CHAPTER III

MATERIALS AND METHODS

I. Materials

1. Reagents

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

1.2 Eudragit S-100 (Pharma Polymer, Germany), Lot No. 1951205603.

1.3 Suppocire® AM (Rhodia Co.Ltd, Bangkok, Thailand), Lot No. 4226-2.

1.4 Diclofenac sodium (Department of Pharmacy), Lot No. DFSJ036.

1.5 Polyethylene glycol 4000 (ICI Surfactants, Bangkok,Thailand), Lot No. K702.

1.6 Polyethylene glycol 6000 (NOF Corporation, Japan),Lot No.712207.

1.7 Polyethylene glycol 1500 (BASF Co.Ltd, Germany), Lot No. 32-2662.

1.8 Hydroxypropyl methylcellulose phthalate (HP55), (Shin-Etsu Chemical Co.Ltd, Japan), Lot No. 411461.

1.9 Ethanol AR grade (BDH Labolatory Supplies, England), Lot No. L768107.

1.10 Acetone AR grade (Labscan Ltd, Ireland), Lot No. 98 09 1020.

1.11 Methanol AR grade (Labscan Ltd, Ireland), Lot No. 98 06 0049.

1.12 Acetonitrile HPLC grade (Labscan Ltd, Ireland), Lot No. 98 08 0006.

1.13 Methanol HPLC grade (Labscan Ltd, Ireland), Lot No. 97 10 0011.

1.14 Sodium hydroxide GR (E. Merck, Darmstadt, Germany), Lot No. B870498.

1.15 Potassium dihydrogen phosphate GR (E. Merck, Darmstadt, Germany), Lot No. A987363.

1.16 Sodium acetate trihydrate GR (E. Merck, Darmstadt, Germany), Lot No. A404865.

1.17 Chloroform AR grade (BDH Laboratory Supplies, England) Lot No. K20567741.

1.18 Glacial acetic acid AR grade (E. Merck, Darmstadt, Germany), Lot No. K23679303.

2. Apparatus

2.1 Analytical balance (Sartorius,Germany).

2.2 Dissolution apparatus (Sotax AT7, Switzerland)

2.3 Spectrophotometer (Jasco 7800, Jasco Corp., Japan).

2.4 pH meter (Beckman 50, Beckman Instrument, Inc., USA).

2.5 Centrifuge (Labofuge 610, Heraeus-Christ GMBH, Germany).

2.6 Vortex mixer (Vortex Genies-2, Scientific Industries, Inc., USA).

2.7 High performance liquid chromatography (HPLC) equipped with the following.

2.7.1 A turnable absorbance detector (Waters, USA).

2.7.2 A constant flow pump (570, Waters, USA).

2.7.3 An integrator (745B, Waters, USA).

2.7.4 An autoinjector (712WISP, Waters, USA).

2.7.5 μ-Bondapak C_{18} stainless steel column (30 cm x 3.9 mm I.D., 10 μm packing) and μ-Bondapak C_{18} guard column (Waters, USA).

2.8 Hot air oven (Memmert 100, Memmert GMBH, Germany).

2.9 Micropipette (Eppendorf, Germany).

2.10 Ultrasonic bath (Transonic Digital, Diethelm & Co. Ltd, Germany).

2.11 Speed vacuum concentrator (Maxi Dry Plus, Heto, Denmark).

2.12 Incubator (Memmert U10, Memmert GMBH, Germany).

II. Methods

A. Formulations of Ketoprofen Rectal Suppositories.

1. Selection of suppository bases.

1.1 Hydrophilic suppository bases.

Three hydrophilic suppository bases were selected from Remington's Pharmaceutical Sciences:

Base 1, R_x

polyethylene glycol 1000	96 %
polyethylene glycol 4000	4 %

Base 2, R_x

polyethylene glycol 1000	75 %
polyethylene glycol 4000	25 %

Base 3, R_x

polyethylene glycol 1500	70 %
polyethylene glycol 6000	30 %

1.2 Hydrophobic suppository bases.

For ketoprofen suppository commercially available, the Suppocire® AML was used as suppository bases.This base was not available in Thailand. In order to obtain a formulation which was closely similar to the commercial one, the Suppocire®AM which was a semisynthetic glyceride was used as Base 4 as reference base.

