CHAPTER III
MATERIALS AND METHODS

Materials

Drug: -

Glucosamine Hydrochloride : 99.84 % assay, Lot no.20060322, Zhe jiang Chitin Bioengineering, Yuhuan, China

Standard Glucosamine Hydrochloride : 99% assay, Lot 125K0024, Sigma, Shanghai, China

Chemical:-

Acetonitrile HPLC grade (Lot no.G 3AAIH, Honeywell Burdick & Jackson, Seoul, Korea)

Brilliant blue dye (Lot no.5-1183, Butterfield food ingredients limited, England)

Butylalcahol (Lot no.382647/1, Fluka, Buchs, Switzerland)

Cetyltrimethylammonium bromide (CTAB) (Lot no.kgig 72428 528, BDH Laboratory Supplies, England)

Hydroxypropyl methylcellulose (HPMC) viscosity 15 cP (Lot no.0503-55, Chem sources, Bangkok, Thailand)

Hydroxypropyl methylcellulose (HPMC) viscosity 5000 cP (Lot no.1540335, Chem sources, Bangkok, Thailand)

Isopropyl myristate (IPM) (Lot no.405657/1, Fluka, Buchs, Switzerland)

Methanol HPLC grade (Lot no.G8LGIH, Burdick & Jackson, Seoul, Korea)

n-hexane (Lot no.05010072, Labscan, Dublin, Ireland)

Paracetamol powder USP 24 (Lot no.6088903D798, Srichard United Dispensary co., Bangkok, Thailand)

Phenyl isothiocyanate (PITC) (Lot no.1110115, Fluka, Tokyo, Japan)

Phosphatidylcholine (PC, lecithin) (Lot no.70060P, Nattermann phospholipid MMBH, Cologne, France)

Poloxamer 188 (Lutrol F68) (Lot no.60-0480, BASF, Ludwigshafen, Germany)
Poloxmer 407 (Lutrol F127) (Lot no. WPEZ 555C, BASF, Ludwigshafen, Germany)
Poly-ethylene glycol 35 castor oil (Cremophore EL) (Lot no. 72188324VO, BASF, Ludwigshafen, Germany)
Poly-ethylene glycol 400 (Lutrol E400) (Lot no.82-1765, BASF, Ludwigshafen, Germany)
Polyoxyethelene 20 sorbitan monooleate (Tween 80) (Lot no.392141/1, Fluka, Buchs, Switzerland)
Potassium dihydrogen orthophosphate (Lot no.AF 401428, Ajax Finechem, Taren Point, Australia)
Sorbitan monolaurate (Span 20) (Lot no.SGA 03, Srichard United Dispensary co., Bangkok, Thailand)
Sorbitan monopalmitate (Span 40) (Lot no.SGB 03, Srichard United Dispensary co., Bangkok, Thailand)
Sorbitan monostearate (Span 60) (Lot no.303288, Srichard United Dispensary co., Bangkok, Thailand)
Soduim acetate (Lot no.F3D144, Ajax Finechem, Taren Point, Australia)
Soduim bis (2-ethylhexyl) sulfosuccinate (AOT) (Lot no.456162/1, Fluka, Seelse, Germany)
Sodium hydroxide (Lot no.B247498 249, Merck, Darmstadt, Germany)
12-Hydroxystearic acid- polyethylene glycol polymer (Solutol HS 15) (Lot no.59-1768, BASF, Ludwigshafen, Germany)

Membrane:-

Pig skin was generously purchased from market in Chainat, Thailand

Equipment:-

Analytical balance (AG 204, Metter Toledo, Switzerland)
High Performance Liquid Chromatography (HPLC) (LC 10, Shimadzu, Japan)
HPLC column (Aquasil C18, Thermo Hypersil, UK)

Light microscope attached with camera and polarize microscope (Eclipse, Nikon, Japan)

Micropipette (822 0200, Socorex, Swithzerland)

Multipoint magnetic stirrer (MPS-9, Biosan, Latvia)

pH meter (210, Thermo orion, USA)

Scanning electron microscope (JSM-5410 LV, JEOL electron microscope, Japan)

Transmission electron microscope (JEM-2100, JEOL electron microscope, Japan)

