CHAPTER II

Experimental

Materials

- Chitosan (Kyowa Technos Co.,Ltd.,Japan, supplied by G.T.Chemical, Thailand)
- HPMC,Methocel® E 15 LV Premium (Colorcon Ltd.,England)
- MC,Methocel® A 15 LV Premium (Colorcon Ltd.,England)
- Citric acid anhydrous (Fluka Chemie AG,Switzerland)
- PVA average molecular weight 70,000-100,000 (Sigma Chemical Co.,St.Louis, USA.)
- Triacetin (Sigma Chemical Co.,St.Louis, USA.)
- PVP K90 (GAF,Singapore)
- 4-Dimethylaminobenzaldehyde AR grade (E.Merck, Darmstadt,Germany)
- Glutaraldehyde grad II 25% aqueous solution (Sigma Chemical Co., St.Louis, USA.)
- Simethicone (Pharmaceutical traders Co.,Ltd.,Bangkok, Thailand)
- Isosorbide dinitrate/Lactose 40/60 (SIFA, Ltd.,supplied by General Drugs House, Bangkok, Thailand)
- Corn starch (Pharmaceutical Sciences, Bangkok, Thailand)
- Methanol HPLC grade (JT Baker Incorporation, Phillipsberg, New Jersy, USA.)

- Polyethylene glycol 1450
  (Supplied by Srichand United Dispensary,Co.,Ltd.,Thailand)
- Polyethylene glycol 400 (Supplied by Srichand United Dispensary,Co.,Ltd.,Thailand)
- Silicone paste (Fluka Chemie AG,Switzerland)

Apparatus

- Analytical Balance (Sartorius model A200S, Sartorius Ltd. ,Co., Germany)
- Incubator (Memmert model BM600, Germay)
- Shaking Water-Bath (Hotech shaker bath model 905, Hotech Instruments Corporation, Taipei, Taiwan)
- Sonicator (Bransonic 321, Smith Kline, CT,USA.)
- Vortex Mixer (Vortex Genie-2, model G-560E, Scientific Industries Inc.,Bohermia, New York, USA.)
- Magnetic Stirrer (Nuova 7 stir-plate, Sybron Termolyne, USA.)
- Micrometer (Teclock Corp.,Japan)
- Micropipet (20-100 µl;Pipetman®,Gilson, UK.)
- Micropipet (200-1000 µl;Socorex®,Switzerland)
- HPLC (Millipore Waters Chromatography Division, Milford, Massachusetts, USA.) composed of :-
  : Model 600E multisolvent delivery system
  : Water 746 data module
  : Water 484 tunable absorbance detector
  : Model 712 Waters Intelligent Sample Processor (WISP™)
- HPLC (Milton Roy, LDC division, Florida, USA.) composed of :-

: Model CM 4000 multiple solvent delivery system

: Model SM 4000 programmatic wavelength detector

: Model 4100 computor integrator

- Scanning electron microscope (model JSM 35 CF, Jeol, Japan)
- Thermal analyzer (DSC model 200, Netzsch, Germany)
- Fourier transform infrared spectrometer
  (model 1760X,Perkin Elmer, USA.)
- Dissolution apparatus (Hanson Research model SR 2, USA.)
- Tensometer, Instron (model4301,serial No.H333,
  Instron Corp.,Canton.MA,USA.)

Methods

1. Solubility of ISDN

Each 500 mg of 40% ISDN in lactose equivalent to ISDN 200 mg was accurately weighed and put into a glass vial containing 30 ml of various solvents including reversed osmosis treated water, 20% PEG400 in reversed osmosis treated water and 30% PEG400 in reversed osmosis treated water. These suspensions were equilibrated by constant shaking in a shaking water bath at 37^{o}C for 24 hours. Sample solutions were then withdrawn, appropiately diluted and analyzed by the HPLC method (USPXXII). All solubility determinations were carried out in triplicate.

