

## Validation of HPLC Method for Timolol Maleate Determination in Nano-Polymeric Formula

Muhammad Sulaiman<sup>1</sup>, Endang Lukitaningsih<sup>2\*</sup>, Ronny Martien<sup>3</sup>, Retno Danarti<sup>4</sup> and Yohanes Widodo Wirohadidjojo<sup>4</sup>

<sup>1</sup> Doctoral Program, Faculty of Pharmacy, Universitas Gadjah Mada, Skip Utara 55281 Yogyakarta, Indonesia.

<sup>2</sup> Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Skip Utara 55281 Yogyakarta, Indonesia.

<sup>3</sup> Department of Pharmaceutics, Faculty of Pharmacy, Universitas Gadjah Mada, Skip Utara 55281 Yogyakarta, Indonesia.

<sup>4</sup> Department of Dermatology and Venerology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Skip Utara 55281 Yogyakarta, Indonesia.

### Article Info

**Submitted:** 29-05-2024

**Revised:** 30-08-2024

**Accepted:** 02-09-2024

\*Corresponding author  
Endang Lukitaningsih

Email:  
Lukitaningsih\_end@ugm.ac.id

### ABSTRACT

In this study, a sensitive, selective, and precise HPLC method for the detection of timolol maleate (TM), one of the anti-haemangioma  $\beta$ -blockers, in nano-polymeric formulation was developed. Chromatographic separation was achieved using a mobile phase of 10 mM ammonium acetate buffer pH=3.5: acetonitrile (80:20, v/v) in isocratic mode of elution at a flow rate of 1.0 ml/min on Luna Phenomenex C18 ODS column (150mm x 4.6mm, 5 $\mu$ m). Validation was performed on the method's system suitability, selectivity, linearity, accuracy, precision, and robustness. The results showed that the precision and uniformity values of the nanoparticle samples (TMNP) were within an acceptable range for both between and within days. The retention time of TM was observed to be around 6.8 minutes in the absence of any peak intervention. The standard curve exhibited a strong linear relationship, and the recovery values satisfied the specified range criteria consistently over a period of three consecutive days. After different ways of making sample preparations, which should affect the amount of contained drug, the employed technique successfully determined the percentage of drug encapsulation in a polymeric nanoparticle formulation with dependable outcomes. The penetration profile of TMNP and free drugs on mouse skin membranes was successfully determined using the same approach that had previously undergone a verification step. The improved and validated method has proven its reliability and suitability in measuring the amount of timolol maleate in nano-polymer products to evaluate the efficiency of encapsulation and its penetration profile, which is a crucial factor in building drug delivery systems using nanotechnology.

**Keywords:** Timolol maleate, Validation, RP-HPLC, Polymeric nanoparticle, Penetration study

### INTRODUCTION

Infantile hemangioma (IH) is a form of vascular tumor that is quite common in neonates and has a worldwide frequency of up to 10% (Ames & Sykes, 2015). In 2008, propranolol, a non-specific beta blocker, was inadvertently discovered to be effective against IH (Kim et al., 2017). Since then, subsequent studies have focused on the utilization of different beta blockers, including timolol maleate

(TM), which has recently gained attention as a potential medicine for treating infantile hemangioma (IH) due to its comparable mechanism of action (Itinteang et al., 2014; Püttgen et al., 2016). Timolol maleate or (S)-1-tert-butylamino-3-(4-morpholino-1,2,5-thiadiazol-3-yl)oxy)propan-2-ol-hydrogen maleate has molecular weight of 432.50 g/mol with short half life and moderate bioavailability when given

systemically (Morsi et al., 2016; Singh & Dhawan, 2024). Moreover, the absence of targeting capability also gives rise to its own concerns, particularly if the majority of patients are newborns (Tiemann & Hein, 2020). It has been demonstrated that low timolol plasma levels result in significant systemic  $\beta$ -blocking activity, albeit with just a small drop in heart rate and still can be controlled (Alasmari et al., 2023). Considering the location of the lesion, the limited oral bioavailability, and the propensity for systemic adverse effects, topical use of such an efficacious medicine is preferable to systemic delivery for the treatment of IH. Despite its potency, topical timolol application has been observed to give good response only in superficial and uncomplicated lesions (Püttgen et al., 2016).

Numerous studies have demonstrated that nanoparticles can improve the topical penetration of anti-hemangioma medications or even allow them to be actively deposited at the target location, allowing for the use of a lower dose while avoiding systemic side effects (M. El-Sayaad et al., 2017). One of the required characteristics to take into account while optimizing a nanoparticle formula is a maximum percentage of encapsulation efficiency (EE), which can be modified due to its correlation with constituents' aspects (Modena et al., 2019). Many different materials have been used to make nanoparticles, each of which has a different capacity for loading the desired active substance (Gopi et al., 2018). The utilization of natural substances in drug delivery strategies has recently gained prominence due to its inherent advantages, notably biocompatibility and safety. Chitosan, among several potential natural therapies, emerges as a promising choice for facilitating the transdermal delivery of various chemicals owing to its inherent properties as a natural permeation enhancer. Measuring the amount of drug that is successfully formed on the nano meter scale as a result of encapsulation or adsorption on the nanoparticle surface is essentially how encapsulation efficiency is determined (Shen et al., 2017). This stage poses significant challenges due to the physicochemical qualities of each material utilized, which result in distinct interaction characteristics with various active compounds. Considering the significance of this factor, accurate techniques for estimating encapsulation efficiency must be guaranteed.

