methanol and acetonitrile. The results of each extraction method are presented in Table 2. The results showed that a higher amount of solvent used in the extraction process was able to increase the extraction efficiency. Additionally, it was also observed that methanol provided a higher extraction recovery than acetonitrile. Regarding the extraction of CAB using methanol, the results showed that methods C and D exhibited the highest extraction recovery percentages, which are 91.57% ± 0.66% and 92.12% ± 2.43%, respectively. Moreover, both of these methods showed no significant difference (p <0.05). Therefore, in this study, method C (5 mL methanol) was chosen as the most suitable extraction method as it provided a minimum amount of solvent with optimum extraction recovery.

Table 2. Mean extraction recovery of CAB of each method with methanol and acetonitrile from vaginal tissue (n = 3)

Organic Solvent	Methods	Volume (mL)	%Extraction Recovery ± SD	%RSD
Methanol	A	1	12.30 ± 1.16	9.44
	B	3	38.11 ± 3.40	8.92
	C	5	91.57 ± 0.66	0.72
	D	7	92.12 ± 2.43	2.64
Acetonitrile	A	1	8.80 ± 1.15	13.04
	B	3	18.32 ± 5.04	27.49
	C	5	34.96 ± 1.17	3.36
	D	7	59.66 ± 1.09	1.83

3.2 Selectivity of UV-Vis spectrophotometry method

The specificity test aimed to ensure the absence of interferences between CAB and other compounds present in the gel formulations and vaginal tissue during the analysis using the UV-Visible spectrophotometry [18,24]. As mentioned previously, the analytical method in methanol was used to determine the CAB concentration in the formulation. Moreover, the developments of the analytical method in SVF and vaginal tissue was carried out to determine the CAB in *in vitro* and *ex vivo* studies. A well-defined peak of CAB was each observed at 276 nm, 278 nm and 305 nm in methanol, SVF and vaginal tissue, respectively. The absorption spectra of blank thermosensitive and mucoadhesive gel (Figure 1. (A)) exhibited no additional peak at 276 nm. These results indicated no interference occurred due to the presence of other gel constituents [18]. Nonetheless, the absorption spectrum of blank vaginal tissue (in Figure 1. (B)) showed a peak appeared at 270 nm, indicating possible interference with CAB peak at 276 nm. Alternatively, another absorption peak of CAB was shown at 305 nm. At this wavelength, there was no peak observed at blank vaginal tissue spectra. Therefore, the developed method in this study has been specific at the appropriate wavelength.

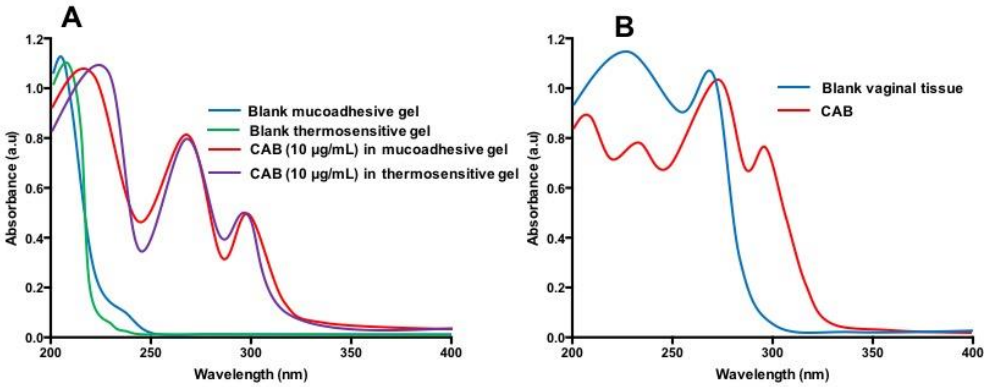

Figure 1. Representative UV-Spectra of CAB-MeOH, blank thermosensitive and mucoadhesive gel, (A); CAB-SVF and blank vaginal tissue (B)

3.3 Linearity, LOD, and LLOQ

In an attempt to assess the linearity and determine LOD and LLOQ values of the analytical method, a calibration curve was generated by measuring a set concentration of the standard solutions of CAB in methanol, SVF and vaginal tissue, respectively, using the optimized UV-Vis spectrophotometric. The spectrum of CAB standard solutions in methanol, SVF and vaginal tissue are shown in Figure 2. The linearity, LOD and LLOQ properties of CAB are summarized in Table 3 and Figure 3.

Table 3. Properties of the calibration curve for analysis of CAB with LOD and LLOQ values

Matrices	Concentration range (µg/mL)	r^2	LOD (µg/mL)	LLOQ (µg/mL)
MeOH	0.5 – 16	0.9990	0.71	2.15
SVF	0.5 – 16	0.9989	0.74	2.22
Vaginal Tissue	1 – 32	0.9985	1.69	5.13

It was observed that following the construction of the calibration curve, all samples showed bilinear curves. The low concentrations showed a slightly higher slope than the higher concentrations of CAB. The difference in slope values in bilinear could decrease the sensitivity of the methods since the calculation of LOD and LOQ values of the analytical method depends on the slope values of the calibration curves (Equation 1 and Equation 2). Despite this, no significant difference ($p > 0.05$) was found in the slope values in low and high concentrations of CAB. Importantly, a linear connection was obtained between the absorbance and the concentration in the range of 0.5 – 16 µg/mL for CAB-MeOH and CAB-SVF, and 1 – 32

µg/mL for CAB-vaginal tissue. The correlation coefficient values (r^2) of three regression equations of CAB in methanol, SVF, and vaginal tissue were 0.9990, 0.9989, and 0.9985, respectively, indicating the acceptable linearity. Furthermore, the LOD and LLOQ values of CAB in methanol were 0.71 and 2.15 µg/mL, in SVF were 0.74 and 2.22 µg/mL, and in vaginal tissue were 1.69 and 5.13 µg/mL.

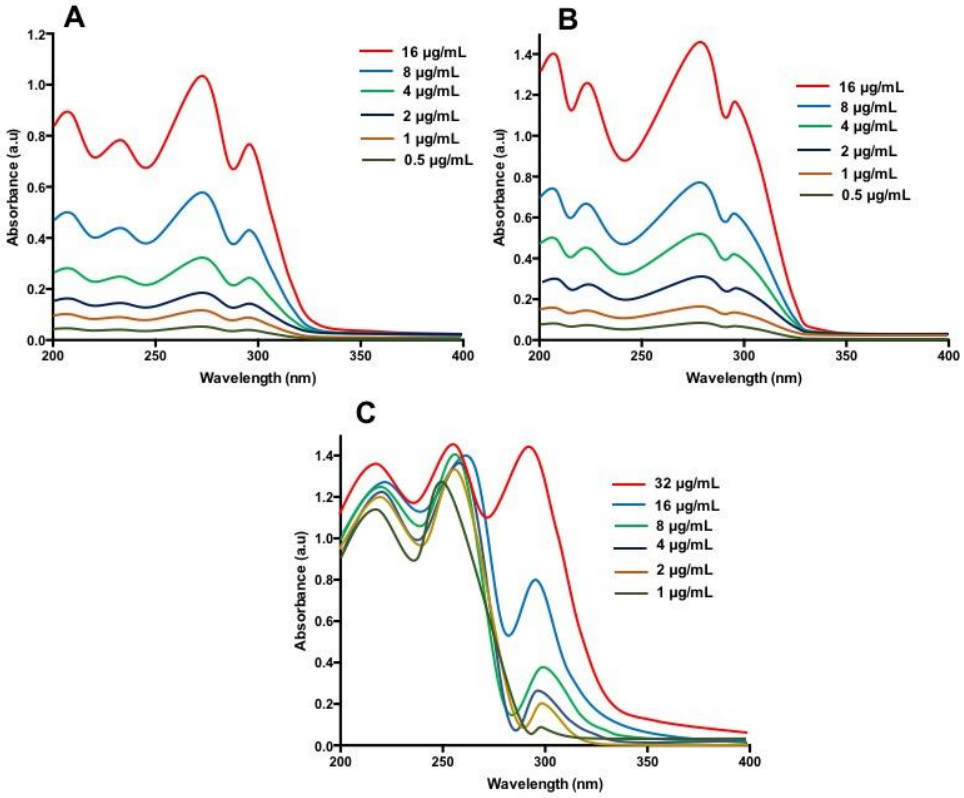

Figure 2. Spectrum of CAB standard solutions in MeOH (A), SVF (B) and vaginal tissue (C)

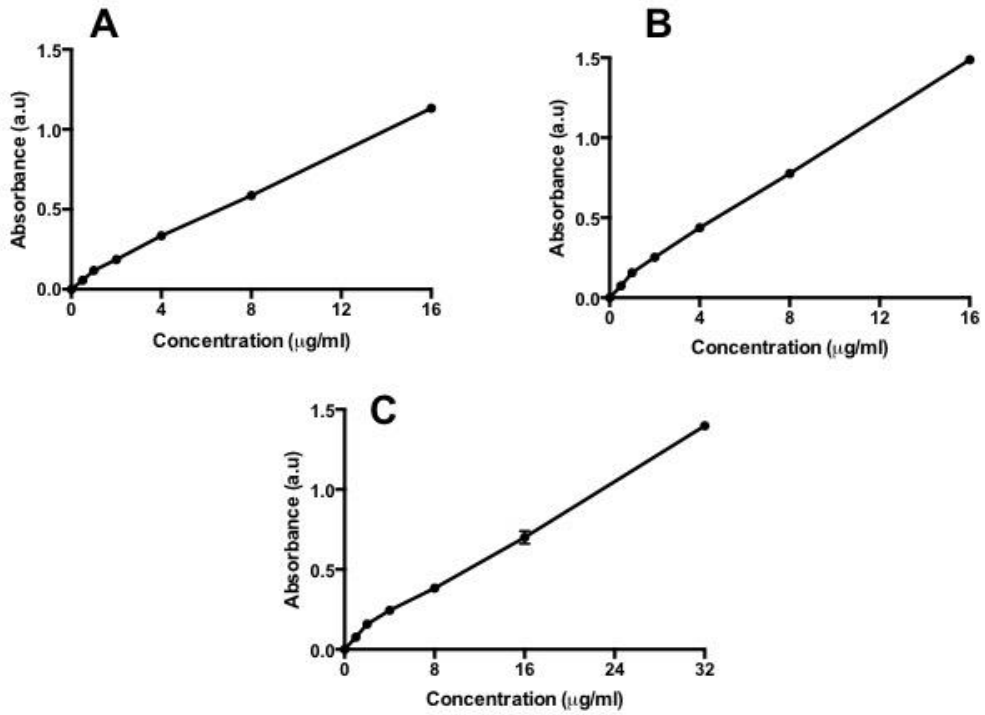

Figure 3. Calibration curve in MeOH (A), SVF (B) and Vaginal tissue (C) (mean ± SD, n= 3)

3.4 Accuracy and precision

The determinations of accuracy in the intra-day and inter-day measurements using the developed methods were found to be accurate for methanol (Table 4), SVF (Table 5), and vaginal tissue (Table 6) with the percentage of error value below 15%, which met the requirements from ICH guidelines. The precision of intra-day and inter-day was also found to be acceptable. The intra-day and inter-day precision in all solvents showed %RSD value that ranged of 0.5% - 12% and 0.5% - 10.5% which fulfill the limit from ICH guideline (15%). Consequently, the developed method using UV-Vis spectrophotometry for CAB was found to be accurate and precise.