2. Formulation of crosslinked chitosan-polymer membrane

The prepared formulae of crosslinked chitosan-polymer membrane were shown in Tables 2 to 6. The amount of total polymer in casting solution was 2.5% w/w. The ratios of chitosan : polymer were 1:4,2:3 and 3:2. The

investigated polymers were PVA, PVPK90, HPMC15, corn starch and MC15. Glutaraldehyde of 2.5% solution in the amount of 5-10% w/w of total weight was used as crosslinking agent. Plasticizer, PEG1450 or triacetin, in a concentration of 10% w/w of total polymer was also incorporated in some formulations.

3. Method of preparing crosslinked chitosan-polymer membranes

3.1 Crosslinked chitosan-polyvinylalcohol membranes

A known amount of polyvinylalcohol was dissolved in deionized water at 100^{o}C. Chitosan was dissolved in 8% citric acid solution. An aqueous solution of polyvinylalcohol was mixed with chitosan in 8% citric acid solution. The polymer blend solution was stirred at room temperature, followed by the addition of the known amount of other additives according to the formula. The mixture was stirred for two and a half hours. The casting solutions were filtered through filter paper No.1 in order to remove dust. The casting solution of appropiate volume was poured onto the clean glass plate kept on the level surface, then stored at room temperature to remove the solvent. After 3 days the dried membrane was peeled off from the glass surface and stored in a desiccator. The dried membranes easily taken were selected for furthur evaluation.

3.2 Crosslinked chitosan-polyvinylpyrrolidone K90 membranes

An aqueous solution of desired amount of PVP K90 was prepared and mixed with chitosan in 8% citric acid solution. The later methods were also operated by the same methods as described in 3.1.

Table 2 Formulations of Crosslinked Chitosan-PVA Membrane Casting Solutions

Ingredients	Composition (% w/w)																	
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	A16	A17	A18
Chitosan	.5	1	1.5	.5	1.0	1.5	.5	1.0	1.5	.5	1	1.5	.5	1	1.5	.5	1.0	1.5
PVA	2	1.5	1	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0
PEG1450	.25	.25	.25	-	-	-	-	-	-	.25	.25	.25	-	-	-	-	-	-
Triacetin	-	-	-	-	-	-	.25	.25	.25	-	-	-	-	-	-	.25	.25	.25
Glutaraldehyde, 2.5% solution	5	5	5	5	5	5	5	5	5	10	10	10	10	10	10	10	10	10
8% Citric acid solution	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Deionized water q.s. to	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Table 3 Formulations of Crosslinked Chitosan-PVPK90 Membrane Casting Solutions

Ingredients	Composition (% w/w)																	
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18
Chitosan	.5	1	1.5	.5	1.0	1.5	.5	1.0	1.5	.5	1	1.5	.5	1	1.5	.5	1.0	1.5
PVP K90	2	1.5	1	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0
PEG1450	.25	.25	.25	-	-	-	-	-	-	.25	.25	.25	-	-	-	-	-	-
Triacetin	-	-	-	-	-	-	.25	.25	.25	-	-	-	-	-	-	.25	.25	.25
Glutaraldehyde, 2.5% solution	5	5	5	5	5	5	5	5	5	10	10	10	10	10	10	10	10	10
8% Citric acid solution	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Deionized water q.s. to	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Table 4 Formulations of Crosslinked Chitosan-HPMC15 Membrane Casting Solutions

Ingredients	Composition (% w/w)																	
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18
Chitosan	.5	1	1.5	.5	1.0	1.5	.5	1.0	1.5	.5	1	1.5	.5	1	1.5	.5	1.0	1.5
HPMC 15	2	1.5	1	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0
PEG1450	.25	.25	.25	-	-	-	-	-	-	.25	.25	.25	-	-	-	-	-	-
Triacetin	-	-	-	-	-	-	.25	.25	.25	-	-	-	-	-	-	.25	.25	.25
Glutaraldehyde, 2.5% solution	5	5	5	5	5	5	5	5	5	10	10	10	10	10	10	10	10	10
8% Citric acid solution	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Deionized water q.s. to	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Table 5 Formulations of Crosslinked Chitosan-Corn starch Membrane Casting Solutions

