CHAPTER III

MATERIALS AND METHODS

Materials

Reagents

1. Acetonitrile HPLC grade (Labscan Asia Co., Ltd., Ireland) Batch No. 01120107

2. Diclofenac sodium (Department of Pharmacy) Lot No. DFSJ 036

3. Dimethyl Isosorbide (DMI), (Donated from East Asiatic Pubic Company., Ltd., Thailand)

4. Gelatin powder (Srichand United Dispensary Co., Ltd., Thailand) Lot No. GA 1143

5. Glacial acetic acid AR grade (E. Merck, Germany) Lot No. K 14090663

6. Hard gelatin capsule No.0 (Donated from Capsule Product Co., Ltd., Thailand)

7. Hydroxy propylmethylcellulose (Methocel$^®$ E5), (Donated from Rama Production Co., Ltd., Thailand)

8. Ketoprofen (Biolab Co. Ltd., Thailand), potency 100.25%, Lot No. 1997 17605 A

9. Methanol HPLC grade (Labscan Asia Co., Ltd., Ireland) Batch No. 2 K 070103

10. Oruvail$^®$ (Olic Co., Ltd., Thailand) Batch No. 10602

11. Polyethylene glycol 1500 (PEG 1500), (S. Tong Chemical Co., Ltd., Thailand)

12. Polyoxyethylene (20) Sorbitan Monooleate (Tween 80$^®$), (Donated from East Asiatic Pubic Company., Ltd., Thailand)

13. Polyoxyethylene (20) Sorbitan Monostearate (Tween 60$^®$), (Donated from East Asiatic Pubic Company., Ltd., Thailand)

14. Potassium dihydrogen phosphate (E. Merck, Germany) Lot No. A 315973 127

15. Propylene glycol (PG), (S. Tong Chemical Co., Ltd., Thailand)

16. Sodium acetate trihydrate GR (E. Merck, Germany) Lot No. A 404865

17. Sodium chloride (Fluka, Switzerland) Lot No. EEC 2315983

18. Sodium hydroxide (Mallinckrodt Baker, Mexico) Lot No. 7708 MVHV

19. Triethyl citrate (TEC), (Fluka, Switzerland) Lot No. 371165/1

20. 95% Ethanol (Excise Department, Thailand)

Apparatus

1. Analytical balance (Sartorius, Germany)
2. Centrifuge (Labofuge 610, Heraeus-Christ GMBH, Germany)
3. Desiccator
4. Disintegration apparatus (Model ZT 31, Erweka, Germany)
5. Dissolution apparatus (Sotax AT 7, Switzerland)
6. Fluidized Bed Coater (Aeromatic, Model STREA, Nitro, Switzerland)
7. High performance liquid chromatography (LC-10 AD)
   - Communications bus module (CBM-10A, Shimadzu, Japan)
   - Pumps (LC-10A)
   - UV detector (SPD-10A)
   - Autosampler (SIL-10A)
8. Hygro-thermometer (Sato Keiryoki MFG. Co., Ltd., Japan)
9. Incubator (Memmert U 10, Memmert GMBH, Germany)
10. Micropipette (Eppendorf, Germany)
11. pH meter (Beckman 50, Beckman Instrument, Inc., USA)
12. Spectrophotometer (Jasco 7800, Jasco Corp., Japan)
13. Speed vacuum concentration (Maxi Dry Plus, Heto, Denmark)
14. Ultrasonic bath (Transonic Digital, Diethelm & Co., Ltd., Germany)
15. Vortex mixer (Vortex Genies-2, Scientific Industries, Inc., USA)

