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Short communication

In vitro studies of the hemolytic activity of microemulsions in human erythrocytes

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Abstract

Hemolytic activity in human erythrocytes as alternative to in vivo testing was used as a potential screening method to evaluate irritant potential of microemulsions for possible application in pharmaceutical and cosmetic formulations. Microemulsions were prepared by mixing surfactants and oil and slowly titrating the mixtures with aliquots of phosphate buffer saline or water. All microemulsions were characterized by dynamic light scattering to determine both the mean droplet size and droplet distribution. Microemulsion droplet size decreased as aqueous component increased. No differences in droplet size were observed between formulations containing phosphate buffered saline or water. The hemolytic activity was measured photometrically by the RBC assay, based in the cell membrane lysis, to estimate the potential irritation of both surfactants and microemulsions selected with water or PBS as aqueous component. The most hemolytic microemulsions corresponded to those containing the surfactant Labrasol®, with or without butyl lactate, and no differences were found between the hemolytic activity between these components and microemulsions containing them. The highest hemolytic activity of microemulsions in this study may be attributed to the excipient used in the formulations. We should avoid the use of high amounts of Labrasol® and butyl lactate in microemulsions because they may be potential irritants.

Keywords: Microemulsions; Surfactants; In vitro models; Toxicology; Excipients; Light scattering

1. Introduction

Various approaches have been used for the formulation of drugs with poor water solubility in pharmaceutical preparations. Oil-in-water microemulsions have gained increasing acceptance for use in the administration of these drugs over recent years. The main advantage of such systems is the potential to increase the solubility as well as the bioavailability of drugs.

Microemulsions are transparent, isotropic, thermodynamically stable dispersions of water and oil [1]. They can be stabilised either by a single surfactant, a mixture of surfactants, or co-surfactant/surfactant combinations [2,3]. Such systems have received recent interest as potential vehicles both for transdermal and oral drug delivery [4–6], and parenteral formulations [7,8].

Cell lysis by surfactants is a process of fundamental and practical importance. Much research has been undertaken to understand the mechanism underlying this process, mostly using erythrocytes as a convenient model system. However, despite advances in the understanding of this process, its mechanism is still unclear, and the dependence on surfactant type, initial surfactant concentration, and experimental conditions is not fully understood [9,10]. Two processes have been suggested to explain cell lysis by surfactants: (a) solubilization and (b) osmotic lysis. It is usually assumed that at sufficiently high concentration, the principal mechanism of cell lysis is solubilization, whilst at low concentrations there is an intercalation of the surfactant into the membrane that

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induces changes in membrane permeability [11]. In general, the in vitro hemolytic test is used as a screening method for the toxicity of both surfactants and formulations by estimating the erythrocyte damage that they will induce in vivo [12,13].

The aim of the present work was to study the potential hemolytic activity of microemulsions prepared with varying types of surfactants and oils and different ratios of aqueous to nonaqueuos. The compositions that were identified as having a high proportion of aqueous component and reduced hemolytic activity may be suitable for use in drug delivery systems.

2. Experimental

2.1. Materials

Polyoxyethylene-35-ricinoleate (Cremophor® EL) and purified polyoxyethylene-35-ricinoleate (Cremophor® EL-P) were purchased from Basf Española (Barcelona, Spain). Polysorbate 80 (Tween® 80), medium chain triglycerides (Miglyol® 812), and isopropyl myristate (IPM) were kindly supplied by Acofarma (Barcelona, Spain). Caprylocaproyl macrogol-8 glycerides (Labrasol®) and diethylene glycol monoethyl ether (Transcutol® P) were kindly supplied by Gattefossé España, S.A. (Barcelona, Spain). Butyl lactate and polyethylene glycol 400 (PEG 400) were supplied by Sigma–Aldrich (Madrid, Spain). Phosphate-buffered saline (PBS) was supplied by Reactiva (Beit Haemek, Israel) and sodium dodecyl sulphate (SDS) was purchased from Merck (Darrmstadt, Germany). Finally deionized ultrapure distilled water was obtained with 'Milli-Q plus' equipment (Millipore, Barcelona, Spain).

2.2. Preparation of microemulsions

The boundaries of the microemulsion domains were determined, with the aid of partial pseudoternary phase diagrams, for the six systems listed in Table 1, for a surfactant/co-surfactant/oil ratio from 99.5:0.5 to 70:30 w/w. The aqueous component was isotonic phosphate buffered saline (PBS) pH 7.4 in all the systems studied. Clear and isotropic water-rich samples were selected.

Samples (Table 2) were prepared by mixing surfactant, co-surfactant and oil with a vortex. Then, the mixtures were slowly titrated with aliquots of PBS or water and maintained at 25 °C. Samples were checked visually for transparency and through cross polarizers for optical isotropy.