There are a number of analytical methods in the literature for the determination of timolol maleate in either drug formulation or physiological

fluid, either pure or in combination with other medicines (Eickner et al., 2018; Marley & Connolly, 2014; Singh & Dhawan, 2024; Walash & El-Shaheny, 2016). Reversed-phase high-performance liquid chromatography (RP-HPLC), liquid chromatography mass spectrophotometry (LCMS), spectrophotometry, and other techniques are among those used (Hassib et al., 2016; Swamy et al., 2020; Tambe et al., 2024). To determine EE and skin penetration profile, the HPLC technique was applied to a recently produced nanoparticle containing timolol maleate. To the best of our knowledge, HPLC method development to assess TM in polymeric nanoparticle formulation has not yet been investigated. The purpose of this research was to establish a rapid, sensitive, and economically viable technique for quantifying TM in polymeric nanoparticles. Following the International Harmonization Conference (ICH) Guidelines, the performance of the created technique was validated (ICH, 2022). As recommended by the guidelines, this study reported the system selectivity, suitability, linearity, accuracy, precision, LODs, LOQ, and robustness. The penetration test was conducted in accordance with the in vitro procedure outlined in The Organization for Economic Cooperation and Development (OECD) 428 (OECD Guideline for the testing of chemicals, Skin absorption: in vitro method).

## **MATERIALS AND METHODS**

### **Materials**

Analytical grade Timolol maleate was purchased from BPOM/The National Agency of Drug and Food Control of Indonesia, as Farmakope Indonesia Chemical Reference Substance (Purity 99.46%). Other reagents, such as ammonium acetate powder, were obtained from PT. Smart Lab (Semarang, Indonesia), acetic acid from Merck (New Jersey, USA). Freshly triple distilled water was used during the experiment. The molecular weight of chitosan medium (CH) and acacia gum (AG) were used as nanoparticle matrix and purchased from local companies in Indonesia (CV Bio Chitosan, Surabaya, Indonesia and Rofa Lab Centre, Bandung, Indonesia subsequently).

### **Instrumentation**

The HPLC system was run on Shimadzu LC-2050C equipped with PDA detectors, column oven, and automatic 20  $\mu$ L injector. Analytical balance (Ohaus, New Jersey, USA), vortex mixer (Thermo scientific, Massachusetts, USA), pH meter (Ohaus Starter 3100, New Jersey, USA), 0.45  $\mu$ m PTFE

membrane filter, 0.45  $\mu\text{m}$  nylon syringe filter, glassware (Pyrex), Vivaspin ultracentrifugation tube polyethersulfone (PES) membrane pore 100 kDa MWCO (Sartorius, Germany), Milli-Q Water purified system (Merck, New Jersey, USA), and ultrasonic bath (Branson, Branson Ultrasonics, USA) were employed during the experiment.

Permeation studies were conducted using an automated system (PermeGear®ILC-07) equipped with seven in-line flow-through diffusion cells. The cells were continuously pumped by a peristaltic pump (Ismatec, Switzerland) to ensure automatic sampling into receptor chambers with a maximum capacity of 3 mL. The diameter of the diffusional area measured 0.785  $\text{cm}^2$  and the temperature of the cells was maintained at  $32 \pm 1$  °C using a water bath.

### Chromatographic condition

HPLC system was run with isocratic elution using mobile phase consisting of 10 mM ammonium acetate buffer with pH 3.5 and acetonitrile in the volume ratio of 80:20. The samples were monitored at a detection wavelength of 297 nm with an injection volume of 20  $\mu\text{L}$  and a flow rate of 1 mL/min at a constant temperature of 30° Celcius through a Luna Phenomenex C18 (250 x 4.6 mm, 5  $\mu\text{m}$ ) column. Before being used in the study, the mobile phase was filtered through a 0.45  $\mu\text{m}$  membrane filter and degassed for 10 minutes by an ultrasonic sonicator. On the PDA detector, wavelength scanning was performed, and a maximum wavelength for timolol maleate detection was chosen for analysis.

### Preparation of stock solutions, sample, and matrix/blank nanoparticle

Timolol maleate stock solution (1000  $\mu\text{g}/\text{mL}$ ) was prepared by diluting 10 mg Timolol maleate in distilled water using 10 mL volumetric flask. The flask was then shaken vigorously, followed by 15-minute sonication to homogenize the solution and final volume was made up to 10 mL. The stock solution was protected from direct sunlight and stored at a temperature of 4 °C for further use. The stock solution was further diluted with distilled water to prepare a series of concentrations ranging from 22.5 to 90  $\mu\text{g}/\text{mL}$  for calibration curve preparation.

The nanoparticles bearing the drug samples (timolol maleate nanoparticle, abbreviated TMNP) were prepared using ionic gelation technique where an accurate amount of CH solution was first mixed thoroughly with TM, then added with a

solution of AG. Other formula was prepared the same way with further sodium tripolyphosphate (STPP) addition. The formulas contained a theoretical concentration of TM at 0.02% w/v or 200  $\mu\text{g}/\text{mL}$ . Each sample was then transferred into a Vivaspin 6 tube with a 100 KDa MWCO polyether sulfone (PES) membrane filter (Sartorius, Germany). The samples were then centrifuged for 3 minutes at 2000 rpm to separate untrapped drug. The filtrate at the bottom of the tube was collected and mixed with mobile phase to reach proper dilution and filtered with 0.45  $\mu\text{m}$  membrane filter prior to injection into the HPLC system. The data were collected in triplicate and the result was presented in mean  $\pm$  SD.

For the EE determination, nanoparticle matrix was used as blank and prepared using the same method employed in the production of TMNP formula without the contained drug. The matrix solution was later processed using the same method as in the TMNP preparation. To ensure that the measured concentration of the sample fell within the range of the calibration curve, a similar approach was employed as the treatment of the TMNP sample, whereby the nanoparticle matrix was diluted by a factor of 10. Furthermore, the blank solution utilized in the drug determination in the penetration test was a mixture of blank nanoparticle and pumped receptor solution that were collected just prior to conducting the sample penetration test.

