CHAPTER II

EXPERIMENTAL

Materials

- Chitosan average molecular weight 30,000-70,000 (Kyowa Technos Co., Ltd., Japan, supplied by G. T. Chemical, Thailand)
- Polyvinyl alcohol. Airvol® average molecular 30,000, 70,000 and 100,000
- Corn starch (Pharmaceutical Science, Bangkok, Thailand)
- Potato starch (Fluka Chemie AG, Switzerland)
- Tapioca starch (Supplied by Maxway, Thailand)
- Glutaraldehyde grade II 25% aqueous solution (Sigma Chemical Co., St. Louis, USA)
- Triacetin (Sigma Chemical Co., St., Louis, USA)
- Methanol HPLC grade (JT Baker Incoorporation, Phillisberg, New Jersy, USA)
- Isosorbide dinitrate/Lactose 40/60 (SIFA, Ltd., supplied by General Drugs House, Bangkok, Thailand)
- 4-Dimethylaminobenzaldehyde AR grade (E.Merck, Darmstadt, Germany)
- Polyethylene glycol 400 (Supplied by Sirchand United Dispensary, Co., Ltd., Thailand)

- Simethicone (Pharmaceutical Traders Co., Ltd., Bangkok, Thailand)
- Silicone paste (Fluka Chemie AG, Switzerland)
- Lactic acid BP 1980 (Supplied by Srichand United Dispensary, Co., Ltd., Thailand)

Apparatus

- Analytical Balance (Sartorius model A200S, Sartorius Ltd., Co., Germany)
- Incubator (Memmert model BM600, Germany)
- Sonicator (Transsonic Digitals, Elma, Germany)
- Magnetic Stirrer (Bransted Thermolyne Model No. SP46920-26, USA)
- Micrometer (Teclock Corp., Japan)
- Micropipet (10-100 µl ; Socorex, Switzerland)
- Micropipet (100-1000 µl ; Socorex, Switzerland)
- Dissolution apparatus (Hanson Research model SR2, USA)
- Tensometer (Instron model 4301, serial No. H333, Instron Corp., Canton. MA, USA)
- HPLC (Millipor Waters Chromatograph Division, Milford, Massachusetts, USA) composed of :
  - Model 600 E multisolvent delivery system
  - Water 746 data module
  - Water 484 tunable absorbance detector

- Model 712 Waters Intelligent Sample Processor (WISP^{TM})
- Scanning electron microscope (model JSM-T220A, Jeol, Japan)
- Fourier transform infrared spectrometer (Model 1760X, Perkin Elmer, USA)
- Thermal analyzer (DSC model 200, Netzsch, Germany)
- Surface area measurement (Micromeritics model 2300FC, USA)

Methods

1. Formulation of crosslinked chitosan-polymer membrane

The formulations of crosslinked chitosan-polymer membrane are shown in Tables 5 to 10. Each formulation consisted of the fixed amount of the total polymer (2%w/w). The ratios of chitosan : polymer were varied from 9 : 1 to 1 : 9. The polymer to be used with chitosan were polyvinyl alcohol (PVA), with average molecular weight of 30,000, 70,000 and 100,000 corn starch, potato starch and tapioca starch. Glutaraldehyde grade II of 25% aqueous solution in the amount of 1.25% to 10% w/w of total polymer was used as a crosslinking agent. In some formulations of crosslinked chitosan-corn starch membrane, triacetin in a concentration of 10% w/w of total polymer was used as plasticizer. The thickness of the prepared membrane was controlled to be about 100$\pm$10 μm. Lactic acid was used for dissolution of chitosan. The amount of lactic acid was varied in the formulation to control pH of casting solution to 4-5.

Table 5. Formulation of Crosslinked Chitosan-PVA 30,000 Casting Solution

Ingredients (g)	Formulation CPL																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Chitosan	1.8	1.8	1.8	1.8	1.8	1.6	1.6	1.6	1.6	1.6	1.4	1.4	1.4	1.4	1.4	1.2	1.2	1.2	1.2	1.2	1.0	1.0	1.0
PVA, 30,000	0.2	0.2	0.2	0.2	0.2	0.4	0.4	0.4	0.4	0.4	0.6	0.6	0.6	0.6	0.6	0.8	0.8	0.8	0.8	0.8	1.0	1.0	1.0
Lactic acid	0.9	0.9	0.9	0.9	0.9	0.8	0.8	0.8	0.8	0.8	0.7	0.7	0.7	0.7	0.7	0.6	0.6	0.6	0.6	0.6	0.5	0.5	0.5
Glutaraldehyde	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3
Deionized water	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Ingredients (g)	Formulation CPL																						
	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	
Chitosan	1.0	1.0	0.8	0.8	0.8	0.8	0.8	0.6	0.6	0.6	0.6	0.6	0.4	0.4	0.4	0.4	0.4	0.2	0.2	0.2	0.2	0.2	
PVA, 30,000	1.0	1.0	1.2	1.2	1.2	1.2	1.2	1.4	1.4	1.4	1.4	1.4	1.6	1.6	1.6	1.6	1.6	1.8	1.8	1.8	1.8	1.8	
Lactic acid	0.5	0.5	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	
Glutaraldehyde	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	
Deionized water	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	

