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Study of the Effects of Polyethylene Glycol Sorbitan Esters Surfactants Group on Biological Membranes

[1,2]Gholamreza Dehghan Noudeh, [1]Payam Khazaeli and [2]Pedram Rahmani
[1]Department of Pharmaceutics, School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran
[2]Pharmaceutics Research Center, Kerman University of Medical Sciences, Kerman, Iran

Abstract: The aim of this study is the evaluation of the effect of one group of surfactants including polyethylene glycol sorbitan esters (Tweens: 20, 40, 60 and 80) on Red Blood Cells (RBC) as a model for biological membranes. Also in this study some of physicochemical properties including Emulsification index (E_{24}) and Foam producing activity (F_h) were studied. In this study the hemolytic effect of four surfactants from Tween category were evaluated. Surfactants solutions were prepared in McIvan's buffer in specific concentration. 0.2 mL of RBC was mixed with 0.2 mL of one of surfactants solution incubated in four different temperatures for two different times. The absorbance of the samples was determined by UV spectrophotometer. Each test was done nine times. The results were shown by mean±SD. E_{24} and F_h were also determined for each surfactant solutions. In comparison of the four studied surfactants, Tween 20 have the highest hemolytic effect and the Tween 80 is the lowest one. The values of E_{24} and F_h have good correlation with Hydrophilic-Lipophilic Balance (HLB) values. Increasing in HLB value lead to increasing in those parameters.

Key words: Tween, biological membrane, hemolysis and HLB

INTRODUCTION

Surfactants have many characteristics comprising groups with hydrophilic and hydrophobic characters with different usages in pharmaceutical formulations, such as co-solvent, humectant, emulsifying, solubilizing agent and enhancer (Sajadi and Mamagani, 2001).

Regarding their hydrophilic part, they are divided into four groups, anionic, cationic, amphoteric and non-ionic (Porter and Mitgi, 1994).

Absorption enhancing ability of surfactants in formulations with low absorption like peptides or proteins is used for drug delivery in non-injectable formulations. A board spectrum of surfactants is used as enhancers including bile salts, anionic detergents, glycerides and lysophospholipids (lysolecithins); however, the efficacy of non-ionic surfactants with moderate polarity is better. On the other hand, it is reported that non-ionic polar surfactants do not have toxicity, while surfactants with moderate polarity showed toxic effects (Gould *et al*., 2000).

Morphologic and biochemical studies on membrane of absorption sites showed that surfactants enhance membrane transport followed by acute toxicity but these effects were reversed after a long time. As a result, there is a pivotal relationship between permeability enhancement activity and acute toxicity; moreover, permeability enhancing effect of surfactants is not only related to their nature, but also depends on other characteristics like electrical charge, polarity and the membrane (Golembeck *et al*., 1998; Gould, 1996).

Permeability enhancers are agents that decrease or remove extra cellular layer resistance reversibly and allow the drug to pass trough and between epithelial cells toward blood and lymph. Recently, enhancing drugs permeability trough cellular membrane becomes one of the main topics in pharmaceutical researches (Muranishi, 1990).

According to chemical structure enhancers consist of surfactants, steroidal or bile salts detergents, salicylates, chelators and enamines (N-acetyl amino acids) (Vinardell and Infante, 1999).

In recent years permeability enhancing effects of some ionic and non-ionic surfactants were studied. Gould *et al*. (2000) showed that some of non-ionic surfactants could increase mucosal absorption of drugs with low absorption. One of the suggested mechanisms is inducing partial but reversible gap within cells membranes' and consequently increasing the permeability by surfactants or other enhancers. Various models exist for evaluation of membrane toxicity of surfactants including single cell models using erythrocytes, erythrocyte ghosts,

Corresponding Author: Gholamreza Dehghan Noudeh, Department of Pharmaceutics, School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran
Tel: ++98-341-3220090-95 Fax: ++98-341-3220799

or liposomes. The erythrocyte model has been widely used as it presents a direct indication of toxicity of injectable formulations as well as general indication of membrane toxicity. Another advantage of erythrocytes model is that blood is readily available and that cells are easy to isolate from the blood; moreover, its membrane has similarities with other cell membrane (Robertis and Robertis, 1995).

