International Research Journal of Pharmaceutical and Applied Sciences (IRJPAS)
Available online at **www.irjpas.com**
Int. Res J Pharm. App Sci., 2013; 3(4):32-39

Research Article

A MICROEMULSION SYSTEM FOR CONTROLLED CORNEAL DELIVERY OF TIMOLOL

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Abstract: **Purpose**: This research was aimed to formulate and characterize microemolsion systems as an ocular delivery system of timolol for treatment of glaucoma. **Methods:**microemulsion(ME) formulations were prepared by mixing of appropriate amount of surfactants including Tween 80 and Labrasol , co-surfactant such as propylene glycol (PG) and oil phase including isopropyl myristate – Transcutul P (10:1 ratio). The prepared MEs were evaluated regarding their particle size, stability, scanning electron microscopy (SEM), release and permeation of the drug through rabbit cornea. **Results:** The results showed that the maximum oil was incorporated in ME system that was contained surfactant to co-surfactant ratio (Km) of 4:1.The mean droplets sizes of ME formulations were in the range of 2.48-46.2 nm .Drug release profile showed that 13.5% of the drug released in the first 8 hours of experiment. Also, micellar structure was seen in the SEM photographs of the MEs .**Conclusion:** characterization, physicochemical properties and *in vitro* release and permeation were dependent on the contents of S+C (surfactant+cosurfactant) , water and oil in formulations. Also, ME-8may be preferable for ocular timolol formulation

Key**words:** microemulsion- timolol- ocular delivery system- glaucoma- rabbit cornea

Introduction

Since the introduction of timolol ophthalmic solution in 1974its clinical usefulness has been under extensive investigation.[1] This beta-adrenergic blocking agent reduces intraocular pressure in patients with chronic open angle glaucoma.[2] Although no adverse reactions were noted at 1.5% concentration,"0.5% was found to be the peak of the dose-response curve. Incidence of side effects is important not only for reasons of compassion for patients, but because of its positive effect on the level of obtained patient compliance.[3]

Ocular absorption of topically applied ophthalmic drugs via the cornea is in constant competition with systemic absorption via the conjunctival and nasal mucosae. Since 10% or less of a dose is usually absorbed topically into the eye, theoretically 90% or more would be available for absorption into the bloodstream, potentially eliciting systemic side effects.
Relatively little has been known about the influence of formulation composition on the systemic absorption of ocularly applied drugs [4]

In vitro permeability rates of timolol from MEs through rabbit corneahave been previouslyinvestigated. ME showed a reservoir effect. [(5)]

The present work is concerned with the permeated of timolol to rabbits cornea membrane, using aoil-in-waterME.
The aims of the work were to examine the influence ofoil-in-water MEto the corneal absorption of timolol in rabbits, and to investigate the effect of the ME on the duration of absorption of timolol. [6]

Experimental section

Timolol was purchased from Sinadaroo Company (Iran),Caprylocaproylmacrogoglycerides (Labrasol) and diethyleneglycolmonoethyleether (Transcutul P) were kindly gifted from GattefosseCompany (France), isopropyl myristate, Tween 80 and PG were obtainedfrom Merck (Germany). All chemicals and solvents were of analytical grade. Freshly distilled water was used in the experiments.

Solubility of timolol

Solubility of timolol was determined in different oil (oleic acid, isopropyl myristate, Transcutul P), surfactants (Span 80, Labrasol, Tween 80) and co-surfactant (Propylene glycol) by dissolving an excess amount of timolol in 3ml of oil, and other components using a stirrer at 37 ± 0.5^{0}C for 72 h. [(7)] The equilibrated samples were then centrifuged at 10000rpm for 30 min to remove the undissolved drug. The solubility of timolol was determined by analyzing the filtrate spectrophotometrically using a nano-specterophotometer (Biochrom WPA Bioware) after dilution with phosphate buffer pH=7at 306 nm.

Pseudo-ternary phase diagram construction

To investigate concentration range of components for the existing boundary of MEs, pseudo-ternary phase diagrams were constructed using the water titration method. Three phase diagrams were prepared with the 2:1, 3:1, and 4:1 weight ratios of (Labrasol /Tween 80) Propylene glycol respectively. Oil phase (Isopropylmirystate-Transcutul P) and the surfactant mixture were then mixed at the weight ratios of 3.52:6.48, 3.1:6.9, 0.58:9.42, 0.52:9.48, 0.52:9.48, 0.58:9.42, 3.1:6.9 and 3.52:6.48.[8]These mixtures were diluted dropwise with double distilled water, under moderate agitation. When they appeared as clear liquids, the

samples were classified as MEs. [9] Full factorial design was used concerning with 3 variables at 2 levels for formulations Major variables take part in determination of ME's properties includes surfactant/cosurfactant ratio (S/C), percentage of oil (% oil) and water percentage (%w). Eight different formulations with low and high values of oil (5% and 30%), water (5% and 15%), and S/Co mixing ratio (3:1and 4:1) were prepared for formulating the ME.