2. Preparation of rectal suppositories.

2.1 Determination of the displacement value

The displacement value of ketoprofen, Eudragit S-100 and hydroxypropyl methylcellulose phthalate (HP55) were determined in each hydrophilic suppository base and only ketoprofen was determined in Suppocire®AM (Appendix A).

2.2 Preparation of rectal suppositories

Each suppository contained ketoprofen 100 mg. 20 rectal suppositories of each formulation were prepared. Methods of preparation depended upon types of suppository base and prolonged release carriers (poorly water soluble carriers). They were described as follow:

2.2.1 Conventional rectal suppositories

These rectal suppositories were those with three hydrophilic (Base 1, Base 2 and Base 3) and one hydrophobic (Suppocire®AM) suppository bases.

They were prepared by fusion method in water bath at 48°C for PEG mixtures and 48°C for Suppocire®AM. Ketoprofen was added after molten base was formed and stirred until nearly to congeal. The mixtures were then poured into steel mold and allowed to solidify in room temperature.

2.2.2 Matrix rectal suppositories

The matrix suppositories were formulated using various proportions of ketoprofen to prolonged release carrier weight by weight in each conventional hydrophilic suppository base. They were prepared by fusion method. Method of preparation depended on types of prolonged release carrier.

2.2.2.1 Eudragit S-100

The proportions of ketoprofen to Eudragit S-100 were 1:1, 1:1.5, and 1:2, respectively. The method of preparation was described as follow:

The PEG mixtures were heated at 60°C in water bath. After melting, the specific portion of Eudragit S-100 was added and stirred until clear homogeneous molten mixtures were formed. Ketoprofen (100 mg) was incorporated into the Eudragit S-100 – PEG molten mixtures and continued stirring nearly to congeal. The mixtures were poured into steel molds and allowed to solidify at room temperature.

2.2.2.2 Hydroxypropyl methylcellulose phthalate (HP55)

The proportions of ketoprofen to HP55 were 1:3, and 1:4, respectively. The method of preparation was described as follow:

The PEG mixtures were heated in a thermostatic oven at 140 °C. After melting, the specific portion of HP55 was added. With occasional stirring until clear homogeneous molten mixtures were formed. Ketoprofen was then added into the HP55 – PEG molten mixtures and stirred until nearly to congeal. The mixtures were poured into steel molds and allowed to solidify at room temperature.

After preparing, all suppositories were wrapped individually in aluminum foil and stored in the refrigerator at 4°C .

B. *In Vitro* studies.

1. Evaluation of physical and chemical properties of suppositories.

The prepared rectal suppositories in each formulation were observed for their appearance and evaluated for uniformity of weight and uniformity of content according to the BP 1993.

1.1 Uniformity of weight.

Weigh individually 20 suppositories taken at random and determine the average weight. Not more than two of the individual weight deviate from the average weight by more than 5 % and none deviated by more than 10%.

1.2 Uniformity of content.

Determine the content of ketoprofen of each of 10 suppositories taken at random using a suitable analytical method. The preparation being examined complied with the test if not more than one of the individual values thus obtained is outside the limits 85 – 115% of the average value and if none is outside the limits 75 – 125% of the average value. If two or three individual values are outside the limits 85 – 115% of the average value and none is outside the limits 75 – 125%, repeated the determination using another 20 suppositories taken at random. The preparation being examined complied with the test if, in the total sample of 30 suppositories, not more than three individual values are outside the limits 85 – 115% and none is outside the limits 75 – 125% of the average value.

