BUKTI SUBMIT MANUSKRIP

Universitas Hasanuddin

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

Submission to Microchemical Journal - manuscript number

1 message

Microchemical Journal <em@editorialmanager.com> Tue, Jul 6, 2021 at 12:07 AM
Reply-To: Microchemical Journal <microc@elsevier.com>
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Manuscript Number: MICROC-D-21-01793
New and Rapid HPLC-UV analytical method for quantification of metronidazole: Application to ex vivo ocular kinetic assessments following the application of thermosensitive ocular in situ gel

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BUKTI REVIEW DARI REVIEWERS

Universitas Hasanuddin

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

Decision on submission to Microchemical Journal

1 message

Microchemical Journal <em@editorialmanager.com> Wed, Sep 22, 2021 at 2:47 PM
Reply-To: Microchemical Journal <support@elsevier.com>
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Manuscript Number: MICROC-D-21-01793
New and Rapid HPLC-UV analytical method for quantification of metronidazole: Application to ex vivo ocular kinetic assessments following the application of thermosensitive ocular in situ gel

Dear Dr. Permana,

Thank you for submitting your manuscript to Microchemical Journal.

I have completed my evaluation of your manuscript. The reviewers recommend reconsideration of your manuscript following major revision. I invite you to resubmit your manuscript after addressing the comments below. Please resubmit your revised manuscript by Nov 21, 2021.

When revising your manuscript, please consider all issues mentioned in the reviewers' comments carefully: please outline every change made in response to their comments and provide suitable rebuttals for any comments not addressed. Please note that your revised submission may need to be re-reviewed.

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Microchemical Journal values your contribution and I look forward to receiving your revised manuscript.

Kind regards,

Justyna Płotka-Wasylka
Editor
Microchemical Journal

Editor and Reviewer comments:

Reviewer 1: The authors presented HPLC-UV method for quantification of metronidazole and its application. The language of article is very poor. Introduction section is too large. There are so many spelling mistakes in the entire paper. Kindly do necessary changes. Please resubmit the entire paper after appropriate corrections.

1. Application word repeated in the title of the manuscript. Kindly reframe it.
2. Kindly remove rapid word from the title because there are so many papers on metronidazole which has lower chromatographic run time compared to the present method.
3. Spelling mistake in line no. 43, Kindly correct it. i.e. "nd". It is actually "and".
4. Kindly use multiplication symbol instead of X in the column dimension.
5. Literature is incomplete. Kindly cite some paper which has used HPLC and LC-MS/MS for the quantification of metronidazole in various biological matrices.

Reviewer 2: 1. In the abstract: However, its effectiveness has been hampered by several limitations of eye drop preparations due to rapid elimination from the eye", this sentence signifies that several limitations are only due to "rapid elimination", which is not true, correction in this sentence is needed. Line 51: what is ocular "cokinetics"? Put the Trade Mark on "Xselect" CSHTM C18 HPLC column, like Xselect™.
2. In highlights, what does it mean "ex vivo HPLC method", I do not think we should call it ex vivo, it is simple HPLC analytical method.
3. The Poloxamers used to prepare the thermo sensitive in situ ocular gels is not new (although the method

development for metronidazole for ocular use can give a little novelty). Therefore, the formulations and findings in the present study are different from the previous reports where these polymers were used for ocular application. Justify your findings in comparison to the previous reports with proper references such as:
PMID: 20500130 or DOI: 10.3109/10717544.2010.483255
PMID: 9519158 or DOI: 10.1021/js970090e
https://www.sciencedirect.com/science/article/pii/S1319016420301523

4. Page 9, line 240: As mentioned "The treated cornea was then placed between the chambers in the diffusion cells", what was the treatment of the cornea? I think here freshly excised cornea not the treated one was used. Line 244: What do you mean by "Kornea"?
5. In the equations of LOD and LLOQ, specify the term "Syx". Mention the values in the text also.
6. Line 171-172: what was the v/v ratio of the mobile phase mixture (acetonitrile and 20 mM acetate buffer)?
7. Line 297: I think, the unit of time in the value 61.33 is missing.
8. In Table S3. Mention the unit of excipients used.
9. Line 226-227: "2.6 Application of validated HPLC method to assess ocular kinetics evaluation of formulated from thermosenstivie gel formulation" recheck this subheading.
10. Mention all the excipients with exact weights used in the preparation of stimulated tear fluid.
11. Although the main of this work is the development and validation of a new and rapid HPLC-UV analytical method for metronidazole quantification, then maximum data and tables related to method validation should be presented in the main manuscript rather than in Supplementary files.
12. The drug was analyzed only in the corneal extract, so it is better to mention in the manuscript that the thermosensitive ocular gel improved metronidazole concentration in corneal tissues rather than "ocular tissue", because ocular tissue includes iris, conjunctiva, retina etc.

*****

Data in Brief (optional):
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BUKTI SUBMIT HASIL REVIEW

Universitas Hasanuddin

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

Confirming submission to Microchemical Journal

1 message

Microchemical Journal <em@editorialmanager.com> Mon, Sep 27, 2021 at 12:45 AM
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Manuscript Number: MICROC-D-21-01793R1

New HPLC-UV analytical method for quantification of metronidazole: Application to ex vivoocular kinetic assessments following the administration of thermosensitive ocular in situgel

Dear Dr. Permana,

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Microchemical Journal

New HPLC-UV analytical method for quantification of metronidazole: Application to ex vivoocular kinetic assessments following the administration of thermosensitive ocular in situgel

--Manuscript Draft--

Manuscript Number:	MICROC-D-21-01793R1
Article Type:	Research Paper
Section/Category:	Chromatography and separation techniques
Keywords:	Metronidazole; keratitis; HPLC; thermosensitive in situ gel; ocular kinetic; Method Validation
Corresponding Author:	Andi Dian Permana INDONESIA
First Author:	Nur Asma
Order of Authors:	Nur Asma
	Nurul Muhlisah Maddeppungeng
	Muhammad Raihan
	Arini Putri Erdiana
	Achmad Himawan
	Andi Dian Permana
Abstract:	Metronidazole eye drops have been used to treat Acanthamoebakeratitis. However, ophthalmic preparations also have some limitations, one of which is the rapid elimination of the drug, that reducing the effectiveness of the drug. Accordingly, an alternative delivery approach can be applied to overcome this issue. Additionally, as one of critical steps in the formulation development, analyitical methods that allow the quantification of metronidazole in ex vivo corneal permeation and deposition should also be developed. Here, we report a validated high-performance liquid chromatography method (HPLC-UV) according to ICH guidelines for the measurements of metronidazole concentrations following formulation of thermosensitive ocular in situ gel and its administration in ex vivoporcine corneas. The development of extraction techniques and optimization of HPLC conditions were optimized using analytical quality by design. Xselect™ CSHTM C18 HPLC column (Water, 3.0 × 150 mm, particle size 3.5 m) was used to separate all analytes by isocratic elution with mobile phases of acetate buffer and acetonitrile with LLOQ value of 0.08 µg/mL. The resulting method proved to be selective, precise, and accurate and was succefully applied to determine ocular kinetic profiles of metronidazole from thermosensitive ocular in situ gel in ex vivoporcine corneas, showing that this approach was able to improve the concentration of metronidazole in the corneal tissues. We, therefore, suggested that HPLC-UV approach developed in this study has the potential to be used in drug release evaluation, therapeutic drug control research, ocular kinetics, and toxicological evaluation.

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The Editor
Microchemical Journal

September 26, 2021

Dear Sir/Madam,
I wish you to consider our manuscript for publication in *The Microchemical Journal*. Following the reviewer comments, we have changed out title from "**New and rapid HPLC-UV analytical method for quantification of metronidazole: application to *ex vivo* ocular kinetic assessments following the application of thermosensitive ocular in situ gel**" to "**New HPLC-UV analytical method for quantification of metronidazole: Application to *ex vivo* ocular kinetic assessments following the administration of thermosensitive ocular *in situ* gel**".
Importantly, we have addressed all comments from all reviewers as shown in "Response to Reviewer" file. Also, we have corrected all technical issues in our manuscript.
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 *The Microchemical Journal* 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

Manuscript Number: MICROC-D-21-01793

New and Rapid HPLC-UV analytical method for quantification of metronidazole: Application to ex vivo ocular kinetic assessments following the application of thermosensitive ocular in situ gel

Response to Reviewers

We are very thankful to the reviewers for taking the time to provide helpful comments for improvements for our manuscript. We have addressed each of the reviewers' comments in detail below.

Reviewer 1

The authors presented HPLC-UV method for quantification of metronidazole and its application. The language of article is very poor. Introduction section is too large. There are so many spelling mistakes in the entire paper. Kindly do necessary changes. Please resubmit the entire paper after appropriate corrections.

Response to Reviewer

We thank the Reviewer for noticing our spelling mistakes and the large Introduction. We deliberately corrected our poor language and deleted some sentences that we considered not necessary for the article. We believe that the English of our manuscripit has now been improved significantly.

Comments to the Author:

1. Application word repeated in the title of the manuscript. Kindly reframe it.
2. Kindly remove rapid word from the title because there are so many papers on metronidazole which has lower chromatographic run time compared to the present method.

Response to Reviewer

We thank the reviewer for this valuable suggestion. We have reframe the title so that the repeating word is avoided. We have also removed the word 'rapid' as we also agree that our method is comparable to previous studies.

3. Spelling mistake in line no. 43, Kindly correct it. i.e. "nd". It is actually "and".

Response to Reviewer

Thank you for your suggestion. We have corrected the misspelling word.

4. Kindly use multiplication symbol instead of X in the column dimension.

Response to Reviewer

We thank the Reviewer for suggesting all the detail in our manuscript. We noticed the mistake and change X in the column dimention to ×

5. Literature is incomplete. Kindly cite some paper which has used HPLC and LC-MS/MS for the quantification of metronidazole in various biological matrices.

Response to Reviewer

Thank you for your suggestion. We have added some literatures in Introduction.

Reviewer 2

Comment to authors:

1. In the abstract:

a. However, its effectiveness has been hampered by several limitations of eye drop preparations due to rapid elimination from the eye", this sentence signifies that several limitations are only due to "rapid elimination", which is not true, correction in this sentence is needed.

Response to Reviewer

We thank reviewers for this valuable suggestion. We have revised the sentences to address your concerns and hope that it is now clearer. We have changed “However, its effectiveness has been hampered by several limitations of eye drop preparations due to rapid elimination from the eye" to “However, ophthalmic preparations also have some limitations, one of which is the rapid elimination of the drug, that reducing the effectiveness of the drug.”

b. Line 51: what is ocular "cokinetics"? Put the Trade Mark on "Xselect" CSHTM C18 HPLC column, like Xselect™.

Response to Reviewer

Thank you for your suggestion. We have corrected the misspelling word that contain "cokinetics" to “kinetics” and put the Trade Mark on Xselect.

2. In highlights, what does it mean "ex vivo HPLC method", I do not think we should call it ex vivo, it is simple HPLC analytical method.

Response to Reviewer

We thank reviewers for this valuable suggestion. We have revised the sentences to address your concerns and hope that it is now clearer.

3. The Poloxamers used to prepare the thermo sensitive in situ ocular gels is not new (although the method development for metronidazole for ocular use can give a little novelty). Therefore, the formulations and findings in the present study are different from the previous reports where these polymers were used for ocular application. Justify your findings in comparison to the previous reports with proper references such as:

PMID: 20500130 or DOI: 10.3109/10717544.2010.483255

PMID: 9519158 or DOI: 10.1021/js970090e

https://www.sciencedirect.com/science/article/pii/S1319016420301523

Response to Reviewer

We thank reviewers for this valuable input. Indeed, Poloxamers is not new in the field of thermosensitive preparation and thus, we agree to incorporate the suggested articles on our manuscript draft. We have read the articles and justified our findings using the suggeted citations. More specifically, we have added the our justification in the line 416-420 and 446-457. We again highly appreciated the suggestion of the reviewers in improving the quality of this work.

4. Page 9, line 240: As mentioned "The treated cornea was then placed between the chambers in the diffusion cells", what was the treatment of the cornea? I think here freshly excised cornea not the treated one was used. Line 244: What do you mean by "Kornea"?

Response to Reviewer

We thank the Reviewer for this question. In this study, the treated cornea determined by freshed cornea with a scleral tissue size of 2-4 mm then washed with cold normal saline. To avoid misconception we change the “treated” word. There was spelling mistake in the line 244. We have clarified this in the method.

5. In the equations of LOD and LLOQ, specify the term "Syx". Mention the values in the text also.

Response to Reviewer

We thank the Reviewer for pointing this out. In the measurement of LOD and LOQ, the value were expressed as 3.3 × Syx/*b* and 10.0 × Syx/*b*, respectively, where Syx is residual variance due to regression and *b* is the mean slope of the linear regression curves. We have clarified this in the method.

6. Line 171-172: what was the v/v ratio of the mobile phase mixture (acetonitrile and 20 mM acetate buffer)?

Response to Reviewer

We thank the Reviewer for reviewing the manuscript in detail and giving the comments about the v/v ratio of mobile phase. There are 3 variable that we use to optimize the chromatographic conditions including mobile phase, flow rate, mobile phase pH, and acetonitrileconcentration. The optimized using The Composite Central Design (CCD) under response surface methodology, as we can see in the supplementary section Table S2 for Acetonitrile concentration.

7. Line 297: I think, the unit of time in the value 61.33 is missing.

Response to Reviewer

We thank you Reviewer for reviewing our manuscript in detail. We rewrote the sentence to improve its clarity.

8. In Table S3. Mention the unit of excipients used.

Response to Reviewer

We thank the reviewer for pointing this out. We added the unit of excipient used as (% w/v).

9. Line 226-227: "2.6 Application of validated HPLC method to assess ocular kinetics evaluation of formulated from thermosenstivie gel formulation" recheck this subheading.

Response to Reviewer

We thank the Reviewer for informing us to recheck the subheading. We realized that the previous subheading was not quite right so we changed the subheading to "Optimization and ocular kinetics evaluation of formulated metronidazole gels"

10. Mention all the excipients with exact weights used in the preparation of stimulated tear fluid.

Response to Reviewer

We thank the Reviewer for reviewing the manuscript in detail and giving the comments about all the excipients with exact weights used in the preparation of stimulated tear fluid. In this study, simulated tear fluid consisting of 6.7 g NaCl, 2.0 g $NaHCO_3$ and 0.08 g $CaCl_2 \cdot 2H_2O$ was intially prepared by dissolving all components in 1 L deionized water (final pH of 7.4). We have clarified this in the method.

11. Although the main of this work is the development and validation of a new and rapid HPLC-UV analytical method for metronidazole quantification, then maximum data

and tables related to method validation should be presented in the main manuscript rather than in Supplementary files.

Response to Reviewer

We thank the Reviewer for reviewing the manuscript in detail and giving the comments about our validation tables. We combined Table S8, S9, S10 that related to method validation with Table 1 in the main manuscript, respectedly.

12. The drug was analyzed only in the corneal extract, so it is better to mention in the manuscript that the thermosensitive ocular gel improved metronidazole concentration in corneal tissues rather than "ocular tissue", because ocular tissue includes iris, conjunctiva, retina etc.

Response to Reviewer

We would like to thank the Reviewer for the comments. Following the comments, we have changed "ocular tissue" to “corneal tissue" in our manuscript.

