Drug Development and Industrial Pharmacy, 24(2), 157-162 (1998)

RESEARCH PAPER

Preformulation Studies on Freund's Incomplete Adjuvant Emulsion

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ABSTRACT

Freund's Incomplete Adjuvant (FIA), which is used in vaccine therapy, is a water-in-oil emulsion delivery system consisting of an aqueous internal phase containing an antigenic protein dispersed in an external phase containing a mixture of mannide monooleate and light mineral oil. Preformulation studies are reported in this investigation for FIA emulsion. The preformulation studies included the determination of the critical micelle concentration (CMC) of the formulations investigated, the surface activity of mannide monooleate at the interface between the oil phase and the aqueous phase containing ovalbumin as the model antigenic protein, and the effect of ovalbumin on the surface activity at the interface. The influence of the concentration of mannide monooleate and/or ovalbumin on the interfacial tension between light mineral oil and either purified water or 0.9% w/v normal saline solution was measured by the DuNouy Ring Method at 25°C. The CMC was determined experimentally from the relationship between the concentration of the surface active agent in each formulation and the interfacial tension. The number of moles of the surface active agent per unit area at the interface (surface excess concentration) was calculated from the Gibbs' Adsorption equation. The results indicated that mannide monooleate was an effective surface active agent since the formulation containing only mannide monooleate provided the lowest magnitude of CMC. The presence of the surface active agent, mannide monooleate and/or ovalbumin, in the formulations studied reduced the interfacial tension between the two phases. The surface activity was influenced by the presence of an electrolyte (sodium chloride), a protein (ovalbumin), or mannide monooleate in the formulation. The presence of antigenic proteins in the aqueous phase of a water-in-oil emulsion influenced the effectiveness of a surface active agent in the formulation.

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INTRODUCTION

The use of purified vaccines in a clinical setting causes less immunogenic effect to patients (1). The rate and amount of antibody response following the administration of a vaccine containing a protein antigen is enhanced when an adjuvant is present in the formulation (2). Toullet et al. reported that adjuvants containing muramyl dipeptides (MDP) or MDP analogs produce a more effective response in vaccine therapy (3). Freund's adjuvants are considered to be the new adjuvants used in water-in-oil (w/o) emulsion delivery systems (4,5). When the vaccine is injected subcutaneously, the w/o emulsion acts as an inert depot from which the emulsified antigen is slowly released to the site of antibody production (6). Therefore the physical stability of the emulsion vehicle influences the adjuvant response by regulating the amount of antigen released from the emulsion.

Freund's Incomplete Adjuvant (FIA) is a w/o emulsion consisting of an aqueous internal phase dispersed in an external phase composed of mannide monooleate and light mineral oil in a 1:9 ratio. Mannide monooleate is a nonionic surfactant included in the formulation to reduce the interfacial tension between the aqueous and oil phases. Bollinger (7) investigated the metabolic fate of mannide monooleate in mineral oil adjuvants using C-labeled tracers, and found that it must be pure and free of nonesterified components (e.g., free oleic acid, mannitol, and their altered forms). He concluded that this was necessary in order to prolong the retention time of mannide monooleate at the site of injection and enhance the mobilization of the mineral oil component. Berlin (8) reported that light mineral oil with a low viscosity was preferred because it caused no adverse events and produced more effective emulsions.

The objective of the present study was to determine the critical micelle concentration (CMC) and surface activity of mannide monooleate, and to investigate the influence of varying levels of ovalbumin on various modifications of the FIA emulsion formulation as part of a preformulation investigation.

MATERIALS AND METHODS

Light mineral oil (Drakeol® 6VR) was purchased from Penreco (Los Angeles, CA). Mannide monooleate (Arlacel A) and ovalbumin (Albumin, chicken egg grade V; molecular weight approximately 44,287; aqueous solubility about 40 mg/ml) were purchased from Sigma Chemical Company (St. Louis, MO). Normal saline was prepared by dissolving sodium chloride (EM Science, Gibbstown, NJ) in purified water (Milli QUV Plus water system; Millipore, Molsheim, France).