Table 6. Formulation of Crosslinked Chitosan-PVA 70,000 Casting Solution

Ingredients (g)	Formulation CPM																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Chitosan	1.8	1.8	1.8	1.8	1.8	1.6	1.6	1.6	1.6	1.6	1.4	1.4	1.4	1.4	1.4	1.2	1.2	1.2	1.2	1.2	1.0	1.0	1.0
PVA, 70,000	0.2	0.2	0.2	0.2	0.2	0.4	0.4	0.4	0.4	0.4	0.6	0.6	0.6	0.6	0.6	0.8	0.8	0.8	0.8	0.8	1.0	1.0	1.0
Lactic acid	0.9	0.9	0.9	0.9	0.9	0.8	0.8	0.8	0.8	0.8	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.6	0.6	0.6
Glutaraldehyde	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3
Deionized water	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Ingredients (g)	Formulation CPM																						
	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	
Chitosan	1.0	1.0	0.8	0.8	0.8	0.8	0.8	0.6	0.6	0.6	0.6	0.6	0.4	0.4	0.4	0.4	0.4	0.2	0.2	0.2	0.2	0.2	
PVA, 70,000	1.0	1.0	1.2	1.2	1.2	1.2	1.2	1.4	1.4	1.4	1.4	1.4	1.6	1.6	1.6	1.6	1.6	1.8	1.8	1.8	1.8	1.8	
Lactic acid	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.4	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	
Glutaraldehyde	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	
Deionized water	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	

Table 7. Formulation of Crosslinked Chitosan-PVA 100,000 Casting Solution

Ingredients (g)	Formulation CPH																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Chitosan	1.8	1.8	1.8	1.8	1.8	1.6	1.6	1.6	1.6	1.6	1.4	1.4	1.4	1.4	1.4	1.2	1.2	1.2	1.2	1.2	1.0	1.0	1.0
PVA, 100,000	0.2	0.2	0.2	0.2	0.2	0.4	0.4	0.4	0.4	0.4	0.6	0.6	0.6	0.6	0.6	0.8	0.8	0.8	0.8	0.8	1.0	1.0	1.0
Lactic acid	0.9	0.9	0.9	0.9	0.9	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.7	0.7	0.7	0.7	0.7	0.6	0.6	0.6
Glutaraldehyde	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3
Deionized water	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Ingredients (g)	Formulation CPH																						
	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	
Chitosan	1.0	1.0	0.8	0.8	0.8	0.8	0.8	0.6	0.6	0.6	0.6	0.6	0.4	0.4	0.4	0.4	0.4	0.2	0.2	0.2	0.2	0.2	
PVA, 100,000	1.0	1.0	1.2	1.2	1.2	1.2	1.2	1.4	1.4	1.4	1.4	1.4	1.6	1.6	1.6	1.6	1.6	1.8	1.8	1.8	1.8	1.8	
Lactic acid	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.4	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	
Glutaraldehyde	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	0.1	0.2	0.3	0.4	0.8	
Deionized water	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	

Table 8. Formulation of Crosslinked Chitosan-Corn Starch Casting Solution

Ingredients (g)	Formulation CC																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Chitosan	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
Corn starch	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Lactic acid	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Glutaraldehyde	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4
Triacetin	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2
Deionized water	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Ingredients (g)	Formulation CC																							
	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
Chitosan	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Corn starch	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Lactic acid	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Glutaraldehyde	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4
Triacetin	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2
Deionized water	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Table 9. Formulation of Crosslinked Chitosan-Potato Starch Casting Solution

Ingredients (g)	Formulation CP																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Chitosan	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
Potato starch	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Lactic acid	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Glutaraldehyde	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4
Triacetin	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2
Deionized water	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Ingredients (g)	Formulation CP																							
	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
Chitosan	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Potato starch	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Lactic acid	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Glutaraldehyde	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4
Triacetin	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2
Deionized water	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Table 10. Formulations of Crosslinked Chitosan-Tapioca Starch Casting Solution

Ingredients (g)	Formulation CT																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Chitosan	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
Tapioca starch	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Lactic acid	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Glutaraldehyde	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4
Triacetin	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2
Deionized water	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Ingredients (g)	Formulation CT																							
	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
Chitosan	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Tapioca starch	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Lactic acid	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Glutaraldehyde	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4	0.1	0.1	0.2	0.2	0.3	0.3	0.4	0.4
Triacetin	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2
Deionized water	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

2. Methods of preparing crosslinked chitosan-polymer membranes

Most membranes were prepared by casting technique.