Preparation of timolol MEs

Various MEs were selected from the pseudoternary phase diagram with 3:1, and 4:1 weight ratio of Labrasol /Tween 80/Propylene glycol. Timolol(0.5%) was added to oil phase , then adding S/C (surfactant/cosurfactant) mixture and an appropriate amount of double distilled water was added to the mixture drop by drop and the MEs containing timolol were obtained by stirring the mixtures at ambient temperature.[10-11]The phases percentage of 8 formulations are showed in table 2.

Characterization

Scanning electron microscopy (SEM) was used to characterize microstructure of emulsions. SEM images of samples were taken by LED 1455VP, Germany.

The average droplet size of samples was measured at 25 °C by Scatter Scope 1 Quidix (South Korea) and their refractory indices were also calculated.

The stability of MEs was studied regarding the temperature stability and the stability after centrifugation. MEs were kept in various temperature ranges (4 °C, 25 °C and 37 °C) for 3 months and inspected for phase separation, flocculation or precipitation.[12] Also, MEs were centrifuged by high speed brushless centrifuge (MPV-350R, Poland at 10000 rpm for 30 minute at 25 °C and inspected for any change in their homogeneity.[13]

The pH value of MEs was determined at 25 °C by pH meter (Mettler Toledo seven easy, Switzerland). All measurements were carried out in triplicate. The viscosity of MEs was measured at 25 °C with a Brookfield viscometer (DV-II+Pro Brookfield., USA) using spindle no. 34. With shear rate 100 rpm [14]. DSC measurements were carried out by means of a Metller Toldo DSC1 starR system equipped with refrigerated cooling system (Hubert Tc45). Approximately 5-10mg of ME samples were weighted into hermetic aluminum pans and quickly sealed to prevent water evaporation from ME samples. Simultaneously an empty hermetically sealed pan was used as a reference. ME samples were exposed in a temperature ranging from $+30^0$C to - 50^0C (scan rate: 100C/min).

Release study

Franz diffusion cells (area 3.4618 cm^2) with a cellulose membrane were utilized to determine the release rate of timolol from different ME formulations. The cellulose membrane (molecular weight G12 000) was first hydrated in distilled water at 25^0C for 24 hours and then was clamped between the donor and receptor compartments of the cells. Each diffusion cell was filled with 25 ml of phosphate buffer (pH = 7). The receptor phase was constantly stirred by externally driven magnetic bars at 600 rpm throughout the experiment. Timolol ME (3 g) was accurately weighted and placed in donor compartment. At 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h time intervals, 2 ml sample was removed from buffer for spectrophotometric determination and replaced immediately with an equal volume of fresh receptor solution. Samples were analyzed by UV visible spectrophotometer (BioWaveII, WPA) at 306 nm. The results were plotted as cumulative released drug percent versus time.

Permeability study

Drug permeability was investigated using ophthalmic diffusion cells (area=0.7 cm) to determine the Permeability rate of timolol from different ME formulations. The cornea was extract from rabbit eyes by surgery. First we kill rabbit by diethylethere, and then the cornea withthickness of0.035 cm was extracted from its eyes and put in the Glutathione Buffer. They were clampedbetween the donor and receptor compartments of the diffusion cells. Cells were filled with 11 ml buffer and the receptor fluid was constantly stirred by externally driven magnetic bars at 600 rpm throughout the experiment. Timolol ME (0.5 g) was accurately weighted and placed in donor compartment. At 0.5, 1, 2, 3, 4, 5, 6 time intervals, 2 ml sample was removed from receptor for spectrophotometric determination and replaced immediately with an equal volume of fresh receptor solution. Samples were analyzed by UV visible spectrophotometer (BioWaveII, WPA) at 306 nm. The results were plotted as cumulative permeated drug percent versus time. The solution of timolol was used as positive control in this work.

Statistical methods

All the experiments were repeated three times and data were expressed as the mean value ± SD. Statistical data were analyzed by one-way analysis of variance (T-Test) and $P < 0.05$ was considered to be significant with 95% confidence intervals. Sigma plot software was applied for providing tertiary phase diagrams.