### Preparation of calibration plot

To create the calibration curve, the stock solution was diluted with distilled water, followed by nanoparticle matrix addition and mobile phase dilution to reach a final concentration of 7.5-30  $\mu\text{g}/\text{mL}$ . A separate calibration curve was also generated for low concentrations (0.25 - 0.7  $\mu\text{g}/\text{mL}$ ) using the same method. This involved introducing a blank solution consisting of a mixture of nanoparticle matrix and receptor compartment fluid (phosphate buffer with pH 5.5) to the stock solution. Each resulting solution (n=6 and n=9) was then vortexed and filtered with 0.45 microns membrane filter before injected into the HPLC system. On the basis of the linear relationship between peak area and matching concentration, a calibration curve was generated.

### System suitability testing

Prior to the analysis, six replicates of the highest concentration of the standard solution were injected as a system suitability test to

determine whether the established system was appropriate and relevant for quantification (ICH, 2022). The measured parameters were % RSD of peak area and retention time, tailing factor, and theoretical plates which should conform with acceptance criterion of  $\leq 2\%$ ,  $\leq 2$ , and  $\geq 2000$ , respectively.

### Selectivity study

Selectivity of the proposed approach was tested by comparing the chromatograms of the timolol maleate and nanoparticle matrix (including chitosan, acacia gum, and tripolyphosphate solubilized in acetate buffer media). The matrix chromatogram had to be shown to have a minor effect on the timolol maleate chromatogram at the detection wavelength. By doing so, the effects of the other components, such as excipients or drug matrices, can also be investigated.

### Calibration curve and linearity study

The calibration curve was used to find out if the relationship between peak area and concentration was linear. The HPLC system was injected with six concentration levels (7.5 - 30  $\mu\text{g/mL}$ ) of TM working standard solution dispersed in nanoparticle matrix to determine if the peak area of the tested sample corresponded to the relevant concentration. After each injection, the area of the chromatogram values was plotted against corresponding concentrations and a calibration curve was created. The equation and coefficient of determination ( $R^2$ ) value of the line were determined. The second calibration curve was generated specifically for the low concentration range of 0.25 - 0.7  $\mu\text{g/mL}$  to accurately estimate the drug concentration during the penetration study step.

### Accuracy and precision study

This method's accuracy was evaluated using six measurements at three concentration levels: 15, 20, and 25  $\mu\text{g/mL}$ , which corresponded to a specified range of 80%, 100%, and 120% of the theoretical concentrations of the analyte. In a theoretical scenario, it can be postulated that when the encapsulation effectiveness of the nanoparticle sample being evaluated was exceedingly low, the resulting concentration of the analyte was anticipated to reside within the range of  $\pm 200$   $\mu\text{g/mL}$  which was further diluted 10 times using mobile phase prior to the measurements. The accuracy was represented as a percentage of recovery based on measurements of a known

analyte concentration applied to the nanoparticle matrix (Equation 1). Following the ICH Q14 guidelines, the reportable range for %recovery should be between 80 - 120% (ICH, 2022).

$$\% \text{Recovery} = \frac{(C_{\text{practical}})}{(C_{\text{theoretical}})} \times 100 \quad \dots\dots\dots(1)$$

$C_{\text{practical}}$  = concentration obtained from measurements;

$C_{\text{Theoretical}}$  = calculated concentration

On the basis of intra- and inter-day precisions expressed as %RSD, the proposed method's precision was evaluated. To evaluate the intra- and inter-day precisions, the produced samples were evaluated on the same day and three consecutive days, respectively, by injecting three concentrations of drug (15, 20, and 25  $\mu\text{g/mL}$ ). The low %RSD values ( $< 2\%$ ) of recovery demonstrate the acceptable precision of the offered procedures. During the evaluation of method verification on the second calibration curve for penetration study, three concentration levels were assessed: 0.35, 0.5, and 0.65  $\mu\text{g/mL}$ .

### Limit of detection and quantification

The lowest concentration at which the examined chemical may be determined to have sufficient accuracy and reproducibility is known as the limit of detection (LOD). The lowest amount of substance that can be reliably measured using a certain technique is called the limit of quantification (LOQ). LOD and LOQ were calculated based on ICH guidelines Q2 (R1) using formula as depicted in Equations 2 and 3 where  $\sigma$  is the standard deviation of the sample and S refers to slope of the selected calibration curve.

$$\text{LOD} = (3.3 \times \sigma) / S \quad \dots\dots\dots(2)$$

$$\text{LOQ} = (10 \times \sigma) / S \quad \dots\dots\dots(3)$$

### Robustness

Modifications to the ideal method parameters were made to test the robustness of the developed RP-HPLC method. Chromatogram area was measured and analysed as a function of each of the different flow rates (0.8 and 1.2 ml/min), different column temperatures (25 and 35  $^{\circ}\text{C}$ ), and different mobile phase pH (3 and 4).

### Application of the proposed method

The amount of TM contained in the nanoparticle formula was subsequently determined using the proposed approach. In our

study, three formulas were chosen to study the effect of polymer concentration on encapsulated drugs. The chosen formulas, namely TMNP1, TMNP2, and TMNP5, represented the weight ratio of the CH/AG employed in the synthesis, i.e., 20, 4.25, and 5 subsequently plus one formula (TMNP1') which had the same CH/AG ratio to study the effect of tripolyphosphate (STPP) addition. The encapsulation efficiency value (%EE) was determined using formula based on Equation 4 where C<sub>0</sub> is the added total drug concentration and C<sub>1</sub> is the determined free drug concentration.