UV visible spectrophotometer (V530, Jasco, Japan)

Viscometer (Bookfield DV –II+, Bookfield Engineering Laboratories, Inc, USA)

Vortex mixer (VELP cod.F 20220170, Scientifica, Europe)

Water bath (28L/8/SH/C, Polyscience co., Ltd., USA)

Method

1. Determination of solubility of glucosamine hydrochloride

Solubility of glucosamine hydrochloride (GS HCl) was determined by dissolving an excess of GS HCl in 2 ml of defined solvent (water, ethanol, phosphate buffer solution pH 7.4 and isopropyl myristate (IPM). The dispersion was heated at 60°c for 15 minutes then equilibrated at 32°c for 48 hours (http://www.skin-forum.org.uk/abstracts/ismaeil-tekko.php). The supernatant was withdrawn and analyzed for GS HCl by HPLC method as described in section 3.3.

2. Preparation of transdermal formulations

In this study, four types of dosage forms were investigated for the effect of formulation components on permeation of GS HCl across pig skin. There were aqueous solution, hydroalcoholic solution, gel and microemulsion which were formulated as the following.

2.1 Glucosamine hydrochloride solution

GS HCl solution was prepared by dissolving GS HCl in water. The formulations of GS HCl are shown in Table 3-1.

Table 3-1 Glucosamine hydrochloride solution composition (batch size 20 g.)

Composition (%)	Formulation		
	F1	F2	F3
Glucosamine HCl	0.5	2	10
Water	99.5	98	90

2.2 Glucosamine hydrochloride hydroalcoholic solution

GS HCl hydroalcoholic solution dosage form were prepared by mixing ethanol and water, then dissolving GS HCl in this hydroalcoholic solution to obtain a clear solution. The formulations of GS HCl hydroalcoholic solution are shown in Table 3-2.

Table 3-2 Glucosamine hydrochloride hydroalcoholic solution composition (batch size 20 g.)

Composition (%)	Formulation	
	F4	F5
Glucosamine HCl	2	2
Ethanol	9.8	58.8
Water	88.2	39.2

2.3 Glucosamine hydrochloride gel

GS HCl gel was prepared by dispersing HPMC 15 cP and HPMC 5000 cP in boiled water while undergoing continuous stirring. GS HCl was dissolved in water and then this drug solution was slowly added to HPMC dispersion. The mixture was stirred until clear gel was obtained. The formulations of GS HCl gel are shown in Table 3-3.

Table 3-3 Glucosamine hydrochloride gel composition (batch size 20 g.)

Composition (%)	Formulation
	F6
Glucosamine HCl	2
HPMC 15 cP	2
HPMC 5000 cP	3
Water	93

The pH and viscosity of the solution, hydroalcoholic solution and gel were measured and reported.

2.4 Glucosamine hydrochloride microemulsion

2.4.1 The pseudo-ternary phase diagram

In preliminary studies the water titration method was used to select the surfactants, which can produce the microemulsion when isopropyl myristate (IPM) was used as oil phase in the system. The surfactants used in this study were (1) AOT anionic surfactant; (2) CTAB cationic surfactant; (3) zwittering ionic surfactant lecithin (PC) and (4) non-ionic surfactant, cremophore EL, Lutrol E400 Lutrol F68, Lutrol F127, span 20, span 40, span 60, solutol HS 15 and Tween 80. The amounts of each surfactant (10 g.) and IPM (10 g.) were kept constant and mixed together with an amount of water that was titrated to the mixture. Each addition was thoroughly mixed for one minute using magnetic stirrer and observed for microemulsion formation. If microemulsion was shown, the surfactants would be selected to form the pseudo-ternary phase diagram.

The pseudo-ternary phase diagram were prepared by keeping constant the amount of water, IPM and the selected surfactant according to Table 3-4. The clear-turbid boundaries and the viscosity were observed by naked eyes for each system; and the results were plotted in the pseudo-ternary phase diagram as shown in Figure 3-1.