2.3. Microemulsion characterization

Mean droplet size (*Z* average) as well as droplet size distribution (polidispersity) of the different compositions selected were determined by photon correlation spectroscopy (PCS) using a Malvern 4700 c equipment equipped with an argon laser, $\lambda = 480$ nm. All measurements were performed at 25 °C without diluting. The intensity of the scattered light was observed at a fixed angle of 90°.

2.4. Red blood cell (RBC) hemolysis

Human red blood cells (RBC), from the Blood Bank of the Hospital Clínic i Provincial (Barcelona, Spain), were obtained by centrifugation (10 min at 3000 rpm). The supernatant was discarded and the erythrocytes were resuspended in isotonic PBS containing 22 mM Na_2HPO_4, 5.6 mM KH_2PO_4, and 123.3 mM NaCl in distilled water (pH 7.4) to remove white cells and any traces of plasma. The washing step was repeated three times and then the erythrocyte stock dispersion was suspended in PBS at a cell density of 8×10^9 cells/ml. The density was chosen on the basis that the stock solution yielded an optical density at $\lambda = 575$ nm of approximately 2 after total hemolysis in the assay (control, 100% hemolysis): haemoglobin concentration and resulting absorption exhibit a linear relationship at absorption values below 2 [14].

Different amounts of excipients and microemulsions ranging from 10 to 80 μl/ml for liquids, and 10 to 80 μg/ml for solids (SDS), were introduced into eppendorf tubes to assay various concentrations. Aliquots of 25 μl of erythrocyte suspension were added to the tubes and the volume adjusted to 1 ml with PBS. The tubes were then incubated at room temperature for 10 min with constant rotation. After incubation, the tubes were centrifuged (2 min at 10000 rpm) and finally the percentage of hemolysis was determined by comparing the absorbance ($\lambda = 540$ nm) of the supernatant with that of control samples totally hemolyzed with distilled water [15]. From the hemolysis results, the dose-response curve was determined, and the concentration that induces 50% hemolysis of the erythrocytes (HC_{50}) was subsequently calculated.

3. Results and discussion

3.1. Pseudoternary phase diagrams

The phase behavior of the systems studied is shown in Table 1. The aqueous component was PBS in all cases and the region of microemulsions studied is shown in Fig. 1. The results obtained agree with those previously reported with the same systems using water as aqueous component [16]. We are interested in compositions with a high proportion of an aqueous component and low amounts of oil, as they are suitable for different drug administration routes [17] and have demonstrated good drug solubility [16,18]. So we only considered formulations ranging from surfactant/oil 99.5:0.5 to 70:30 w/w at different PBS concentrations.

From the diagram selected, 11 isotropic monophasic formulations were selected with a ratio of aqueous component

Table 1
Composition of phase diagrams studied

System	AQ[a]	Surfactant	Oil component
A	PBS or water	Tween® 80	IPM[b]
B	PBS or water	Labrasol®	IPM[b]
C	PBS or water	Labrasol®	IPM[b]:Transcutol® P (1:9)
F	PBS or water	Labrasol®	IPM[b]:butyl lactate (1:9)
D	PBS or water	Cremophor® EL	Miglyol® 812
E	PBS or water	Cremophor® EL:PEG 400 (1:1)	Miglyol® 812

[a] AQ: aqueous component.
[b] IPM: isopropyl myristate.

ranging from 50% to 95%. Twenty-two compositions were then prepared with varying proportions of aqueous component; 11 were prepared with PBS, and 11 with water, to allow a comparison between them. All the compositions used are shown in Table 2.

3.2. Particle size evaluation

The prepared formulations were characterised by Photon Correlation Spectroscopy (PCS), without diluting, and the values corresponding to the *Z* average were considered as mean values of the diameter of droplets. Polydispersities above 0.25 were not considered because they represent polydisperse populations or interactions among droplets that cannot be quantified. The droplet size was less than 50 nm in all the compositions studied.

As shown in Table 3, in all the preparations studied, the size of droplets diminished with increasing proportion of aqueous component. However, significant differences in droplet size between the formulations with PBS and the same formulation with water were not observed.

3.3. Red blood cell (RBC) hemolysis

Table 4 shows the results of hemolysis observed with the different excipients tested. The excipients used in this study were non-ionic surfactants such as Cremophor® EL, Cremophor® EL-P, Tween® 80, Labrasol® and PEG 400 and the oils and solvents Miglyol® 812, isopropyl myristate (IPM), Transcutol® P and butyl lactate. The ionic surfactant SDS was used as positive control. The HC_{50} values were expressed as μl/ml, except in the case of SDS, which is shown as μg/ml because it is a solid surfactant. The HC_{50} of the non-hemolytic surfactants at the tested concentrations was not calculated as they were assumed to be higher than the highest concentration tested at 80 μl/ml.

