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A novel approach to the preparation of injectable emulsions by a spontaneous emulsification process

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Summary

Submicronic emulsions of muramyltripeptide-cholesterol (MTP-Chol), diazepam and amphotericin B were prepared using an original and simple method. This procedure is based on a spontaneous emulsification process, comprising the following steps: (1) dissolving the oil phase containing phospholipid in the alcoholic solution; (2) optionally dissolving the drug into the oily alcoholic phase and (3) mixing the product of step (2) with an aqueous preparation of surfactant, forming an oil-in-water emulsion, and finally, (4) removing at least most of any co-solvent which is present. The emulsions exhibited a mean droplet size in the range 200–300 nm. The results indicated that emulsions prepared by this method are very stable. They can also be sterilized by heat sterilisation or by filtration. In the case of MTP-Chol, the emulsion retained biological activity. Taken together, these results suggest that an emulsion obtained by spontaneous emulsification process would be very suitable for use as a drug carrier.

Introduction

Intravenous emulsions containing emulsified vegetable oils have been in clinical use for nearly 30 years (Waddell et al., 1957; Schuberth and Wretlind, 1961). They were originally used for parenteral nutrition. These were only resorted to when a patient was unable to obtain sufficient nourishment orally. During the past few years, such emulsions have been investigated as a vehicle for parenteral drug delivery (Jeppsson, 1972; Jeppsson and Rossner, 1975; Nakamoto et al., 1975; Mizushima et al., 1981; Attwood and Florence, 1983; Burnham et al., 1983; Mizushima et al., 1983; Von Dardel et al., 1983; Singh and Ravin, 1986), and for radio-opaque diagnostic agents (Kunz et al., 1965; Grimes et al., 1979; Laval-Jeantet et al., 1982), and even, if the oil phase is a suitable fluorocarbon, as blood substitutes (Clark et al., 1970; Davis, 1974). Interest has also been shown in the use of such emulsions to improve the delivery of drug by the oral route (Kakemi et al., 1972; Carrigan and Bates, 1973; Noguchi et al., 1975).

There exist a wide variety of techniques for the formation of emulsions. The techniques usually

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used to prepare such injectable emulsions involve the utilization of ultrasound and/or two-stage homogenizations (Hansrani et al., 1983; Hazane et al., 1983; Eberth and Merry, 1983; Groves, 1985; Yalabik-Kas et al., 1985), and more recently with the microfluidizer (Washington and Davis, 1988; Lidgate et al., 1990). The objectives of the manufacturing stages are to prepare an emulsion whose particles mimic both chemically and physically the properties of the natural lipid particles. These natural particles or chylomicra appear after ingestion of a fatty meal and are cleared within a few hours after a meal (Hallberg, 1964). These methods were often difficult to use on the laboratory scale and limited the use of this form. An ideal method for the preparation of an injectable emulsion should be simple, reproducible, rapid and easy to scale-up.

The present paper describes an original and simple method for the preparation of injectable emulsions. The procedure is based on the spontaneous emulsification process of an oil phase containing phospholipid, when an organic solution is mixed, under moderate mechanical agitation, with an excess aqueous phase containing surfactant. The organic solvent is removed by evaporation under reduced pressure. As examples, a fat emulsion and three drug-containing emulsions were prepared. The fat emulsion (10–20% soybean oil emulsion) had a mean particle diameter of 300 nm. The drug-containing emulsions (diazepam emulsion, amphotericin B emulsion and muramyltripeptide-cholesterol emulsion) had mean particle diameters between 200 and 300 nm. The use of emulsion formulations of diazepam has been shown clinically to reduce pain at the site of injection (Von Dardel et al., 1983). The emulsion containing muramyltripeptide-cholesterol (MTP-Chol) can inhibit the growth of tumours in vitro and in vivo (Barratt et al., 1989; Yu et al., 1991). The emulsion containing amphotericin B is less toxic to red blood cells than Fungizone®, which consists of a solubilized formulation of amphotericin B in the natural surfactant material sodium deoxycholate (Davis and Washington, 1988).

Incorporation of amphotericin B into liposomes has also be found to increase its therapeutic index, and several formulations of this type are on the market in some countries (Lopez-Berestein, 1987; Bradjburg et al., 1990; Pisarik et al., 1990; Ralph et al., 1991).