Highlights:

- We developed HPLC method of metronidazole for quantification in corneal tissue
- We validated the HPLC method according ICH guidelines
- We successfully applied the method in *ex vivo* ocular kinetic assay
- Thermosensitive ocular gel improved metronidazole concentration in ocular tissue

New HPLC-UV analytical method for quantification of metronidazole: Application to *ex vivo* ocular kinetic assessments following the administration of thermosensitive ocular *in situ* gel

Nur Asma[1], Nurul Muhlisah Maddeppungeng[1], Muhammad Raihan[2], Arini Putri Erdiana[3], Achmad Himawan[3], Andi Dian Permana[3]*

1. Department of Pharmacy, Faculty of Medicine and Health Sciences Faculty, Alauddin Islamic State University, Samata Gowa, 92113, Indonesia
2. Department of Phytochemistry, Faculty of Pharmacy, Hasanuddin University, Makassar, 90245, Indonesia
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Andi Dian Permana

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Abstract

Metronidazole eye drops have been used to treat *Acanthamoeba* keratitis. However, ophthalmic preparations also have some limitations, one of which is the rapid elimination of the drug, that reducing the effectiveness of the drug. Accordingly, an alternative delivery approach can be applied to overcome this issue. Additionally, as one of critical steps in the formulation development, analyitical methods that allow the quantification of metronidazole in *ex vivo* corneal permeation and deposition should also be developed. Here, we report a validated high-performance liquid chromatography method (HPLC-UV) according to ICH guidelines for the measurements of metronidazole concentrations following formulation of thermosensitive ocular *in situ* gel and its administration in *ex vivo* porcine corneas. The development of extraction techniques and optimization of HPLC conditions were optimized using analytical quality by design. Xselect™ CSHTM C18 HPLC column (Water, 3.0 × 150 mm, particle size 3.5 m) was used to separate all analytes by isocratic elution with mobile phases of acetate buffer and acetonitrile with LLOQ value of 0.08 μg/mL. The resulting method proved to be selective, precise, and accurate and was succefully applied to determine ocular kinetic profiles of metronidazole from thermosensitive ocular *in situ* gel in *ex vivo* porcine corneas, showing that this approach was able to improve the concentration of metronidazole in the corneal tissues. We, therefore, suggested that HPLC-UV approach developed in this study has the potential to be used in drug release evaluation, therapeutic drug control research, ocular kinetics, and toxicological evaluation.

Keywords:

Metronidazole, keratitis, HPLC, thermosensitive *in situ* gel, ocular kinetic, method validation

1. Introduction

Infection that presents with corneal ulcerations known as Acanthamoeba keratitis is caused by *Acanthamoeba* species. It was first reported in 1973. This condition occurs commonly when someone uses a contact lens washing solution prepared with non-sterile salt tablets and water [1].

Metronidazole is a derivative of 5-Nitroimidazole known to act as a powerful antibacterial and antiprotozoal. For decades, eye preparations has become a fast growing pharmaceutical technology [2]. In ophthalmology, general eye drops and topical eye preparations are the most widely used preparations. It is considered the most efficient form of treating infections of the foreground structures generally caused by anaerobic bacteria [3]. Acanthamoeba keratitis can be treated using 0.5% metronidazole eye drops mixed with other antiprotozoal preparations [4]. Metronidazole eye drops have long been a formulary drug in pharmacies as an alternative to the lack of a commercially available form of eye medications. In ophthalmology, preparations with 0.1% and 0.5% metronidazole solutions have been used to treat Acanthamoeba keratitis [5–7].

Topical administration of the drug is more prevalent among patients and safer than the intraocular injection, but topical use of this drug has the disadvantage that the drug cannot be adequately absorbed in the eye and reaches the posterior segment. As a part of trends in topical formulation, thermosensitive gels have been utilized in overcoming these problems. The term thermosensitive refers to its ability to form gel or solution reversibly upon at a particular temperature. This can be achieved by taking advantages of unique properties of some gelling agent such as N-acrylamide based co-polymers and poly(propylene oxide) (PPO)/ polyethylene oxide (PEO) block co-polymers [8]. However, previous reports showed that combination of two co-polymers or its interaction with other gelling agents is required to obtained a relatively precise temperature for the gel to form [9–11]. To our best understanding, this is the first time the technology of thermosensitive gels is applied to administer metronidazole in an ocular drug delivery. Therefore, it is also an important task to assess whether the newly incorporated gel bases is able to release metronidazole upon application in the eye condition.

Over the last few decades, many improved methods have been undertaken to find and explore more effective ways of administering eye medications and treatments for various eye diseases [12]. In transcorneal penetration studies, *in vitro* cell culture models have been widely selected. The development of an organotypic corneal construction technique also studies the

bioavailability of ophthalmic drugs using primary cell cultures and immortalized cell lines. There is great potential for research into corneal penetration in artificially cultivated human corneas. Using a research model using cut animal corneal tissue to study drug penetration is one technique with promising results, although it is necessary to consider the significant variation between species [13]. One of the developments in the new *ex vivo* model is to use a porcine cornea disc. It is considered to be more cost-effective for candidates for transcorneal penetrating topical therapy [14].

Method development and validation have tremendous importance in the QC of the drug. In recent years because of its importance, the development of new testing methods for drug determination has received considerable attention in determining potency of active ingredients in eye drops. Hence, HPLC is the analytical method of choice for measuring metronidazole [7]. The HPLC method validation test will be conducted according to guidelines recommended by the USFDA (2001) [15]. Comparison between the retention times of metronidazole detected in the drug-free plasma assay and metronidazole derived from drug injection extracted from spiked plasma will be investigated as a test of specificity and selectivity [7].

Several previous studies related to HPLC and LC-MS/MS for the quantification of metronidazole in various biological matrices have been carried out. A study by Silva et al (2009) showed simple, fast, sensitive, and selective results using liquid chromatography (LC)-tandem mass spectrometry (MS-NONA)[16]. In addition, HPLC and LC-MS/MS for metronidazole quantification have also been performed on saliva, plasma, and gingival crevicular fluid [17].

Transcorneal penetration of metronidazole using *ex vivo* porcine corneal model has never been developed. The development of extraction method before having HPLC assay is essential to produce maximum drug concentration. Optimization and development of analytical methods, also recognized as the analytical quality by design (AQbD), are now widely applied to quality by design (QbD). This concept is considered necessary since the first time it was first introduced by the USFDA [18]. This concept is known to require less time for experimentation because it uses the experimental design method (DOE) to obtain possible combinations of parameters and has also been recommended in robustness testing [18,19]. Implementation of AQbD applications in HPLC method development has been done in many studies, Rozet et al reported using AQbD approach for analytical method by HPLC [20]. Prior to analyzing metronidazole with HPLC, we developed an optimised method for extracting drug form matrix using various sonication time and strirring

time. This aimed to maximise the number of metronidazole that were measured using HPLC method. The validated HPLC method will be used to assess the *ex vivo* ocular kinetics of the drug after topical application to the eye.

2. Materials and methods

2.1. Chemicals and materials

Metronidazole (≥99.9%, purity), analytical grade trifluoroacetic acid, and HPLC-grade Methanol (MeOH) (Sigma-Aldrich Pte Ltd, Singapore), Xselect CSH ™ C_{18} HPLC column (particle size 3.5 µm, 3, 0 × 150 mm; Waters, Dublin, Ireland), chemical reagent (Sigma - Aldrich Pte Ltd, Singapore). Other reagents were analytical grade and obtained from standard commercial supplier.

2.2. Stock Preparation, Calibration Standards and Quality Control Samples

Metronidazole stock solution was prepared by dissolving 50 mg in 50 ml methanol so that a concentration of 1 mg/mL was obtained. The working solutions were diluted into serial concentrations for standard calibration, and quality control (QC) solutions. In the calibration of the test method, a concentration series was made using the stock solution diluted with porcine eye corneal tissue matrices. The final concentrations obtained ranged from 0.01 to 10 µg/mL. QC solutions were made in three types of concentrations, namely 0.15 µg/mL, 3.5 µg/mL and 7.5 µg/mL for low QC, medium QC and high QC, respectively. All QC solutions were prepared in porcine eye corneal tissue matrices.

2.3. Preparation and Analytes Extraction of Samples

Simulated tear fluid consisting of 6.7 g NaCl, 2.0 g NaHCO3 and 0.08 g CaCl2·2H2O was intially prepared by dissolving all components in 1 L deionized water (final pH of 7.4). Preparation of the sample was conducted by mixing porcine eye corneal 2,5 g with 2,5 g STF (stimulated tear fluid) pH 7,4 to make porcine eye corneal fluid, 2 mg of metronidazole was added with 2 mL aquadest. The 100 µL of the mixture was centrifuged with 900 µL porcine eye corneal fluid. The extraction volume, stirring time and sonication time were evaluated. The response surface methodology to optimize sonication time, vortex time, and methanol volume (**Table S1**) used Composite Central Design (CCD) by Design Expert Software

version 11 (State-Ease, Minneapolis, MN, USA). The response parameters were extraction performance and fluid evaporation time.

The supernatant liquid was put into a small glass container and then dried up in a fume hood for about 3 hours. This is done in order to obtain dried residue. The residual mixture and the 100 µL mobile phase were stored in a 0.5 mL centrifuge tube. For 30 seconds, the mixture was stirred and then rotated at 14,000 rpm × g for 15 minutes. A total of 10 µL of the treated supernatant solution was injected into the HPLC column. The work steps are illustrated in **Figure 1.**

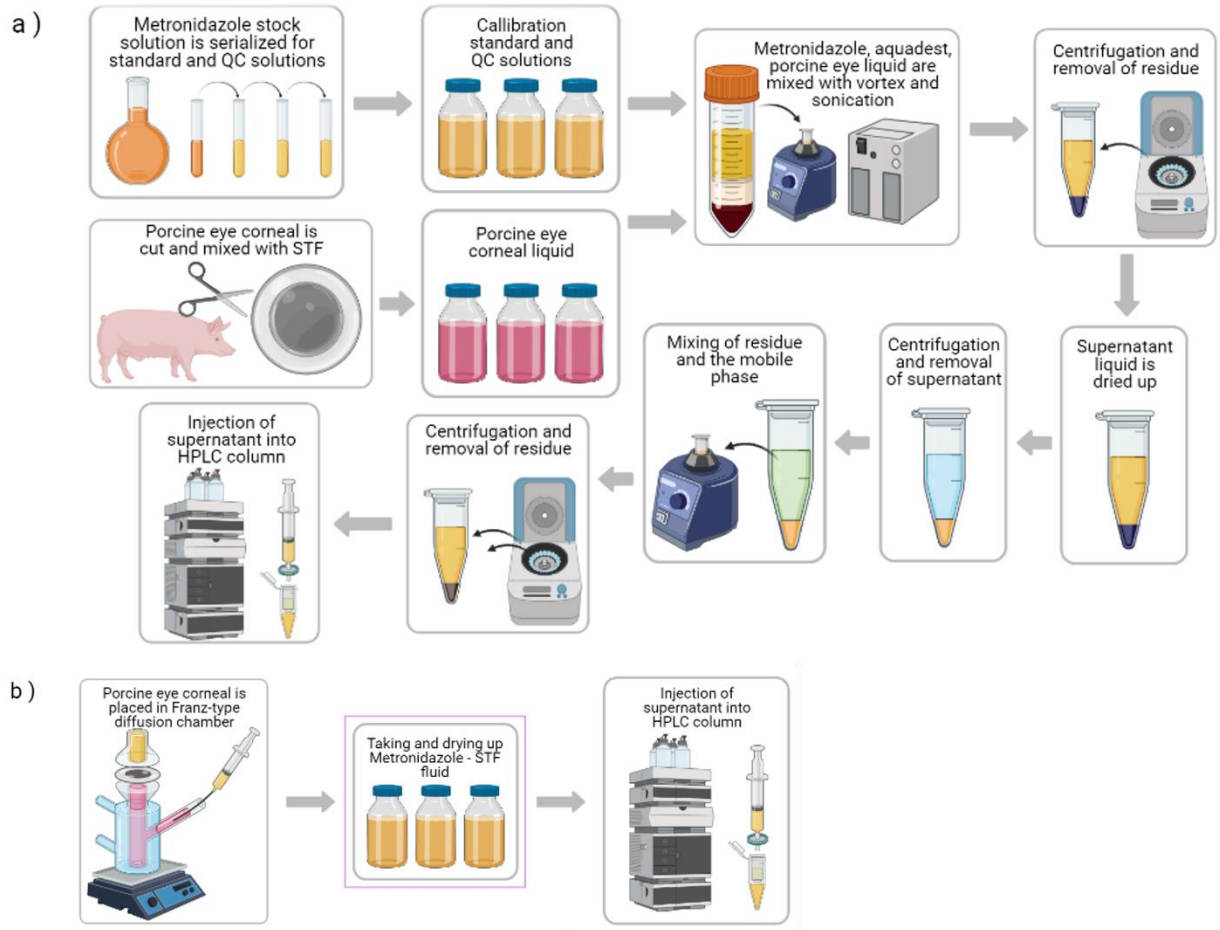

Figure 1. Illustration of the work steps

2.4. Instrumentation and Optimisation of HPLC–UV conditions

Simultaneous analysis of the entire analyte applied using HPLC system (Shimadzu Prominence, Shimadzu, Kyoto, Japan) equipped with PDA detector; acetonitrile and 20 mM

acetate buffer were utilized as the mobile phases; the separation of analytes using Xselect CSH ™ C18 column (Waters, 3.0 × 150 mm); 3.5 µm particle size with guard cartridge.

CCD was used in the response surface methodology to optimize mobile phase, flow rate, mobile phase pH, and acetonitrile concentration (**Table S2**). Design Expert® Software version 11 (State-Ease, Minneapolis, MN, USA) was used in processing the data and performed statistical analyses. Parameters recorded response were the retention time (RT), the theoretical plates and tailing factors.

2.5. Analytical method validation

Based on US FDA and ICH guidelines, the developed bioanalytical method was validated [15,18]. Validated parameters were precision, accuracy, linearity, selectivity, carry over, extraction recovery, lower limit of quantification (LLOQ), dilution integrity, and stability.

2.5.1. Linearity, LOD and LLOQ

Linearity was measured by a calibration curve derived from a sample with a working standard solution. The calibration curve was made of seven levels of concentration with three separate times.

The limits of detection and limits of quantification were determined by the regression of the obtained standard curves. LOD and LOQ were represented as 3.3 × Syx/*b* and 10.0 × Syx/*b*, respectively, where Syx is residual variance because of regression and *b* is the slope mean of the linear regression curves.

2.5.2. Accuracy and Precision

Accuracy and precision measurements are carried out at three concentrations: low, medium, and high concentrations in six replicates for the LLOQ and QC samples. Intra-day and inter-day accuracy and precision evaluations were carried out. The precision test was carried out to observe the relative standard deviation (RSD) of the responses of all samples, while the accuracy test was carried out by determining the relative error (RE). The RSD and RE values were set to be 15% for each replication of the sample [15,18].

2.5.3. Carry-over and dilution integrity

Evaluation of carry-over was accomplished by incorporating the QC samples at high concentrations. After that, the empty solution was injected. The response of blank solution was looked after, and the response obtained should not be more than 20% of the sample solution at a concentration of LLOQ [21].

In this study, all samples were prepared at a concentration 250 µg/mL for each metronidazole. The solutions were dissolved 5 and 10 times with porcine eye liquid. Accuracy and precision were finally calculated. To evaluate the integrity of the dilution, samples are analyzed using concentrations of analyte above the higher concentration of the calibration standard solution.

2.5.4. Extraction recovery

In order to measure the recovery of the extracted metronidazole from the sample matrix, the values were measured for all analytes from the extracted QC samples, such as the LLOQ, low, medium and high concentrations. The exact concentration of samples prepared in the mobile phase will be used.