Table 4. The results of precision and accuracy evaluations of the UV-Vis spectrophotometry method for analysis of CAB in MeOH (mean ± SD, n= 3)

Intra-day Precision and Accuracy				
Replication	**Concentration added (µg/mL)**	**Concentration found (µg/ mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	2.15	2.07 ± 0.10	4.63	-3.50
	4	3.91 ± 0.15	3.94	-2.19
	7.5	7.63 ± 0.11	1.39	1.76
	12	11.54 ± 0.18	1.53	-3.86
2	2.15	2.12 ± 0.12	5.87	-1.23
	4	4.13 ± 0.09	2.28	3.31
	7.5	7.71 ± 0.18	2.32	2.80
	12	12.19 ± 0.18	1.46	1.60
3	2.15	2.18 ± 0.08	3.56	1.28
	4	4.09 ± 0.18	4.51	2.33
	7.5	7.85 ± 0.12	1.52	4.63
	12	12.06 ± 0.48	3.95	0.50
Inter-day Precision and Accuracy				
Day	**Concentration added (µg/mL)**	**Concentration found (µg/ mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	2.15	2.41 ± 0.08	3.35	11.96
	4	4.23 ± 0.26	6.21	5.63
	7.5	7.50 ± 0.17	2.22	-0.07
	12	12.43 ± 0.35	2.81	3.56
2	2.15	2.10 ± 0.15	7.00	-2.36
	4	4.02 ± 0.24	5.86	0.62
	7.5	7.63 ± 0.09	1.15	1.76
	12	11.70 ± 0.39	3.34	-2.47
3	2.15	2.07 ± 0.12	5.78	-3.50
	4	3.99 ± 0.19	4.86	-0.35
	7.5	7.66 ± 0.41	5.32	2.09
	12	11.90 ± 0.38	3.16	-0.84

Table 5. The results of precision and accuracy evaluations of the UV-Vis spectrophotometry method for analysis of CAB in SVF (mean ± SD, n= 3)

Intra-day Precision and Accuracy				
Replication	**Concentration added (µg/mL)**	**Concentration found (µg/ mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	2.2	1.89 ± 0.14	7.37	-13.88
	4	3.97 ± 0.08	2.00	-0.68
	7.5	7.43 ± 0.12	1.66	-0.90
	12	12.20 ± 0.10	0.83	1.64
2	2.2	2.31 ± 0.20	8.46	4.91
	4	4.05 ± 0.09	2.11	1.28
	7.5	7.48 ± 0.12	1.61	-0.20
	12	12.16 ± 0.18	1.45	1.33
3	2.2	2.28 ± 0.25	10.79	3.56
	4	4.15 ± 0.07	1.62	3.79
	7.5	7.68 ± 0.25	3.27	2.38
	12	12.10 ± 0.14	1.17	0.86
Inter-day Precision and Accuracy				
Day	**Concentration added (µg/mL)**	**Concentration found (µg/ mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	2.2	2.11 ± 0.15	6.92	-4.06
	4	3.97 ± 0.08	2.00	-0.68
	7.5	7.66 ± 0.37	4.80	2.18
	12	12.92 ± 0.39	2.98	7.66
2	2.2	2.09 ± 0.20	9.65	-5.08
	4	3.95 ± 0.14	3.49	-1.33
	7.5	7.86 ± 0.08	1.02	4.86
	12	12.65 ± 0.07	0.59	5.45
3	2.2	1.96 ± 0.08	4.04	-10.83
	4	4.08 ± 0.08	1.92	1.93
	7.5	7.48 ± 0.10	1.29	-0.30
	12	12.14 ± 0.31	2.52	1.17

Table 6. The results of precision and accuracy evaluations of UV-Vis spectrophotometry method for analysis of CAB in vaginal tissue (mean ± SD, n= 3)

Intra-day Precision and Accuracy				
Replication	**Concentration added (µg/mL)**	**Concentration found (µg/ mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	5.13	5.29 ± 0.34	6.36	3.21
	7.5	7.41 ± 0.65	8.76	-1.20
	15	14.31 ± 0.49	3.43	-4.60
	24	25.19 ± 0.33	1.30	4.96
2	5.13	4.88 ± 0.62	12.66	-4.91
	7.5	7.22 ± 0.46	6.33	-3.76
	15	14.56 ± 0.48	3.30	-2.95
	24	24.89 ± 0.54	2.18	3.73
3	5.13	5.29 ± 0.33	6.27	3.06
	7.5	7.37 ± 0.53	7.15	-1.73
	15	15.26 ± 1.05	6.88	1.75
	24	24.24 ± 1.75	7.22	0.99
Inter-day Precision and Accuracy				
Day	**Concentration added (µg/mL)**	**Concentration found (µg/ mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	5.13	5.21 ± 0.24	4.62	1.50
	7.5	7.67 ± 0.28	3.60	2.33
	15	14.15 ± 1.15	8.16	-5.67
	24	23.99 ± 1.46	6.08	-0.05
2	5.13	5.00 ± 0.25	4.94	-2.57
	7.5	7.17 ± 0.40	5.55	-4.40
	15	14.18 ± 0.75	5.29	-5.46
	24	24.68 ± 1.35	5.46	2.82
3	5.13	5.13 ± 0.37	7.16	-0.07
	7.5	7.39 ± 0.74	10.03	-1.52
	15	13.64 ± 0.62	4.58	-9.04
	24	24.23 ± 1.72	7.11	0.95

3.5 Dilution integrity

In an attempt to analyze the effect of dilution integrity of the analytical method, high concentrated samples were diluted 5 and 10 times using the appropriate matrices. This experiment showed satisfactory results for all dilution integrity in methanol, SVF, and vaginal tissue with of less than 15% bias. The precision parameter was found to be acceptable with %RSD value between 3.5% – 6.7%. Therefore, these results showed that CAB concentrations higher than the upper range of the calibration standards could be analyzed with suitable dilution.

3.6 Extraction Recovery

Extraction recovery from the method used in this study was obtained by comparing the sample concentrations of LLOQ, LQC, MQC, and HQC extracted from vaginal tissue with the concentrations obtained from measuring samples at the same concentrations. The results of the average extraction recovery are exhibited in Table 7. The %RSD values obtained were in the range of ±15%. Thus, the extraction technique was found to be precise, consistent and repeatable.

Table 7. Mean extraction recovery of CAB in vaginal tissue (n = 3)

Sample	Concentration (µg/mL)	%Extraction Recovery ± SD	%RSD
Vaginal Tissue	LLOQ (5,13)	90.38 ± 1.24	1.37
	QC (7,5)	93.34 ± 4.61	4.94
	MQC (15)	93.39 ± 4.34	4.65
	HQC (24)	92.16 ± 5.46	5.92

3.7 Application of the analytical method

The validated spectrophotometry UV-Visible method was further used to determine the amount of CAB in the thermosensitive and mucoadhesive gels. As depicted in Figure 4(A), the result showed that the recovery values of CAB were 99.24 ± 2.88 % and 99.19 ± 1.34% from the thermosensitive and mucoadhesive gels, respectively. These findings indicated that the formulation of CAB into thermosensitive and mucoadhesive gels did not affect the CAB concentration. According to the ICH recommendation for acceptable recovery percentage, all formulations also showed acceptable recovery percentage, within the range of 95 – 105% [25].

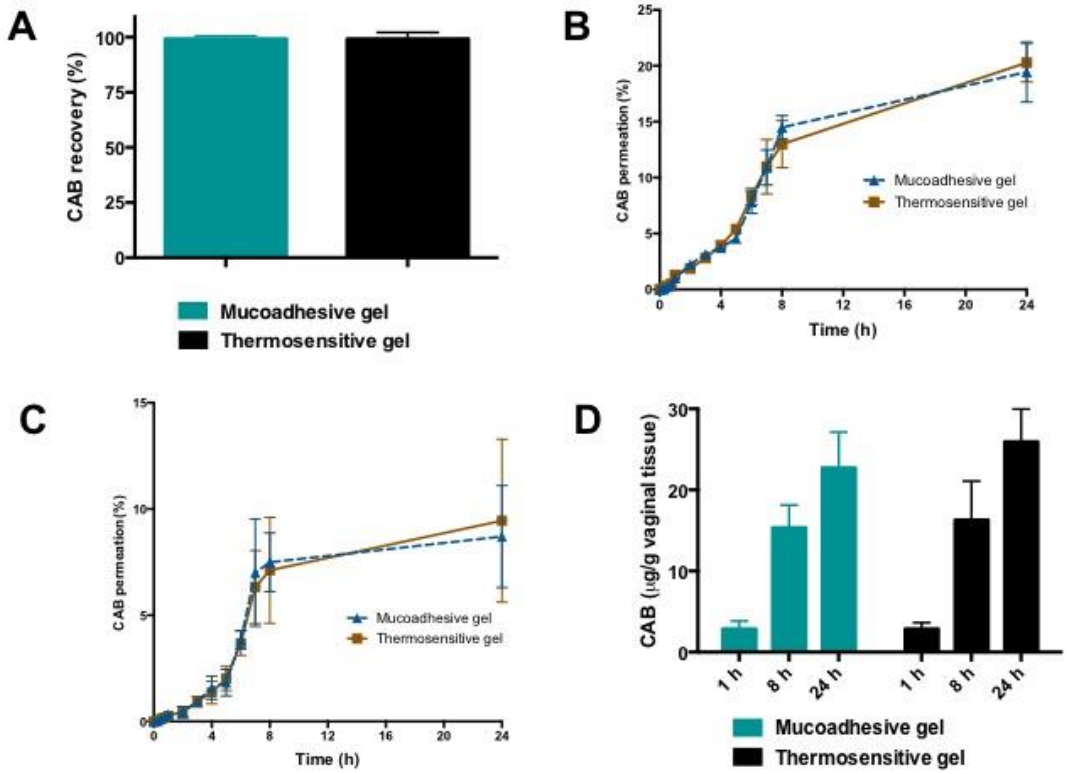

Figure 4. (A) CAB recovery (%) from gels, (B) *In vitro* permeation profile of CAB from thermosensitive and mucoadhesive gel, (C) *Ex vivo* permeation profile of CAB from thermosensitive and mucoadhesive gel, (D) *Ex vivo* retention of CAB (mean ± SD, n= 3)