Results and Discussion

The maximum solubility of timolol was found in isopropylmyristate: Transcutul P (10:1) (0.344 ± 0.09). In addition, the highest drug solubility of timolol in surfactants were found in Labrasol (25 ± 0.23), and Tween 80 (35 ± 0.12). Based on the solubility studies of timolol in oil, surfactant and co-surfactant and the preformulation studies it was found IPM-Transcutul P, Labrasol, Tween 80 and propylene glycol were the most appropriate combination for preparation ofthe ME. (Table1)In order to develop ME formulations the optimum oil was selected by determining the concentration of timolol that would be dissolved. Based on the solubility studies of timolol in oil, surfactant and co-surfactant and the preformulation studies it was found isopropylmyristate, Transcutul P, Labrasol, Tween 80 and propyleneglycol could be the most appropriate combination for preparation of ME.

Table 1. Solubility of Timolol in differentials, surfactants and co-surfactants (mean±SD n=3)

Phase type	Excipient	Solubility (mg/ml)
Oil	Oleic Acid myristateIsopropyl Transcutul P	0.145± 0.3 0.344±0.09 0.678±0.04
Surfactant	Tween 80 Labrafac Labrasol	35 ± 0.12 13.697 ± 0.536 25 ± 0.23
Co-surfactant	PropyleneGlycol	14.120 ± 0.368

Pseudo-ternary phase diagrams of the investigated quaternary system water/ isopropylmyristate, Transcutul P (10:1)/ Labrasol -Tween 80/ PG is showed in figure 3. MEs were formed at ambient temperature. It seems that phase behavior depended on surfactant and cosurfactant properties. The weight ratio of surfactant/cosurfactant is a critical and important parameter affecting phase behaviors of the 8 ME. Theextent of ME area increasing with increasing of relative concentration of surfactant was reported.

The phase diagrams clearly indicated that ME formulation region increased with increase in the weight ratio of surfactant/cosurfactant. [13]

The Viscosity, pH and mean Particle size of timolol MEs are illustrated in Table4.ME formulations had particle size in the range of 2.48-46.2 nm. The ME 7 formulation had the lowest particle size 2.48 nm. The SEM of ME-1 is showed in figure 4-7. There was no significant difference observed in average particle size with ratio of S+C and oil and water.

Figure 1 shows the release profile of timolol ME formulations. The cumulative amount of timolol that had permeated through the cellulose membrane (%) was plotted as a function of time (hours). In this study, ME-1 and ME-6showed the highest and lowest accumulative release percent, respectively. Table 2 shows release percent and kinetic of release of timolol ME formulations.*In vitro* release studies with an artificial hydrophobic membrane can provide information about the diffusion of a drug, which depends on the physicochemical properties of components and the MEs structure[15, 16] It seems that release percent enhanced with decrease in the S+C percent and increase in S/C ratio.[19] It may appear because of increase the solubility of drug with surfactant.[13] The release profile of MEs was calculated by fitting the experimental data to equations describing different kinetic models. Linear regression analyses were made for zero-order (Mt/M0 = kt), first-order (ln (M0–Mt) = kt), Higuchi (Mt/M0 = (kt)1/2), Log Wagner, Linear wagner, Weibul, Second root of mass, Three-Seconds root of mass, and Pepas kinetics. The slowest release was observed for ME-6 with Log Wagner kinetic and the highest release was observed for ME-1 with first kinetic.The highest release amount percentage was observed for ME-1 with micellar structures.[19]

Table 2 .Percent release and kinetic of release of selected MEs

Formulation	release %	kinetic model	R^2	Intercept
ME-1	13.5457	First	0.6310	-0.0263
ME-2	9.15314	Log Wagner	0.9880	-2.1151
ME-3	8.87492	Log Wagner	0.9474	-2.0110
ME-4	7.86755	Log Wagner	0.9328	-2.2026
ME-5	9.3183	Log Wagner	0.9477	-2.0260
ME-6	5.16344	Log Wagner	0.9701	-2.4292
ME-7	6.68861	Log Wagner	0.9654	-2.2908
ME8	7.70015	Higuchi	0.9702	-0.0241