Evaluating the toxicity of permeability enhancers using biological membranes plays an important role. Consequently, in present study we decided to determine the effects of poly ethylene glycol lorate (Tween 20), poly ethylene glycol palmitate (Tween 40), combination of poly ethylene glycol stearate and palmitate (Tween 60) and poly ethylene glycol oleate (Tween 80) on cellular membrane using erythrocyte model.

MATERIALS AND METHODS

Materials: All materials were of reagent grade unless otherwise mentioned. Tween 20, 40, 60 and 80 were prepared from Fluka, (Netherlands). Sodium chloride, di-sodium hydrogen phosphate, citric acid (monohydrate), di-sodium phosphate and liquid paraffin were purchased from Merck (Germany). Drabkin's agent was supplied from Chimi-Daru (Iran).

Buffer and reagents preparation

McIlvaine's buffer was prepared as follows: Solution 1, containing 21 g of citric acid (100 mmol) and 8.775 g of sodium chloride (150 mmol) made up to 1000 mL with deionized water, was mixed with solution 2, containing 28.4 g of di-sodium hydrogen phosphate (200 mmol) and 8.775 g of sodium chloride (150 mmol) made up to 1000 mL with deionized water, to produce the required pH of 7.0. Solution's pH was measured by electrical pH-meter (TWT Metrohm, Germany).

Preparation of red blood cells suspension: Human blood was collected from a healthy individual with 46.7% hematocrit and added to four heparinized tubes. After centrifuging at 3000 rpm for 10 min (Hermle 230 ZA, Germany), plasma and buffy coat were removed and the erythrocytes were washed three times in at least five times of their volume with McIlvaine's buffer, pH = 7.0. Afterward, by adding McIlvaine's buffer, an erythrocyte suspension with 12% hematocrit were prepared and kept in 4°C for experiments (Gould *et al*., 2000).

Hemolytic method: A suspension of erythrocyte (200 μL) within a micro-tube was incubated for the required times with an equal volume of the test sample of surfactants mixture, including Tween 20, 40, 60, or 80, prepared in McIlvaine's buffer, at 25, 30, 37, or 42°C. After incubation, the mixture were spun in a microcentrifuge at 3000 rpm for 35 sec (Spectrafuge 161M, England) and 200 μL of the resulting supernatants was added to 3 mL of Drabkin's reagent. To assay for the amount of hemoglobin released, the absorbance of samples were assessed in 540 nm wavelength using spectrophotometer (Shimadzu, 3100, Japan). Positive controls consisted of 200 μL of uncentrifuged mixtures of erythrocyte suspensions and 200 μL of buffer, which were added to 3 mL Drabkin's reagent to obtain a value for 100% haemolysis. A negative control, included to assess the level of spontaneous haemolysis, comprised 200 μL buffer mixed with 200 μL erythrocytes and after centrifugation for 35 sec, a 200 μL sample of supernatant was added to 3 mL of Drabkin's reagent. Haemolysis percentage for each sample were calculated by dividing sample's absorbance on positive control absorbance (compete haemolysis) multiplied 100 (Gould *et al*., 2000).

Determination of emulsification index: For estimation of the emulsification index, 5 mL of liquid paraffin was added to 5 mL of different concentrations of surfactants in a graduated tube and vortexed at high speed for 2 min. The emulsion stability was determined after 24 h. The E_{24} was calculated by measuring the emulsion layer formed (Carrillo *et al*., 1996).

Foam formation activity: Different concentrations of surfactants were dissolved to 5 mL Disodium phosphate buffer and shaked with vibrator for 5 sec. The samples put aside at 25°C for one minute. Foam activity was measured as foam height in graduated cylinder (Porter, Mitgi, 994).