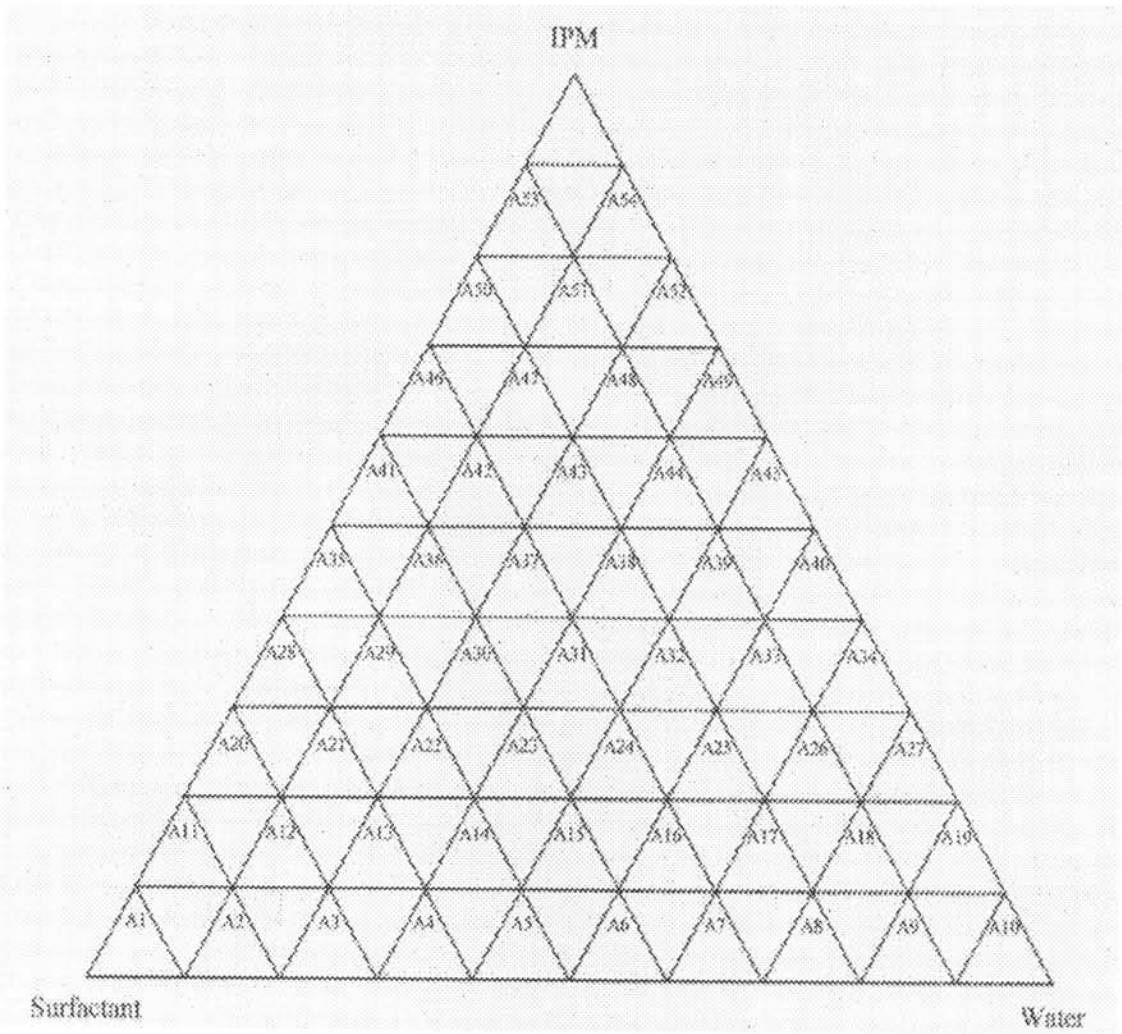


Figure 3-1 The pseudo-ternary phase diagram for plotting the results

Table 3-4 The amount (g.) of IPM, water and surfactant for preparation the pseudo-ternary phase diagram