The cumulative amount of CAB permeation following *in vitro* and *ex vivo* permeation studies and *ex vivo* retention studies of the thermosensitive and mucoadhesive gels were also determined by the validated analytical method. Figure 4(B) represents the *in vitro* permeation profile of CAB. After 24 hours, 20.29 ± 1.72 % of CAB permeated from the thermosensitive gel and 19.44 ± 2.69% of CAB permeated from the mucoadhesive gel. Figure 4(C) depicts the results of *ex vivo* permeation studies, showing that after 24 hours, 9.45 ± 3.83 % and 8.71 ± 2.40 % of CAB were able to permeate through vaginal tissue from the thermosensitive and mucoadhesive gels, respectively. The permeation of drugs from Pluronic based gel could occur through diffusion from micelles formed. Higher concentrations of Pluronic produce lower diffusion of the drug due to the increased number and size micelles formed in the gel structure [26]. Analyzed statistically, the release profiles between thermosensitive and mucoadhesive gels were not significantly different ($p < 0.05$).

The result of the *ex vivo* retention study is revealed in Figure 4 (D). The amount of CAB retained in the vaginal mucosa after 24 hours from the thermosensitive and mucoadhesive gels were 22.71 ± 4.44 µg/g vaginal tissue and 25.94 ± 4.04 µg/g vaginal tissue, respectively.

Despite non-significant difference ($p > 0.05$), it was found that mucoadhesive gel has a slightly higher concentration of CAB localized in vagina mucosa. This might be due to the presence of Carbopol as a mucoadhesive polymer. Carbopol has carboxyl groups that bind strongly through a hydrogen bond with the oligosaccharide chain of mucin [27].

The results obtained from the *in vitro* and *ex vivo* permeation studies were further fitted to five mathematical models in order to explain CAB release behavior from both thermosensitive and mucoadhesive gels. For the *in vitro* permeation of CAB from the thermosensitive gel, the value of coefficient correlation was 0.8613, 0.8936, 0.8240, 0.9247, 0.8836 for ZO, FO, Higuchi, KP, and HC, respectively. For the *in vitro* permeation of CAB from the mucoadhesive gel, the value of coefficient correlation was 0.8116, 0.8461, 0.7977, 0.8850, 0.8354 for ZO, FO, Higuchi, KP, and HC, respectively. Furthermore, the *ex vivo* permeation study showed coefficient correlation values of 0.7794, 0.7939, 0.7338, 0.8318, 0.7892 for thermosensitive gel and 0.6838, 0.7015, 0.7036, 0.7689, 0.6957 for mucoadhesive gel for Zero order, First order, Higuchi, Korsmeyer Peppas, and Hixson-Crowell, respectively. The result obtained clearly showed that all formulations tested in the *in vitro* and *ex vivo* permeation studies followed Korsmeyer-Peppas kinetic models. This model has been used to describe drug release from the polymeric matrix based on relaxation and diffusion [28].

Based on the results obtained, the validated analytical methods using spectrophotometry UV-visible were successfully applied to determine the amount of CAB in the thermosensitive and mucoadhesive gels. Moreover, the methods were also be able to determine the concentration of CAB following *in vitro* and *ex vivo* permeation tests, as well as *ex vivo* retention tests. Moving forward, *in vivo* studies using suitable animal models for both types of gels are essential to be carried out to obtain pharmacokinetic and pharmacodynamic profiles of CAB.

4. Conclusion

This study was conducted to develop and validate spectrophotometry UV-visible methods for the analysis of CAB. The proposed method was validated in the parameter of selectivity, accuracy and precision, linearity, LOD and LLOQ, and dilution integrity, as well as extraction recovery of CAB and vaginal tissue. Additionally, the suitable extraction method of CAB from vaginal tissue was also determined. The results showed that all validation parameters were well-established and met the requirements of ICH guidelines. Moreover, the validated analytical method was successfully employed to evaluate the percentage recovery, permeation profiles, and retention of CAB following each appropriate study. In conclusion, the validated

method was able to be used for various studies of CAB in thermosensitive and mucoadhesive gel formulations.

Disclosure of interest

The authors declare no conflicts of interest.

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Credit Author Statement

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Reviewers' comments:

Reviewer #1: Overall, the paper is significantly improved after the revision by authors. However, as method validation is one of the major goals of this paper, the additional explanations on calculation of Table 3 limits of detection and quantitation (LOD and LLOQ) are still required, in my opinion. Please see the comment 1 below. Comments 2 and 3 are optional.

1) Lines 311-317. I commend the authors for taking my previous comment seriously. However, the provided explanations, in my opinion, are confusing and, therefore, should still be updated. For example, it is still not clear to me which slope the authors ultimately used to calculate the detection and quantitation limits, LOD and LLOQ, presented in Table 3. In addition, while statistically comparing the slopes, the authors claim "p>0.05" in line 316. If this is a typing error, it must be fixed. If p-value exceeds 0.05 indeed, then it is not clear why authors concluded that these slopes are not statistically different.

Anyway, as method validation rather than advanced statistics is the main point of the paper, my suggestions for the authors are:

Option 1: make the explanation as simple as possible - just mention which of "bilinear" slopes (or combined slope) they used for LOD and LLOQ calculations presented in Table 3, and why authors think that the selected slope provides more accurate estimate of the detection and quantitation limits than the other slope. The additional details about the statistical significance or statistical difference between the slopes can be omitted for the sake of simplicity.

Option 2: if the authors choose to include the proof that the observed slopes are not statistically different, then full information about both datasets that are being compared, description of the statistical test or method, as well as the exact p-value (especially, if this value exceed 0.05 as stated in line 316) must be included.

2) Lines 95, 98, 99, 104 - "Spectrophotometer UV-visible" is suggested to be replaced with "UV-visible spectrophotometer."

3) Line 263 - term "statistical significance" rather than "statistical difference" is preferred to be used for p-value.

Reviewer #2: The title is still not appropriate, not clear. Is the formulation intended for ex vivo vaginal delivery of the CAB-loaded gel(s)? Please, re-write

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Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue in ex vivo permeation and retention studies from thermosensitive and mucoadhesive gels

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Abstract:	Cabotegravir (CAB) is an antiretroviral therapy (ARV) used for Human Immunodeficiency Virus (HIV) treatment. CAB has low solubility, which affects its bioavailability in oral therapy. Moreover, the injection form of CAB has difficulty in the administration process. Therefore, it is essential to develop a new drug delivery system for CAB. Vaginal drug delivery system offers many advantages such as a large surface area, increased drug bioavailability, and improved drug delivery. CAB was developed in thermosensitive and mucoadhesive vaginal gel preparations that provided optimal distribution in the vaginal mucosa. To support the process of formulation development, in this study, UV-visible spectrophotometry method was validated in methanol, simulated vaginal fluid (SVF) and vaginal tissue to quantify the amount of CAB in the gel preparations, in vitro , and ex vivo studies, respectively. The developed analytical method was subsequently validated according to ICH guidelines. The calibration curves in these matrices were found to be linear with correlation coefficient values (R^2) ≥ 0.998. The LLOQ values in methanol, SVF and vaginal tissue were 2.15 µg/mL,2.22 µg/mL,and 5.13 µg/mL, respectively. The developed method was found to be accurate and precise without being affected by dilution integrity. These methods were successfully applied to quantify the amount of CAB in gel preparations, in vitro , and ex vivo studies, showing uniformity of drug content and controlled release manner in the permeationprofile for 24 hours for both thermosensitive and mucoadhesive vaginal gels. Further analytical method is required to be developed for the quantification of CAB in in vivo studies.

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The Editor
Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

October 20, 2021

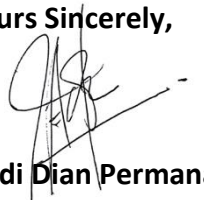
Dear Sir/Madam,

I wish you to consider our manuscript for publication in **Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy**. Following the reviewer comments, we have changed our title from from "**Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue for *ex vivo* vaginal delivery of thermosensitive and mucoadhesive vaginal gels**" to "**Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue in *ex vivo* permeation and retention studies from thermosensitive and mucoadhesive gels**". Importantly, we have addressed all comments from all reviewers as shown in "Response to Reviewer" file.

The manuscript has not been previously published in any language anywhere and it is not under simultaneous consideration by another journal. We have no conflicts of interest.

We appreciate your attention. We hope you will now consider publishing our research in **Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy** and look forward to hearing from you in due course.

Yours Sincerely,



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Title: Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue in *ex vivo* permeation and retention studies from thermosensitive and mucoadhesive gels

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

Reviewer #1: Overall, the paper is significantly improved after the revision by authors. However, as method validation is one of the major goals of this paper, the additional explanations on calculation of Table 3 limits of detection and quantitation (LOD and LLOQ) are still required, in my opinion. Please see the comment 1 below. Comments 2 and 3 are optional.

