CHAPTER III

MATERIALS AND METHODS

Materials

1. Ketoprofen powder (Menarini Co., Ltd. gifted from Biopharm Co., Ltd., Thailand)
2. Tween 80 (BDH Laboratory supplies, England)
3. Propylene glycol (S. Tong Chemical Co., Ltd., Bangkok, Thailand)
4. L-Lactic acid (Wako Chemical Inc., Japan)
5. Ethanol (Merck Co., Ltd., Germany)
6. Triethanolamine (May and Baker Ltd., England)
7. Methanol (LAB-SCAN Ltd., Inc., Ireland)
8. Acetonitrile (LAB-SCAN Ltd., Inc., Ireland)
9. Acetic acid (Merck Co., Ltd., Germany)
10. Monobasic potassium phosphate (Merck Co., Ltd., Germany)
11. Dibasic potassium phosphate (BDH Laboratory supplies, England)
12. Sodium chloride (Vidhayasom Co., Ltd., Thailand)
13. Hydroxypropylmethylcellulose E4M (Colorcon Co., Ltd., USA)
14. Carbopol 980 NF (BF Goodrich Co., Ltd., USA)
15. Carbopol 2020 ETD (BF Goodrich Co., Ltd., USA)
16. Full thickness abdominal Wistar rat skin

Apparatus

1. High Performance Liquid Chromatography (HPLC, HP1100, Vectra XM series 4, Hewlett Packard, USA)
2. Chromatographic column (Neucleosil 100-5,C18, 5 μm, size 125x4.0 mm., Lot number 7224, Hewlett Packard, USA)
3. pH meter Model 345 (Mettler Co.,Ltd., UK)
4. Sonicator (Vibra cell, Sonic & Material Inc., USA)
5. Centrifuge (Beckman Avanti ™ 30 Centifuge, USA)
6. Micrometer (Fowler®)
7. Modified Franz® diffusion cell (Japan, Figure 4)
8. Brookfield Viscometer DVIII (Brookfield Engineering Laboratories Inc., USA)
9. UV spectrophotometer Spectronic, Genesys 5 (Milton Roy Co.,Ltd., USA)

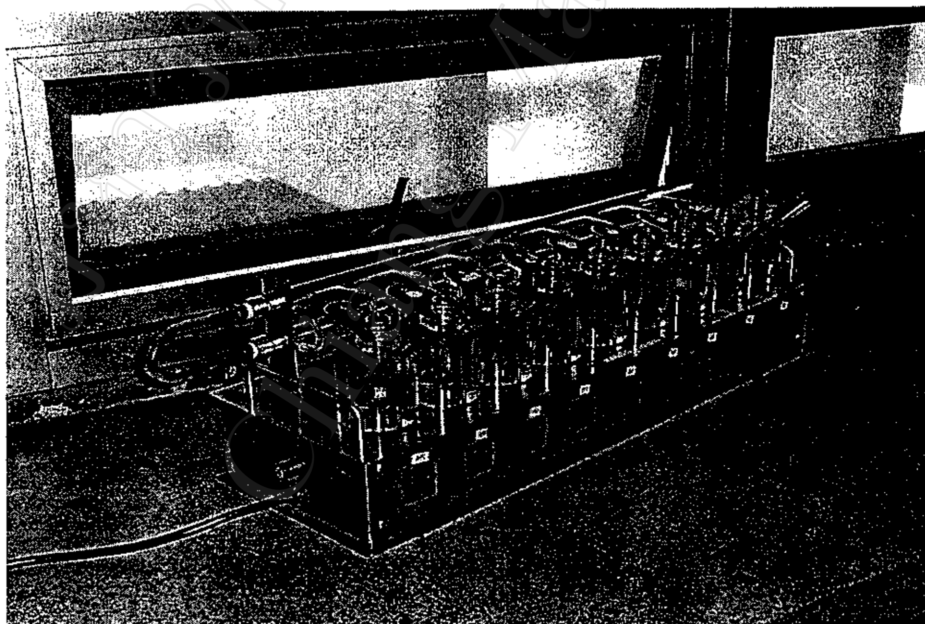

Figure 4 Modified Franz® diffusion cells

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Methods

1. Preparation of KP Gels

KP gels were prepared in two steps as follow.

1.1 Preparation of 5%w/w Gel Base

The 5% (w/w) gel bases were prepared in the composition shown in Table 2. The gelling agent powder was slowly added into hot water while stirring continuously. Triethanolamine(TEA) was added to the well-dispersed Carbopol® mixture until the gel was formed. After that, the Carbopol® gel base was kept in the refrigerator for 24 hours before use. In the case of HPMC mixture, the dispersion was kept in the refrigerator for 24 hours before use.