2.1 Crosslinked chitosan-polyvinyl alcohol membranes

A required amount of chitosan was dispersed in a part of deionized water and lactic acid was added. This dispersion was stirred until clear solution. A known amount of polyvinyl alcohol was dissolved in a residual deionized water at 90-100°C. Then the polyvinyl alcohol solution was left to stand until cool. An aqueous solution of chitosan was mixed with polyvinyl alcohol solution. Then a required amount of glutaraldehyde 25% aqueous solution was added in this mixture. This blending solution was stirred at room temperature for about one hour. This resultant viscous solution was filtered through filter paper number 1. in order to remove dust and left to stand until air bubbles has disappeared. The air bubble-free liquid was poured on a clear dry glass petri dish in a dust-free atmosphere, kept on the level surface and allowed to dry at room temperature. The dried membrane was peeled from the glass petri dish and stored in desicator to selected for further evaluation.

2.2 Crosslinked chitosan-starch membranes

An aqueous solution of chitosan was prepared as above. A viscous colloidal mixture of a known amount of starch was prepared by dispersing starch in a part of deionized water, heating and agitating to gelatinized. The colloidal mixture was left to stand until cool. Then a colloidal mixture of starch was mixed with an aqueous solution of chitosan. In some of formulations, a known amount of triacetin was added to act as plasticizer. After a required amount of glutaraldehyde 25%

aqueous solution was added, the mixture solution was stirred about one hour. The casting solution was filtered through a cloth in order to remove dust and left to stand until air bubbles had disappeared. The later processes were consecutively performed as in 2.1.

3. Physicochemical properties of membranes

3.1 Determination of membrane thickness

The thickness of membrane was measured by using a micrometer. The free films were cut into 4.3 cm diameter circular pieces. Membrane thickness at five different points, one point at center membrane and the other points around the central point were recorded and averaged.

3.2 Determination of water sorption

Test membranes, diameter of 4.3 cm, were cut from the selected membranes and dried to constant weight in desicator at room temperature. Then they were immersed in deionized water at 37°C in an incubator for 24 hours. The resultant wet membranes were blotted with filter paper to remove excess surface water and weight again. Water sorption could be calculated from the following formula :

$$\frac{(\text{wet weight of membrane} - \text{dried weight of membrane}) \times 100}{\text{dried weight of membrane}}$$

In this experiment, water sorption was performed in triplicate.

3.3 Determination of mechanical properties by measuring ultimate tensile strength and percent elongation at break

The apparatus to be used for measuring ultimate tensile strength and percent elongation at break of test membranes was a tensile tester (Instron). Membrane specimens were cut out using a standard template. The thickness of each specimem was the average value of four separate measurements taken along the middle 4 cm section of the specimens using micrometer. The cross-section area of the test film was calculated by multiplying the mean thickness with gauge width (6.25 mm). Then the test membrane was clamped by an upper and lower grip. The condition to be used in this test was as following :

Temperature	=	$25 \pm 5^{\circ}C$
Relative humidity	=	$45 \pm 5\%$
Rate of grip separation	=	12.5 mm/min
Loading weight	=	100 Newton

In this experiment, three specimens were subjected to the test for one membrane formulation. After the test membrane was broken, the using force was recorded by the digital system. For the measurement of percent elongation at break, extensometer was not used due to the protection of the test membrane from fracture. The gauge length was marked at 2.0 cm in the middle section of the test membrane, which was calibrated to zero percent elongation. Then percent elongation at break was measured visually by using ruler scale and recorded from the different in the length at the breaking point of the specimem.

The ultimate tensile strength and percent elongation at break were calculated from the below formula.

$$\text{Ultimate tensile strength} : \frac{\text{breaking load}}{\text{cross-section area of the test specimem}}$$

$$\text{Percent elongation at break} : \frac{(\text{different in the length at breaking point}) \times 100}{\text{original length of the test specimen}}$$

3.4 Surface morphology

The surface morphology of selected crosslinked chitosan-polymer membranes was observed by using a scanning electron microscope. Membrane were mounted on a metal stub and coated with gold. The using electron beam was 10 kV and the using extension of photograph was 350 and 750.