The penetration study included the collection of rat skin using a method permitted by the ethical clearance provided by the Faculty of Veterinary Medicine at Universitas Gadjah Mada in Yogyakarta, Indonesia (no: 72/EC-FKH/Eks./2023). The isolated skin was affixed to the cells and put between the donor and receptor chambers, with the test substances (TM and TMNP) applied to the top of the donor chamber. The receptor fluid was administered at a flow rate of 4.8 mL/h for a duration of 6 h. Prior to the study, each formula was homogeneously mixed into a freshly produced gel matrix made of HPMC to ease the application of the sample. As a result, the theoretical concentration of TM was 100 µg/mL. The penetration profile of the TM and TMNP gel was established by measuring the drug concentration that effectively penetrated the mouse skin membrane at a sampling interval of 0.5 hours (n=12). The data were then processed further to create a curve plot illustrating the relationship between drug accumulation per unit time. Each data sample was measured in triplicate and reported as a mean ± SD.

## RESULTS AND DISCUSSION

### Method validation

The production of Nanoparticles necessitates the confirmation of several characteristics, one of which is EE. Regarding the measurement of the desired entrapped drugs, HPLC method must be able to separate, detect, and quantify the drugs independently from the ingredients. Consequently, understanding the performance characteristics and limitations of the method is crucial for method validation. Validation of analytical methods should include evaluations of linearity, selectivity, accuracy (recovery), precision, LOD, LOQ, and measurement uncertainty for this purpose. With a Luna Phenomenex C18 ODS column (250 mm x 4.6 mm, 5 µm), different chromatographic conditions were tested. In an

isocratic mode, based on several studies, different mobile phase mixtures like phosphate buffer, ammonium acetate buffer, methanol, and acetonitrile, were tested in different compositions. In contrast to other studies, our research revealed that the presence of polymer elements, particularly CH, in the organic phase of methanol can lead to precipitation and column-related issues. This leads to discrepancies in the resulting retention times. In addition, CH exhibits solubility in an acidic environment, hence when the optimization process is conducted at elevated pH levels, it can lead to a blockage within the column due to precipitation. To mitigate interference from polymer matrices like CH, the author promotes the use of buffers for maintaining an acidic atmosphere. TM has chemical stability across a wide range of pH values, with the exception of alkaline environments, where the presence of various breakdown products has been observed (Sharma et al., 2012). For the majority of reverse phase applications, low buffer concentrations (0.01M) are mostly sufficient when precipitation issues due to high concentrations of organic modifiers utilized in the mobile phase are avoided at this concentration. During this experiment, the utilization of phosphate buffer resulted in the appearance of peaks that exhibited significantly longer retention times, exceeding 15 minutes. It is important to consider this result with regards to efficiency. The acetate buffer was checked subsequently, and while interference from the existence of a maleic acid peak was typically present in tests conducted at low pH values, none was discovered in this investigation (Elshanawane et al., 2014). To obtain the best separation and good resolution, several percentages of organic solvents were attempted with acetate buffer. Adequate separation and resolution were achieved with 20% acetonitrile in the mobile phase. Using a 10 mM ammonium acetate buffer with a pH of 3.5 produced better peaks, higher stability, and a shorter retention time. The maximum wavelength value of 297 nm was established by scanning the UV spectra of the TM in the mobile phase throughout our research. The UV spectrum of detection wavelength obtained from a set of calibration curves ranging from the lowest to the highest concentrations was also tested (Supplementary data).

From our current study, the optimized nanoparticle was formed by interaction between two main polymers: CH and AG with percentages subsequently ranging from 0.34 – 0.4% and 0.02 – 0.08% (w/v) and 0.02% STPP in one formula. The

method's selectivity was evaluated by determining the target analytes in the sample without interference from any matrix components. This was supported by the lack of interference at the TM retention time (RT) (about 6.8 min) from six independent batches of drug-free matrix components in the mobile phase used for analysis. Chromatogram example of standard and placebo are shown in Figures 1a and 1b where the placebo showed no peaks at the same RT with standard or sample; hence this method can be justified to be specific for TM. Unlike previous investigations, the maleic acid signal was not noticeable at the detecting wavelength in this analysis (Elshanawane et al., 2014). The observed phenomenon can be attributed to variations in multiple factors, including the column, mobile phase type and composition, and the pH utilized. In another work employing a column of comparable design, the timolol maleate peak was observed at 3.6 min using a combination of triethylamine with pH 3.5 (adjusted with acetic acid) and acetonitrile 20:80 v/v. Effective separation was achieved between timolol and brinzolamide using a PDA detector at a wavelength of 260 nm (Mandour et al., 2023).

There are many factors for different test results, including how long does the same type of column last, what does the analyte look like, what are the composition and pH of the mobile phase, and so on. Therefore, the parameters of the system suitability test are very important for figuring out the results of the analysis because the system suitability test provides assurance on the performance of the chromatographic system. This indicates the ability to establish a valid conclusion about the test result based on the analytical data obtained from the test. The final system suitability test parameters were determined to be satisfactory and within the acceptance range according to the ICH Guidelines. The RSD of retention time and area was <2%, the tailing factor was <2 and the number of theoretical plates was >2000 (Table I).

ICH recommends using at least five concentrations to generate a calibration curve. In the concentration range of 7.5 to 30 µg/mL, the linearity of the method was observed, confirming its usefulness. After mapping the Chromatographic area value to the corresponding concentration, the calibration curve was constructed. The equation of the line and the coefficient of determination ( $R^2$ ) were obtained.  $R^2=0.9976$  over the concentration range studied showed a functional linear connection between analyte concentration and area under the peak. The Calibration graph, as

shown in Figure 1d, also confirmed its linearity. The linearity criteria were also met during the verification of the second calibration curve, which varied from 0.25 to 0.7 µg/mL. This confirms its suitability for use in the penetration experiment to quantify the drug concentration in the receptor compartment (data not shown). Furthermore, the LOD and LOQ for this technique were 0.03475 µg/mL and 0.11581 µg/mL, respectively. This approach has been demonstrated to be more sensitive when compared to prior research, which had LOD and LOQ values of 0.09 and 0.29 µg/mL, respectively (Elshanawane et al., 2014).

