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Research Article

Development and Evaluation of Lorazepam Microemulsions for Parenteral Delivery

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Abstract. The objective of this investigation was to develop lorazepam (LZM) microemulsions as an alternative to the conventional cosolvent based formulation. Solubility of LZM in various oils and Tween 80 was determined. The ternary diagram was plotted to identify area of microemulsion existence and a suitable composition was identified to achieve desired LZM concentration. The LZM microemulsions were evaluated for compatibility with parenteral fluids, globule size, *in vitro* hemolysis and stability of LZM. Capmul MCM demonstrated highest solubilizing potential for LZM and was used as an oily phase. LZM microemulsions were compatible with parenteral dilution fluids and exhibited mean globule size less than 200 nm. The *in vitro* hemolysis studies indicated that microemulsions were well tolerated by erythrocytes. The LZM microemulsions containing amino acids exhibited good physical and chemical stability when subjected to refrigeration for 6 months.

KEY WORDS: capmul MCM; lorazepam; parenteral microemulsion; poor aqueous solubility.

INTRODUCTION

Lorazepam (LZM) is a poorly water-soluble 1,4-benzodiazepine derivative which can be used as a tranquillizer, muscle relaxant, sleep inducer, sedative and antiepileptic agent (1). Since conditions like epilepsy require immediate treatment, most of the important antiepileptic agents including lorazepam are formulated in a suitable parenteral dosage form. Due to poor aqueous solubility of LZM, cosolvents such as polyethylene glycol 400, propylene glycol and benzyl alcohol are employed for the development of parenteral formulation. Currently, LZM is being marketed as Ativan® (Wyeth-Ayerst) which contains aforementioned cosolvents and LZM at a final strength of 2 mg/ml or 4 mg/ml (2). However, cosolvent based parenteral formulations suffer from several disadvantages such as pain and tissue damage at the site of injection and precipitation of the drug on dilution in several cases (3). Furthermore, parenteral administration of the organic cosolvents can also cause hemolysis. Yalin *et al.*, (4) observed that the conventional LZM solution results in considerable *in vitro* hemolysis of human and rabbit blood (>80%). Hence, it is desirable to develop a suitable parenteral dosage form of LZM using novel delivery approaches. Researchers have explored the potential of emulsions (4,5) and cyclodextrins (1) in improved parenteral delivery LZM. However, both these approaches have their own limitations. Emulsions suffer from various disadvantages such as poor physical stability on long term storage, risk of emboli formation, need for strict aseptic handling and rapid growth of microorganisms (6) whereas relatively high concentrations of cyclodextrin derivatives are required (15–30% w/v) to yield suitable parenteral LZM formulation equivalent to the marketed formulation (1).

Recently, microemulsions have gained considerable interest in parenteral delivery of hydrophobic drugs and are being preferred over emulsions in several cases (3). Microemulsions are thermodynamically stable, transparent, isotropic, low-viscosity colloidal dispersions consisting of microdomains of oil and/or water stabilized by an interfacial film of alternating surfactant and cosurfactant molecules. They include swollen micellar (oil-in-water, O/W), reverse micellar (water-in-oil, W/O) and bicontinuous structures and have globule size below 200 nm (7). The various advantages such as ability to solubilize hydrophobic dugs, spontaneity of formation (zero energy input), optical transparency, long-term physical stability, ease of manufacture and scale-up and self-preserving nature (8) give them an edge over conventional emulsions. In view of this, suitability of microemulsions for the parenteral delivery of LZM has been attempted in this investigation.

MATERIALS AND METHODS

Materials

Lorazepam (LZM) was kindly provided by Themis Medicare Ltd, (Mumbai, India). Capmul MCM (Abitec Corp., USA) was received as a gift sample from Indchem International (Mumbai, India). Tween 80, alanine, arginine, methionine, glycine, sodium chloride, oleic acid, soybean oil, dextrose (AR grade) and methanol (HPLC grade) were purchased from s.d. Fine Chemicals (Mumbai, India). All the excipients and reagents were used as received. Double distilled water was prepared freshly whenever required.