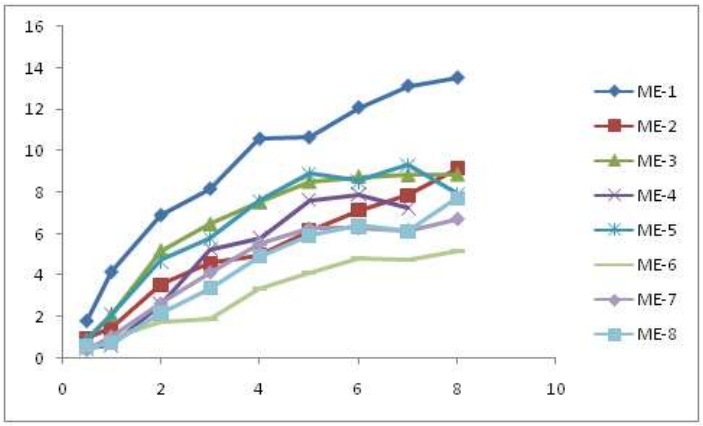


Figure 1 . The release profile of 8 Timolol ME formulations (release%-time)

The visual inspection test was performed for 3 months by drawing ME sample at weekly interval for the first month and monthly interval for the subsequent months. The visual observation showed no evidence of phase separation or any flocculation or precipitation. These samples also revealed no sign of phase separation under stress when subjected to centrifugation at 10000 rpm for 30 min. The centrifugation tests revealed that MEs were remained homogenous without any phase separation throughout the test indicates good physical stability of preparations. [17]

Figure 2 represents DSC cooling thermograms of timolol MEs. In cooling curves of the samples, bulk water (free water) and bound water are obtained in -2 to -7^{0}C and -11 to -13^{0}C, respectively. Table 5 showed the results of thermogram analysis of MEs in cooling program temperature. DSC results indicate important information about water state in MEs. When water is mixed in to a ME system it can be either bound (interfacial) or free (bulk) water depending of its state in the system. In cooling curves of the sample ME-1, DSC thermograms showed one exothermic peak at around -10 to -12^{0}C that indicate the freezing of bound water in this formulation and a peak at around -16 to -18^{0}C that indicate the freezing of oil phase in

this formulation and a peak at around -26^{0}C that indicate the bound water interacts with surfactant. In ME-2 implies three exothermic peak at around -10 to -12^{0}C (bound water), -17^{0}C that indicate the freezing of the oil phase and a peak around -25 to -260C (bound water with surfactant. Decrease of ΔH of first and last peak with ME-1 was because of decrease in water phase in this formulation. In cooling curves of ME-3, DSC thermograms showed two exothermic peak at -4^{0}C (free water) and -30^{0}C which indicates bound water with surfactant . In cooling curves of ME-4, DSC thermograms showed two exothermic peak at -5^{0}C (free water) and -32^{0}C which indicates bound water with surfactant . DSC thermograms of ME-5 showed two exothermic peak at -2^{0}C (bulk water) and -33^{0}C (bound water with surfactant) which means water was bounded to surfactant strongly .DSC thermograms of ME-6 showed two exothermic peaks at -7^{0}C (bulk water) and -29^{0}C (bound water with surfactant). In cooling curves of the sample ME-7, DSC thermograms showed one exothermic peak at around -13^{0}C that indicate the freezing of bound water in this formulation and a peak at around -18^{0}C that indicate the freezing of oil phase in this formulation and a peak at around -26^{0}C that indicate the bound water interacts with surfactant. DSC thermograms of ME-8 showed one exothermic peak at around -11^{0}C that indicate the freezing of bound water in this formulation and a peak at around -20^{0}C that indicate the freezing of oil phase in this formulation and a peak at around -25^{0}C that indicate the bound water interacts with surfactant.[18]

Figure 2 shows the permeation profile of timolol ME formulations. The amount of timolol that had permeated through the rabbit cornea membrane per the area of ocular cells (mg/cm^2) was plotted as a function of time (hours). In this study, ME-1 and ME-6 have the highest and lowest accumulative release percent, respectively. Table 3showsJ_{SS}, TLag, $D_{app,}$ P, ER_{Flux}, ER_D, ER_P intimolol ME formulations.

Results give from t-test analyzer

All the MEs have P-value under 0.05 which means all of them have significant difference with solution of timolol eye drop in amount of timolol maleate permeated through cornea membrane just ME-2 has P-vale=0.1463 which means this formulation has not significant difference with timolol solution eye drop in permeation. The highest permeation is observed in ME-8. All the formulations have significant difference with timolol solution eye drop in amount timolol permeated through area of cornea membrane (mg/cm^2) ($p<0.05$) just ME-2 has P-vale=0.1463 which means this formulation has not significant difference with timolol solution eye drop in permeation.