Response to Reviewer

We are very thankful to the reviewers for taking the time to provide helpful comments for improvements to our manuscript. We are grateful that the reviewer thinks that our manuscript is significantly improved after the revision. We have addressed each of the reviewers' comments in detail below.

1) Lines 311-317. I commend the authors for taking my previous comment seriously. However, the provided explanations, in my opinion, are confusing and, therefore, should still be updated. For example, it is still not clear to me which slope the authors ultimately used to calculate the detection and quantitation limits, LOD and LLOQ, presented in Table 3. In addition, while statistically comparing the slopes, the authors claim "p>0.05" in line 316. If this is a typing error, it must be fixed. If p-value exceeds 0.05 indeed, then it is not clear why authors concluded that these slopes are not statistically different.

Anyway, as method validation rather than advanced statistics is the main point of the paper, my suggestions for the authors are:

Option 1: make the explanation as simple as possible - just mention which of "bilinear" slopes (or combined slope) they used for LOD and LLOQ calculations presented in Table 3, and why authors think that the selected slope provides more accurate estimate of the detection and quantitation limits than the other slope. The additional details about the statistical significance or statistical difference between the slopes can be omitted for the sake of

simplicity.

Option 2: if the authors choose to include the proof that the observed slopes are not statistically different, then full information about both datasets that are being compared, description of the statistical test or method, as well as the exact p-value (especially, if this value exceed 0.05 as stated in line 316) must be included.

Response to Reviewer

We thank the reviewer for this valuable suggestion. The range values of 0.5 – 16 µg/mL for CAB-MeOH and CAB-SVF, and 1 – 32 µg/mL for CAB-vaginal tissue were used for the calculation of LOD and LLOQ. Following the suggestion, we used the suggestion in option 2 in the revised manuscript. We have included the full information suggested by the reviewer, as follows:

"It was observed that following the construction of the calibration curve, all samples showed bilinear curves. The low concentrations (0.5 - 2 µg/mL for MeOH and SVF, and 1 - 4 µg/mL for vaginal tissue) showed a slightly higher slope than the higher concentrations (2 -16 µg/mL for MeOH and SVF, and 4 - 16 µg/mL for vaginal tissue) of CAB. The difference in slope values in bilinear could decrease the sensitivity of the methods since the calculation of LOD and LOQ values of the analytical method depends on the slope values of the calibration curves (Equation 1 and Equation 2). Despite this, analyzed statistically using an unpaired *t-test*, no significant difference (p = 0.092, 0.369 and 0.114 for MeOH, SFV and vaginal tissue, respectively) was found in the slope values in low and high concentrations of CAB. Importantly, a linear connection was obtained between the absorbance and the concentration in the range of 0.5 – 16 µg/mL for CAB-MeOH and CAB-SVF, and 1 – 32 µg/mL for CAB-vaginal tissue. The correlation coefficient values (r^2) of three regression equations of CAB in methanol, SVF, and vaginal tissue were 0.9990, 0.9989, and 0.9985, respectively, indicating the acceptable linearity. Therefore, the range values of 0.5 – 16 µg/mL for CAB-MeOH and CAB-SVF, and 1 – 32 µg/mL for CAB-vaginal tissue could be used for the calculation of LOD and LLOQ."

We have included these explanations in the revised manuscript (Line 311-326)

2) Lines 95, 98, 99, 104 - "Spectrophotometer UV-visible" is suggested to be replaced with "UV-visible spectrophotometer."

Response to Reviewer

We have corrected this.

3) Line 263 - term "statistical significance" rather than "statistical difference" is preferred to be used for p-value.

Response to Reviewer

We have corrected this.

Reviewer #2: The title is still not appropriate, not clear. Is the formulation intended for ex vivo vaginal delivery of the CAB-loaded gel(s)? Please, re-write

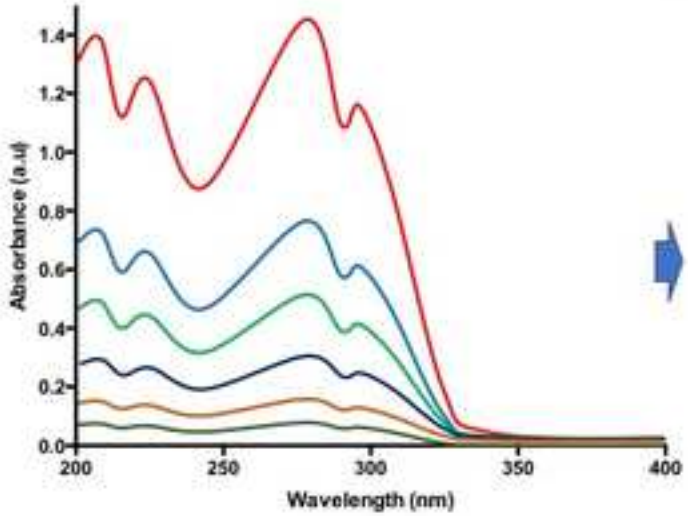
Response to Reviewer

We thank the reviewer for the suggestion and question. In this study, we evaluated the *ex vivo* permeation and retention studies of CAB from thermosensitive and mucoadhesive gels. Accordingly, we have changed our title to "Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue in *ex vivo* permeation and retention studies from thermosensitive and mucoadhesive gels".

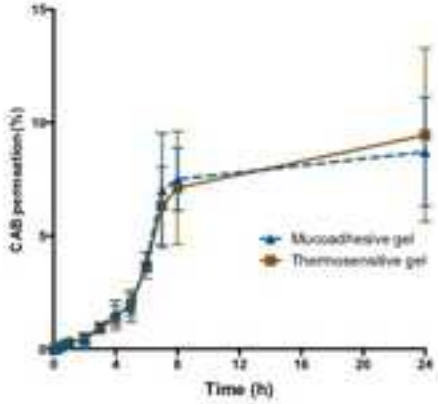
Graphical Abstract

Thermosensitive and mucoadhesive vaginal gels of cabotegravir

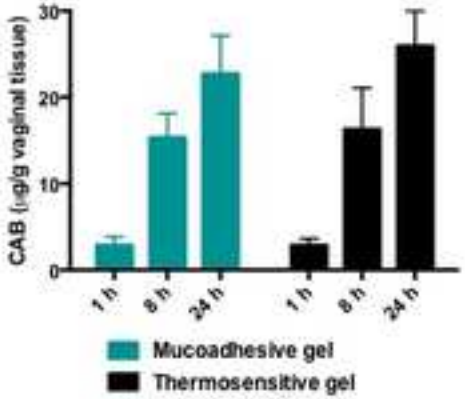
Spectrophotometric validation



CAB spectrum in vaginal tissue



***Ex vivo permeation* study**



***Ex vivo retention* study**

Highlights:

- Spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue was developed
- The analytical method was validated according to ICH guidelines
- The validated method was applied in *in vitro* study and *ex vivo* vaginal delivery of thermosensitive and mucoadhesive vaginal gels

Validation of spectrophotometric method to quantify cabotegravir in simulated vaginal fluid and porcine vaginal tissue for *ex vivo* vaginal delivery of thermosensitive and mucoadhesive vaginal gels

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Abstract

Cabotegravir (CAB) is an antiretroviral therapy (ARV) used for Human Immunodeficiency Virus (HIV) treatment. CAB has low solubility, which affects its bioavailability in oral therapy. Moreover, the injection form of CAB has difficulty in the administration process. Therefore, it is essential to develop a new drug delivery system for CAB. Vaginal drug delivery system offers many advantages such as a large surface area, increased drug bioavailability, and improved drug delivery. CAB was developed in thermosensitive and mucoadhesive vaginal gel preparations that provided optimal distribution in the vaginal mucosa. To support the process of formulation development, in this study, UV-visible spectrophotometry method was validated in methanol, simulated vaginal fluid (SVF) and vaginal tissue to quantify the amount of CAB in the gel preparations, *in vitro*, and *ex vivo* studies, respectively. The developed analytical method was subsequently validated according to ICH guidelines. The calibration curves in these matrices were found to be linear with correlation coefficient values (R^2) ≥ 0.998. The LLOQ values in methanol, SVF and vaginal tissue were 2.15 µg/mL, 2.22 µg/mL, and 5.13 µg/mL, respectively. The developed method was found to be accurate and precise without being affected by dilution integrity. These methods were successfully applied to quantify the amount of CAB in gel preparations, *in vitro*, and *ex vivo* studies, showing uniformity of drug content and controlled release manner in the permeation profile for 24 hours for both thermosensitive and mucoadhesive vaginal gels. Further analytical method is required to be developed for the quantification of CAB in *in vivo* studies.

Keywords: Cabotegravir, UV-Vis spectrophotometry, mucoadhesive, thermosensitive, validation

1. Introduction

Cabotegravir (CAB) is known as an HIV-1 integrase inhibitor, one of the antiretroviral medications for HIV patients. It is categorized as Biopharmaceutics Drug Disposition Classification System (BDDCS) II, which has low solubility and high permeability, affecting the absorption of the drug [1,2]. It has been reported that the solubility of CAB in the aqueous solution is limited to 4.8 µg/mL [3]. Furthermore, the log P value of CAB is 1.04 [4]. Currently, CAB is available in tablet and injection forms. However, following oral administration, many HIV patients were found to develop oral candidiasis, causing difficulty in swallowing. [5]. On the other hand, the long-acting injectable form of CAB also possesses several limitations. Despite having less frequency of administration, CAB injection needs to be administered by healthcare professionals. Additionally, the injection form is not comfortable and painful, which could lead to a decrease in the patient compliance to the medications [6].

In order to increase patient compliance and obtain sustained drug delivery, in this study, CAB was separately developed in the form of thermosensitive and mucoadhesive vaginal gels. Thermosensitive dosage forms would enable easy administration due to the liquid form of the formulation, which would transform to semisolid form in the vaginal temperature [7]. Furthermore, the mucoadhesive preparations offer the advantage, mainly due to their ability to prolong the contact of the drugs in the vaginal tissue [8]. The delivery of CAB through vaginal delivery can be an alternative therapy as it provides various advantages, including large surface area and rich blood supply, resulting in higher drug bioavailability. Importantly, due to the presence of receptors and coreceptors of HIV, such as CD4+ T cells and CCR5, vagina is the main route of HIV infection [9]. Therefore, vaginal administration of CAB could be an alternative delivery approach for the treatment and prevention of HIV.