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Table I. Composition of Various Lorazepam (LZM) Microemulsions

Component	Composition (% *w/v*)				
	ME1	ME2	ME3	ME4	ME5
Lorazepam	0.2%	0.2%	0.2%	0.2%	0.2%
Capmul MCM	3.0%	3.0%	3.0%	3.0%	3.0%
Tween 80	21.0%	21.0%	21.0%	21.0%	21.0%
Glycine		0.8%	–	–	–
Methionine	–	–	0.8%	–	–
Arginine	–	–	–	0.8%	–
Alanine	–	–	–	–	0.8%
SWFI q.s.[a]	100 ml	100 ml	100 ml	100 ml	100 ml

SWFI sterile water for injection
[a] For *in vitro* hemolysis 0.9% saline solution was used instead of SWFI

Solubility Studies

The solubility of LZM in various oils and surfactant (Tween 80) was determined by using shake flask method. Briefly, an excess amount of LZM was added to each vial containing 1 ml of the selected vehicle i.e. either oil or surfactant. After sealing, the mixture was vortexed using a cyclomixer for 10 min in order to facilitate proper mixing of LZM with the vehicles. Mixtures were shaken for 24 h in an isothermal shaker (Remi, Mumbai, India) maintained at 37± 1°C. Mixtures were centrifuged at 5000 rpm for 15 min, followed by filtration through membrane filter (0.22 μ, 13 mm, Pall Life sciences, Mumbai, India). The concentrations of LZM were then determined by high-performance liquid chromatography (HPLC) method.

HPLC Analysis of LZM

The solubility of LZM in various excipients was determined by a validated reverse-phase HPLC method developed in house. The HPLC apparatus consisted of Jasco PU-2080 Plus Intelligent HPLC pump (Jasco, Japan) equipped with a Jasco UV-2075 Intelligent UV/VIS detector (Jasco, Japan), a Rheodyne 7725 injector (Rheodyne, U.S.A.), a Jasco Borwin Chromatography Software (version 1.50) integrator software and a Spherisorb ODS 2 RP-18 (4.6 mm×250 mm and 5 μ particle size) column. The mobile phase consisted of a mixture of methanol: ammonium acetate (0.05M) buffer pH 6.5 (60:40 *v/v*) at a flow rate of 0.8 ml/min that led to retention time of 6.22 min when detection was carried out at 240 nm. The assay was linear (r^2=0.9996) in the concentration range 0.25–40 μg/ml with the lowest detection limit of 190 ng/ml of LZM. The method was validated with respect to accuracy and inter- and intra-day precision as per ICH guidelines and the relative standard deviation was less than 2% in both the cases.

Phase Diagrams

An oil titration method was employed in present investigation to construct phase diagrams (9). Briefly, mixtures of the double distilled water with tween 80 were prepared at ratios (%wt/wt) of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 into different vials. A small amount of Capmul MCM in 0.5% (w/w) increment was added into the vials. Following each addition, the mixtures in vials were vortexed for 2–3 min and were allowed to equilibrate at 25°C for 30 min. After equilibration, the mixtures were examined visually for phase separation, transparency and flow properties. In addition, the mixtures were observed through crossed polarizers (fabricated in house by using polarizing lenses, Nikkon, Japan) for determining the optical isotropy of the systems. The point at which the mixture

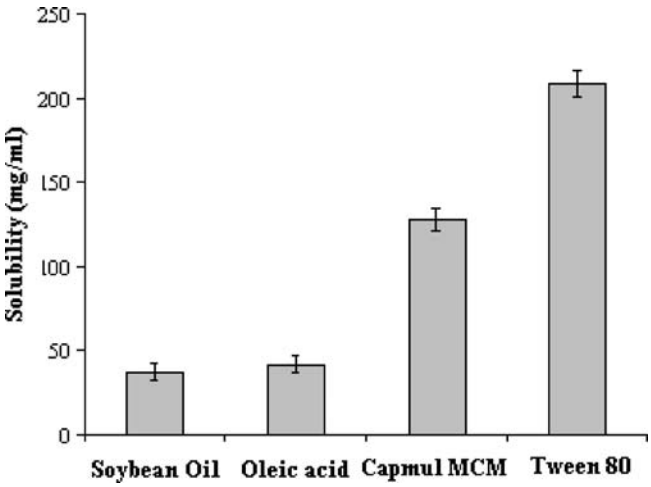

Fig. 1. Solubility of LZM in various oils and Tween 80 (n=3)

