IN VITRO SAFETY AND PERMEATION ASSESSMENTS OF LIDOCAINE LOADED IN DISSOLVING MICRONEEDLE PATCH FOR OCULAR ADMINISTRATION

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Cosmetic Science
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การประเมินความปลอดภัยและการซึมผ่านแบบนอกกายของลิโดเคนที่บรรจุในแผ่นไมโครนีเดิลชนิดละลายได้เพื่อการให้ยาทางตา

น.ส.ศิริกุล ธรรมเนียม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	IN VITRO SAFETY AND PERMEATION ASSESSMENTS OF LIDOCAINE LOADED IN DISSOLVING MICRONEEDLE PATCH FOR OCULAR ADMINISTRATION
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ศิริกุล ธรรมเนียม : การประเมินความปลอดภัยและการซึมผ่านแบบนอกกายของลิโดเคนที่บรรจุในแผ่นไมโครนีเดิลชนิดละลายได้เพื่อการให้ยาทางตา. (IN VITRO SAFETY AND PERMEATION ASSESSMENTS OF LIDOCAINE LOADED IN DISSOLVING MICRONEEDLE PATCH FOR OCULAR ADMINISTRATION) อ.ที่ปรึกษาหลัก : อ. ภญ. ดร.จิตติมา ลัคนากุล, อ.ที่ปรึกษาร่วม : ผศ. ภญ. ดร.วิภาพร พนาพิศาล

สิ่งที่ท้าทายในการนำส่งตัวยาทางตาได้รับความสนใจทางเภสัชวิทยา โดยการพยายามปรับปรุงชีวปริมาณการออกฤทธิ์ที่ต่ำให้ดีขึ้น เนื่องจากข้อจำกัดทางสรีระวิทยาในการนำส่งยาทางตา การให้ยาทางตาแบบนอกกายในรูปแบบสารละลายได้รับความนิยมในการรักษาโรคทางตา แต่ตัวยาสามารถลงไปสู่บริเวณที่ต้องการให้ออกฤทธิ์ได้เพียง 5% ดังนั้นลิโดเคนที่บรรจุในแผ่นไมโครนีเดิลชนิดละลายได้จึงเป็นอีกทางเลือกหนึ่งในการให้ยาทางตา จุดประสงค์ในงานวิจัยนี้คือทำการศึกษาความเป็นพิษของลิโดเคนที่บรรจุในแผ่นไมโครนีเดิลชนิดละลายได้และศึกษาการซึมผ่านของตัวยานอกกาย ในแง่ของการศึกษาความเป็นพิษต่อเซลล์ปฐมภูมิของมนุษย์บริเวณกระจกตาชั้นเนื้อเยื่อบุผิว (Human corneal epithelial cells) โดยพิจารณาความเป็นพิษของสารที่มีต่อเซลล์ดังนี้ สารละลายลิโดเคน สารละลายมอลโทส และสารละลายของแผ่นมอลโทสไมโครนีเดิลที่บรรจุตัวยาลิโดเคน และทำการย้อมสีเพื่อดูการมีชีวิตรอดของเซลล์พบว่ามีค่า IC_{50} 11.23, 22592.96 และ 9.50 มิลลิกรัมต่อมิลลิลิตรตามลำดับ ในส่วนของการวิเคราะห์การมีชีวิตรอดของเซลล์ด้วยการย้อมสีด้วย Calcein AM กับ Propidium iodide พบว่าสีเขียวแสดงการรอดชีวิต ในขณะที่สีแดงการไม่รอดชีวิต ในที่นี้ใช้กระบวนการทดสอบการซึมผ่านของตัวยา (Modified-franz diffusion method) ในการศึกษาค่าสัมประสิทธิ์การซึมผ่าน (permeation partition coefficient) ของสารละลายลิโดเคนและแผ่นไมโครนีเดิลที่บรรจุตัวยาลิโดเคน อีกทั้งยังทำการวิเคราะห์ตัวยาที่อยู่ในเนื้อเยื่อตาขาว การทดสอบการซึมผ่านบริเวณเนื้อเยื่อตาขาวเป็นการยืนยันถึงความไม่เป็นพิษต่อเซลล์หรือการตรวจไม่พบตัวยาที่ผ่านชั้นเนื้อเยื่อตาขาว ในส่วนของสารละลายลิโดเคนพบตัวยาอยู่บนเนื้อเยื่อตาขาวสูงถึง 80 เปอร์เซ็นต์หรือมากกว่า 7.19 มิลลิกรัมต่อตารางเซนติเมตรและไม่พบตัวยาบริเวณเนื้อเยื่อตาขาว ในที่นี้ไม่สามารถคำนวณหาค่าสัมประสิทธิ์การซึมผ่านได้เนื่องจากไม่พบปริมาณตัวยาที่อยู่ในบริเวณส่วนที่บรรจุสารละลายชั้นล่าง (receptor chamber) ในขณะที่ลิโดเคนที่บรรจุในแผ่นไมโครนีเดิลชนิดละลายได้พบตัวยาบนเนื้อเยื่อตาขาวในช่วง 2.68 – 3.36 มิลลิกรัมต่อตารางเซนติเมตรและพบตัวยาในชั้นเนื้อเยื่อตาขาวช่วง 4.18 – 4.51 มิลลิกรัมต่อตารางเซนติเมตร สิ่งที่ค้นพบอีกอย่างในงานวิจัยนี้คือปริมาณตัวยาในเนื้อเยื่อตาขาวไม่แปรผันตามความหนาตาขาวของสุกร ทำให้สามารถสรุปได้ว่าลิโดเคนที่บรรจุในแผ่นไมโครนีเดิลชนิดละลายมีความปลอดภัยและประสิทธิภาพในการใช้เป็นยาชา

สาขาวิชา วิทยาศาสตร์เครื่องสำอาง ลายมือชื่อนิสิต .....
ปีการศึกษา 2562 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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KEYWORD: Human corneal epithelial cells, Ocular drug delivery, Dissolving microneedle, Lidocaine

Sirikool Thamnium : IN VITRO SAFETY AND PERMEATION ASSESSMENTS OF LIDOCAINE LOADED IN DISSOLVING MICRONEEDLE PATCH FOR OCULAR ADMINISTRATION . Advisor: Jittima Luckanagul, Ph.D. Co-advisor: Asst. Prof. VIPAPORN PANAPISAL, Ph.D.

The challenge in ocular drug delivery have brought much attention in pharmaceutical research. Efforts have been made to improve the low bioavailability causing by physiological limitations of ophthalmic drug administration. Topical lidocaine solution is a popular choice for ocular treatment, but less than 5% of topically applied dose reached to the target site. Lidocaine loaded in dissolving microneedle patch was considered a good alternative approach to treating eyes. The study aimed to investigate the cytotoxicity of the lidocaine loaded in dissolving microneedle administration against human corneal epithelial cells (HCECs) as well as ex vivo drug target localization with tissue penetration. In the case of cytotoxicity, HCECs cell viability was compared when given with different treatments; i.e., lidocaine solution, maltose solution and dissolved matrix of lidocaine microneedle patch. HCECs were analyzed for percent viability and stained for Dead/Life cell showed IC50 were 11.23, 22592.96 and 9.50 mg/mL, respectively. In Dead/Life cell evaluation using calcein AM and propidium iodide, live cells showed green fluorescent and dead cell showed red fluorescent. Form modified-franz diffusion method used to study permeation partition coefficient of lidocaine solution and lidocaine microneedle patch. The drug retained on the sclera was also determined. Sclera permeation tests were ensured the non-toxic or neglectable amount of drug getting permeated out of porcine sclera. In the case of lidocaine solution, we found that more than 80 Percent or 7.19 mg/cm^2 of the drug retained on the sclera with undetectable amount of lidocaine permeated through the membrane. The permeation coefficient of lidocaine hydrochloride solution could not be determined because of drug absent in the receptor chamber. However, lidocaine loaded in dissolving microneedle patch showed drug was adsorbed in the range 2.68 – 3.36 mg/cm^2 and localized in sclera tissue in the range 4.18 – 4.51 mg/cm^2. Another interesting observation is that the amount of drug adsorbed on the surface of sclera tissue was independent with the thickness of porcine sclera. In the conclusion, lidocaine loaded in dissolving microneedle was shown to be safe and exhibited excellent permeation property or targeted drug delivery.

Field of Study:	Cosmetic Science	Student's Signature .....
Academic Year:	2019	Advisor's Signature .....
		Co-advisor's Signature .....

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List of abbreviations

% w/v	=	Percent weight by volume
µg	=	Microgram
µg/mL	=	Microgram per milliliter
°C	=	Degree Celsius
ANOVA	=	Analysis of variance
CO_2	=	Carbon dioxide
Conc.	=	Concentration
HPLC	=	High performance liquid chromatography
Hr.	=	Hour
Min.	=	Minute
mL	=	Milliliter
mm	=	Millimeter
HCECs	=	Human corneal epithelial cell
S.D.	=	Standard deviation

INTRODUCTION

The ideal properties of local anesthetics include high efficiency, rapid onset of action, and no side effects [1]. Lidocaine hydrochloride can block the nerve system and reduce pain. Lidocaine hydrochloride structure contains 2,6-xylidine linked with diethylglycine by amide bond [2], The molecule contains both hydrophilic and lipophilic groups from its tertiary amine and aromatic ring structure, respectively, as shown in Figure 1.

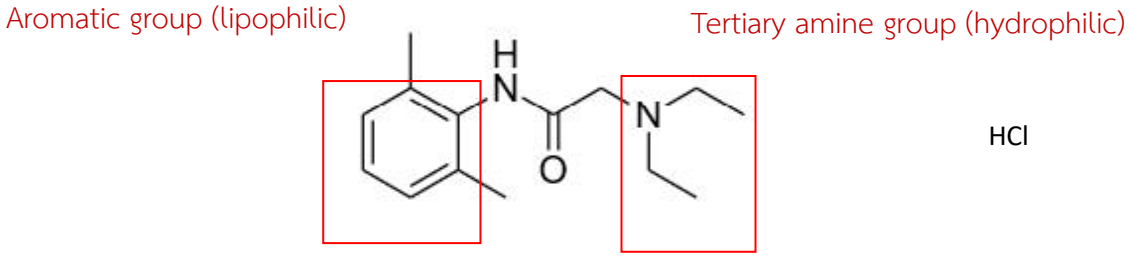

Figure 1: The structure of lidocaine hydrochloride

Normally lidocaine hydrochloride has been used in ophthalmology due to its efficiency and rapid onset of action [3]. The typical administration for the drug is via hypodermic needle, because it is cheap, quick and direct to the target site [4]. There was a report with the injection of 4 mg/kg lidocaine hydrochloride to mouse's vein showed that the drug distribution appeared between 45 – 60 seconds with the onset of action between 10 – 20 minutes. The half-life was 10 minutes. The elimination half-life reported to be 1 – 2 hours [5]. Another report by Ikeda *et. al*. studied the bioavailability of lidocaine injection with repeated injections of the drug aiming for the prolonged effect [6]. For the drug to reach the effective dose delivered, the repeated injections could cause significant pain to the patients. So, the researchers have put efforts in dosage form design of lidocaine for improving drug efficiency in a non-invasive way. Olsen et al. studied the process for reducing pain from injection needle [7]. The researcher tried to decrease the pore of injection needle to micro scale called microneedles. These microneedles were hollow microneedles. Each microneedle was sized with the length of 0.5 - 3 mm. and 0.1 – 0.25 mm diameter [8]. Microneedles were loaded with 2% lidocaine hydrochloride. The result showed that the hollow microneedles caused significant less pain than 26-gauge needle

when tested on dorsum and forearm. Similar to Nicoli et al. study that clearly showed small pore of hollow microneedles caused significantly less pain than hypodermic needle [9]. However, with the hollow microneedles, the post-administration removal of the patch is required. The device retraction may result in tissue injuries with concerns on cross-contamination and infection from repeatedly use of hollow microneedles. Dissolving microneedles has gained more attention due to soluble ability, high dose release, with no residue left after used and sterility of the single-use design. By far, dissolving microneedles have been fabricated from polyvinylpyrrolidone (PVP) [10], 4-n-butylresorcinol [11], and maltose [12]. In this research, maltose was chosen for microneedles fabrication, because it has been used as an excipient in pharmaceutical formulations. Maltose is a crystalline form, quickly dissolve and shows high efficiency as delivery vehicle [12]. Moreover, Convention et al. studied the efficacy of maltose dissolving microneedle and showed that microneedles could poke the skin and were readily soluble [13].

The challenge in ocular drug delivery is the administration of drug to the target site, because of low drug bioavailability and limitation of ocular drug delivery system. The limitations include, for example, barriers of eye, blinking, tear turnover and nasolacrimal drainage [14]. So only less than 5% of applied ocular dose passed through ocular tissues. Currently, conventional eye drops, and injection needles are the methods of administration for ophthalmology purposes [15]. Including the treatments of glaucoma, allergic conjunctivitis, anterior uveitis, cataract, age-related macular degeneration (AMD) and diabetic retinopathy [16].

The eye is very complex in anatomy and physiology [16]. The physiology of human eye is similar to porcine eye in regard to the following parameters; water content, embryonic development, protein sequence, negatively charged and isoelectric point. However, the thickness of porcine sclera is averagely twice of the human's. So, porcine eye is the best model for *in vitro* study. Each porcine eyeball has different thickness depending on the size of porcine. Olsen *et. al.* showed the means limbus thickness of porcine eyeball in 3 groups (small, medium and large) were 0.83 ± 0.2 mm., 0.91 ± 0.17 mm., and 1.12 ± 0.23 mm., respectively[7].

Nevertheless, the means limbus thickness of human eyeball is approximately 0.5 mm. However, Nicoli *et al.* informed that thickness of sclera did not have an effect on permeation of small molecules [9]. Therefore, we exploited porcine eyeballs as a model to monitor localized drug deposition along with ocular adsorption.

Regarding the safety of topical lidocaine delivery on the sclera, the cytotoxicity test is extremely crucial. There was cytotoxicity report of lidocaine to human corneal endothelial cells line that present concentration of lidocaine more than 1.25 g/L could decrease viability of human corneal endothelial cells line [3]. Similarly, Zhou *et al.* also informed that the lidocaine concentrations in range of 0.31 g/L to 20.00 g/L could decrease human corneal stromal cells line and also changed cell morphology [17].

The primary human corneal epithelial cells (HCECs) were chosen as the most biorelevant model for a safety assessment of ophthalmic lidocaine. Moreover, to confirm safety of lidocaine solution and lidocaine in dissolving microneedle patch, sclera permeation test with lidocaine solution and lidocaine in dissolving microneedle patch were ensured the non-toxic or neglectable amount of drug getting permeated to deeper tissue through porcine sclera. The lidocaine loaded in maltose dissolving microneedle patch as a topical anesthesia for ophthalmology surgery has not been reported. Therefore, the aim of the study was to investigate the cytotoxicity on primary human corneal epithelial cells and the sclera permeation of lidocaine loaded in dissolving microneedle patch compared with lidocaine solution.

Objectives

General Objectives

The aim of the study was to investigate the cytotoxicity of lidocaine loaded in dissolving microneedle patch for ocular administration on primary human corneal epithelial cell and the sclera permeation assessment.

Specific Objectives

1. To compare the cytotoxicity of lidocaine solution and lidocaine loaded maltose microneedles after dissolving by cell viability assay and cell morphology.
2. To study the sclera permeation assessment of lidocaine solution and lidocaine loaded microneedle patch.

CHAPTER II

LITERATURE REVIEW

Local Anesthesia

Topical anesthetic agents usually provide shorter duration of administration, comfort, lower cost, and less complication. Topical anesthetics are applied directly to the site of action; such as inside of mouth, nose and throat as well as on the eye.

Lidocaine Hydrochoride

Lidocaine hydrochloride is one of local anesthesia, which blocks the nerve system and reduces pain. Lidocaine hydrochloride structure contains 2,6-xylidine linked with diethylglycine by amide bond [2], The molecule contains both hydrophilic and lipophilic groups from its tertiary amine and aromatic ring structure, respectively, as shown in Figure 2. It could be quickly absorbed in the tissue with rich blood supply and blocked nerve signals. Anesthesiologists have been used lidocaine to reduce pain during surgery.

1.1 General Properties of Lidocaine Hydrochloride

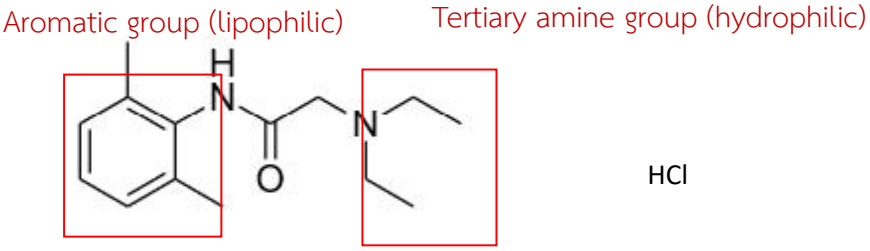

Figure 1: The structure of lidocaine hydrochloride

Chemical name : 2 - (diethylamino) – N - (2,6-dimethylphenyl) acetamide hydrochloride

Molecular formula : $C_{14}H_{22}N_2O$. HCl .H_2O

Molecular weight : 288.81 g/mol

Physicochemical properties : White crystalline powder, Odourless

pH : 4.0 – 5.5 (0.5% solution in H_2O)

pKa : 7.86

Solubility : Very soluble in water, freely soluble in alcohol, soluble in chloroform, and insoluble in ether.

Melting point : Between 74 and 79°C

Storage : Lidocaine hydrochloride should be stored at controlled room temperature and protected from freezing. The condition should avoid excessive heat.

Adverse effects: Allergic reaction of lidocaine hydrochloride was rare [18]. Lidocaine hydrochloride may cause side effects in some people.

1.2 Pharmacology

Lidocaine hydrochloride is the hydrochloride salt from the lidocaine. It interacts with sodium channels (Na+ channels) in nerve cell membrane and the nerve is transiently blocked.

Onset of Action

Drug effect of lidocaine hydrochloride is achieved within 5 min. depending on the area of application.

Hemodynamics

Lidocaine hydrochloride has effects on excitable membranes in the brain and heart. If excessive amount of drug reach systemic circulation rapidly, signs of toxicity will appear.

1.3 Pharmacokinetics

The rate of absorption depended on concentration and total dose administration.