Ingredients	Composition (% w/w)																	
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18
Chitosan	.5	1	1.5	.5	1.0	1.5	.5	1.0	1.5	.5	1	1.5	.5	1	1.5	.5	1.0	1.5
Corn starch	2	1.5	1	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0
PEG1450	.25	.25	.25	-	-	-	-	-	-	.25	.25	.25	-	-	-	-	-	-
Triacetin	-	-	-	-	-	-	.25	.25	.25	-	-	-	-	-	-	.25	.25	.25
Glutaraldehyde, 2.5% solution	5	5	5	5	5	5	5	5	5	10	10	10	10	10	10	10	10	10
8% Citric acid solution	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Deionized water q.s. to	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Table 6 Formulations of Crosslinked Chitosan-MC15 Membrane Casting Solutions

Ingredients	Composition (% w/w)																	
	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18
Chitosan	.5	1	1.5	.5	1.0	1.5	.5	1.0	1.5	.5	1	1.5	.5	1	1.5	.5	1.0	1.5
MC 15	2	1.5	1	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0	2	1.5	1.0
PEG1450	.25	.25	.25	-	-	-	-	-	-	.25	.25	.25	-	-	-	-	-	-
Triacetin	-	-	-	-	-	-	.25	.25	.25	-	-	-	-	-	-	.25	.25	.25
Glutaraldehyde, 2.5% solution	5	5	5	5	5	5	5	5	5	10	10	10	10	10	10	10	10	10
8% Citric acid solution	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40	40
Deionized water q.s. to	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

3.3 Crosslinked chitosan-hydroxypropylmethylcellulose 15 membranes

A clear viscous colloid solution of the desired amount of HPMC 15 was prepared and mixed with chitosan in 8% citric acid solution. The subsequent procedures were run as described in 3.1.

3.4 Crosslinked chitosan-corn starch membranes

A viscous colloidal mixture of a known amount of corn starch was prepared and mixed with chitosan in 8% citric acid solution. The consecutive steps were run as previously described and then the casting solution was filtered through a cloth in order to remove dust. The later procedures were carried out as described in 3.1.

3.5 Crosslinked chitosan-methylcellulose 15 membranes

The desired amount of methylcellulose 15 was dissolved, swelled in cold water and left overnight to produce a clear viscous-colloid solution and then mixed with chitosan in 8% citric acid solution. The later steps were consecutively performed as in 3.1.

4. Characterization of membranes

4.1 Determination of water sorption

Test samples, diameter of 4.3 cm, were cut from the selected dried membranes and dried to constant weight in a desiccator at room temperature. Then the test membranes were immersed in deionized water at 37^{o}C in incubator for 24 hours, blotted with filter paper for one minute to remove excess surface water and weighed again.

Water sorption were calculated using the following formula.

$$\textbf{water sorption(\%)} = \frac{\text{(wet weight of membrane-dry weight of membrane)}}{\text{dry weight of membrane}} \times 100$$

The experiments were performed in triplicate.

4.2 Determination of membrane thickness

The free membranes were cut into 4.3 cm diameter circular pieces. Membrane thickness at five different points, one point at membrane center and the others around the central point, were measured by using a thickness tester and recorded as the mean of fives measurements.

4.3 To determine the mechanical properties of membranes

Ultimate tensile strength and percent elongation at break of test membranes were measured on a tensile tester, at $25\pm1^{o}C$ and $50\pm2\%$ relative humidity. The method used for determination was based on the guidelines of the American Society for Testing Materials method D 882-80 a. Membrane specimens were cut out using a standard template. The thickness of each membrane specimen was the mean value of four separate measurements taken along the middle 4 cm section of the specimens using a micrometer. The cross-sectional area of the film was calculated by multiplying the mean thickness with gauge width (6.25 mm). Three specimens were subjected to the test for one membrane formulation. The test specimen was clamped by an upper and lower grip. Perfect alignment of the test specimens between the upper and the lower grips was checked visually before the test was started. To avoid any damage to the test specimens which were very fragile, extensometer was not used. The gauge

length was marked at 2.0 cm. in the middle section of the test specimen, which was calibrated to zero percent elongation. The test was performed using rate of grip separation 12.5 mm/min. The elongation at break was measured by using test scales and recorded as different length at the breaking point of the test specimens. Breaking tensile strength was recorded from the digital display.

