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Data Article

# Validation of spectrophotometric method for quantification of cefazoline in simulated tear fluid and porcine ocular tissue from thermosensitive-mucoadhesive *in situ* ocular gel preparation

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## ABSTRACT

In the formulation development, it is necessary to validate the analytical method as a critical step. Here, we validated the spectrophotometric method of cefazoline (CFZ) from thermosensitive-mucoadhesive *in situ* gels in simulated tear fluid (STF) and ocular tissue for *in vitro* and *ex vivo* studies, respectively. The method was validated as per ICH guidelines, showing excellent linearity with the correlation coefficient values of 0.9997 in STF and 0.9981 in ocular tissue with acceptable precision and accuracy. The LOD values were 0.496 µg/mL and 2.390 µg/mL in STF and ocular tissue, respectively. Moreover, LOQ values were 1.504 µg/mL in STF and 7.242 µg/mL in ocular tissue. The method was utilized in the *in vitro* and *ex vivo* studies, showing that the concentration of CFZ localized in the ocular tissue was significantly improved following its incorporation into thermosensitive mucoadhesive *in situ* gels. *In vivo* studies with suitable analytical method must be performed.

## 1. Rationale

## Specifications Table

Subject area	Spectroscopy, Analytical Method Validation
Compounds	Cefazoline
Data category	Spectral, <i>in vitro</i> release, <i>ex vivo</i> delivery, drug concentration
Data acquisition format	UV-Vis Spectrum
Data type	Analyzed
Procedure	We developed and validated the spectrophotometric method of CFZ in simulated tear fluid (STF) and ocular tissue for <i>in vitro</i> and <i>ex vivo</i> studies, respectively. The method was successfully validated as per the ICH guidelines.
Data accessibility	Data is with this article

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Bacterial keratitis is a potentially blinding corneal infection and has been categorized as a neglected tropical disease. Prior to blindness, there are several important signs and symptoms associated with the disease, including acute ocular pain, decreased vision, corneal ulceration, and infiltrates in the anterior chamber [1]. Several studies around the world have consistently reported Gram-positive bacteria as the most common etiology of this infection [2,3]. Several risk factors have been identified, including contact lens use, trauma, ocular surface and eyelid diseases, post ocular surgery, use of topical steroids, and systemic immunosuppression [3]. The pathogenesis associated with each risk factor occurs in a complex and multifactorial manner that begins with the accumulation and attachment of microbes to the corneal surface. For example, the use of contact lenses as a risk factor most often occurs due to impaired tear exchange, resulting in stagnation under the contact lens hence facilitating the adherence and accumulation of commensal bacteria at a later timepoint [4].

Topical antibiotic has become the first line in the treatment of bacterial keratitis [5]. Cefazoline (CFZ) is a first-generation cephalosporin antimicrobial used for keratitis infections therapy by inhibiting bacterial cell wall synthesis. CFZ is available in eye drop preparations with a dose of 3,5 mg/mL [6]. However, when applied *via* ocular route, only 5% of the applied drug could penetrate the ocular tissue due to short contact of the preparations in the ocular tissue [7]. Additionally, many drug companies promote drugs at a scale 50  $\mu$ L as the maximum volume for the application of eye drops. However, generally, 20  $\mu$ L of these eye drops do not touch the eye surface, and only 30  $\mu$ L remains on the ocular surface area. Thus, the application of eye drop preparations is not optimal due to the excessive application volume required. Consequently, various new drug delivery systems have been created to overcome these issues. For example, structural changes of pharmaceutical preparations from liquid to gel in an *in situ* condition, well-known as thermosensitive *in situ* gel, has attracted the attention of researchers to employ this approach to increase the retention time of drugs on the surface of the eye [8]. Several studies have shown that the formulation of active substances in thermosensitive preparations was able to improve the concentration of drugs in the ocular tissue [8,9]. In addition, the presence of mucosa in the ocular tissue could improve the ocular contact by using the mucoadhesive system [8]. Accordingly, the combination of thermosensitive and mucoadhesive could be an alternative approach to deliver CFZ to the ocular tissue for keratitis treatment.

In the formulation development, *in vitro* and *ex vivo* assessments shall be conducted before performing *in vivo* evaluation. *In vitro* study is used to predict the release manner of the formulated drugs. Importantly, in ocular delivery studies, *ex vivo* is performed by using corneal tissue of animals to mimic the *in vivo* conditions [10]. By performing the *ex vivo* studies, some formulations might be eliminated from the *in vivo* studies and, thus, could minimize the use of animals in the *in vivo* studies. In the *in vitro* and *ex vivo* evaluation, analytical methods to quantify active substances should be developed and validated. Analytical procedures allowing the precise determination of drugs in the development studies is crucial. Essentially, analytical validation procedures must be in place to ensure reliability, traceability or comparability of results. Thus, developing and validating the analysis procedure to determine the amount of cefazolin in the *in vitro* and *ex vivo* studies is the important steps for the development of new dosage forms [11].

In terms of the analytical method, several techniques have been developed to quantify CFZ, including high-performance liquid chromatography and mass spectroscopy. Alternatively, the UV-Vis spectrophotometry method is usually preferable with its advantages. Analytical methods other than UV-Vis spectrophotometry are expensive, in contrast to the UV-Vis spectrophotometry method which has the advantages of being time-efficient, economical, widely available, easy and simple to use, relatively accurate, and results are obtained satisfactory. Essentially, no residue is produced when the analysis uses reagents or solvents [12,13]. Spectrophotometry is a method used mainly in routine analysis or research for identification. Additionally, in the developing countries, spectrophotometric method could potentially be a great favor to be applied. Several studies have shown the effectiveness of the spectrophotometry method to quantify several drugs in the biological matrices [14–16]. Furthermore, the spectrophotometry method has also been applied to quantify drugs in fruits, food and beverages products [17–19]. Accordingly, this has shown that despite its simplicity, this method has been reported to be effective in the several analytical purposes. Therefore, in this study, as part of the formulation development process, we validated the method for the quantification of CFZ in thermosensitive-mucoadhesive ocular preparation. The validated method was utilized to analyze the drug content in the formulation, *in vitro* permeation, *ex vivo* permeation and *ex vivo* retention in the ocular tissues.

## 2. Procedure

### 2.1. Material

Cefazolin sodium salt (CFZ) (purity 89.1–110.1%) was obtained from Sigma Aldrich Pte Ltd. (Singapore, Singapore). Pluronic® F127 and F68 were kindly provided by BASF Indonesia (Jakarta, Indonesia). Sodium chloride (NaCl). Sodium bicarbonate (NaHCO<sub>3</sub>) and calcium chloride dihydrate (CaCl<sub>2</sub>•2H<sub>2</sub>O) were obtained from Merck (Jakarta, Indonesia). Other materials were analytical grade.

### 2.2. Preparation of stock solution

Simulated tear fluid (STF) was prepared by dissolving 6.7 g NaCl, 2.0 g NaHCO<sub>3</sub> and 0.08 g CaCl<sub>2</sub>•2H<sub>2</sub>O in 1 L deionized water. The final pH was adjusted to 7.4 [20]. CFZ stock solution was prepared by weighing 10 mg of CFZ and put into a 10 mL volumetric flask. STF was added to dissolve the CFZ and then made up to the mark to obtain a final concentration of 1000  $\mu$ g/mL.