Number	IPM	Surfactant	Water	Number	IPM	Surfactant	Water	Number	IPM	Surfactant	Water
A1	1.0	9.0	-	A19	2.0	-	8.0	A37	5.0	3.0	2.0
A2	1.0	8.0	1.0	A20	3.0	7.0	-	A38	5.0	2.0	3.0
A3	1.0	7.0	2.0	A21	3.0	6.0	1.0	A39	5.0	1.0	4.0
A4	1.0	6.0	3.0	A22	3.0	5.0	2.0	A40	5.0	-	5.0
A5	1.0	5.0	4.0	A23	3.0	4.0	3.0	A41	6.0	4.0	-
A6	1.0	4.0	5.0	A24	3.0	3.0	4.0	A42	6.0	3.0	1.0
A7	1.0	3.0	6.0	A25	3.0	2.0	5.0	A43	6.0	2.0	2.0
A8	1.0	2.0	7.0	A26	3.0	1.0	6.0	A44	6.0	1.0	3.0
A9	1.0	1.0	8.0	A27	3.0	-	7.0	A45	6.0	-	4.0
A10	1.0	-	9.0	A28	4.0	6.0	-	A46	7.0	3.0	-
A11	2.0	8.0	-	A29	4.0	5.0	1.0	A47	7.0	2.0	1.0
A12	2.0	7.0	1.0	A30	4.0	4.0	2.0	A48	7.0	1.0	2.0
A13	2.0	6.0	2.0	A31	4.0	3.0	3.0	A49	7.0	-	3.0
A14	2.0	5.0	3.0	A32	4.0	2.0	4.0	A50	8.0	2.0	-
A15	2.0	4.0	4.0	A33	4.0	1.0	5.0	A51	8.0	1.0	1.0
A16	2.0	3.0	5.0	A34	4.0	-	6.0	A52	8.0	-	2.0
A17	2.0	2.0	6.0	A35	5.0	5.0	-	A53	9.0	1.0	-
A18	2.0	1.0	7.0	A36	5.0	4.0	1.0	A54	9.0	-	1.0

2.4.2 Drug incorporation

The selected formulations from the "clear" region of the pseudo ternary-phase diagram which were stable, not separated into two phases, were studied for drug loading.

For GS HCl in AOT and lecithin microemulsion, the drug solution (aqueous phase) was slowly added to the blend of surfactant, n-butanol (co-surfactant that was used in system of lecithin microemulsion) and IPM (oil phase) under continuous agitation until the microemulsion was formed. N-butanol was introduced to the system of lecithin so that microemulsion could form.

For GS HCl in CTAB and Tween 80 microemulsion, the drug in aqueous solution was added to the blend of surfactant, n-butanol (co-surfactant that was used in system of CTAB microemulsion) and water (aqueous phase) under continuous agitation. Then IPM (oil phase) was slowly added and the dispersion was stirred until the microemulsion was formed.

The result of drug loading was used to determine the drug content in the formulations to study their potential as drug delivery system.

2.4.3 Evaluation of microemulsion dosage form

2.4.3.1 Physical properties

Appearance

The color and clarity of GS HCl microemulsions were observed with naked eyes after preparation for 1, 3, 7 and 30 days for any change in the physical appearance of these GS HCl microemulsions.

Viscosity

The viscosities of GS HCl microemulsions were measured with Brookfield DV –II+ (Spindle number 63 and 64), at ambient temperature under varying shear rate, and 70-80% touge range.

pH

The pH of GS HCl microemulsions were measured with pH meter.

2.4.3.2 Determination the type of microemulsion

Dye technique

The type of the GS HCl microemulsion was determined by adding 1% Brilliant blue, a water soluble dye, into the microemulsion. If the dye disperses homogenously in the microemulsion, the type of the microemulsion is considered as oil in water microemulsion.

Polarized light microscopy

Liquid crystalline phase in GS HCl microemulsions were examined under polarized light microscope after preparation. A small amount (two drops) of each sample was transferred onto a glass slide and covered with a cover slip. The appearance of the sample was observed between crossed polarizers.

Transmission electron microscopy (TEM)

The size and morphology of GS HCl microemulsions as described above were observed using a JEOL electron microscope. The GS HCl microemulsions samples were fixed in 1% phosphotungstic acid (PTA) before being viewed under the transmission electron microscope.

3. Permeation through pig ear skin

The *in vitro* permeation study was used as a tool for determining the most suitable system, which could provide the highest permeability coefficient and flux of GS HCl for delivery GS HCl through skin.