The percent elongation at break (%EB) and ultimate tensile strength were calculated using the following relationships.

$$\%EB = \frac{\text{(difference in the length at breaking point)}}{\text{original length of the test specimen}} \times 100$$

$$\text{ultimate tensile strength} = \frac{\text{breaking load}}{\text{cross-sectional area of the test specimen}}$$

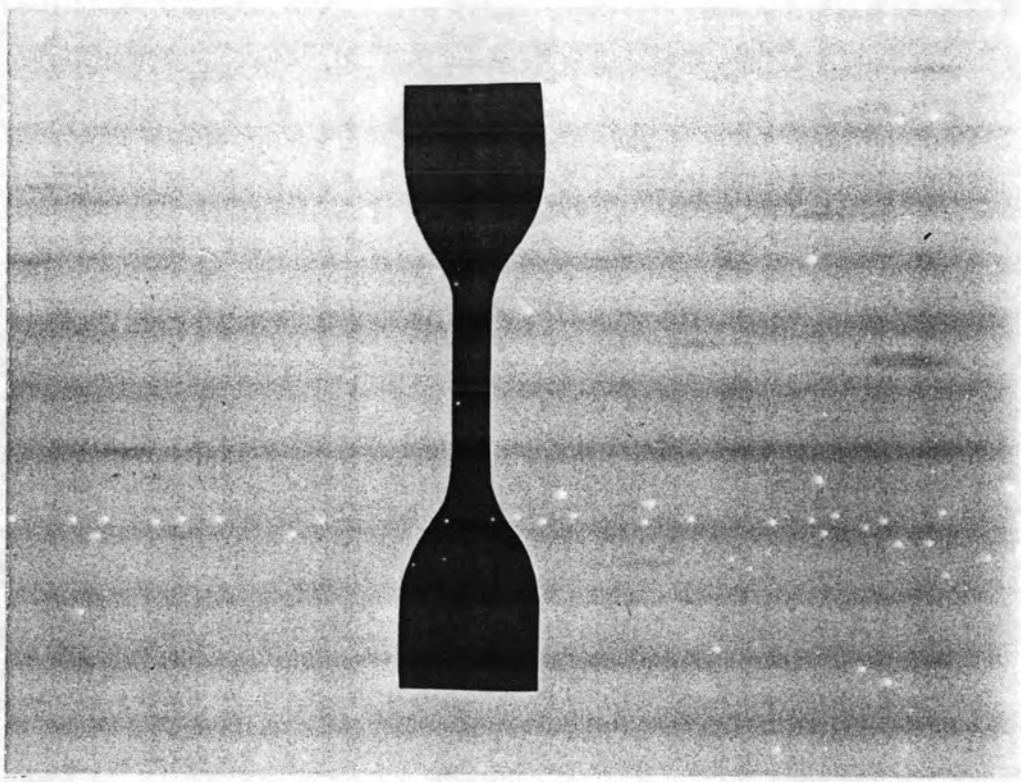

Figure 2 A standard template for tensile testing.

4.4 Surface morphology

Free films were mounted on a metal stub, coated with gold and examined using a scanning electron microscope. The free film was imaged using a 20 kV electron beam. Then the surface morphology was observed at an appropriate magnification.

4.5 Infrared spectrometry

Infrared spectra were examined by using a Fourier transform infrared spectrometer. The measurement was made by the KBr disc method and scanning from wavenumber 400-4000. Samples that were examined were pure substances and selected crosslinked chitosan-polymer membranes. All samples were directly used for recording the infrared spectra.

4.6 Differential scanning calorimetry

The DSC curves were determined by using a differential scanning calorimeter. The baseline was obtained using an empty aluminium pan. Each sample was investigated for its melting point. Samples, including pure substances and selected crosslinked chitosan-polymer membranes, were accurately weighed,and put into the equipment using a given condition.