Materials and Methods

Materials

MTP-Chol was supplied by the Institut Choay (France). Amphotericin B was obtained from Sigma (France). The following chemicals were obtained from commercial sources and used without further purification: soyabean oil was from Bertin (France), Miglyol 812 was from Dyna France S.A. (Paris), ethyl oleate was from Aldrich Chemical Co. (U.S.A.), soyabean lecithin (Epikuron 170, Epikuron 200) was from Lucas Meyer (Germany), and block copolymer of ethylene oxide and propylene oxide (Synperonic PE-F28) was from BASF, and glycerol was from Labosi (France). All other chemicals were of reagent grade.

Preparation of injectable emulsion

The method of preparation was as follows: 1 ml of oil (soybean oil, ethyl oleate or Miglyol 812), 100 mg of soyabean lecithin (Epikuron 170 or 200) and 0.2 ml of glycerol were dissolved in 25 ml of ethanol or 50 ml of methanol (in the case of amphotericin B). 200 mg of the block copolymer of ethylene oxide and propylene oxide (Synperonic) was dissolved in 50 ml of water. The oily alcoholic solution was then slowly injected into the aqueous phase under moderate magnetic stirring. The aqueous phase immediately turned milky with bluish opalescence as result of the formation of an emulsion. The ethanol was then removed under reduced pressure at 45–50°C. The colloidal emulsion was concentrated to the desired final volume (5–20 ml) by removal of water under the same conditions.

The drug-containing emulsions were made by adding the drug to the oily alcoholic phase.

Morphological examination of emulsions

Morphological examination of emulsion was performed using a transmission electron microscope Philips EM 301 following negative staining

with sodium phosphotungstate solution (2.0%) (Du Plessis et al., 1988).

Measurement of particle size

The particle size of the emulsion was observed with a Coulter Nanosizer (Type N4) by laser light scattering.

Stability of emulsion

The physical stability of each emulsion was monitored by estimation of the particle size distribution and the centrifugation technique (Hamill et al., 1963; Chiang et al., 1978; Pope, 1980a,b; Burnham et al., 1983; Kuroda and Kawata, 1985; Idson, 1988).

The influence of pH on stability was also examined by diluting the emulsions in buffers of different pH.

Biological evaluation

In vitro Rat alveolar macrophages were harvested and incubated with the preparations to be tested for 24 h as described by Barratt et al., (1989). The cells were then washed and fresh medium was added, either with or without syngeneic tumour cells and a radioactive DNA precursor. Inhibition of tumour cell proliferation due to macrophage activation was estimated according to Yu et al. (1991). In the wells without tumour cells, nitrite and citrulline levels were