The surface tension of the pure components and the interfacial tension of the various aqueous and oil phase combinations were determined by the DuNouy ring method (Fisher Surface Tensiomat Model 21, Pittsburg, PA). A platinum–iridium ring measuring 6.000 cm in mean circumference was cleaned with benzene and acetone, and lightly flamed with a Bunsen burner prior to each measurement. Each determination was performed in duplicate. For the surface tension determinations, a 25-ml aliquot of each pure ingredient was placed into a beaker at 25°C. The ring was positioned just below the surface of the liquid and the tensiometer was started to obtain the surface tension (9). The surface tension was determined for purified water, light mineral oil, normal saline,and mannide monooleate. For the interfacial tension determinations, a 25-ml aliquot of the aqueous phase was placed into a beaker at 25°C. The ring was lowered into the aqueous phase and 25 ml of the oil phase was carefully poured over the aqueous phase. The phases were equilibrated for 30 sec. The ring was positioned just below the interface and the tensiometer was started to obtain the interfacial tension (9,10). Table 1

Table 1

Composition of the Formulations Investigated in the Preformulation Study

Formulation	Aqueous Phase	Oil Phase
A	Purified water	Mannide monooleate and light mineral oil
B	0.9% w/v Normal saline solution	Mannide monooleate and light mineral oil
C	Purified water and Ovalbumin	Light mineral oil
D	Purified water and 0.125% w/v ovalbumin	Mannide monooleate and light mineral oil
E	Purified water and 0.250% w/v ovalbumin	Mannide monooleate and light mineral oil
F	Purified water and 0.420% w/v ovalbumin	Mannide monooleate and light mineral oil

lists the composition of each of the formulations investigated in this study.

The CMC of each formulation was determined experimentally from a graph of the logarithm of the concentration of surface active agent plotted as a function of the interfacial tension. The concentration corresponding to the intersection of the tangents drawn to the points of the two linear segments represented the CMC (11,12). The surface excess concentration was calculated according to Gibbs' adsorption equation, and is presented here as Eq (1) (11,13):

$$\Gamma = -\frac{1}{2.303RT}\frac{dr}{d\log c} \quad (1)$$

where Γ is the surface excess concentration (moles/cm^2), R is the gas constant (8.314×10^7 ergs/deg · mole), T is the absolute temperature (K), and $dr/d \log c$ is the slope of logarithm of the concentration of surface active agent plotted as a function of interfacial tension.

RESULTS AND DISCUSSION

Mannide monooleate is a nonionic surface active agent used to decrease the interfacial tension between the oil and aqueous phases in order to augment the formation of the w/o emulsion, promote dispersion of aqueous droplets containing a model protein throughout the oil phase, and enhance the physical stability of the emulsion system.

The results presented in Table 2 show the values of the interfacial tension (dynes/cm) of the materials investigated. The results indicated that the interfacial tensions at the interface between purified water and air (65.98 dynes/cm), light mineral oil and air (33.47 dynes/cm), and normal saline solution and air (68.39 dynes/cm) were similar in magnitude to values previously reported (10,14). The addition of sodium chloride to purified water to make normal saline solution resulted in an increase in interfacial tension between the aqueous and air phases. Similarly, the interfacial tension between the normal saline solution and mineral oil (49.96 dynes/cm) was greater in magnitude than that between the purified water and mineral oil (45.67 dynes/cm). This was due to the sodium and chloride ions that tended to concentrate in the bulk of the aqueous phase and cause an increase in the interfacial tension. Therefore, the CMC of the surface active agent in the formulation containing purified water and mineral oil was lower than that of the formulation containing normal saline solution and mineral oil. Also, the results presented in Table 2 indicate

Table 2

Interfacial Tension Values for the Materials Studied

Components	Interfacial Tension (dynes/cm)
Purified water/air	65.98
Light mineral oil/air	33.47
0.9% w/v Normal saline solution/air	68.39
Mannide monooleate/air	28.73
Purified water/light mineral oil	45.67
0.125% w/v Ovalbumin in purified water/ light mineral oil	39.27
0.250% w/v Ovalbumin in purified water/ light mineral oil	31.54
0.420% w/v ovalbumin in purified water/ light mineral oil	20.27
0.9% w/v Normal saline solution/ light mineral oil	49.96

that an increase in the ovalbumin concentration in the aqueous phase from 0 to 0.420% w/v resulted in a decrease in the interfacial tension between the two phases from 45.67 to 20.27 dynes/cm. Ovalbumin is a surface active agent and thereby reduced the interfacial tension between the aqueous and oil phases (15).