Experimental animals

Twelve male white New ZeaLand rabbits, weighing between 2.6-3.8 kg were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornprathom, Thailand. They were housed individually per cage at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok and acclimatized for at least 1 week before the experiment. All of them were allowed freely to assess standard
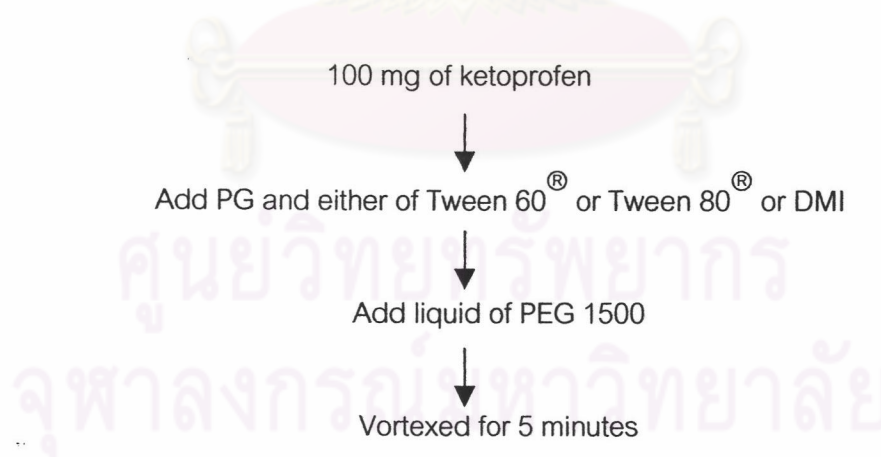
food (C.P. Co., Ltd., Thailand) and drinking water. Light / dark period and temperature were controlled at 12/12 hour cycle and $25^{o}C$, respectively.

Methods

A. Formulation of ketoprofen liquid – filled hard gelatin capsule.

Procedures of ketoprofen liquid preparation

One hundred milligrams of ketoprofen was transferred into 10 mL test tube. Propylene glycol (PG) and either of polyoxyethylene (20) sorbitan monostearate (Tween 60®) or polyoxyethylene (20) sorbitan monooleate (Tween 80®) or dimethyl isosorbide (DMI) were added. Then, polyethylene glycol 1500 (PEG 1500) was heated until it became liquid. PEG 1500 liquid was added into test tube containing ketoprofen and specified vehicles. The mixture was blended together using vortexed mixer about 5 minutes. All liquid formulae were prepared, on a weight by weight (W/W) basis, the amount of ingredients used in each system were presented in Table 1. The schemes of preparing was described as follows.

100 mg of ketoprofen

↓

Add PG and either of Tween 60® or Tween 80® or DMI

↓

Add liquid of PEG 1500

↓

Vortexed for 5 minutes

Physical evaluation for selection of the formula for capsule filling

The characteristics of each liquid formula was observed after consecutively 24 hours storing at room temperature, in refrigerator and at room temperature, respectively.

Table 1. Liquid formulae of 100 mg Ketoprofen

Formula No.	The amount of cosolvent (100 mg) PEG1500 : PG : A*	Formula No.	The amount of cosolvent (150 mg) PEG1500 : PG : A*	Formula No.	The amount of cosolvent (200 mg) PEG1500 : PG : A*
1	80:10:10	109	120:15:15	214	160:20:20
2	70:10:20	110	105:15:30	215	140:20:40
3	70:20:10	111	105:30:15	216	140:40:20
4	60:10:30	112	90:15:45	217	120:20:60
5	60:20:20	113	90:30:30	218	120:40:40
6	60:30:10	114	90:45:15	219	120:60:20
7	50:10:40	115	75:15:60	220	100:20:80
8	50:20:30	116	75:30:45	221	100:40:60
9	50:30:20	117	75:45:30	222	100:60:40
10	50:40:10	118	75:60:15	223	100:80:20
11	40:10:50	119	60:15:75	224	80:20:100
12	40:20:40	120	60:30:60	225	80:40:80
13	40:30:30	121	60:45:45	226	80:60:60
14	40:40:20	122	60:60:30	227	80:80:40
15	40:50:10	123	60:75:15	228	80:100:20
16	30:10:60	124	45:15:90	229	60:20:120
17	30:20:50	125	45:30:75	230	60:40:100
18	30:30:40	126	45:45:60	231	60:60:80
19	30:40:30	127	45:60:45	232	60:80:60
20	30:50:20	128	45:75:30	233	60:100:40
21	30:60:10	129	45:90:15	234	60:120:20
22	20:10:70	130	30:15:105	235	40:20:140
23	20:60:20	131	30:30:90	236	40:40:120
24	20:30:50	132	30:45:75	237	40:60:100
25	20:40:40	133	30:30:60	238	40:80:80
26	20:50:30	134	30:75:45	239	40:100:60
27	20:60:20	135	30:90:30	240	40:120:40
28	20:70:10	136	30:105:15	241	40:140:20
29	10:10:80	137	15:15:120	242	20:20:160
30	10:20:70	138	15:30:105	243	20:40:140
31	10:30:60	139	15:45:90	244	20:60:120
32	10:40:50	140	15:60:75	245	20:80:100
33	10:50:40	141	15:75:60	246	20:100:80
34	10:60:30	142	15:90:45	247	20:120:60
35	10:70:20	143	15:105:30	248	20:140:40
36	10:80:10	144	15:120:15	249	20:160:20