2.5.5. Stability studies

Metronidazole stability studies were performed with porcine eye corneal fluid under different storage and treatment conditions. Within 48 hours, an autosampler was carried out to evaluate the stability of all analyte solutions. The bench-top stability evaluation was carried out at room temperature for 24 hours, while the long-term stability evaluation was carried out at -20 ° C for 2 weeks. Evaluation of freeze-thaw stability was carried out in three cycles with a storage temperature of -20 ° C. Each response obtained was then compared with the initial response of each solution.

2.6 Application of validated HPLC method to assess ocular kinetics of metronidazole from thermosenstivie gel formulation in corneal tissue

2.6.1 Thermosensitive in situ ocular gel optimization

Different thermosensitive formulation containing metronidazole were prepared using Pluronic® F127 (PF127), Pluronic® F88 (PF88), and benzalkonium chloride. In this formulation,

gelling agent was used at different concentrations (**Table S3**). The responses to be examined were physical characteristics of the ocular gel which were gelation temperature and viscosity.

2.6.2 Ex vivo ocular kinetics evaluation of formulated metronidazole gels in corneal tissue

A Franz-type diffusion chamber on the porcine cornea was used in the experiment to determine the ocular kinetics and drug absorption through the cornea. Immediately after sacrificing the animal, the eyes of the porcine were collected and the corneas were carefully separated to avoid damage. This aims to get the porcine's eyeballs that were still fresh; The cornea with a scleral tissue size of 2-4 mm was carefully removed, washed with cold normal saline, then stored.

The fresh cornea was then placed between the chambers in the diffusion cells. Epithelial surface faced upwards, whereas the endothelial surface faced down towards the receptor compartment. STF at pH 7.4 was filled into the receptor compartment and then stirred gently with a magnetic stirrer. Thermosensitive *in situ* gel containing metronidazole was put into the donor compartment. At predetermined times, the cornea was collected and processed using the optimized extraction method.

2.7. Statistical analysis

Data were displayed as mean ± standard deviation (SD). Calculations of mean, SD, % RSD, and % RE were calculated using Microsoft Excel® 2016 (Microsoft Corporation, Redmond, USA). The calculation of ocular kinetic parameters (the maximum metronidzaole concentration in corneal tissue (C_{max}), the time needed to achive the maximum concentration (t_{max}), the metronidazole concentration time in the corneal tissue curve from 0 to 72 h (AUC), the mean half-life ($t_{1/2}$) of metronidazole in corneal tissue and the mean residence time (MRT)) was used by PK Solver using non-compartment pharmacokinetic analysis [22]. Curves for comparison of drug concentration and time profiles were created. Statistical data analysis with $p < 0.05$ as a significant difference was used GraphPad Prism® version 8.3.0 (GraphPad Software Inc., San Diego, California).

3. Result and Discussion

3.1 Sample preparation and analytes extraction

One-step protein precipitation was used in this study to extract and prepare metronidazole from biological matrices. Permana et al. [23] have reported that this procedure is simple to be applied and used. Any metabolite from biological matrices are removed to avoid analytical error or column damage. The sample preparation and extraction method were optimized using a central composite experimental design. As seen in **Table S4.**, two parameters, namely extraction efficiency and evaporation time, were observed in this optimization process. The results showed that extraction efficiency and evaporation time in three parameters fitted to the quadratic model and the value of F for the efficiency of extraction and evaporation time were determined respectively 10.64 and 1162.7878.

Extraction efficiency and evaporation time was found to be significantly affected (p <0.001) this optimizing, and p-values represent it was <0.00. **Figure 2** depicts a representative three-dimensional graph showing the response of the selected factors to the extraction efficiency and evaporation time. The model indicated that the extraction performance was directly proportional to the volume of methanol, vortex time, and sonication time. The high extraction efficiency resulted from increasing the amount of methanol volume, vortex time, and sonication time in the extraction method resulting in higher extraction efficiency.

Finally, according to the CCD analysis, the methanol volume of 525 ml, vortex time of 15 minutes, and 10.091 minutes of sonication time were recommended for the extraction method to obtain the optimum extraction efficiency evaporation time (**Table S5**).

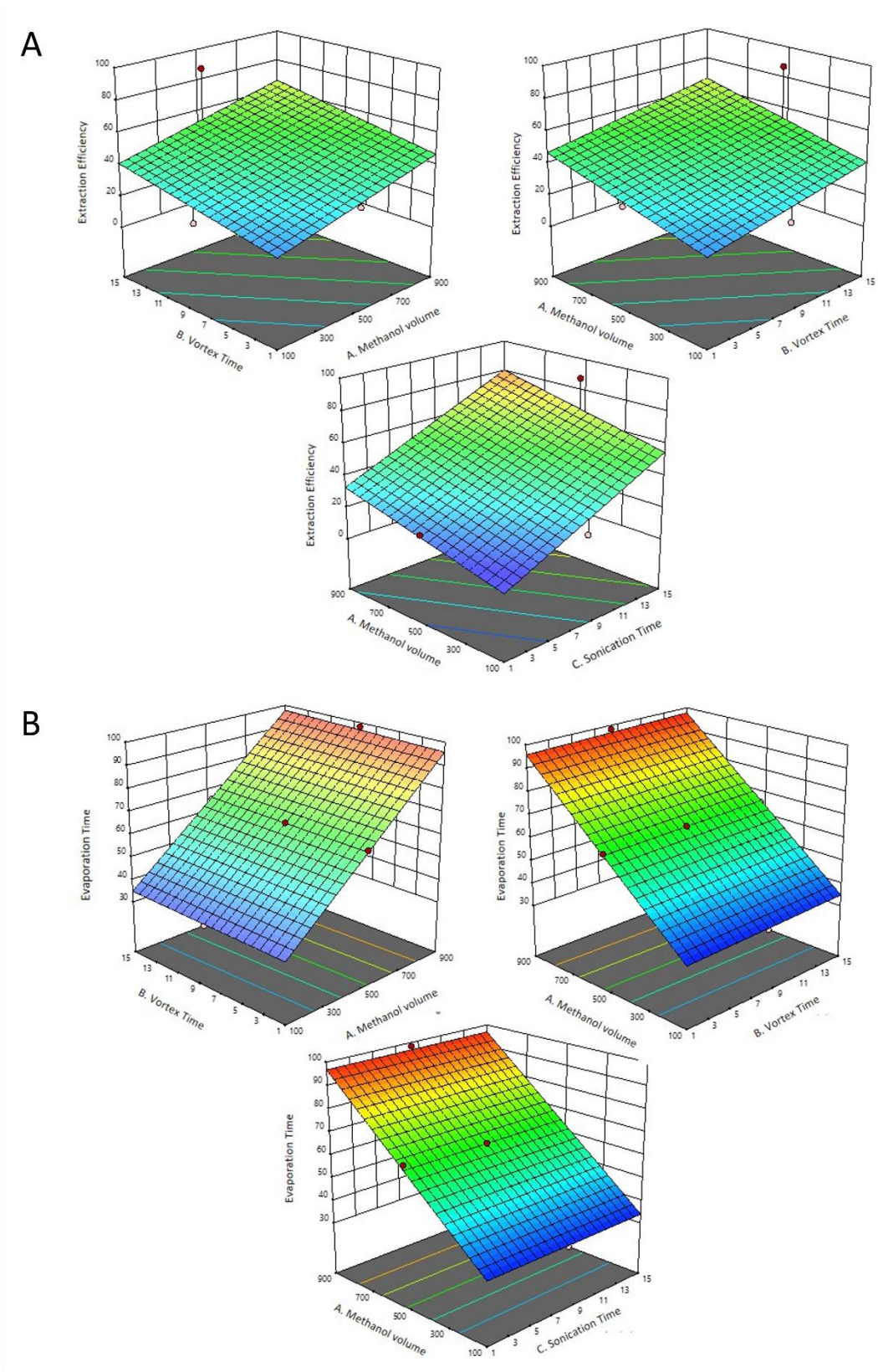

Figure 2. Illustrations of representative response surface plots illustrating the impact of the selected factors on the : A. Extraction Efficiency; B. Evaporation time.

3.2 Instrumentation and optimization of HPLC–UV conditions

The RP-HPLC isocratic process was used in this study. **Table S6** represented the overview result of the response surface analysis. The aim of this process is to optimize the mobile phase conditions including the concentration of acetonitrile, pH, and flow rate. **Figure 3** shows HPLC chromatograms of porcine corneal tissue fluid with and without metronidazole at a retention time of 7.32 minutes, analyzed at 313 nm. The C18 column demonstrated improved column efficiency and analyte elution with good resolution, tailing factor, and theoretical plate count. The entire separation of analytes was done by reverse C18 column due to its flexibility and suitability in analyzing and separating metronidazole [24].

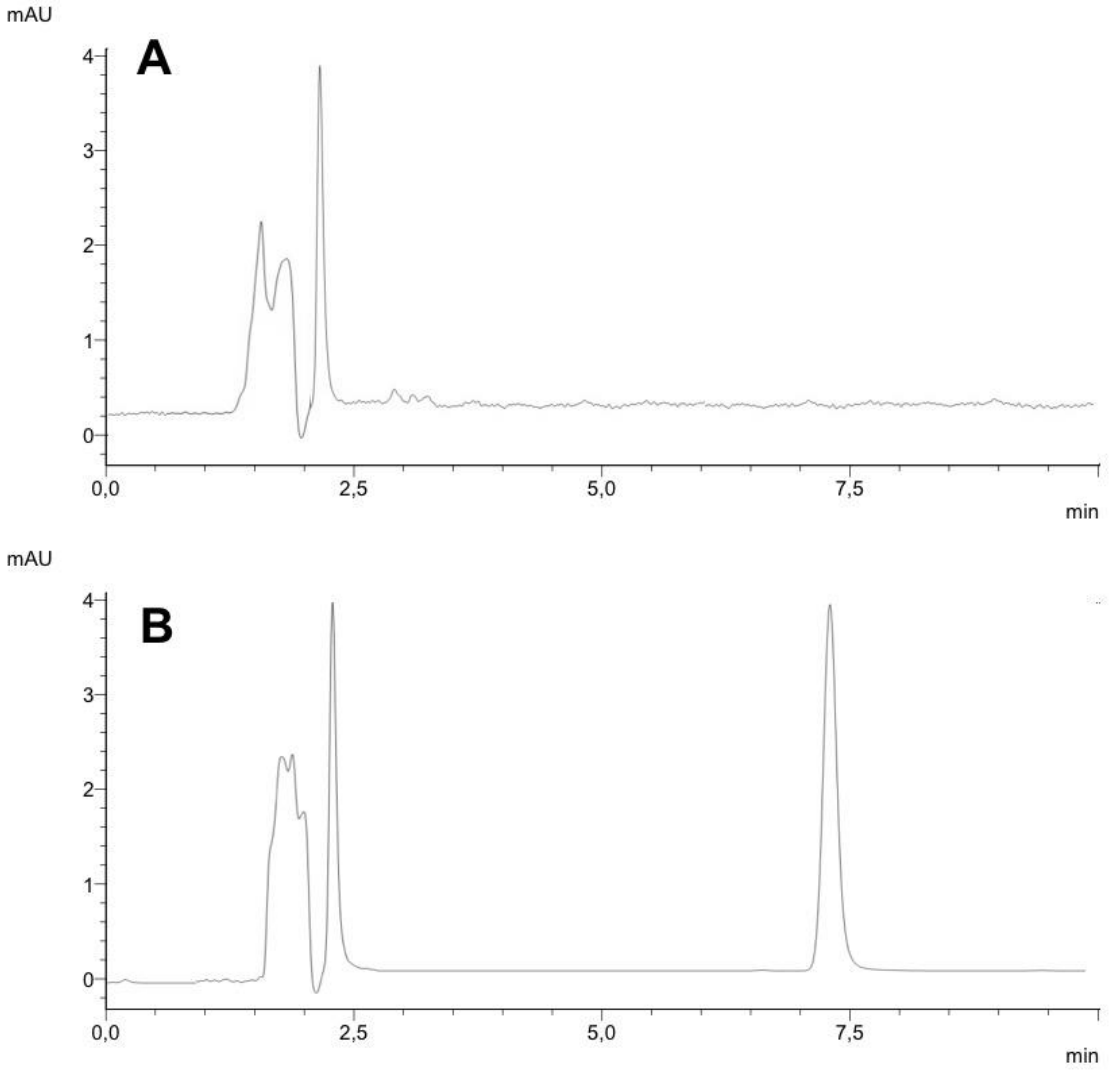

Figure 3. Representative HPLC-UV chromatograms of blank porcine eye corneal tissue fluid (A) and metronidazole with porcine eye corneal tissue fluid (B)

The optimization process was carried out by observing effects of the theoretical plate, tailing factors, and retention time fitted in the quadratic model. Our analysis revealed that all of these parameters gave p-values of less than 0.001 when compared using the F-values from each of the parameters. The results showed that the F value for tailings factor, theoretical plate, and retention time were 15.65, 10.97, and 61.33, respectively. Therefore, we are confident that all of these factors were important to the optimisation process. The effect of the selected factors on retention time, tailings factor, and theoretical plates is shown in **Figures 4** in the form of a representative 3D graph. The graph shows that the combination of acetonitrile concentration, pH and flow rate is directly proportional to the tailings factor, theoretical plate and retention time.

The mobile phase pH of an ionizable analyte has a significant impact on the retention activity of the analyte. It can be said that the pH of the mobile phase is one of the critical parameters and needs to be considered during the development of the method. One of them is when determining the pH value to achieve the desired separation. Problems in liquid chromatography often occur when pH of the mobile phase is close to or equal to the pKa of the compound being analyzed, which is indicated by a prominent peak. Process accuracy can also be reduced by a broad peak accompanied by a long tail [25]. Optimization of the mobile phase can be done with this method by selecting the pH of the mobile phase which is one unit lower than the pKa of the compound under investigation [23]. In this analysis, an acetate buffer at a concentration of 20 mM was used. The recommended results under HPLC-UV conditions to obtain optimal tailings factor, theoretical plates, and retention time using software are acetonitrile concentration of 21,000 %, pH 4.505, and recommended flow rate of 1.020 ml/min (**Table S7**).