RESULTS AND DISCUSSION

The results of haemolysis induced by surfactants were showed in Table 1-8 that Table from 1 to 4 are related to haemolysis after 15 min and from 5 to 8 are related to haemolysis after 30 min. Each point stands for mean of haemolysis percentage repeated in nine experiments; lines represent standard deviation that may interfere with some signs. Results of emulsification index and foaming formation are presented in Table 9 and 10, respectively. Despite the fact that all about surfactants hemolytic activity is not fully known, but it's proposed that it may consist of according processes:

Absorption of surfactant molecules on cellular surface, penetration of surfactant molecules into cellular membrane, induction of alterations within cellular membrane, increasing permeability of cellular membrane,

Table 1: Hemolysis induced by Tween 20, 40, 60 and 80 after 15 min at 25°C (n = 9)

	Surfactants							
	Tween 20		Tween 40		Tween 60		Tween 80	
Conc. (mM)	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.001	2.301	0.025	2.301	0.021	1.255	0.014	1.674	0.008
0.004	8.159	0.171	7.113	0.085	4.603	0.097	3.766	0.041
0.008	14.017	0.196	11.925	0.155	12.762	0.242	10.669	0.149
0.012	29.707	0.683	26.569	0.558	26.360	0.663	24.895	0.523
0.016	44.142	0.574	40.795	0.775	37.238	0.782	32.636	0.816
0.02	69.665	1.115	64.854	1.038	60.669	1.335	48.954	0.881
0.04	79.707	1.913	78.033	1.717	69.665	1.951	53.766	1.183
0.06	84.937	1.869	84.100	2.003	68.619	2.127	54.812	1.699
0.08	85.983	2.322	83.473	2.103	71.548	1.789	53.975	1.133
0.1	86.402	1.642	81.799	2.290	72.176	1.660	56.067	1.065

Table 2: Hemolysis induced by Tween 20, 40, 60 and 80 after 15 min at 30°C (n = 9)

	Surfactants							
	Tween 20		Tween 40		Tween 60		Tween 80	
Conc. (mM)	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.001	3.393	0.041	2.595	0.029	1.597	0.019	1.633	0.018
0.004	10.379	0.187	14.172	0.241	6.986	0.147	4.356	0.074
0.008	17.166	0.412	22.954	0.528	18.762	0.281	12.886	0.412
0.012	36.128	0.397	38.124	0.534	35.329	0.459	30.490	0.427
0.016	43.313	0.736	47.106	1.036	46.307	1.019	40.472	0.890
0.02	76.248	1.677	74.052	1.333	63.273	1.645	57.713	0.923
0.04	85.030	2.381	86.028	2.495	70.659	0.848	58.258	1.689
0.06	94.212	2.261	89.421	2.146	73.453	2.277	59.710	1.851
0.08	91.417	2.651	86.228	2.673	71.657	1.576	61.162	1.407
0.1	93.014	3.162	88.024	2.025	72.056	1.657	58.439	1.403

Table 3: Hemolysis induced by Tween 20, 40, 60 and 80 after 15 min at 37°C (n = 9)

	Surfactants							
	Tween 20		Tween 40		Tween 60		Tween 80	
Conc. (mM)	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.001	5.492	0.044	3.432	0.017	1.831	0.020	1.831	0.025
0.004	14.416	0.173	9.382	0.169	4.119	0.058	5.034	0.171
0.008	20.824	0.312	19.222	0.250	7.094	0.085	15.103	0.196
0.012	37.071	0.704	30.664	0.491	29.519	0.708	29.977	0.683
0.016	51.259	0.666	45.995	1.012	48.284	1.352	44.622	0.574
0.02	76.430	1.758	72.998	1.971	69.336	1.456	58.352	1.115
0.04	82.838	2.568	81.007	1.944	71.625	1.934	59.954	1.913
0.06	91.991	2.944	89.703	1.884	73.684	2.505	61.327	1.869
0.08	93.822	2.533	92.220	2.582	72.998	1.606	60.641	2.322
0.1	95.423	2.386	91.076	2.095	74.828	1.571	62.014	1.640