The criteria for selection of the formula from those with each cosolvent for capsule filling was based on:

1. The formulation with clear solution was formed.
2. The formulation without precipitate when temperature was changed.
3. The formulation with the least amount of propylene glycol was employed.

   A* = either polyoxyethylene 20 sorbitan monostearate (Tween 60[®])

   or polyoxyethylene 20 sorbitan monooleate (Tween 80[®])

   or dimethyl isosorbide (DMI)

B. *In Vitro* studies

Three hundred milligrams of selected liquid formula was transferred into each capsule body No.0 using dropper, and individual cap was then secured manually by hand. The release pattern of ketoprofen from hard gelatin capsules was studied according to the following method in order to select the best formulae.

1. Release characteristics of ketoprofen from hard gelatin capsules

Since an official *in vitro* release method is not available for the test of drug release from rectal dosage form. In this study, *in vitro* release of the drug from ketoprofen capsule was thus, carried out using the USP paddle dissolution apparatus (USP 24). Each capsule was placed in the sinker and immersed into a flask containing 1,000 mL of 0.05 M phosphate buffer solution (pH 7.4) equilibrated at 37 ± 0.5^{o}C. The paddle was rotated at 50 rpm. A 5 mL of sample was withdrawn at appropriate time intervals and equal volume of warmed dissolution medium at 37°C was replaced at once to maintain a constant volume. The concentrations of ketoprofen were quantified using a calibration curve. The release versus time profiles of ketoprofen from ketoprofen capsules was constructed.

Calibration curve: 50 mg of ketoprofen was accurately weighed and transferred into a 50 mL volumetric flask. The drug was dissolved and adjusted to volume with methanol to produce the stock solution. Standard ketoprofen solutions with known concentrations of 3, 4.6, 6.2, 7.8, 9.4, 11, 12.6, 14.2 and 15.8 µg/mL were then prepared by dilution of the stock solution with phosphate buffer pH 7.4 The drug was assayed by UV spectrophotometry at the maximum wavelength of 260 nm.

2. Selection of the best formulation from those with each cosolvent

Only two formulations were selected for further *in vivo* study. One was from those with group of polyoxyethylene sorbitan fatty acid ester and another was from formulations with dimethyl isosorbide. The criteria for selection was as follows:

2.1 The formulation with fast release of ketoprofen within 30 minutes

2.2 The formulation with the least amount of Tween 60$^®$, Tween 80$^®$ or DMI

For the two selected liquid formulae, again, 300 mg of each liquid formula was transferred into each capsule body No.0 using dropper, and individual cap was then secured by hand. Then, these capsules were sealed and coated, respectively according to following method.

C. Sealing and coating of hard gelatin capsule

1. Sealing of hard gelatin capsule

Gelatin solution was used to uniformly seal at the contact area of body and cap by manual painting to prevent leaking of the filled liquid. The gelatin solution was prepared by dissolving 5 g of gelatin powder in 15 mL of hot water and stirred until gelatin powder was dissolved.