3.1 Skin source and preparation of skin membrane

Fresh pig-ears were collected from a local market in Chainat province, Thailand. Pig - ear skins were cleaned with water and prepared by heating them in a water bath at 60°C for 45 seconds. Then the whole skin was removed carefully from the underlying cartilage. The skin was cleaned and rinsed with the phosphate buffer saline pH 7.4 (PBS). The skin specimen was then cut into the size of 4x4 cm^2 wrapped in aluminium foil and stored at -20°c for up to 7 days (Dick and Scott, 1992). The frozen skin was thawed to room temperature prior to use.

3.2 *In vitro* permeation study

The *in vitro* permeation study of GS HCl formulation system across pig ear skin was conducted using franz-diffusion cells. The experiment was carried out in six replicates for each formulation. The diffusion cell possessed an available diffusion area of 2.72 cm^2. The amount of sample apply to pig skin membrane were 2 g. The receptor compartment of diffusion cell was filled with phosphate buffer saline pH 7.4 (PBS). The fact that the volume of some of the receptor compartment varied slightly, the weight of the filled volume was used in calculation. The receptor medium was maintained at a constant temperature of 32°C and stirred at speed of 900 rpm. The excised skin was mounted between the donor and the receptor compartment. The sample of 3 ml was collected at 0, 3, 12, 18 and 24 hours and 3 ml of fresh medium was replaced after the sample was collected. The samples were then analyzed for GS HCl by HPLC.

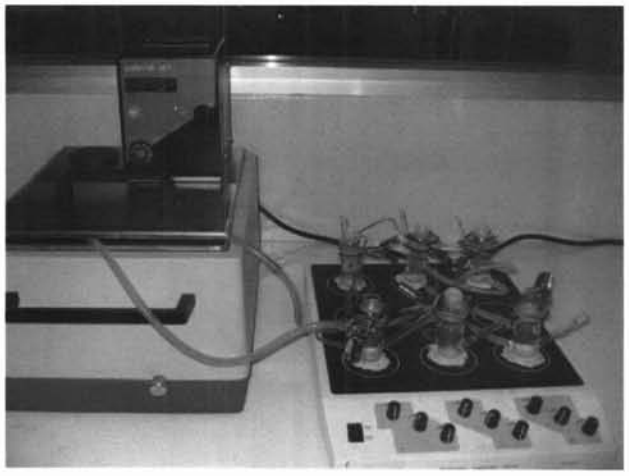
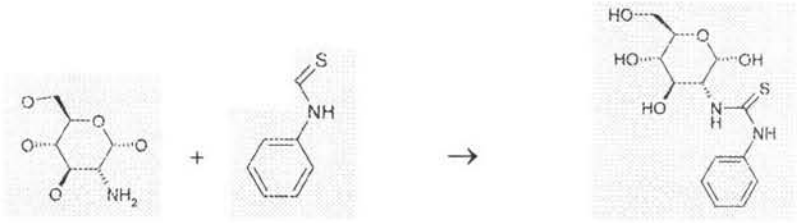


Figure 3-2 Modified Franz-diffusion cells for *In vitro* permeation studies

3.3 High-Performance Liquid Chromatographic Technique for drug analysis

The maximum UV wavelength of GS HCl is relatively low at 190 nm. In preliminary studies it was found that composition of the formula could interfere with

the absorbance of GS HCl at this wavelength. Therefore, the HPLC method was developed and validated.



Glucosamine PITC phenylthiocarbonyl-glucosamine

Figure 3-3 Glucosamine and phenylisothiocyanate reaction

According to the equation, the derived product of GS HCl and phenyisothiocyanate (PITC) is phenylthiocarbonyl-glucosamine which has maximum wavelength at 245 nm. The analyses of this derivative can avoid the interference of the formulation composition.

3.3.1 High –Performance Liquid Chromatographic Technique for glucosamine hydrochloride analysis by pre -column PITC derivatization

GS HCl standard solutions were prepared from an aqueous stock solution in concentrations of 1.25, 2.5, 12.5, 25, 125 and 250 μg/ml

Paracetamol, used as intenal standard were prepared from a methanolic stock solution of paracetamol 1 mg/ml, and by dilution to produce solution of 0.1 mg/ml. Phenylisothiocyanate (PITC) were freshly prepared in methanol at 100 μg/ml immediately before the derivatisation step.