### 2.3. Determination of maximum wavelength

250  $\mu$ L of the stock solution of CFZ was transferred into a volumetric flask prior to dilution with STF for up to 10 mL (concentration

25 µg/ml). The absorbance of the solution was measured at a wavelength of 200–400 nm using a UV–Vis spectrophotometer (HALO XB-10, Dynamica Scientific Ltd. United Kingdom). The maximum wavelength was determined from the absorption in the sample.

#### 2.4. Preparation calibration standards and quality control samples

In this study, calibration standards and quality control samples were prepared in two different matrices, namely STF and porcine ocular tissue. STF was used in the drug content in the formulation, *in vitro* permeation and *ex vivo* permeation studies. Porcine ocular tissue was used in the *ex vivo* retention in the ocular tissues.

For STF, the stock solution was diluted with STF to obtain a calibration standard solution with a concentration from 0.625 to 20 µg/mL. Then, quality control samples were prepared by diluting CFZ stock solution using STF to achieve low quality control (LQC) concentration (2 µg/mL), medium quality control (MQC) concentration (10 µg/mL), and high quality control (HQC) concentration (15 µg/mL).

To prepare porcine tissue matrices, initially, the tissue was crushed, then added to distilled water in a ratio of 9:1. This mixture was then homogenized for 10 min. After obtaining the tissue matrices, a series concentration (40 – 1.25 g/mL) were made by spiking the stock solution to the tissue matrices [21]. Afterwards, the LQC (10 µg/mL), MQC (20 µg/mL), and HQC (30 µg/mL) of CFZ in tissue matrices were also prepared in a similar fashion.

#### 2.5. Sample preparation and extraction of cfz from ocular tissues

For quantification of CFZ in the ocular tissue for the *ex vivo* studies, sample extractions using two organic solvents, methanol and acetonitrile, were carried out [22,23]. In this study, 1 g of tissue spiked CFZ was used. To optimize the extraction process, several volumes of each solvent 1 mL, 3 mL, 5 mL and 7 mL, were used. Briefly, the solvent was mixed with the tissue and vortexed for 10 min. Following this step, the mixture was centrifuged at 7000 rpm for 15 min (LC-04S Centrifuge, Zenith Lab (Jiangsu) Co., LTD. China). The supernatant was collected, and the organic solvent was evaporated in a fume hood. Afterwards, 1 mL of STF was added into the dry extract and vortexed for 5 min. The samples were then centrifuged at 7000 rpm for 15 min and the clear supernatant was measured using spectrophotometric.

#### 2.6. Preparation of cfz thermosensitive-mucoadhesive gel

CFZ thermosensitive-mucoadhesive gel was prepared using the cold method by dispersing a combination of Pluronic® F127 16% and Pluronic® F68 4% w/w in cold distilled water (at 4 °C) and maintained under constant stirring to form poloxamer solution and then kept in the refrigerator overnight until a clear solution was observed. Hyaluronic acid 0.2% w/w was dissolved in hot water until a clear solution was formed and dissolved in poloxamer solution. CFZ 0.35% was dissolved in distilled water and dissolved in poloxamer solution. Benzalkonium chloride 0.01% was added to the poloxamer solution and stirred until a clear solution was formed [24].

#### 2.7. Method validation

##### 2.7.1. Specificity

In an attempt to perform the specificity test, CFZ solution in gel formulation, blank gel formulation, CFZ in ocular tissue matrices and blank ocular tissue matrices were used. All samples were scanned using spectrophotometry UV–Vis at the wavelength between 200 nm and 400 nm. Specificity was evaluated by observing the peak of CFZ in each sample.

##### 2.7.1. Linearity, LOD and LOQ

The correlation coefficient values of the calibration curves obtained from STF and ocular tissue were used as a parameter for linearity. Furthermore, the calibration curve was also used to calculate limit of detection (LOD) and limit of quantification (LOQ) using the following equation [25]:

$$\text{LOD} = \frac{3.3 \sigma}{S} \quad (1)$$

$$\text{LOQ} = \frac{10 \sigma}{S} \quad (2)$$

Where  $\sigma$  = the SD of the response of the data and  $S$  = the slope of the calibration curve.

##### 2.7.2. Accuracy and precision

Accuracy and precision were determined in intra-day and inter-day measurements. Intra-day was measured three times in a day, while inter-day was measured three days in a row. After the measurement for intra-day and inter-day the relative standard deviation (RSD,%) and relative errors (RE,%) were calculated to interpret the values of accuracy and precision, respectively. LOQ, LQC, MQC, and HQC were used in this method, where the values should be  $\pm 15\%$  [26].

### 2.7.3. Determination of extraction recovery

The extraction recovery was obtained by comparing the measured value of matrix concentrations with the extracts of the blank matrices with equivalent analyte [27].

### 2.7.4. Dilution integrity

The effect of sample dilution should be determined during the validation of the quantitative method. To perform this, CFZ was prepared in the concentrations of 75 µg/mL in STF and 200 µg/mL in ocular tissue. Each sample was diluted 5 times and 10 times, and the RSD and RE values were then calculated.

## 2.8. Application of the method

### 2.8.1. Determination of drug content

The thermosensitive-mucoadhesive gel was weighed accurately and diluted with STF to obtain a final concentration of 10 µg/ml and tested for drug content using a UV-Vis spectrophotometer. In this study, the result of the drug content formula was also compared with the result of 10 µg/ml CFZ solution [28].

### 2.8.2. In vitro, ex vivo permeation and ex vivo retention study of CFZ from thermosensitive-mucoadhesive gel

In this study, both *in vitro* and *ex vivo* evaluations were carried out using Franz cell diffusion, where the difference between the two methods was the membranes used. The *in vitro* test used a dialysis membrane (Spectra-Por®, 12,000 - 14,000 MWCO dialysis membrane) [29], while the *ex vivo* test used porcine cornea tissue [30]. Prior to the experiment, 24 mL of STF was filler in the inside receiver compartment of the Franz cell diffusion, then stirred at 100 rpm and accurately maintained the temperature at 37 °C.

After the pretreatment steps, the formulation was weighed accurately equal to 10 mg of CFZ. The sample was put above the membrane and covered with the top part of Franz cell diffusion. The diffusion area of the Franz diffusion cell was 4.9 cm<sup>2</sup>. For every certain time span, 1 ml was sampled from the cell diffusion arm, and 1 ml of fresh STF was added again. Sampling was carried out at 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7, 8, and 24 h. The absorbances of samples were measured the absorbance using spectrophotometry with an appropriate dilution.

Following the *ex vivo* permeation study, after 1 h, 6 h and 24 h, the ocular tissue was removed from the cell diffusion, and the excess of the formulation was removed. The extraction solvent was mixed with the ocular tissue using the optimized volume obtained from the method described in Section 2.5. The mixture was vortexed for 10 min and centrifuged for 15 min at 7000 rpm. The clear supernatant was dried under a fume hood at 25 °C and the dry extract was reconstituted using 1 mL of STF. The mixture was vortexed for 5 min and centrifuged for 15 min at 7000 rpm. CFZ in the supernatant was measured using spectrophotometry. In both *in vitro* and *ex vivo* studies, CFZ solution was used as a control.

The release kinetic profile was calculated using the following equations [31]

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

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

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

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

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

Where  $C_t$  is the concentration of cefazoline released at time  $t$ ,  $C_0$  is the initial amount of CFZ in STF (at  $t = 0$ ),  $k_0$  is constant of zero-order kinetics,  $k_1$  is constant of first-order kinetics,  $k_H$  is constant of Higuchi model,  $k_{KP}$  is constant of Korsmeyer-Peppas model, and  $k_{HC}$  is constant of Hixson-Crowell model.