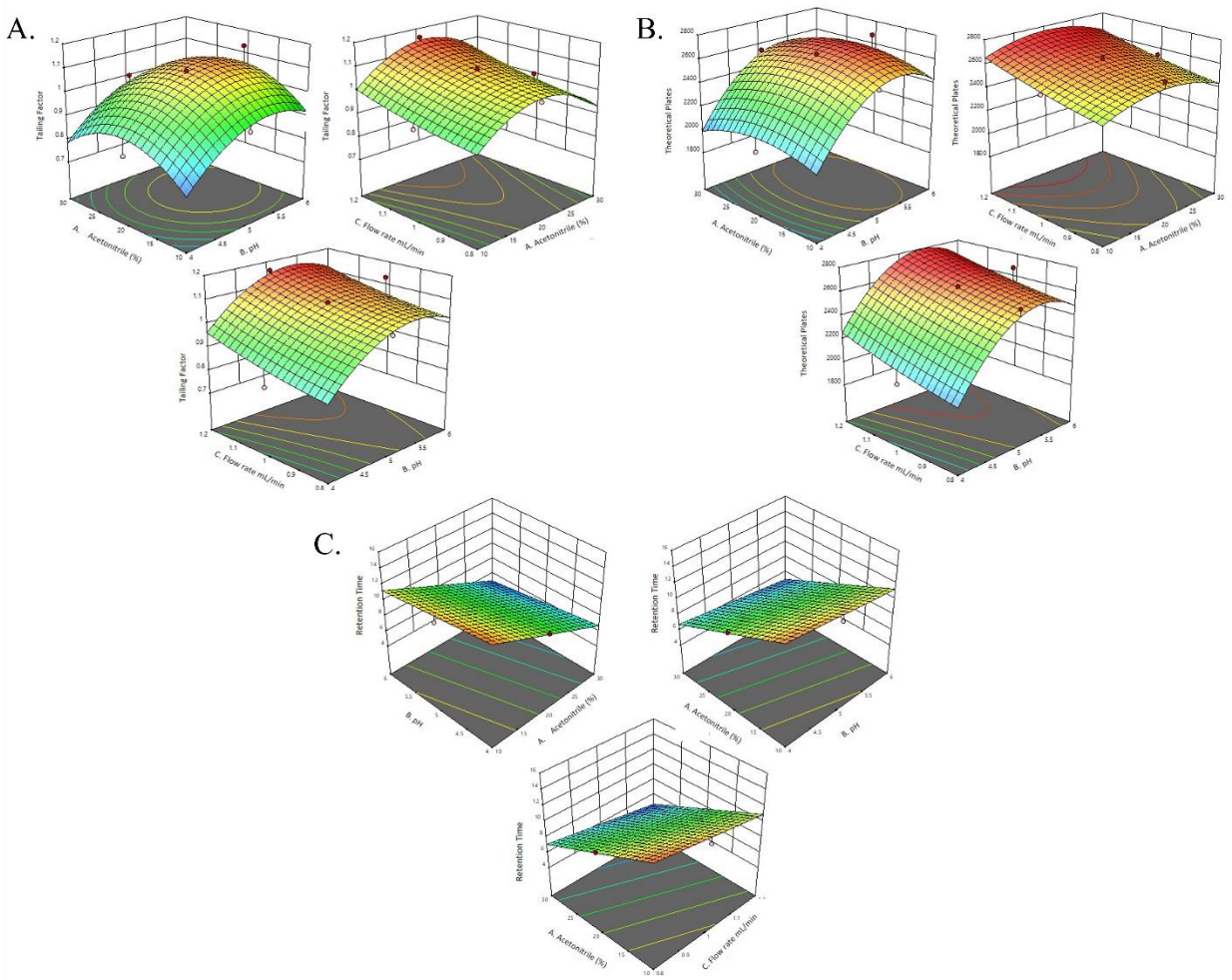

Figure 4. Illustrations of representative response surface plots illustrating the impact of the selected parameters on : A. Tailing factor; B. Theoretical plates and retention time; C. Retention time

3.3 Analytical method validation

3.3.1 Linearity, LOD and LLOQ

The results of the linearity of the methods, LOD, and LLOQ are shown in **Table 1**. This method is known to obtain linear values (R = 0.999) at concentrations ranging from 0.01 to 10 µg/ml. Excellent sensitivity was also shown by the LOD and LLOQ values as we found that metronidazole can be detected at relatively low concentration.

3.3.2 Accuracy and precision

Intra and inter-day measurements were performed to assess the precision and accuracy of this method. Metronidazole was tested on three different days as a method of evaluating accuracy and

precision between days. Meanwhile, metronidazole measured on the same day with three repetitions as part of the intra-day evaluation. Based on the research, it is known that the method of measuring metronidazole concentrations in intra-day and inter-day measurements with good accuracy is at concentrations between 0.08 to 7.5 μg/mL as indicated by the %RSD value of not more than ± 15% (**Table 1**). In addition, this method is also considered to have good precision, indicated by the %RSD which decreases between 2.16 and 14.39% at the exact concentration measurements as above. It meets the criteria for precision measurement since it did not exceed ± 15% of the %RSD limit (**Table 1**).

3.3.3 Carry-over and dilution integrity

Evaluation of the carry-over effect was carried out to observe the possibility of metronidazole signaling in the subsequent measurement of the blank solution by injecting high concentrations of metronidazole into the HPLC column. The tested blank solution has not more than 20% LLOQ in the corresponding retention time of metronidazole. The result obtained in this experiment was that metronidazole was not detected in the sequence of blank solutions. This indicated the absence of carry-over effects in the HPLC profile.

The dilution integrity was checked by evaluating the consistency of the concentration of metronidazole after the solution was diluted 5 and 10 times lower than the concentrated solution of metronidazole. The results of the metronidazole dilution, when analyzed using the method we developed, showed recoveries between 98.21 ± 5.19% and 102.01 ± 9.31% with a precision of 4.14%-7.42%. Based on the standard satisfaction range of accuracy (85 - 115%) and precision (± 15%), it can be considered that the results showed excellent dilution integrity.

3.3.4 Recovery of Extraction

Evaluation of the extraction recovery of porcine eye fluid and tissue was carried out by adding fluid at three different concentrations: low (0.08 and 0.15 μg/mL), medium (3.5 μg/mL), and high (7.5 μg/mL). In **Table 1**, it can be seen that the spiked sample recoveries were between 94.19 ± 8.76% and 96.51 ± 7.84% with a range of %RSD values from 8.12 to 9.80%. These results of the %RSD value is below ±15% of the RSD, hence it is considered that the method is precise and consistent for the determination of metronidazole concentrations from porcine eye fluid.

3.3.5 Stability studies

Table 1 shows that metronidazole was stable under all storage conditions when added to swine eye fluid. In addition, all concentration levels (low, medium, and high) showed values that were acceptable to the ICH standard for stability validation [26] which were indicated by recoveries values above 95% with an SD percentage of not more than 15%. Based on the literature searched, there were no publications regarding the stability of metronidazole in porcine eye fluid. However, there have been studies regarding the stability of metronidazole in human plasma under storage conditions similar to those carried out in this study [27].

Table 1. The results of the HPLC validation of metronidazole (n=6)

Linearity, LOD and LLOQ values				
Slope	y-intercept	R	LOD (µg/mL)	LLOQ (µg/mL)
3762.3	32.796	0.999	0.05	0.08
Intra-day Precision and Accuracy				
Replication	Concentration added (µg/mL)	Concentration found (µg/mL) ± SD	Precision (%RSD)	Accuracy (%RE)
1	0.08	0.073 ± 0.004	5.48	-8.75
	0.15	0.14 ± 0.003	2.16	-7.33
	3.5	3.57 ± 0.21	5.88	2.00
	7.5	7.63 ± 0.54	7.08	1.73
2	0.08	0.082 ± 0.003	3.66	2.50
	0.15	0.15 ± 0.021	13.64	2.67
	3.5	3.87 ± 0.37	9.56	10.57
	7.5	7.82 ± 0.69	8.82	4.27
3	0.08	0.076 ± 0.006	7.89	-5.00
	0.15	0.13 ± 0.015	11.36	-12.00
	3.5	3.94 ± 0.42	10.66	12.57
	7.5	7.43 ± 0.84	11.31	-0.93
Inter-day Precision and Accuracy				
Day	Concentration added (µg/mL)	Concentration found (µg/mL) ± SD	Precision (%RSD)	Accuracy (%RE)
1	0.08	0.076 ± 0.005	6.58	-5.00
	0.15	0.13 ± 0.019	14.39	-12.00
	3.5	3.84 ± 0.32	8.33	9.71
	7.5	7.66 ± 0.63	8.22	2.13
2	0.08	0.085 ± 0.006	7.06	6.25
	0.15	0.17 ± 0.021	12.43	12.67
	3.5	3.39 ± 0.43	12.68	-3.14
	7.5	7.65 ± 0.84	10.98	2.00
3	0.08	0.084 ± 0.004	4.76	5.00
	0.15	0.13 ± 0.012	9.16	-12.67
	3.5	3.29 ± 0.41	12.46	-6.00
	7.5	7.59 ± 0.54	7.11	1.20
Extraction recoveries				
Concentration added (µg/mL)		% Extraction Recovery ± SD	% RSD	
0.08		94.19 ± 8.76	9.30	
0.15		96.51 ± 7.84	8.12	
3.5		92.01 ± 9.02	9.80	
7.5		93.93 ± 8.89	9.46	
% Stability recoveries (mean ± SD)				
Concentration added (µg/mL)	Autosampler (48 h)	Bench-top (24 h)	Long-term (2 weeks)	Freeze-thaw (3 cycles)
0.08	98.31 ± 8.52	96.95 ± 9.04	103.21 ± 5.42	99.18 ± 8.32
0.15	101.02 ± 7.43	98.72 ± 6.03	97.64 ± 9.51	98.13 ± 8.43
3.5	99.43 ± 8.03	99.31 ± 9.43	98.39 ± 8.05	100.92 ± 7.94
7.5	96.53 ± 6.05	100.93 ± 5.65	99.53 ± 6.99	98.53 ± 9.91

3.4 Physical properties of the formulated gels and evaluation of their ocular kinetics

3.4.1 Gelation temperature and viscosity of the formulated in-situ gels

Factors such as gelation temperature and viscosity, particularly at different conditions, are some of the most important aspects of studying thermosensitive gel preparations. Hence, we assessed these properties and compared all four formulations to see the influence of Pluronic F127 and F88 in affecting the physical characteristics of the gels. Since the eye temperature ranges from 33.5 to 35.5 [28], our goal is to formulate a thermosensitive metronidazole gel that forms a gel around these points.

In the results showing the gelation temperature (**Figure 5.IA**), it was found that all preparations formed gel at different temperatures ($p<0.05$). Formulations A and B formed gels above body temperature while formulation C showed gelation at around 34.87 ± 3.12 °C. Formulation D, which contains Pluronic F127 alone (20%), formed gels even lower at approximately 23.64 ± 2.19 °C. The data obtained in this experiment indicated that Pluronic F88 as a combination of the gelling agent is required to obtain a suitable gelation temperature for eye applications. This finding is also in line with the previous report stating that a combination of longer hydrophobic chain poloxamers such as Pluronic F127 with more hydrophilic co-polymers is essential to the formation of gelling state at the desired temperature [9,29]. The interactions of Pluronic F127 with the other materials such as polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), methylcellulose (MC), and hydroxypropyl methylcellulose (HPMC) have also been demonstrated as an important factor in order to control the gelation temperature of the formulated ophthalmic sol-gel preparation [30].

In terms of viscosity, a further investigation on the influence of Pluronic F127 and F88 toward the rheology of formulated gels also provides insight into the importance of this combination on the physical properties of the gels upon application in eye or storage. As seen in the **Figure 5.IB**, the viscosity of the gels increased as the temperature elevated. For instance, the viscosity of formulation A and B at 4 °C was 131 ± 9.32 cPs and 143 ± 8.54 cPs, respectively. When the gels are tested at room temperature, the viscosity increases, but the gelling state has not been acquired. Consistent with gelation temperatures, the viscosity of Formulation C increased significantly ($p<0.05$) up to 26483 ± 2409 cPs when applied at eye temperature. In addition, this formulation did not form gel upon storage at 4 and 25 °C which is considered beneficial for storage conditions.

On the other hand, Formulation D was already in the gel form when temperature reached around 25 °C, indicated by its viscosity (up to 23498 ± 2192 cPs) and even increased to around 36218 ± 3381 cPs at around eye temperature. No formulation was observed to form gel at 4 °C. Based on these results, we agree that combination of Pluronic F127 and F88 used in formulation C is the most suitable mixture of these co-polymers to obtain a thermosensitive gel for ocular administration. However, we are also aware that this experiment is limited to the shorter time of storage and hence a thoroughly designed stability studies should be carried out to establish effects of more prolonged or even extreme storage conditions to the formulated preparations.

Pluronic F127 has longer hydrophobic poly(propylene oxide) (PPO) blocks compared to its hydrophilic poly(ethylene oxide) (PEO) blocks. In contrast, Pluronic F88 has 80% PEO relative to the other block chains, making this co-polymer more hydrophilic than Pluronic F127. Hydrophobic PPO blocks are responsible for lowering the gelation temperature while hydrophilic PEO blocks, otherwise, elevate the temperature [31,32]. A careful NMR study revealed that dehydration of PPO-PEO micelles in the solution is the key for the gelation of these co-polymers when applied at the specific temperature [33].

Although our findings mainly suggested that the use of Pluronic F127 and F88 is important in the solution-to-gel formation, it is interesting to point out that other copolymers have also been long utilized. For instance, in the formulation of Azithromycin thermosensitive gel, 20-22% of Poloxamer 404 which contain hydrophobic PPO blocks were incorporated with 5% of Poloxamer 188 and 3-4% Carbopol 974P NF in order to obtain suitable gelation temperature of the oplthalmic application [34]. Alkholief, M. et.al (2010) also demonstrated this effect using a similar gel base in which the poloxamer and carbopol ratio was the factors that determine the slight changes in the gelation temperature [35]. Another example of applicable copolymers is the use of poly-(DL-lactic acid-co-glycolic acid) (PLGA)–polyethylene glycol (PEG)–PLGA as a thermosenstive gel base to deliver drugs as an ophthalmic preparations [36]. All of these evidences again supported nature of these polymer combinations where longer hydrophobic blocks copolymers interact with other materials to obtain desired properties of the gel bases. Therefore, our study also confirmed that physical characteristics such as gelation temperature and viscosity of thermosensitive in-situ gels prepared using block co-polymers depend on the influence of hydrophobic and hydrophilic blocks from the co-polymers.

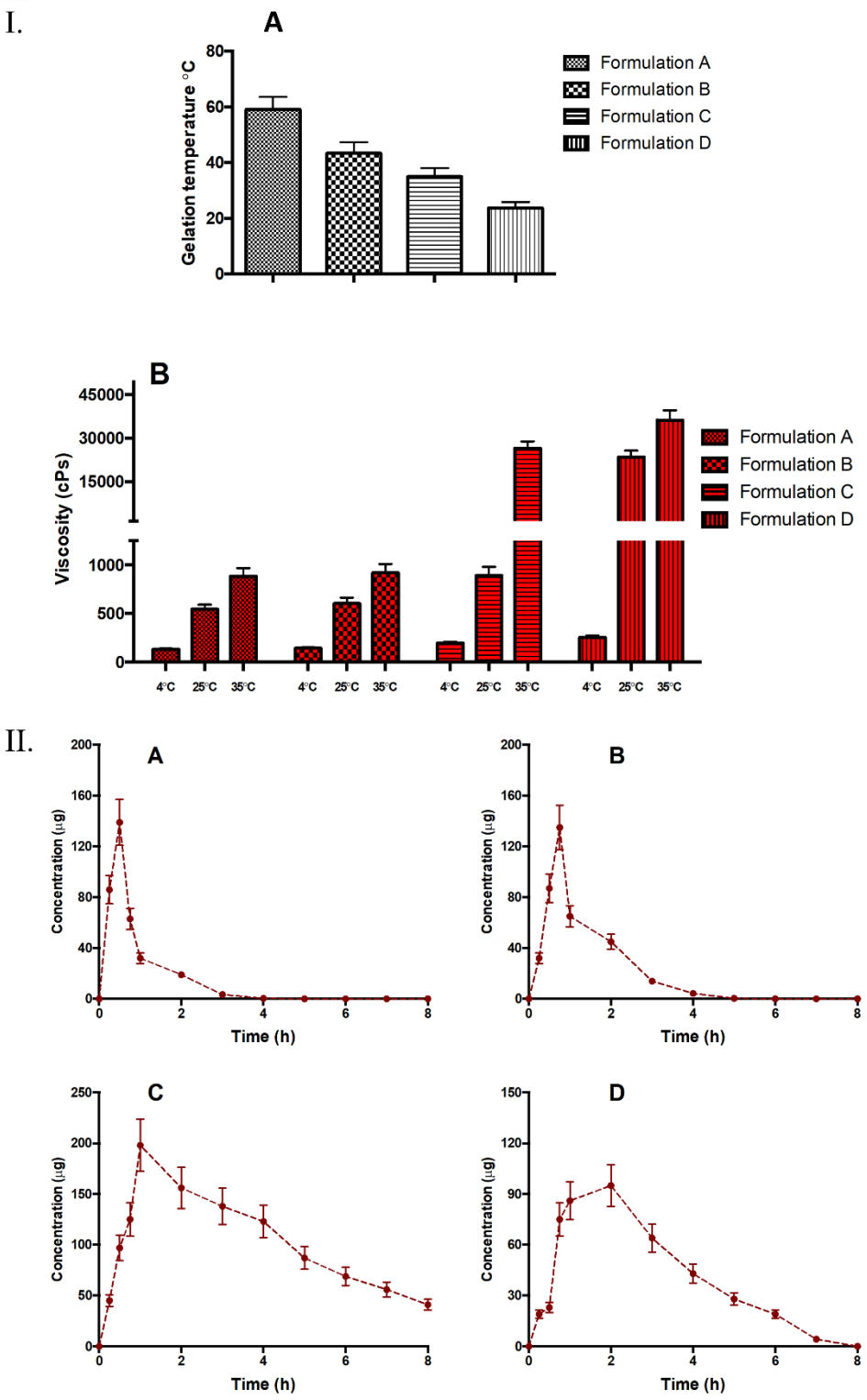

Figure 5. I. Overall physical characteristic evaluation of thermosensitive gel preparations: **(**A) gelation temperature (n=3) and (B) viscosity of formulation A-D measured at 4, 25 and 35 °C (n=3); **II.** Ocular kinetic study of metronidazole thermosensitive gels showing concentration of metronidazole (µg) in corneal matrix over the measured time (hours). A-D correspond to formulation A-D which contain various types of Pluronic F127 and F88 combinations.