Table 4: Hemolysis induced by Tween 20, 40, 60 and 80 after 15 min at 42°C (n = 9)

	Surfactants							
	Tween 20		Tween 40		Tween 60		Tween 80	
Conc. (mM)	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.001	4.762	0.048	3.896	0.027	1.948	0.010	1.948	0.021
0.004	13.203	0.172	9.307	0.140	8.225	0.181	4.545	0.064
0.008	22.944	0.275	19.697	0.217	13.636	0.327	18.831	0.395
0.012	38.095	0.876	33.766	0.912	34.416	1.067	32.684	1.046
0.016	50.000	1.550	58.009	1.798	55.628	1.168	51.299	1.077
0.02	80.303	1.686	69.697	1.255	61.905	1.671	59.307	1.957
0.04	91.342	1.553	87.879	1.230	67.749	1.084	65.584	1.377
0.06	96.537	2.800	91.126	2.369	70.130	1.613	66.234	0.795
0.08	98.701	2.073	94.589	2.176	72.727	1.600	67.316	1.481
0.1	98.052	2.647	95.022	2.090	73.377	1.834	68.182	1.705

Table 5: Hemolysis induced by Tween 20, 40, 60 and 80 after 30 min at 25°C (n = 9)

	Surfactants							
	Tween 20		Tween 40		Tween 60		Tween 80	
Conc. (mM)	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.001	1.674	0.018	1.883	0.009	1.674	0.020	1.674	0.013
0.004	7.322	0.095	6.904	0.083	4.812	0.063	4.393	0.062
0.008	13.389	0.281	12.134	0.133	13.808	0.290	11.506	0.161
0.012	31.590	0.758	26.987	0.729	26.569	0.691	26.778	0.482
0.016	45.816	0.779	42.469	0.679	38.285	0.689	33.891	0.746
0.02	70.293	2.249	70.293	1.546	62.134	1.367	49.791	1.145
0.04	80.544	1.450	79.079	1.898	69.247	1.662	54.603	0.819
0.06	85.983	2.064	84.937	1.954	70.921	1.135	54.812	1.809
0.08	85.356	1.878	85.565	2.653	72.385	1.954	53.347	1.440
0.1	86.820	1.476	85.983	2.322	73.640	1.841	54.603	1.583

Table 6: Hemolysis induced by Tween 20, 40, 60 and 80 after 30 min at 30°C (n = 9)

	Surfactants							
	Tween 20		Tween 40		Tween 60		Tween 80	
Conc. (mM)	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.001	3.792	0.023	2.994	0.033	1.796	0.020	1.597	0.021
0.004	12.176	0.146	14.571	0.204	8.184	0.115	4.990	0.070
0.008	17.764	0.284	23.752	0.523	19.162	0.230	16.367	0.360
0.012	36.727	0.845	42.315	0.592	36.327	0.763	31.737	0.476
0.016	48.902	0.685	48.703	1.120	47.106	0.848	46.108	0.784
0.02	77.645	2.096	75.250	1.956	64.471	0.774	62.076	1.304
0.04	86.028	1.893	87.625	0.964	71.457	0.786	64.271	1.028
0.06	92.814	3.063	88.024	2.729	71.058	1.634	64.072	1.346
0.08	92.415	2.310	89.022	2.582	72.655	1.962	63.074	1.135
0.1	94.611	2.271	90.020	2.881	73.852	1.772	65.469	1.375

Table 7: Hemolysis induced by Tween 20, 40, 60 and 80 after 30 min at 37°C (n = 9)

	Surfactants							
	Tween 20		Tween 40		Tween 60		Tween 80	
Conc. (mM)	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.001	4.805	0.043	4.348	0.057	1.602	0.013	1.831	0.013
0.004	14.188	0.255	10.069	0.232	4.348	0.061	4.348	0.052
0.008	21.053	0.442	19.680	0.216	16.934	0.288	15.561	0.249
0.012	37.529	0.863	31.579	0.600	31.808	0.350	31.350	0.752
0.016	52.860	0.581	47.826	1.004	49.428	0.939	46.224	0.508
0.02	76.888	1.845	74.142	2.150	71.625	0.788	59.725	1.374
0.04	85.355	2.646	84.211	2.863	75.744	1.591	59.039	1.063
0.06	93.364	2.054	91.076	1.275	76.430	1.681	62.471	1.687
0.08	95.881	13.807	89.474	2.058	75.973	2.051	63.158	1.389
0.1	96.110	2.691	92.906	2.323	78.032	2.419	66.590	2.131