## 2.9. Statistical analysis

GraphPad Prism® version 6 (GraphPad Software, San Diego, California, USA) was applied to perform the statistical analysis.  $p$  values < 0.05 represent a statistical difference between data analyzed.

## 3. Data, value and validation

### 3.1. Determination of maximum wavelength and specificity

As a critical step in the development of formulation, the validation of the analytical method should initially be developed. In this study using UV-Vis spectrophotometry, the maximum wavelength of CFZ in the two matrices used, namely STF and porcine ocular tissue, was firstly determined. As shown in Fig. 1, one spectrum peak in STF was observed at 272 nm, and two spectral peaks at 220 nm and 272 nm were observed from the ocular tissue matrices.

To decide the wavelength used for the analysis process, the specificity test was carried out. In this step, the spectrum of the possible interferences was also observed. Blank thermosensitive formulation and blank tissue were scanned, and it was found that both samples showed peaks around 220 nm (Fig. 2). Therefore, 272 nm was selected as the wavelength used in the further steps.

### 3.2. Preparation of samples and extraction of CFZ from ocular tissue

To determine the concentration of CFZ localized in the ocular tissue after *ex vivo* studies, the extraction method was screened. The results showed that the extraction recoveries when methanol was used were  $11.83 \pm 0.78\%$ ,  $37.79 \pm 2.28\%$ ,  $91.44 \pm 0.49\%$  and  $93.05 \pm 2.30\%$  for 1 mL, 3 mL, 5 mL and 7 mL of extraction volumes, respectively. On the other hand,  $8.81 \pm 0.60\%$ ,  $17.74 \pm 5.20\%$ ,  $33.75 \pm 0.82\%$  and  $56.91 \pm 0.90\%$  of extraction recoveries were found when acetonitrile was used with volumes of 1 mL, 3 mL, 5 mL and 7 mL, respectively. It was found that methanol was more effective than acetonitrile. In our study, despite the higher extraction recovery obtained from 7 mL methanol, the recovery obtained from 5 mL was not statistically ( $p > 0.05$ ) different compared to 5 mL. Therefore, 5 mL methanol was selected for further studies.

### 3.3. Linearity, LOD, and loq

In this evaluation, the calibration curves in STF and ocular tissue were found to be linear, with the correlation coefficient values of 0.9997 and 0.9981, respectively. It has been reported in a previous study that the acceptable linearity of the calibration curve in the analytical validation was  $\geq 0.998$  [26,32,33]. The curves of calibration solutions of CFZ in STF and ocular tissue are shown in Fig. 3. Moreover, the spectrums of all samples in the calibration solution are depicted in Fig. 4. Generally, the absorbance values of the samples analyzed using spectrophotometer are between 0.2 and 0.8 [34]. However, depending on the instrument used, an absorbance value of  $< 1$  was also found. Several studies have shown that the calibration curves which were not between 0.2 and 0.8 could result in acceptable linearity, and importantly, have been successfully applied to quantify several analytes [15,17]. Accordingly, as the calibration curves found in our study showed the acceptable linearity, the curves could be precisely used in the further validation steps. Furthermore, the LOD and LOQ were determined in STF and ocular tissues. These values were obtained from calibration curves. It was found that the LOD of CFZ in STF and ocular tissue were calculated to be  $0.496 \mu\text{g/mL}$  and  $2.390 \mu\text{g/mL}$ , respectively. Moreover, LOQ values were  $1.504 \mu\text{g/mL}$  and  $7.242 \mu\text{g/mL}$  in STF and ocular tissue, respectively.

### 3.4. Accuracy and precision

Accuracy and precision were evaluated in STF and ocular tissue. LOQ, LQC, MQC, and HQC were used for inter-day and intra-day measurements with three replications for each measurement to determine the value of accuracy and precision. After measuring inter-day and intra-day, the relative standard deviation (RSD) and relative error (RE) values were calculated. Where precision was denoted by RSD and accuracy was denoted by RE. For STF, it can be seen in Table 1 that intra-day RSD values were in the range of 1.35% - 12.13%, and RE values were in the range of -3.76% - 9.45%. In Table 2, it can be seen that the accuracy and precision values for inter-day, where the RSD values were in the range of 1.2% - 11.66% and the RE values were in the range of -9.86% - 9.81%.

For ocular tissue matrices, it can be seen in Table 3 that for intra-day, the RSD values were between 1.28% and 8.6%, and the RE values were between -6.76% and 8.16%. While on the inter-day, the RSD values were in the range of 3.43% - 9.52% and the RE values were in the range of -10.98% - 7.79% (Table 4.). According to the requirement from ICH [35], supported by several studies reporting that the accuracy and precision values should be  $\pm 15\%$  [22,23,36], the analytical method using a spectrophotometer for the determination of CFZ in STF and ocular tissue developed in this study was found to be precise and accurate.

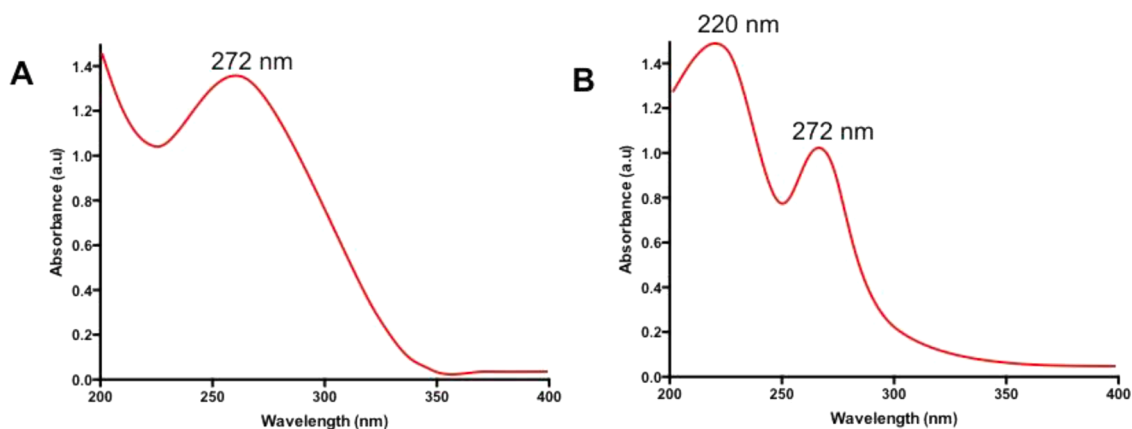


Fig. 1. Maximum UV absorption of CFZ in STF (A) and ocular tissue (B).