Table 2 Formulation of gel bases

Components (g)	Formulation	
	Carbopol®	HPMC
Carbopol®	5.0	0.0
HPMC	0.0	5.0
Triethanolamine	2.0	0.0
DI water qs.	100	100

1.2 Preparation of KP Gels

KP gels were prepared by dissolving KP in ethanol (ETOH) and then the single or mixed enhancers were added to the drug solution. The enhancers used were

propylene glycol (PG), Tween® 80 (TW80), L-lactic acid (L-LA). Next, the KP solution was incorporated into the gel base and adjusted to desired pH using TEA.

All of KP gels were kept at ambient temperature for 24 hours before measuring the pH, viscosity, drug content and in vitro permeability.

The following variables were studied:

1. Difference gelling agents: CBP2020, CBP980, and HPMC.
2. Various concentrations of gelling agents: 1.5, 2, and 3%w/w of CBP2020 and CBP980. In case of HPMC only 2 and 3%w/w of HPMC were used.
3. Various concentrations of ETOH were added to the formulations.
4. Gel with various pHs were prepared by adding different concentrations of TEA.
5. Single or combined penetration enhancers of different concentration were added to the gel formulations.

All the KP gel formulations are shown in Table 3.

2. Solubility of KP

The solubility of KP was determined by adding excess amounts of KP powder into 3 ml of deionized water, 5%w/w PG, 0.5%w/w TW80, and 1%w/w LA in deionized water. The solution was stirred continuously at 25±2 °C for 48 hours. The saturated solutions were then centrifuge at 15,000 rpm for 15 minutes. One ml of the supernatant was diluted with 24 ml of deionized water and the concentration of KP was measured by UV spectrophotometer.

Table 3 Formulations of KP gels

Formulation	Gelling Agents	Composition (g)							
		KP	ETOH	GA	PG	TW80	L-LA	TEA	DI water qs
F-1	C2	2.5	35.5	3	0	0	0	2.7	100
F-2	C2	2.5	35.5	2	0	0	0	2.3	100
F-3	C2	2.5	35.5	1.5	0	0	0	2.1	100
F-4	C9	2.5	35.5	3	0	0	0	3.2	100
F-5	C9	2.5	35.5	2	0	0	0	2.8	100
F-6	C9	2.5	35.5	1.5	0	0	0	2.6	100
F-7	HP	2.5	35.5	3	0	0	0	1.5	100
F-8	HP	2.5	35.5	2	0	0	0	1.5	100
F-9	HP	2.5	30	2	0	0	0	1.5	100
F-10	HP	2.5	40	2	0	0	0	1.5	100
F-11	HP	2.5	30	2.5	0	0	0	0	100
F-12	HP	2.5	30	2.5	0	0	0	1	100
F-13	HP	2.5	30	2.5	0	0	0	1.5	100
F-14	HP	2.5	30	3	0	0	0	1.5	100
F-15	HP	2.5	30	3	5	0	0	1.5	100
F-16	HP	2.5	30	3	0	0.5	0	1.5	100
F-17	HP	2.5	30	3	0	0	1	1.5	100
F-18	HP	2.5	30	3	5	0.5	0	1.5	100
F-19	HP	2.5	30	3	5	0	1	1.5	100
F-20	HP	2.5	30	3	5	0.5	1	1.5	100

Note; GA is Gelling agent, PG is propylene glycol, TW80 is Tween®80, L-LA is L-Lactic acid , C2 is Carbopol® 2020 ETD, C9 is Carbopol® 980 NF, HP is HPMC E4M, TEA is Triethanolamine, and DI water is deionized water.

3. Spectrophotometric Assay

3.1 Analysis of KP in methanol

The calibration curve of KP in methanol was constructed by adding 10 mg of KP dissolved in methanol and the volume was adjusted to 100 ml. The concentration of the standard solution was 100 μg/ml. The standard solution was diluted with methanol to give the KP concentrations of 1, 2, 4, 6, 8, 10, 12 and 14 μg/ml. The amount of KP was determined by UV spectrophotometer at 255 nm. The calibration curve was plotted between the absorbance versus the concentration of KP in standard solution. The estimated linear regression line, as shown in Table 4, was used to calculate the content of KP in KP gels.

3.2 Analysis of KP in deionized water

KP was weighed to 5 mg dissolved in deionized water and the volume was adjusted volume to 100 ml. The concentration of standard solution was 50 μg/ml. The standard solution was diluted with deionized water to give the KP concentrations of 2, 5, 10, 15, and 20 μg/ml. The standard solutions were measured at 255 nm by UV spectrophotometer. The calibration curve was plotted between the absorbance of KP versus the concentration of KP in standard solution The estimated linear regression line, as shown in Table 4, was used to calculate the amount of KP in the solution (solubility).