Table 8: Hemolysis induced by Tween 20, 40, 60 and 80 after 30 min at 42°C (n = 9)

	Surfactants							
	Tween 20		Tween 40		Tween 60		Tween 80	
Conc. (mM)	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD	Hemolysis (%) (Mean)	±SD
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.001	5.195	0.062	3.896	0.027	1.948	0.014	1.948	0.010
0.004	14.286	0.157	9.957	0.119	8.009	0.176	5.195	0.068
0.008	22.727	0.500	20.996	0.294	15.368	0.292	18.398	0.442
0.012	38.312	0.881	35.498	0.745	35.931	0.431	32.900	0.362
0.016	59.307	0.712	58.658	0.763	66.667	1.600	52.165	1.617
0.02	82.251	1.481	70.779	1.769	73.377	1.101	59.524	0.655
0.04	92.424	2.126	87.446	2.099	74.459	0.968	58.658	1.642
0.06	97.186	1.361	92.641	2.594	75.974	2.127	61.688	0.802
0.08	96.320	2.986	93.506	1.309	75.325	1.582	64.286	1.479
0.1	97.619	2.538	96.104	2.306	78.355	2.037	63.203	1.770

Table 9: Emulsification index at different concentrations of Tween 20, 40, 60 and 80 (n = 9)

	Surfactants							
	Tween 20		Tween 40		Tween 60		Tween 80	
Conc. (mM)	E24 (Mean)	±SD	E24 (Mean)	±SD	E24 (Mean)	±SD	E24 (Mean)	±SD
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.001	1.850	0.015	1.850	0.022	1.920	0.027	1.890	0.025
0.004	1.850	0.020	1.850	0.011	2.000	0.046	1.890	0.026
0.008	1.920	0.023	1.920	0.027	2.000	0.044	1.920	0.035
0.012	1.920	0.013	1.920	0.025	1.920	0.054	3.850	0.035
0.016	1.920	0.023	1.920	0.021	3.770	0.064	5.770	0.075
0.02	1.920	0.021	2.000	0.032	4.000	0.052	14.280	0.200
0.04	2.000	0.026	5.360	0.118	16.000	0.256	26.920	0.485
0.06	3.850	0.081	5.770	0.121	30.770	0.585	33.330	0.533
0.08	3.700	0.104	7.410	0.141	32.000	0.672	33.330	0.500
0.1	4.850	0.116	7.690	0.115	31.770	0.572	32.560	0.358

Table 10: Foam formation activity at different concentrations of Tween 20, 40, 60 and 80 (n = 9)

	Surfactants			
	Tween 80	Tween 60	Tween 40	Tween 20
Conc. (mM)	Foam height (mm) (Mean±SD)	Foam height (mm) (Mean±SD)	Foam height (mm) (Mean±SD)	Foam height (mm) (Mean±SD)
0	0	0	0	0
0.001	1.0±0.11	0	0	0
0.004	2.0±0.14	0	0	0
0.008	3.5±0.08	1.0±0.11	1.0±0.11	1.0±0.13
0.012	4.0±0.05	2.0±0.21	1.5±0.12	1.5±0.21
0.016	5.0±0.14	3.0±0.24	2.0±0.50	1.2±0.17
0.02	7.0±0.10	5.0±0.03	4.0±0.21	3.5±0.12
0.04	9.0±0.20	7.0±0.14	6.0±0.17	5.0±0.08
0.06	12.0±0.08	9.0±0.17	6.0±0.42	5.0±0.24
0.08	12.0±0.11	9.0±0.21	6.0±0.24	5.0±0.12
0.1	12.0±0.13	9.0±0.11	7.0±0.27	6.0±0.61