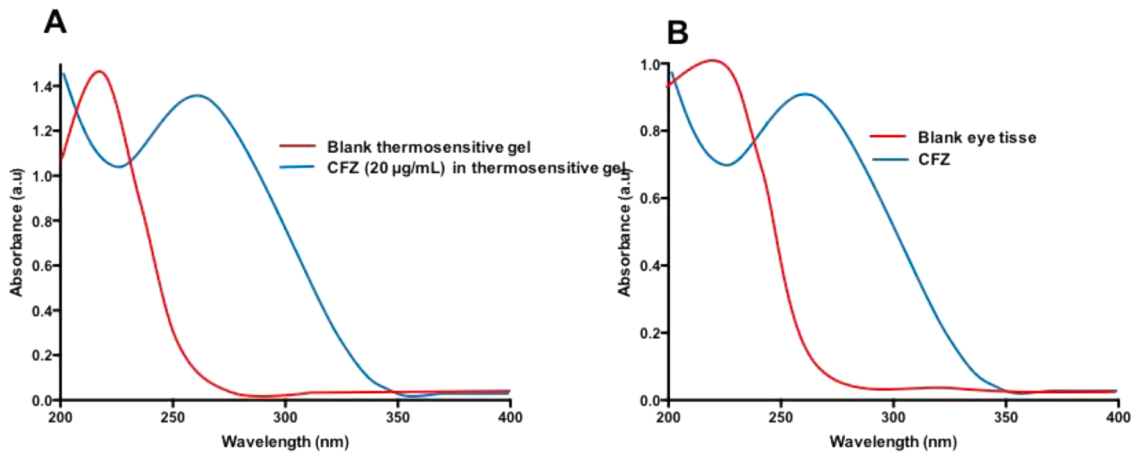


Fig. 2. Representative UV-Spectra of CFZ in thermosensitive gel and blank formulation (A) and blank ocular tissue and CFZ solution (B).

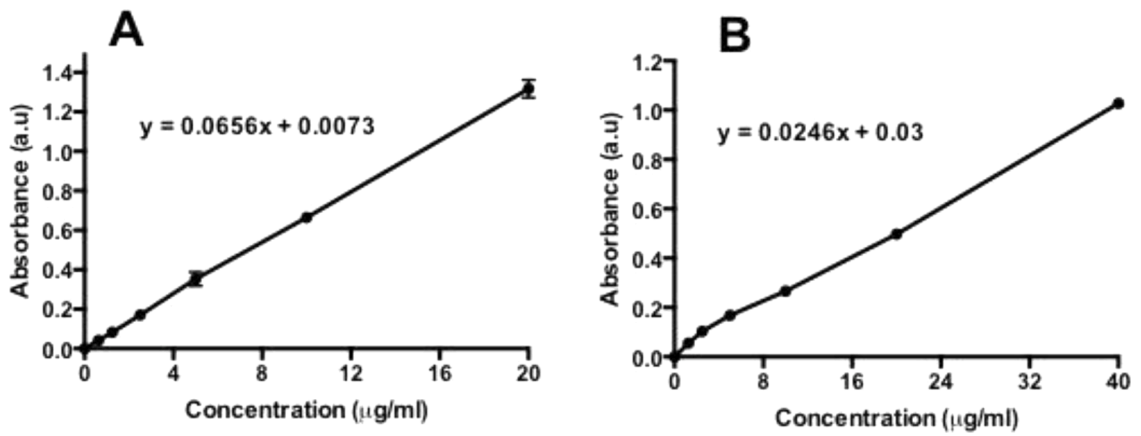


Fig. 3. The calibration curve of CFZ in STF (A) and ocular tissue (B).

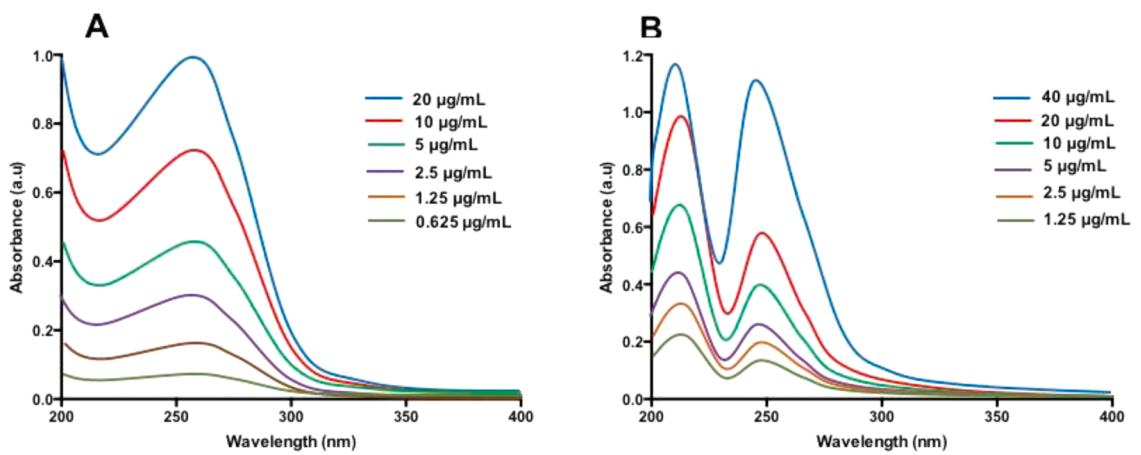


Fig. 4. Spectrum of CFZ standard solutions in STF (A) and ocular tissue (B).

**Table 1**Intra-day precision and accuracy data of spectrophotometry for quantification of CFZ in STF ( $n = 3$ ).

Intra-day Precision and Accuracy				
Replication	Concentration added ( $\mu\text{g/mL}$ )	Concentration found ( $\mu\text{g/mL}$ ) $\pm$ SD	Precision (%RSD)	Accuracy (%RE)
1	1.5	1.44 $\pm$ 0.13	8.82	-3.76
	2	2.03 $\pm$ 0.25	12.13	1.45
	8	8.54 $\pm$ 0.18	2.14	6.76
	15	15.47 $\pm$ 0.73	4.72	3.12
2	1.5	1.61 $\pm$ 0.11	7.00	7.42
	2	1.96 $\pm$ 0.09	4.78	-1.91
	8	8.72 $\pm$ 0.18	2.10	9.05
	15	15.32 $\pm$ 0.21	1.35	2.10
3	1.5	1.64 $\pm$ 0.09	5.27	9.45
	2	1.98 $\pm$ 0.20	10.13	-0.99
	8	8.30 $\pm$ 0.27	3.25	3.72
	15	15.31 $\pm$ 0.23	1,50	2.05

**Table 2**Inter-day precision and accuracy data of spectrophotometry for quantification of CFZ in STF ( $n = 3$ ).

Inter-day Precision and Accuracy				
Day	Concentration added ( $\mu\text{g/mL}$ )	Concentration found ( $\mu\text{g/mL}$ ) $\pm$ SD	Precision (%RSD)	Accuracy (%RE)
1	1.5	1.55 $\pm$ 0.08	5.18	3.35
	2	2.06 $\pm$ 0.05	2.24	2.97
	8	8.09 $\pm$ 0.28	3.51	1.12
	15	15.18 $\pm$ 0.55	3.62	1.19
2	1.5	1.57 $\pm$ 0.18	11.66	4.88
	2	2.14 $\pm$ 0.15	6.79	6.94
	8	8.43 $\pm$ 0.47	5.60	5.32
	15	15.12 $\pm$ 0.43	2.87	0.78
3	1.5	1.35 $\pm$ 0.13	9.42	-9.86
	2	2.08 $\pm$ 0.16	7.94	3.89
	8	8.79 $\pm$ 0.11	1.20	9.81
	15	14.98 $\pm$ 0.86	5.73	-0.13

**Table 3**Intra-day precision and accuracy data of spectrophotometry for quantification of CFZ in ocular tissue ( $n = 3$ ).