There are two ways to determine accuracy: the spiked-placebo recovery simulation method and the standard addition method. The reliability and validity of the proposed method were tested using the spiked-placebo recovery method. In this method, a number of pure substance analytes are added to a mixture of pharmaceutical preparations (placebo). The combination is subsequently examined, and the results are compared to the concentrations of added analytes (actual levels). This aims to examine the impact of these excipients, with the results expressed as recovery values. Sextuplicate measurements were performed on the samples containing known concentrations of standard drug in nanoparticle matrix components (15, 20, and 25 µg/mL) (supplementary data). The recoveries were between 100.97 and 102.43 percents, suggesting that nanoparticle preparation excipients did not interfere with the analyte test. The value of %recovery demonstrates the accuracy and dependability of the proposed method in accordance with the previous method for timolol determination in pharmaceutical preparation. The suggested method was evaluated for the Precision study by calculating intra- and inter-day precisions as %RSD on recoveries. The intra- and inter-day precisions were obtained by analysing the samples collected on the same day and three consecutive days, respectively. The low %RSD values of the peak areas indicate that the precision of the offered approaches is adequate (Table II). This study also demonstrated high precision, as indicated by the lower value of HorRat, within the acceptable range of 0.5 - 2.0 for Horwitz ratio (HorRat) (Table II) (Horwitz & Albert, 2006). The results of this study did not exhibit a major disparity compared to other methods described in literature. The comparison of the chosen parameters between the suggested approach and prior method which employed more effective column types (Table IV).

Table I. Observed parameters on system suitability testing

Parameters	Average (n=6)	RSD (%)	Acceptance criterion
Retention time	6.695±0.010	0.150	RSD ≤ 2%
Area	640951±246	0.038	
Tailing Factor	1.377±0.001	0.046	≤ 2
Theoretical Plates	7833±31	0.394	≥ 2000

\*RSD= (Standard deviation/Mean concentration measured) x 100

Table II. Intermediate precision study of timolol maleate

Day	Actual conc (µg/mL)	Measured Conc (µg/mL) (n=6)	% RSD
1	14.95	15.09 ± 0.129	0.86
	19.93	20.37 ± 0.075	0.37
	24.91	25.52 ± 0.071	0.28
2	14.95	15.03 ± 0.176	1.17
	19.93	20.36 ± 0.069	0.34
	24.91	25.30 ± 0.085	0.33
3	14.95	15.37 ± 0.098	0.64
	19.93	20.60 ± 0.068	0.33
	24.91	25.63 ± 0.158	0.62

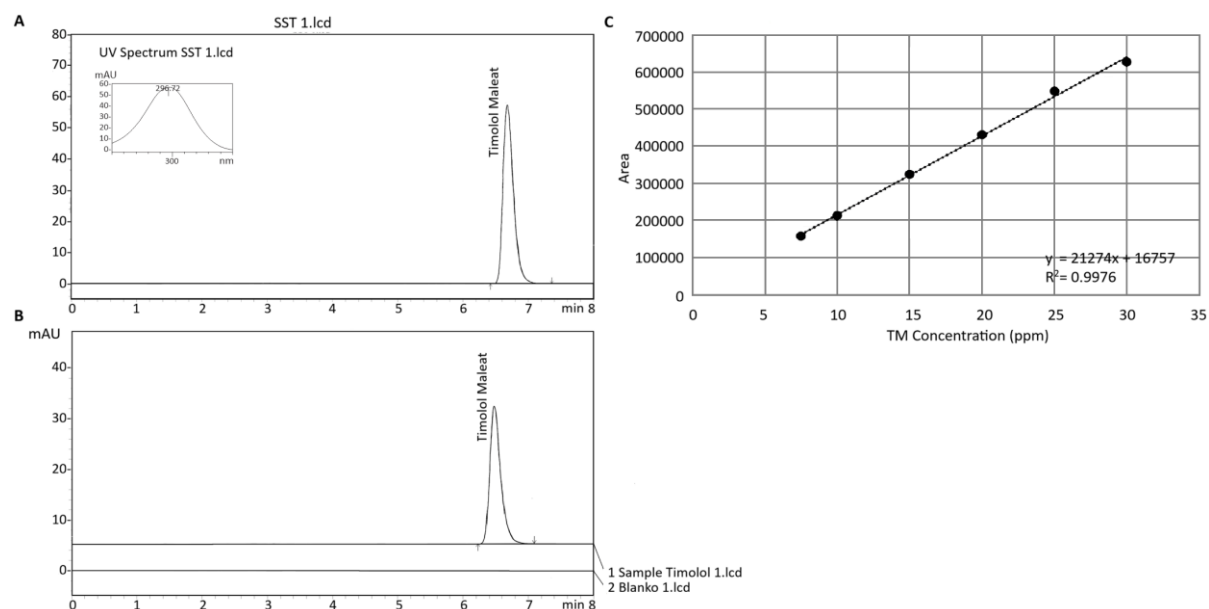


Figure 1. Validation results of HPLC method showed (a) chromatogram of timolol standard, (b) overlay chromatogram of placebo (blank nanoparticle) and timolol nanoparticle sample (abbreviated TMNP) and (c) standard calibration curve.

Table II. Results on percent (%) encapsulation efficiency (EE).