The analytical methods used for determination of ketoprofen were developed and depended on types of suppository base and poorly water soluble carriers employed for preparing of ketoprofen rectal suppositories as follows:

1.2.1 Conventional rectal suppositories.

1.2.1.1 Hydrophilic suppository bases

The content of ketoprofen in each suppository was determined by dissolving each suppository in phosphate buffer pH 7.2 at 37°C in water bath and adjusted to volume in a 50 mL volumetric flask. A portion of 0.5 mL of the solution was transferred and adjusted to volume in the other 10 mL volumetric flask and finally 1 mL of the diluted solution was transferred again and adjusted to

volume in a 10 mL volumetric flask. This final solution was assayed by UV spectrophotometry at the maximum wavelength of 260 nm.

1.2.1.2 Hydrophobic suppository bases. (Suppocire ® AM)

The content of ketoprofen was determined following the same method as described in 1.2.1.1 except chloroform was used instead of phosphate buffer pH 7.2 and the final solution was assayed by UV spectrophotometry at the maximum wavelength of 255 nm.

1.2.2 Matrix rectal suppositories.

1.2.2.1 Eudragit S-100

The content of ketoprofen was determined following the same method as described in 1.2.1.1 except methanol was used instead of phosphate buffer pH 7.2 and the solution was assayed by UV spectrophotometry at the maximum wavelength of 255 nm.

1.2.2.2 Hydroxypropyl methylcellulose phthalate (HP55)

Each suppository was dissolved in acetone and 0.5 mL of the solution was transferred into 10 mL test tube. Acetone was then evaporated by Maxi Dry Plus at 40°C for 3 hours. The residue was reconstituted with methanol and adjusted to volume in a 10 mL volumetric flask. A portion of 0.5 mL was then transferred and adjusted to volume in a 10 mL volumetric flask. The final solution was assayed by UV spectrophotometry at the maximum wavelength of 255 nm.

Only solution of hydroxypropyl methylcellulose phthalate (HP55) in methanol gave absorbance value at the wavelength of 255 nm whereas others did not. Therefore the blank suppository containing only HP55 was prepared and determined for the absorbance value to be used to obtaining the net absorbance of

the samples. The final absorbances of all samples were converted to ketoprofen concentrations using the calibration curve.

Calibration curve: 30 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 solutions with known concentrations of 3.2, 4.8, 6.4, 8.0, 9.6, 11.2, 12.8 and 14.4 μg/mL were then prepared by dilution of the stock solution with phosphate buffer pH 7.2 for hydrophilic suppository base, and with methanol for Eudragit S-100 and HP55.

In case of Suppocire® AM, chloroform was used as solvent to prepare the stock solution. The calibration curve was prepared as follow:

30 mg of ketoprofen was accurately weighed and transferred into a 50 mL volumetric flask. The drug was dissolved and adjusted to volume with chloroform to produce the stock solution. Standard solutions with known concentrations of 2.4, 4.8, 7.2, 9.6 and 12.0 μg/mL were then prepared by dilution of the stock solution with chloroform.

1.3 Validation of analytical methods for *in vitro* studies.

Validations of the quantitative determination of ketoprofen from all types of suppositories were performed. The accuracy in term of percent recovery and precisions in term of percent coefficient of variation of the analytical methods were evaluated (Vanderwielen and Hardwidge, 1982).

1.3.1 Accuracy.

Three sets of the calibration curves were analyzed by spectrophotometry at specified wavelengths. Inversely estimated concentrations were determined and the percent recovery of each concentration was calculated.

1.3.2 Precision.

1.3.2.1 Within run precision.

Within run precision was determined by analyzing three sets of calibration curves in the same day. Absorbance values of ketoprofen were compared, and the percent coefficient of variation (%C.V.) of each concentration was calculated.

1.3.2.2 Between run precision.

Between run precision was determined by analyzing three sets of the calibration curves on different days. Absorbance values of ketoprofen were compared and the percent coefficient of variation (%C.V.) of each concentration was calculated.

2. Release characteristics of ketoprofen from rectal suppositories.

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 rectal suppository was thus, carried out using the USP rotating basket dissolution apparatus. Each suppository was placed in the basket and immersed into a flask containing 900 mL of phosphate buffer solution (pH 7.2) equilibrated at 37±0.5 °C. The basket 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 rectal suppositories were constructed.