Drug detection and quantification are essential parts of the development of novel drug delivery systems. Accordingly, an appropriate analytical method is crucial to be developed for this purpose. In this study, calculation of drug content in the formulations and measurement of drug concentration in *in vitro* and *ex vivo* studies were critical evaluations during the development of CAB thermosensitive and mucoadhesive vaginal gels. *In vitro* studies are an initial step to evaluate drug release behavior using a suitable release medium. Besides, *ex vivo* studies are performed using tissues from organisms by mimicking natural conditions. *Ex vivo* studies are advantageous for evaluating the drug behavior in specific organs/tissues as they can eliminate other physiological factors, resulting in more controlled conditions compared to living organisms. This indicates the importance of *ex vivo* experiments before proceeding to *in vivo* studies [10]. As the gel was intended for vaginal application, the concentration of CAB

was measured both in the simulated vaginal fluid (SVF) and vaginal mucosa. To the best of our knowledge, analytical methods to quantify CAB in both of these media have not been reported previously. Various analytical methods have been developed to quantify CAB in various matrices, including HPLC-UV [11] and HPLC-MS/MS [12]. However, the reported methods are expensive, time-consuming and require sophisticated equipment and, thus, are difficult to apply in the middle- and low-income countries/laboratories. On the other hand, spectrophotometer UV-visible for CAB detection and quantification in *in vitro* and *in vitro* studies would be promising due to its simplicity, cost-effectiveness and high adaptability in small laboratories. Therefore, in the present study, for the first time, CAB quantification methods were developed in SVF and vaginal mucosa using a spectrophotometer UV-visible. Several previous studies have reported the ability of spectrophotometer UV-visible in quantifying numerous types of drugs in both *in vitro* and *ex vivo* studies [13,14]. Following the development of analytical procedures, method validation must be carried out to ensure reliability, traceability, and comparability of the results.

This study aimed to develop and validate analytical methods of CAB in thermosensitive and mucoadhesive vaginal gels using a spectrophotometer UV-Visible. The developed analytical method was subsequently validated according to the International Conference Harmonization (ICH) guidelines. Linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) were established. The validated method was finally applied to determine the drug content in vaginal preparations, as well as the *in vitro* and *ex vivo* permeation profiles.

2. Materials and methods

2.1 Materials

Cabotegravir (CAB) was kindly provided by ViiV Healthcare Ltd. (Research Triangle Park, NC, USA). Pluronic® F127 and F68 were kindly gifted by BASF Indonesia, Jakarta. Other materials were analytical grade.

2.2 Preparation of simulated vaginal fluid

Simulated vaginal fluid (SVF) was prepared by weighing 5 g of glucose, 0.4 g of urea, 3.51 g of NaCl, 2 g of lactic acid, 1.4 g of KOH, 1 g of acetic acid, 0.22 g of $Ca(OH)_2$, and 0.016 g glycerin. Deionized water (800 mL) was added to dissolve the mixture, and the pH was adjusted to 4.2 [15]**.** Deionized water was then added to obtain 1 L of SVF. In this study, as SVF was used in the *in vitro* and *ex vivo* permeation studies, 20% v/v of methanol was added into SVF to achieve the sink condition during the experiment.

2.3 Preparation of CAB stock solution

An amount of 10 mg of CAB was carefully weighed and placed into a 10 mL volumetric flask. Afterwards, methanol was added to dissolve CAB, obtaining the concentration of 1000 µg/mL.

2.4 Determination of maximum UV light absorption wavelength, preparation of calibration standards and quality control samples

The maximum UV light absorption wavelength was determined using a UV-Visible spectrophotometer (Dynamica, HALO XB-10). CAB solutions, with the concentration of 50 µg/mL, in methanol (CAB-MeOH) and SVF (CAB-SVF) were scanned between 200-400 nm at room temperature. Furthermore, the calibration solutions were prepared in six different concentrations in triplicate in the range concentration between 0.5 µg/mL and 16 µg/mL by spiking the stock solution with methanol and SVF. In addition, quality control (QC) samples were prepared using each solvent in four different levels, namely lower limit of quantification (LLOQ), low quality control (LQC), medium quality control (MQC), and high quality control (HQC). For CAB-MeOH, the QC samples included LLOQ – 2.15 µg/mL, LQC - 4 µg/mL, MQC – 7.5 µg/mL, and HQC - 12 g/mL. For CAB-SVF, the QC samples were LLOQ – 2.2 µg/mL, LQC - 4 µg/mL, MQC – 7.5 µg/mL, and HQC - 12 µg/mL. All samples were prepared and measured in triplicate.

To prepare the calibration standards of CAB in vaginal tissue (CAB-VT) for *ex vivo* studies, fresh vaginal tissue of porcine was used. Vaginal mucosa matrices were initially prepared by mixing vaginal tissue with deionized water (9:1) using UltraTurrax homogenizer for 10 min. The calibration standard solutions were made by mixing 200 mL of drug stock solutions into 1.8 g of blank vaginal matrices to obtain the concentrations in the range of 1 µg/mL – 32 µg/mL. In addition, Quality Control (QC) samples in vaginal tissue include LLOQ – 5.13 µg/mL, LQC – 7.5 µg/mL, MQC – 15 µg/mL, and HQC 24 µg/mL were prepared.

2.5 Sample preparation and CAB extraction from vagina samples

The preparation of the vaginal mucosa sample was carried out to precipitate proteins and other molecules in the organ to avoid any interferences during the measurement. The CAB extraction method was performed using methanol and acetonitrile. The volumes of methanol and acetonitrile used to extract the drug were varied, as shown in Table 1. Initially, 1 g of the matrices-spiked CAB were mixed with the extraction solvent. The mixture was then homogenized for 10 minutes using a vortex mixer and centrifuged for 15 minutes, 14000 x g. The supernatant obtained was then placed at room temperature to allow the evaporation of the organic solvent. Finally, 1 mL of methanol was added to reconstitute the dry extract,

homogenized, and centrifuged as previously mentioned. The supernatant obtained was then measured using spectrophotometry UV-visible.

Table 1. Volume of organic solvent for CAB extraction from vaginal samples

Organic Solvent	Methods	Volume (mL)
Methanol	A	1
	B	3
	C	5
	D	7
Acetonitrile	A	1
	B	3
	C	5
	D	7

2.6 Preparation of thermosensitive and mucoadhesive vaginal gels

CAB thermosensitive gel was prepared by dissolving 16% w/w of Pluronic® F127 and 4% w/w of Pluronic® F68 in cold water using a magnetic stirrer to produce a gel base. Then, 1% w/w of CAB was dispersed in 5% w/w of (poly(ethylene glycol) (PEG) 400 before being added into the gel base. Finally, after all mixtures were homogenous, 0.1% w/w of DMDM hydantoin was added into the mixture and homogenized for 15 minutes at 1000 rpm.

CAB mucoadhesive gel was prepared by hydrating 0.5% w/w of Carbopol 940 in distilled water for 24 hours. Afterwards, 2% w/w of triethanolamine (TEA) was added into Carbopol 940, and the mixture was homogenized for 15 minutes, 1000 rpm to obtain a gel base. The 1% w/w of CAB was first dispersed in 5% w/w of PEG 400 before being added into the gel base. After all mixtures were homogenous, 0.1% w/w of DMDM hydantoin was added into the mixture and homogenized for 15 minutes at 1000 rpm.

2.7 Validation of analytical method

2.7.1 Specificity

Specificity was determined by comparing the UV spectra of blank thermosensitive gel, mucoadhesive gel and vaginal tissue with appropriate CAB standard solution after scanning between 200 – 400 nm. This parameter was assessed to identify any possible interferences between the responses of the analyte and other compounds at the relevant wavelength.

2.7.2 Linearity

Linearity was evaluated using six different concentrations of each CAB-MeOH, CAB-SVF and CAB-VT. The sample solutions were analyzed in triplicate using UV-Vis spectrophotometry at 276 nm for CAB-MeOH, 278 nm for CAB-SVF and 305 nm for CAB-VT. The calibration curve, which consisted of six different concentrations versus absorbance was analyzed to obtain the value of correlation coefficient (r^2), slope, and y-intercept [16].

2.7.3 Limit of detection (LOD)

LOD expresses the smallest concentration of analyte which can be identified in a sample [17]. LOD was determined by using eq. (1), where sy is the standard deviation of the blank (without analyte) and b is the slope obtained from the regression equation of the calibration curve

$$LOD = \frac{3.3sy}{b} \quad \text{(Equation 1)}$$

2.7.4 Lower limit of quantification (LOQ)

LLOQ is expressed as the smallest concentration of samples which can be determined accurately with satisfactory accuracy and precision [16]. LLOQ was determined by using eq. (2), where b is the slope obtained from the regression equation of calibration curve and sy is the standard deviation of the blank (without analyte) [18].

$$LLOQ = \frac{10sy}{b} \quad \text{(Equation 2)}$$

2.7.5 Accuracy and precision

Accuracy and precision demonstrate the closeness to the reference value and degree of scattering between a series of measurements attained from numerous testing in an analytical method. These parameters were evaluated by intra-day and inter-day measurements of QC samples (HQC, MQC, LQC, and LLOQ). Percentage of relative error (%RE) and relative standard deviation (%RSD) were calculated to represent the values of accuracy and precision, respectively [19].

2.7.6 Dilution integrity

Dilution integrity was evaluated by preparing 75 µg/mL for both CAB-MeOH and CAB -SVF and 150 µg/mL for CAB-VT. Each of the solutions was diluted 5 and 10 times with appropriate solvents. The experiment was in triplicate and the absorbance of the analyte was observed [17].

2.7.7 Extraction recovery

The extraction recovery determination was conducted by comparing the values obtained from all samples at LLOQ, LQC, MQC, and HQC extracted from vaginal tissue with the measured values of the concentrations of the same samples (LLOQ, LQC, MQC, and HQC) [20].

