CHAPTER III

EXPERIMENTAL

Materials

Chemicals:-

Cephalexin monohydrate BP , Batch no. FT1908, Swiss chemical, Swiss.

Methanol HPLC grade, Batch no. I424607, E. Merck, Germany.

Sodium dihydrogen phosphate, Batch no.143 A606846, E. Merck, Germany.

Sodium acetate, Batch no. 306 TA 404965, E. Merck, Germany.

Glacial acetic acid, Batch no. K14090663, E. Merck, Germany.

Acetonitrile HPLC grade, Batch no. B24P2300, J.T. Baker, USA.

Cyclohexane, Batch no. 2L 527092N, Carlo Erba, Italy.

Dichloromethane, Batch no. K.20245050 340, E. Merck, Germany.

Triacetin, Batch no. 326952/1 993, Fluka, Germany.

Triethyl citrate, Batch no. 328224/1 1293, Fluka, Germany.

Ethylcellulose, Batch no. MM92828-2, Colorcon, England.

Eudragit RL 100®, Batch no. 6732-80, Rohm pharma, Germany.

Eudragit RS 100®, Batch no. 6732-86, Rohm pharma, Germany.

All chemicals were analytical or pharmaceutical grades and were used as received.

Equipments:-

Modified Franz Diffusion apparatus.

pH meter, Model SA 520, Orion, USA.

Analytical balance, Sartorius GMPH, range 300g/mg, Germany.

Thermostatted shaker bath, Julabovsw1, Juchheim Labortechnik HG, Germany.

Vortex mixer, Scientific industries, Inc.

High Performance Liquid Chromatography, equipped with

- a tunable absorbance detector, Water 484 Model M484, Serial No. 484-PRA 902, USA.
- an automatic injector, Millipore, Water 712 WISP, Serial No. 712-007617, USA.
- a constant flow pump, Water 510 HPLC pump, Millipore, USA.
- an integrator, Water 745B Data Modules, Serial No. 7BE/400678, USA.
- a Spherisorb HPLC cartridge column phase separation S5 ODS 2(250 x 4.6 mm) , 5μ, Phase separation Co. Ltd., USA.
- UV Spectro photometer, Hitashi, Model U2000, Serial No. 0402-040, Japan.

Fluidized bed granulator, Glatt, Germany.

Spray dryer, Buchi 190 , Buchi Laborator-Techniques Co. Ltd., Switzerland.

Ultrasonic bath, Bransonic 221, Branson, Smithkline company, USA.

Hot air oven, Memmert, Germany.

Micrometer, Mitutoyo, 0-25 mm, 0.01, Mitutoyo MFG. Co. Ltd., Japan.

Scanning Electron Microscope, JSM T100, Joel Co. LTD., Japan.

Methods

1. Calibration Curve of Cephalexin Using UV Spectrophotometry.

Standard solutions containing an accurate amount of cephalexin RS (5, 10, 20, 30, 40 and 50 μg/ml) in water were prepared. Absorbances of cephalexin solutions were measured at a wavelength of 262 nm. The plot of absorbance versus known concentrations was made. The linearity of the relation between absorbance and concentration was determined by linear regression.

2. The Solubility of Cephalexin in Water at 33 ± 1°C.

The solubility of cephalexin was determined by UV spectrophotometry. An excess amount of cephalexin was added into a screw-capped tube containing 5 ml of water. The test tubes were vigorously shaken and swirled by using the vortex mixer and then placed in the thermostatted shaker bath for 24, 48 and 72 hours at a temperature of 33 ± 1° C. The supernatant solution was withdrawn and diluted to a suitable concentration. The absorbance was determined spectrophotometrically at 262 nm. All measurements were done in duplicate. The cephalexin concentration was calculated by using the calibration curve performed previously. From this solubility data the concentration of cephalexin was used for further study as a donor solution.

3. Preparation of Membranes

3.1 Preparation of ethylcellulose membranes

Membranes were prepared by using the 5 different plasticizers at five levels. The appropriate levels of plasticizer were determined during experiments including 0, 10, 20, 30 and 40% (w/w) plasticizer evaluated as a percentage of the ethylcellulose weight thus, for each membrane prepared, 1 g of ethylcellulose was used and the amount of plasticizer added was 0.1 g (10%), 0.2 g (20%), 0.3 g (30%), and 0.4 g (40%). The 5 different plasticizers were used in this study are polyethylene glycol 6000, polyethylene glycol 1450, castor oil, triacetin and triethyl citrate (Steurenagel, 1989).