Heating rate	=	10^{o}C per min
Temperature	=	30 - 270^{o}C
Atmosphere	=	N_2 15 ml per min
Sample cell	=	Aluminium closed pan

5. In vitro evaluation

5.1 Selection of crosslinked chitosan-polymer membranes

In order to furthur experiment, the suitable formulae of crosslinked chitosan-polymer membrane were selected depending on the ease to

remove dried membrane from the glass plate, water sorption and mechanical properties of dried membrane.

5.2 Permeation study

5.2.1 Fabrication for TDDs

The selected crosslinked chitosan-polymer membranes, which had good physical and mechanical properties, were used as rate-controlling membrane in ISDN transdermal drug delivery system. For fabrication of this ISDN transdermal patch, the procedure was as followed.A circular piece of aluminium foil with 3.5 cm. in diameter was used as backing layer. The uniform dispersion of drug in simethicone equivalent to ISDN 40 mg which acted as drug reservoir, was weighed into aluminium backing layer (Bhalla and Khanolkar,1985, Takada, Yoshikawa, and Muranishi,1990). The ratio of ISDN : simethicone was 1:1. A circular piece of selected membrane with 4.3 cm in diameter was placed next to the drug reservoir layer.Consecutively, the circular edge of membrane was adhered to circular edge of adhesive layer of 5.2 cm in diameter.

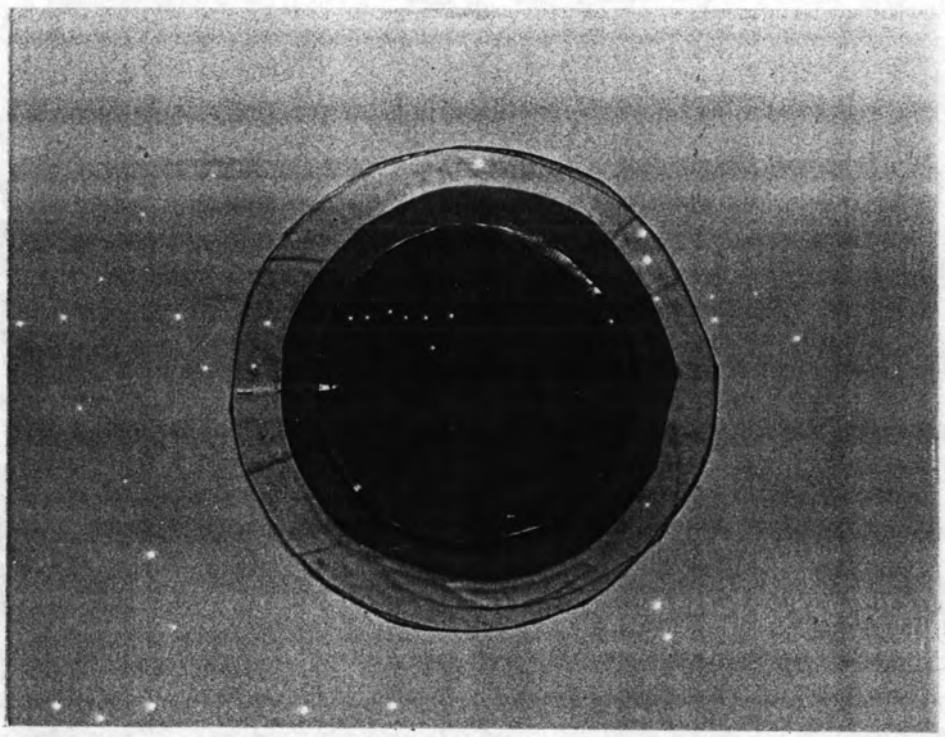

Figure 3 Isosorbide dinitrate transdermal patch.

5.2.2 Skin permeation cell

An in-vitro skin permeation cell which was selected to be used throughout the experiment for the investigation of long term skin permeation kinetic of ISDN transdermal delivery system was the USP XXII dissolution apparatus II with the addition of a plastic disk assembly designed for holding the transdermal system at the bottom of the vessel. A plastic disk assembly consisted of two major parts, an upper piece with 3.2 cm in internal circular hole at the center and a plane lower piece.