2.8 Application of the analytical method

2.8.1 Drug content measurement

A total of 0.1 g of gel was dissolved in methanol up to 10 mL in a volumetric flask, obtaining a CAB concentration of 100 µg/mL. The solution was then diluted by taking 1 mL of the gel solution and diluted with methanol up to 10 mL. The absorbance of the final solution was determined using UV-Vis spectrophotometry at 276 nm. Measurements were performed in triplicate.

2.8.2 In vitro permeation, ex vivo permeation and ex vivo retention studies of CAB from thermosensitive and mucoadhesive gels

The permeation behavior of CAB from thermosensitive and mucoadhesive gels was evaluated using Franz diffusion cells using a dialysis membrane (Spectra-Por®, 12,000 - 14,000 MWCO dialysis membrane) and porcine vaginal mucosa for *in vitro* and *ex vivo* studies, respectively. The average thickness of the vaginal mucosa was 2.18 ± 0.13 mm. The tissues were obtained from female pigs (three- to four-month-old). The receptor compartment was filled with 24 mL of the receptor media and the temperature was maintained at 37ºC. In this study, SVF containing 20% v/v of methanol was used as the receptor media to maintain the sink condition during the experiments. Furthermore, 1 g of formulation (equal to 10 mg of CAB) was placed in the donor compartment with a diffusion area of 4.9 cm^2. A sample of 1 ml from the receptor compartment was taken at the predetermined time points (0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, and 24 h) and replaced by the same volume of fresh medium. The absorbances of the samples were then measured using UV-Vis spectrophotometry at 278 nm. The calculated cumulative amount of CAB permeating through the dialysis membrane and the porcine vaginal mucosa, was plotted versus time [21].

Following the *ex vivo* permeation study, the concentration of CAB retained in the vaginal tissue was also determined. After 1 h, 8 h and 24 h, the vaginal tissue was removed from Franz cells and the gel remained on the surface of the tissue was carefully removed. Afterwards, CAB was extracted using the method described previously.

2.8.3 Mathematical Modelling for In Vitro and Ex Vivo Permeation Studies

The data obtained from the *in vitro* and *ex vivo* permeation studies were fitted to five different mathematical models, such as zero-order kinetics (ZO), first-order kinetics (FO), Higuchi, Korsmeyer – Peppas (KP), and Hixson – Crowell (HC) to determine the release kinetics of

CAB from thermosensitive gels and mucoadhesive gels. The equations of each model are described below [22]:

$$Zero\ Order\ Kinetics: C_t = C_0 + k_0 t \quad \text{(Equation 3)}$$

$$First\ Order\ Kinetics: \ln C_t = \ln C_0 + k_1 t \quad \text{(Equation 4)}$$

$$Higuchi\ Model: C_t = k_H \sqrt{t} \quad \text{(Equation 5)}$$

$$Korsmeyer - Peppas\ Model: C_t = k_{KP} t^n \quad \text{(Equation 6)}$$

$$Hixson - Crowell\ Model: C_t^{1/3} = C_0^{1/3} k_{HC} t \quad \text{(Equation 7)}$$

C_t represents CAB concentration at time t, C_0 represents the initial concentration of CAB in the media (t = 0), k_0 denotes the zero-order constant, k_1 denotes the first-order constant, k_H denotes the Higuchi constant, k_{KP} denotes the Korsmeyer - Peppas constant, and k_{HC} denotes the Hixson-Crowell constant. All calculations were carried out using the DD-solver software. The release kinetics were determined from the value of correlation coefficient (r^2) [23].

2.9 Statistical Analysis

All data were expressed as means ± standard deviation (SD). The values of mean, SD, relative standard deviation (RSD) and reduction of error (RE) were calculated utilizing Microsoft Excel® 2019 (Microsoft Corporation, Redmond, USA). To analyze the data statistically, GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA) was applied, where *p* value < 0.05 indicates a statistical difference.

3. Results and discussion

3.1 Selection of sample preparation method and drug extraction

In this work, CAB extraction from vaginal tissue was performed using organic solvents, which were methanol and acetonitrile. The results of each extraction method are presented in Table 2. The results showed that a higher amount of solvent used in the extraction process was able to increase the extraction efficiency. Additionally, it was also observed that methanol provided a higher extraction recovery than acetonitrile. Regarding the extraction of CAB using methanol, the results showed that methods C and D exhibited the highest extraction recovery percentages, which are 91.57% ± 0.66% and 92.12% ± 2.43%, respectively. Moreover, both of these methods showed no significant difference (p <0.05). Therefore, in this study, method C (5 mL methanol) was chosen as the most suitable extraction method as it provided a minimum amount of solvent with optimum extraction recovery.

Table 2. Mean extraction recovery of CAB of each method with methanol and acetonitrile from vaginal tissue (n = 3)

Organic Solvent	Methods	Volume (mL)	%Extraction Recovery ± SD	%RSD
Methanol	A	1	12.30 ± 1.16	9.44
	B	3	38.11 ± 3.40	8.92
	C	5	91.57 ± 0.66	0.72
	D	7	92.12 ± 2.43	2.64
Acetonitrile	A	1	8.80 ± 1.15	13.04
	B	3	18.32 ± 5.04	27.49
	C	5	34.96 ± 1.17	3.36
	D	7	59.66 ± 1.09	1.83

3.2 Selectivity of UV-Vis spectrophotometry method

The specificity test aimed to ensure the absence of interferences between CAB and other compounds present in the gel formulations and vaginal tissue during the analysis using the UV-Visible spectrophotometry [18,24]. As mentioned previously, the analytical method in methanol was used to determine the CAB concentration in the formulation. Moreover, the developments of the analytical method in SVF and vaginal tissue was carried out to determine the CAB in *in vitro* and *ex vivo* studies. A well-defined peak of CAB was each observed at 276 nm, 278 nm and 305 nm in methanol, SVF and vaginal tissue, respectively. The absorption spectra of blank thermosensitive and mucoadhesive gel (Figure 1. (A)) exhibited no additional peak at 276 nm. These results indicated no interference occurred due to the presence of other gel constituents [18]. Nonetheless, the absorption spectrum of blank vaginal tissue (in Figure 1. (B)) showed a peak appeared at 270 nm, indicating possible interference with CAB peak at 276 nm. Alternatively, another absorption peak of CAB was shown at 305 nm. At this wavelength, there was no peak observed at blank vaginal tissue spectra. Therefore, the developed method in this study has been specific at the appropriate wavelength.

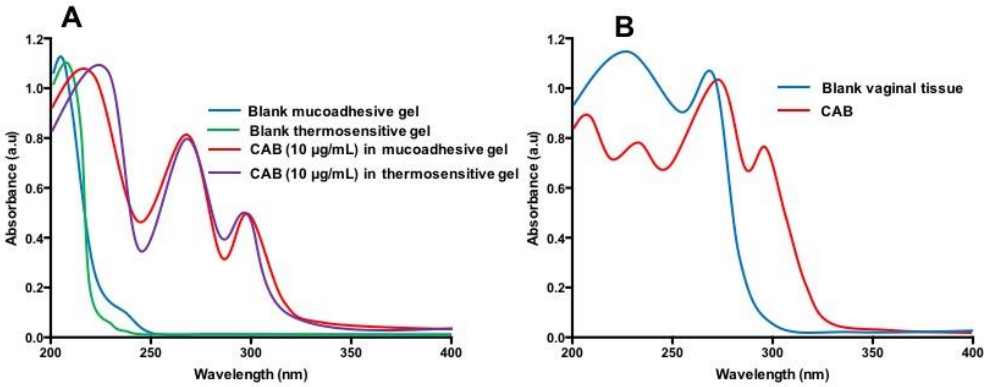

Figure 1. Representative UV-Spectra of CAB-MeOH, blank thermosensitive and mucoadhesive gel, (A); CAB-SVF and blank vaginal tissue (B)

3.3 Linearity, LOD, and LLOQ

In an attempt to assess the linearity and determine LOD and LLOQ values of the analytical method, a calibration curve was generated by measuring a set concentration of the standard solutions of CAB in methanol, SVF and vaginal tissue, respectively, using the optimized UV-Vis spectrophotometric. The spectrum of CAB standard solutions in methanol, SVF and vaginal tissue are shown in Figure 2. The linearity, LOD and LLOQ properties of CAB are summarized in Table 3 and Figure 3.

Table 3. Properties of the calibration curve for analysis of CAB with LOD and LLOQ values

Matrices	Concentration range (µg/mL)	r^2	LOD (µg/mL)	LLOQ (µg/mL)
MeOH	0.5 – 16	0.9990	0.71	2.15
SVF	0.5 – 16	0.9989	0.74	2.22
Vaginal Tissue	1 – 32	0.9985	1.69	5.13

It was observed that following the construction of the calibration curve, all samples showed bilinear curves. The low concentrations showed a slightly higher slope than the higher concentrations of CAB. The difference in slope values in bilinear could decrease the sensitivity of the methods since the calculation of LOD and LOQ values of the analytical method depends on the slope values of the calibration curves (Equation 1 and Equation 2). Despite this, no significant difference ($p > 0.05$) was found in the slope values in low and high concentrations of CAB. Importantly, a linear connection was obtained between the absorbance and the concentration in the range of 0.5 – 16 µg/mL for CAB-MeOH and CAB-SVF, and 1 – 32

µg/mL for CAB-vaginal tissue. The correlation coefficient values (r^2) of three regression equations of CAB in methanol, SVF, and vaginal tissue were 0.9990, 0.9989, and 0.9985, respectively, indicating the acceptable linearity. Furthermore, the LOD and LLOQ values of CAB in methanol were 0.71 and 2.15 µg/mL, in SVF were 0.74 and 2.22 µg/mL, and in vaginal tissue were 1.69 and 5.13 µg/mL.