The results presented in Fig. 1 show the relationship of interfacial tension (dynes/cm) as a function of the logarithm of surface active agent concentration (% w/v) for formulations A and B. In formulations A and B containing purified water and containing normal saline

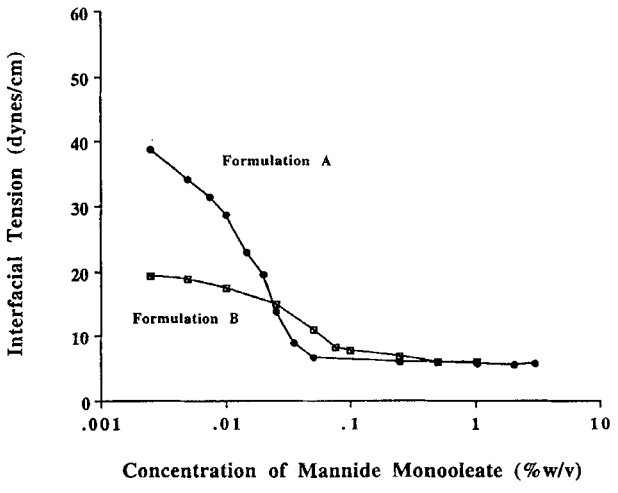

Figure 1. Plot of interfacial tension versus concentration of mannide monooleate in formulation A (purified water) and formulation B (0.9% w/v normal saline solution).

solution as the aqueous phase; respectively, the increase in concentration of mannide monooleate resulted in an initial decrease in the interfacial tension, followed by a plateau where the interfacial tension remained constant. At the point where the plateau was reached the interface was saturated with the surface active agent. The addition of more surface active agent above this concentration did not influence the interfacial tension as indicated by the plateau region. The excess surface active agent remained in the bulk solution and formed micelles. The two formulations compared in Fig. 1 demonstrated that an increase in concentration of mannide monooleate caused an 85% decrease in the interfacial tension of formulation A, but only a 65% decrease for formulation B.

The results presented in Table 3 show the surface properties derived for the formulations investigated, including the CMC and surface excess concentration. The surface excess concentration is derived from the Gibbs' adsorption equation and represents the number of moles of the surface active agent per unit area at the interface. It is a useful parameter to describe the effectiveness of adsorption of the surface active agent at the interface (11). In formulation A, mannide monooleate was an efficient surface active agent since at a very low concentration, as indicated by a CMC of 0.051% w/v, the interface was saturated. Subsequently micelles were formed when the amount of mannide monooleate was continually increased in the formulation above the CMC. The presence of electrolytes from the 0.9% w/v sodium chloride solution in formulation B resulted in a higher magnitude of the CMC of 0.137% w/v. This may have been due to the replacement of molecules of the surface active agent, mannide monooleate, at the interface by the sodium and chloride ions. As previously discussed, the addition of the sodium and chloride ions in the solution tended to concentrate in the bulk of the medium and cause an increase in the interfacial tension. Therefore, the CMC of the formulation containing ions was higher than the formulation containing only purified water as the aqueous phase. Additionally, the sodium and chloride ions reduced the effectiveness of adsorption of mannide monooleate molecules at the interface, as indicated by the decrease in surface excess concentration from 5.26×10^{-10} moles/cm^2 in formulation A to 3.54×10^{-10} moles/cm^2 in formulation B. Similar results have been reported in a previous study (11).