2. Coating of hard gelatin capsule

2.1. Preparation of film coating solution

Cellulose coating solution was prepared by dissolving 5% of hydroxypropyl methylcellulose polymer (HPMC) in the mixture of ethanol: purified water (1:1). One percent of triethyl citrate (TEC) was incorporated as plasticizer. The solution was stirred at least one hour and kept over night in order to complete swelling (Peeracha Thanawnttanawanich, 1999).

2.2. Coating capsule with fluidized bed coater

Liquid – filled hard gelatin capsules of 45 g were coated in each batch using bottom spraying with the coating solution. The capsule were warmed for 5 minutes in stainless steel container, with inlet temperature set up at 45°C when outlet temperature reached 40°C spraying was then operated. The coating solution was continuously fed into a spraying nozzle by a peristaltic pump at flow rate of 6 - 8 mL/min. Atomized pressure was approximately 1.1 bar with fluid bed pressure setting at No.9 - 10. These conditions were found to be optimal because there were no blockage of the spray nozzle, no aggregate of capsule, no sliding between body and cap and completely dried. Total coating time was about 25 minutes for one batch. After finishing, capsules were dried in the chamber for 5 minutes (Peeracha Thanawnttanawanich, 1999).

The two formulations of coated ketoprofen rectal capsule were reevaluated for its specification and performance using the following tests; dissolution profile, disintegration time, uniformity of content, stability and *in vivo* study.

3. Evaluation of physical and chemical properties of coated ketoprofen rectal capsule

3.1. Dissolution profile of coated capsule

They were determined as described in B (1).

3.2. Disintegration time.

Determination of disintegration time was based on BP disintegration test for rectal capsule (BP 1999) using disintegration testing apparatus. Six capsules were test by placing a capsule in each tube of the basket, then immersed in water maintained at $37 \pm 2^{\circ}C$. A disk was added to each tube to prevent floating of capsule. Disintegration was considerably achieved when gelatin shell was ruptured, allowing release of the content. Average disintegration time and standard deviation of 6 capsules were calculated.

3.3. Uniformity of content

Content of ketoprofen of each of 10 capsules taken at random was determined using the method of BP 1993. It was analyzed by sampling one capsule and dissolving in 50 mL of 75% methanol. A 0.5 mL of the supernatant liquid was diluted to 10 mL with 75% methanol and the absorbance of the resulting solution was measured at the maximum wavelength of 258 nm. The final absorbances of all samples were converted to ketoprofen concentrations using the calibration curve.

Calibration curve: 50 mg of ketoprofen was accurately weighed and transferred into a 50 mL volumetric flask. The drug was dissolved and adjusted to volume with 75% of methanol to produce the stock solution. Standard solutions with known concentrations of 3, 5, 7, 9, 11, 13 and 15 µg/mL were then prepared by dilution the stock solution with 75 % of methanol.

D. Stability of coated ketoprofen rectal capsule

The two selected formulations of coated hard gelatin capsules were kept in tight, light-resistant glass vials. This preparation was stored over saturated sodium chloride

solution in the desiccator, which would give the 75% RH (Nyqvist, 1983). The desiccator was then stored in the incubator at 40°C for 3 months.

The rectal capsule was evaluated for physical appearance and the average content of ketoprofen was determined by HPLC method at 0, 0.5, 1, 1.5, 2, 2.5 and 3 months. The procedure was described as follows.

1. Preparation of sample

The amount of ketoprofen in each of six selected capsules was determined by dissolving each capsule in 100.0 mL of 50% ethanol. After centrifugation 0.5 mL of supernatant was transferred into 10 mL volumetric flask. 2.0 mL of 1000 μg/mL of diclofenac sodium was added as an internal standard. Finally the solution was adjusted to volume with mobile phase.