Table 4 Estimated linear regression line and correlation coefficient of KP in methanol and deionized water

solvents	Estimated linear regression line	Correlation coefficient
Methanol	$Y = 0.067X + 0.0469$	0.9975
Deionized water	$Y = 0.072X - 0.0042$	0.9999

Where X = KP concentration in μg/ml

Y = Absorbance of KP

4. High Performance Liquid Chromatographic Assay

The amount of KP permeated through rat skin was analyzed by high performance liquid chromatography (HPLC). HPLC parameters are as follows:

Column	Neucleosil 100-5, C18
Column size	125 x 4 mm.
Particle size	5 μm
Mobile phase	Acetonitrile : DI water : Acetic acid, ratio 70 : 26 : 4
Flow rate	1.5 ml/min
Absorbance	255 nm
Injection volume	20 μl
Temperature	Ambient temperature
Pressure	144 bar
Retention time	2.000 ± 0.200 min
Run time	3 min
Method	External standard

The calibration curve was constructed by adding 10 mg of KP in phosphate buffer saline pH 7.4 (PBS). Its volume was adjusted to 100 ml. The concentration of stock solution was 100 μg/ml. The stock solution was diluted with PBS to prepare concentrations 0.2, 0.5, 1, 2, 4, 6, 8, 10, 12, 14, 16, 30, 50 and 100 μg/ml. The standard solutions were filtered through a 0.45 μm membrane filter before analysis by HPLC. The standard curve was obtained by plotting the area under the peak as a function of KP concentration. The estimated linear regression line was used to calculate the permeated of KP through Wistar rat skin from KP gels.

5. Evaluation of KP gels

5.1 Drug Content Analysis.

A known amount of gel was placed in a 25 ml volumetric flask. Methanol was added to dissolve the gel and adjusted to the volume. The drug content was determined spectrophotometrically. The assay was carried out in triplicate.

5.2 The pH Measurement

The pH of KP gel was directly measured using pH meter three times at room temperature and the average pH was reported.

5.3 Viscosity Measurement

The exactly amount of gel was placed in the chamber of small sample adapter of Brookfield viscometer and it was sheared under the following conditions:

Spindle number	SCS-28
Temperature	25$\pm$0.5 ^{o}C
Speed	50 rpm
ShearingTime	1 min

6. *In vitro* Permeation of KP

6.1 Preparation of Skin Membrane

The skin membrane was obtained from male Wistar rats, weight 180-250 g. The abdominal hair was removed using electric clipper one day before the excision. The full-thickness skin was excised and cleaned with normal saline. Excess fat was

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carefully removed from the skin using scissors. The skin was free of obvious defects. Each skin membrane was wrapped with plastic wrap, sealed in a vacuum plastic bag, and stored at –40°C until used (not more than one month). The skin was thawed at room temperature before mounted individually between the donor and receiver compartment of a diffusion cell.

6.2 Preparation of Receiver Medium

The PBS, which used as a receiver medium was prepared by dissolving 1.816 grams of monobasic potassium phosphate in deionized water, and then 4.40 grams of sodium chloride and 9.292 grams of dibasic potassium phosphate were added. The solution volume was adjusted to 1000 ml with deionized water. The pH of this solution was 7.4±0.05.

6.3 Permeation Experiments

A vertical diffusion cell was utilized for the *in-vitro* permeation experiment. The modified Franz® diffusion cell consisted of two compartments; the donor compartment and the receiver compartment. The capacity of the receiver compartment was 10.3 ml. Full-thickness skin membrane samples were carefully mounted between the two compartments except where the experiment studied the effects of temperature, a cellophane membrane was used as a barrier. The stratum corneum of the skin faced upward into the donor compartment and the dermal faced downward into the receiver compartment. The receiver compartment was filled with PBS and stirred with constant stirring at a rate of 600 rpm at 25±2 °C. A known weight sample of KP gel was placed in the donor compartment in contact with skin.

One ml of sample solution was withdrawn periodically (10, 20, 30, 60, 90, 120, and 180 minutes) from the receptor compartment and the same volume of PBS was

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added after sampling. The KP concentration in each sample was then determined by HPLC. All permeation experiments were carried out in triplicate and the results are expressed as mean ±SD.

7. Stability Studies

7.1 Heating and Cooling Stability Studies

All KP gels, in well-closed jars, were kept at 45 °C in a hot air oven for 48 hours and then cooled to 4 °C in a refrigerator for 48 hours for each testing cycle. They were kept for six cycles. Then the drug content (%w/w), pH and viscosity (cP) were analyzed. This procedure was done in triplicate.

7.2 At Room Temperature Stability Studies

All KP gels were kept at room temperature (25-27°C) for six months. Then the drug content (%w/w) was analyzed. This experiment was measured in triplicate.

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