Table 3

Critical Micelle Concentration and Surface Excess Concentration Determined for the Formulations Investigated

Formulation	Critical Micelle Concentration (% w/v)	Surface Excess Concentration, Γ ($\times 10^{-10}$), (moles/cm^2)
A	0.051	5.26
B	0.137	3.54
C	0.477	5.34
D	0.075	3.26
E	0.089	2.10
F	0.147	1.83

The results shown in Fig. 2 illustrate the influence of mannide monooleate (formulation A) and ovalbumin (formulation C) levels on the interfacial tension between the aqueous and mineral oil phases. The increase of mannide monooleate and the ovalbumin concentrations in the formulations resulted in an initial decrease in the interfacial tension, followed by a plateau that occurred as the interfacial tension became constant as a function of concentration. The CMC of formulation A containing mannide monooleate as the surface active agent was 0.051% w/v, while the CMC of formulation C containing ovalbumin was 0.477% w/v. The results indicated that ovalbumin possessed some surface activity. Ovalbumin reduced the interfacial tension between the two phases at a concentration much greater than the CMC of mannide monooleate. However, the effectiveness of the surface active agent, as indicated by the surface excess concentration of both formulations, was not significantly different ($p > 0.05$). For the two formulations containing different surface active agents, mannide

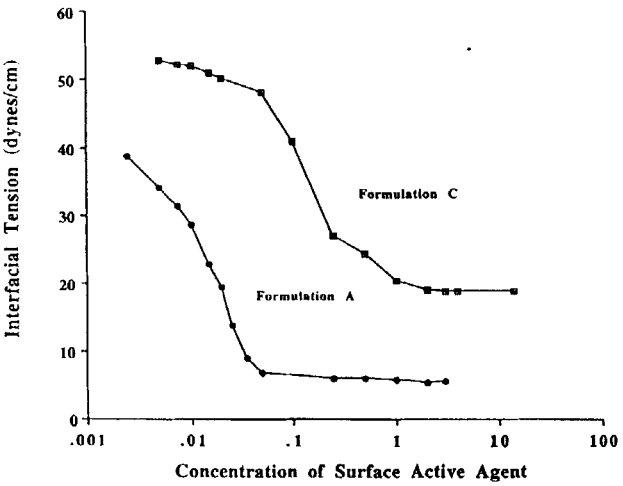

Figure 2. Influence of mannide monooleate (formulation A) and ovalbumin (formulation C) on the interfactal tension.

monooleate was more effective as a surface active agent in reducing the interfacial tension between the aqueous and oil phases than ovalbumin.

The effect of the presence of mannide monooleate on the lowering of the interfacial tension between the aqueous phase containing varying levels of ovalbumin and the mineral oil phase is shown in Fig. 3. Formulations A, D, E, and F contained ovalbumin at concentrations of 0, 0.125, 0.250, and 0.420% w/v, respectively. It was found that the increase in mannide monooleate concentration for each formulation influenced the initial lowering phase of the interfacial tension plot up to the point where a plateau was reached. The results presented in Table 3 indicated that an increase in ovalbumin concentration in the formulations containing varying levels of mannide monooleate resulted in higher magnitudes of the CMC of 0.051, 0.075, 0.089, and 0.147% w/v for formulations A, D, E, and F, respectively. In addition, the comparison of the surface excess concentrations between formulations A, D, E, and F indicated that higher concentrations of ovalbumin retarded the effectiveness of adsorption of mannide monooleate at the interface between the two phases. The surface excess concentration of formulation A was 5.26×10^{-10} moles/cm^2 and formulation F was 1.83×10^{-10} moles/cm^2. This was due to an increase in hydrophilicity of the formulation due to the increase in ovalbumin concentration. The result was a reduction in the effectiveness of adsorption of mannide monooleate at the interface. Similarly, a previous study found that the increase of hydrophobicity by modification of the ovalbumin structure played a significant role in reduction of surface tension of various o/w formulations, and could therefore influence the formation and stabilization of an o/w emulsion (15). The mannide monooleate and ovalbumin did not act synergistically in lowering the interfacial tension of the formulations containing both ingredients. Garti et al. found that bovine serum albumin and Span 80 acted synergistically as an interfacial complex in the inner phase of a w/o/w multiple emulsion system, and the protein/nonionic surface active agent combination could enhance emulsion stability (16). This may have been due to differences in physicochemical properties of the surface active agents and the two proteins. In order to establish the formulation of a stable FIA emulsion, the presence of some antigenic proteins must be investigated since their inclusion could retard the effectiveness of a surface active agent in the formulation and stability of the emulsion.