The apparent volume of distribution : 1L/kg

Bioavailability : 35% for oral

: 3% for topical

Protein binding rate : 51%

Onset of action : within 1.5 minutes

Elimination half – life : 1.5 – 2 hours

Duration of action : 10 – 20 minutes (IV)

Duration of action : 0.5 – 3 hours (local)

Pharmacokinetics of lidocaine showed short half-life resulting in very quickly duration of action. To maintain the effective therapeutic concentration, patients were repeatably injected a small dose of lidocaine. Repeated injections could cause pain , inconvenience and lead to side effects [5]. In Table 1 summarized the recommended dosages of lidocaine hydrochloride injection for anesthetic procedures. The volumes and concentration to be used depend on a lot of factors such as type of surgical procedure, depth of anesthesia, duration required and condition of patient.

Table 1: Recommended dosages of lidocaine hydrochloride injection

Procedure	Concentration (%)	Volume (ml)	Total dose (mg)
Percutaneous	0.5 or 1	1 to 60	5 to 300
Intravenous regional	0.5	10 to 60	50 to 300
Brachial	1.5	15 to 20	225 to 300
Dental	2	1 to 5	20 to 100
Intercostal	1	3	30
Paravertebral 1 3 to 5 30 to 50	1	3 to 5	30 to 50
Pudendal (each side) 1 10 100	1	10	100
Obstetrical analgesia (each side)	1	10	100
Cervical (stellate ganglion)	1	5	50
Lumbar	1	5 to 10	50 to 100
Thoracic	1	20 to 30	200 to 300
Analgesia	1	25 to 30	250 to 300
Anesthesia	1.5	15 to 20	225 to 300
	2	10 to 15	200 to 300
Obstetrical analgesia	1	20 to 30	200 to 300
Surgical anesthesia	1.5	15 to 20	225 to 300

Transdermal drug delivery system

Table 2: Comparison between topical cream, transdermal patch, hypodermic needle, and microneedle drug delivery systems.[19]

	Topical cream	**Transdermal patch**	**Hypodermic needle**	**Microneedle**
Description	Emulsion Emulgel Cream Ointment	Adhesive patch to be placed on the skin	Fine, hollow tube having a sharp tip with small opening at the end	Micron size needles are aligned on the surface of a small patch
Onset of action	Slow	Slow	Faster	Faster
Pain	Painless	Painless	Painful	Painless
Bioavailability	Poor	Insufficient	Sufficient	Sufficient
Patient compliance	Less	Better	Less	Better
Self-administration	Possible	Possible	Not possible	Possible
Mechanism of drug delivery	Permeation through skin pores.	Drug has to cross stratum corneum barrier, thus poor diffusion of large molecules	Drug placed directly in the dermis	Bypass stratum corneum and drug placed directly into epidermis or dermis hence enhanced permeability

Table 2 above showed microneedle is the best transdermal drug delivery. Microneedle patch could completely deliver drug without causing pain.

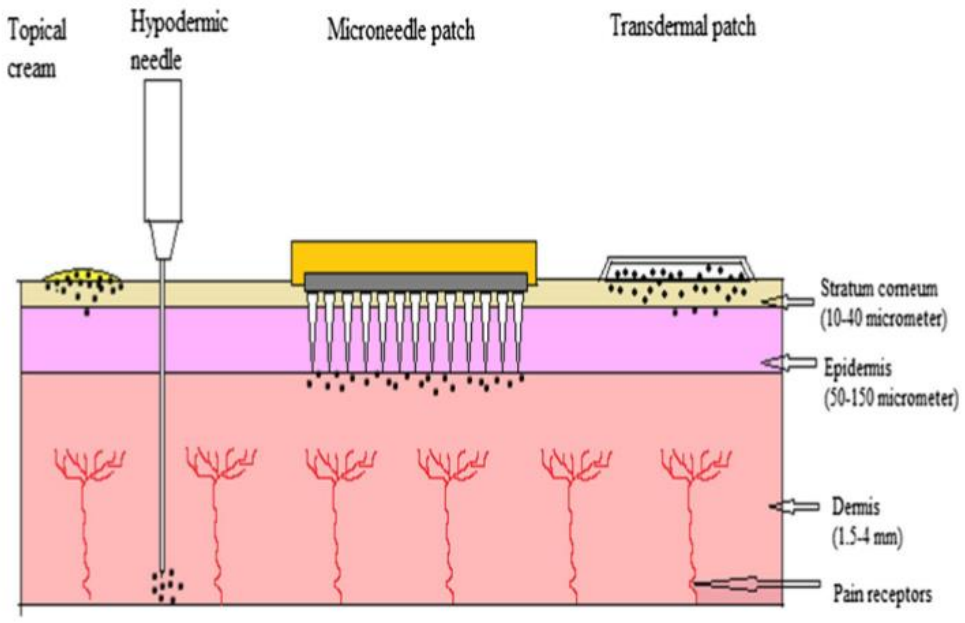

Figure 2: Comparison between topical cream, Transdermal patch, hypodermic needle, and microneedle drug delivery systems.[19]

Microneedles

Hypodermic needles have been widely used methods for transdermal administration of the drug. However, needles are less accepted by patients due to painful. Olsen et al. [7] studied the process for reducing painful from needle injection. They tried to decrease the pore of injection needle to micro scale. It was called microneedles. Microneedles were developed for drug delivery system, which was liked traditional needle [20]. However, microneedles created micron size pathways that lead drugs directly to the target site. The microneedles was a novel transdermal permeation technique, that was painless and provided proper dose of drugs [21]. Microneedles considered as microscopic applicators used to deliver the drug. Microneedles have many advantages such as, needle phobia prevention, painless and rapid onset of action.

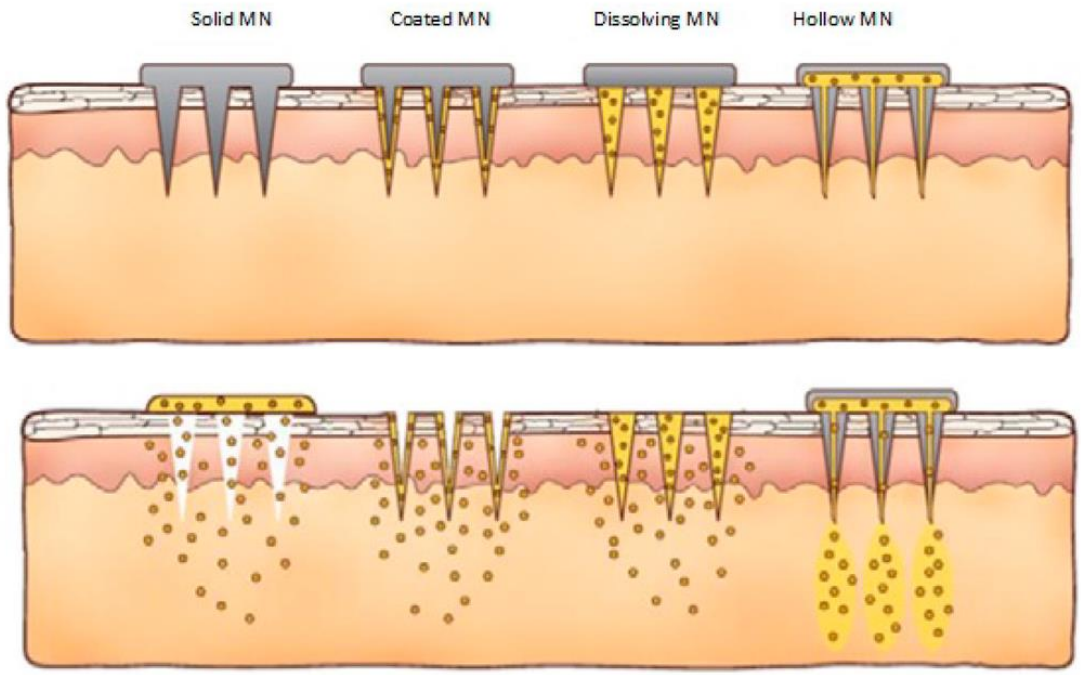

Figure 3: Different types of microneedles: solid, coated, dissolving and hollow [22]

Type of microneedle

Microneedles, a transdermal drug delivery system, drugs were encapsulated in the microneedles. Types of microneedles are solid, coated, hollow and dissolving microneedles. Each type of microneedle has its own drug delivery mechanism to the target site.

Solid microneedle

Solid microneedles are used to pre-treatment the target by forming pores. Micron-sized channels are created and directly lets drugs to enter the target.

Coated microneedles

Microneedles are coated with drug solution or drug dispersion and appears as thin layer film [22]. The quantities of drug are depended on the coating thickness and the size of microneedles.

Hollow microneedles

Hollow microneedles have holes in the center of each needle. Drug dispersion or solution can be filled and transfer directly to the skin.

Dissolving microneedles (DMN)

Dissolving microneedles (DMN) are fabricated with biodegradable polymers and drug is encapsulated in the polymer. DMN are designed to dissolve after skin penetration and then the encapsulated drug releases into the target site. DMN does not need to be removed out after insertion. Different kinds of sugars are mostly used as the matrix. Main materials composed of sugars (Maltose) [23], sucrose or a PVA/sucrose [24] , 4-n-Butylresorcinol [11], hyaluronan [25] and carboxy methyl cellulose [26] etc.

Drug delivery mechanisms of microneedles

Several mechanisms are involved in drug delivery of microneedles. Firstly, microneedles poke the target site and create pores. This process creates a direct transport pathway for drug to travel into the target site. Secondly, drugs can be coated on the microneedles surface and later dissolves after inserted. Thirdly, microneedles are dipped into the solution containing drug and scrape the needles on the target site. Finally, microneedles are fabricated with biodegradable polymer, where drug is incorporated into the polymer. Drug will be delivered after poking and dissolving.

Applications of microneedles

The microneedles have many advantages such as oligonucleotide delivery, Vaccine therapy, Peptide delivery, Hormone delivery, Cosmetics, Lidocaine delivery, Pain therapy, Ocular delivery, and Cancer therapy.

Eye anatomy

Eye is the specific organ. It separates in two segments: anterior segment and posterior segment. Anterior segment occupies around one-third of eye including pupil, cornea, iris, lens, and aqueous humor. Posterior segment composes of vitreous humor, retina, macula, choroid and optic nerve (Figure 5). The eye is very complex in anatomy and physiology [16]. The physiology of human eye is similar to porcine eye in regard to the following parameters; water content, embryonic development, protein sequence, negatively charged and isoelectric point. However, the thickness of porcine sclera is averagely twice of the human's. So, porcine eye is the best model for *in vitro* study. Each porcine eyeball has different thickness depending on the size of porcine. Olsen *et. al.* [7] showed the means limbus thickness of porcine eyeball in 3 groups (small,

medium and large) were 0.83 ± 0.2 mm., 0.91 ± 0.17 mm., and 1.12 ± 0.23 mm., respectively. Nevertheless, the means limbus thickness of human eyeball is approximately 0.5 mm. However, Nicoli *et al.* informed that thickness of sclera did not have an effect on permeation of small molecules [9]. Therefore, we exploited porcine eyeballs as a model for the human eye to study localized drug deposition along with ocular adsorption.

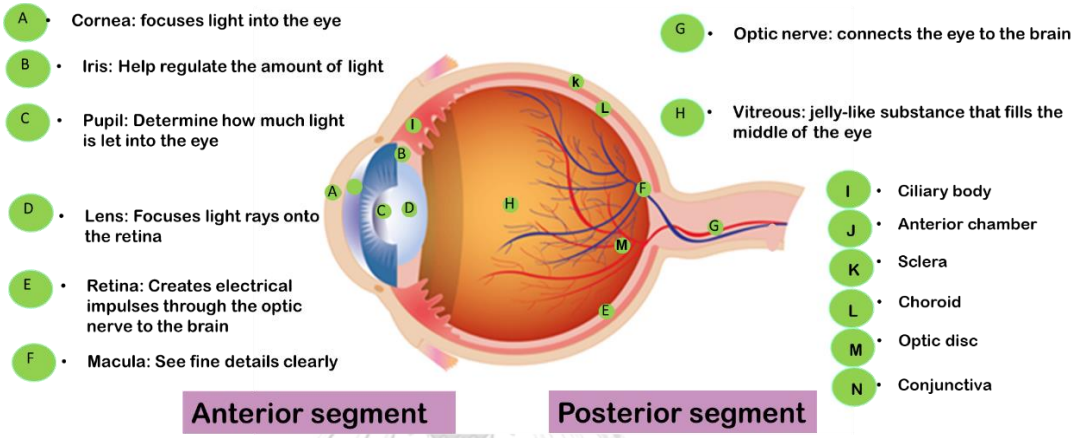

Figure 4: Eye anatomy

Cornea

Cornea is very important mechanical and chemical barrier. It can limit the substances getting into the eye. The human cornea consists of five layers: corneal epithelium, basement membrane, Bowman's layer, stroma, Descemet's membrane and endothelium.

Conjunctiva

Conjunctiva is a mucous membrane that consists of epithelium, adenoid and fibrous. The anterior segment of sclera is covered by bulbar conjunctiva. The conjunctiva epithelium plays an important role as a protective barrier on the ocular surface.

Blood-aqueous barrier

The blood-aqueous barrier locates at the anterior part of the eye.

Blood-retinal barrier

The blood-retinal barrier locates at the posterior part of the eye.

Ocular drug delivery system

The challenge in ocular drug delivery is the administration of drug to the target site, because of low drug bioavailability and limitations of ocular drug delivery system. The limitations include, for example, barriers of eye, blinking, tear turnover and nasolacrimal drainage [14]. So only less than 5% of applied ocular dose of drug passed through ocular tissues [27]. Topical drugs are the most favorite non-invasive route of drug administration to treat infections. Currently, conventional eye drops and injection needles are the methods of administration widely implemented in ophthalmology purpose [15]. They are used as the treatment of diseases located at either anterior segment or posterior segment such as glaucoma, allergic conjunctivitis, anterior uveitis, cataract, age-related macular degeneration (AMD) and diabetic retinopathy [16].

Most ocular drugs were applied into eye but only a small fraction of drug was absorbed into the eye. Drug could enter to the anterior segment mainly through the cornea, whereas hydrophilic molecules were absorbed through the conjunctiva and sclera.

Ocular drug delivery and ocular bioavailability could be improved by drug delivery systems. The systems have been developed in many formulations such as gel, ointments, emulsions, suspensions and microneedles.

Delivery routes

Anatomical and physiological barriers obstruct drug from getting to the posterior segment of eye especially choroid and retina [27]. Topical drugs were absorbed by corneal route (cornea → aqueous humor → intraocular tissue) or non-corneal route (conjunctiva → sclera → choroid). The route depended on the corneal permeability of drug molecule.

Cell culture models of the ocular barriers

Ocular tissues in the anterior and posterior parts of the eye are essential for normal visual function. The barriers of ocular are cornea, conjunctiva, blood-retinal barrier. In this research study, the drug's transport into ocular tissues was investigated along with pathological ocular conditions and toxicological screening of lidocaine loaded microneedles using *in vitro* tests as an alternative to in vivo toxicity tests.

Animal experimentation is an essential part of the research and development of ocular drug delivery systems. The rabbit is the most commonly used animal model, but the larger animals are very rare to use, and the small eye size of mice and rat limits their value in ocular studies.

Cell culture and cell analysis

Normally immortalized cell lines were used to replace primary cells [28].
Immortalized cell lines were used to study biological processes. However, the results of immortalized cell lines were not accurate when compared with those from primary cells.

Table 3: The comparison of primary cells and cell lines.

Comparisons	Primary Cells	Cell Lines
1. Biologically relevant tools for studying human and animal biology.	More suitable	Less suitable
2. Lifespan	Limited (Can passage about 5-6 depend on type of cells)	Infinite (Can continually passage)
3. Genotypic Characteristics	Heterogeneous	Homogeneous

4. Phenotypic Characteristics	Heterogeneous	Homogeneous
5. True characteristics of the original tissue	Do not lost the true characteristics of the original tissue	Lost the true characteristics of the original tissue
6. Tissue characteristics	Resembles	Loses
7. Convenience	No	Yes

In vitro permeation study

Modified franz diffusion cells were used to evaluate drug permeation. The cumulative permeation profiles of microneedle treated and untreated were compared [21].Nicoli et al. found permeation assessment of porcine scleras and human scleras were very similar toward small molecule and high molecular weight compound, eventhough a statistically significant difference was determined for the tissue thicknesses. This observation was rationalized that human sclera and porcine sclera had a similar histology and collagen bundle organization[9].

Olsen et al. showed similarities between human eyes and porcine eyes in terms of anatomy of intraocular and thickness of sclera [7, 29]. These similarities are absent in other animal model such as cow and rabbit. However, porcine sclera is easily obtained in large amounts from slaughterhouses.