3. Chromatographic condition

Column	:	μBondapak C18 with particle size of 10 μm, 300 x 3.9 mm i.d.
Mobile phase	:	Acetonitrile: sodium acetate buffer pH 4.2 = 1:1
Flow rate	:	1.0 mL/min
Injection volume	:	20 μL
Detector	:	UV, 260 nm
Attenuated	:	2^3
Retention time	:	About 6.5 min for ketoprofen
		About 10.0 min for diclofenac sodium
Temperature	:	Ambient

3. Calibration curve

100 mg of ketoprofen was accurately weighed and transferred into a 100 mL volumetric flask. The drug was dissolved and the solution was adjusted to volume with 50 % of ethanol to produce the stock solution. Standard ketoprofen solutions with known concentrations of 10, 20, 30, 40, 50, 60 and 70 µg/mL were then prepared by dilution of stock solution with mobile phase.

4. Assay validation

The high performance liquid chromatographic (HPLC) technique of Panvipa Tuntisak (1993) was modified for analysis of ketoprofen remaining in preparations.

4.1 Accuracy of method

Three sets of quality control samples (low, medium and high) of ketoprofen solution were prepared for analysis of ketoprofen in term of percent recovery. Percent recovery of each concentration was calculated from the ratio of inversely estimated concentration to known concentration of ketoprofen multiplied by 100. The accuracy was determined using three determinations per concentration.

4.2 Within run precision

Within run precision was determined by analyzing three sets of quality control samples (low, medium and high) in the same day. The percent coefficient of variation (% C.V) of the estimated concentration of ketoprofen of each concentration was determined. The precision was determined using three determinations per concentration.

4.3 Between run precision

Between run precision was determined by comparing the estimated concentration of ketoprofen of three sets of quality control samples (low, medium and high) for three different days. The percent coefficient of variation (% C.V.) of each concentration was determined.

Acceptance criteria:

For accuracy, the percent recovery should be within ± 15 percent of each nominal concentration whereas the percent coefficient of variations for both the within run and between run should be less than 2 percent (Wainer, 1985).

E. *In Vivo* studies

1. Experimental rectal capsule

Following the previously mentioned *in vitro* evaluation, two formulations of 100 mg coated ketoprofen rectal capsule were selected for *in vivo* bioavailability evaluation in rabbits. Both formulations were freshly prepared and conformed to the requirements for percent labeled amount and uniformity of content. The ketoprofen formulation (100 mg/ 2 mL) for intramuscular injection (Oruvail[®]) was assigned as a reference product for bioavailability study.

2. Subjects and drug administration.

Twelve male white New Zealand rabbits, weighing between 2.6 - 3.8 kg were used as subjects in this study. They were acclimatized to the research facility for one week prior to the study. Each of them received a single dose of ketoprofen after being fasted for 24 hours with freely water ingestion. For the intramuscular injection formulation, only 50 mg of ketoprofen could be administered because the rabbits could not tolerate with the dose excessively than this.

3. Experimental design

The study was conducted in a randomized crossover manner. One of each formulation was given to each subject with a washout period at least one week between each treatment.

4. Sample collection

3 mL of blood sample was collected from a marginal ear vein using a disposable syringe and immediately transferred to heparinized tubes containing 20 μL of 5,000 I.U./mL heparin solution. Blood samples were collected immediately before drug administration and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8,10 and 12 hours post dose. They were immediately centrifuged at 5,000 rpm for 10 minutes. The plasma was separated and kept at -20°C until subsequent analysis.

5. Determination of ketoprofen in plasma

Concentrations of ketoprofen in plasma were determined by high performance liquid chromatographic method modified from that described by Panvipa Tuntisak (1993). The procedure was described as follows.

5.1 Preparation of plasma sample

0.5 mL of plasma sample

Add 20 μL of water containing
9 mg/mL of diclofenac sodium
as an internal standard

Vortexed for 10 seconds

Add 2.0 mL of acetonitrile

Centrifuged for 20 minutes at 5,000 rpm

20 μL of supernatant was injected into HPLC

5.2 Chromatographic condition

Column	:	µBondapak C18 with particle size of 10 µm, 300 x 3.9 mm.i.d.
Mobile phase	:	Acetonitrile: Sodium acetate buffer pH 4.2 = 1:1
Flow rate	:	1.0 mL/min
Injection volume	:	20 µL
Detector	:	UV, 260 nm
Attenuated	:	2^3
Retention time	:	About 6.3 min for ketoprofen About 9.6 min for diclofenac sodium
Temperature	:	Ambient

The area under the peak of ketoprofen and that of the internal standard were calculated by the integrator. Peak area ratios of ketoprofen to the internal standard were then determined. Concentrations of ketoprofen in plasma samples were quantified using a calibration curve.