An accurate weight of each plasticizer was added into 50 ml of ethanol and stirring for 5 minutes using a magnetic stirrer, 1 g of ethylcellulose was added into prior solution and stirring for 30 minutes to get a clear solution. Each of solution was casting on a glass plate. The ethanol was allowed to evaporate at one of 4 different temperatures for 24 hours by using a controlled-temperature hot air oven. The 4 different temparatures were room temperature (33 $\pm$ 1°C), 40°C, 50°C and 60°C. When a membrane was dried, it was then removed from the glass plate and was cut into uniform piece of circle which had diameter of 3.5 cm . Membrane thickness was determined using the micrometer. The selected membrane was equilibrated in 0.1% tween 80 in water for at least twelve hours at room temperature prior to use in diffusion study to help membrane wetability.

3.2 Preparation of Eudragit® membranes.

Membranes were prepared by varying the type and amount of plasticizers (which are 0, 10, 20, 30, and 40%), drying temperature as same as

preparation of ethylcellulose membranes. Two grams of Eudragit RL 100® and Eudragit RS 100® and the combination in various ratios (0:5, 1:4, 2:3, 3:2, 4:1, and 5:0) were seperately dissolved in 10 ml of acetone and homogeneously mixed with different amounts of the plasticizers and stirring for 15 minutes then casting on a teflon plate (Lin, Lee and Lin, 1991). The acetone was allowed to evaporated at one of 4 different temperatures which were room temperature (33±1°C), 40°C, 50°C, and 60°C for 24 hours. The polymeric membrane was removed and cut into uniform piece of circle by the same procedure as ethylcellulose membrane. Measuring of membrane thickness was done. The selected membrane was equilibrated in water at room temperature for 12 hours prior to use in diffusion study.

4. Evaluation of cast film.

The cast film were evaluated for different physical characteristics such as appearance, colour, transparancy, easily detachable, crack on folding and stickiness. Surface topography of cast film were obtained by using scanning electron microscope.

5. Diffusion Experiments.

A membrane was mounted and clamped in place between the donor and receptor compartments. The receptor fluid that was distilled water was carefully filled into receptor compartment to prevent air bubbles under the membrane. The receptor fluid had been continually stirred with a magnetic stirring bar rotating at 300 ± 5 rpm with syncronous motor. The temperature was maintained at 37 ± 1°C by thermostatically controlled water which was circulated through a jacket surrounding the receptor cell body throughout the time of diffusion studies. The receiving volumes varied between 12.214 and 12.745 ml (Fig. 10).

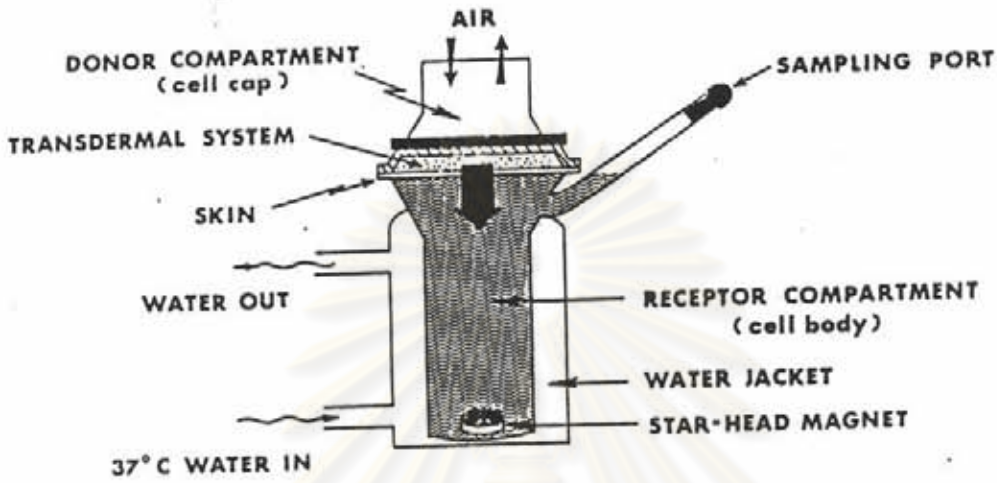

Fig. 10: Schematic of Modified Franz Diffusion Cell.