CHAPTER III

MATERIALS AND METHODS

Materials

- **Chemical for hplc analysis**
- Lidocaine Hydrochloride BP (Batch no. 2252, Strongchemicals)
- Acetonitrile HPLC grade (Lot no. LC1005-G4L, RCI Labscan, V.S. chem house, Bangkok, Thailand)
- Di – Sodium – hydrogen phosphate dodecahydrate (Lot no. 0B215170F, Cario erba Reagenti SpA)
- Dimethyl sulfoxide Analytical reagent grade (Lot no. 1690734, Fisher BioReagents , Fisher Scientific)
- Ethanol (Lot no. AR1069-G2. 5L, RCI Labscan, V. S. chem house, Bangkok, Thailand)
- Methanol HPLC grade (Lot no. Q9AG1H, Honeywell Burdick & Jackson, Seoul, Korea)
- Potassium chloride (Lot no. TA822436 037, Merck KGaA, 64271 Darmstadt, Germany)
- Potassium dihydrogen orthophosphate (Lot no. AF501339, Ajax Finechem, Taren Point, Australia)
- Sodium dihydrogen phosphate (Lot no. 480087, Cario erba Reagenti SpA)
- Sodium hydroxide (Lot no. B0998898 406, Merck KGaA, 64271 Darmstadt, Germany)

Chemical for cell culture

- Primary human corneal epithelial cell; Normal, Human ATCC ® PCS-700-010 ™ (Lot no. 63901614)

- Apo-Transferrin PCS-999-005 (Lot no. 3004163)
- CE Growth Factor PCS 999-044 (Lot no. 3004199)
- Corneal epithelial cell basal medium ATC.PCS-700-030 (Lot no. 80829222)
- Corneal epithelial cell growth kit PCS-700-040 (Lot no. 80519100)
- Epinephrine PCS-999-008 (Lot no. 3004187)
- Extract-P PCS-999-009 (Lot no. 3004192)
- Fetal Bovine Serum Standard (FBS, Qualified) (Lot no. 42G7283K, gibco, Life Technologies Corporation, N.Y., U.S.A.)
- Hydrocortisone PCS-999-013 (Lot no. 3004162)
- L-glutamine PCS 999-015 (Lot no. 3004191)
- Penicillin Streptomycin (Pen Strep) (Lot no. 1970743, gibco, Life Technologies Corporation, N.Y., U.S.A.)
- rh insulin PCS-999-022 (Lot no. 3004184)
- Dulbecco's Phosphate Buffered Saline, D-PBS, 1X Without Calcium, Without Magnesium Sterile-filtered ATCC 30-2200 (Lot no. 80608180)
- Trypsin – EDTA solution for Primary Cells (ATCC PCS-999-003) containing 0.05% Trypsin and 0.02% EDTA (Lot no. 80829333)
- Trypsin Neutralizing Solution, ATCC PCS-999-004 (Lot no. 80901180)

Membrane:

- Filtration membranes Part No: N14745 Membrane Disc Nylon 47 mm (Lot no. N06081807021)
- Porcine eyes were obtained from slaughterhouse
- Preclean 13 mm syringe filter, nylon membrane, 0.45 um, white (Lot no. 12731000, Anpel labolatory technologies, Shianghai)

Equipment and special tools:

- Analytical balance (XS 105, Mettler Toledo, Switzerland)
- Auto clave (Harayama, HVE-25/50)
- Clearcut slit knife (Lot no. 981142M, Alcon, Novaris company)
- High Performance Liquid Chromatography (LC 10, Shimadzu, Japan)
- HPLC column (ZORBAX C18, Agilent Technology)
- Microplate reader, cario star, BMG Labtech , Germany
- Light microscope attached with camera (Eclipse, Nikon, Japan)
- pH – Meter (SevenCompact™ S220, Mettle Toledo, Switzerland)
- Vernier caliper (Fowler Sylvac Ultra-Cal Mark IV IP65 Electronic Caliper, Switzerland)

Method

1. High - performance liquid chromatography conditions (HPLC conditions)

The lidocaine hydrochloride was analyzed by using HPLC with reversed phase C18 column 150 ×4.6 mm (ZORBAX C18) and UV detection at 254 nm. The mobile phase was acetonitrile and buffer solution A pH 3.4 containing 930 mL. ultrapure water and 50 mL. acetic acid. The flow rate of 0.8 mL/min and 20 µL injection volume were used. Lidocaine hydrochloride was used as the standard solution to construct the calibrate curve in the range of 2.5 - 20 µg/ mL of lidocaine hydrochloride. HPLC method was verified according to the USP monograph [30] with parameters including specificity, accuracy, precision, limit of detection, limit of quantitation and linearity. Each study was done in triplicate.

Specificity

The peaks of other components must not interfere the peak of lidocaine hydrochloride. The validation was compared among the chromatograms of lidocaine hydrochloride solution, lidocaine in phosphate buffer saline and lidocaine dissolving microneedles respectively.

Accuracy

The accuracy was determined by analyzing each concentration of lidocaine hydrochloride solution. Percent recovery of lidocaine hydrochloride for each concentration was calculated using the constructed standard curve.

Precision

The precision was performed as within run precision and between run precision. The precision was determined by analyzing each concentration of lidocaine hydrochloride solution. Percent recovery of each lidocaine hydrochloride concentration was calculated using the constructed standard curve.

Limit of detection (LOD)

The lower limit of detection of lidocaine hydrochloride solution was defined at the signal - to - noise ratio of 3:1 [31] by injecting the low concentration of lidocaine

hydrochloride solution 10 times and the percent coefficient of variation (% CV) was calculated to confirm the limit of detection at this concentration.

Limit of quantitation (LOQ)

The lower limit of quantitation of lidocaine hydrochloride solution was defined at the signal - to - noise ratio of 10:1 [31] by injecting the low concentration of lidocaine hydrochloride solution 10 times and the percent coefficient of variation (% CV) was calculated to confirm the limit of quantitation at this concentration.

Linearity

The linearity of series lidocaine hydrochloride solutions with 5 concentrations was prepared and injected to obtain the corresponding integrated peak area. The calibration curve was plotted between lidocaine concentrations and peak areas. Linear regression was fitted to obtain the coefficient of determination (r^2) and percent precision.

2. Preparation of ocular tissue

Ocular tissues were prepared from porcine eyeballs. The porcine eyeballs were kindly provided by a butcher shop in Bangkok, Thailand. Eyeballs were used within 24 hrs. after procurance or frozen at -20°C until use. After thawing, the tissue was removed from the eye bulb, and the anterior segment of the eye was circumferentially cut behind the limbus. The eye was then cut into two halves; the vitreous humor and lens were removed, and the anterior sclera was isolated, soaked in phosphate buffer saline (at pH 7.4) for 60 min and stored in a sealed container at -20°C until use. The frozen tissues were used within 3 months.

3. Fabrication of microneedles

Microneedles were fabricated with maltose and lidocaine hydrochloride in 2 % W/V. Explain briefly how microneedles were fabricated: 500 µL solution was added into a mold, vacuumed 15 minutes for twice, and heated at 65 °C for 24 hours, after that microneedles were removed from the mold and kept them in desiccator.
Microneedles were fabricated as described in Apiwat Jaroonpol's master thesis {Jaroonpol, 2017 #64} entitled of "Fabrication and characterization of maltose microneedles for transdermal drug delivery".

4. Characterization of microneedle geometry

Microneedles were imaged by Dino-Lite USB microscope camera and analyzed by DinoCapture 2.0: Microscope Imaging Software to determine the height, width and tip thickness.

5. Drug content in dissolving microneedle patch

The lidocaine content in dissolving microneedle patch was quantified with the area of 1 cm^2. The patch was placed in phosphate buffer saline pH 7.4 and sonicated in a sonicator bath for a period of 10 minutes. The lidocaine content was determined using HPLC.

6. Cell culture study

Primary human corneal epithelial cell; Normal, Human (ATCC ® PCS-700-010 ™) was purchased from American Type Culture Collection (ATCC). Cells were plated in 25 cm^2 culture flask with corneal epithelial cell basal medium ATC.PCS-700-030 (Lot no. 80829222) and corneal epithelial cell growth kit PCS-700-040 (Lot no. 80519100) at a 37°C humidified 5% CO_2 atmosphere. The culture medium was changed every two days and typsinized with trypsin – EDTA solution for Primary Cells (ATCC PCS-999-003) containing 0.05% Trypsin and 0.02% EDTA (Lot no. 80829333). Cells were grown to confluence and transferred to 96-well plates kept for 24 hrs.

7. Cell viability study

The sample was dissolved in cell culture medium at various concentrations and was filtered using 0.22 µm filter. Then cells were grown onto 96-well tissue culture plate at a density of 10,000 cells per well for 24 hrs. prior to use. Cell culture medium was removed and replaced with 100 µL of the basal and 50 µL of the sample. Then incubated for 24 hrs. After that, the monolayer cells were washed with phosphate buffer saline (at pH 7.4) and replaced with basal medium (resazurin reagent at 0.01 mg/mL). The cells were further incubated for 4 hrs. and measured for fluorescence intensity using a microplate reader at the excitation wavelength 560 nm. and emission wavelength 590 nm. The results of treated cells were compared with the control (untreated cells).

8. Dead stain/ Life stain

Viability of human corneal epithelial cells was analyzed by histological staining to confirm viability of cells. Cells were grown until confluence more than 80% of the area after which the cells were harvested. The cell suspension was seeded onto 96-well tissue culture plate at the density of 10,000 cells per well to ensure the cell coverage. After that, the basal media was removed and replaced with the treatment sample with different pre-defined concentrations in culture media then incubated at 37°C 5% CO_2 for 24 hrs. The treatment was removed, then washed with the phosphate buffer saline pH 7.4, The cells were stained with calcein AM (AM stand for acetoxymethyl), and then fixed with glutaraldehyde. After that the cells were stained with propidium iodide. The resulting human corneal epithelial cells were analyzed by invert fluorescence microscope for dead/ life cells.

9. Permeation through porcine sclera

9.1 Permeation through porcine sclera by using lidocaine solution

In vitro permeation studies were conducted using a modified Franz diffusion cell with porcine eyeballs. The porcine eyeballs were kindly provided by a butcher shop in Bangkok, Thailand. Porcine eyes were cleaned with phosphate buffer saline (pH 7.4) and extracted the sclera according to this criteria: limbus thicknesses of 0.83 ± 0.2, 0.91 ± 0.17 mm., and 1.12 ± 0.23 mm. and extracted space from limbus were 5, 5, and 6 mm., respectively. [7] The sclera was carefully mounted onto the donor compartment of the diffusion cell. The receptor chamber was filled with phosphate buffer saline (pH 7.4), under sink conditions, and maintained 37°C ± 1°C. The studies were conducted using a group of six cells [9] with 1000 µL was applied to each cell. At predetermined time points, 1 mL solution was withdrawn from the receptor compartment, and the withdrawn volume was replaced by an equal volume of 10mM phosphate buffered saline at 5 minutes, 10 minutes, 30 minutes and every hr. until 4 hrs. The solution was analyzed using high-performance liquid chromatography (HPLC) [11]. The cumulative amount of lidocaine permeated per unit area was plotted as a function of time, and the steady-state permeation rate (Jss) was calculated from the slope. Drug remaining in the sclera was analyzed. At the end of the permeation study, the remained formulation on the porcine sclera was collected

and the donor compartment was rinsed with PBS pH 7.4. Each sample was analyzed for lidocaine hydrochloride solution using HPLC. The permeation area of porcine sclera was cut to small pieces and extracted with methanol using homogenizer at 8000 rpm for 30 minutes. The methanolic extract was evaporated under nitrogen purge until dryness. After drying, Lidocaine hydrochloride residual was reconstituted with methanol and quantified using HPLC. The experiment was performed in 6 replicates.

9.2 Permeation through porcine sclera by using lidocaine loaded in dissolving microneedle patch

After the porcine eyeballs were prepared as described in criteria 9.1., the porcine sclera was clamped between donor and receptor compartments. PBS pH 7.4 was used as receptor fluid. The porcine sclera and receptor fluid were equilibrated to 37 ± 1°C for 60 minutes. After equilibration, lidocaine loaded in dissolving microneedle patch was forced 5 minutes until all tips disappeared to each donor compartment. The receptor fluid was magnetically stirred at 400 rpm and maintained at 37 ± 1°C throughout the experiment. Samples were withdrawn at 5 minutes, 10 minutes, 30 minutes and every hr. until 4 hrs. and an equal of fresh PBS was immediately added to the receptor compartment to keep the constant volume. Lidocaine hydrochloride concentration of each sample was analyzed using HPLC. The experiment was performed in 6 replicates. At the end of the permeation study, the remained formulation on the porcine sclera was collected and the donor compartment was rinsed with PBS pH 7.4. Each sample was analyzed for lidocaine hydrochloride solution using HPLC. The permeation area of porcine sclera was cut to small pieces and extracted will methanol by homogenizer at 8000 rpm for 30 minutes. The methanolic extract was evaporated under nitrogen purge until dryness. After drying, Lidocaine hydrochloride residual was reconstituted with methanol and quantified using HPLC. The experiment was performed in 6 replicates.

10. Statistical analysis

The data were expressed as a mean value ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) with multiple comparisons between deposition data using SPSS software. A p-value < 0.05 was considered statistically significant.

CHAPTER IV

RESULTS AND DISCUSSION

1. High - performance liquid chromatography methods

The HPLC analytical method was verified and complied under specificity, linearity, precision, and accuracy according to USP monograph [30]. The verification results were given in APPENDIX A. The calibration curve of lidocaine hydrochloride showed in Figure 5. This analytical assay showed good precision and good accuracy. According to described assay conditions, the peaks of lidocaine and methylene blue were not overlapped or no interference from methylene blue.

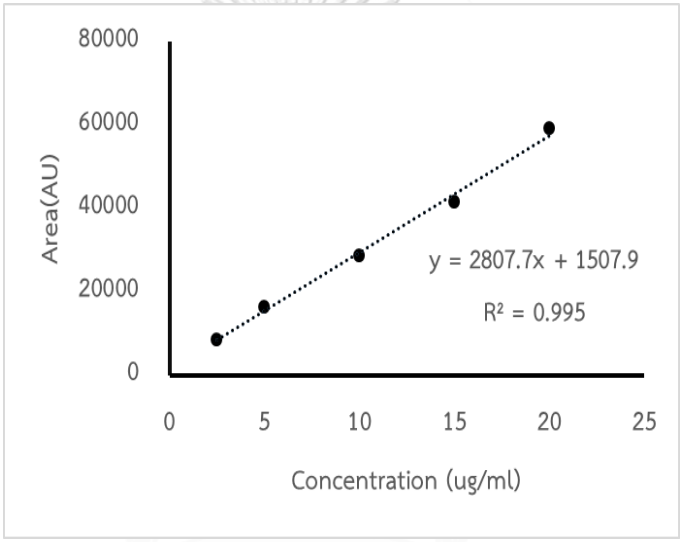

Figure 5: The calibration curve properties for analytical methods of lidocaine hydrochloride in the range of 2.5 – 20 µg/mL.

Specificity

Specificity study aimed to ensure that there was no interference from other components in the sample. The HPLC chromatograms of lidocaine hydrochloride solution, lidocaine on phosphate buffer saline and lidocaine dissolving microneedle are presented in Figure 26 - 30 (Appendix A)

Accuracy

Accuracy criteria presented as average recovery percentage and the acceptance criteria was set at 100 ± 20% for each concentration. The percent recoveries from the accuracy study are presented in Table 5 and falled within the range of 86.17 – 114.05%.

Precision

Precision criteria presented as instrument precision (RSD) of ≤5% and intra-assay precision of ≤15%. The concentrations of 2,5, 5, 10, 15, 20 µg/mL for lidocaine hydrochloride were chosen for both precision and accuracy studied. The results of precision and accuracy study are presented in Table 5.

Limit of detection (LOD)

The LOD is the lowest concentration of the sample that can be detected, but not necessarily quantitated. The LOD for lidocaine hydrochloride was found to be 1 µg/mL.

Limit of quantitation (LOQ)

The LOQ is the lowest concentration of the sample that can be detected with an acceptable level of accuracy and precision. The LOQ for lidocaine hydrochloride was found to be 2.5 µg/mL.

Linearity

Linear relationship was showed between lidocaine hydrochloride concentrations and integrated areas as presented in Table 4 with the coefficient of determination (R^2) > 0.9 within the concentration range of 2.5 – 20 µg/mL. The results indicated that the method could be used in this study to quantify lidocaine hydrochloride.

Table 4: Precision and accuracy of lidocaine hydrochloride

Drug	Concentration range (µg/ml)	Slope	y-Intercept	Coefficient of determination (R^2)	LOD (µg/ml)	LOQ (µg/ml)
Lidocaine hydrochloride	2.5 - 20	2807.7	1507.9	0.995	1	2.5

Table 5: The linearity data of lidocaine hydrochloride.

Data	Lidocaine hydrochloride
Precision (RSD, %)	
Within day precision	4.04
Between day precision	3.86 – 10.95
Accuracy	86.17 – 114.05

2. Characterization of microneedle geometry

Microneedles were imaged by Dino-Lite USB microscope camera and analyzed by DinoCapture 2.0: Microscope Imaging Software to determine the height, width and tip thickness. From the Figure 6 showed the image of an intact microneedle. Most microneedles were 0.495 mm. long, 0.234 mm. wide, and 0.091 mm. tip thickness. The total number of tips were 100 tips. Microneedles tip with triangular shape could create narrow transport pathway when they penetrated into the skin and readily dissolved. From the Figure 7 shows some damage microneedles which may cause by removing microneedle form the mold. Only the intact microneedles were used for *in vitro* permeation study. To assure that the drug did not pass through tissue sclera by comparing the length of microneedles with the thickness of porcine sclera. Regarding to comparison data, microneedles potentially delivered drug molecule through the human sclera because the thickness of human sclera near limbus appeared to be around 0.54 ± 0.14 mm.

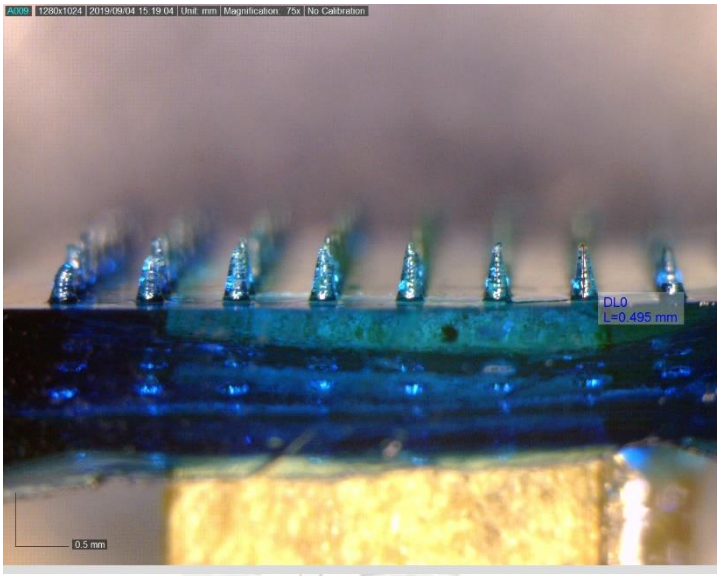

Figure 6 Representative image of an intact microneedles

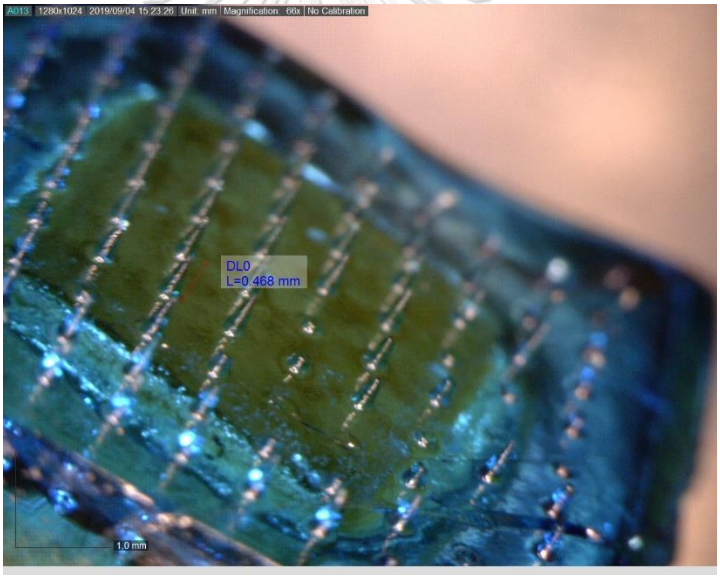

Figure 7: Representative image of a damage microneedles

3. Analysis of drug content in dissolving microneedle

The lidocaine content in a dissolving microneedle patch was quantified in triplicate. Average lidocaine content was 19.89 ± 0.15 mg lidocaine per patch. However, for *in vitro* permeation study theoretical lidocaine amount (20 mg) was used. Lidocaine loaded dissolving microneedle patch could not be weighted because the lidocaine microneedles started to melt when getting into contact with ambient air.