3.5 Infrared spectrometry

Fourier transform infrared spectrometer was employed to examine infrared spectra. The pure substances and selected crosslinked chitosan-polymer membranes were examined. They were scanned from wave number 450-4000. The measurement was made by potassium bromide disk method.

3.6 Differential scanning calorimetry

The differential scanning calorimetry thermograms were observed by using the differential scanning calorimeter. The principle on

which differential scanning calorimeter was that the samples, which were pure substances and selected crosslinked chitosan-polymer membranes, were accurately weighed in an aluminium pan, and a reference, which did not undergo a thermal transition in the temperature range of interest (empty aluminium pan), were placed into the apparatus and heated by individual electric heaters. The temperature of sample and reference, monitored by thermocouples, was then gradually raised in such a manner that the temperature of sample and reference remained the same. Then if the sample suddenly absorbed heat, its heater would supply loss heat. In this way, transition temperature could be very accurately measured by monitoring the electric current going to the heaters (Robinson and Lee, 1987).

For the experimental, each sample was investigated for its melting point and put into the equipment using condition as follows :

Heating rate	=	10°C/min
Temperature	=	50-250°C
Atmosphere	=	nitrogen gas 10 ml/min
Sample and reference aluminium pan	=	Shimadzu aluminium closed pan

3.7 Surface area measurement

After selected crosslinked chitosan-polymer membranes were dried to constant weight in vacuum desicator. These membranes were cut into small pieces and heated about one hour at 70°C according to

driving residual gas and water. Then they were measured surface area by surface area equipment (Micromeritics). The principle of this apparatus was nitrogen gas absorption on surface area of test material. Thereby, the resultant record would show surface morphology of material. The method used for determination was based on the guidelines of the American Society for Testing Material method D4567-86.

4. Stability study of crosslinked chitosan-polymer membranes

The selected crosslinked chitosan-polymer membranes were kept in incubator at 40°C and 75% relative humidity for one week. Then test membranes were observed for physical characteristics, water sorption and mechanical properties (ultimate tensile strength and percent elongation at break).

5. Permeation study

5.1 Skin permeation cell used for in vitro release study

According to the assumption that the release of drug obtained from the in vitro study was similar to drug release obtained from in vivo study, the system of permeation study was designed to provide a mimic in vivo situation and modified to achieve a convenience and suitable pattern for each diffusion system proposed. For this experiment, USP XX III dissolution apparatus II with the addition of a plastic disk assembly designed for holding the transdermal system at the bottom of vessel was selected to be used out through the experiment. 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 lower piece.

5.2 Dissolution medium

It was important to ensure that the release profile of drug from the permeation was not limited by the solubility of drug in receptor medium. Therefore, the receptor medium in permeation cell is essentially acted as a perfect sink condition. In permeation study of isosorbide dinitrate, a very slightly soluble in water, a volume of dissolution medium used in this study was limited of 250 ml in order to obtain sample of detectable concentration of isosorbide dinitrate for the HPLC analysis, so it is necessary to find a solvent capable of dissolving isosorbide dinitrate completely.

Isosorbide dinitrate was freely soluble in acetone, ether, chloroform and alcohol. These solvent were carcinogenic agents. It was considered that co-solvent could be used to increase the solubility of drug. Therefore, 20% polyethylene glycol 400 in reversed osmosis treated water was selected as dissolution medium because it had been used in several isosorbide dinitrate permeation studies (Vyas et al., 1994, Leesajakul, 1995).

5.3 The skin used in permeation study

Shed snake skin of ***Elaphe obsoleta*** was used as a model skin for in vitro permeation study. Shed snake skins were stored at -20°C prior to use and the dorsal portion was cut into 4.3 cm diameter circular piece, hydrated in 100 ml dissolution medium at room temperature about fourteen hours.