3.4.2 Ex vivo ocular kinetics evaluation of formulated metronidazole gels in corneal tissue

The validated method obtained in this study was then applied in studying the ocular kinetic of the formulated metronidazole gels. The kinetic profiles were obtained by evaluating the concentration of metronidazole from each formulated preparation in the corneal matrix. The results showing the summary of the kinetics properties of these formulations was given in **Table 2**. As indicated by the data, formulation C revealed the most significant AUC value meaning the metronidazole in porcine corneal availability is achieved better than the other formulations. It also showed a significantly higher C_{max} ($p<0.05$) which was reached at approximately 1 hour after administration.

Meanwhile, this formula's half-life (T½) was 2.54 ± 0.31 hours, a specific resident time of metronidazole in the examined compartment. Overall, metronidazole concentrations from formulation A and B were not detected at 5 hours after administration while formulation C and D can extent the drug release longer (**Figure 5.II**). It is also interesting to note that formulation A which contains 5% of Pluronic F127 and 15 % of Pluronic F88 reached the maximum concentration of 110.81 ± 9.32 µg/ml in approximately 30 minutes after application and the longest time to reach maximum concentration was achieved by formulation D consisting of 20% of Pluronic F127 and 0% Pluronic F88. The data indicate correlation between the concentration of Pluronic combination (F127 and F88) and the availability of metronidazole in the cornea. Increasing the concentration of Pluronic F127 in the formulation A-D seems to cause the T_{max} value to be obtained at a longer time. However, there is an influence of Pluronic 127 combined with F88 in enhancing the peak corneal concentration, AUC and MRT of metronidazole since the half-life of formulation D which contains no Pluronic F88 cannot be solely improved by simply increasing Pluronic F127 concentration. Therefore, the data presented in this study is in line with our hypothesis that the combination of these two poloxamers is required in such preparations to obtain better kinetic outcomes of metronidazole.

Table 2. *Ex vivo* ocular kinetic parameters of metronidazole thermosensitive *in-situ* gels (n=3).

Parameters	Formulation A	Formulation B	Formulation C	Formulation D
C_{max} (µg)	110.81 ± 9.32	89.08 ± 8.71	171.72 ± 18.21	30.07 ± 4.31
T_{max} (h)	0.5	0.75	1	2
AUC_{0-t} (µg.h)	104.83 ± 10.21	189.59 ± 19.21	843.85 ± 90.29	37.83 ± 4.32
AUC_{0-INF} (µg.h)	104.83 ± 11.02	191.28 ± 20.12	991.36 ± 91.21	38.04 ± 3.92
$T_{1/2}$ (h)	0.25 ± 0.02	0.78 ± 0.08	2.54 ± 0.31	0.38 ± 0.05
MRT (h)	0.69 ± 0.07	1.62 ± 0.17	4.56 ± 0.43	0.93 ± 0.12

The effect of poloxamer matrices in enhancing bioavailability of metronidazole eye preparations has not been widely available, but some evidence shows that Poloxamer-based gel is useful in improving ocular drug delivery of other drugs. Our previous report observed that gels developed using the combination of Pluronic F127 and F68 enhance the ocular kinetic of Itraconazole in an optimized thermosensitive gel preparation whether it is applied in an infected or a normal eye model [9]. Pluronic F127 with other poloxamers is also known to improve the availability of other incorporated drugs [37]. In terms of metronidazole delivery into eye tissue, Vanderbilj [6] reported that metronidazole gel showed significant diffusion rate in human and rabbit eyes compared to the eye solution. Further investigations also revealed that trans-corneal diffusion of metronidazole in human and rabbit eyes is not affected by the presence of chemical preservatives such as benzalkonium chloride [38]. In contributing to the previous knowledge available, the experiment carried out in this study provides a new insight on the relationship between poloxamer based thermosensitive gels and the kinetics of metronidazole in the eye tissue. However, it is beyond our scope to evaluate the kinetic profiles of these formulations in human or animal eye models even though factors such as tissue temperature and physiological conditions highly influence this type of pharmaceutical preparation. Therefore, we suggested that further research is carried out in addressing whether these factors are important to establish excellent kinetic profiles of metronidazole in Pluronic based thermosensitive gels.

4. Conclusions

The development of a validation method using this new high performance liquid chromatography (HPLC-UV) method was established. The new approach developed in this study in which metronidazole concentrations were measured after *ex vivo* administration in porcine cornea, had high precision and accuracy, simplicity, and high sensitivity and selectivity for use in therapeutic drug control research and ocular kinetics evaluation.

As our findings suggested, the formulation of thermosensitive gels represented in this study revealed influences of PF127 and PF88 combination to produce gels with an excellent gelation temperature and viscosity and their ocular kinetic properties. The method we developed here was also applicable in examining the concentration of metronidazole in the formulated thermosensitive in-situ gel preparations. However, further research is required to fully understand whether this analysis method can also be implemented in studying the drug administration under pathological conditions.

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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.

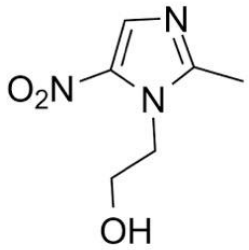
☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author Contribution Statement

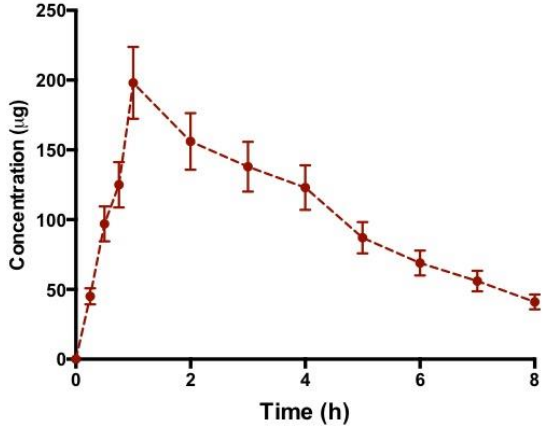
Nur Asma: Conceptualization, Methodology, Writing - Original Draft, Review & Editing**; Nurul Muhlisah Maddeppungeng:** Methodology, Writing - Original Draft, Review & Editing**; Muhammad Raihan:** Writing - Original Draft, Review & Editing**; Arini Putri Erdiana:** Methodology, Investigation, Data Curation**;**, **Achmad Himawan:** Data Curation, Review & Editing, Validation, Supervision**; Andi Dian Permana:** Conceptualization, Review & Editing Project Administration, Funding Acquisition, Validation, Supervision**.**

Graphical Abstract

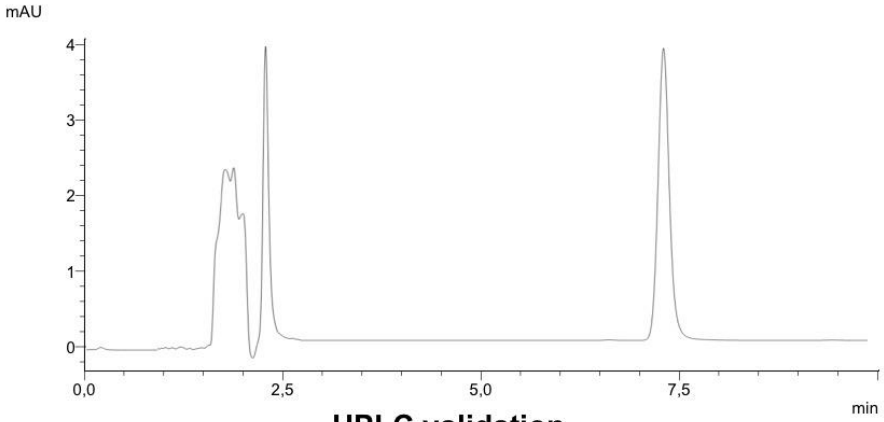
Thermosensitive *in situ* gel



Metronidazole



***Ex vivo* ocular kinetic study**



HPLC validation

New HPLC-UV analytical method for quantification of metronidazole: Application to *ex vivo* ocular kinetic assessments following the administration of thermosensitive ocular *in situ* gel

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Abstract

Metronidazole eye drops have been used to treat *Acanthamoeba* keratitis. However, ophthalmic preparations also have some limitations, one of which is the rapid elimination of the drug, that reducing the effectiveness of the drug. Accordingly, an alternative delivery approach can be applied to overcome this issue. Additionally, as one of critical steps in the formulation development, analyitical methods that allow the quantification of metronidazole in *ex vivo* corneal permeation and deposition should also be developed. Here, we report a validated high-performance liquid chromatography method (HPLC-UV) according to ICH guidelines for the measurements of metronidazole concentrations following formulation of thermosensitive ocular *in situ* gel and its administration in *ex vivo* porcine corneas. The development of extraction techniques and optimization of HPLC conditions were optimized using analytical quality by design. Xselect™ CSHTM C18 HPLC column (Water, 3.0 × 150 mm, particle size 3.5 m) was used to separate all analytes by isocratic elution with mobile phases of acetate buffer and acetonitrile with LLOQ value of 0.08 µg/mL. The resulting method proved to be selective, precise, and accurate and was succefully applied to determine ocular kinetic profiles of metronidazole from thermosensitive ocular *in situ* gel in *ex vivo* porcine corneas, showing that this approach was able to improve the concentration of metronidazole in the corneal tissues. We, therefore, suggested that HPLC-UV approach developed in this study has the potential to be used in drug release evaluation, therapeutic drug control research, ocular kinetics, and toxicological evaluation.

Keywords:

Metronidazole, keratitis, HPLC, thermosensitive *in situ* gel, ocular kinetic, method validation

1. Introduction

Infection that presents with corneal ulcerations known as Acanthamoeba keratitis is caused by *Acanthamoeba* species. It was first reported in 1973. This condition occurs commonly when someone uses a contact lens washing solution prepared with non-sterile salt tablets and water [1].

Metronidazole is a derivative of 5-Nitroimidazole known to act as a powerful antibacterial and antiprotozoal. For decades, eye preparations has become a fast growing pharmaceutical technology [2]. In ophthalmology, general eye drops and topical eye preparations are the most widely used preparations. It is considered the most efficient form of treating infections of the foreground structures generally caused by anaerobic bacteria [3]. Acanthamoeba keratitis can be treated using 0.5% metronidazole eye drops mixed with other antiprotozoal preparations [4]. Metronidazole eye drops have long been a formulary drug in pharmacies as an alternative to the lack of a commercially available form of eye medications. In ophthalmology, preparations with 0.1% and 0.5% metronidazole solutions have been used to treat Acanthamoeba keratitis [5–7].

Topical administration of the drug is more prevalent among patients and safer than the intraocular injection, but topical use of this drug has the disadvantage that the drug cannot be adequately absorbed in the eye and reaches the posterior segment. As a part of trends in topical formulation, thermosensitive gels have been utilized in overcoming these problems. The term thermosensitive refers to its ability to form gel or solution reversibly upon at a particular temperature. This can be achieved by taking advantages of unique properties of some gelling agent such as N-acrylamide based co-polymers and poly(propylene oxide) (PPO)/ polyethylene oxide (PEO) block co-polymers [8]. However, previous reports showed that combination of two co-polymers or its interaction with other gelling agents is required to obtained a relatively precise temperature for the gel to form [9–11]. To our best understanding, this is the first time the technology of thermosensitive gels is applied to administer metronidazole in an ocular drug delivery. Therefore, it is also an important task to assess whether the newly incorporated gel bases is able to release metronidazole upon application in the eye condition.

Over the last few decades, many improved methods have been undertaken to find and explore more effective ways of administering eye medications and treatments for various eye diseases [12]. In transcorneal penetration studies, *in vitro* cell culture models have been widely selected. The development of an organotypic corneal construction technique also studies the

bioavailability of ophthalmic drugs using primary cell cultures and immortalized cell lines. There is great potential for research into corneal penetration in artificially cultivated human corneas. Using a research model using cut animal corneal tissue to study drug penetration is one technique with promising results, although it is necessary to consider the significant variation between species [13]. One of the developments in the new *ex vivo* model is to use a porcine cornea disc. It is considered to be more cost-effective for candidates for transcorneal penetrating topical therapy [14].

Method development and validation have tremendous importance in the QC of the drug. In recent years because of its importance, the development of new testing methods for drug determination has received considerable attention in determining potency of active ingredients in eye drops. Hence, HPLC is the analytical method of choice for measuring metronidazole [7]. The HPLC method validation test will be conducted according to guidelines recommended by the USFDA (2001) [15]. Comparison between the retention times of metronidazole detected in the drug-free plasma assay and metronidazole derived from drug injection extracted from spiked plasma will be investigated as a test of specificity and selectivity [7].

Several previous studies related to HPLC and LC-MS/MS for the quantification of metronidazole in various biological matrices have been carried out. A study by Silva et al (2009) showed simple, fast, sensitive, and selective results using liquid chromatography (LC)-tandem mass spectrometry (MS-NONA)[16]. In addition, HPLC and LC-MS/MS for metronidazole quantification have also been performed on saliva, plasma, and gingival crevicular fluid [17].

Transcorneal penetration of metronidazole using *ex vivo* porcine corneal model has never been developed. The development of extraction method before having HPLC assay is essential to produce maximum drug concentration. Optimization and development of analytical methods, also recognized as the analytical quality by design (AQbD), are now widely applied to quality by design (QbD). This concept is considered necessary since the first time it was first introduced by the USFDA [18]. This concept is known to require less time for experimentation because it uses the experimental design method (DOE) to obtain possible combinations of parameters and has also been recommended in robustness testing [18,19]. Implementation of AQbD applications in HPLC method development has been done in many studies, Rozet et al reported using AQbD approach for analytical method by HPLC [20]. Prior to analyzing metronidazole with HPLC, we developed an optimised method for extracting drug form matrix using various sonication time and strirring

time. This aimed to maximise the number of metronidazole that were measured using HPLC method. The validated HPLC method will be used to assess the *ex vivo* ocular kinetics of the drug after topical application to the eye.