Intra-day Precision and Accuracy				
Replication	Concentration added ( $\mu\text{g/mL}$ )	Concentration found ( $\mu\text{g/mL}$ ) $\pm$ SD	Precision (%RSD)	Accuracy (%RE)
1	7.24	7.54 $\pm$ 0.65	8.60	4.14
	10	9.90 $\pm$ 0.82	8.32	-1.02
	20	18.65 $\pm$ 0.62	3.34	-6.76
	30	32.45 $\pm$ 0.73	1.28	8.16
2	7.24	7.35 $\pm$ 0.46	6.21	1.49
	10	9.65 $\pm$ 0.58	6.00	-3.46
	20	18.96 $\pm$ 0.61	3.21	-5.18
	30	32.07 $\pm$ 0.69	2.15	6.91
3	7.24	7.50 $\pm$ 0.53	7.02	3.59
	10	9.85 $\pm$ 0.67	6.79	-1.52
	20	19.86 $\pm$ 1.33	6.71	-0.71
	30	31.24 $\pm$ 2.22	7.10	4.13

### 3.5. Extraction recovery

The extraction recovery of CFZ from the ocular tissue matrix is summarized in Table 5. This study was performed to evaluate whether the extraction method could be applied in the determination of CFZ retained in the ocular tissue in the *ex vivo* studies. The obtained recovery values were in the range of 90.38–93.90%, with RSD values less than 15%. In some studies, regardless the values of the extraction recovery, it has been reported that RSD values of the average of the extraction recovery should be  $< 15\%$  [22,23,36]. Therefore, the extraction method was found to be precise for the application in the further *ex vivo* studies.

### 3.6. Dilution integrity

In the analysis process, due to a low sample volume, it could result in highly concentrated samples. Accordingly, the samples with excessively high concentrations above the set calibration range might be found and should be adjusted accordingly. To bring the

**Table 4**Inter-day precision and accuracy data of spectrophotometry for quantification of CFZ in ocular tissue ( $n = 3$ ).

Inter-day Precision and Accuracy				
Day	Concentration added ( $\mu\text{g/mL}$ )	Concentration found ( $\mu\text{g/mL}$ ) $\pm$ SD	Precision (%RSD)	Accuracy (%RE)
1	7.24	7.48 $\pm$ 0.41	5.49	3.35
	10	10.23 $\pm$ 0.35	3.43	2.34
	20	18.45 $\pm$ 1.46	7.94	-7.77
	30	30.92 $\pm$ 1.85	5.98	3.08
2	7.24	7.80 $\pm$ 0.28	3.54	7.79
	10	9.59 $\pm$ 0.50	5.26	-4.07
	20	18.49 $\pm$ 0.95	5.15	-7.57
	30	31.80 $\pm$ 1.71	5.37	6.00
3	7.24	7.30 $\pm$ 0.40	5.45	0.83
	10	9.87 $\pm$ 0.94	9.52	-1.32
	20	17.80 $\pm$ 0.79	4.45	-10.98
	30	31.23 $\pm$ 2.18	6.99	4.10

**Table 5**Mean extraction recovery of CFZ in ocular tissue ( $n = 3$ ).

Concentration	%Extraction Recovery $\pm$ SD	%RSD
4.24 $\mu\text{g/mL}$	90.38 $\pm$ 1.24	1.37
7.5 $\mu\text{g/mL}$	92.51 $\pm$ 3.33	3.60
15 $\mu\text{g/mL}$	93.90 $\pm$ 3.77	4.01
24 $\mu\text{g/mL}$	92.08 $\pm$ 5.45	5.92

analyte concentration within a validated concentration range and allow reanalysis of the samples, a dilution integrity test was carried out [37]. In this evaluation, all samples in the STF and porcine ocular tissues showed the dilution integrity with RSD and RE values of  $\pm 15\%$ . The dilution integrity showing the RSD and RE values which were  $\pm 15\%$  was found to be optimized in several analytical validation process [22,23,36]. Therefore, even though the absorbances of samples were above the absorbance of the calibration standard solution, it could be determined by the appropriate dilution.

### 3.7. Application of the analytical method

#### 3.7.1. Drug content determination of CFZ in the thermosensitive-mucoadhesive *in situ* gel formulation

The spectrophotometer method was initially applied to determine the amount of CFZ recovered from the thermosensitive-mucoadhesive formulation. In this study, CFZ solution with a similar concentration was also determined for the drug content. As shown in Fig. 5A, the % recovery of CFZ from the thermosensitive-mucoadhesive gel was  $99.19 \pm 4.04\%$ , and % recovery of CFZ from the control eye drop was  $99.04 \pm 2.39\%$ . The ICH has recommended that the acceptable recovery percentage in the formulation product lies between 95 and 105% [38]. Therefore, it can be concluded that no intervention between each excipient with CFZ could affect the recovery of CFZ from the formulation [39].

#### 3.7.2. *In vitro*, *ex vivo* permeation and *ex vivo* retention study of CFZ from thermosensitive-mucoadhesive gel

In the *in vitro* and *ex vivo* permeation studies, the percentage of CFZ permeated through the membrane and ocular tissue was determined [40]. For the topical treatment of the ocular cornea, the amount of drug concentration in the ocular tissue is very influential for the effectiveness of treatment for bacterial keratitis. The formulation of thermosensitive-mucoadhesive gel was developed to localize the drug in the ocular tissue with a low permeation manner. Initially, *in vitro* permeation between thermosensitive mucoadhesive *in situ* gel and control eye drop was compared. It can be seen that after 24 h, *in vitro* permeation of CFZ from the control solution was  $100.77 \pm 8.048\%$  and from the thermosensitive mucoadhesive *in situ* gel was  $50.67 \pm 4.12\%$  (Fig. 5B). Furthermore, using the same method, the *ex vivo* permeation study through ocular tissue of CFZ from the thermosensitive mucoadhesive gel was compared to the control solution. The percentage of permeated drugs are depicted in Fig. 5C. Based on the results, following 24 h,  $99.58 \pm 10.64\%$  and  $39.4 \pm 3.21\%$  of CFZ were permeated from control solution and thermosensitive mucoadhesive gel, respectively. Therefore, the formulation of CFZ into thermosensitive mucoadhesive gel was able to sustain the release of CFZ in a controlled manner.

It was important to determine the release mechanism of CFZ from the thermosensitive mucoadhesive *in situ* gel formulation *in vitro* and *ex vivo* permeation study. The release mechanism determination was performed by fitting the data result to different mathematical kinetic models [41]. The release mechanism was determined based on the correlation coefficient ( $R^2$ ) value nearly to 1. Based on the result, *in vitro* and *ex vivo* studies showed the same release mechanism of CFZ from thermosensitive mucoadhesive *in situ* gel formulation (Table 6). Both studies depicted that the release mechanism followed Higuchi's model. This model indicates that the release of CFZ from the formulation was potentially caused by the gradual swelling of the polymers [42].