5.2.3 Shed snake skin preparation

Shed snake skin of ***Elaphe obsoleta*** (Black rat snake) was used as model membrane for in vitro permeation studies. Shed snaked skin was stored at freezing temperature, -20^{o}C, prior to use. The dorsal portion was cut into 5.2 cm circular piece, hydrated overnight in a beaker containing 100 ml dissolution medium at ambient temperature and then mounted on transdermal patch for skin permeation study.

5.2.4 Permeation procedure

In order to permeation studies, the USP XXII dissolution apparatus II with the addition of a plastic disk assembly designed for holding the transdermal system was used. The test ISDN transdermal patch with selected membrane to act as rate-controlling membrane was placed over the lower piece of disk assembly. The circular edge of the TDDs patch was greased by using silicone paste in order to protect the patch from direct contact to the dissolution medium. Then shed snake skin was mounted on TDDs patch and covered with the upper part of disk assembly. The whole assembly was securely clamped together with screws at four different points. For this purpose, a six-station dissolution

tester with paddles was employed. The paddle was adjusted to rotate at 50 rpm. Two hundred and fifty milliliters of solution of 20% PEG400 in reversed osmosis treated water was used as dissolution medium. The disk assembly including the ISDN TDDs patch and shed snake skin was placed into the dissolution vessel containing dissolution medium at 37°C. Samples were withdrawn at predeterminal intervals over a peroid of 24 hours (0.5,1,2,4,6,8,10,12,16,20 and 24 hours) and filtered through membrane filter of 0.45 μm. The exact volume withdrawn from each vessel at each time interval was replaced immdiately with the equal volume of fresh dissolution medium. The amount of permeated ISDN was determined by modified HPLC method (Gelber and Papas,1983, USP XXII,1992).

It was essential to use small receptor volume in this experiment in order to obtain sample of detectable concentration of ISDN for the HPLC analysis. All permeation studies were conducted in triplicated run.

6. Analytical quantitation of ISDN

6.1 Chromatographic conditions

HPLC system was setted to various parameter for analysis as following.

column : Spherisorb 10 ODS,Phenomenex®,size 250 x 4.6 mm.

mobile phase : Degassed mixture of methanol and reversed osmosis treated water as 55:45

internal standard : 4-Dimethylaminobenzaldehyde

injection volumn : 200 μl

flow rate : 1 ml/min

pressure : 1900 psi

chart speed : 2.5 mm/min

detector : UV detector set at 220 nm, peak area was calculated by using an integrator, Millipore Water 484 tunable absorbance detector.

column temperature : Room temperature 25±1°C

The mobile phase was freshly prepared, consisted of a mixture of methanol and reversed osmosis treated water in proportion of 55:45. The mixture solution was filtered through a 0.45 μm membrane filter, type HV, Milipore, and then was degassed by sonication for 15 min prior to use.

6.2 Preparation of calibration curve

The calibration curve of ISDN was performed within the concentration range of 0.06-1.00 μg/ml. Standard solutions containing 0.06, 0.07, 0.09, 0.10, 0.20, 0.30, 0.70, 1.00 μg/ml of ISDN and 0.392 μg/ml of internal standard in each dilution, in mobile phase were prepared. A 200 μl aliquot standard solution was injected into HPLC. An autoinjector was used for injection. Calibration curve was constructed by plotting the ratio of peak areas under curve of ISDN and internal standard against the concentration of ISDN and a least square fitted of linear regression equation was used to calculate the concentration of ISDN in each elution sample.

6.3 Sample preparation

Each sample of elution solution had to be appropiate dilution for assaying the ISDN concentration. The sample was accurately pipetted into a 5 ml volumetric flask, then 20 μl of 98 μg/ml of internal standard in methanol was added, the mixture solution was then diluted with freshly mobile phase to achieve the corrected volume of volumetric flask. The final solution was agitated with a

vortex mixer for one minute. A 200 μl aliquot of sample solution was injected into a HPLC column for quantitation by using an autoinjector. The ratio of peak under curve of ISDN and internal standard was calculated to determine the concentration of ISDN in each sample from the calibration curve in that day. The amount of ISDN permeating through the shed snake skin was then calculated from ISDN concentration in the receptor solution at each sampling time after correcting for the total volume of the solution and the amount of ISDN withdrawn for assay.