Sample code	Free drug ( $\mu\text{g/mL}$ )	Entrapped drug ( $\mu\text{g/mL}$ )	Total drug ( $\mu\text{g/mL}$ )	%EE
TMNP1	$17.716 \pm 0.003$	$3.736 \pm 0.003$	$21.452 \pm 0.009$	$17.41 \pm 0.015$
TMNP2	$17.523 \pm 0.003$	$3.929 \pm 0.003$	$21.452 \pm 0.009$	$18.31 \pm 0.017$
TMNP5	$18.107 \pm 0.013$	$3.360 \pm 0.013$	$21.452 \pm 0.009$	$15.66 \pm 0.062$
TMNP1'	$10.608 \pm 0.009$	$10.842 \pm 0.009$	$21.452 \pm 0.009$	$50.54 \pm 0.042$

Table III. Comparison between the proposed method and other published method.

Parameters	Proposed method	n	Previously proposed method (Elshanawane et al., 2014)	n
Accuracy and Precision	LQC	$100.97 \pm 0.86$	$100.53 \pm 1.06$	3
	Intra-day MQC	$102.22 \pm 0.37$	$100.01 \pm 1.02$	3
	HQC	$102.43 \pm 0.28$	$100.57 \pm 0.72$	3
Inter-day	$102.57 \pm 0.65$	18	$99.56 \pm 1.67$	9
LOD/LOQ	$0.034/0.1$ ( $\mu\text{g/mL}$ )		$0.09/0.9$ ( $\mu\text{g/mL}$ )	
Mobile phase	Ammonium acetate (pH3,5)-Acetonitrile (80:20)		Ammonium acetate (pH5)-Methanol (40:60)	
Flow rate	1 mL/min		1.5 mL/min	
Column type	Luna Phenomenex @ C18 ODS- (150mm $\times$ 4.6mm, 5 $\mu\text{m}$ )		BDS HIPERSIL Cyano (250mm $\times$ 4.6mm, 5 $\mu\text{m}$ )	

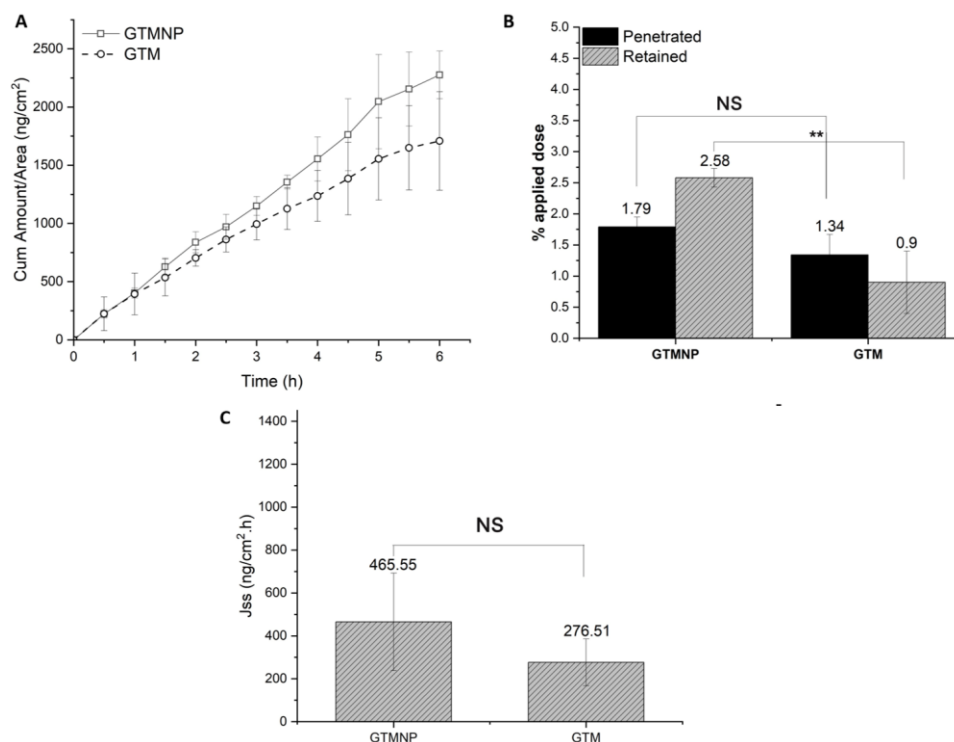


Figure 2. Application of the proposed HPLC method on timolol maleate nanoparticle gel (GTMNP) and timolol maleate gel (GTM) penetration profile comparison using Sprague Dawley rat skin after duration study of six hours. Data collected on triplicate and served as  $\pm$ SD. Cumulative amount/area vs time plot graph (Panel a), percent penetration and skin retention comparison (Panel b) and steady state flux ( $J_{ss}$ ) comparison (Panel c). b and c One tail t-test; n=3, NS: not significant  $p > 0.05$ , \*\* $p < 0.01$

The results indicated that the employed method yielded good results, which was likely attributed to the utilization of more efficient mobile phase (Elshanaawane et al., 2014).

To determine the robustness of the proposed method, the influence of minor alterations on relevant chromatographic parameters, such as flow rate, column temperature, and pH of mobile phase, were evaluated. Table VI (supplementary data) depicts the robustness study. Small variations in the pH of the mobile phase, the temperature of the column, and the flow rate were observed to have no effect on the suggested approach. It is noteworthy that employing a lower pH in the mobile phase can lead to a shorter retention time, thereby accelerating the measuring process. Nevertheless, the utilization of such a low pH is in extremely close proximity to the limits of the column, hence posing a potential hazard of column impairment. Furthermore, the increased flow rate leads to heightened pressure applied on the instrument throughout its operation, consequently requiring increased effort from the instrument compared to standard operating settings.