As mentioned previously, the main purpose of the development of this formulation was to improve the concentration of CFZ in the ocular tissue. Therefore, the *ex vivo* retention study was carried out. Fig. 5D presents the concentration of CFZ in the ocular tissue following the application of the thermosensitive mucoadhesive gel in comparison with the control solution. After the administration of

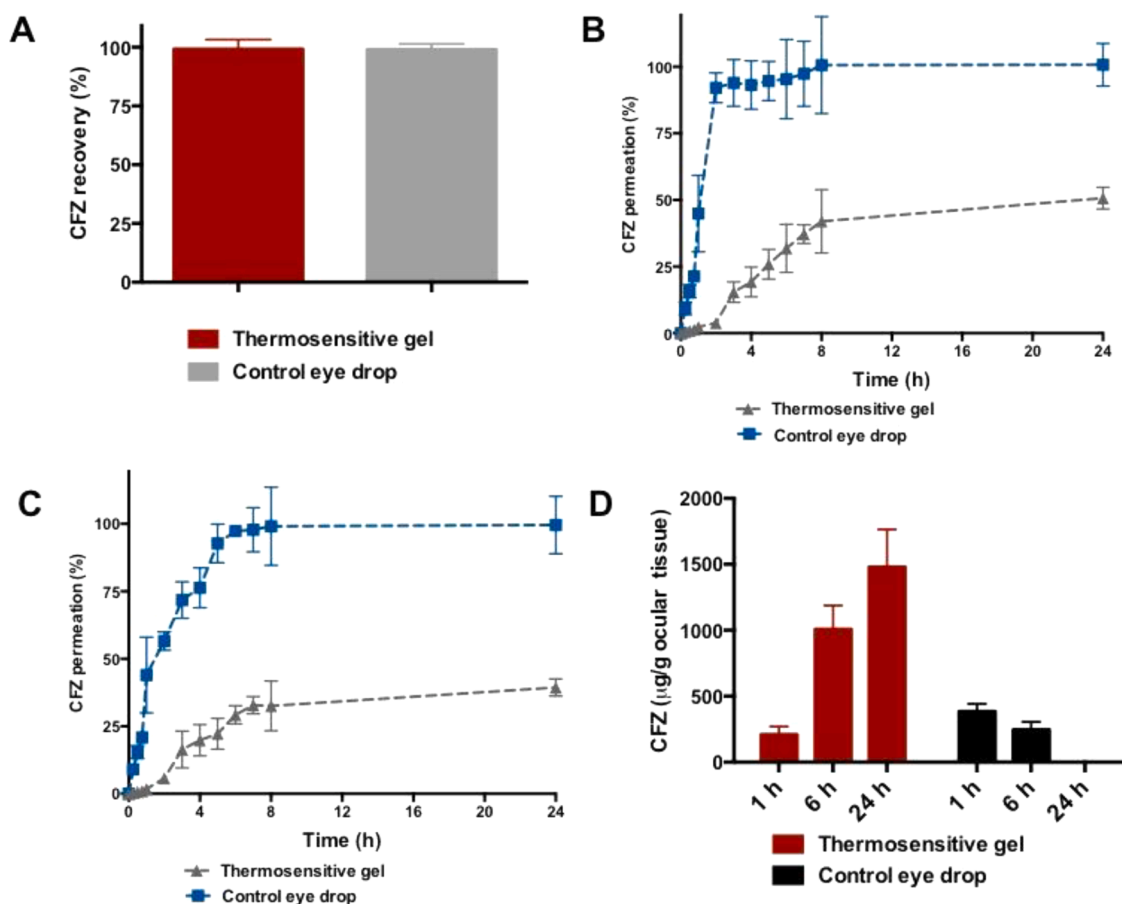


Fig. 5. Recovery percentage of CFZ from thermosensitive gel and solution (A), *in vitro* permeation profile of CFZ from thermosensitive gel in comparison with control solution (B), *ex vivo* permeation profile of CFZ from thermosensitive gel in comparison with control solution (C) and *ex vivo* retention of CFZ in ocular tissue (D). (mean  $\pm$  SD,  $n = 3$ ).

Table 6

*In vitro* and *ex vivo* correlation coefficient of CFZ from thermosensitive mucoadhesive *in situ* gel formulation in different mathematic kinetic models.

Mathematic model	Correlation Coefficient ( $R^2$ )	
	<i>In vitro</i>	<i>Ex vivo</i>
Zero Order Kinetics	0.7043	0.6328
First Order Kinetics	0.8026	0.8026
Higuchi Models	0.8344	0.8344
Krossmeyer – Peppas Model	0.2271	0.2271
Hixson – Crowell Model	0.7330	0.7330

thermosensitive mucoadhesive formulation, the concentrations of CFZ retained were  $208.76 \pm 62.16$   $\mu\text{g/g}$  tissue,  $1007.71 \pm 179.83$   $\mu\text{g/g}$  tissue and  $1479.49 \pm 284.12$   $\mu\text{g/g}$  tissue after 1 h, 6 h and 24 h. On the other hand, in the control solution group, significant lower ( $p < 0.05$ ) concentrations of CFZ were observed. After 1 h and 6 h, only  $384.82 \pm 56.99$   $\mu\text{g/g}$  tissue and  $245.26 \pm 60.78$   $\mu\text{g/g}$  tissue of CFZ were localized in the ocular tissue. Furthermore, no CFZ was detected after 24 h. Thus, thermosensitive mucoadhesive *in situ* gel formulation could increase the retention of CFZ in the ocular tissue and would potentially make the therapy more effective. Moving forward, further study involving *in vivo* studies with appropriate analytical methods must be conducted.

#### 4. Conclusion

The present study showed the development of validation of the analytical method of CFZ in the *in vitro* and *ex vivo* evaluation steps of the formulation of thermosensitive mucoadhesive *in situ* gel. The method was developed in the simulated tear fluid (STF) and ocular tissue. The analytical method developed in this study was based on the spectrophotometry method, showing excellent linearity with

correlation coefficient values of 0.9997 in the STF and 0.9981 in ocular tissue. Furthermore, the inter-day and intra-day precision and accuracy values were not more than 15%, indicating that the method was precise and accurate. Finally, our accurate and precise method was applied to determine CFZ in the drug content determination, the *in vitro* and *ex vivo* permeation study, as well as *ex vivo* retention study from thermosensitive mucoadhesive *in situ* gel formulation.

### Declaration of competing interest

The authors declare no conflicts of interest.

### Author's Role

**Alhidayah:** Conceptualization; Data curation; Investigation; Methodology; Project administration; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing, **Muh. Al Fiqri:** Conceptualization; Methodology; Software; Validation; Visualization; Writing - review & editing, **Nirmayanti:** Methodology; Validation; Visualization, **Ummu Athiyyah:** Methodology; Validation; Visualization, **Tamara Gabriela Angeleve Fadjar:** Methodology; Validation; Visualization, **Firzan Nainu:** Writing - review & editing, **Andi Arjuna:** Writing - review & editing, **Andi Dian Permana:** Conceptualization; Funding acquisition; Project administration; Supervision; Writing - review & editing.

### Declaration of Competing Interest

The authors declare no conflict of interest.