Results give from Minitab software analyzer

The regression equation of J_{SS} is:
Jss = 0.250 - 0.0538 s/c + 0.00076 %oil + 0.00138 %w
P-value for S/C is 0.0467 which means a significant relation was between J_{SS} and S/C.
With decrease the S/C , increasing the J_{SS}

The regression of T_{lag} equation is:
Tlag = - 0.77 + 0.622 s/c + 0.0528 %oil - 0.0684 %w
P-value for %oil is 0.005 which means a significant relation was between T_{lag} and %oil.
With increase the %oil , increasing the T_{lag}.

The regression equation of D is:
D = - 0.0031 + 0.00303 s/c - 0.000148 %oil - 0.000298 %w
Any parameter has not the significant relation with D coefficient. (p.0.05)

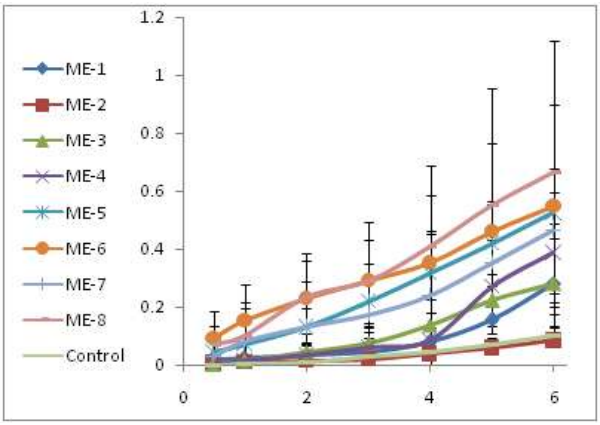
The regression equation is
P = 0.0536 - 0.0116 s/c + 0.000149 %oil + 0.000216 %w
P-value for S/C is 0.0497 which means a significant relation was between P and S/C.
With decrease the S/C , increasing the P.

J_{SS} = The rate of drug permeation which is get from amount drug permeated through area unite- time curve.

P = Permeability coefficient which give from this equation
$J_{SS} = C_0 \times P$
C_0 = The concentration of drug in receptor phase
D= Diffusion coefficient

Table 3.J_{SS},T_{Lag},$D_{app,}$ P,ER_{Flux},ER_D,ER_P of timolol ME formulations (mean±SD, n=3)

formulation	J_{SS}	T_{Lag}	D_{app}	P	ER_{Flux}	ER_D	ER_P
ME-1	0.0991 ±0.103107	2.931829 ±0.506161	0.0000644 ±0.000011	0.018300333 ±0.018277056	3.851086154	0.466289	3.333394
ME-2	0.020833 ±0.00822	1.958941 ±0.97453	0.0001203 ±0.000076	0.004128667 ±0.00158597	0.809595979	0.871748	0.752034
ME-3	0.032367 ±0.01004	0.670214 ±0.41881	0.000465 ±0.000434	0.006507 ±0.002031	1.257788	3.369494	1.185307
ME-4	0.097167 ±0.097152	2.262564 ±1.995237	0.013265 ±0.022876	0.019578 ±0.019846365	3.7759556	96.11916	3.56612
ME-5	0.093433 ±0.029921	0.604799 ±0.290763	0.000349 ±0.000207	0.018621 ±0.006264	3.630876	2.529397	3.391803
ME-6	0.0962 ±0.061057	0.401494 ±0.335049	0.00118 ±0.001414	0.019492 ±0.012333	3.73839	8.548961	3.550455
ME-7	0.11795 ±0.029812	3.122708 ±0.58026	0.000073 ±0.000016	0.025005333 ±0.008156258	4.583609	0.52513	4.554706
ME-8	0.157033 0.1124	1.20498 0.527474	0.000191 0.000128	0.031663 0.021792	6.102411	1.384791	5.767395
Positive Control	0.025733 ±0.016187	1.806989 ±0.994449	0.000138004 ±0.000077	0.00549 ±0.003453	-	-	-



Figur 2 . The permeation profile of 8 formulation (mg/cm^2-time)(Mean±SD)

