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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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Keywords:
Metronidazole
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Method validation

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.

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

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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

mobile phase, flow rate, mobile phase pH, and acetonitrile concentration (Table S2). Design Expert® Software version 11 (State-Ease, Minneapolis, MN, USA) was

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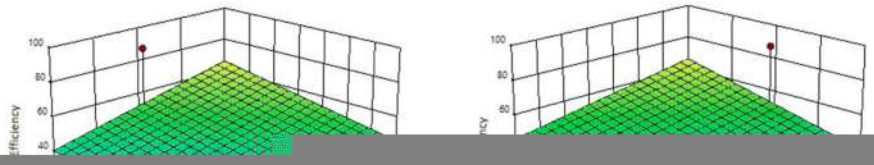
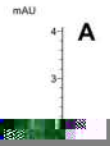


2.6. Application of validated HPLC method to assess ocular kinetics of metronidazole from thermosenstivie gel formulation in corneal tissue



2.6.1. Thermosensitive in situ ocular gel optimization

Different thermosensitive formulation containing metronidazole were prepared using Pluronic® F127 (PF127),

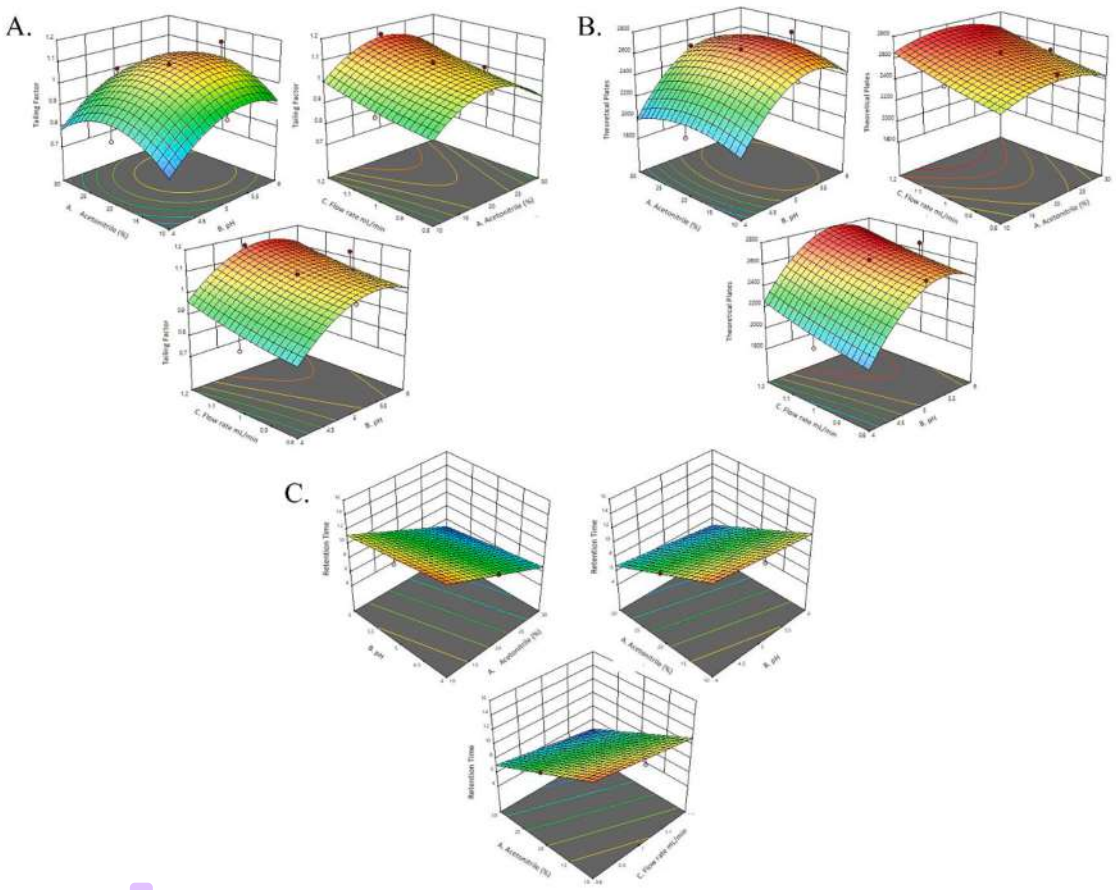


Fig. 4. Illustrations of representative response surface plots illustrating the impact of the selected parameters on : A. Tailing factor; B. Theoretical plates and retention time; C. Retention time.

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).

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 and 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

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

Linearity, LOD and LLOQ values				
Slope	y-intercept	R	LOD (µg/	LLOQ

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 value

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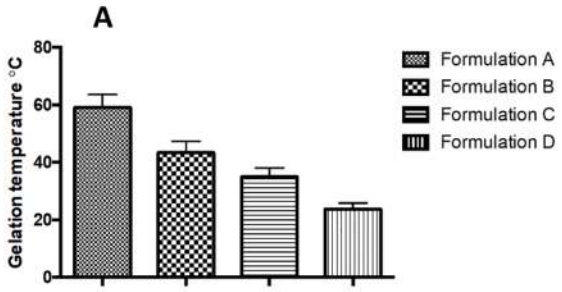
A
Gelation temperature °C
80
60
40
20
0
Formulation A
Formulation B
Formulation C
Formulation D


B
45000

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
$AUC_{0-\infty}$ (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

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.

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.

CRediT authorship contribution statement

Nur Asma: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Nurul Muhlisah Maddeppungeng:** Methodology, Writing – original draft, Writing – review & editing. **Muhammad Raihan:** Writing – original draft, Writing – review & editing. **Arini Putri Erdiana:** Methodology, Investigation, Data curation. **Achmad Himawan:** Data curation, Writing – review & editing.

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MRT (h)	0.69 ± 0.07	1.62 ± 0.17	4.56 ± 0.43	0.93 ± 0.12

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 opthalmic 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 thermosensitive 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.

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 h after administration.

Meanwhile, this formula's half-life ($T_{1/2}$) was 2.54 ± 0.31 h, a specific resident time of metronidazole in the examined compartment. Overall, metronidazole concentrations from formulation A and B were not detected at 5 h after administration while formulation C and D can extent the drug release longer (Fig. 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 min after application and the longest time to reach maximum concentration was achieved by formulation D consisting of

Declaration of Competing Interest

The authors declare that they have no known competing financial

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