5.4 The permeability of isosorbide dinitrate through shed snake skin

The permeability of isosorbide dinitrate through shed snake skin was measured in in vitro to examine the permeation profile of isosorbide dinitrate. The saturated isosorbide dinitrate solution was prepared by dissolving 0.1000 g of drug in 20.00 ml of reversed osmosis treated water. This dispersion was kept in protected light container at ambient temperature about 48 hours. After the appropriate amount of saturated isosorbide dinitrate solution (0.40 ml) was poured into the lower piece with circular hole of plastic disk assembly, the circular edge of lower piece was greased by using silicone paste in order to protect the dispersion from direct contact to the dissolution medium. Then shed snake skin was mounted carefully over the hole of lower piece of plastic disk assembly according to protect the production of air bubbles and covered with the upper part of disk assembly. The whole assembly was clamped together with screws at four different points. The disk assembly including the dispersion of isosorbide dinitrate and shed snake skin was placed into the dissolution vessel containing 250 ml dissolution medium at 37°C. For this study, the dissolution apparatus with paddles was used. The paddle was adjusted to rotate at 50 rpm. The exact volume of samples were withdrawn at 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 10 hours and filtered through membrane filter of 0.45 μm. Then the equal volume of fresh dissolution medium was replaced immediately. The amount of permeated isosorbide dinitrate was observed by modified HPLC method (Gelber and Papas, 1983, USP XX III, 1995). The permeation study was conducted in triplicate run.

5.5 The permeation study of isosorbide dinitrate transdermal patches

5.5.1 Preparation of isosorbide dinitrate transdermal patches

Membranes with good stability and physico-chemical properties were used as rate-controlling membranes in isosorbide dinitrate transdermal patches. The preparation of transdermal patch was as follows. Drug reservoir was prepared by uniform dispersion of isosorbide dinitrate in simethicone in a ratio of 1 : 1. Each preparation composed of isosorbide dinitrate 40 mg (Bhalla and Khanolkar, 1985, Takada, Yoshikawa and Muranishi, 1990). The uniform dispersion of drug was then weighed on circular piece of aluminium foil with 3.2 cm in diameter which adhered with a circular piece of aluminium foil with about 5 cm in diameter. The circular piece of membrane with 4.3 cm in diameter was placed next to the drug reservoir layer. Consecutively, the circular edge of membrane was closed with circular edge of adhesive layer.

5.5.2 The permeation study of isosorbide dinitrate transdermal patches

Before in vitro permeation study, the prepared transdermal patch was kept in room temperature about 12 hrs. The method of permeation study of isosorbide dinitrate transdermal patch was same method as 5.4. The saturated solution of isosorbide dinitrate was replaced by the test isosorbide dinitrate transdermal patch with selected crosslinked chitosan-polymer membrane to act as rate-controlling membrane. For this study, samples were withdrawn at various time intervals over a period of

24 hours (1, 2, 4, 6, 8, 12, 16, 20 and 24 hours). All the permeation studies were conducted in triplicate run.

a

b

Figure 6. Isosorbide dinitrate transdermal patch

(a) commercial TDDS

(b) prepared transdermal patch

6. Analytical quantitation of isosorbide dinitrate

6.1 HPLC analysis of isosorbide dinitrate

The modified isocratic reversed-phase HPLC technique was used for this study. Various parameters for setting the analysis were shown 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 volume	: 200 µl
Flow rate	: 1 ml/min
Pressure	: 1800 psi
Chart speed	: 0.25 cm/min
Detector	: UV detector wavelength was set at 220 nm, peak area was calculated by using an integrator, Millipore water 484 tunable absorbance detector.
Column temperature	: Room temperature 25 ± 5°C

The mobile phase was freshly prepared, and was filtered through a 0.45 µm membrane filter, type HV, Millipore®. Then the mobile phase was degassed by sonicating for 15 min prior to use.

6.2 Calibration curve

The standard solution containing 0.10, 0.30, 0.50, 0.80 and 1.00 μg/ml of isosorbide dinitrate and 0.15 μg/ml of internal standard in each dilution, in mobile phase were prepared. A 200 μl of standard solution was injected into HPLC column for quantitation by autoinjection. The calibration curve of isosorbide dinitrate was constructed by plotting the ratio of the peak area under curve of isosorbide dinitrate and 4-dimethylaminobenzaldehyde (internal standard) versus the concentration of isosorbide dinitrate. A linear regression equation was used to calculate the concentration of isosorbide dinitrate in each elution sample.

6.3 Sample preparation

The sample was accurately pipetted into a 10 ml volumetric flack, then 10 μl of 150 μg/ml 4-dimethylaminobenzaldehyde in mobile phase was added. The mixture solution was diluted with freshly mobile phase to the corrected volume of volumetric flask. Each sample of elution solution had to be appropriate dilution for assaying the amount of isosorbide dinitrate. A 200 μl of sample solution was injected by using autoinjection. The peak area ratio of isosorbide dinitrate and 4-dimethylaminobenzaldehyde calculated was used to determine the concentration of isosorbide dinitrate in the sample through the linear regression equation obtained from the calibration curve in that day. Then the cumulative amount of isosorbide dinitrate permeating through shed snake skin was calculated from isosorbide dinitrate concentration in each sampling.