4. Cell viability study

In order to evaluate any potential cytotoxicity of lidocaine hydrochloride solution on HCECs cells, the cells were treated with different concentrations of lidocaine hydrochloride solution ranging from 0.781 mg/mL to 100 mg/mL for 24 hrs. The effect on cell viability was measured using the Resazurin conversion assay. *In vitro* cytotoxicity results are presented in Figure 8. Results showed that, at 24 hrs., the percent viability was below 20% for the tested concentrations above 12.5 mg/mL. On the other hand, the lower concentrations of lidocaine solutions (below 12.5 mg/mL) were considered safe. The half maximal inhibitory concentration (IC_{50}) is a quantitative measurement showing drug effect on the cells. Result showed IC_{50} of lidocaine is 11.23 mg/mL. The high concentration of lidocaine could cause dramatically decrease in viability of cell.

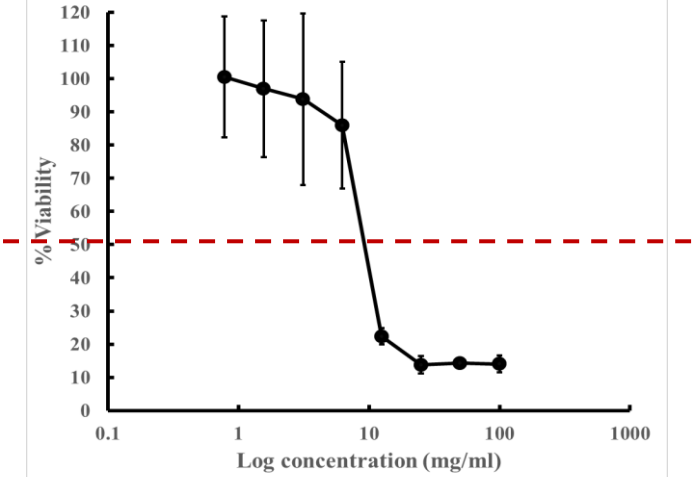

Figure 8: Represent the % viability of lidocaine solution

In order to evaluate any potential cytotoxicity of maltose solution on HCECs cells, the cells were treated with different concentrations of maltose solution ranging from 0.781 mg/mL to 100 mg/mL for 24 hrs. The effect on cell viability was measured using the Resazurin conversion assay. *In vitro* cytotoxicity results are presented in Figure 9. Results showed that, at 24 hrs., the percent viability were above 50% for all

tested concentrations. On the other word, maltose solution was considered safe in the concentrations ranging from 0.781 mg/mL to 100 mg/mL. Result showed IC_{50} of maltose is 22592.96 mg/mL.

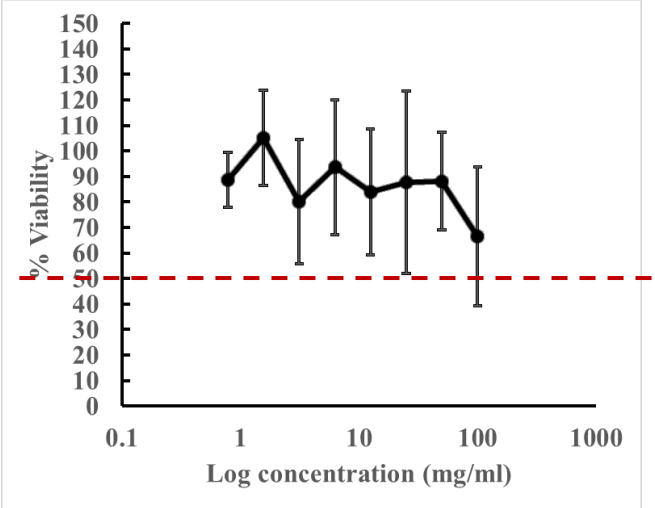

Figure 9: Represent the %viability of maltose solution

In order to evaluate any potential cytotoxicity of lidocaine loaded dissolving microneedles patch solution on HCECs cells, the cells were treated with different concentrations of lidocaine loaded dissolving microneedles patch solution ranging of lidocaine concentration from 2.371 mg/mL to 76.07 mg/mL along with maltose concentration from 23.93 mg/mL to 97.629 mg/mL for 24 hrs. The effect on cell viability was measured using the Resazurin conversion assay. *In vitro* cytotoxicity results are presented in Figure 10. Results showed that, at 24 hrs., the percent viability were below 50% for the tested concentration of lidocaine concentration in range 19.01 – 76.07 mg/mL along with maltose concentration in range 80.99 – 23.93 mg/mL. In conclusion, the toxicity results of dissolved matrix of lidocaine loaded in dissolving microneedle patch were aligned with lidocaine solution results where there was no significant different between lidocaine in the form of microneedles and lidocaine solution.

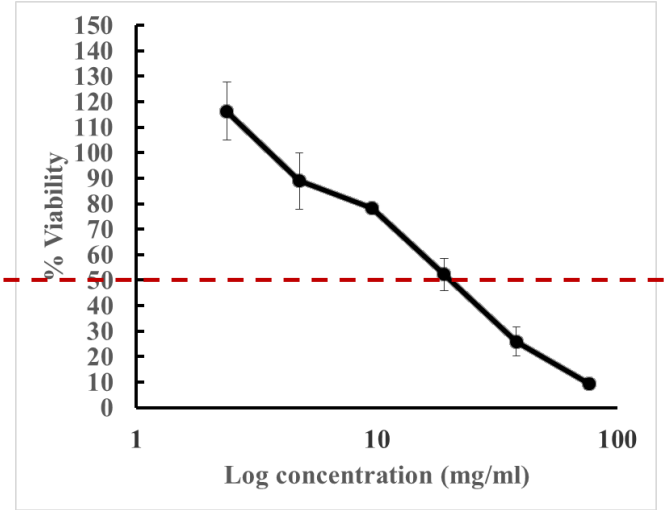

Figure 10: Represent the %viability of lidocaine loaded dissolving microneedles patch solution

5. Morphology

The transmission light microscope picture showed regular cell and irregular morphology of HCECs at 4X objective lens and 10X objective lens. At the high concentration lidocaine solution and lidocaine mixed with maltose, the cell morphology change was observed. On the other hand, HCECs cells that were treated with high concentration of maltose showed unchanged morphology.

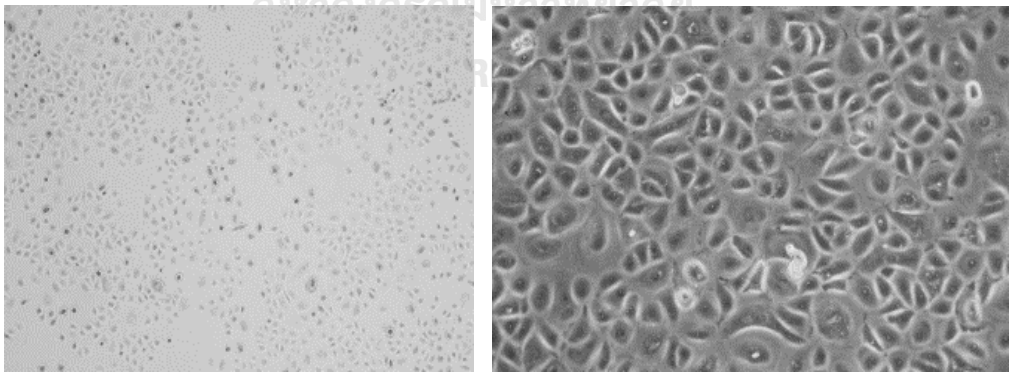

Figure 11: The transmission light microscope picture showed regular cell morphology of HCECs at 4X objective lens (left panel) and 10X objective lens (right panel).

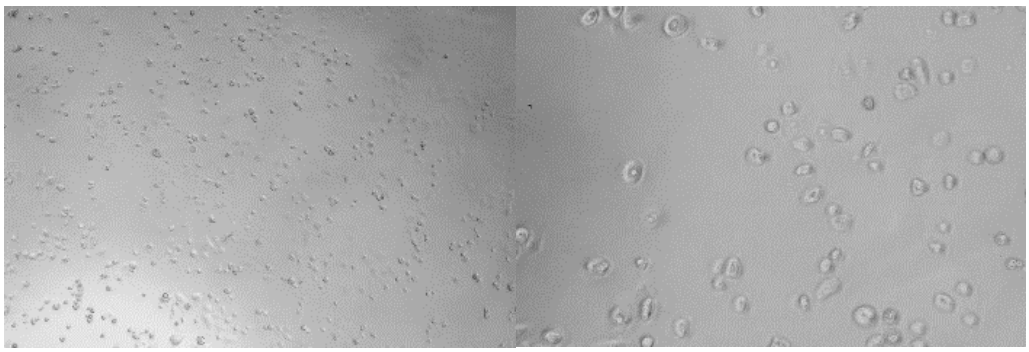

Figure 12: The transmission light microscope picture showed irregular cell morphology of HCECs after treated with high concentration (100 mg/mL) of lidocaine at 4X objective lens (left panel) and 10X objective lens (right panel).

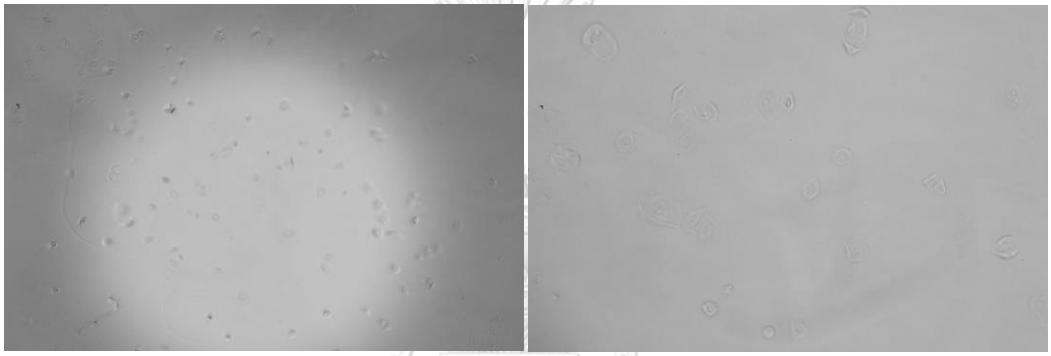

Figure 13: The transmission light microscope picture showed irregular cell morphology of HCECs after treated with high concentration (76.07 mg/mL) of lidocaine loaded in dissolving microneedle solution at 4X objective lens (left panel) and 10X objective lens (right panel).

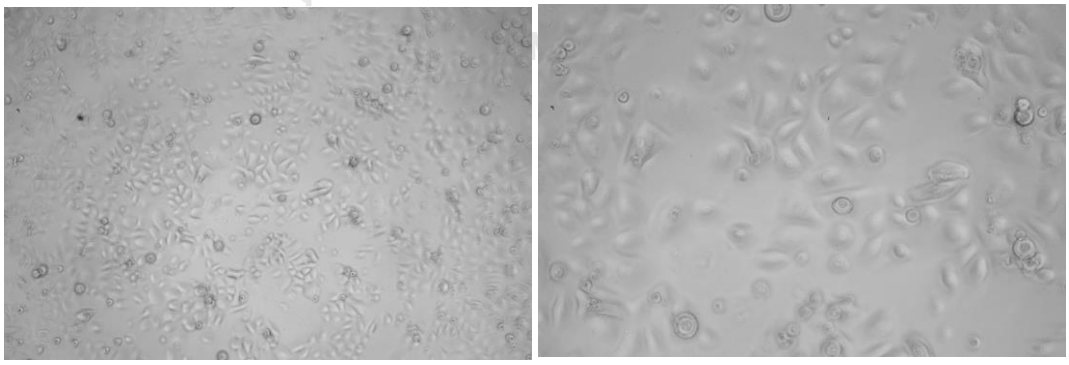

Figure 14: The transmission light microscope picture showed unchanged morphology of HCECs after treated with high concentration (100 mg/mL) of maltose at 4X objective lens (left panel) and 10X objective lens (right panel)

6. Dead stain/ Life stain

HCECs dead stain/ life stain results were compared when given with different treatments; i. e. , lidocaine solution, maltose solution and dissolved matrix of lidocaine loaded in dissolving microneedle patch. The different treatment showed in Figure 15-17. Results showed that cell viability of HCECs cultures was determined by calcein AM and PI. Pictures were analyzed with ImageJ program. The last column showed the dark spot, which presented amount of cell survived in each treatment. First, the percent total live-cell spot area of each lidocaine treatment group including control (no treatment), 12.5, 1.68, 0.84, and 0.42 mg/mL were 17.857, 1.302, 3.204, and 5.161, respectively. Second, the percent total live-cell spot area of each maltose treatment group including control (no treatment), 100, 50, 25, and 12.5 mg/mL were 6.642, 13.119, 14.606, and 19.045, respectively. The last, the percent total live-cell spot area of each lidocaine mixed with maltose treatment group including control (no treatment), 76.07, 38.03, 19.01, and 9.50 mg/mL were 17.857, 0.653, 0.689, 0.739, and 8.795, respectively. In summary at high concentration of lidocaine and lidocaine mixed with maltose treatment group showed decreasing the number of cells. However, at high concentration of maltose did not show decreasing the number of cells.

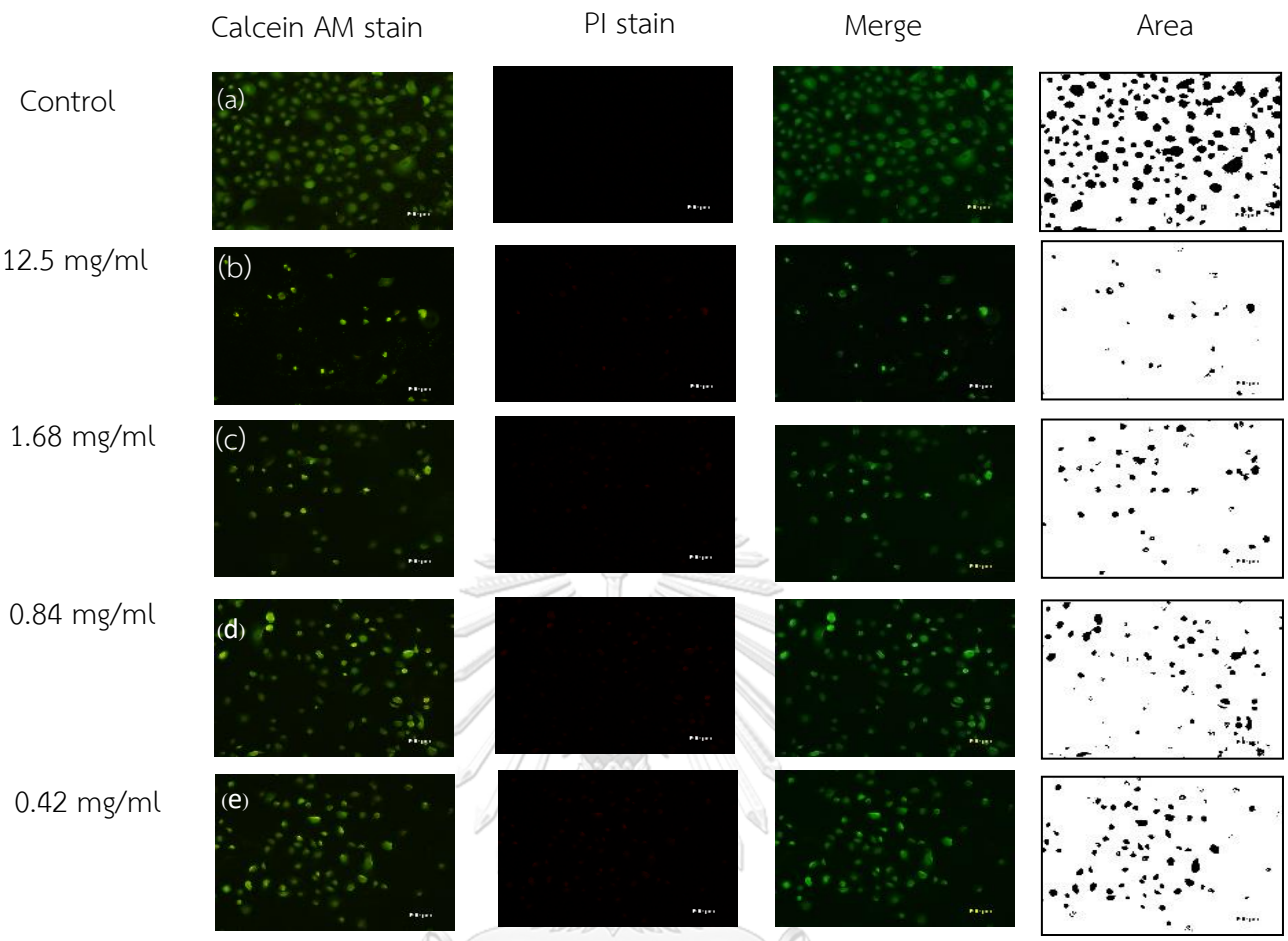

Figure 15: Cell viability of HCECs cultures was determined by Calcein AM and PI. The live cells fluoresced green, while dead cells fluoresced red. Fluorescent images of cells after 24 hrs. of direct contact with different concentrations of lidocaine hydrochloride including (a) control (without test materials) (b) 12.5 mg/mL, (c) 1.68 mg/mL, (d) 0.84 mg/mL, (e) 0.42 mg/mL