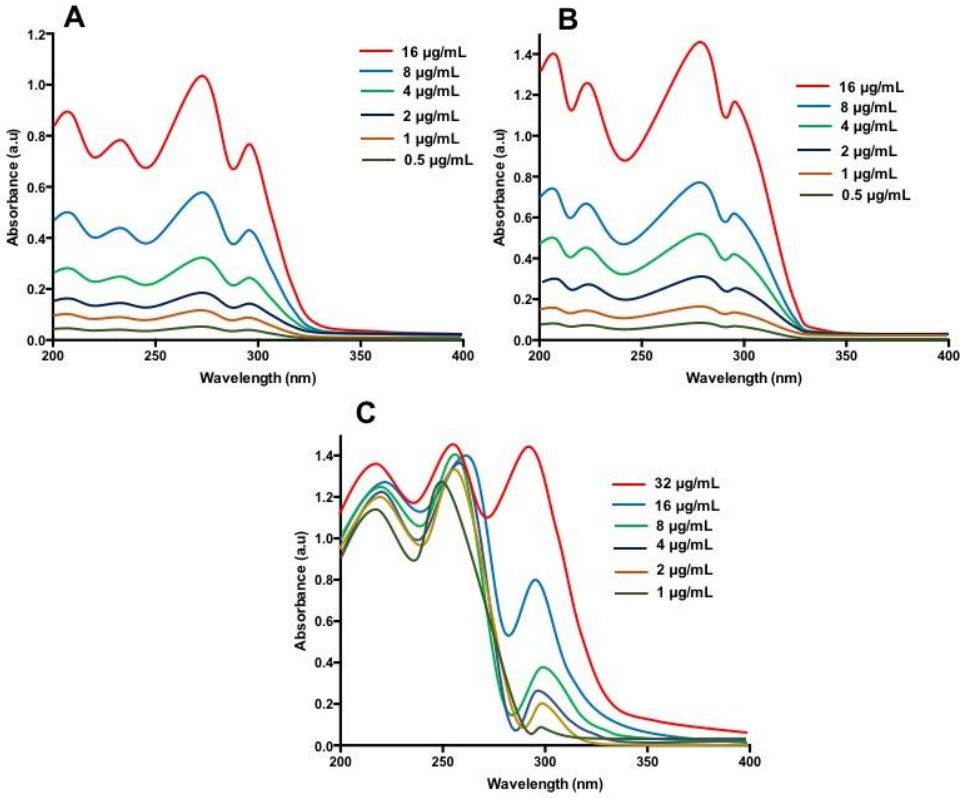

Figure 2. Spectrum of CAB standard solutions in MeOH (A), SVF (B) and vaginal tissue (C)

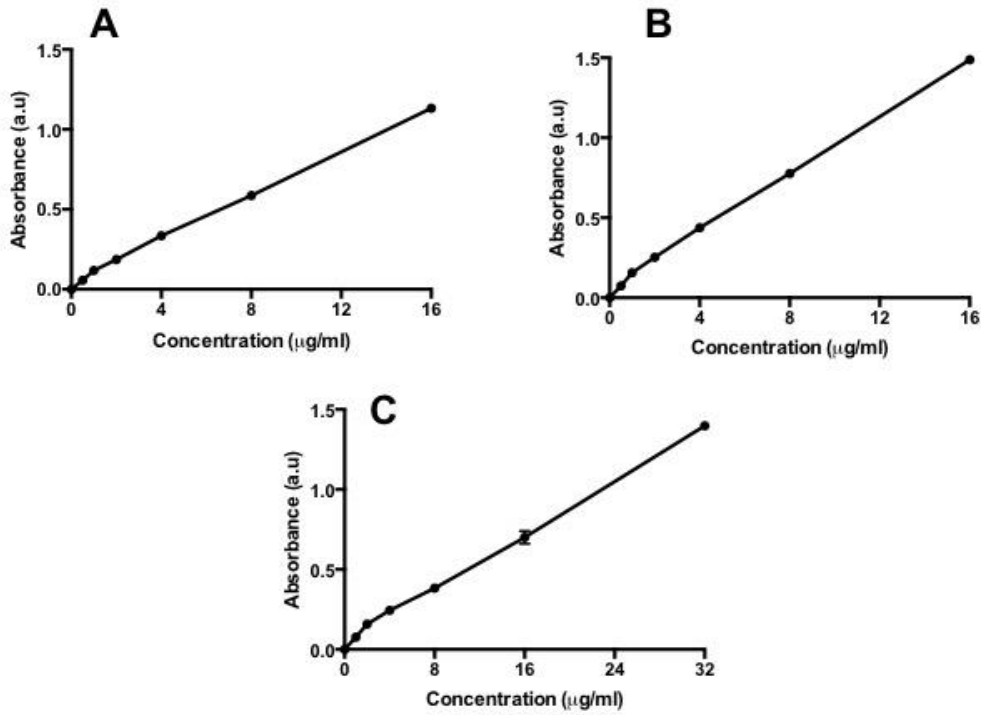

Figure 3. Calibration curve in MeOH (A), SVF (B) and Vaginal tissue (C) (mean ± SD, n= 3)

3.4 Accuracy and precision

The determinations of accuracy in the intra-day and inter-day measurements using the developed methods were found to be accurate for methanol (Table 4), SVF (Table 5), and vaginal tissue (Table 6) with the percentage of error value below 15%, which met the requirements from ICH guidelines. The precision of intra-day and inter-day was also found to be acceptable. The intra-day and inter-day precision in all solvents showed %RSD value that ranged of 0.5% - 12% and 0.5% - 10.5% which fulfill the limit from ICH guideline (15%). Consequently, the developed method using UV-Vis spectrophotometry for CAB was found to be accurate and precise.

Table 4. The results of precision and accuracy evaluations of the UV-Vis spectrophotometry method for analysis of CAB in MeOH (mean ± SD, n= 3)

Intra-day Precision and Accuracy				
Replication	**Concentration added (µg/mL)**	**Concentration found (µg/ mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	2.15	2.07 ± 0.10	4.63	-3.50
	4	3.91 ± 0.15	3.94	-2.19
	7.5	7.63 ± 0.11	1.39	1.76
	12	11.54 ± 0.18	1.53	-3.86
2	2.15	2.12 ± 0.12	5.87	-1.23
	4	4.13 ± 0.09	2.28	3.31
	7.5	7.71 ± 0.18	2.32	2.80
	12	12.19 ± 0.18	1.46	1.60
3	2.15	2.18 ± 0.08	3.56	1.28
	4	4.09 ± 0.18	4.51	2.33
	7.5	7.85 ± 0.12	1.52	4.63
	12	12.06 ± 0.48	3.95	0.50
Inter-day Precision and Accuracy				
Day	**Concentration added (µg/mL)**	**Concentration found (µg/ mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	2.15	2.41 ± 0.08	3.35	11.96
	4	4.23 ± 0.26	6.21	5.63
	7.5	7.50 ± 0.17	2.22	-0.07
	12	12.43 ± 0.35	2.81	3.56
2	2.15	2.10 ± 0.15	7.00	-2.36
	4	4.02 ± 0.24	5.86	0.62
	7.5	7.63 ± 0.09	1.15	1.76
	12	11.70 ± 0.39	3.34	-2.47
3	2.15	2.07 ± 0.12	5.78	-3.50
	4	3.99 ± 0.19	4.86	-0.35
	7.5	7.66 ± 0.41	5.32	2.09
	12	11.90 ± 0.38	3.16	-0.84

Table 5. The results of precision and accuracy evaluations of the UV-Vis spectrophotometry method for analysis of CAB in SVF (mean ± SD, n= 3)

Intra-day Precision and Accuracy				
Replication	**Concentration added (µg/mL)**	**Concentration found (µg/ mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	2.2	1.89 ± 0.14	7.37	-13.88
	4	3.97 ± 0.08	2.00	-0.68
	7.5	7.43 ± 0.12	1.66	-0.90
	12	12.20 ± 0.10	0.83	1.64
2	2.2	2.31 ± 0.20	8.46	4.91
	4	4.05 ± 0.09	2.11	1.28
	7.5	7.48 ± 0.12	1.61	-0.20
	12	12.16 ± 0.18	1.45	1.33
3	2.2	2.28 ± 0.25	10.79	3.56
	4	4.15 ± 0.07	1.62	3.79
	7.5	7.68 ± 0.25	3.27	2.38
	12	12.10 ± 0.14	1.17	0.86
Inter-day Precision and Accuracy				
Day	**Concentration added (µg/mL)**	**Concentration found (µg/ mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	2.2	2.11 ± 0.15	6.92	-4.06
	4	3.97 ± 0.08	2.00	-0.68
	7.5	7.66 ± 0.37	4.80	2.18
	12	12.92 ± 0.39	2.98	7.66
2	2.2	2.09 ± 0.20	9.65	-5.08
	4	3.95 ± 0.14	3.49	-1.33
	7.5	7.86 ± 0.08	1.02	4.86
	12	12.65 ± 0.07	0.59	5.45
3	2.2	1.96 ± 0.08	4.04	-10.83
	4	4.08 ± 0.08	1.92	1.93
	7.5	7.48 ± 0.10	1.29	-0.30
	12	12.14 ± 0.31	2.52	1.17

Table 6. The results of precision and accuracy evaluations of UV-Vis spectrophotometry method for analysis of CAB in vaginal tissue (mean ± SD, n= 3)

Intra-day Precision and Accuracy				
Replication	**Concentration added (µg/mL)**	**Concentration found (µg/ mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	5.13	5.29 ± 0.34	6.36	3.21
	7.5	7.41 ± 0.65	8.76	-1.20
	15	14.31 ± 0.49	3.43	-4.60
	24	25.19 ± 0.33	1.30	4.96
2	5.13	4.88 ± 0.62	12.66	-4.91
	7.5	7.22 ± 0.46	6.33	-3.76
	15	14.56 ± 0.48	3.30	-2.95
	24	24.89 ± 0.54	2.18	3.73
3	5.13	5.29 ± 0.33	6.27	3.06
	7.5	7.37 ± 0.53	7.15	-1.73
	15	15.26 ± 1.05	6.88	1.75
	24	24.24 ± 1.75	7.22	0.99
Inter-day Precision and Accuracy				
Day	**Concentration added (µg/mL)**	**Concentration found (µg/ mL) ± SD**	**Precision (%RSD)**	**Accuracy (%RE)**
1	5.13	5.21 ± 0.24	4.62	1.50
	7.5	7.67 ± 0.28	3.60	2.33
	15	14.15 ± 1.15	8.16	-5.67
	24	23.99 ± 1.46	6.08	-0.05
2	5.13	5.00 ± 0.25	4.94	-2.57
	7.5	7.17 ± 0.40	5.55	-4.40
	15	14.18 ± 0.75	5.29	-5.46
	24	24.68 ± 1.35	5.46	2.82
3	5.13	5.13 ± 0.37	7.16	-0.07
	7.5	7.39 ± 0.74	10.03	-1.52
	15	13.64 ± 0.62	4.58	-9.04
	24	24.23 ± 1.72	7.11	0.95

3.5 Dilution integrity

In an attempt to analyze the effect of dilution integrity of the analytical method, high concentrated samples were diluted 5 and 10 times using the appropriate matrices. This experiment showed satisfactory results for all dilution integrity in methanol, SVF, and vaginal tissue with of less than 15% bias. The precision parameter was found to be acceptable with %RSD value between 3.5% – 6.7%. Therefore, these results showed that CAB concentrations higher than the upper range of the calibration standards could be analyzed with suitable dilution.