gradual increase of osmotic phenomenon and followed by destruction of cellular membrane and haemolysis. According to above explanation, two different effects from surfactants in hemolytic studies can be observed, the first one is increasing cellular membrane permeability and the latter is cellular lysis. Surfactants which induce haemolysis can alter the membrane permeability for hemoglobin. This alteration occurs in a specific spectrum of the surfactant concentration and in lower concentrations hemolytic effects can not be seen; in these concentrations cellular membrane is permeable for low molecular weight molecules. Destruction due to surfactants is the result of cellular membrane breakage by alteration of structural molecules of the membrane; subsequently, the membrane permeability for macro molecules similar to smaller molecules increase. In this chain reaction mechanism, surface active agents adhere to erythrocyte surface and enter inside, change the molecular structure of the membrane which results in colloid-osmotic swelling of the erythrocyte and ultimately cellular rupture. Micelle production from surfactant molecules and membrane phospholipids lead to increase in membrane permeability and colloid-osmotic lysis of erythrocyte. Above mechanism highly depends on surfactant concentration and temperature and by increase in these factors the level of haemolysis increases. These mentioned effects support the idea of surfactant usage as absorption enhancer (Bonarska *et al*., 2005).

Haemolysis is due to red blood cells destruction which resulted from lysis of membrane lipid bilayer emulsion and cellular membrane destruction. As this haemolysis relates to concentration and potency of surfactants, the model can be used for evaluation of surfactants potency. Biological membrane consists of a lipid bilayer which surrounds whole cell surface and proteins. Lipid bilayer structure is stabilized by non-covalent bonds among acyl groups and ionic bonds between polar heads and aqua. In non-ionic surfactants the interaction with biological membrane needs hydrophobic interaction between alkyl chains of surfactant and lipoprotein parts of membrane (Swensones and Curatio, 1992).

In this study, the hemolytic effects of surfactants increased as temperature increased. Note that liquid characteristic and fluidity of bilayer lipid is one of its special features. Therefore, some parts of the membrane can easily move throughout the surface and this characteristic is due to membrane phospholipids which covert to jelly in temperatures lower than physiologic temperature. This conversion of phospholipids helps in

more stabilized and regular membrane and increases its resistance. As a result, the amount of haemolysis in is 42°C more than 25°C; the reason is that with increase in temperature the membrane fluidity and accordingly its permeability increase (Boris *et al*., 2002).

Also, in solutions with higher concentration of surfactants haemolysis amount were more. This result can be easily described by Fick's law that according to this law, the diffusion flux from a membrane is proportional to concentration difference of both sides (Muranishi, 1990).

In other words, the concentration of intra-membrane surfactant is related to its extra-membrane concentration and by increasing the latter concentration the first one increases until reaching to a specific concentration which leads to membrane destruction and hemolytic effects (Boris *et al*., 2002).

The first step in surfactant-membrane interaction is membrane saturation with surfactant's monomers; following the process cellular lysis is possible. The onset is followed by destruction and deconstruction of surfactant-protein-lipid complexes and surfactant-lipid mixture micelles. Adding more surfactant enriches the surfactant-protein-lipid complexes and more mixture micelle production. At the extremity and in cmc the amount of protein-surfactant complexes, mixture micelles and surfactant's micelles become balanced with free surfactant (Swensones and Curatio, 1992). Our results showed that hemolytic effects of surfactants increase as the latency of incubation and the amount of contact duration with erythrocytes increase (Table 1-8). It is reported that the more is the contact duration of erythrocytes with a solution, including a surface active agent solution, the more is the amount of cellular lysis (Tragner and Csordes, 1987).

Adherence of surfactants to erythrocyte's membrane which is followed by their entrance leads to alteration of the molecular structure of cell membrane, osmotic-colloid swelling and erythrocyte membrane rupture. Above mechanism depends on surfactant concentration, temperature and duration of contact with erythrocyte and by increasing these factors membrane permeability and haemolysis, that happen due to micelle production from surfactant and membrane phospholipids bilayer, increase (Araki and Rifkind, 1981).