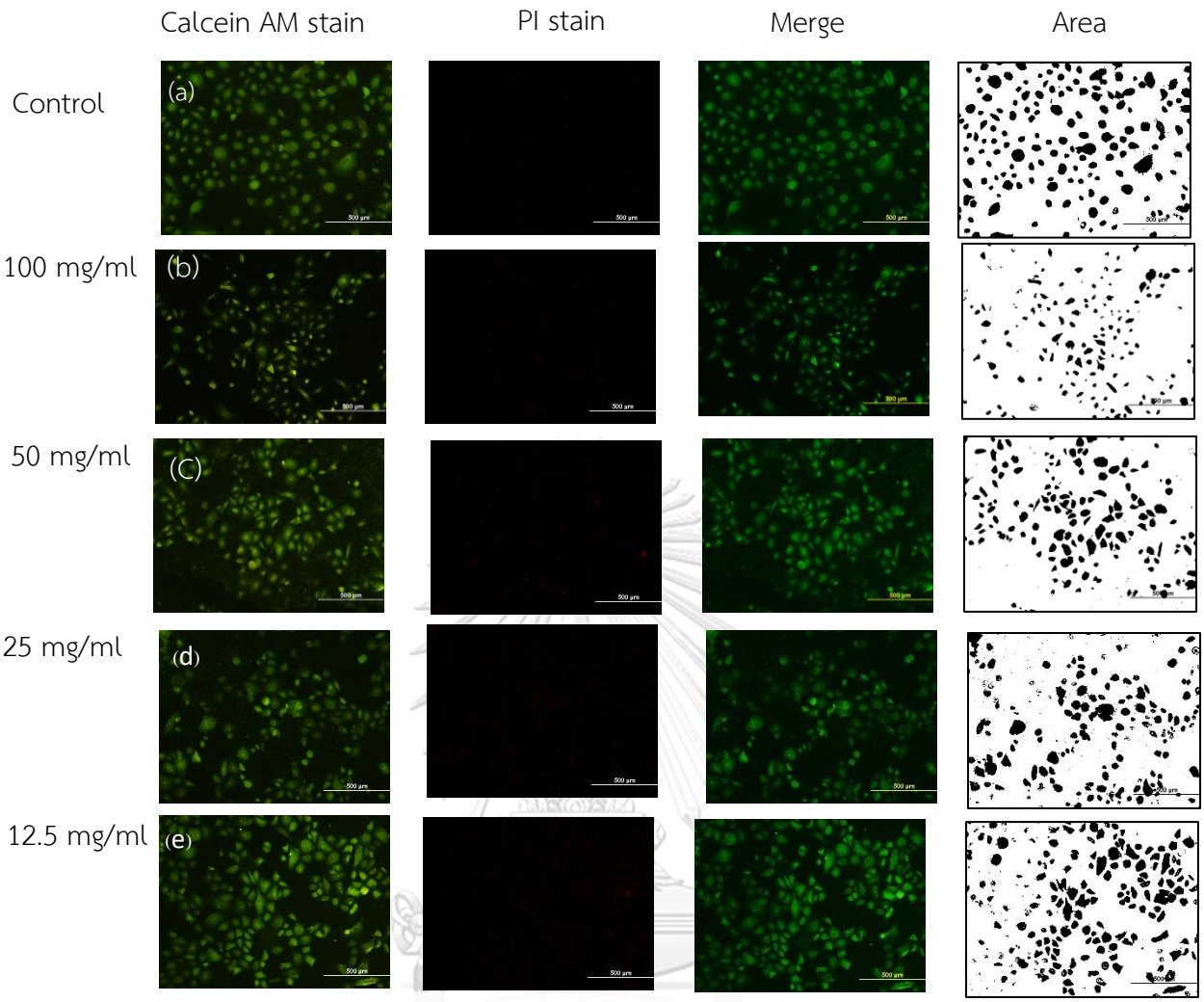

Figure 16: Cell viability of HCECs cultures was determined by Calcein AM and PI. The live cells fluoresced green, while dead cells fluoresced red. Fluorescent images of cells after 24 hrs. of direct contact with different concentrations of maltose including (a) control (without test materials) (b) 100 mg/mL, (c) 50 mg/mL, (d) 25 mg/mL, (e) 12.5 mg/mL

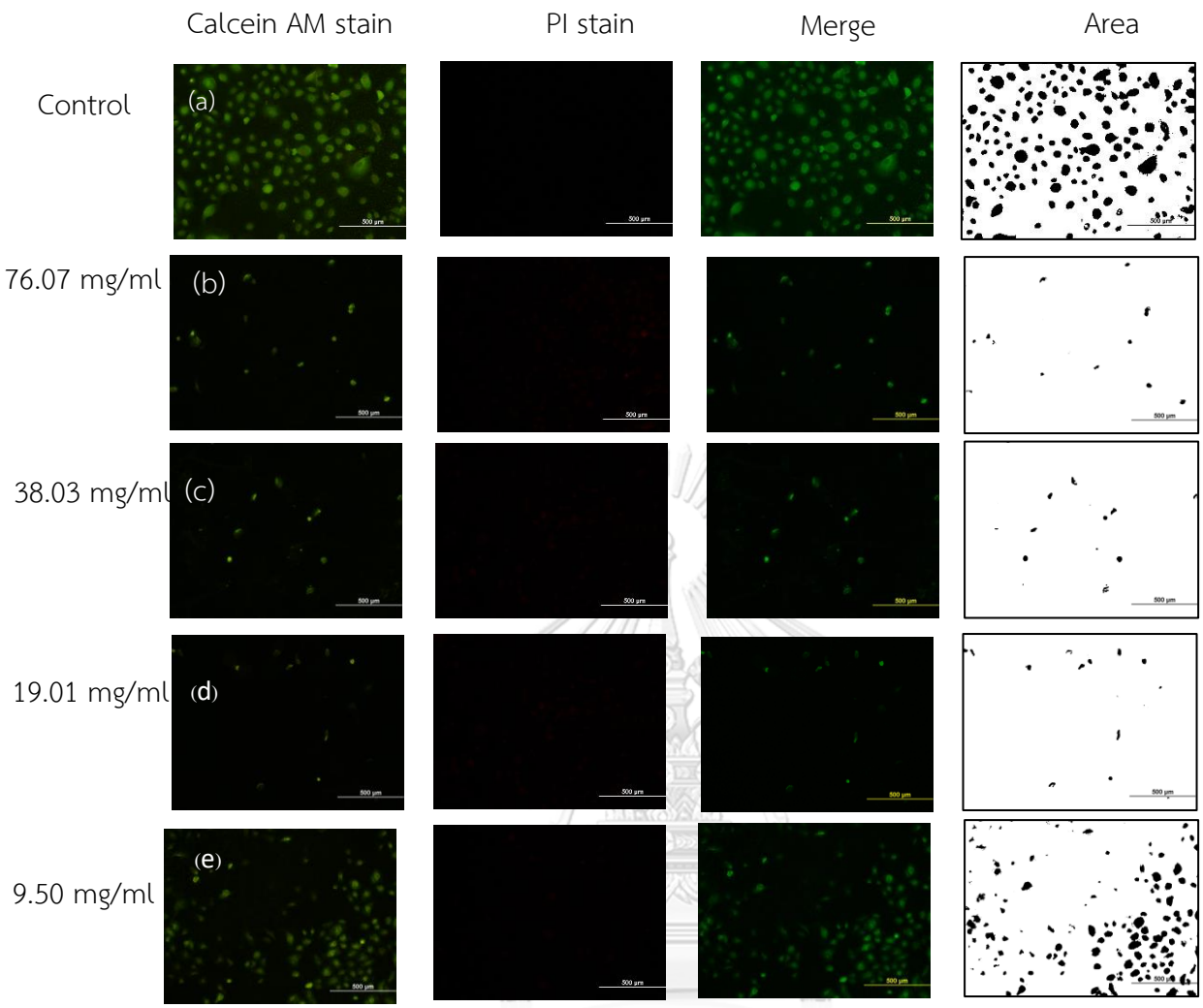

Figure 17: Cell viability of HCECs cultures was determined by Calcein AM and PI. The live cells fluoresced green, while dead cells fluoresced red. Fluorescent images of cells after 24 hrs. of direct contact with different concentrations of lidocaine loaded in maltose dissolving microneedle patch solution including (a) control (without test materials) (b) 76.07 mg/mL, (c) 38.03 mg/mL, (d) 19.01 mg/mL, (e) 9.50 mg/mL

Regarding the safety of topical lidocaine delivery on the sclera, the cytotoxicity test is extremely crucial. There was cytotoxicity report of lidocaine to human corneal endothelial cells line that presented the concentration of lidocaine more than 1.25 g/L could decrease viability of human corneal endothelial cells line [3]. Similarly, Zhou *et al.* also informed that the lidocaine concentrations in range of 0.31 g/L to 20.00 g/L could decrease human corneal stromal cells line and also changed cell morphology [17]. In this result, the type of cell used was different from previous studies. Herein, human primary corneal epithelial was used, as the primary cell is more suitable biological relevant tools for studying human and animal biology than the cell-line counterparts. The primary cells possess limited lifespan and heterogeneous genotypes, it also expressed heterogeneous phenotype with no loss of true characteristics of the original tissue.

From this result, there might have been clinical concerns that the high concentration of lidocaine solution, lidocaine mixture, and maltose solution could cause toxicity. The issue of cell-study when each material was used to treat the cells for 24 hrs. can be considered extreme by a much longer contact time comparing with the estimated regular use.

7. *In vitro* permeation studies

The *in vitro* permeation studies were selected as the tool for determining the amount of drug through porcine sclera. The important factors of the *in vitro* permeation experiment were shown in Table 6 and Table 7 permeation parameters of porcine sclera treatment by solution and lidocaine loaded in dissolving microneedle patch form. The in vitro permeation studies were conducted by franz-diffusion cell. After the sample was collected at 5 minutes, 10 minutes, 30 minutes and every hr. until 4 hrs. The amount of permeated drug and drug adsorbed on porcine sclera were calculated. The mean thicknesses of scleras (measured after thawing of the frozen tissues) in case of lidocaine solution and lidocaine loaded in dissolving microneedle were 1.075 ± 0.19 mm., 0.86 ± 0.12 mm, respectively.

In vitro permeation results of lidocaine hydrochloride solution 2% w/v (20mg/mL) are showed in Table 8. that the drug located on the surface of sclera tissue within the range of 7.42 – 8.71 mg/cm^2 However, no drug appeared in the sclera. In vitro permeation results of lidocaine loaded in dissolving microneedle patches are showed in Table 9. that the drug adsorbed on the surface of sclera tissue within the range of 2.68 – 3.36 mg/cm^2. and absorbed in the sclera tissue in the range 4.18 – 4.51 mg/cm^2. Another interesting observation could conclude that the amount of drug adsorbed on the surface of sclera tissue and absorbed in the sclera tissue were independent with the thickness of porcine sclera.

Dissolving microneedles successfully delivered lidocaine into the sclera tissue and did not cause unwanted systemic absorption of lidocaine when comparing with no sclera absorption of lidocaine solution.

Porcine sclera was used to analyze permeation assessment as a model representing human eyes due to similar histology and tissue water content. The histology between human and porcine sclera possess no significant differences. The water content of tissues from the two species were compared. Even though, human sclera was two-fold thinner than porcine sclera, the human's contained 71.6% ± 0.63% moisture comparable to that observed with porcine's (69.6% ± 1.18%). Nicoli *et al.* found permeation assessment of porcine scleras and human scleras were very similar toward small molecule and high molecular weight compound [9]. In this case

lidocaine hydrochloride is a low molecular weight molecule. The results showed lidocaine hydrochloride cannot pass through porcine sclera. However, this result may need to be confirmed by other model of permeation study or *in vivo* study, because the diffusion cells are known for their limitation to reproduce the natural intraocular pressure, that can influence the permeation of drugs [32]. Additionally, the thickness of sclera near limbus was measured. Statistically analysis was performed using SPSS, version 19.0. In a first step, sclera thickness was calculated the mean values ± standard deviations and paired t test. In this study the thickness of each sclera was not equal. The sclera thickness of solution group and microneedle group were significant different with p-value less than 0.05 according to pair t-test. Lidocaine solution group had thicker sclera in the range of 0.71 – 1.31 mm. while microneedle group had sclera thicknesses in the range of 0.64 - 0.98. Therefore, lidocaine of the solution sample may be harder to get absorbed into the sclera due to thicker sclera.

Table 6: Permeation parameters of porcine sclera treatment by solution

Parameter	Value
Duration for loading sample	4 hrs.
Surface area of porcine sclera	2.23 cm^2
Thickness of porcine sclera	0.71 – 1.31 mm.
Volume of solution in receptor compartment	13 mL
Donor concentration	20 mg (Theoretical concentration)

Table 7: Permeation parameters of porcine sclera treatment by lidocaine loaded in dissolving microneedle patch

Parameter	Value
Duration for loading sample	4 hrs.
Surface area of porcine sclera	2.23 cm^2
Thickness of porcine sclera	0.64 – 0.98 mm.
Volume of solution in receptor compartment	13 mL
Donor concentration	20 mg (Theoretical concentration)

Table 8: Model parameters for analytical tissue deposition

Porcine sclera treatment by Solution	Permeation parameters				% recovery
	Thickness (mm)	Drug adsorbed on the surface of sclera tissue (mg/cm^2)	Drug absorbed into the sclera. the surface of sclera tissue (mg/cm^2)	Drug in receptor chamber (mg/cm^2)	
1	1.17	7.68	-	-	85.53%
2	1.09	8.23	-	-	91.57%
3	1.31	8.71	-	-	96.92%
4	0.71	7.43	-	-	82.65%
5	1.00	7.88	-	-	87.69%
6	1.17	7.57	-	-	84.26%

* (-) represent data that could not be detected by High - performance liquid chromatography

Table 9: Model parameters for analytical the tissue deposition

Porcine sclera treatment by lidocaine loaded in dissolving microneedle patch	Permeation parameters				% recovery
	Thickness (mm)	Drug adsorbed on the surface of sclera tissue (mg/cm^2)	Drug absorbed into the sclera. the surface of sclera (mg/cm^2)	Drug in receptor chamber (mg/cm^2)	
1	0.87	3.36	4.18	-	83.95%
2	0.64	2.68	4.51	-	80.02%
3	0.97	3.00	4.20	-	80.10%
4	0.84	3.27	4.35	-	84.80%
5	0.85	2.84	4.49	-	81.57%
6	0.98	2.97	4.23	-	80.14%

* (-) represent data that could not be detected by High - performance liquid chromatography

CHAPTER V

CONCLUSION

Lidocaine hydrochloride chemical assay was optimized and validated using high performance liquid chromatography following ICH guideline [28]. HPLC methods were verified with parameters including specificity, accuracy, precision, limit of detection, limit of quantitation and linearity. The primary human corneal epithelial cells (HCECs) was chosen as the most biorelevant model for a safety assessment of ophthalmic lidocaine. Moreover, to confirm safety of lidocaine solution and lidocaine in dissolving microneedle patch, the cytotoxicity of the lidocaine microneedle administration against human corneal epithelial cells (HCECs) as well as ex vivo drug target localization with tissue penetration were investigated. Tissue penetration tests were ensured for the non-toxic or neglectable amount of drug getting permeated. To our knowledge, the lidocaine loaded in maltose dissolving microneedle patch as a topical anesthesia ophthalmology surgery has not been reported, thus far. Here, we reported that cells treated with lidocaine solution showed decreases in cell viability with the concentration above 12.5 mg/mL. The treatment of cells with dissolved matrix of lidocaine microneedle patch could cause significant decrease in viability with the tested formulations containing 19.02-76.07 mg/mL lidocaine with 80.98-97.629 mg/mL maltose. However, the maltose-only solution was considered safe within the concentration range of 0.781-100 mg/mL. Result showed IC50 of maltose is 22592.96 mg/mL. Irregularities in cell morphology were observed with high concentration treatment of lidocaine and dissolved matrix of lidocaine microneedle patch. In contrary, maltose solution did not cause any morphological change of HCECs. Investigation of sclera penetration experiment via modified Franz diffusion method showed that lidocaine loaded in dissolving microneedle patch was adsorbed and localized in sclera tissue, while no detectable amount was penetrated through the tissue into the acceptor chamber. Another interesting observation was that the amount of drug adsorbed on the surface of sclera tissue was independent with the thickness of porcine sclera. In conclusion, safety assessment of lidocaine loaded in dissolving microneedle was investigated in this study. The formulation of

microneedles patch could be optimized to avoid the toxicity that could occur with high level of lidocaine. This report could unlock the potentiality in formulating the dissolving microneedle as local anesthetic formulation with biocompatibility. This study suggested that the 2% lidocaine loaded in maltose dissolving microneedle patch might help surpassing the ocular delivery limitation when comparing with 2% lidocaine solution with the better localized drug in the sclera.

APPENDICES

Appendix A

High - performance liquid chromatography analytical

HPLC method was fully validated according to ICH guideline. In terms of specificity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ) and linearity.

Pumping conditions

In high-performance liquid chromatography, two pumps were used to mix the mobile phases in a close system and to prevent solvent evaporation causing ratio of mobile phases changed. The following data shows the influence of pumping conditions:

One pump

Table 10: The relation of concentration and area using one pump

Concentration of lidocaine	Area	Predict Area	% Recovery
20	59096	59550	99.23761545
20	60034	59550	100.8127624
20	60831	59550	102.1511335
10	30311	35136	86.26764572
10	44204	35136	125.8082878
10	30817	35136	87.70776412
5	25342	22929	110.5237908
5	21794	22929	95.04993676
5	16671	22929	72.7070522
1	13663	13163.4	103.795372
1	4104	13163.4	31.17735539
1	17031	13163.4	129.3814668
0.5	2446	11942.7	20.48113073
0.5	39994	11942.7	334.8823968
0.5	1835	11942.7	15.36503471
		Mean	80.27941109
		S.D.	49.1020557
		%CV	61.16394606

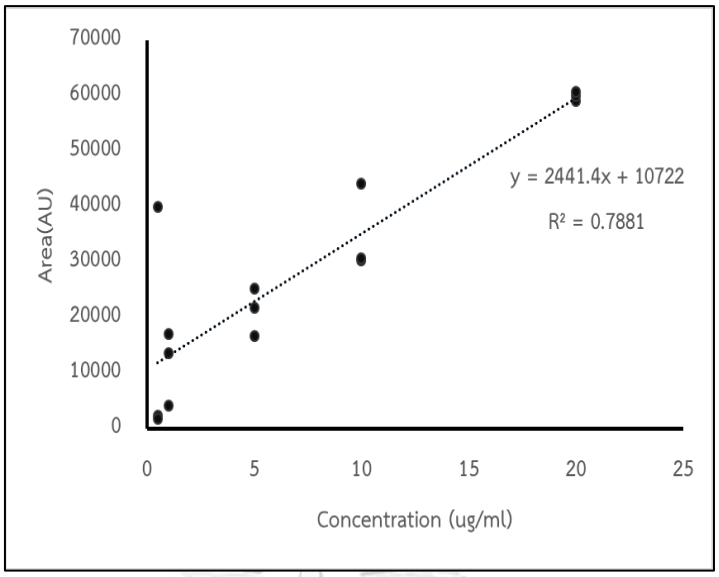

Figure 18: System linearity corresponding to the concentration range of 0.5 - 20µg/mL of lidocaine hydrochloride standard solution using one pump showed varies area Two pumps

Table 11: The relation of concentration and area using two pumps

Concentration of lidocaine	Area	Predict Area	% Recovery
20	51814	51966.3	99.70692545
20	51890	51966.3	99.85317408
20	51604	51966.3	99.3028174
10	26464	26226.3	100.9063421
10	26675	26226.3	101.710878
10	26795	26226.3	102.168434
5	13236	13356.3	99.09930145
5	13387	13356.3	100.2298541
5	13430	13356.3	100.5517995
1	2903	3060.3	94.85998105
1	2761	3060.3	90.21991308
1	2869	3060.3	93.74897886
		Mean	91.98444597
		S.D.	1.764532889
		%CV	1.918294849

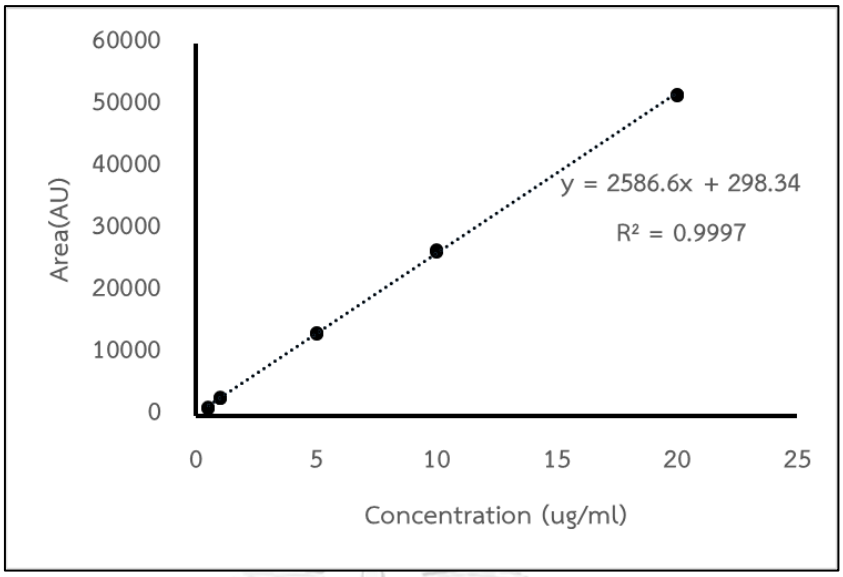

Figure 19: System linearity corresponding to the concentration range of 0.5 - 20µg/mL of lidocaine hydrochloride standard solution using two pumps.