2. Materials and methods

2.1. Chemicals and materials

Metronidazole (≥99.9%, purity), analytical grade trifluoroacetic acid, and HPLC-grade Methanol (MeOH) (Sigma-Aldrich Pte Ltd, Singapore), Xselect CSH ™ C_{18} HPLC column (particle size 3.5 µm, 3, 0 × 150 mm; Waters, Dublin, Ireland), chemical reagent (Sigma - Aldrich Pte Ltd, Singapore). Other reagents were analytical grade and obtained from standard commercial supplier.

2.2. Stock Preparation, Calibration Standards and Quality Control Samples

Metronidazole stock solution was prepared by dissolving 50 mg in 50 ml methanol so that a concentration of 1 mg/mL was obtained. The working solutions were diluted into serial concentrations for standard calibration, and quality control (QC) solutions. In the calibration of the test method, a concentration series was made using the stock solution diluted with porcine eye corneal tissue matrices. The final concentrations obtained ranged from 0.01 to 10 µg/mL. QC solutions were made in three types of concentrations, namely 0.15 µg/mL, 3.5 µg/mL and 7.5 µg/mL for low QC, medium QC and high QC, respectively. All QC solutions were prepared in porcine eye corneal tissue matrices.

2.3. Preparation and Analytes Extraction of Samples

Simulated tear fluid consisting of 6.7 g NaCl, 2.0 g NaHCO3 and 0.08 g CaCl2·2H2O was intially prepared by dissolving all components in 1 L deionized water (final pH of 7.4). Preparation of the sample was conducted by mixing porcine eye corneal 2,5 g with 2,5 g STF (stimulated tear fluid) pH 7,4 to make porcine eye corneal fluid, 2 mg of metronidazole was added with 2 mL aquadest. The 100 µL of the mixture was centrifuged with 900 µL porcine eye corneal fluid. The extraction volume, stirring time and sonication time were evaluated. The response surface methodology to optimize sonication time, vortex time, and methanol volume (**Table S1**) used Composite Central Design (CCD) by Design Expert Software

version 11 (State-Ease, Minneapolis, MN, USA). The response parameters were extraction performance and fluid evaporation time.

The supernatant liquid was put into a small glass container and then dried up in a fume hood for about 3 hours. This is done in order to obtain dried residue. The residual mixture and the 100 µL mobile phase were stored in a 0.5 mL centrifuge tube. For 30 seconds, the mixture was stirred and then rotated at 14,000 rpm × g for 15 minutes. A total of 10 µL of the treated supernatant solution was injected into the HPLC column. The work steps are illustrated in **Figure 1.**

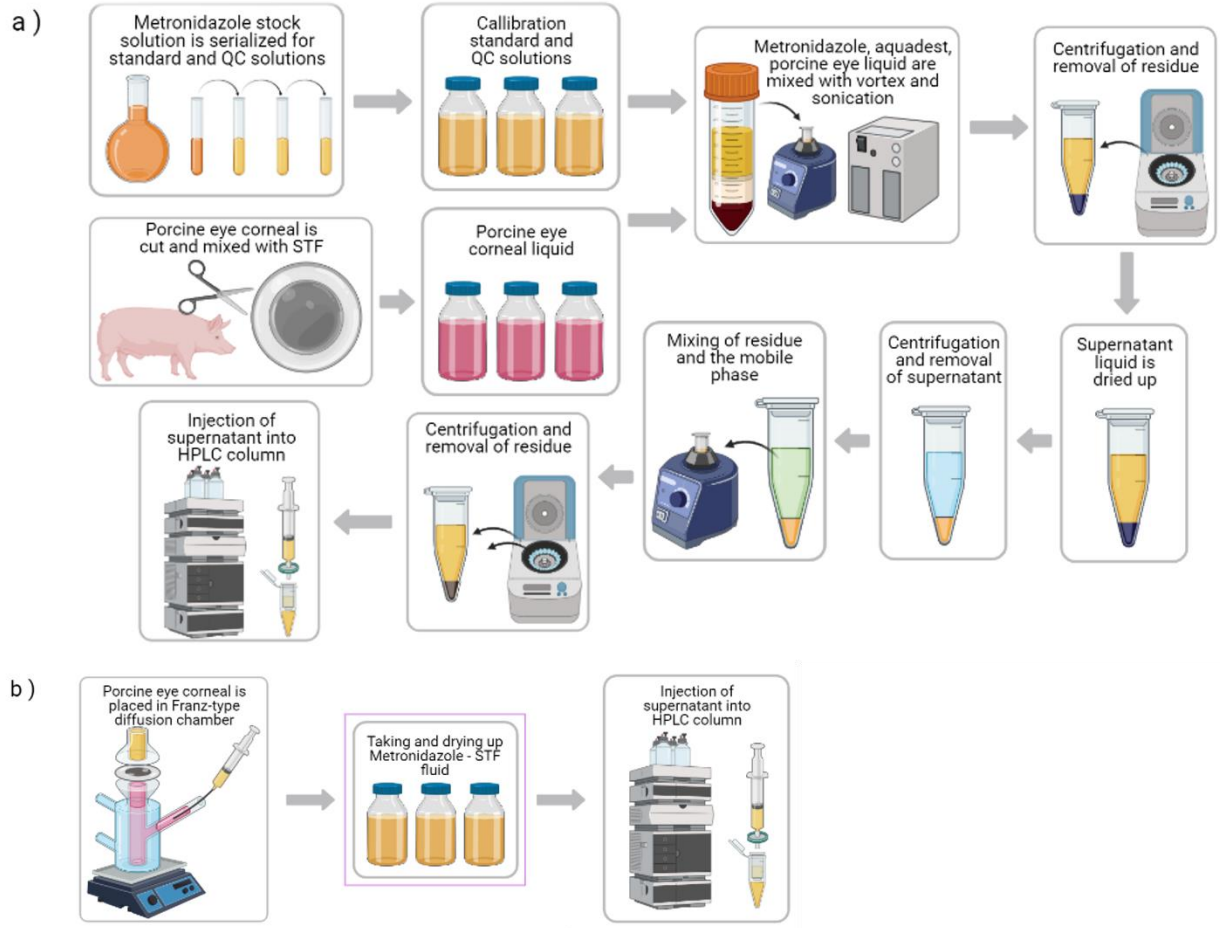

Figure 1. Illustration of the work steps

2.4. Instrumentation and Optimisation of HPLC–UV conditions

Simultaneous analysis of the entire analyte applied using HPLC system (Shimadzu Prominence, Shimadzu, Kyoto, Japan) equipped with PDA detector; acetonitrile and 20 mM

acetate buffer were utilized as the mobile phases; the separation of analytes using Xselect CSH ™ C18 column (Waters, 3.0 × 150 mm); 3.5 µm particle size with guard cartridge.

CCD was used in the response surface methodology to optimize mobile phase, flow rate, mobile phase pH, and acetonitrile concentration (**Table S2**). Design Expert® Software version 11 (State-Ease, Minneapolis, MN, USA) was used in processing the data and performed statistical analyses. Parameters recorded response were the retention time (RT), the theoretical plates and tailing factors.

2.5. Analytical method validation

Based on US FDA and ICH guidelines, the developed bioanalytical method was validated [15,18]. Validated parameters were precision, accuracy, linearity, selectivity, carry over, extraction recovery, lower limit of quantification (LLOQ), dilution integrity, and stability.

2.5.1. Linearity, LOD and LLOQ

Linearity was measured by a calibration curve derived from a sample with a working standard solution. The calibration curve was made of seven levels of concentration with three separate times.

The limits of detection and limits of quantification were determined by the regression of the obtained standard curves. LOD and LOQ were represented as 3.3 × Syx/*b* and 10.0 × Syx/*b*, respectively, where Syx is residual variance because of regression and *b* is the slope mean of the linear regression curves.

2.5.2. Accuracy and Precision

Accuracy and precision measurements are carried out at three concentrations: low, medium, and high concentrations in six replicates for the LLOQ and QC samples. Intra-day and inter-day accuracy and precision evaluations were carried out. The precision test was carried out to observe the relative standard deviation (RSD) of the responses of all samples, while the accuracy test was carried out by determining the relative error (RE). The RSD and RE values were set to be 15% for each replication of the sample [15,18].

2.5.3. Carry-over and dilution integrity

Evaluation of carry-over was accomplished by incorporating the QC samples at high concentrations. After that, the empty solution was injected. The response of blank solution was looked after, and the response obtained should not be more than 20% of the sample solution at a concentration of LLOQ [21].

In this study, all samples were prepared at a concentration 250 µg/mL for each metronidazole. The solutions were dissolved 5 and 10 times with porcine eye liquid. Accuracy and precision were finally calculated. To evaluate the integrity of the dilution, samples are analyzed using concentrations of analyte above the higher concentration of the calibration standard solution.

2.5.4. Extraction recovery

In order to measure the recovery of the extracted metronidazole from the sample matrix, the values were measured for all analytes from the extracted QC samples, such as the LLOQ, low, medium and high concentrations. The exact concentration of samples prepared in the mobile phase will be used.

2.5.5. Stability studies

Metronidazole stability studies were performed with porcine eye corneal fluid under different storage and treatment conditions. Within 48 hours, an autosampler was carried out to evaluate the stability of all analyte solutions. The bench-top stability evaluation was carried out at room temperature for 24 hours, while the long-term stability evaluation was carried out at -20 ° C for 2 weeks. Evaluation of freeze-thaw stability was carried out in three cycles with a storage temperature of -20 ° C. Each response obtained was then compared with the initial response of each solution.

2.6 Application of validated HPLC method to assess ocular kinetics of metronidazole from thermosenstivie gel formulation in corneal tissue

2.6.1 Thermosensitive in situ ocular gel optimization

Different thermosensitive formulation containing metronidazole were prepared using Pluronic® F127 (PF127), Pluronic® F88 (PF88), and benzalkonium chloride. In this formulation,

gelling agent was used at different concentrations (**Table S3**). The responses to be examined were physical characteristics of the ocular gel which were gelation temperature and viscosity.

2.6.2 Ex vivo ocular kinetics evaluation of formulated metronidazole gels in corneal tissue

A Franz-type diffusion chamber on the porcine cornea was used in the experiment to determine the ocular kinetics and drug absorption through the cornea. Immediately after sacrificing the animal, the eyes of the porcine were collected and the corneas were carefully separated to avoid damage. This aims to get the porcine's eyeballs that were still fresh; The cornea with a scleral tissue size of 2-4 mm was carefully removed, washed with cold normal saline, then stored.

The fresh cornea was then placed between the chambers in the diffusion cells. Epithelial surface faced upwards, whereas the endothelial surface faced down towards the receptor compartment. STF at pH 7.4 was filled into the receptor compartment and then stirred gently with a magnetic stirrer. Thermosensitive *in situ* gel containing metronidazole was put into the donor compartment. At predetermined times, the cornea was collected and processed using the optimized extraction method.

2.7. Statistical analysis

Data were displayed as mean ± standard deviation (SD). Calculations of mean, SD, % RSD, and % RE were calculated using Microsoft Excel® 2016 (Microsoft Corporation, Redmond, USA). The calculation of ocular kinetic parameters (the maximum metronidzaole concentration in corneal tissue (C_{max}), the time needed to achive the maximum concentration (t_{max}), the metronidazole concentration time in the corneal tissue curve from 0 to 72 h (AUC), the mean half-life ($t_{1/2}$) of metronidazole in corneal tissue and the mean residence time (MRT)) was used by PK Solver using non-compartment pharmacokinetic analysis [22]. Curves for comparison of drug concentration and time profiles were created. Statistical data analysis with p <0.05 as a significant difference was used GraphPad Prism® version 8.3.0 (GraphPad Software Inc., San Diego, California).

3. Result and Discussion

3.1 Sample preparation and analytes extraction

One-step protein precipitation was used in this study to extract and prepare metronidazole from biological matrices. Permana et al. [23] have reported that this procedure is simple to be applied and used. Any metabolite from biological matrices are removed to avoid analytical error or column damage. The sample preparation and extraction method were optimized using a central composite experimental design. As seen in **Table S4.**, two parameters, namely extraction efficiency and evaporation time, were observed in this optimization process. The results showed that extraction efficiency and evaporation time in three parameters fitted to the quadratic model and the value of F for the efficiency of extraction and evaporation time were determined respectively 10.64 and 1162.7878.

Extraction efficiency and evaporation time was found to be significantly affected (p <0.001) this optimizing, and p-values represent it was <0.00. **Figure 2** depicts a representative three-dimensional graph showing the response of the selected factors to the extraction efficiency and evaporation time. The model indicated that the extraction performance was directly proportional to the volume of methanol, vortex time, and sonication time. The high extraction efficiency resulted from increasing the amount of methanol volume, vortex time, and sonication time in the extraction method resulting in higher extraction efficiency.

Finally, according to the CCD analysis, the methanol volume of 525 ml, vortex time of 15 minutes, and 10.091 minutes of sonication time were recommended for the extraction method to obtain the optimum extraction efficiency evaporation time (**Table S5**).

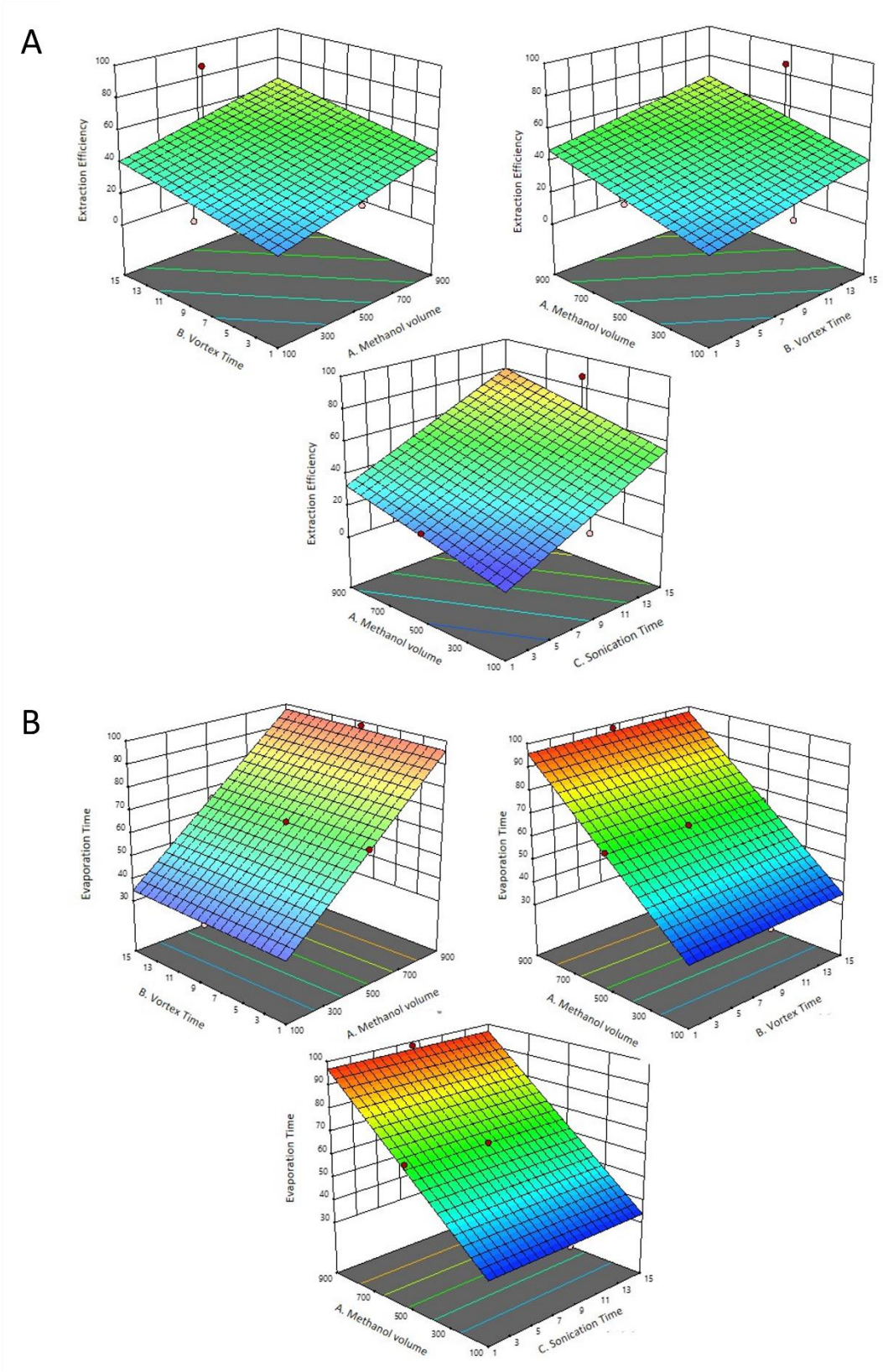

Figure 2. Illustrations of representative response surface plots illustrating the impact of the selected factors on the : A. Extraction Efficiency; B. Evaporation time.