The membrane and the receptor solution were equilibrated and warmed for twenty minutes. After equilibration, an excess amount of the donor solution, which is 75% saturated concentration of cephalexin in water, was added on top of the membrane and the donor cell was covered with a parafilm to prevent the evaporation of donor vehicle. The ambient temperature of donor solution was about $33 \pm 1^{\circ}C$.

The sampling time during each study was continued for a sufficient time period to ensure that steady-state permeation was achieved and perfect sink conditions were maintained throughout experiment. At appropriate time intervals, the entire receiving solution was drawn using a syringe fitted with a piece of flexible tubing through sampling side arm of the cells. The receptor compartments

were immidiately replaced with fresh distilled water with an equal in volume and the run was continued. All drug concentrations in the receiving solution withdrawn was determined using the HPLC assay. The dilution of the samples was made with an appropriate volume of distilled water prior to analysis.

6. HPLC Analysis.

The liquid chromatography was composed of a constant flow pump (Water 510), a variable wavelength UV absorption detector (Water 484), and an integrater (Water 745 B). Injections were made, using an automatic injector (Water 712 WISP), with a fix 20 μl volume. An internal standard was used to determine the drug concentrations. The condition for analyzing cephalexin content by HPLC techniques is the followings.

Column	:	Spherisorb C18, 4.6 mn x 25 cm., 5μ.
Mobile phase	:	A mixture of 25% methanol and 75% 0.01 M sodium dihydrogen phosphate buffer, pH 5, by volume.
Detector wavelength	:	262 nm.
Flow rate	:	1.3 ml/min.
Internal Standard	:	0.25 μg/ml pyrazinamide in water.

6.1 Standard Solution.

6.1.1 Preparation of internal standard solution.

Twenty five mg of pyrazinamide was accurately weighed into a 50 ml volumetric flask. Water was added and the mixture was swirled unitl

pyrazinamide was completely dissolved and then adjusted to volume. One ml of this solution was transfered to a 200 ml volumetric flask and diluted with water to volume so that the final concentration of the stock solution of internal standard was 2.5 μg/ml.

One millitier of the 2.5 μg/ml internal standard was pipetted into a 10 ml volumetric flask of each concentration of cephalexin standard solution, then diluted to volume so the final concentration of internal standard before injected was 0.25 μg/ml.

6.1.2 Preparation of standard solutions.

Fifty mg of cephalexin RS was accurately weighed into a 100 ml volumetric flask. Water was added and the mixture was swirled unitl cephalexin was completely dissolved and then adjust to volume. One ml of this solution was transferred to another 100 ml volumetric flask and diluted with water to volume. The stock solution had a final concentration of 5 μg/ml (S1). Each concentration of cephalexin standard solution was prepared by pipetting S1 0.2, 0.4, 0.8, 1.6, 2.0, 3.0 and 5.0 ml to 7 volumetric flasks (10 ml) and made it to volume by amount of water. The final concentration of standard solutions were 0.1, 0.2, 0.4, 0.8, 1.0, 1.5 and 2.5 μg/ml, respectively.

6.1.3 Preparation of Sample Solutions.

Samples were diluted with an appropriate amount of water, if necessary, to obtain peak area ratios in the range of standard curvre. The appropriate amount of internal standard was added to the sample solution prior to the HPLC analysis.

7. Preparation of Cephalexin Microcapsules.

The membranes which had an appropriate release rate were selected as the walls of microcapsules. Ethylcellulose with 30% triacetin based on ethylcellulose weight gave a good sustained release properties , the combination of Eudragit RL 100® and Eudragit RS 100® in the ratios of 3:2 and 2:3 containing 20% triacetin as a plasticizer were prepare. The techniques used to prepare microcapsules were coacervation, fluidization and spray drying technique. These three techniques were investigated with the variation of the wall materials and the core:wall ratio which were described as follows.

7.1 Coacervation techniques

7.1.1. Ethylcellulose

Cephalexin microcapsules were prepared by modified coacervation technique described by Tirkonen S. and Paronen P. (1993) and Hasan M. et al (1992). Ethylcellulose (Ethocel® , 10 cps) was used as the wall material. With stirring at a rate of 1000 rpm ethylcellulose was added to cyclohexane solution containing 30% w/w triacetin evaluated as a percentage of the ethyl cellulose weight. The mixture was heated to 80°C. Ten grams of cephalexin was suspended in the solution, with the variation in the core : wall ratios. The ratio of cephalexin : ethylcellulose were 2:1, 1:1, and 1:2. The suspension was homogenized with homogenizer at a speed of 3000 rpm for 5 minutes then the suspension was allowed to cool slowly at a controlled stirring rate of 1000 rpm to 40°C for 1 hour. The mixture was then cooled on an ice bath to 25°C. The stirring was continued for a further 20 minutes. The microcapsules were then seperated from the solution by vacuum filtration on a Buchner funnel. The filtered microcapsules were then washed 3 times with 25 ml of fresh cyclohexane to remove any empty polymer

coats. The microcapsules were then collected and dried at room temperature for 12 hours and stored in a dessicator for further studies.