Flow rate optimization

High performance liquid chromatography at flow rate 0.8 mL/min was suitable to use. Although, coefficient of variation (%CV) of flow rate 0.8 mL/min was higher than flow rate 1 mL/min. but the proper retention time was seen at 0.8 mL/min and the flow rate was selected for the study.

Flow rate 1mL/min

Table 12: The relation of concentration and area at flow rate 1mL/min

Concentration of lidocaine	Area	Predict Area	% Recovery
40	74267	74213.7812	100.0717101
20	36927	37103.7812	99.52354937
10	18714	18548.7812	100.8907259
5	9221	9271.2812	99.45766719
1	1858	1849.2812	100.4714697
		Mean	100.0830245
		S.D.	0.549087138
		%CV	0.548631639

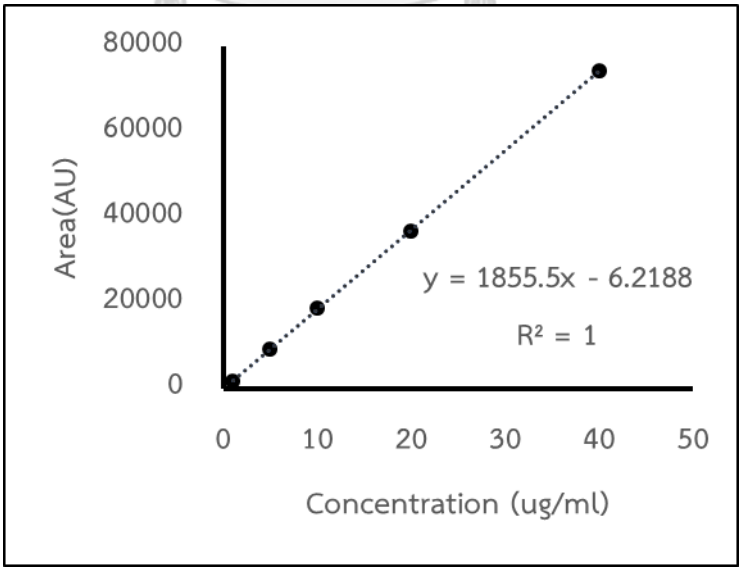

Figure 20: System linearity corresponding to the concentration range of 1-40µg/mL of lidocaine hydrochloride standard solution at flow rate 1 mL/min

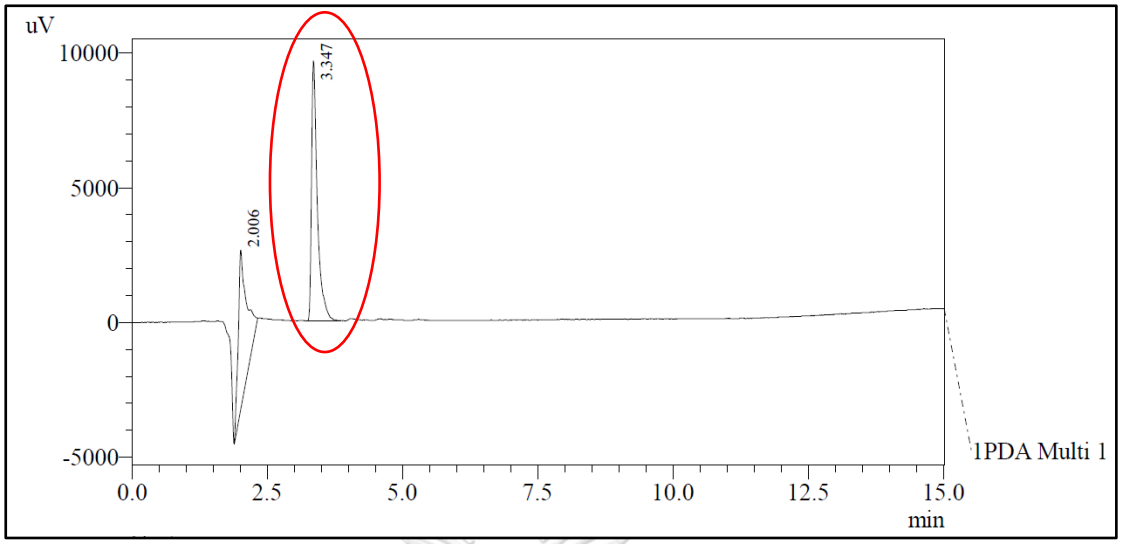

Figure 21: Representative chromatogram of the standard solution of lidocaine. The retention time was about 3.347; Flow rate 1 mL/min; detection wavelength, 254 nm; injection volume 20 µL.

Flow rate 0.8 mL/min

Table 13: The relation of concentration and area at flow rate 0.8 mL/min

Concentration of lidocaine	Area	Predict Area	% Recovery
40	75105	78017.5	96.2668632
20	46236	40657.5	113.7207157
10	23363	21977.5	106.3041747
5	11403	12637.5	90.23145401
1	2346	5165.5	45.416707
		Mean	90.38798293
		S.D.	23.89453065
		%CV	26.43551706

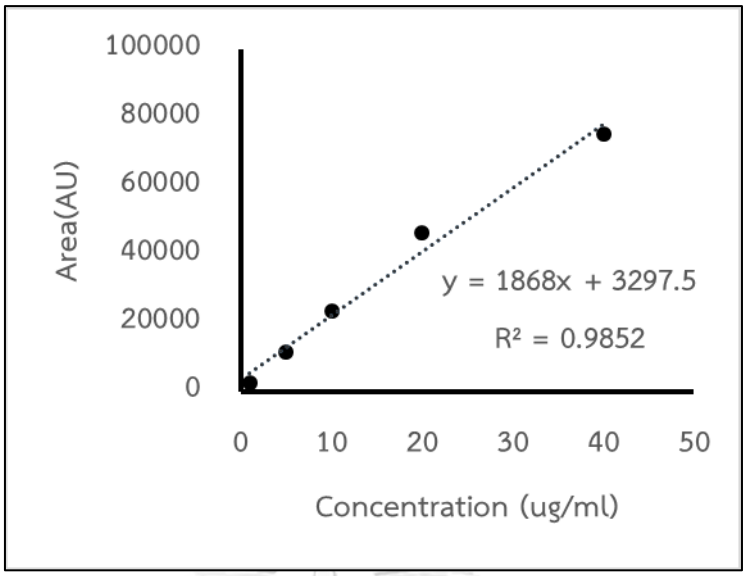

Figure 22: System linearity corresponding to the concentration range of 1-40µg/mL of lidocaine hydrochloride standard solution at flow rate 0.8 mL/min

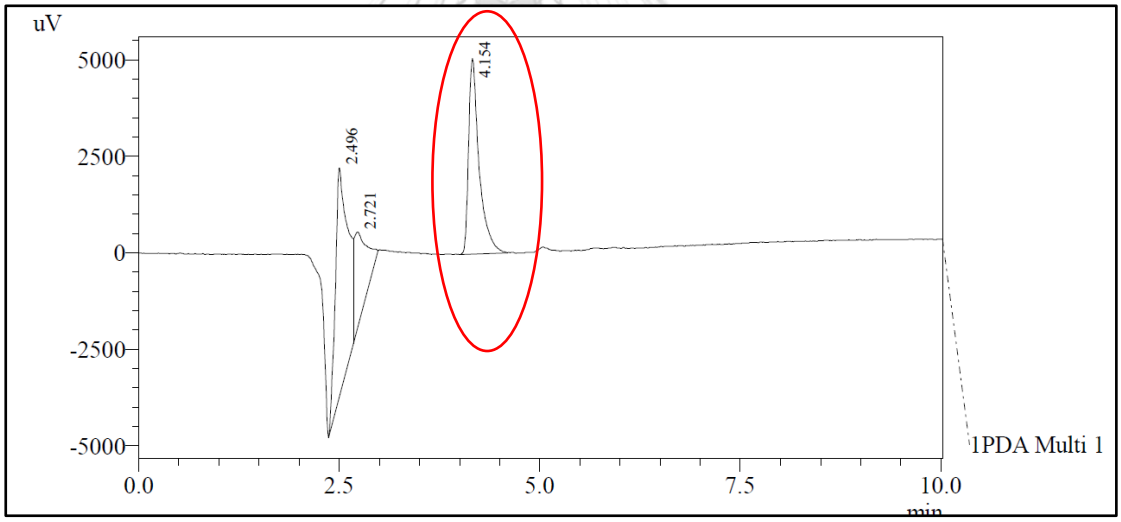

Figure 23: Representative chromatogram of the standard solution of lidocaine. The retention time was about 4.154; Flow rate 0.8 mL/min; detection wavelength, 254 nm; injection volume 20 µL

HPLC chromatogram

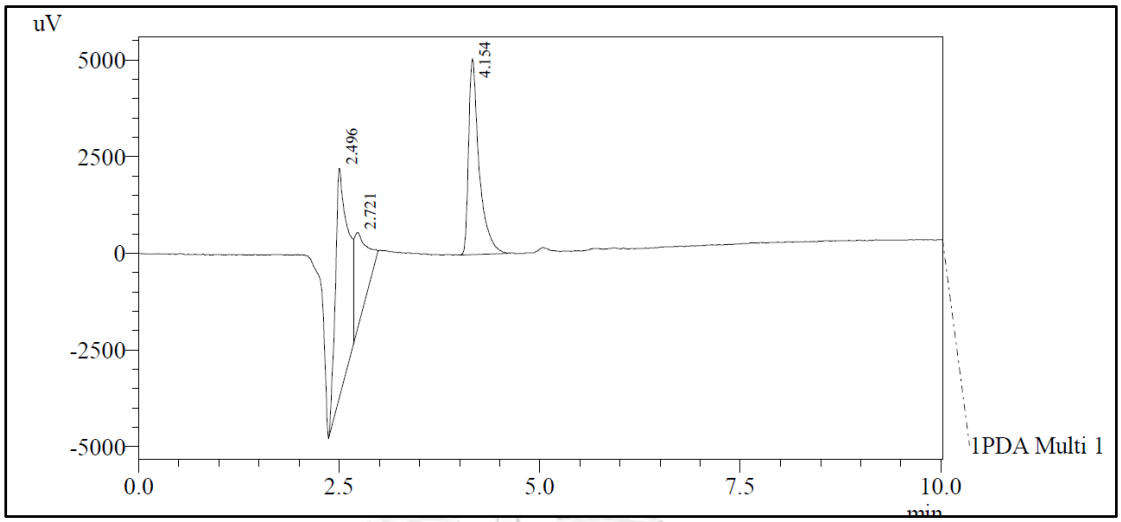

Figure 24: HPLC chromatogram of the standard lidocaine hydrochloride

Calibration curve of lidocaine hydrochloride

Table 14: Data for calibration curve of lidocaine hydrochloride

Concentration of lidocaine (µg/mL)	Area	Predict Area	% Recovery
20	59270	57661.9	102.7888432
15	41614	43623.4	95.39375656
10	28854	29584.9	97.52948295
5	16540	15546.4	106.3911902
2.5	8668	8527.15	101.6517828
		Mean	100.7510112
		S.D.	3.894975999
		%CV	3.86594234

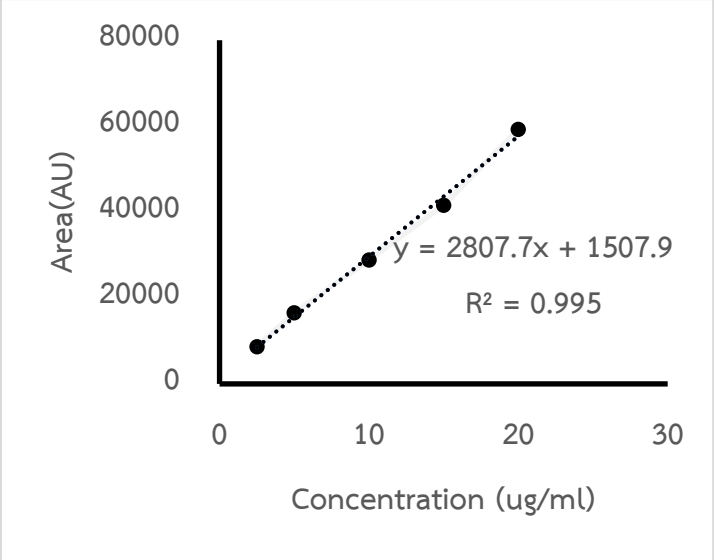

Figure 25: Standard of lidocaine hydrochloride

Specificity

The specificity was determined by comparing the HPLC chromatograms among the lidocaine hydrochloride solution, lidocaine in phosphate buffer saline and spiked lidocaine hydrochloride solution with methylene blue (colorant used while fabricating microneedles). The peak of phosphate buffer saline solution and methylene blue did not interfere the peak of lidocaine hydrochloride. These results show in Figure 26 – Figure 30.

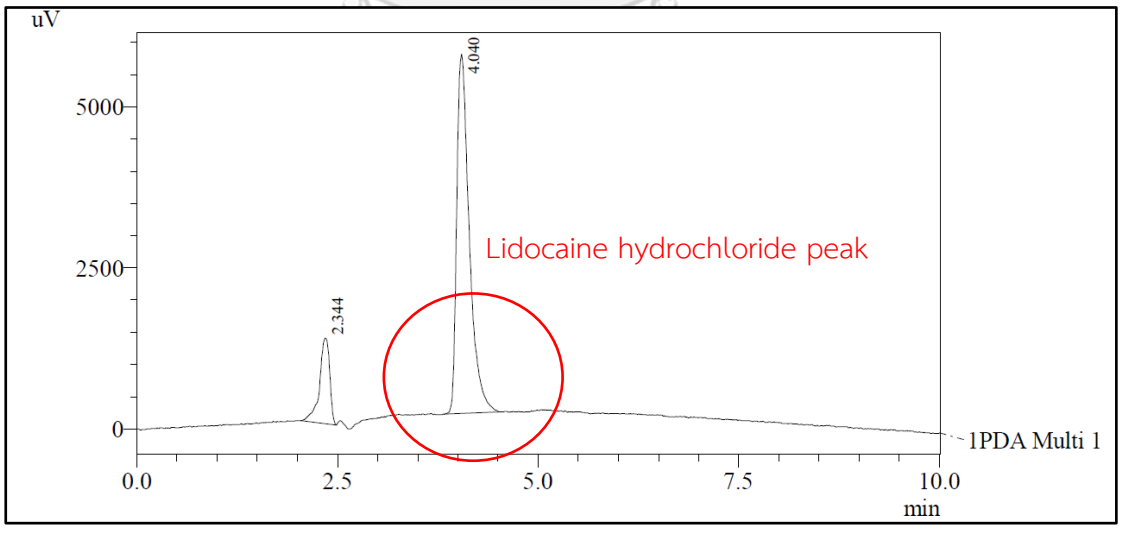

Figure 26: The chromatogram of lidocaine hydrochloride

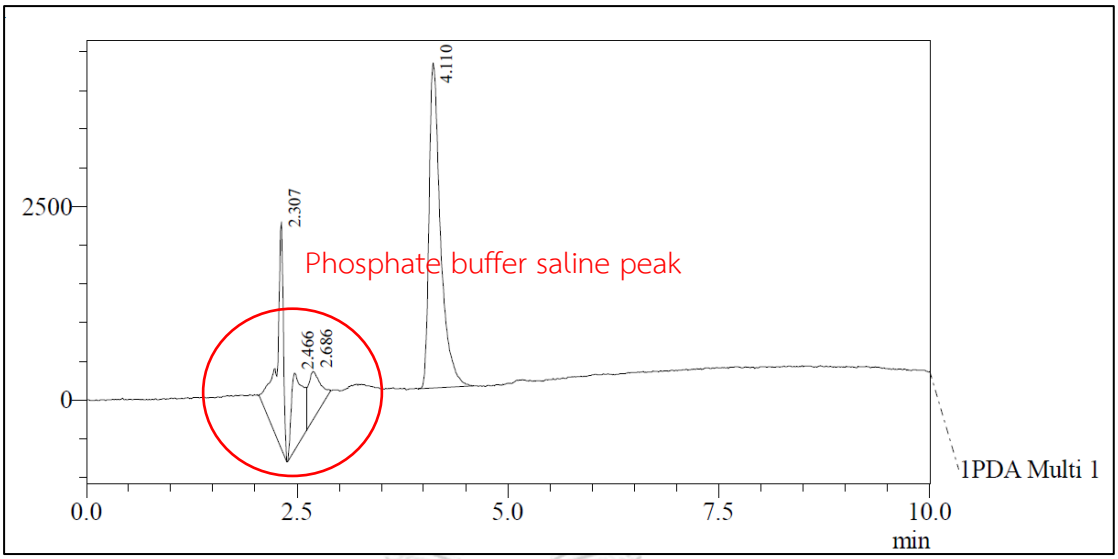

Figure 27: The chromatogram of lidocaine hydrochloride in phosphate buffer saline solution