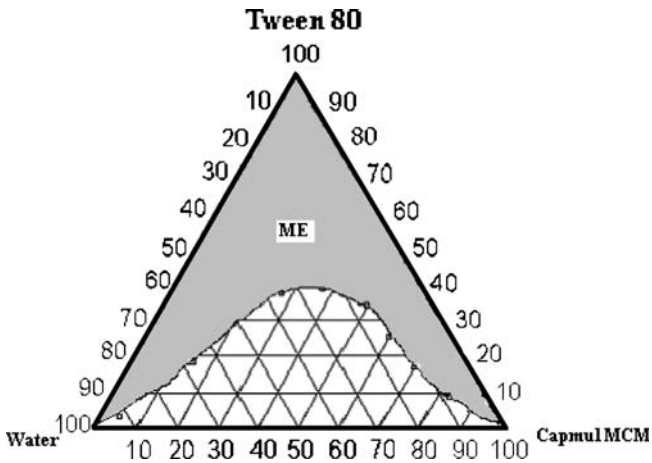

Fig. 2. Ternary diagram of Tween 80-Capmul MCM–Water system

became turbid or showed signs of phase separation was considered as the end point of the titration. The area of microemulsion existence was determined and denoted as ME.

Formulation of Microemulsions

Based on the phase diagrams and solubility of LZM, a microemulsion composition that could solubilize LZM to yield a concentration of 2 mg/ml was selected. Briefly, a suitable quantity of LZM was dissolved in a mixture of Capmul MCM and Tween 80. This homogenous mixture was diluted with water to yield a microemulsion. Microemulsions containing amino acids like glycine, alanine, methionine and arginine were also prepared. The composition of various microemulsions is listed in Table I.

Effect of Various Vehicles on Globule Size and pH of the Microemulsions

The effect of various vehicles viz. water, 5% w/v dextrose solution and 0.9% w/v saline solution on the globule size and pH of the microemulsions was assessed. All the aforementioned vehicles (except water) are isoosmotic to blood and are employed for the development of parenteral formulations. Based on their effect on globule size and pH, the suitable vehicle was selected and used as an aqueous phase in further investigation.

Globule Size Analysis

The average globule size and polydispersity index (P.I.) of microemulsions were determined (n=3) by the photon correlation spectroscopy (PCS; Beckman Coulter N4, Wipro, India). Microemulsions were diluted with double distilled water to ensure that the light scattering intensity (between 6e+004 to 1e+006), was within the instrument's sensitivity range. Measurements were made at an angle of 90° for all the microemulsions.

In Vitro Hemolysis

The hemolytic activity has been suggested as a toxicity screen *in vitro* and it also serves as a simple and reliable measure for estimating the membrane damage caused by formulation *in vivo*. The *in vitro* haemolytic potential of the microemulsion (ME1) and its individual components (at the concentration used in the formulation) was studied by using the method proposed by Jumaa *et al*. (10). The samples tested for erythrocyte toxicity were as follows

1. ME1
2. Capmul MCM
3. 21% tween 80 solution in PBS

Blood was obtained from two human volunteers. Both volunteers signed written consent forms. Fresh blood was collected in a vial containing EDTA (anticoagulant). The blood was centrifuged for 5 min to remove WBC debris and suspended red blood cells (RBCs) were taken out. The RBCs were washed three times with isotonic saline solution (0.15M NaCl and pH 7.4) before diluting with buffer to prepare erythrocyte stock dispersion. The washing step was repeated in order to remove debris and serum protein. The stock solution was refrigerated for a period of 24 h. Test sample (1 ml) was added to a 100 μl aliquot of the erythrocyte stock dispersion. Incubation was carried at 37°C for a period of 1 h. After incubation under shaking, debris and intact erythrocytes were removed by centrifugation and 100 μl of resulting supernatant was dissolved in 2 ml of an ethanol/HCl mixture (ratio 39:1 99% ethanol, and HCl, w/v). This mixture dissolved all components and avoided the precipitation of haemoglobin. The absorbance of the mixture was determined at 398 nm by spectrophotometer monitoring against a blank sample. Control sample of 0% lysis (in buffer) and 100% lysis (in Triton X 100) were employed in the experiment.