7.1.2 Combination of Eudragit RL 100® and Eudragit RS 100®

Eudragit RL 100® and Eudragit RS 100® in the ratio of 3:2 and 2:3, were used as the wall of microcapsules. Microencapsution of cephalexin was prepared by modifying the coacervation technique described by Watts, Devies and Melia (1991). Microcapsules were produced by using an emulsification-solvent evaporation technique. An accurate amount of Eudragit RL 100® combined with Eudragit RS 100® was dissolved in methylene chloride to produce a 10% w/v solution and 20% w/w triacetin based on dried solid was added as a plasticizer. Ten grams of cephalexin was added to the polymer solution and the dispersion was aided by sonication for 20 minutes. The drug-polymer-sovent phase was poured into 200 ml of an aqueous solution with agitation using a controlled stirring rate at 1000 rpm. Stirring was continued at 40°C until the methylene chloride was completely evaporate out (typically 1 hour). The resulting microcapsules were collected by centrifugation at 3000 rpm for 15 minutes and dried at room temperature for 12 hours and stored in a dessicator for further studies.

7.2 Fluidization techniques

7.2.1 Ethylcellulose

The polymer solution was prepared by adding ethylcellulose into ethanol, containing 30% triacetin as the plastcizer, to make 10% w/v polymer solution. About 200 g of cephalexin was introduced into a product container of fluidized bed apparatus (Fig. 11), the ratio of cephalexin : polymer were 2:1, 1:1, and 1:2. , the warm air was applied to the powder to raise its temperature. Inlet air

temperature was 75-80°C and outlet temperature was 40-50°C. After cephalexin powder was warmed, spraying of polymer solution was atomized at the top. The atomizing air pressure was 1.5 bar and the fludizing air velocity was 30-70 M^3/hr. Polymer solution was feed at a constant rate of 10 ml/min. In process, the product collector was shaking 5 times every 8 minutes to prevent the powder attaching to the sides of expansion chamber. At the end of spraying, microcapsules were received and let it dry in a process for 10 minutes. Finished microcapsules were collected and stored in a dessicator for further use.

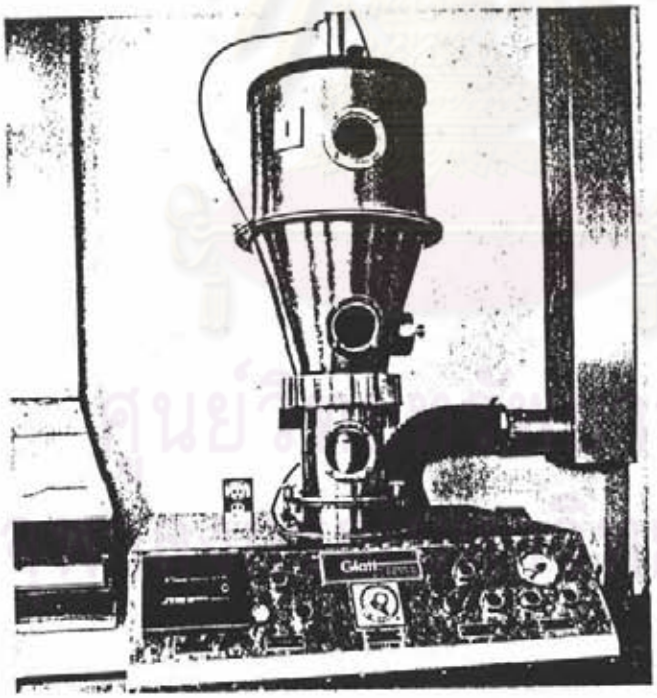

Fig. 11: Schematic of fluidized bed apparatus.