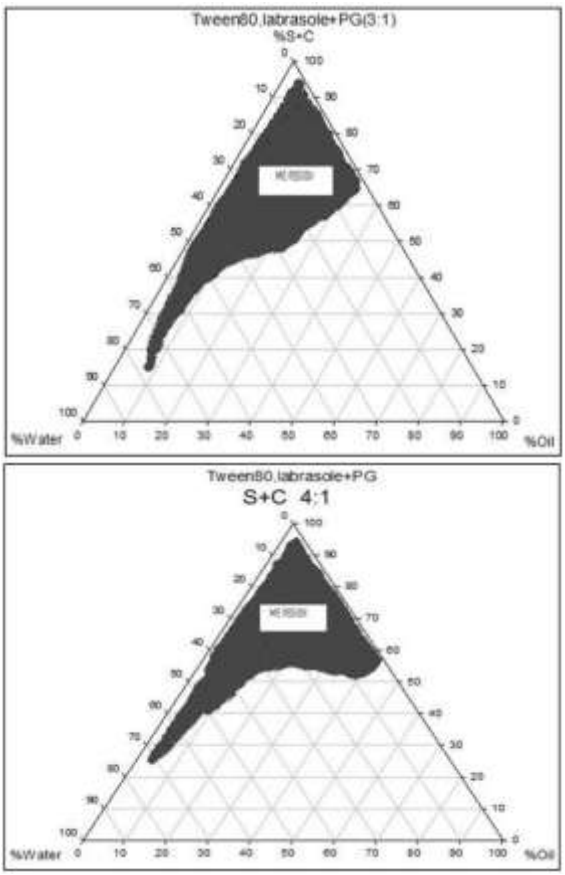
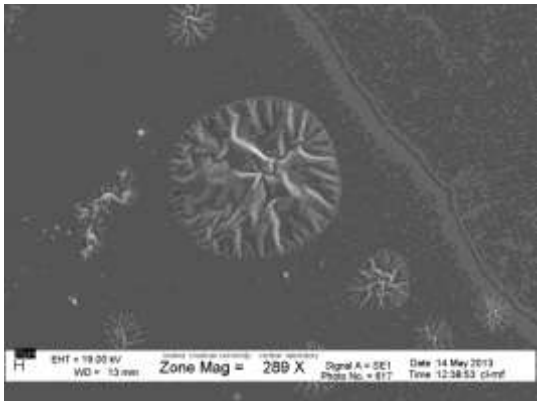
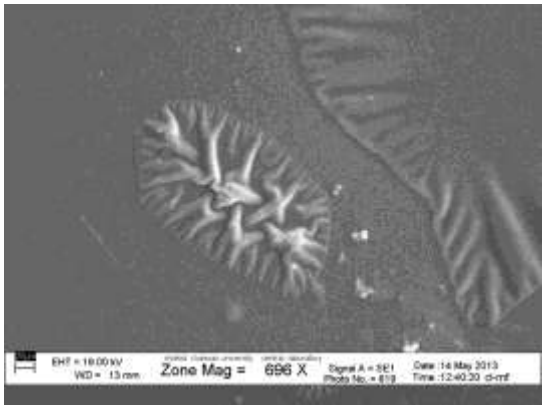
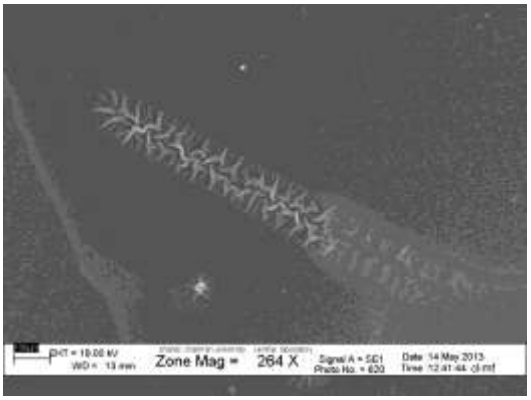


Figure 3.The pseudo-ternary phase diagrams of the oil-surfactant/cosurfactant mixture–water system at the3:1 and 4:1 weight ratio of Labrasol /Tween 80/ Propylene glycol at ambient temperature, dark area show MEs zone.