3.2 Instrumentation and optimization of HPLC–UV conditions

The RP-HPLC isocratic process was used in this study. **Table S6** represented the overview result of the response surface analysis. The aim of this process is to optimize the mobile phase conditions including the concentration of acetonitrile, pH, and flow rate. **Figure 3** shows HPLC chromatograms of porcine corneal tissue fluid with and without metronidazole at a retention time of 7.32 minutes, analyzed at 313 nm. The C18 column demonstrated improved column efficiency and analyte elution with good resolution, tailing factor, and theoretical plate count. The entire separation of analytes was done by reverse C18 column due to its flexibility and suitability in analyzing and separating metronidazole [24].

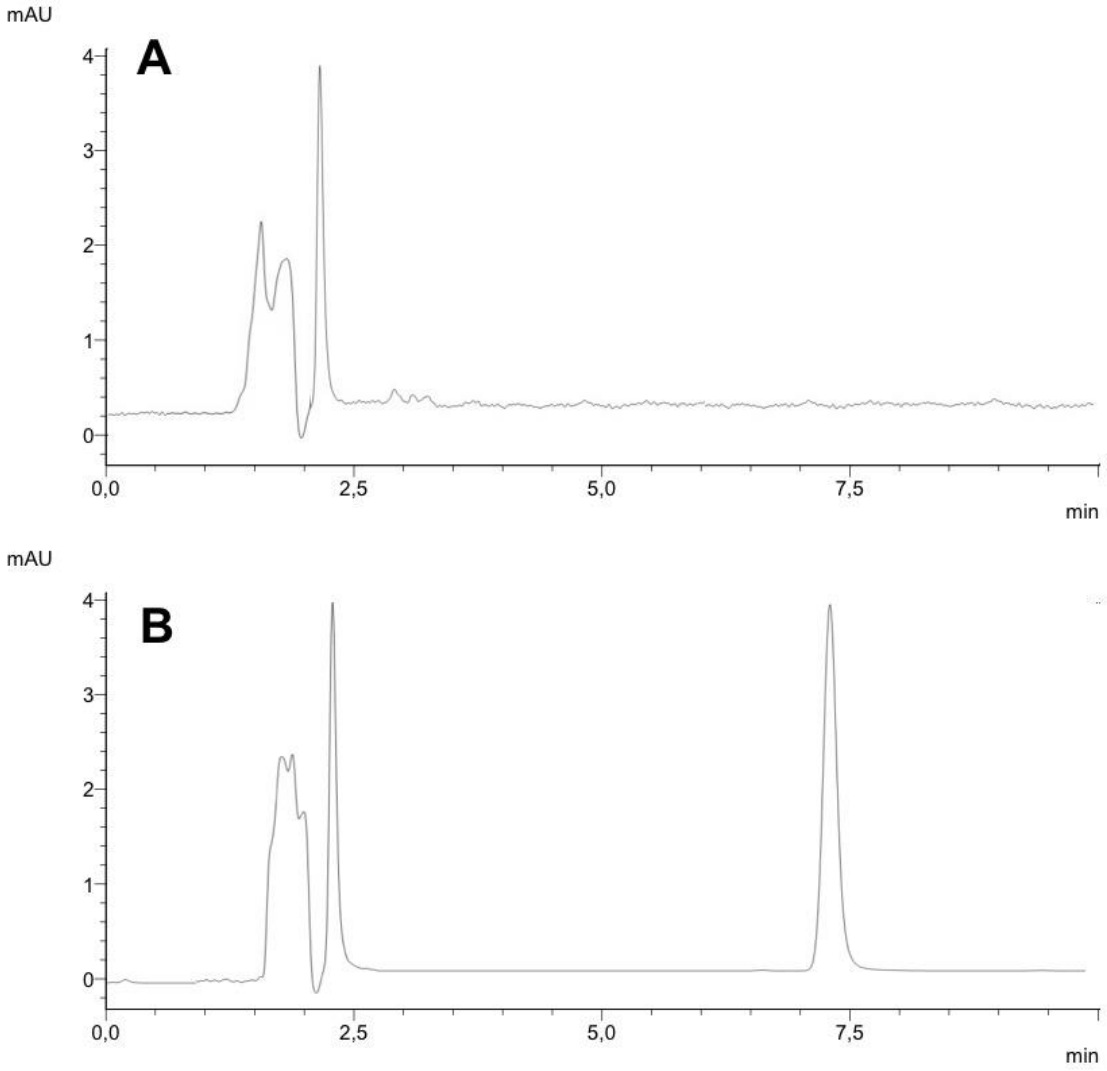

Figure 3. Representative HPLC-UV chromatograms of blank porcine eye corneal tissue fluid (A) and metronidazole with porcine eye corneal tissue fluid (B)

The optimization process was carried out by observing effects of the theoretical plate, tailing factors, and retention time fitted in the quadratic model. Our analysis revealed that all of these parameters gave p-values of less than 0.001 when compared using the F-values from each of the parameters. The results showed that the F value for tailings factor, theoretical plate, and retention time were 15.65, 10.97, and 61.33, respectively. Therefore, we are confident that all of these factors were important to the optimisation process. The effect of the selected factors on retention time, tailings factor, and theoretical plates is shown in **Figures 4** in the form of a representative 3D graph. The graph shows that the combination of acetonitrile concentration, pH and flow rate is directly proportional to the tailings factor, theoretical plate and retention time.

The mobile phase pH of an ionizable analyte has a significant impact on the retention activity of the analyte. It can be said that the pH of the mobile phase is one of the critical parameters and needs to be considered during the development of the method. One of them is when determining the pH value to achieve the desired separation. Problems in liquid chromatography often occur when pH of the mobile phase is close to or equal to the pKa of the compound being analyzed, which is indicated by a prominent peak. Process accuracy can also be reduced by a broad peak accompanied by a long tail [25]. Optimization of the mobile phase can be done with this method by selecting the pH of the mobile phase which is one unit lower than the pKa of the compound under investigation [23]. In this analysis, an acetate buffer at a concentration of 20 mM was used. The recommended results under HPLC-UV conditions to obtain optimal tailings factor, theoretical plates, and retention time using software are acetonitrile concentration of 21,000 %, pH 4.505, and recommended flow rate of 1.020 ml/min (**Table S7**).

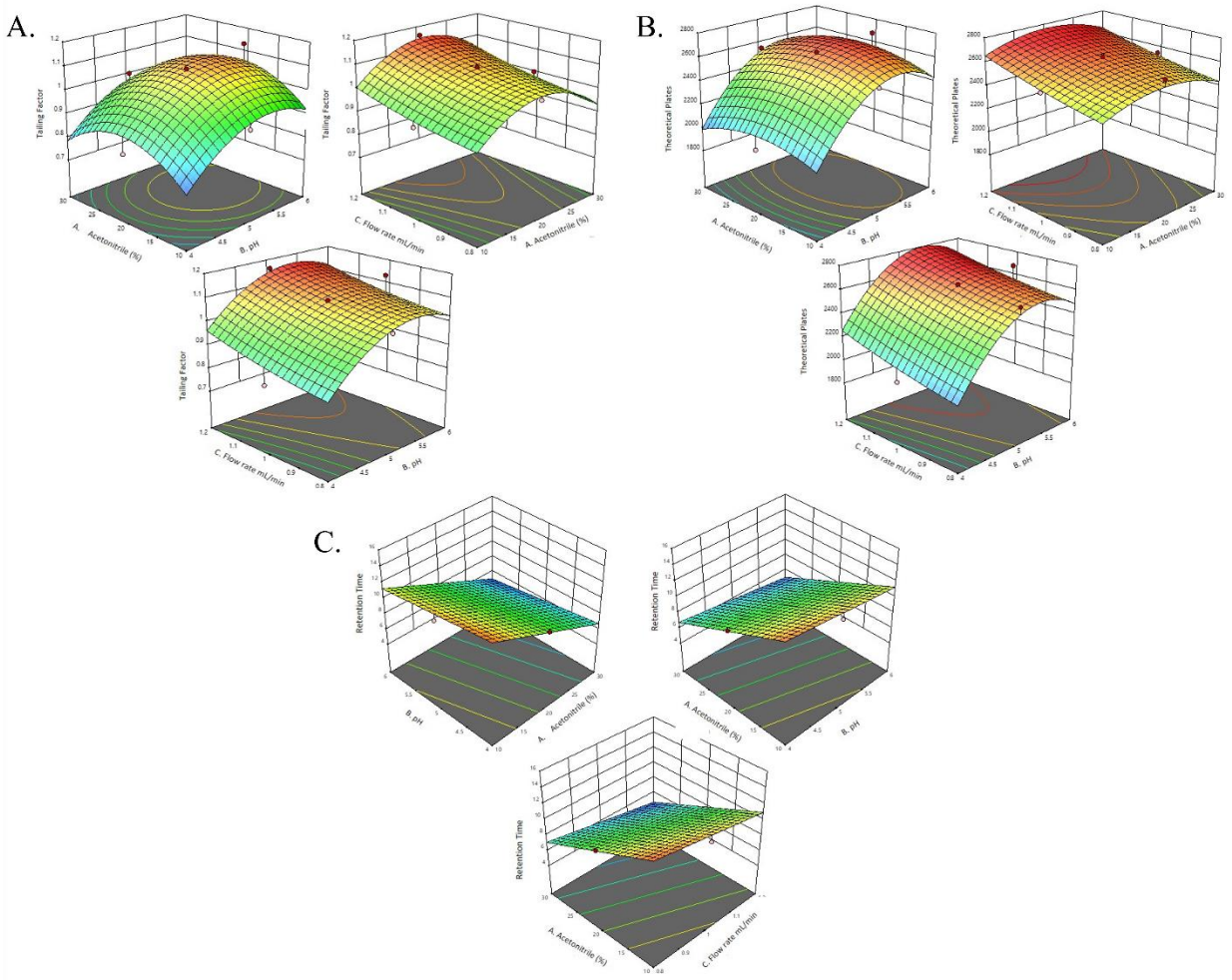

Figure 4. Illustrations of representative response surface plots illustrating the impact of the selected parameters on : A. Tailing factor; B. Theoretical plates and retention time; C. Retention time

3.3 Analytical method validation

3.3.1 Linearity, LOD and LLOQ

The results of the linearity of the methods, LOD, and LLOQ are shown in **Table 1**. This method is known to obtain linear values (R = 0.999) at concentrations ranging from 0.01 to 10 µg/ml. Excellent sensitivity was also shown by the LOD and LLOQ values as we found that metronidazole can be detected at relatively low concentration.

3.3.2 Accuracy and precision

Intra and inter-day measurements were performed to assess the precision and accuracy of this method. Metronidazole was tested on three different days as a method of evaluating accuracy and

precision between days. Meanwhile, metronidazole measured on the same day with three repetitions as part of the intra-day evaluation. Based on the research, it is known that the method of measuring metronidazole concentrations in intra-day and inter-day measurements with good accuracy is at concentrations between 0.08 to 7.5 μg/mL as indicated by the %RSD value of not more than ± 15% (**Table 1**). In addition, this method is also considered to have good precision, indicated by the %RSD which decreases between 2.16 and 14.39% at the exact concentration measurements as above. It meets the criteria for precision measurement since it did not exceed ± 15% of the %RSD limit (**Table 1**).

3.3.3 Carry-over and dilution integrity

Evaluation of the carry-over effect was carried out to observe the possibility of metronidazole signaling in the subsequent measurement of the blank solution by injecting high concentrations of metronidazole into the HPLC column. The tested blank solution has not more than 20% LLOQ in the corresponding retention time of metronidazole. The result obtained in this experiment was that metronidazole was not detected in the sequence of blank solutions. This indicated the absence of carry-over effects in the HPLC profile.

The dilution integrity was checked by evaluating the consistency of the concentration of metronidazole after the solution was diluted 5 and 10 times lower than the concentrated solution of metronidazole. The results of the metronidazole dilution, when analyzed using the method we developed, showed recoveries between 98.21 ± 5.19% and 102.01 ± 9.31% with a precision of 4.14%-7.42%. Based on the standard satisfaction range of accuracy (85 - 115%) and precision (± 15%), it can be considered that the results showed excellent dilution integrity.

3.3.4 Recovery of Extraction

Evaluation of the extraction recovery of porcine eye fluid and tissue was carried out by adding fluid at three different concentrations: low (0.08 and 0.15 μg/mL), medium (3.5 μg/mL), and high (7.5 μg/mL). In **Table 1**, it can be seen that the spiked sample recoveries were between 94.19 ± 8.76% and 96.51 ± 7.84% with a range of %RSD values from 8.12 to 9.80%. These results of the %RSD value is below ±15% of the RSD, hence it is considered that the method is precise and consistent for the determination of metronidazole concentrations from porcine eye fluid.

3.3.5 Stability studies

Table 1 shows that metronidazole was stable under all storage conditions when added to swine eye fluid. In addition, all concentration levels (low, medium, and high) showed values that were acceptable to the ICH standard for stability validation [26] which were indicated by recoveries values above 95% with an SD percentage of not more than 15%. Based on the literature searched, there were no publications regarding the stability of metronidazole in porcine eye fluid. However, there have been studies regarding the stability of metronidazole in human plasma under storage conditions similar to those carried out in this study [27].