Another aspect of this study was to evaluate the membrane toxicity of surfactants. As the agents or any other substance which have the ability to destruct the erythrocytes membrane can have similar effects on other cells membranes, evaluating erythrocytes membrane stability is a proper criterion for determination of surfactant toxicity. According to our result, in 0.016 mM concentration almost all surfactants caused about 50% haemolysis of erythrocytes. Further haemolysis was observed by increasing the incubation period and temperature. In 0.02 mM and temperature of 37°C Tween 80 caused 58-59% of erythrocytes destruction, while Tween 20, 40 and 60 caused 76-77%, 73-74%, 69-71% of destruction, respectively. Moreover, Tween 20 and 40 induced almost 100% haemolysis while Tween 60 and 80 showed the maximal effect of 78 and 69%, respectively. The hemolytic activity of experienced surfactants in this study increase in higher concentrations and in a specific concentration, in critical concentration for micelle formation, reached to its utmost and after this point remained steadily. Hence, the ability to increase membrane permeability and after it osmotic cellular lysis are due to mixture micelle formation in bilayer membrane. Evaluating the erythrocyte haemolysis showed that Tween 80 had lower destruction level and less toxicity on cellular membrane. Erythrocyte haemolysis method is used to evaluate surfactant and cellular membrane interactions, enhancing activity and emulsifying ability. Accordingly, Tween 80 with lower toxicity should be preferred to be used as a surface active agent and needs more studies on its enhancing abilities and formulatory properties. As we showed Tween 20 (HLB = 16.7) with low hydrophobic and high hydrophilic properties has more capability for membrane destruction, while Tween 80 with lower hydrophobic properties has less destruction capability. Tranger et al that evaluated haemolysis effects of some non-ionic surface active agents reported that in a series of surfactants from one family, the ones with higher hydrophobic contents and lower HLB have lower haemolysis (Schott, 1999).

In non-ionic surfactants hemolytic effects depends on HLB and the percentage of lipophilic part. Surfactants hydrophobic part has a great impact on their properties and affects the size of their micelles and micelle-membrane interaction. Micelle size can affect the cellular membrane permeability, followed by colloid-osmotic lysis through mixture micelle production in bilayer membrane (Araki and Rifkind, 1981; Ohnishi and Sagitani, 1993).

Accordingly, it can be concluded that higher content of hydrophobic part may lead to reduction in permeability and hemolytic effects. Another potential property of surfactants is their ability in induction and stabilizing emulsions. Emulsifying index has direct relationship with surface tension and agent ability in micelle production. In this study, increasing the concentration of all surfactants leads to increase in emulsions stability; however, this trend was not the same in all surfactants (Table 9). This effect started in low concentration (0.016 mM) and reached to its maximum effect in 0.1 mM. Emulsifying index in 0.1 mM concentration for Tween 20, 40, 60 and 80

were 4.8, 7.6, 31.7 and 32.5, respectively. The difference between observed data of surfactants was significant ($p<0.01$ for all experiments). According to hemolytic data and emulsifying index, Tween 80 had the least toxicity and the best properties for emulsification to be used in formulations. Foaming ability of surfactants is a propriety which may help proving the existence of surfactants in a solution; furthermore, this ability can be used in order to compare the detergency properties of detergents with high ability of foaming production. Foam production and stability depends on type and concentration of surfactants, more and stable foam is produced by ionic surfactants comparing with non-ionics. In a homolog series of surfactants more foam is produced by increasing the content of hydrophobic parts of surfactant molecule until reaching to a maximum point. Present results also showed that Tween 60 and 80 with higher hydrophobic contents had more ability to produce foam (Table 10).

CONCLUSION

According to the results of this study we must use Tweens at concentrations lower cmc in formulations. According to the results, the use of Tweens with low hemolytic effect like as Tween 80 is preferred in pharmaceutical preparations.

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