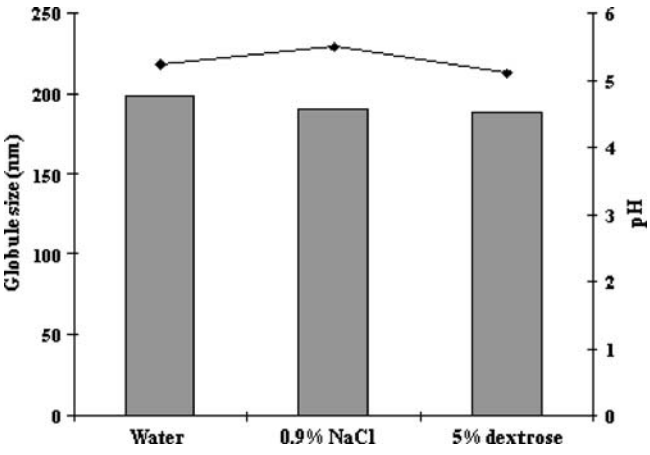

Fig. 3. Effect of various dilution fluids on globule size and pH of LZM microemulsion

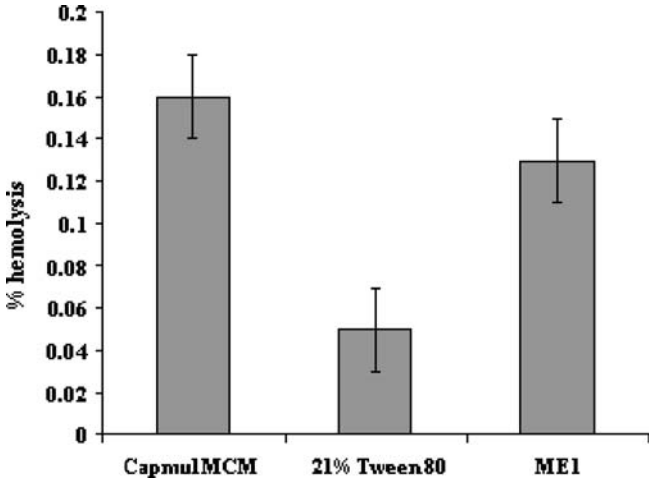

Fig. 4. Results of hemolytic studies

Table II. LZM Content in Microemulsions When Subjected to Refrigeration (n=3)

	Time (months)						
Formulation	0 (%)	1 (%)	2 (%)	3 (%)	4 (%)	5 (%)	6 (%)
ME1	99.61	94.36	85.26	79.78	74.66	65.25	58.27
ME2	100.85	100.44	99.84	100.20	99.26	98.41	98.37
ME3	100.76	99.82	99.09	99.38	98.51	99.88	99.05
ME4	100.61	99.33	99.36	100.09	99.38	99.17	98.35
ME5	99.26	101.02	99.06	99.35	99.03	98.34	99.30

Relative standard deviation was less than 5%

Table III. pH of Various LZM Microemulsions When Subjected to Refrigeration (n=3)

	Time (months)						
Formulation	0	1	2	3	4	5	6
ME1	5.23	5.22	5.15	5.01	4.91	4.76	4.65
ME2	5.22	5.21	5.22	5.22	5.23	5.23	5.22
ME3	5.20	5.20	5.19	5.19	5.19	5.19	5.19
ME4	5.21	5.22	5.23	5.22	5.21	5.22	5.22
ME 5	5.21	5.22	5.23	5.22	5.23	5.22	5.22

Relative standard deviation was less than 5%

The percent haemolysis caused by the test sample (n=3) was calculated by following equation:

$$\% \text{ haemolysis} = \frac{\text{Absorbance of test sample}}{\text{Absorbance at 100\% lysis}} \times 100 \qquad (1)$$

Stability Studies

Chemical and physical stability of the LZM microemulsions was assessed at various storage conditions viz. 5± 3°C and at room temperature (~ 25°C). All LZM formulations were stored in glass vials with rubber stoppers and aluminum-crimped tops. For each formulation, three such vials were stored at various aforementioned storage conditions. The samples subjected to refrigeration were stored up to 6 months whereas samples stored at room temperature were monitored for 15 days with respect to physical stability and LZM content. Samples were removed at 0, 30, 60, 90, 120, 150 and 180 days and were assessed for content of LZM, mean globule size, P.I. and pH. The data obtained at various time points was evaluated by statistical method. The statistical significance of differences in the data was analyzed utilizing analysis of variance (ANOVA) followed by Bonferroni's test (GraphPad InStat Demo Version). Differences were considered statistically significant at $P<0.05$.

Determination of $LD_{50(IV)}$ of Capmul MCM

The protocol for determination of LD_{50} was approved by the Institutional ethical committee of the Institute of Chemical Technology, Mumbai and was in accordance with the OECD (Organization for economic cooperation and development) guidelines. Swiss male mice (n=10; avg. wt: 27.5 g) were injected with different doses of ultrafiltration sterilized Capmul MCM ranging from 0.5 µl (0.181 g/kg) to 25 µl (0.9 g/kg) via tail vein. The dose at which 50% of the population died was considered as LD_{50}.