5.3 Calibration curve

Five hundred milligrams of ketoprofen was accurately weighed and transferred into 50 mL volumetric flask. The drug was dissolved and the solution was adjusted to volume with the mixture of 1:1 acetonitrile and water. The solution was used as stock solution. Standard solutions with known concentration of 50, 1000, 2000, 3000, 4000, 5000, 6000 and 7000 µg/mL were then prepared by dilution of the stock solution with a mixture of 1:1 acetonitrile and water. An exactly 20 µL of each standard solution was individually added to 0.48 mL of pooled rabbit plasma to produce the plasma concentrations of 2, 40, 80, 120, 160, 200, 240 and 280 µg/mL, respectively. These plasma standards were finally clarified and analyzed following the same procedure as

mentioned previously. The peak area ratio of ketoprofen to that of diclofenac sodium were calculated.

Calibration curve was constructed by fitting the peak area ratios of ketoprofen to that of diclofenac sodium against ketoprofen concentrations using linear regression method.

6. Assay validation

The analytical methods for determination of ketoprofen in rabbit plasma were validated under the following conditions for accuracy and precision (Shah et al, 1992).

6.1 Accuracy, in term of percent analytical recovery, was determined by analyzing three sets of quality control samples (low, medium and high) of ketoprofen prepared in rabbit plasma. Percent analytical recovery of each concentration was calculated from the ratio of inversely estimated concentration to known concentration of ketoprofen multiplied by 100. The accuracy was determined using three determinations per concentration.

6.2 Within run precision was determined by analyzing three sets of quality control samples (low, medium and high) in the same day. The percent coefficient of variation (% C.V) of estimated concentration of ketoprofen of each concentration was determined. The within run precision was determined using three determinations per concentration.

6.3 Between run precision was determined by comparing the estimated concentration of ketoprofen of three sets of quality control samples (low, medium and high) for three different days. The percent coefficient of variation (% C.V.) of estimated concentration of ketoprofen of each concentration was determined. The between run precision was determined using three determinations per concentration.

Acceptance criteria:

For accuracy, the percent recovery should be within $\pm$ 15 percent meanwhile the percent coefficient of variation for both the within run and between run should be less than 15 percent (Shah, 1992).

7. Pharmacokinetic analysis

The relevant pharmacokinetic parameters of ketoprofen from each treatment following rectal administration of two formulations of 100 mg coated ketoprofen capsule and intramuscular administration of 50 mg Oruvail[®] were derived from the plasma ketoprofen concentration–time profiles. Peak plasma ketoprofen concentration (C_{max}) and time to peak plasma ketoprofen concentration (t_{max}) were directly observed from the data. Area under the plasma ketoprofen concentration-time curve (AUC) was calculated by linear trapezoidal rule up to the last quantifiable time point and extended to infinite time by adding with C^* / K_{el} term, where C^* was the last measurable concentration and K_{el} was the terminal elimination rate constant. The elimination rate constant (K_{el}) was obtained from slope of the plasma drug concentration – time curve in semilogarithmic scale. The elimination half-life ($t_{1/2}$) was calculated by $0.693 / K_{el}$.

7.1 Statistical evaluation of pharmacokinetic parameters

The comparisons of the corresponding pharmacokinetic parameters of the two formulations of 100 mg coated ketoprofen rectal capsules were established employing a two way analysis of variance according to complete randomize block design at $\alpha = 0.05$. In this analysis, subjects were assigned as block.

7.2 Bioavailability evaluation

Due to unequal doses of the test and reference formulations were given, bioequivalence evaluation was, therefore unable to assess. The relative bioavailability of

individual formulated formulation relatively to the intramuscular injection was determined instead.