7.2.2 Combination of Eudragit RL 100® and Eudragit RS 100®

The required amount of Eudragit RL 100® and Eudragit RS 100® was added into ethanol, containing 20% triacetin (w/w of polymer) as the plasticizer, to make 10% w/v polymer solution. The ratios of cephalexin : polymer were 2:1, 1:1, and 1:2. Both procedure and operative parameters were the same as producing microcapsules by using ethylcellulose as the wall material performed previously.

7.3 Spray-drying technique.

7.3.1 Ethylcellulose.

To a 600 ml beaker equipped with a magnetic stirrer 250 ml of ethanol containing 30% triacetin (w/w of polymer) was added as the plasticizer were mixed. Ethylcellulose was added gradually into the ethanolic solution to produced 2.5% w/v solution and stirred until the clear solution was obtained. Twenty grams of cephalexin was introduced and mixed until homogeneous. The ratios of cephalexin : polymer were 2:1, 1:1, and 1:2. The resultant slurrys were then spray into spray-drying chamber with a constant flow rate of 3-5 ml/min and spraying pressure of 300 Nl/h. The inlet air temperature was 100-120°C and the outlet air temperature was 50-60°C. The aspirator suck the drying air through the instrument and the quantity of the heat drying air was set within a range of 100-120 mmWc to keep a constant outlet temperature. Microcapsules were recovered in a reservior throughout the cyclone collector and kept in a dessicator for further studies.

7.3.2 Combination fo Eudragit RL 100® and Eudragit RS 100®

The accurate amount of Eudragit RL 100® and Eudragit RS 100® was dissolved in ethanol containing 20% triacetin (w/w of polymer) as the plasticizer to obtain 2.5% w/v polymer solution in a 600 ml beaker and then stirred with a constant rate until the clear solution was obtained. Twenty grams of cephalexin was added and stirred vigorously in a sufficient time period to ensure its homogenity. The ratios of cephalexin : polymer were 2:1, 1:1, and 1:2. The dispersion were fed to a spray-dryer with the same procedure and operative parameters as in preparing microcapsules by using ethycellulose as the wall material.

8. Evaluation of Cephalexin Microcapsules.

8.1 Particle Size Analysis.

The optical microscope was used to determine particle size of microcapsules. Six hundred and twenty five particles were measured along an arbitrarily chosen fixed line. The lines are calibrated with the standard line slide (0.01 mm). Cumulative frequency curve was plotted between cumulative percent under size versus particle diameter of microcapsules prepared at different wall materials, core:wall ratios and microencapsulation techniques.

8.2 Surface topography.

Surface characteristic of microcapsules were studied by using scanning electron microscopy. The dry sample of microcapsules was coated with gold, using vacuum deposition coater. The thickness of the coating was depend on the geometry of the sample and was obtained on a trial and error basis.

The surface characteristics of microcapsules prepared by different wall materials, core: wall ratios, and microencapsulation techniques were studied by magnifying photograph 35-1500 times in the electron microscopes (JSM T100, Joel, Japan) and photographing. These were subsequently magnified to 35-1500 times the size.

8.3 Drug content of microcapsules

Fifty grams of microcapsules was dissolved in 2 ml of chloroform to dissolve the polymer. Two ml of water was added and swirled vigorously by using vortex mixer for 15 minutes then centrifuged for 15 minutes at 3000 rpm. Portion of water was seperated in another test tube. This was repeated 5 times for each sample, 10 ml of water portion was collected. The cephalexin extracted in water portion was determined spectrophotometrically at 262 nm.

8.4 Percentage of microcapsule yielded

Prepared microcapsules were accurately weighed. The net weight was divided with the expected weight of microcapsules. The percentage of microcapsule yield was calculated.

8.5 Dissolution studies

Dissolution experiments of pure or microencapsulated cephalexin were carried out in a six-station dissolution tester, 250 mg of the pure drug or an amount of the microencapsulated drug equivalent to 250 mg of pure drug were placed in 900 ml of water. The dissolution apparatus miantained at 37°C and paddle method was used in these studies with a constant paddle stirring rate of 50 ± 2 rpm. Samples of 5 ml were withdrawn at appropriate time intervals and immidiately replaced with a fresh dissolution medium over a period of 10 hours.

The samples were then filtered through a 0.22 μm membrane filter unit, and diluted with water to get an appropriate concentration before determined the cephalexin concentration by using spectrophotometer at wavelength 262 nm. After each time interval, the average value and standard deviation were computed and finally a mean release of cephalexin were plotted versus time to obtain dissolution profiles.