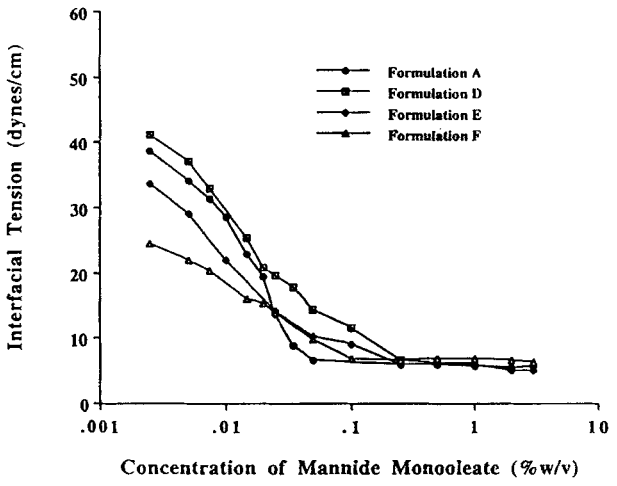

Figure 3. Influence of ovalbumin concentration in the aqueous phase on the interfacial tension of light mineral oil/mannide monooleate and purified water in different formulations (● 0% w/v ovalbumin; □ 0.125% w/v ovalbumin; ◆ 0.250% w/v and Δ, 0.420% w/v ovalbumin).

In conclusion, the presence of mannide monooleate reduced the interfacial tension of formulations composed of light mineral oil and either purified water or normal saline solution. Formulation A containing only mannide monooleate had the lowest CMC (0.051% w/v) and was an efficient surface active agent. The presence of electrolytes from 0.9% w/v sodium chloride solution and ovalbumin in aqueous phase of the formulation resulted in a higher magnitude of the CMC. The effectiveness of adsorption of mannide monooleate at the interface was reduced as indicated by the lower magnitude of the surface excess concentration. Formulation and processing techniques must be investigated in order to optimize the biological activity and physical stability of the emulsion vehicle. The activity of the vaccine is dependent on the physical and chemical properties of the adjuvant emulsion.

REFERENCES

1. L. Chedid and E. Lederer, Biochem. Pharmacol., 27, 2183 (1978).
2. J. Freund, Ann. Rev. Microbiol., 1, 291 (1947).
3. F. Toullet, F. Audibert, G. A. Voisin, and L. Chedid, Ann. Immunol., 128C, 267 (1977).
4. A. C. Allison and N. E. Byars, J. Immunol. Meth., 95, 157 (1986).
5. D. M. Lidgate, R. C. Fu, N. E. Byars, L. C. Foster, and J. S. Flietman, Pharm. Res., 6, 748 (1989).
6. S. S. Davis, J. Hadgraft, and K. J. Palin, in *Encyclope-*

dia of Emulsion Technology (P. Becher, eds.), Vol. 2, Marcel Dekker, New York, 1983, p. 188.
7. J. N. Bollinger, J. Pharm Sci., 59, 1092 (1970).
8. B. S. Berlin, J. Immunol., 85, 81 (1959).
9. Fisher Scientific, Instruction Manual for Fisher Surface Tensiomat Model 21, p. 9.
10. S. Silvestri, N. Ganguly, and E. Tabibi, Pharm. Res., 9, 1347 (1992).
11. M. J. Rosen, in *Surfactants and Interfacial Phenomena*, John Wiley & Sons, New York, 1978, p. 55.
12. H. Schott, J. Pharm. Sci., 69, 852 (1980).
13. A. Martin, in *Physical Pharmacy*, 4th ed., Lea & Febiger, Philadelphia, 1993, p. 374.
14. D. R. Lide, *CRC Handbook of Chemistry and Physics 1992/1993*, 73rd ed., CRC Press, Inc., 1992.
15. S. Magdassi and A. Stawsky, J. Dispersion Sci. Technol., 10, 213 (1989).
16. N. Garti, A. Aserin and Y. Cohen, J. Controlled Release, 29, 41 (1994).