Derivatisation procedure

Four hundred microlitre of sample were transferred into a 1 ml centrifugal tube. Then, 250 microlites of 0.1 M sodium acetate and 200 microlites of methanol were added, shaken and left for 15 minutes before 250 μl of PITC methanolic solution was added. This solution was then vortexed for 30 seconds before being placed in a water bath at 80°c for 30 minutes. The samples were then left at room temperature.

Then, 100 microlitre of 0.1 mg/ml paracetamol internal standard and 200 microlites of n-hexane were added. This solution was vortexed for 1 minute. Then the lower part of this solution was removed for analysis by HPLC.

Instrumentation and chromatographic conditions

Separation of phenylthiocarbonyl-glucosamine and paracetamol internal standard adducts from other compounds in the formulation, was achieved on a reverse -phase column 250×4.6 mm internal diameter (Aquasil C18) using a high performance liquid chromatography system. Sample of 20 µl were injected. Analysis was carried out using acetonitrile : water : phosphoric acid (10/90/0.1 v/v/v) as the mobile phase delivered at 1.5 ml/min. The UV detector was operated at 245 nm.

After 10-12 injections, the column was flushed with 100% acetonitrile to remove unreacted PITC build up on column. Flushing of column was carried out for 15 minutes.

3.3.2 Validation for the quantitative determination of glucosamine hydrochloride released by HPLC

The parameters evaluated to ensure the acceptability of the performance of the selected analytical method were specificity, linearity, precision and accuracy.

3.3.2.1 Specificity

Under the condition selected for *In vitro* GS HCl release studies, the peaks of other components must not interfere with the peak of GS HCl. The validation was made by comparing the chromatogram of GS HCl, internal standard and the receptor solution taken from receptor compartment.

3.3.2.2 Linearity

Linearity regression analysis of the absorbance versus the corresponding concentration was performed, and the coefficient of the determination was calculated

3.3.2.3 Precision

Within run precision

The within run precision was determined by analyzing in six replicates (n=6) for each of three concentrations of GS HCl standard solution, prepared in the receptor solution on the same day. The percent coefficient of variation (% CV) for each comcentration was calculated.

Between run precision

The between run precision was determined by analyzing six replicates (n=6) for one concentration of GS HCl standard solution, prepared in the receptor solution for two days. The percent coefficient of variation (% CV) for each concentration was calculated.

3.3.2.4 Accuracy

The accuracy was determined by analyzing in three replicates (n=3) for each of three concentrations of GS HCl in placebo microemulsion. The percent recovery of glucosamine for each concentration was calculated.

3.4 Determination of glucosamine hydrochloride permeability in the skin.

The amount of permeated GS HCl was calculated by multiplying GS HCl concentration with the receptor volume. For each skin specimen, drug permeated was

plotted against time. The permeability coefficient (k_p, cm/sec) of GS HCl was calculated from equation.

$$k_p = \frac{Jss}{c_d} = \frac{1}{A} \cdot \frac{\Delta Q}{\Delta t} \cdot \frac{1}{c_d}$$

Where A is the effective area (cm^2)

c_d is the saturated solubility of Glucosamine in the microemulsion system (μg/ml)

Q is the cumulative mass of Glucosamine that passes through the membrane in time t (μg)

t is time (second)

3.5 Determination the effect of Glucosamine HCl microemulsion on the pig-ear skin membrane by light microscope

The effects of GS HCl microemulsion on pig-ear skin was viewed under light microscope (LM). The skin material that had been obtained from same ear and prepared the same in the permeation study as described above. The skin sample were divided into three groups: the fresh pig-ear skin, experimental and control groups. The control group is the skin that was treated with PBS 7.4 instead of microemulsion experiment. The control and experimental group were taken to franz-diffusion cells and mounted between the donor and the receptor compartment. The phosphate buffer pH 7.4 and GS HCl microemulsion were applied on the skin sample respectively. Receptor medium, temperature, the stirring speed were kept constant as those in *in vitro* permeation study described previously. The time of study was 24 hours. After that the skin sample were prepared for observation under the light microscope.