Table 1. The results of the HPLC validation of metronidazole (n=6)

Linearity, LOD and LLOQ values				
Slope	y-intercept	R	LOD (µg/mL)	LLOQ (µg/mL)
3762.3	32.796	0.999	0.05	0.08
Intra-day Precision and Accuracy				
Replication	Concentration added (µg/mL)	Concentration found (µg/mL) ± SD	Precision (%RSD)	Accuracy (%RE)
1	0.08	0.073 ± 0.004	5.48	-8.75
	0.15	0.14 ± 0.003	2.16	-7.33
	3.5	3.57 ± 0.21	5.88	2.00
	7.5	7.63 ± 0.54	7.08	1.73
2	0.08	0.082 ± 0.003	3.66	2.50
	0.15	0.15 ± 0.021	13.64	2.67
	3.5	3.87 ± 0.37	9.56	10.57
	7.5	7.82 ± 0.69	8.82	4.27
3	0.08	0.076 ± 0.006	7.89	-5.00
	0.15	0.13 ± 0.015	11.36	-12.00
	3.5	3.94 ± 0.42	10.66	12.57
	7.5	7.43 ± 0.84	11.31	-0.93
Inter-day Precision and Accuracy				
Day	Concentration added (µg/mL)	Concentration found (µg/mL) ± SD	Precision (%RSD)	Accuracy (%RE)
1	0.08	0.076 ± 0.005	6.58	-5.00
	0.15	0.13 ± 0.019	14.39	-12.00
	3.5	3.84 ± 0.32	8.33	9.71
	7.5	7.66 ± 0.63	8.22	2.13
2	0.08	0.085 ± 0.006	7.06	6.25
	0.15	0.17 ± 0.021	12.43	12.67
	3.5	3.39 ± 0.43	12.68	-3.14
	7.5	7.65 ± 0.84	10.98	2.00
3	0.08	0.084 ± 0.004	4.76	5.00
	0.15	0.13 ± 0.012	9.16	-12.67
	3.5	3.29 ± 0.41	12.46	-6.00
	7.5	7.59 ± 0.54	7.11	1.20
Extraction recoveries				
Concentration added (µg/mL)		% Extraction Recovery ± SD	% RSD	
0.08		94.19 ± 8.76	9.30	
0.15		96.51 ± 7.84	8.12	
3.5		92.01 ± 9.02	9.80	
7.5		93.93 ± 8.89	9.46	
% Stability recoveries (mean ± SD)				
Concentration added (µg/mL)	Autosampler (48 h)	Bench-top (24 h)	Long-term (2 weeks)	Freeze-thaw (3 cycles)
0.08	98.31 ± 8.52	96.95 ± 9.04	103.21 ± 5.42	99.18 ± 8.32
0.15	101.02 ± 7.43	98.72 ± 6.03	97.64 ± 9.51	98.13 ± 8.43
3.5	99.43 ± 8.03	99.31 ± 9.43	98.39 ± 8.05	100.92 ± 7.94
7.5	96.53 ± 6.05	100.93 ± 5.65	99.53 ± 6.99	98.53 ± 9.91

3.4 Physical properties of the formulated gels and evaluation of their ocular kinetics

3.4.1 Gelation temperature and viscosity of the formulated in-situ gels

Factors such as gelation temperature and viscosity, particularly at different conditions, are some of the most important aspects of studying thermosensitive gel preparations. Hence, we assessed these properties and compared all four formulations to see the influence of Pluronic F127 and F88 in affecting the physical characteristics of the gels. Since the eye temperature ranges from 33.5 to 35.5 [28], our goal is to formulate a thermosensitive metronidazole gel that forms a gel around these points.

In the results showing the gelation temperature (**Figure 5.IA**), it was found that all preparations formed gel at different temperatures ($p<0.05$). Formulations A and B formed gels above body temperature while formulation C showed gelation at around 34.87 ± 3.12 °C. Formulation D, which contains Pluronic F127 alone (20%), formed gels even lower at approximately 23.64 ± 2.19 °C. The data obtained in this experiment indicated that Pluronic F88 as a combination of the gelling agent is required to obtain a suitable gelation temperature for eye applications. This finding is also in line with the previous report stating that a combination of longer hydrophobic chain poloxamers such as Pluronic F127 with more hydrophilic co-polymers is essential to the formation of gelling state at the desired temperature [9,29]. The interactions of Pluronic F127 with the other materials such as polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), methylcellulose (MC), and hydroxypropyl methylcellulose (HPMC) have also been demonstrated as an important factor in order to control the gelation temperature of the formulated ophthalmic sol-gel preparation [30].

In terms of viscosity, a further investigation on the influence of Pluronic F127 and F88 toward the rheology of formulated gels also provides insight into the importance of this combination on the physical properties of the gels upon application in eye or storage. As seen in the **Figure 5.IB**, the viscosity of the gels increased as the temperature elevated. For instance, the viscosity of formulation A and B at 4 °C was 131 ± 9.32 cPs and 143 ± 8.54 cPs, respectively. When the gels are tested at room temperature, the viscosity increases, but the gelling state has not been acquired. Consistent with gelation temperatures, the viscosity of Formulation C increased significantly ($p<0.05$) up to 26483 ± 2409 cPs when applied at eye temperature. In addition, this formulation did not form gel upon storage at 4 and 25 °C which is considered beneficial for storage conditions.

On the other hand, Formulation D was already in the gel form when temperature reached around 25 °C, indicated by its viscosity (up to 23498 ± 2192 cPs) and even increased to around 36218 ± 3381 cPs at around eye temperature. No formulation was observed to form gel at 4 °C. Based on these results, we agree that combination of Pluronic F127 and F88 used in formulation C is the most suitable mixture of these co-polymers to obtain a thermosensitive gel for ocular administration. However, we are also aware that this experiment is limited to the shorter time of storage and hence a thoroughly designed stability studies should be carried out to establish effects of more prolonged or even extreme storage conditions to the formulated preparations.

Pluronic F127 has longer hydrophobic poly(propylene oxide) (PPO) blocks compared to its hydrophilic poly(ethylene oxide) (PEO) blocks. In contrast, Pluronic F88 has 80% PEO relative to the other block chains, making this co-polymer more hydrophilic than Pluronic F127. Hydrophobic PPO blocks are responsible for lowering the gelation temperature while hydrophilic PEO blocks, otherwise, elevate the temperature [31,32]. A careful NMR study revealed that dehydration of PPO-PEO micelles in the solution is the key for the gelation of these co-polymers when applied at the specific temperature [33].

Although our findings mainly suggested that the use of Pluronic F127 and F88 is important in the solution-to-gel formation, it is interesting to point out that other copolymers have also been long utilized. For instance, in the formulation of Azithromycin thermosensitive gel, 20-22% of Poloxamer 404 which contain hydrophobic PPO blocks were incorporated with 5% of Poloxamer 188 and 3-4% Carbopol 974P NF in order to obtain suitable gelation temperature of the oplthalmic application [34]. Alkholief, M. et.al (2010) also demonstrated this effect using a similar gel base in which the poloxamer and carbopol ratio was the factors that determine the slight changes in the gelation temperature [35]. Another example of applicable copolymers is the use of poly-(DL-lactic acid-co-glycolic acid) (PLGA)–polyethylene glycol (PEG)–PLGA as a thermosenstive gel base to deliver drugs as an ophthalmic preparations [36]. All of these evidences again supported nature of these polymer combinations where longer hydrophobic blocks copolymers interact with other materials to obtain desired properties of the gel bases. Therefore, our study also confirmed that physical characteristics such as gelation temperature and viscosity of thermosensitive in-situ gels prepared using block co-polymers depend on the influence of hydrophobic and hydrophilic blocks from the co-polymers.

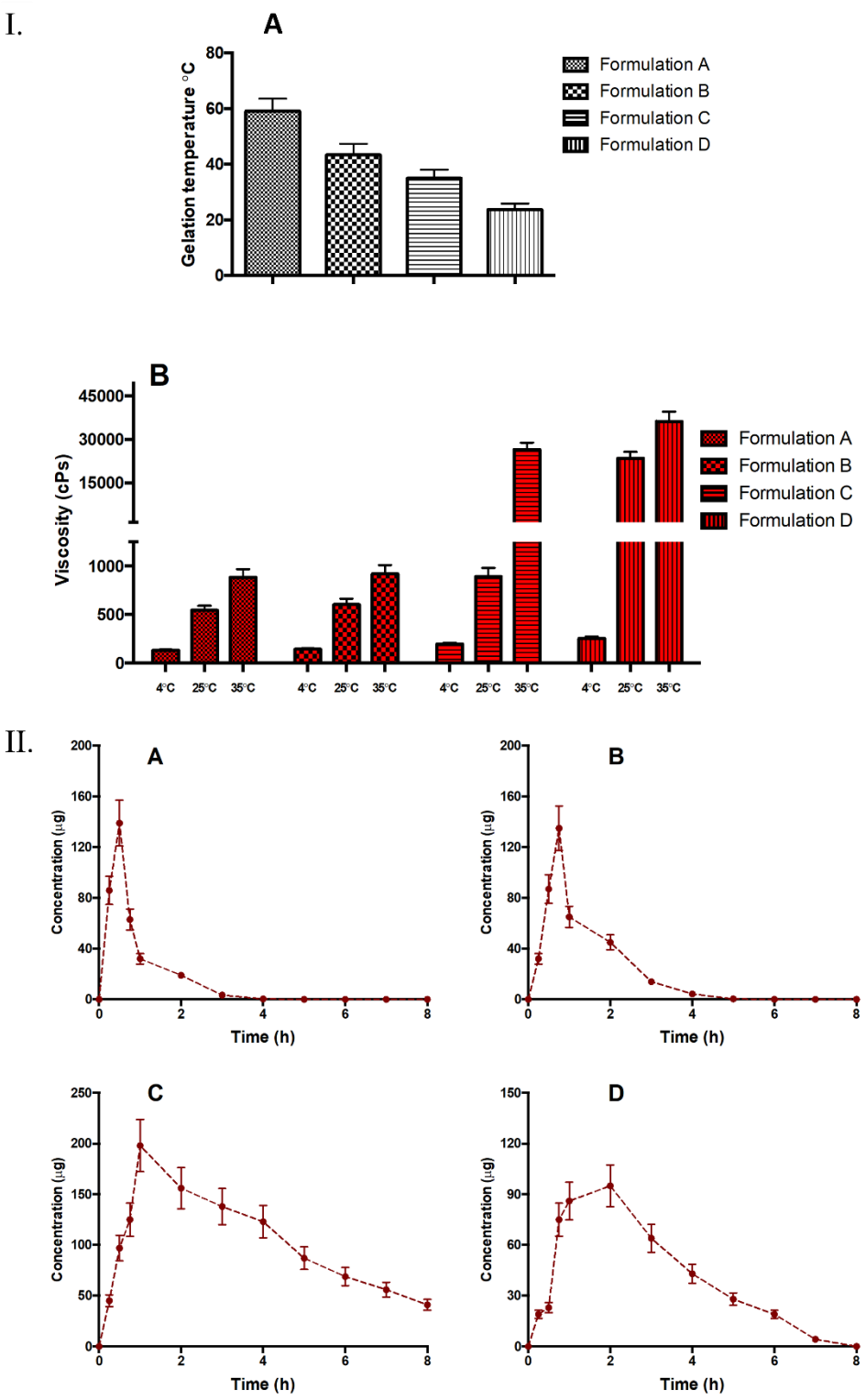

Figure 5. I. Overall physical characteristic evaluation of thermosensitive gel preparations: **(**A) gelation temperature (n=3) and (B) viscosity of formulation A-D measured at 4, 25 and 35 °C (n=3); **II.** Ocular kinetic study of metronidazole thermosensitive gels showing concentration of metronidazole (µg) in corneal matrix over the measured time (hours). A-D correspond to formulation A-D which contain various types of Pluronic F127 and F88 combinations.

3.4.2 Ex vivo ocular kinetics evaluation of formulated metronidazole gels in corneal tissue

The validated method obtained in this study was then applied in studying the ocular kinetic of the formulated metronidazole gels. The kinetic profiles were obtained by evaluating the concentration of metronidazole from each formulated preparation in the corneal matrix. The results showing the summary of the kinetics properties of these formulations was given in **Table 2**. As indicated by the data, formulation C revealed the most significant AUC value meaning the metronidazole in porcine corneal availability is achieved better than the other formulations. It also showed a significantly higher C_{max} ($p<0.05$) which was reached at approximately 1 hour after administration.

Meanwhile, this formula's half-life (T½) was 2.54 ± 0.31 hours, a specific resident time of metronidazole in the examined compartment. Overall, metronidazole concentrations from formulation A and B were not detected at 5 hours after administration while formulation C and D can extent the drug release longer (**Figure 5.II**). It is also interesting to note that formulation A which contains 5% of Pluronic F127 and 15 % of Pluronic F88 reached the maximum concentration of 110.81 ± 9.32 µg/ml in approximately 30 minutes after application and the longest time to reach maximum concentration was achieved by formulation D consisting of 20% of Pluronic F127 and 0% Pluronic F88. The data indicate correlation between the concentration of Pluronic combination (F127 and F88) and the availability of metronidazole in the cornea. Increasing the concentration of Pluronic F127 in the formulation A-D seems to cause the T_{max} value to be obtained at a longer time. However, there is an influence of Pluronic 127 combined with F88 in enhancing the peak corneal concentration, AUC and MRT of metronidazole since the half-life of formulation D which contains no Pluronic F88 cannot be solely improved by simply increasing Pluronic F127 concentration. Therefore, the data presented in this study is in line with our hypothesis that the combination of these two poloxamers is required in such preparations to obtain better kinetic outcomes of metronidazole.

Table 2. *Ex vivo* ocular kinetic parameters of metronidazole thermosensitive *in-situ* gels (n=3).

Parameters	Formulation A	Formulation B	Formulation C	Formulation D
C_{max} (µg)	110.81 ± 9.32	89.08 ± 8.71	171.72 ± 18.21	30.07 ± 4.31
T_{max} (h)	0.5	0.75	1	2
AUC_{0-t} (µg.h)	104.83 ± 10.21	189.59 ± 19.21	843.85 ± 90.29	37.83 ± 4.32
AUC_{0-INF} (µg.h)	104.83 ± 11.02	191.28 ± 20.12	991.36 ± 91.21	38.04 ± 3.92
$T_{1/2}$ (h)	0.25 ± 0.02	0.78 ± 0.08	2.54 ± 0.31	0.38 ± 0.05
MRT (h)	0.69 ± 0.07	1.62 ± 0.17	4.56 ± 0.43	0.93 ± 0.12

The effect of poloxamer matrices in enhancing bioavailability of metronidazole eye preparations has not been widely available, but some evidence shows that Poloxamer-based gel is useful in improving ocular drug delivery of other drugs. Our previous report observed that gels developed using the combination of Pluronic F127 and F68 enhance the ocular kinetic of Itraconazole in an optimized thermosensitive gel preparation whether it is applied in an infected or a normal eye model [9]. Pluronic F127 with other poloxamers is also known to improve the availability of other incorporated drugs [37]. In terms of metronidazole delivery into eye tissue, Vanderbilj [6] reported that metronidazole gel showed significant diffusion rate in human and rabbit eyes compared to the eye solution. Further investigations also revealed that trans-corneal diffusion of metronidazole in human and rabbit eyes is not affected by the presence of chemical preservatives such as benzalkonium chloride [38]. In contributing to the previous knowledge available, the experiment carried out in this study provides a new insight on the relationship between poloxamer based thermosensitive gels and the kinetics of metronidazole in the eye tissue. However, it is beyond our scope to evaluate the kinetic profiles of these formulations in human or animal eye models even though factors such as tissue temperature and physiological conditions highly influence this type of pharmaceutical preparation. Therefore, we suggested that further research is carried out in addressing whether these factors are important to establish excellent kinetic profiles of metronidazole in Pluronic based thermosensitive gels.

4. Conclusions

The development of a validation method using this new high performance liquid chromatography (HPLC-UV) method was established. The new approach developed in this study in which metronidazole concentrations were measured after *ex vivo* administration in porcine cornea, had high precision and accuracy, simplicity, and high sensitivity and selectivity for use in therapeutic drug control research and ocular kinetics evaluation.

As our findings suggested, the formulation of thermosensitive gels represented in this study revealed influences of PF127 and PF88 combination to produce gels with an excellent gelation temperature and viscosity and their ocular kinetic properties. The method we developed here was also applicable in examining the concentration of metronidazole in the formulated thermosensitive in-situ gel preparations. However, further research is required to fully understand whether this analysis method can also be implemented in studying the drug administration under pathological conditions.

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