#### **Application of the proposed method**

The nanoparticle formulation's encapsulation efficiency value, expressed as a percentage, can be a useful parameter to a good nanoparticle drug delivery system. The results were determined using an indirect method based on the measurement of the free drug on the formula. Similar retention times were observed in the untrapped TM chromatograms of the nanoparticle samples (Figure 1c). The encapsulation efficiency of TMNP1, TMNP2, TMNP5, and TMNP1' are depicted in Table III. From the results, only little difference can be observed in the TM concentration between the tested formula except for the TMNP' sample. This phenomenon was probably due to the formation of more compact structure of the nanoparticles (due to the addition of STPP) that probably can hinder drug leakage during storage and free drug separation step by a centrifugation technique.

The utilization of chitosan, a natural permeation enhancer, in the nano-formulation is expected to boost the transdermal administration of drugs, surpassing the efficacy of over-the-counter drugs. Timolol maleate has limited skin penetration due to its physicochemical characteristics. Upon examining the disparities in the penetration profile curves of different formula on rat skin, it becomes evident that the penetration

profile of TMNP gel (nanoparticles) exceeded that of TM gel as depicted in the curve (Figure 2a). During the final three hours of the test, the disparity in the amounts of accumulation became increasingly evident. There was a major difference in the amount of drugs that effectively crossed and remained in the skin membrane between the two formulae, with TMNP demonstrating superiority (Figure 2b). Moreover, there was also a significant difference observed in the steady state flux (Jss) between the TMNP and the TM group (Figure 2.c). Based on the aforementioned results, it can be concluded that the nanoparticle formulation enhances the rate at which TM medicines penetrate. This study presents evidence that CH, a natural polymer, acts as a penetration enhancer during drug administration and also extends the duration of drug presence in the epidermal layer through various processes (Ma et al., 2022).

Consequently, nanotechnology has the potential to be employed in the specific treatment of IH illness by topical application. In conclusion, the HPLC method developed and validated in this study has been demonstrated to be robust and dependable for determining both the encapsulation efficiency value, which is an important quality control step in designing nano-formulations, and for assisting in the establishment of a precise and accurate penetration profile in skin permeation studies.

#### **CONCLUSION**

Since 2008, topical beta-blockers have been studied extensively to replace oral therapy in the treatment of superficial IH (Satterfield & Chambers, 2019). In this work, a new validated method was used to measure the timolol maleate %EE in nano-formula and its penetration profile against rat skin. The isocratic elution method exclusively employs a blend of the two solvents utilized, resulting in a user-friendly and resilient technique that exhibits selectivity, accuracy, and precision in accordance with the ICH rules. It can be considered as a viable option to other methods, hence suitable for quality control for the routine determination of timolol in pharmaceuticals and nanoparticle manufacturing.

#### **ACKNOWLEDGMENTS**

We expressed our gratitude for the implementation of this research which was carried out through a funding by The Final Project Recognition Grant Gadjah Mada University (Number 5075/UN1.P.II/Dit-Lit/PT.01.01/2023) in 2023.

### CONFLICT OF INTEREST

The authors declared no conflict of interest.

### REFERENCES

- A Elshanawane, A., Abdelaziz, L. M., Mohram, M.S., Havez, H.M. (2014). Development and validation of HPLC method for simultaneous estimation of brimonidine tartrate and timolol maleate in bulk and pharmaceutical dosage form. *Journal of chromatography & separation techniques*, 05(03). <https://doi.org/10.4172/2157-7064.1000230>
- Alasmari, B., Alkhenazian, A., & Al-Khenazian, S. (2023). Hyperkalemia due to topical timolol for hemangioma. *JAAD Case Reports*, 39, 53–54. <https://doi.org/10.1016/j.jcdr.2023.07.006>
- Ames, J. A., & Sykes, J. M. (2015). Current trends in medical management of infantile hemangioma. *Current Opinion in Otolaryngology and Head and Neck Surgery*, 23(4), 286–291. <https://doi.org/10.1097/MOO.00000000000000170>
- Eickner, T., Kopp, F., Brietzke, A., Kischkel, S., Oschatz, S., Schmitz, K. P., Guthoff, R., & Grabow, N. (2018). Quantification method for timolol from in viv samples for the development of a new glaucoma drug depot. *Current Directions in Biomedical Engineering*, 4(1), 225–227. <https://doi.org/10.1515/cdbme-2018-0055>
- Gopi, S., Amalraj, A., Sukumaran, N. P., Haponiuk, J. T., & Thomas, S. (2018). Biopolymers and their composites for drug delivery: a brief review. *Macromolecular Symposia*, 380(1), 1–14. <https://doi.org/10.1002/masy.201800114>
- Hassib, S. T., Elkady, E. F., & Sayed, R. M. (2016). Simultaneous determination of timolol maleate in combination with some other anti-glaucoma drugs in rabbit aqueous humor by high performance liquid chromatography-tandem mass spectroscopy. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 1022, 109–117. <https://doi.org/10.1016/j.jchromb.2016.04.012>
- Horwitz, W., & Albert, R. (2006). The Horwitz ratio (horrat): a useful index of method performance with respect to precision. *Journal of AOAC International*, 89(4): 1095–1109. <https://doi.org/10.1093/jaoac/89.4.1095>
- ICH. (1994). ICH - Q2 (R1) Validation of analytical procedure, test and methodology. *International Conference on Harmonisation*. <https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf>
- ICH. (2022). ICH - Q14 On analytical procedure development. *International Conference on Harmonisation*, 31(0), 65. [https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-q14-analytical-procedure-development-step-2b\\_en.pdf](https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-q14-analytical-procedure-development-step-2b_en.pdf)
- Itinteang, T., Withers, A. H. J., Davis, P. F., & Tan, S. T. (2014). Biology of infantile hemangioma. *Frontiers in Surgery*, 1(September), 1–10. <https://doi.org/10.3389/fsurg.2014.00038>
- Kim, K. H., Choi, T. H., Choi, Y., Park, Y. W., Hong, K. Y., Kim, D. Y., Choe, Y. S., Lee, H., Cheon, J. E., Park, J. Bin, Park, K. D., Kang, H. J., Shin, H. Y., & Jeong, J. H. (2017). Comparison of efficacy and safety between propranolol and steroid for infantile hemangioma: A randomized clinical trial. *JAMA Dermatology*, 153(6), 529–536. <https://doi.org/10.1001/jamadermatol.2017.0250>
- M. El-Sayaad, I., M. Shahin, M., A. E. Abdelmonem, S., T. Mahrous Ahmed El-Shamy, A., H. Gouda, S., & Mahran, Z. (2017). Comparative study between systemic and topical propranolol in treatment of infantile hemangiomas. *Al-Azhar Medical Journal*, 46(2), 363–372. <https://doi.org/10.12816/0038259>
- Ma, J., Wang, Y., & Lu, R. (2022). Mechanism and application of chitosan and its derivatives in promoting permeation in transdermal drug delivery systems: a review. *Pharmaceuticals*, 15(4). <https://doi.org/10.3390/ph15040459>
- Mandour, A. A., Nabil, N., Zaazaa, H. E., Ibrahim, M. M., & Ibrahim, M. A. (2023). Two stability indicating chromatographic methods: tlc densitometric versus hplc method for the simultaneous determination of brinzolamide and timolol maleate in ophthalmic formulation in the presence of probable carcinogenic oxidative degradation product of timolol maleate. *Separations*, 10(1), 1–14. <https://doi.org/10.3390/separations10010037>
- Marley, A., & Connolly, D. (2014). Determination of (R)-timolol in (S)-timolol maleate active pharmaceutical ingredient: validation of a new supercritical fluid chromatography