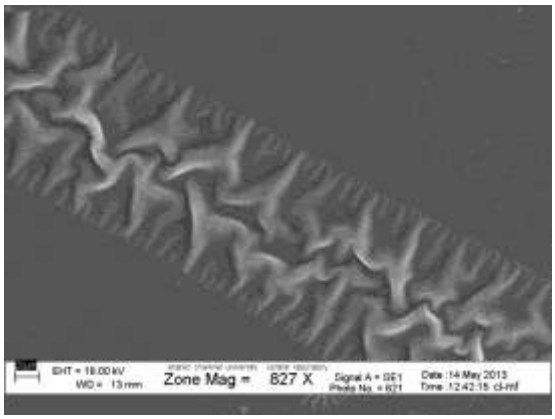


Figure 4-7. Images taken by SEM technology

Table4. Compositions (% w/w) and Particle Size (mean ± SD, n=3)of selected MEs

Formulation	S/C	Oil	S+C	water	Ph of MEs with drug	Viscosity of MEs with drug	Particle size(nm)
ME-1	4:1	30	55	15	6.5± 0.029	215.5± 0.01	22.9
ME-2	4:1	30	65	5	6.83± 0.44	228.4± 0.21	46.2
ME-3	4:1	5	80	15	6.75±0.455	290.1± 1.07	4.1
ME-4	4:1	5	90	5	6.86± 0.121	226.2± 0.081	3.59
ME-5	3:1	5	90	5	7.1± 0.002	253.4± 0.012	22.9
ME-6	3:1	5	80	15	6.78± 1.256	271.3± 1.094	31.3
ME-7	3:1	30	65	5	7.2± 0.18	229.4± 0.031	2.48
ME-8	3:1	30	55	15	6.87± 1.034	343.5± 1.09	9.86

Table 5.Results of thermogram analysis of MEs in cooling program temperature.

Formulation	**Phase change temperature(^{0}C)**	**ΔH (mj)**
ME-1	-12	48.33
	-17	0.87
	-25.5	6.314
ME-2	-12	15.37
	-17	0.22
	-25	1.67
ME-3	-4	0.825
	-30	1.99
ME-4	-5	0.9
	-32	2.06
ME-5	-2	1.09
	-33	2.12
ME-6	-7	0.81
	-29	1.63
ME-7	-13	20.2
	-18	0.32
	-26	2.02
ME-8	-11	7.01
	-20	11.06
	-25	0.81