Calibration curve: The calibration curve to be used for determining the concentrations of ketoprofen were those used for calculation of uniformity of content of hydrophilic and hydrophobic rectal suppositories.

The release rate constant of ketoprofen from suppository was determined by sigma-minus method.

3. Selection of the best formulation from those with each prolonged release carrier.

Only two formulations were selected for further *in vivo* study. One was from those with Eudragit S-100 and another was from various formulations with hydroxypropyl methylcellulose phthalate (HP55). The criteria for selection was as follows:

1. The formulation with prolonged release of ketoprofen within 6-8 hours, decided by the time used to completely release of ketoprofen and release rate constant.

2. The formulation with the least amount of poorly water soluble carrier.

C. *In Vivo* studies.

1. Experimental suppositories.

Three formulations of ketoprofen rectal suppositories were *in vivo* evaluated. The first two formulations were prolonged release ketoprofen rectal suppositories which one with Eudragit S-100 and another with hydroxypropyl methylcellulose phthalate (HP55) as the prolonged release carrier that had been selected. The third formulation was that the conventional hydrophobic suppositories prepared using Suppocire®AM. All formulations were freshly prepared and conformed the requirements for uniformity of weight and uniformity of content. The one with Suppocire® AM was assigned as reference product for bioavailability comparison.

2. Subjects and Drug administration.

Six male and three female New Zealand White 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 rectal suppository after being fasted for 24 hours with water *ad libitum*.

3. Experimental design.

The study was conducted in a crossover fashion using a repeated Latin square design, which had the general rule that every subject received all number of formulations. One suppository of each formulation was given rectally to each subject with a washout period at least one week between each treatment, as shown in Table 3.

Table 3 A three way crossover design for *in vivo* study.

Sequence	Subject	Treatment in Period		
		I	II	III
I	1,2,3	A	B	C
II	4,5,6	B	C	A
III	7,8,9	C	A	B

Where A = Eudragit S-100; B = Suppocire® AM and C = Hydroxypropyl methylcellulose phthalate (HP55).

4. Sample collection.

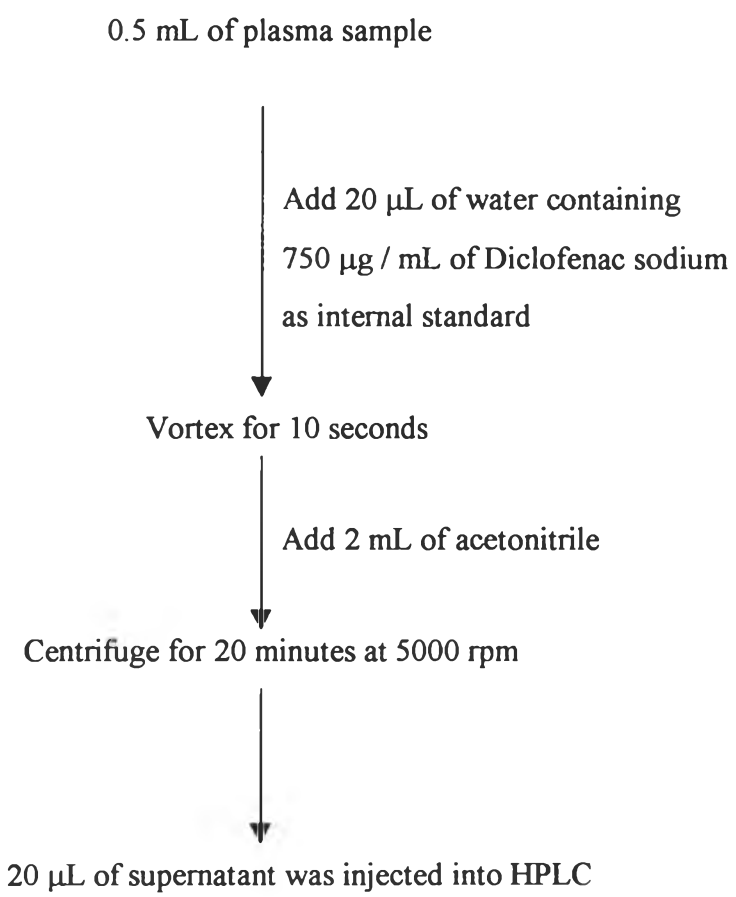
3 mL of blood sample was collected from a marginal ear vein using a disposable syringe and immediately transferred to heparinized tubes containing 20 µL of 5000 I.U. / mL of heparin solution. Blood samples were collected immediately before drug administration and at 0.5, 1, 2, 3, 4, 6, 8, 10, 14,18 and 24 hours post