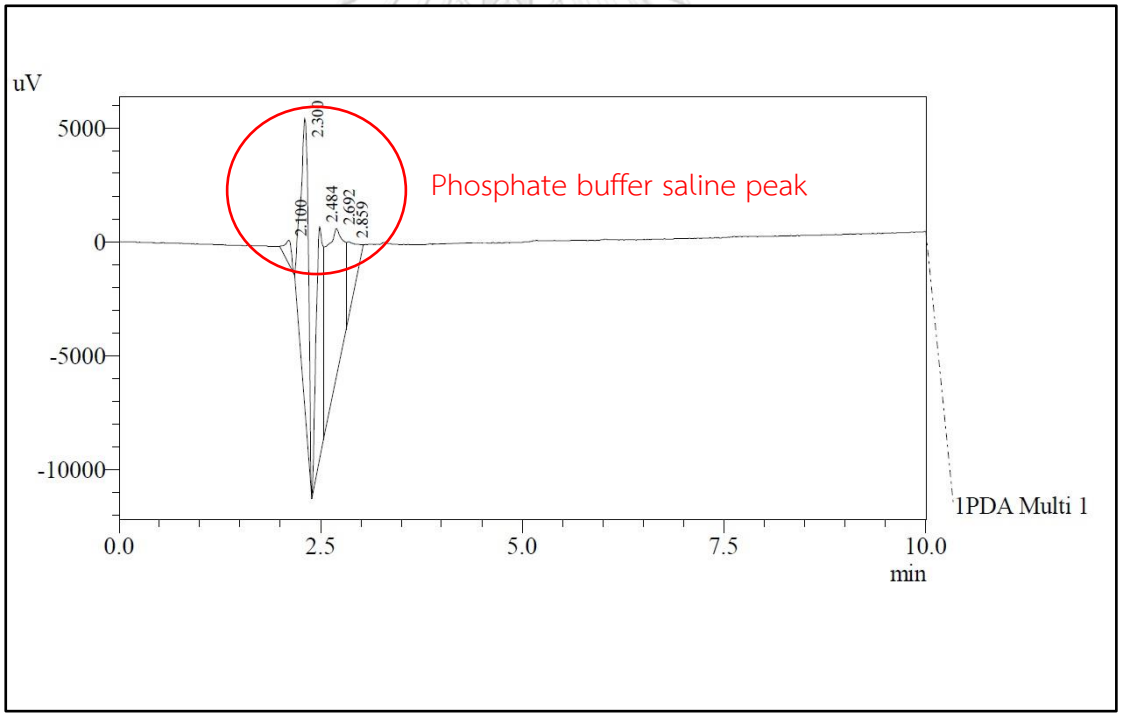

Figure 28: The chromatogram of phosphate buffer saline solution

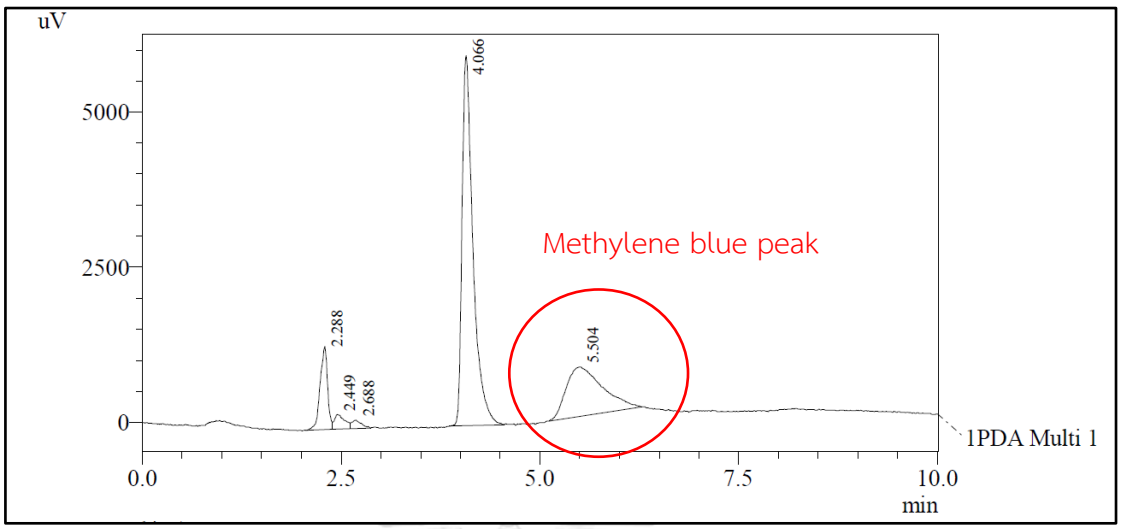

Figure 29: The chromatogram of spiked lidocaine hydrochloride solution with methylene blue (colorant used while fabricating microneedles).

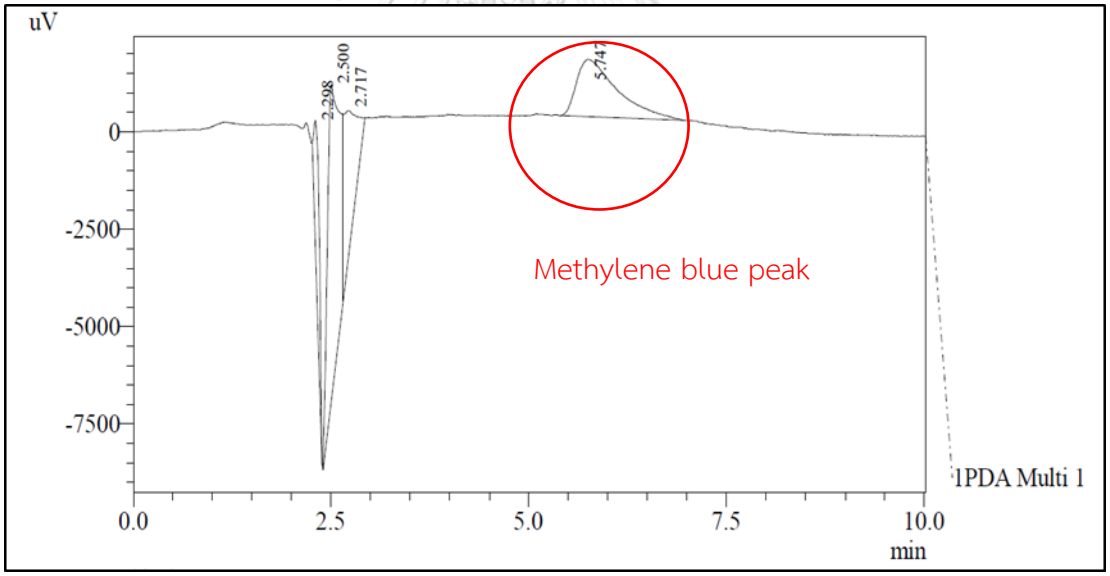

Figure 30: The chromatogram of methylene blue

Table 15: The relation of concentration and area of lidocaine hydrochloride by using phosphate buffer as a mobile phase

Concentration of lidocaine	Area	Predict Area	% Recovery
20	31788	30139.22	105.4705464
20	26639	30139.22	88.38649441
20	31695	30139.22	105.1619783
10	13128	15269.22	85.97688684
10	17348	15269.22	113.6141859
10	15775	15269.22	103.3124154
5	9300	7834.22	118.7099673
5	7566	7834.22	96.57630243
5	7038	7834.22	89.83663977
1	1492	1886.22	79.09999894
1	1862	1886.22	98.71595042
1	1754	1886.22	92.99021323
		Mean	98.15429828
		S.D.	11.16619638
		%CV	11.37616648

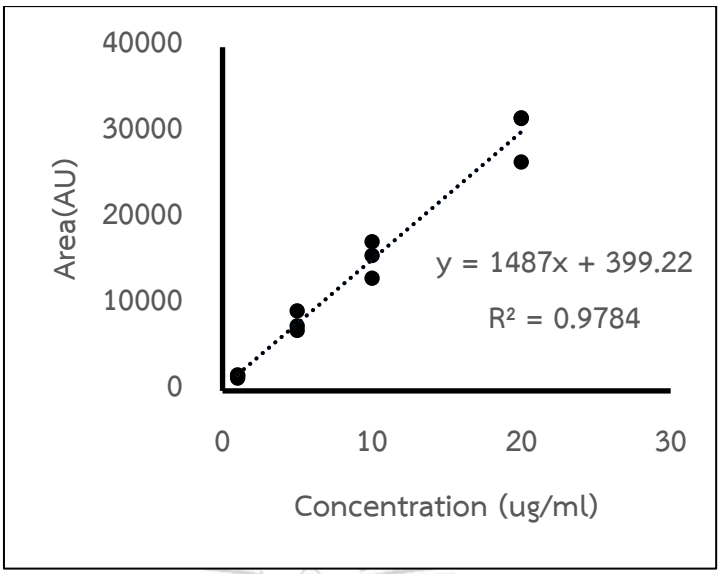

Figure 31: System linearity corresponding to the concentration range of 1-20 µg/mL of lidocaine hydrochloride standard solution by using phosphate buffer as a mobile phase

Table 16: The relation of concentration and area of lidocaine hydrochloride by using phosphate buffer as a mobile phase and spiked with methylene blue

Concentration of lidocaine	Area	Predict Area	% Recovery
20	23470	25368.3	92.51703898
20	26023	25368.3	102.5807799
20	25527	25368.3	100.6255839
10	16328	15984.2	102.150874
10	14763	15984.2	92.35995546
10	19771	15984.2	123.6908948
5	10644	11292.15	94.26017189
5	10111	11292.15	89.54007873
5	11730	11292.15	103.8774724
1	8946	7538.51	118.6706657
1	6411	7538.51	85.04333084
1	6826	7538.51	90.54839749
		Mean	99.65543701
		S.D.	11.19857025
		%CV	11.23728979

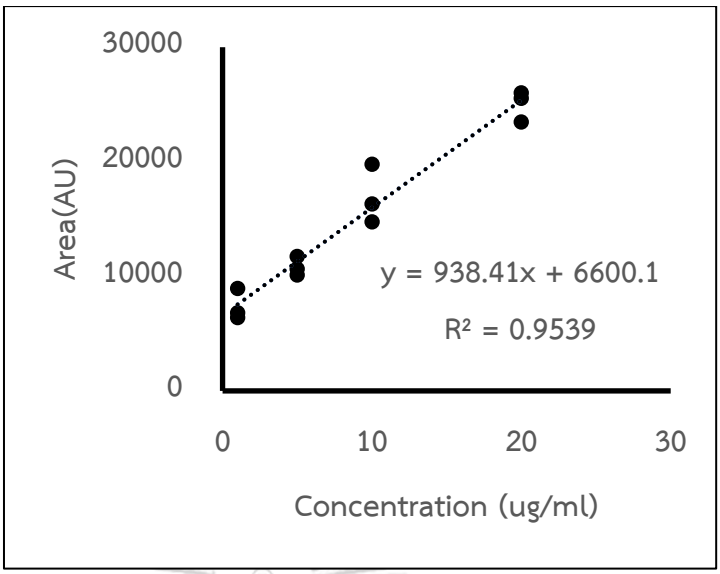

Figure 32: System linearity corresponding to the concentration range of 1-20 ug/mL of lidocaine hydrochloride standard solution by using phosphate buffer as a mobile phase and spiked with methylene blue

Accuracy

The accuracy was determined by analyzing each concentration of lidocaine hydrochloride solution showed in Table 17.

Precision

Within run precision and between run precision was conducted.

Table 17: The relation of concentration and area of lidocaine hydrochloride showed percent recovery

Date	Concentration of lidocaine	Area	Predict Area	% Recovery
27-07-18	20	59270	57661.9	102.7888432
	15	41614	43623.4	95.39375656
	10	28854	29584.9	97.52948295
	5	16540	15546.4	106.3911902
	2.5	8668	8527.15	101.6517828
31-07-18	20	52577	51567.96	101.9567189
	15	38252	38762.46	98.68310732

	10	24262	25956.96	93.4701136
	5	13718	13151.46	104.3078107
	2.5	7377	6748.71	109.3097792
01-08-18	20	59422	56695.18	104.8096152
	15	39734	42761.68	92.91964207
	10	27059	28828.18	93.86301875
	5	16245	14894.68	109.0657872
	2.5	8646	7927.93	109.0574715
09-08-18	20	63114	59789.8	105.5598112
	15	38916	45162.3	86.16921636
	10	32947	30534.8	107.8998389
	5	16629	15907.3	104.5369107
	2.5	8381	8593.55	97.52663335
09-08-18	20	64745	63405.93	102.1119003
	15	46420	47500.43	97.72543112
	10	29978	31594.93	94.88231181
	5	16567	15689.43	105.5933836
	2.5	8217	7736.68	106.2083478
09-08-18	20	54878	48117.9	114.0490337
	15	34113	36949.9	92.32230669
	10	24688	25781.9	95.75710091
	5	13716	14613.9	93.85584957
	2.5	8217	7736.68	106.2083478
09-08-18	20	47354	45699.46	103.6204804
	15	31694	34372.96	92.20619929
	10	23510	23046.46	102.0113284
	5	12154	11719.96	103.7034256
	2.5	6186	6056.71	102.1346573
10-08-18	20	42204	43114.5	97.88818147
	15	32574	33133.5	98.3113767

	10	26168	23152.5	113.0245114
	5	13301	13171.5	100.9831834
	2.5	6507	8181	79.5379538
10-08-18	20	43629	44314.91	98.45219137
	15	34117	33382.41	102.2005302
	10	22915	22449.91	102.0716787
	5	11242	11517.41	97.60875058
	2.5	5811	6051.16	96.03117419

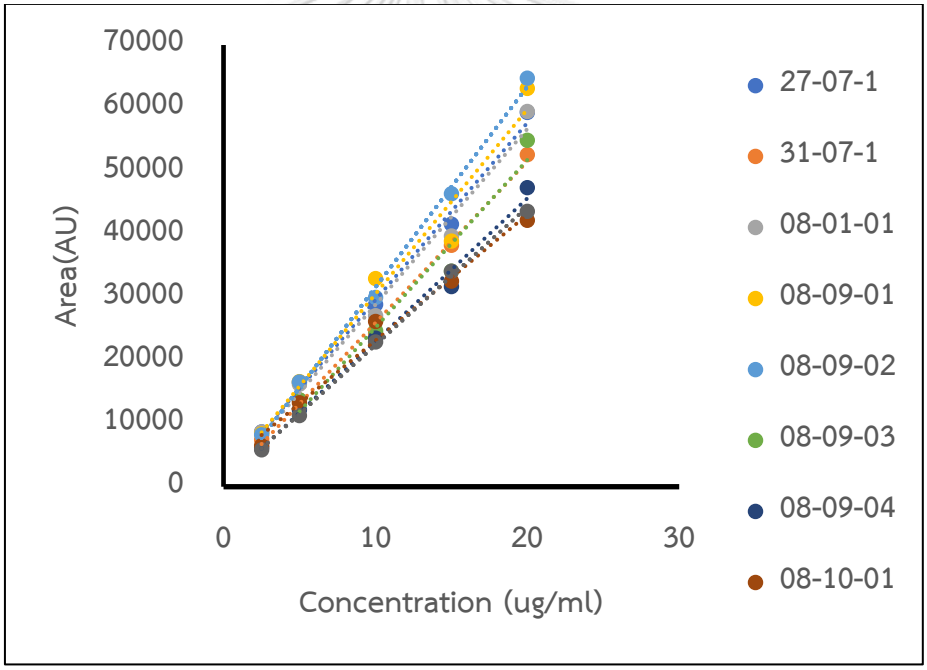

Figure 33: System linearity corresponding to the concentration range of 2.5-20 µg/mL of lidocaine hydrochloride standard solution by using phosphate buffer as a mobile phase

Table 18: The equation, R^2 and %RSD of lidocaine hydrochloride in each day.

Date	Equation	R^2	%RSD
27-07-18	y = 2807.7x + 1507.9	0.995	3.865942
31-07-18	y = 2561.1x + 345.96	0.9964	4.004246
01-08-18	y = 2786.7x + 961.18	0.9863	7.022791
09-08-18-1	y = 2925.5x + 1279.8	0.9688	7.8567784
09-08-18-2	y = 3181.1x - 216.07	0.9968	4.042575
09-08-18-3	y = 2658.2x - 1379	0.9647	8.86458
09-08-18-4	y = 2265.3x + 393.46	0.9903	4.735353
10-08-18-1	y = 1996.2x + 3190.5	0.9843	10.95386
10-08-18-2	y = 2186.5x + 584.91	0.9986	2.023713

Table 19: The relation of concentration and area of lidocaine hydrochloride spiked with methylene blue showed percent recovery

Date	Concentration of lidocaine	Area	Predict Area	% Recovery
09-08-18	20	59021	52528.25	112.3604917
	15	35432	39524.75	89.64509579
	10	26018	26521.25	98.102465
	5	13920	13517.75	102.9757171
	2.5	6308	7016	89.90877993
09-08-18	20	52297	50689.86	103.1705355
	15	30145	38200.86	78.91183602
	10	28350	25711.86	110.2604012
	5	15148	13222.86	114.5591801
	2.5	7585	6978.36	108.69316
10-08-18	20	44130	44766.7	98.57773747
	15	34010	34218.7	99.39009957
	10	25608	23670.7	108.1843798
	5	12823	13122.7	97.71617121
	2.5	7059	7848.7	89.93846115

10-08-18	20	44606	43951.68	101.4887258
	15	31985	32919.18	97.16220149
	10	21823	21886.68	99.70904678
	5	11128	10854.18	102.5227148
	2.5	5406	5337.93	101.2752134

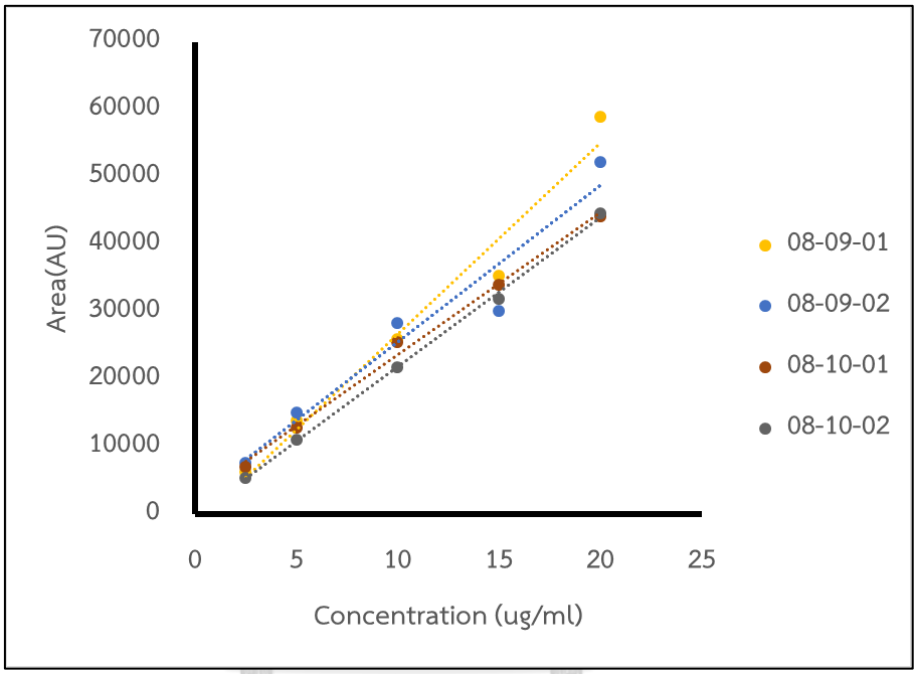

Figure 34: System linearity corresponding to the concentration range of 2.5-20 µg/mL of lidocaine hydrochloride standard solution spiked with methylene blue by using phosphate buffer as a mobile phase

Table 20: The equation, R^2 and %RSD of lidocaine hydrochloride solution spiked with methylene blue in each day.