RESULTS AND DISCUSSION

Solubility Studies

Amongst various oils that were screened, Capmul MCM exhibited highest solubilizing potential for LZM. (Fig. 1). The aqueous solubility of the LZM is reported to be 0.08 mg/ml (1) whereas the solubility of LZM in Capmul MCM showed almost 2000 fold higher than that of water. The Capmul MCM is a mixture of caprylic mono- and di-glycerides and is known for its better solubilization potential as compared to the fixed oils. Furthermore, the low molecular volume modified oils like Capmul MCM are easy to microemulsify as compared to fixed oils and long-chain fatty acids (3,11). It is also noteworthy that the utility of Capmul MCM in parenteral delivery has been established recently (12). Hence, Capmul MCM was chosen as an oily phase for the formulation of microemulsions. Tween 80 is acceptable by parenteral route (2) and it also demonstrated good solubilization potential for LZM.

Ternary Phase Diagram

It is well established fact that non-ionic surfactants can yield microemulsions without use of any cosurfactant (13). The cosolvents used in the parenteral formulations are usually employed as cosurfactants for the formulation of parenteral microemulsions. However, considering the problems associated with the use of cosolvents, formulation of cosolvent(or cosurfactant)-free microemulsion was attempted. Tween 80 has good emulsifying potential for the Capmul MCM (unpublished data) and Capmul MCM also has some self-emulsifying properties. The area of microemulsion existence for Tween 80-Capmul MCM-Water system is shown in Fig. 2. The higher area of microemulsion existence for this ternary system corroborates the aforementioned statements about Tween 80 and Capmul MCM.

Formulation and Evaluation of Microemulsions

Based on the phase diagram and solubilizing potential for LZM, a suitable composition was identified that can yield LZM concentration of 2 mg/ml (Table I). The microemulsions formulated with sterile water for injection (ME1) exhibited

Table IV. Globule Size and P.I. of LZM Microemulsions When Subjected to Refrigeration

	Time (month)						
Formulation	0	1	2	3	4	5	6
LZM1	198.5[a] (0.57)[b]	205.0 (0.8)	199.8 (0.31)	198.8 (0.61)	208.0 (0.29)	199.8 (0.58)	208.0 (0.33)
LZM2	157.6 (0.25)	150.8 (0.65)	147.2 (0.52)	151.2 (0.61)	161.8 (0.57)	166.4 (0.22)	159.2 (0.43)
LZM3	162.8 (0.33)	159.6 (0.45)	160.2 (0.44)	161.8 (0.61)	165.6 (0.88)	159.0 (0.41)	161.0 (0.64)
LZM4	156.8 (0.62)	147.0 (0.27)	149.2 (0.46)	158.6 (0.41)	161.0 (0.67)	164.8 (0.8)	167.8 (0.22)
LZM5	164.6 (0.62)	165.0 (0.27)	169.0 (0.21)	168.8 (0.11)	169.2 (0.71)	170.6 (0.43)	171.8 (0.25)

[a] Particle size expressed as mean, (n=3) where relative standard deviation was <10%
[b] Polydispersity Index (P.I.); Data were expressed as mean, (n=3)

Table V. LZM Content in Various Microemulsions When Stored at Room Temperature (n=3)

Formulation	Time (days)						
	1 (%)	2 (%)	3 (%)	5 (%)	7 (%)	10 (%)	15 (%)
ME1	99.23	98.24	97.77	97.26	89.25	86.94	79.05
ME2	99.28	99.31	98.25	99.33	99.32	95.26	85.43
ME3	100.25	100.01	99.86	99.21	99.25	95.26	88.33
ME4	99.84	99.02	99.33	98.26	98.63	96.34	89.56
ME5	99.88	99.85	99.79	99.89	98.26	96.25	87.51

Relative standard deviation was less than 5%

mean globule size of 198.5 nm with P.I. of 0.57 and a pH value of 5.23. The ME1 could successfully withstand the stress testing such as freeze–thaw cycling and centrifugation at 5000 rpm for 20 min indicating its thermodynamic stability. The other parenteral vehicles like 5% dextrose solution and 0.9% saline solution did not considerably affect the physical stability, pH and globule size of the microemulsions (Fig. 3). Since, LZM is most stable in the pH range of 5–5.2, it was important to maintain the pH of the formulation throughout the storage. The Capmul MCM used in the formulation contains free fatty acids which may catalyze the degradation of LZM on long term storage. Hence, various amino acids were incorporated in the microemulsions. It is known fact that due to zwitter-ionic nature, amino acids can act as a buffer in the presence of an acid or base (14). The incorporation of amino acids in the microemulsions demonstrated considerable effect on the mean globule size of the microemulsions (ME2–ME5). The mean globule size of the amino acid containing microemulsions (ME2–ME5) was significantly lower than that of the ME1. It is possible that amino acids may act as a cosurfactant as short-chain amines are known to have cosurfactant like effect (15).