3.6 Extraction Recovery

Extraction recovery from the method used in this study was obtained by comparing the sample concentrations of LLOQ, LQC, MQC, and HQC extracted from vaginal tissue with the concentrations obtained from measuring samples at the same concentrations. The results of the average extraction recovery are exhibited in Table 7. The %RSD values obtained were in the range of ±15%. Thus, the extraction technique was found to be precise, consistent and repeatable.

Table 7. Mean extraction recovery of CAB in vaginal tissue (n = 3)

Sample	Concentration (µg/mL)	%Extraction Recovery ± SD	%RSD
Vaginal Tissue	LLOQ (5,13)	90.38 ± 1.24	1.37
	QC (7,5)	93.34 ± 4.61	4.94
	MQC (15)	93.39 ± 4.34	4.65
	HQC (24)	92.16 ± 5.46	5.92

3.7 Application of the analytical method

The validated spectrophotometry UV-Visible method was further used to determine the amount of CAB in the thermosensitive and mucoadhesive gels. As depicted in Figure 4(A), the result showed that the recovery values of CAB were 99.24 ± 2.88 % and 99.19 ± 1.34% from the thermosensitive and mucoadhesive gels, respectively. These findings indicated that the formulation of CAB into thermosensitive and mucoadhesive gels did not affect the CAB concentration. According to the ICH recommendation for acceptable recovery percentage, all formulations also showed acceptable recovery percentage, within the range of 95 – 105% [25].

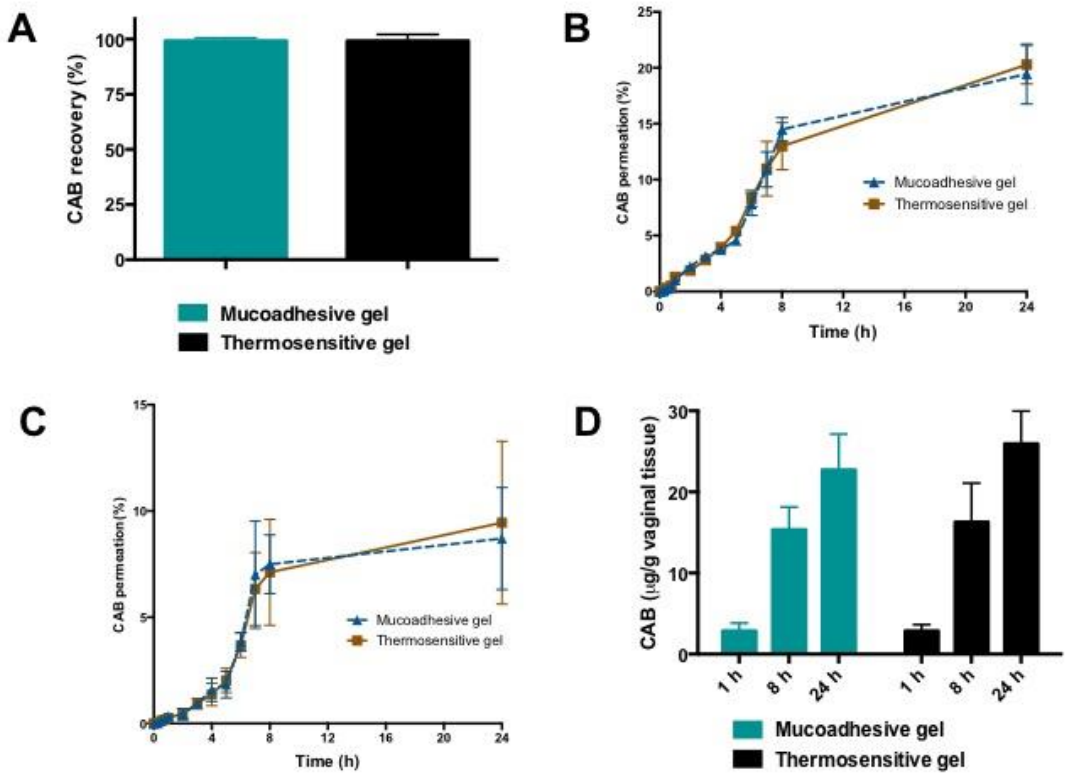

Figure 4. (A) CAB recovery (%) from gels, (B) *In vitro* permeation profile of CAB from thermosensitive and mucoadhesive gel, (C) *Ex vivo* permeation profile of CAB from thermosensitive and mucoadhesive gel, (D) *Ex vivo* retention of CAB (mean ± SD, n= 3)

The cumulative amount of CAB permeation following *in vitro* and *ex vivo* permeation studies and *ex vivo* retention studies of the thermosensitive and mucoadhesive gels were also determined by the validated analytical method. Figure 4(B) represents the *in vitro* permeation profile of CAB. After 24 hours, 20.29 ± 1.72 % of CAB permeated from the thermosensitive gel and 19.44 ± 2.69% of CAB permeated from the mucoadhesive gel. Figure 4(C) depicts the results of *ex vivo* permeation studies, showing that after 24 hours, 9.45 ± 3.83 % and 8.71 ± 2.40 % of CAB were able to permeate through vaginal tissue from the thermosensitive and mucoadhesive gels, respectively. The permeation of drugs from Pluronic based gel could occur through diffusion from micelles formed. Higher concentrations of Pluronic produce lower diffusion of the drug due to the increased number and size micelles formed in the gel structure [26]. Analyzed statistically, the release profiles between thermosensitive and mucoadhesive gels were not significantly different ($p < 0.05$).

The result of the *ex vivo* retention study is revealed in Figure 4 (D). The amount of CAB retained in the vaginal mucosa after 24 hours from the thermosensitive and mucoadhesive gels were 22.71 ± 4.44 µg/g vaginal tissue and 25.94 ± 4.04 µg/g vaginal tissue, respectively.

Despite non-significant difference ($p > 0.05$), it was found that mucoadhesive gel has a slightly higher concentration of CAB localized in vagina mucosa. This might be due to the presence of Carbopol as a mucoadhesive polymer. Carbopol has carboxyl groups that bind strongly through a hydrogen bond with the oligosaccharide chain of mucin [27].

The results obtained from the *in vitro* and *ex vivo* permeation studies were further fitted to five mathematical models in order to explain CAB release behavior from both thermosensitive and mucoadhesive gels. For the *in vitro* permeation of CAB from the thermosensitive gel, the value of coefficient correlation was 0.8613, 0.8936, 0.8240, 0.9247, 0.8836 for ZO, FO, Higuchi, KP, and HC, respectively. For the *in vitro* permeation of CAB from the mucoadhesive gel, the value of coefficient correlation was 0.8116, 0.8461, 0.7977, 0.8850, 0.8354 for ZO, FO, Higuchi, KP, and HC, respectively. Furthermore, the *ex vivo* permeation study showed coefficient correlation values of 0.7794, 0.7939, 0.7338, 0.8318, 0.7892 for thermosensitive gel and 0.6838, 0.7015, 0.7036, 0.7689, 0.6957 for mucoadhesive gel for Zero order, First order, Higuchi, Korsmeyer Peppas, and Hixson-Crowell, respectively. The result obtained clearly showed that all formulations tested in the *in vitro* and *ex vivo* permeation studies followed Korsmeyer-Peppas kinetic models. This model has been used to describe drug release from the polymeric matrix based on relaxation and diffusion [28].

Based on the results obtained, the validated analytical methods using spectrophotometry UV-visible were successfully applied to determine the amount of CAB in the thermosensitive and mucoadhesive gels. Moreover, the methods were also be able to determine the concentration of CAB following *in vitro* and *ex vivo* permeation tests, as well as *ex vivo* retention tests. Moving forward, *in vivo* studies using suitable animal models for both types of gels are essential to be carried out to obtain pharmacokinetic and pharmacodynamic profiles of CAB.

4. Conclusion

This study was conducted to develop and validate spectrophotometry UV-visible methods for the analysis of CAB. The proposed method was validated in the parameter of selectivity, accuracy and precision, linearity, LOD and LLOQ, and dilution integrity, as well as extraction recovery of CAB and vaginal tissue. Additionally, the suitable extraction method of CAB from vaginal tissue was also determined. The results showed that all validation parameters were well-established and met the requirements of ICH guidelines. Moreover, the validated analytical method was successfully employed to evaluate the percentage recovery, permeation profiles, and retention of CAB following each appropriate study. In conclusion, the validated

method was able to be used for various studies of CAB in thermosensitive and mucoadhesive gel formulations.

Disclosure of interest

The authors declare no conflicts of interest.

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Credit Author Statement

Sulistiawati: Conceptualization, Methodology, Funding Acquisition Writing - Original Draft, Review & Editing**; Cindy Kristina Enggi:** Methodology, Writing - Original Draft**; Hansel Tridatmojo Isa:** Methodology, Writing - Original Draft**; Stevens Wijaya:** Methodology, Data Curation**;**, **Komang Agus Rai Ardika:** Data Curation, Validation**; Rangga Meidianto Asri:** Validation, Supervision**; Ryan F. Donnelly**: Review & Editing, Project Administration**; Andi Dian Permana:** Conceptualization, Review & Editing, Project Administration, Funding Acquisition, Validation, Supervision**.**

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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