Date	Equation	R^2	%RSD
09-08-18-1	y = 2829.8x - 1573.2	0.9713	8.864579676
09-08-18-2	y = 2313.7x + 2411.3	0.939	4.735352712
10-08-18-1	y = 2109.6x + 2574.7	0.9946	10.95386186
10-08-18-2	y = 2206.5x - 178.32	0.9986	2.023713067

Limit of detection (LOD)

The lowest lidocaine concentration was determined with acceptable precision and accuracy.

Table 21: The relation of concentration and area of LOD

Concentration of lidocaine	Area	Predict Area	% Recovery
1	2782	2847.51	97.69939351
1	3257	2847.51	114.3806343
1	2992	2847.51	105.0742579
1	3129	2847.51	109.8854789
1	3105	2847.51	109.0426373
1	3124	2847.51	109.7098869
1	3214	2847.51	112.870543
1	2999	2847.51	105.3200867
1	3225	2847.51	113.2568455
1	3202	2847.51	112.4491222
		Mean	108.9688886
		S.D.	4.815460288
		%CV	4.419114804

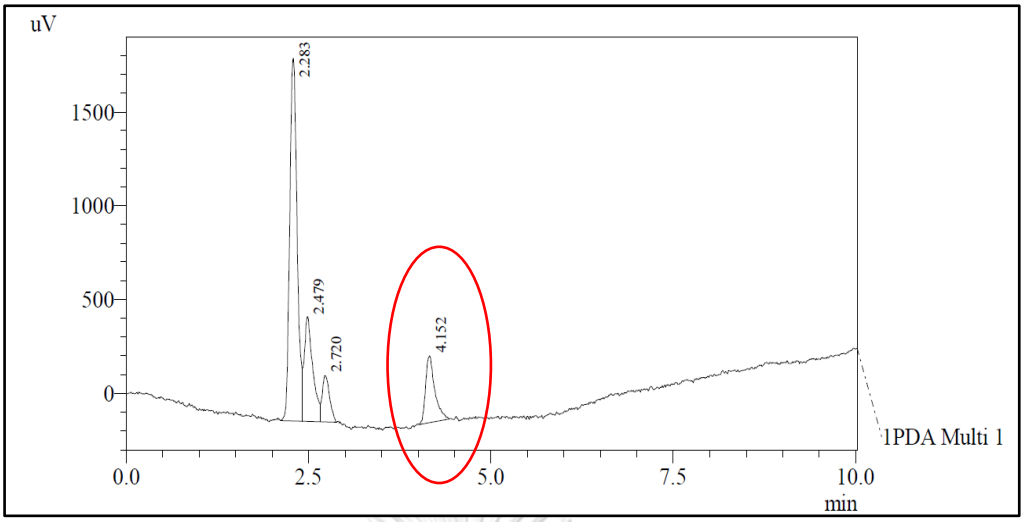

Figure 35: Representative chromatogram of LOD at concentration 1 µg/mL. Flow rate 0.8 ml/min; detection wavelength, 254 nm; injection volume 20 µL.

Limit of quantitation (LOQ)

The lowest quantity of lidocaine was distinguished according to the guideline.

Table 22: The relation of concentration and area of LOD

Concentration of lidocaine	Area	Predict Area	% Recovery
2.5	6495	6363.9	102.0600575
2.5	6231	6363.9	97.91165795
2.5	6234	6363.9	97.95879885
2.5	6575	6363.9	103.3171483
2.5	6199	6363.9	97.40882163
2.5	6172	6363.9	96.9845535
2.5	6460	6363.9	101.5100803
2.5	6070	6363.9	95.38176276
2.5	6163	6363.9	96.84313078
2.5	6763	6363.9	106.2713116
		Mean	99.56473232
		S.D.	3.325524894
		%CV	3.340063109

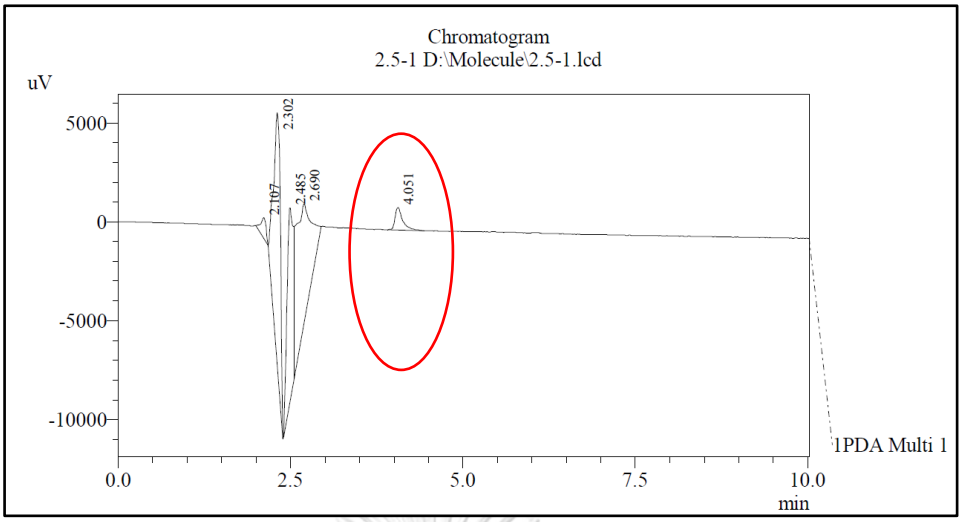

Figure 36: Representative chromatogram of LOQ at concentration 2.5 µg/mL. Flow rate 0.8 ml/min; detection wavelength, 254 nm; injection volume 20 µl

Linearity

The linearity of lidocaine hydrochloride solutions was prepared with 5 series concentration and injected to obtain the peak area. The linear regression was fitted to obtain the coefficient of determination (r^2) and percent precision (% RSD)

Table 23: The equation, R^2 and %RSD of lidocaine hydrochloride in each day.

Date	Equation	R^2	%RSD
1	y = 2807.7x + 1507.9	0.995	3.865942
2	y = 2561.1x + 345.96	0.9964	4.004246
3	y = 2786.7x + 961.18	0.9863	7.022791
4	y = 2925.5x + 1279.8	0.9688	7.8567784
4	y = 3181.1x - 216.07	0.9968	4.042575
4	y = 2658.2x - 1379	0.9647	8.86458
4	y = 2265.3x + 393.46	0.9903	4.735353
5	y = 1996.2x + 3190.5	0.9843	10.95386
5	y = 2186.5x + 584.91	0.9986	2.023713

Appendix B

In vitro cells base assay

Cytotoxicity test

Lidocaine cytotoxicity

Percent cells viability

Concentration (mg/mL)	% viability	SD
0.78125	106.2671	18.22679
1.5625	100.4726	20.57978
3.125	93.79206	25.86423
6.25	85.93711	19.06878
12.5	22.37638	2.486877
25	13.78186	2.68229
50	14.30843	1.283532
100	14.05253894	2.54373394

Maltose cytotoxicity

Percent cells viability

Concentration (mg/mL)	% viability	SD
0.78125	88.65723963	10.73391329
1.5625	105.1052406	18.63852855
3.125	80.00133122	24.37265632
6.25	93.59311906	26.44869479
12.5	83.97651131	24.71672449
25	87.65142663	35.70417355
50	88.11809428	19.08018898
100	66.5145621	27.30542074

Dissolved matrix of lidocaine microneedle patch

Percent cells viability

Concentration (mg/mL)	% viability	SD
2.3771875	116.307483	11.4518579
4.754375	88.92866123	11.09127457
9.50875	78.39942609	1.621523381
19.0175	52.31189078	6.286333864
38.035	25.93296552	5.791496392
76.07	9.429496946	1.985711373

Appendix C

Dead stain/ Life stain

The cell viability of HCECs cultures was determined by calcein AM and PI. Pictures were analyzed with ImageJ program. The column showed that count, total area, average size and present area, which presented amount of cell survived in each treatment.

Dead stain/life stain of lidocaine solution

Table 24: The data of dead stain/life stain of lidocaine solution

Concentration (mg/mL)	Count	Total Area	Average Size	% Area	Mean
12.5	80	0.874	0.011	1.302	42.171
1.68	86	2.152	0.025	3.204	34.165
0.84	151	3.018	0.020	4.493	32.696
0.42	156	3.466	0.022	5.161	31.825
No treat	243	11.993	0.049	17.857	25.040

Dead stain/life stain of maltose solution

Table 25: The data of dead stain/life stain of maltose solution

Concentration (mg/mL)	Count	Total Area	Average Size	% Area	Mean
100	262	4.461	0.017	6.642	23.482
50	295	8.811	0.030	13.119	24.543
25	648	9.810	0.015	14.606	28.774
12.5	349	12.791	0.037	19.045	26.543
No treat	243	11.993	0.049	17.857	25.040

Dead stain/life stain of dissolved matrix of lidocaine microneedle patch

Table 26: The data of dead stain/life stain of dissolved matrix of lidocaine microneedle patch

Concentration (mg/mL)	Count	Total Area	Average Size	% Area	Mean
76.07	31	9097	293.452	0.653	56.490
38.03	23	0.462	0.020	0.689	56.996
19.01	26	0.496	0.019	0.739	44.458
9.50	268	5.907	0.022	8.795	15.231
No treat	243	11.993	0.049	11.857	25.040

Appendix D

In vitro permeation studies

Characterization of porcine eye anatomy

The *in vitro* permeation studies were selected as the tool for determining the amount of drug through porcine sclera. The data of porcine eye anatomy for study permeation showed in Table 27 and Table 28.

Table 27: The data of porcine eye anatomy for study permeation of lidocaine solution.

Number	Equator of eye (mm.)	Hight of eye (mm.)	Width of cornea (mm.)	Thickness of sclera (mm.)	Weight before use (mg)	Weight after use (mg)
1	18.84	20.03	12.17	1.17	1370.12	540.67
2	21.17	18.16	12.84	1.09	1032.31	497.76
3	21.66	20.18	14.98	1.31	1679.42	845.31
4	21.06	20.50	15.18	0.71	1042.77	600.21
5	19.78	16.59	13.86	1.00	1038.07	538.42
6	20.66	15.32	15.26	1.17	1662.69	673.55

Table 28: The data of porcine eye anatomy for study permeation of lidocaine loaded dissolving microneedle patch.

Number	Equator of eye (mm.)	Hight of eye (mm.)	Width of cornea (mm.)	Thickness of sclera (mm.)	Weight before use (mg)	Weight after use (mg)
1	22.02	20.09	14.94	0.83	1439.24	1183.52
2	19.45	19.40	13.82	0.73	1468.99	1370.54
3	20.31	20.11	14.64	0.69	1137.61	866.09
4	22.34	19.53	14.27	0.94	1582.38	1075.24
5	20.21	19.11	15.37	1.04	1581.87	1332.49
6	21.73	18.50	15.40	1.12	1530.87	1291.68

The paired samples test showed p-value less than 0.05. This result was considered statistically significant.

Table 29: Paired Samples test comparing thickness sclera of solution and microneedle

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	ThicknessG1 - ThicknessG2	.21667	.20096	.08204	.00577	.42757	2.641	5	.046

Drug content in dissolving microneedle patch

The lidocaine content in dissolving microneedle patch was quantified in triplicate. The patch was placed in phosphate buffer saline pH 7.4 and sonicated in a sonicator bath for a period of 10 minutes. The lidocaine content was determined using HPLC. The lidocaine content showed concentration 19.89 ± 0.15 mg.

Table 30: The data of HPLC analysis for analyzing drug content

Unknown number	Unknown MN 1	y = Area	Ratio	Conc.(ug/10ml)
1	Y1	15653		
	Y2	15436	1: 1	200355.2136
	Y3	15625	1: 1	100177.6068
	Average	15571.33	1: 1	50088.80341
	conc. at 10 mL	2504.44	1: 9	25044.4017
	µg/10mL	200355.2	1: 9	2504.44017
	Mg	20.03552		
2	Y1	15853		
	Y2	15321	1: 1	197268.4641
	Y3	14890	1: 1	98634.23205
	Average	15354.67	1: 1	49317.11603
	conc. at 10 mL	2465.856	1: 9	24658.55801
	µg/10mL	197268.5	1: 9	2465.855801
	Mg	19.72685		
3	Y1	15659		
	Y2	15333	1: 1	199101.5184
	Y3	15458	1: 1	99550.75922
	Average	15483.33333	1: 1	49775.37961
	conc. At 10 mL	2488.768981	1: 9	24887.68981
	µg/10mL	199101.5184	1: 9	2488.768981
	mg	19.91015184		

HPLC analysis for analyzing permeation of lidocaine hydrochloride solution

Table 31: The data of HPLC analysis for analyzing drug on donor cell

formulation	donor			
	peak area ratio1	peak area ratio2	peak area ratio3	Average
Solution 1	13365	13354	13828	13515.66667
Solution 2	14180	14180	14729	14363
Solution 3	14882	15882	14582	15115.33333
Solution 4	13396	13412	12524	13110.66667
Solution 5	13451	14551	13452	13818
Solution 6	12668	13654	13687	13336.33333

Table 32: The data of HPLC analysis for analyzing amount of drug per area in donor

donor				
sd	dilution factor	cal. conc. (μg/mL)	conc. (mg)	Amount in sample per area (mg/cm^2)
220.8986696	4000	4.2767	17.1069084	7.6845369
258.8010819	4000	4.5785	18.3140649	8.2267996
555.7777334	4000	4.8465	19.3858793	8.7082657
414.8874011	4000	4.1325	16.5299237	7.4253515
518.3094314	4000	4.3844	17.5376287	7.8780193
472.7750228	4000	4.2129	16.8514205	7.5697700

Table 33: The data of HPLC analysis for analyzing SD

SD		
dilution factor	cal. conc. (μg/mL)	conc. (μg/mL)
4000	-0.4584	-1833.5311
4000	-0.4449	-1779.5333
4000	-0.3391	-1356.4444
4000	-0.3893	-1557.1644
4000	-0.3525	-1409.8238
4000	-0.3687	-1474.6946

Table 34: The data of HPLC analysis for analysis drug in skin

skin			
peak area ratio	dilution factor	cal. conc. (μg/mL)	conc. (μg)
-	1	-	-
-	1	-	-
-	1	-	-
-	1	-	-
-	1	-	-
-	1	-	-

* (-) represent cannot detect by High - performance liquid chromatography

HPLC analysis for analyzing permeation of lidocaine loaded in dissolving microneedle patch

Table 35: The data of HPLC analysis for analyzing drug on donor cell

formulation	donor			
	peak area ratio1	peak area ratio2	peak area ratio3	Average
Microneedle 1	43644	45239	41526	43469.66667
Microneedle 2	34358	34798	35690	34948.66667
Microneedle 3	39272	38871	38654	38932.33333
Microneedle 4	44088	42340	40764	42397.33333
Microneedle 5	38114	37445	35473	37010.66667
Microneedle 6	37512	39512	38976	38666.66667

Table 36: The data of HPLC analysis for analyzing amount of drug per area in donor part.

donor				
sd	dilution factor	cal. conc. (μg /mL)	conc. (mg)	Amount in sample per area (ug/cm^2)
1520.83011	500	14.9452	7.4726229	3.3567518
554.1247353	500	11.9104	5.9551887	2.6751103
255.9978299	500	13.3292	6.6646069	2.9937857
1357.622759	500	14.5633	7.2816600	3.2709700
1121.072205	500	12.6448	6.3223932	2.8400610
845.287065	500	13.2346	6.6172965	2.9725335

Table 37: The data of HPLC analysis for analyzing SD

SD		
dilution factor	cal. conc. (μg/mL)	conc. (μg)
500	0.0046	2.3026
500	-0.3397	-169.8499
500	-0.4459	-222.9409
500	-0.0535	-26.7616
500	-0.1378	-68.8870
500	-0.2360	-117.9992

Table 38: The data of HPLC analysis for analysis drug in skin

formulation	Skin			
	peak area ratio1	peak area ratio2	peak area ratio3	Average
Microneedle 1	52547	56453	52478	53826
Microneedle 2	57363	58152	58285	57933.33333
Microneedle 3	56088	53707	52343	54046
Microneedle 4	57654	54672	55252	55859.33333
Microneedle 5	58120	55692	59037	57616.33333
Microneedle 6	54050	53749	55277	54358.66667

Table 39: The data of HPLC analysis for analyzing drug per area in skin.

Skin				
sd	dilution factor	cal. conc. (μg/mL)	conc. (mg)	Amount in sample per area (mg/cm^2)
1520.83011	500	18.6338	9.3168964	4.1852117
554.1247353	500	20.0967	10.0483373	4.5137799
255.9978299	500	18.7121	9.3560744	4.2028107
1357.622759	500	19.3580	9.6789959	4.3478692
1121.072205	500	19.9838	9.9918854	4.4884213
845.287065	500	18.8235	9.4117546	4.2278227

Table 40: The data of HPLC analysis for analyzing SD

SD		
dilution factor	cal. conc. (μg/mL)	conc. (μg)
500	0.1246	62.3078
500	-0.3921	-196.0634
500	0.0141	7.0640
500	-0.0773	-38.6397
500	-0.0344	-17.2084
500	-0.3017	-150.8380

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AWARD RECEIVED	ทุนโครงการสนับสนุนการจัดตั้งห้องเรียนวิทยาศาสตร์ในโรงเรียน โดยการกำกับดูแลของมหาวิทยาลัย ปีที่ 3