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### References

- [1] L. Ung, N.R. Acharya, T. Agarwal, E.C. Alfonso, B. Bagga, P.J.M. Bispo, M.J. Burton, J.K.G. Dart, T. Doan, S.M.J. Fleiszig, P. Garg, M.S. Gilmore, D.C. Gritz, L. D. Hazlett, A. Iovieno, V. Jhanji, J.H. Kempen, C.S. Lee, T.M. Lietman, T.P. Margolis, S.D. McLeod, J.S. Mehta, D. Miller, E. Pearlman, L. Prajna, N. Venkatesh Prajna, G.D. Seitzman, S.S. Shanhag, N. Sharma, S. Sharma, M. Srinivasan, F. Stapleton, D.T.H. Tan, R. Tandon, H.R. Taylor, E.Y. Tu, S.S. Tuli, R.B. Vajpayee, R. N. Van Gelder, S.L. Watson, M.E. Zegans, J. Chodosh, Infectious corneal ulceration: a proposal for neglected tropical disease status, *Bull. World Health Organ.* 97 (2019) 854–856, <https://doi.org/10.2471/BLT.19.232660>.
- [2] L. Ung, P.J.M. Bispo, S.S. Shanhag, M.S. Gilmore, J. Chodosh, The persistent dilemma of microbial keratitis: global burden, diagnosis, and antimicrobial resistance, *Surv. Ophthalmol.* 64 (2019) 255–271, <https://doi.org/10.1016/j.survophthal.2018.12.003>.
- [3] D.S.J. Ting, C.S. Ho, R. Deshmukh, D.G. Said, H.S. Dua, Infectious keratitis: an update on epidemiology, causative microorganisms, risk factors, and antimicrobial resistance, *Eye* 35 (2021) 1084–1101, <https://doi.org/10.1038/s41433-020-01339-3>.
- [4] S.M.J. Fleiszig, A.R. Kroken, V. Nieto, M.R. Grosser, S.J. Wan, M.M.E. Metruccio, D.J. Evans, Contact lens-related corneal infection: intrinsic resistance and its compromise, *Prog. Retin. Eye Res.* 76 (2020), 100804, <https://doi.org/10.1016/j.preteyeres.2019.100804>.
- [5] A. Austin, T. Lietman, J. Rose-Nussbaumer, Update on the management of infectious keratitis, *Ophthalmology* 124 (2017) 1678–1689, <https://doi.org/10.1016/j.ophtha.2017.05.012>.
- [6] USP-NF, U.S., *Pharmacopeia National Formulary, 38th ed.*, The United States Pharmacopeial Convention, United States, 2015.
- [7] U.M. Güven, M.S. Berkman, B. Şenel, Y. Yazan, Development and *in vitro/in vivo* evaluation of thermo-sensitive *in situ* gelling systems for ocular allergy, *Brazilian J. Pharm. Sci.* 55 (2019) 1–11, <https://doi.org/10.1590/s2175-97902019000117511>.
- [8] A.D. Permana, R. Nurul, P. Layadi, A. Himawan, N. Juniarti, Q. Kurnia, E. Utomo, S. Aulia, A. Arjuna, R.F. Donnelly, Thermosensitive and mucoadhesive *in situ* ocular gel for effective local delivery and antifungal activity of itraconazole nanocrystal in the treatment of fungal keratitis, *Int. J. Pharm.* 602 (2021), 120623.
- [9] M.A. dos Santos Ramos, K.C. dos Santos, P.B. da Silva, L.G. de Toledo, G.D. Marena, C.F. Rodero, B.A.F. de Camargo, G.C. Fortunato, T.M. Bauab, M. Chorilli, Nanotechnological strategies for systemic microbial infections treatment: a review, *Int. J. Pharm.* 589 (2020), 119780, <https://doi.org/10.1016/j.jpharm.2020.119780>.
- [10] G. Begum, T. Leigh, E. Courtie, R. Moakes, G. Butt, Z. Ahmed, S. Rauz, A. Logan, R.J. Blanch, Rapid assessment of ocular drug delivery in a novel *ex vivo* corneal model, *Sci. Rep.* (2020) 1–12, <https://doi.org/10.1038/s41598-020-68254-1>.
- [11] J.T. do P. Silva, A.C. da Silva, J.M.T. Geiss, P.H.H. de Araújo, D. Becker, L. Bracht, F.V. Leimann, E. Bona, G.P. Guerra, O.H. Gonçalves, Analytical validation of an ultraviolet–visible procedure for determining lutein concentration and application to lutein-loaded nanoparticles, *Food Chem* 230 (2017) 336–342, <https://doi.org/10.1016/j.foodchem.2017.03.059>.
- [12] M. Rafi, R. Jannah, R. Heryanto, A. Kautsar, D.A. Septaningsih, UV-Vis spectroscopy and chemometrics as a tool for identification and discrimination of four *Curcuma* species, *Int. Food Res. J.* 25 (2018) 643–648.
- [13] C.P. Ferreira, F.T. Techera Antunes, I.N. Rebelo, C.A. da Silva, F.N. Vilanova, D.S. Corrêa, A.H. de Souza, Application of the UV–vis spectrophotometry method for the determination of glutamate in the cerebrospinal fluid of rats, 2020. <https://doi.org/10.1016/j.jpba.2020.113290>.
- [14] I.A. Abdallah, S.F. Hammad, A. Bedair, F.R. Mansour, A green homogeneous liquid-liquid microextraction method for spectrophotometric determination of daclatasvir in human plasma, *Sustain. Chem. Pharm.* 22 (2021), 100498, <https://doi.org/10.1016/j.scp.2021.100498>.
- [15] H. Heidari, C. Mammostaefaei, Spectrophotometric determination of lamotrigine in plasma samples: ultrasound-assisted emulsification-microextraction based on a hydrophobic deep eutectic solvent followed by back-extraction, *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* 247 (2021), 119098, <https://doi.org/10.1016/j.saa.2020.119098>.
- [16] M. Mabrouk, S.F. Hammad, A.A. Abdella, F.R. Mansour, Chitosan-based molecular imprinted polymer for extraction and spectrophotometric determination of ketorolac in human plasma, *Spectrochim. Acta - Part A Mol. Biomol. Spectrosc.* 241 (2020), 118668, <https://doi.org/10.1016/j.saa.2020.118668>.
- [17] N. Khan, M. Tuzen, A new green *in situ* effervescent CO<sub>2</sub>-table-induced switchable hydrophilicity solvent extraction method of Rhodamine B in food and soft drink samples, *J. AOAC Int.* 104 (2021) 384–388, <https://doi.org/10.1093/jaoacint/qsaa107>.
- [18] Ö. Demir, M. Tuzen, N. Altunay, M.R.A. Mogaddam, Air-assisted alkanol-based nanostructured supramolecular liquid–liquid microextraction for extraction and spectrophotometric determination of Morin in fruit and beverage samples, *Food Anal. Methods.* (2021), <https://doi.org/10.1007/s12161-021-02111-3>.