In Vitro Hemolysis

Capmul MCM showed negligible hemolysis of the erythrocytes (0.18%) indicating its suitability in the parenteral formulations. The results are in accordance with the recent report published by Nornoo *et al*., (12) who demonstrated that Capmul MCM has low hemolytic potential. The 21% tween 80 solution (in 0.9% saline) showed a negligible hemolysis of 0.05% whereas ME1 showed hemolysis of 0.13% (Fig. 4). Yalin *et al*., (4) reported that the incorporation of LZM in emulsion showed significantly lesser hemolysis of the human blood (8.88%) as compared to that of cosolvent based formulation (81.98%); thus indicating the suitability of the aqua based emulsion vehicle as compared to the anhydrous cosolvent based formulation. Since, the microemulsion explored in the present investigation resulted in negligible lysis of human blood; it is likely to be advantageous as compared to the currently marketed cosolvent based formulation.

Stability Studies

The results of the physical and chemical stability of the various LZM formulations are depicted in Tables II, III and IV. It is evident that the ME1 resulted in the significant degradation of LZM (P<0.05) on storage. The degradation was evident from the 2nd month and the end of the 6 months around 40% of the LZM degraded at refrigerated condition. This degradation could be due the free fatty acids present in the Capmul MCM and the lowering of pH of ME1 on long-term storage supports this hypothesis. The mean globule size of the ME1 was not affected during the storage (Table IV). On the contrary, amino acid containing microemulsions (ME2–ME5) showed significant improvement in the long-term storage stability of LZM as compared to ME1. The LZM content was greater than 98% in all the formulations at the end of the 6 months. The pH value and the mean globule size of the microemulsions were also not affected during the storage (Tables III and IV). Literature indicates that the emulsion of LZM long-term storage in refrigerated condition resulted in ~30% degradation of LZM (5) which is not observed in case of the microemulsions explored in this investigation. This clearly demonstrates that advantage of the microemulsions over the emulsions. The results of chemical stability of LZM in all the microemulsions (stored at room temperature) are shown in Table V. It is evident that the microemulsions can not provide long-term stability at the room temperature as at the end of 15 days 10–20% degradation of LZM was observed. However, once again, amino acid containing microemulsions (ME2–ME5) showed lesser degradation as compared to that of ME1 (P<0.05).

Determination of $LD_{50(IV)}$ of Capmul MCM

The Capmul MCM has been used as an oily phase in a recent investigation and the authors have established its hemocompatibility (12). However, there have been no attempts to study the tolerable dose of Capmul MCM. In view of this, we tried to determine the $LD_{50(IV)}$ value of Capmul MCM in mice which may provide further information about the *in vivo* acceptability of Capmul MCM and would form the basis of animal studies aimed in future. Capmul MCM was administered by parenteral route at various doses. It was observed that Capmul MCM till the dose of 0.363 g/kg did not show any mortality and any other behavioral or physical

Table VI. Results of $LD_{50(IV)}$ Determination Studies in Mice (n=10)

Volume of Capmul MCM (ml)	Equivalent dose (g/kg)	Mortality (%)
0.005	0.181	0
0.01	0.363	0
0.015	0.545	10
0.020	0.727	40
0.025	0.90	50

changes in the mice whereas at the dose of 0.545 g/kg, 10% mortality was observed (Table VI). The $LD_{50(IV)}$ value of Capmul MCM was found to be 0.9 g/kg. The dose of the Capmul MCM required for the animal studies (calculated on the basis of LZM dose and composition of ME1) is around 0.03 g/kg which is significantly lesser than the dose that showed no adverse effect. Hence, the microemulsions developed in the present study would be suitable for animal studies.

CONCLUSION

The suitability of microemulsion approach for the parenteral delivery of LZM was successfully established in the present investigation. The LZM microemulsions described in the investigation have very low hemolytic potential and exhibit good physical and chemical stability and can be considered as a viable alternative to the currently marketed LZM formulations.

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