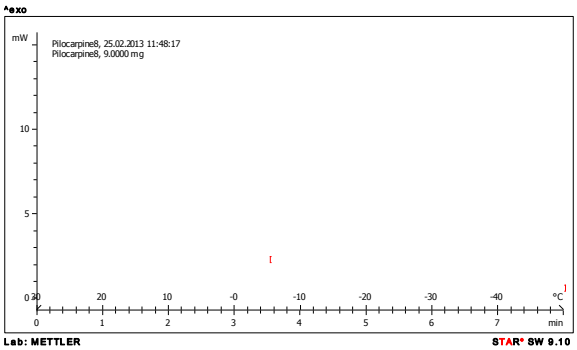


Figure 8. DSC thermogram of ME-1

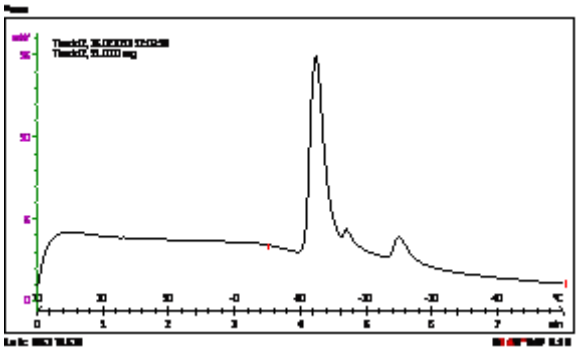


Figure 9. DSC thermogram of ME-2

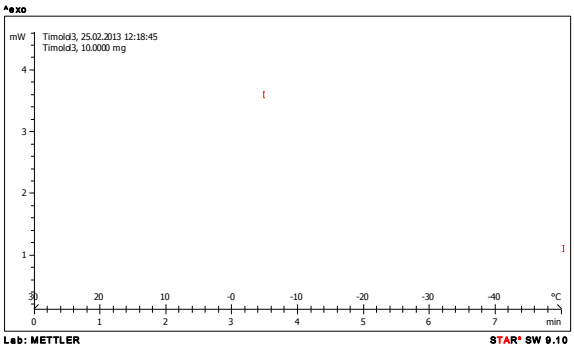


Figure 10. DSC thermogram of ME-3

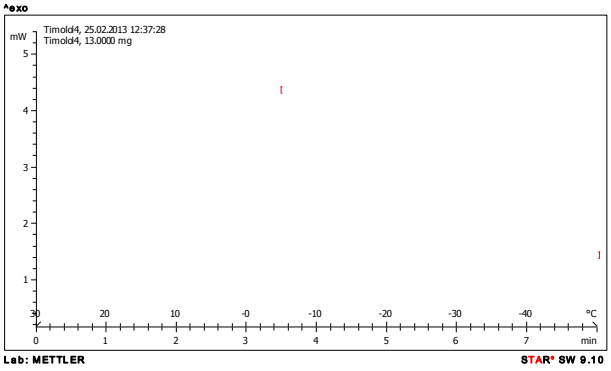


Figure 11. DSC thermogram of ME-4

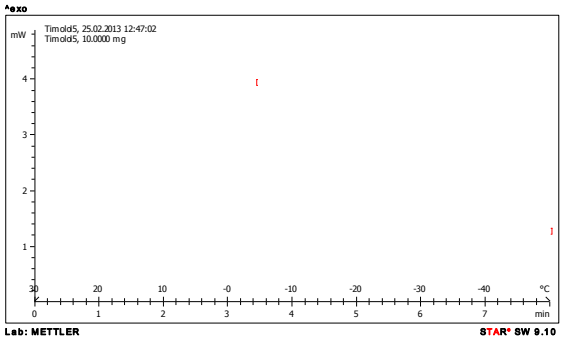


Figure 12. DSC thermogram of ME-5

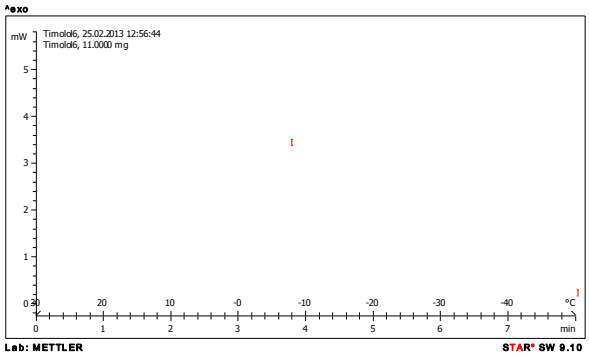


Figure 13. DSC thermogram of ME-6

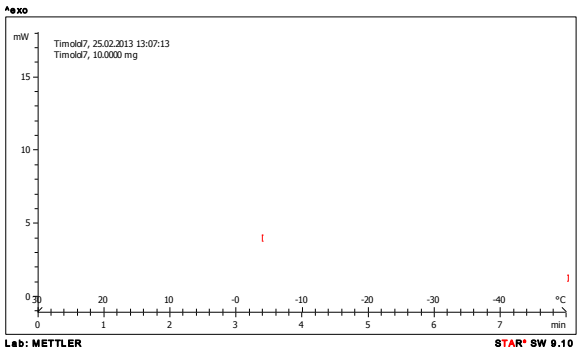


Figure 14. DSC thermogram of ME-7

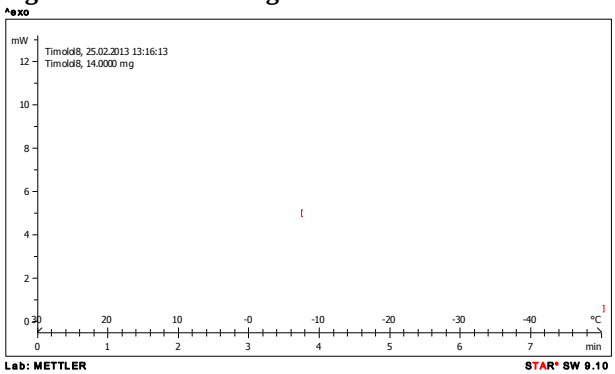


Figure 15. DSC thermogram of ME-8

Conclusion

This study established that physicochemical properties and in vitro release and *invitro* permeabilitywere dependent upon the contents of S+C, oil and water in formulations. Pseudo-ternary Phase diagrams indicated more width ME region with a rise in S/C ratio. By decreasing in S/C ratio and oil percent and increasing in water percent the higher *in* vitro percentage release was obtained. The amount of released drug differs between ME carriers with various internal microstructures. ME-8may be preferable for ocular timolol formulation although the serious work still is needed to be carried out to reveal the mechanisms of drug delivery into the eye.

Acknowledgments

This paper is extracted from Pharm.D.thesis (Saadati, A) and financial support was provided by Ahvaz Jundishapur University of Medical Sciences. The authors are very thankful to Faratin company executive manager (Taheri, M, Iran) for providing gratis sample of Transcutul P, Labrasol from Gattefosse (France) and also GattefosseCompany (France).The authors are very thankful to Sinadaroo Company for providing timolol maleate powder.

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