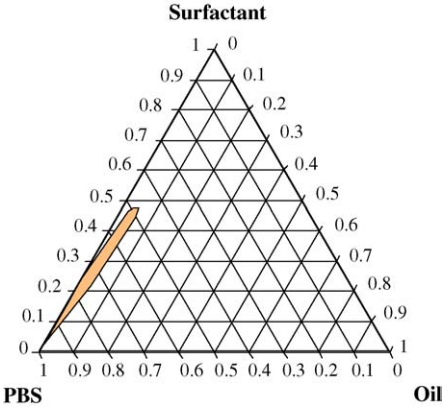

Fig. 1. Pseudoternary phase diagram illustrating the area where all the formulations studied are located. They correspond to the relation from surfactant/oil 90:10 w/w, and different PBS concentrations (50 %, 75%, 80% and 95%).

From the above results it could be clearly deduced that the only components with high hemolytic activity were Labrasol®, butyl lactate and sodium docecyl sulphate (SDS). The HC_{50} of SDS was in agreement with the literature [14,15]. On the other hand, Cremophor® EL showed no hemolytic action, as has been described by other authors [19,20].

The excipients used are generally regarded as non-toxic and non-irritant materials [21]. Hence, they represent a suitable choice of excipients for preparing microemulsions as drug delivery systems. Therefore, selected microemulsions were also tested to determine whether there are differences between the action of single agents and formulations containing different proportions of those agents. In all compositions, both water and isotonic PBS, pH 7.4, were tested as aqueous components of the samples to assess how they affect hemolysis and we could observe that there was no difference in hemolisys of excipients tested.

Fig. 2 shows the hemolytic behavior of some of the excipients and formulations studied. Clearly, increasing the concentration of the excipients induces an increase in the hemolysis, allowing the calculation of the HC_{50}.

Table 5 shows the results of hemolysis for microemulsions. The non-hemolytic or very weakly hemolytic microemulsions correspond to formulations with Tween® 80, Cremophor® EL or IPM, which are non-hemolytic excipients at the concentration used in these formulations, thereby preventing the calculation of their HC_{50}.

The microemulsions with Labrasol®, or Labrasol® and butyl lactate are highly hemolytic, even at the lowest concentration assayed (4.5% in the case of Labrasol®). Although a reduction in the percentage of these components in the formulation reduces the percentage of hemolysis, these

Table 2
Composition in % of the formulations studied

Formulation	AQ[a] (%)		Surfactant (%)				Oil component (%)			
	PBS[b]	H_2O	Tween® 8O	LAS[c]	Cremophor®EL	PEG 400[d]	IPM[e]	Transcutol® P	Butyl lactate	Miglyol®812
A75W-10	–	75	22.5	–	–	–	2.5	–	–	–
A75P-20	75	–	22.5	–	–	–	2.5	–	–	–
A80W-05	–	80	18	–	–	–	2	–	–	–
A80P-18	80	–	18	–	–	–	2	–	–	–
B50W-08	–	50	–	45	–	–	5	–	–	–
B50P-28	50	–	–	45	–	–	5	–	–	–
B80W-02	–	80	–	18	–	–	2	–	–	–
B80P-27	80	–	–	18	–	–	2	–	–	–
B95W-15	–	95	–	4.5	–	–	0.5	–	–	–
B95P-19	95	–	–	4.5	–	–	0.5	–	–	–
C80W-03	–	80	–	18	–	–	0.2	1.8	–	–
C80P-29	80	–	–	18	–	–	0.2	1.8	–	–
C95W-16	–	95	–	4.5	–	–	0.05	0.45	–	–
C95P-30	95	–	–	4.5	–	–	0.05	0.45	–	–
F50W-09	–	50	–	45	–	–	0.5	–	4.5	–
F50P-31	50	–	–	45	–	–	0.5	–	4.5	–
F95W-17	–	95	–	4.5	–	–	0.05	–	0.45	–
F95P-32	95	–	–	4.5	–	–	0.05	–	0.45	–
D80W-06	–	80	–	–	18	–	–	–	–	2
D80P-21	80	–	–	–	18	–	–	–	–	2
E50W-12	–	50	–	–	22.5	22.5	–	–	–	5
E50P-24	50	–	–	–	22.5	22.5	–	–	–	5

The first letter indicates the kind of system (systems A–F), the two first numbers indicate the percentage of aqueous component (50%, 75%, 80%, 95%), the second letter is the aqueous component used (W if water and P if PBS), and the last number is a number at random for the preparation.
[a] AQ: aqueous components.
[b] PBS: phosphate buffer saline.
[c] LAS: Labrasol®.
[d] PEG 400: polyethylene glycol 400.
[e] IPM: isopropyl myristate.