- method with an established normal phase liquid chromatography method. *Journal of Chromatography A*, 1325, 213–220. <https://doi.org/10.1016/j.chroma.2013.12.011>
- Modena, M. M., Rühle, B., Burg, T. P., & Wuttke, S. (2019). Nanoparticle characterization: what to measure? *Advanced Materials*, 31(32), 1–26. <https://doi.org/10.1002/adma.201901556>
- Morsi, N. M., Aboelwafa, A. A., & Dawoud, M. H. S. (2016). Improved bioavailability of timolol maleate via transdermal transfersomal gel: statistical optimization, characterization, and pharmacokinetic assessment. *Journal of Advanced Research*, 7(5), 691–701. <https://doi.org/10.1016/j.jare.2016.07.003>
- Püttgen, K., Lucky, A., Adams, D., Pope, E., McCuaig, C., Powell, J., Feigenbaum, D., Savva, Y., Baselga, E., Holland, K., Drolet, B., Siegel, D., Morel, K. D., Garzon, M. C., Mathes, E., Lauren, C., Nopper, A., Horii, K., Newell, B., Frieden, I. (2016). Topical timolol maleate treatment of infantile hemangiomas. *Pediatrics*, 138(3), e20160355–e20160355. <https://doi.org/10.1542/peds.2016-0355>
- Satterfield, K. R., & Chambers, C. B. (2019). Current treatment and management of infantile hemangiomas. *Survey of Ophthalmology*, 64(5), 608–618. <https://doi.org/10.1016/j.survophthal.2019.02.005>
- Sharma, N., Rao, S. S., & Reddy, A. M. (2012). A novel and rapid validated stability-indicating UPLC method of related substances for dorzolamide hydrochloride and timolol maleate in ophthalmic dosage form. *Journal of Chromatographic Science*, 50(9), 745–755. <https://doi.org/10.1093/chromsci/bms025>
- Shen, S., Wu, Y., Liu, Y., & Wu, D. (2017). High drug-loading nanomedicines: Progress, current status, and prospects. *International Journal of Nanomedicine*, 12, 4085–4109. <https://doi.org/10.2147/IJN.S132780>
- Singh, G., & Dhawan, R. . (2024). Validation of rp-hplc method for the estimation of timolol maleate in fast dissolving tablets. *International Journal For Multidisciplinary Research*, 6(2), 1–6. <https://doi.org/10.36948/ijfmr.2024.v06i02.13862>
- Swamy, N., Reddy, V. K., Thorat, P., & Sengupta, P. (2020). Development and validation of a stability indicating high performance liquid chromatography method for trimethobenzamide. *Brazilian Journal of Pharmaceutical Sciences*, 56(5), 1–11. <https://doi.org/10.1590/s2175-97902019000318817>
- Tambe, S., Das, S. S., Shahane, K., Singh, S. K., Ruokolainen, J., Amin, P., & Kesari, K. K. (2024). First-order derivative spectrophotometric method for simultaneous determination of brinzolamide and timolol maleate in ophthalmic formulation. *Green Analytical Chemistry*, 8(January), 100098. <https://doi.org/10.1016/j.greeac.2024.100098>
- Tiemann, L., & Hein, S. (2020). Infantile hemangioma: A review of current pharmacotherapy treatment and practice pearls. *Journal of Pediatric Pharmacology and Therapeutics*, 25(7), 586–599. <https://doi.org/10.5863/1551-6776-25.7.586>
- Walash, M., & El-Shaheny, R. (2016). Fast separation and quantification of three anti-glaucoma drugs by high-performance liquid chromatography UV detection. *Journal of Food and Drug Analysis*, 24(2), 441–449. <https://doi.org/10.1016/j.jfda.2015.11.006>