dose. They were immediately centrifuged at 5000 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 (1993). The procedure was described as follows.

5.1 Preparation of plasma sample.



5.2 Chromatographic condition.

Column	:	Bondapak C18 with particle size of 10µm, 300 x 3.9 mm.

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	:	2^4
Retention time	:	about 5.65 min for ketoprofen. about 8.17 min for diclofenac sodium.
Temperature	:	ambient.

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

5.3 Calibration curve.

500 mg of ketoprofen was accurately weighed and transferred into 50 mL volumetric flask. The drug was dissolved and adjusted to volume with mixtures of acetonitrile and water ratio 1:1. This solution was used as stock solution. Standard solutions with known concentration of 50, 125, 250, 500, 1250, 2500, 3750 and 5000 μg/mL were then prepared by dilution of the stock solution with a mixture of acetonitrile and water. An exactly 20 μL of each standard solution was individually added to 0.5 mL of pooled plasma to produce the plasma concentrations of 2, 5, 10, 20, 50, 100, 150 and 200 μg/mL, respectively. These plasma standards were finally clarified and analyzed following the same procedure as mentioned previously. The peak area ratios 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 known drug concentrations to a straight line using linear regression.

6. Assay validation.

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

6.1 Accuracy in term of percent recovery was determined by analyzing three sets of calibration curves of ketoprofen prepared in rabbit plasma. Percent recovery of each concentration was calculated from the ratio of inversely estimated concentration to known concentration of ketoprofen multipiled by 100.

6.2 Within run precision was determined by analyzing three sets of calibration curves in the same day. The percent coefficient of variation (%C.V.) of the peak area ratios of ketoprofen to the internal standard of each concentration was determined.

6.3 Between run precision was determined by comparing the peak area ratios of ketoprofen to the internal standard of three sets of calibration curves for three different days. The percent coefficient of variation (%C.V.) of each concentration was determined.

Acceptance criteria:

For accuracy, the percent recovery should not less than 80 meanwhile the percent coefficient of variations for both the within run and between run are not greater than 15.

7. Pharmacokinetic analysis.

The relevant pharmacokinetic parameters of ketoprofen from each treatment following administration of 100 mg prolonged release ketoprofen rectal suppositories were derived from the plasma ketoprofen concentration–time profiles. The peak

plasma ketoprofen concentration (C_{max}) and the time to peak plasma ketoprofen concentration (t_{max}) were directly observed from the data. The 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 (US FDA, 1992). The elimination rate constant (K_{el}), the half-life ($t_{1/2}$), the volume of distribution (V_d) and the clearance (CL) were calculated using non-compartmental method (Gibadi and Perrierr. 1982).

8. Statistical evaluation of pharmacokinetic parameters.

The comparisons of all three formulations of 100 mg prolonged release ketoprofen rectal suppositories were established employing the corresponding pharmacokinetic parameters among all formulations by mean of a three way analysis of variance at $\alpha = 0.05$ (Weiner and Yuh. 1994). If the results showed statistically significant difference, the difference of those values between each pair of treatment would be examined by Duncan's New Multiple Range test.

9. Evaluation of bioequivalence.

The 90% confidence interval (two one-sided test) for the differences of C_{max} and AUC means based on log transformed data of the formulations with Eudragit S-100 and HP55 relative to the one with Suppocire ®AM were constructed (Dighe and Adams. 1991). They were considered to be bioequivalent with the reference formulation when each 90% confidence interval was within 80-125% (Weiner and Yuh. 1994).