Table 3
Results of photon correlation spectroscopy (PCS) for some of the studied formulations

Composition					Formulation	Droplet diameter (nm)	Polydispersity
Surfactant	Oil component	Ratio S/O[a] (w/w)	H_2O (%)	PBS (%)			
Labrasol®	IPM[b]	90/10		80	B80P-27	15.5	0.174
	IPM[b]:Transcutol®		80		C80W-3	15.0	0.158
Labrasol®	P	90/10	80	C80P-29	17.5	0.169	
	(1:9)		95	C95P-30	15.9	0.048	
	IPM[b]:butyl		50		F80W-9	41.0	0.193
Labrasol®	lac-	90/10	50	F50P-31	46.3	0.233	
	tate	95		F95W-17	15.6	0.087	
	(1:9)		95	F95P-32	16.7	0.087	

[a] S/O: surfactant/oil.
[b] IPM: isopropyl myristate.

Table 4
Results of hemolysis test for excipients

Excipient	Range of concentration	Range of hemolysis (%)	HC_{50}
Butyl lactate	10–40 μl/ml	2.7–100	21.1 μl/ml
Cremophor® EL	10–80 μl/ml	0.1	>80 μl/ml
Cremophor® EL-P	10–80 μl/ml	0	>80 μl/ml
Isopropyl myristate	10–80 μl/ml	0	>80 μl/ml
Labrasol®	0.10–0.80 μl/ml	1.2–100	0.5 μl/ml
Miglyol®-812	10–80 μl/ml	0	>80 μl/ml
PEG 400	10–80 μl/ml	0	>80 μl/ml
SDS	10–80 μg/ml	2.9–100	41.0 μg/ml
Transcutol® P	10–80 μl/ml	0	>80 μl/ml
Tween® 80	10–80 μl/ml	0	>80 μl/ml

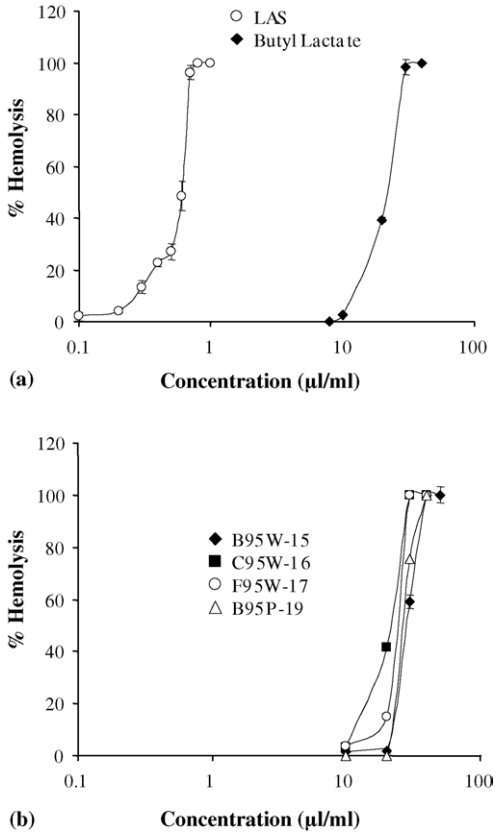

Fig. 2. Graphic representation of induced hemolysis by different concentrations of some excipients (a) and compositions (b) studied. Mean ± S.E. of three independent experiments.

microemulsions are still hemolytic and the HC_{50} is around 20 μl/ml.

The use of buffer with higher ionic strength, dilution of the microemulsions and determination of the maximal effective concentration of the components that do not induce hemolytic effects, should be evaluated to determine if they are appropriate strategies to optimize these formulations.

Table 5
Results of hemolysis for microemulsions

Formulation	Range of hemolysis (%)	HC_{50} (μl/ml)
A75W-10	0	>80
A80W-05	0	>80
B50W-08	100	–
B50P-28	100	–
B80W-02	100	–
B80P-27	100	–
B95W-15	1.5–100	27.2
B95P-19	0–100	27.2
C80W-03	100	–
C80P-29	100	–
C95W-16	3.4–100	20
C95P-30	16.0–100	17
F50W-09	100	–
F50P-31	100	–
F95W-17	3.5–10	21.7
F95P-32	5.1–100	17.6
D80W-06	0	>80
E50W-12	0	>80

4. Conclusions

According to the results of the present work, we conclude that the highest hemolytic activity of microemulsions in this study may be attributed to the excipient used in the formulation, especially Labrasol® and butyl lactate. Also, the aqueous component (water or PBS) had no effect on hemolytic activity.

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