- [19] M. Tuzen, N. Altunay, A. Elik, M.R. Afshar Mogaddam, K. Katin, Experimental and theoretical investigation for the spectrophotometric determination of thiabendazole in fruit samples, *Microchem. J.* 168 (2021), 106488, <https://doi.org/10.1016/j.microc.2021.106488>.
- [20] K. Al Khateb, E.K. Ozhmukhametova, M.N. Mussin, S.K. Seilkhanov, T.K. Rakhypbekov, W.M. Lau, V.V. Khutoryanskiy, In situ gelling systems based on Pluronic F127/Pluronic F68 formulations for ocular drug delivery, *Int. J. Pharm.* 502 (2016) 70–79, <https://doi.org/10.1016/j.ijpharm.2016.02.027>.
- [21] D. Ramadon, A.J. Courtenay, A.D. Permana, I.A. Tekko, E. McAlister, M.T.C. McCrudden, H.O. McCarthy, R.F. Donnelly, A sensitive HPLC-UV method for quantifying vancomycin in biological matrices: application to pharmacokinetic and biodistribution studies in rat plasma, skin and lymph nodes, *J. Pharm. Biomed. Anal.* 189 (2020), 113429.
- [22] A.D. Permana, E. Wahyudin, M.N. Amir Ismail, M. Raihan, Q.K. Anjani, E. Utomo, P. Layadi, R.F. Donnelly, New and sensitive HPLC-UV method for concomitant quantification of antifilaria drugs in rat plasma and organs after simultaneous oral administration, *Anal. Methods*. 13 (2021) 933–945.
- [23] A.D. Permana, I.A. Tekko, H.O. McCarthy, R.F. Donnelly, New HPLC-MS method for rapid and simultaneous quantification of doxycycline, diethylcarbamazine and albendazole metabolites in rat plasma and organs after concomitant oral administration, *J. Pharm. Biomed. Anal.* 170 (2019) 243–253.
- [24] S. Khan, M.U. Minhas, I.A. Tekko, R.F. Donnelly, R.R.S. Thakur, Evaluation of microneedles-assisted *in situ* depot forming poloxamer gels for sustained transdermal drug delivery, *Drug Deliv. Transl. Res.* 9 (2019) 764–782, <https://doi.org/10.1007/s13346-019-00617-2>.
- [25] S.S. Pekamwar, T.M. Kalyankar, B.V. Tambe, S.J. Wadher, Validated UV-visible spectrophotometric method for simultaneous estimation of cefixime and moxifloxacin in pharmaceutical dosage form, *J. Appl. Pharm. Sci.* 5 (2015) 037–041, <https://doi.org/10.7324/JAPS.2015.50107>.
- [26] S.S. Ulhassan, S.F.A. Zaidi, I. Tariq, M.T. Ansari, Development and validation of analytical method for losartan-copper complex using UV-Vis spectrophotometry, *Trop. J. Pharm. Res.* 12 (2013) 407–411, <https://doi.org/10.4314/tjpr.v12i3.20>.
- [27] R.K.T. Kam, M.H.M. Chan, H.T. Wong, A. Ghose, A.M. Dondorp, K. Plewes, J. Tarning, Quantitation of paracetamol by liquid chromatography-mass spectrometry in human plasma in support of clinical trial, *Putur. Sci. OA.* (2018) 4, <https://doi.org/10.4155/foa-2018-0039>.
- [28] S.R. Pandya, M. Singh, Preparation and Characterization of Magnetic Nanoparticles and their Impact on Anticancer Drug Binding and Release Processes Moderated Through 1st Tier Dendrimer, *J. Name.* (2013).
- [29] G. Nava, E. Piñón, L. Mendoza, N. Mendoza, D. Quintanar, A. Ganem, Formulation and *in vitro*, *ex vivo* and *in vivo* evaluation of elastic liposomes for transdermal delivery of ketorolac tromethamine, *Pharmaceutics* 3 (2011) 954–970, <https://doi.org/10.3390/pharmaceutics3040954>.
- [30] S. Manchanda, Nanotechnology Reviews ‘ Just Accepted ’ Papers, *Nanotechnol. Rev.* (2011), <https://doi.org/10.1515/ntrev-2016-0011>.
- [31] A.D. Permana, R.N. Utami, A.J. Courtenay, M.A. Manggau, R.F. Donnelly, L. Rahman, Phytosomal nanocarriers as platforms for improved delivery of natural antioxidant and photoprotective compounds in propolis: an approach for enhanced both dissolution behaviour in biorelevant media and skin retention profiles, *J. Photochem. Photobiol. B Biol.* 205 (2020), 111846.
- [32] Y.S. Chhonker, C. Edi, D.J. Murry, LC-MS/MS method for simultaneous determination of diethylcarbamazine, albendazole and albendazole metabolites in human plasma: application to a clinical pharmacokinetic study, *J. Pharm. Biomed. Anal.* 151 (2018) 84–90, <https://doi.org/10.1016/j.jpba.2017.12.037>.
- [33] S.M. Sunarić, M.S. Denić, Z.Z. Bojanić, V.V. Bojanić, HPLC method development for determination of doxycycline in human seminal fluid, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 939 (2013) 17–22, <https://doi.org/10.1016/j.jchromb.2013.08.035>.
- [34] S. Ahmed, N. Mustaan, M.A. Sheraz, S.A.A. un Nabi, I. Ahmad, Validation of a UV spectrometric method for the assay of tolfenamic acid in organic solvents, *J. Pharm.* 2015 (2015) 1–8, <https://doi.org/10.1155/2015/216249>.
- [35] ICH, Technical requirements for registration of pharmaceuticals for human use: the ICH process, 2005. <https://doi.org/10.1002/9781118532331.ch23>.
- [36] A.D. Permana, S. Stewart, J. Domínguez-Robles, M.N. Amir, M.A. Bahar, R. Donnelly, E.L. Landa, Development and validation of a high-performance liquid chromatography method for levothyroxine sodium quantification in plasma for pre-clinical evaluation of long-acting drug delivery systems, *Anal. Methods*. (2021), <https://doi.org/10.1039/d1ay01049b>.
- [37] S.W. Group, F. Toxicology, V. Methods, M. Development, V. Plan, R. Validation, P. Based, S. Requirements, C. Method, V. Experiments, C. Model, I. Studies, I. Suppression, A.V. Parameters, D. Integrity, R. Revalidation, P.V. Methods, D. Requirements, M. Validation, R. Appendix, B. Validation, E. Appendix, I. Screen, U. Validation, E. Appendix, E. Flowchart, M. Validation, E. Appendix, E. Experiments, Q. Confirmation, I.M. Appendix, E. Experiments, Q.M. This, S.W. Group, F. Toxicology, Scientific working group for forensic toxicology (SWGTOX) standard practices for method validation in forensic toxicology, *J. Anal. Toxicol.* 37 (2013) 452–474, <https://doi.org/10.1093/jat/bkt054>.
- [38] S. Walfish, A statistical perspective on the ICH Q2A and Q2B guidelines for validation of analytical methods, *BioPharm Int* 19 (2006) 28–36.
- [39] M. Attimarad, K.N. Venugopala, B.E. Aldhubaib, N. SreeHarsha, A.B. Nair, Correction: development of UV spectrophotometry methods for concurrent quantification of amlodipine and celecoxib by manipulation of ratio spectra in pure and pharmaceutical formulation (PLoS ONE (2019) 14: 9 (e0222526) DOI: 10.1371/journal.pone.0222526), *PLoS ONE* 14 (2019) 1–15, <https://doi.org/10.1371/journal.pone.0227412>.
- [40] J.R. Jørgensen, F. Yu, R. Venkatasubramanian, L.H. Nielsen, H.M. Nielsen, A. Boisen, T. Rades, A. Müllertz, *In vitro*, *ex vivo* and *in vivo* evaluation of microcontainers for oral delivery of insulin, *Pharmaceutics* 12 (2020) 1–14, <https://doi.org/10.3390/pharmaceutics12010048>.
- [41] J. Zuo, Y. Gao, N. Bou-Chacra, R. Löbenberg, Evaluation of the DDSolver software applications, *Biomed Res. Int.* 2014 (2014), <https://doi.org/10.1155/2014/204925>.
- [42] C. Mircioiu, V. Voicu, V. Anuta, A. Tudose, C. Celia, D. Paolino, M. Fresta, R. Sandulovici, I. Mircioiu, Mathematical modeling of release kinetics from supramolecular drug delivery systems, *Pharmaceutics* (2019) 11, <https://doi.org/10.3390/pharmaceutics11030140>.