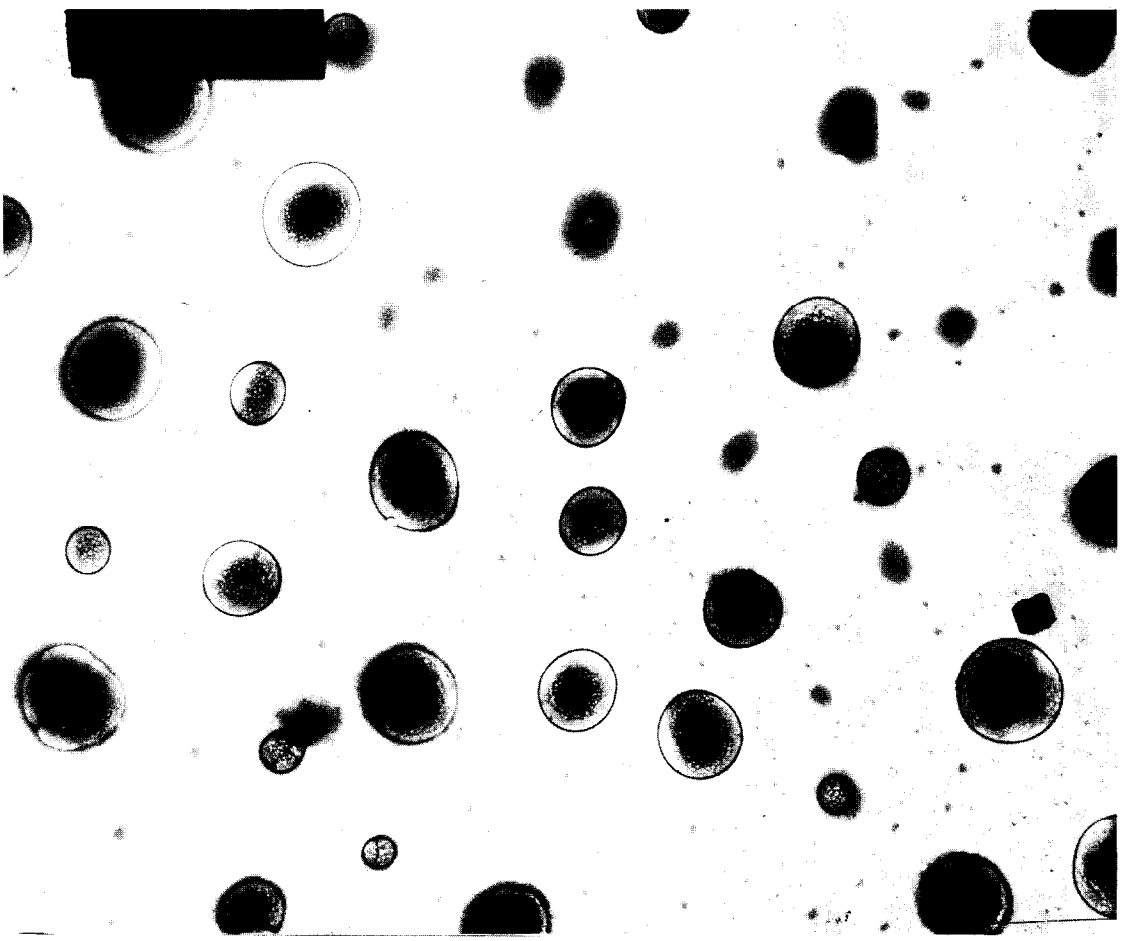

Fig. 1. Empty emulsion.

TABLE 1

Effect of the oil used in the emulsion on the particle size

Emulsion	Oil concentration (v/v)	Size (nm)
Fat emulsion	10–20% soybean oil	298 ± 25
Emulsion A	5% ethyl oleate	175 ± 28
MTP-Chol emulsion	5% ethyl oleate	180 ± 37
Diazepam emulsion	5% Miglyol 812	246 ± 59
Amphotericin B emulsion	5% Miglyol 812	315 ± 83 (81%) 908 ± 210 (19%)

Emulsion A: no drug.

TABLE 2

Effect of sterilization on the particle size

Emulsion	Size (nm)	
	Before sterilization	After sterilization
Fat emulsion	298 ± 25	305 + 37
MTP-Chol emulsion	175 + 28	187 + 32
Diazepam emulsion	246 ± 59	249 + narrow
Amphotericin B emulsion	315 + 83 (81%) 908 ± 210 (19%)	816 + 410 (37%) 3 110 ± 460 (63%)

measured 20 h later by standard spectrophotometric techniques. Results represent the mean of three wells.

In vivo A model of 'artificial' liver metastases induced by i.v. injection of M5076 histiocytosarcoma cells in C57BL/6 mice was used (Yu et al., 1991). Groups of seven mice were given emulsions either intravenously (tail vein) or orally (intragastric cannula) on days −2, 2, 6 and 10 with respect to tumour cell inoculation. They were killed on day 14 and the number of visible liver metastases counted.

Results

Electron microscopic examination

A typical electron microscopic image of an emulsion is shown in Fig. 1. The method of preparation yields a homogeneous emulsion.

Particle size

Particle size distribution determination indicated that the mean diameter size or emulsion is dependent on the oil used. A list of particle sizes of different types of emulsion is presented in Table 1.

Three different emulsions were homogeneous with only one population. The emulsion prepared with soyabean oil had an average size of 298 nm, but, with ethyl oleate, the emulsion size was 180 nm. Addition of drug did not alter the particle size of the emulsion. In the case of amphotericin B, we believe that the surfactant system and the oil concentration are not suitable for the emulsion system because the physico-chemical properties of the drug are very different from those of MTP-Chol and diazepam (Asher et al., 1977). This may explain the presence of two populations.

TABLE 3

Effect of accelerated ageing test on emulsion stability

Emulsion	Ultracentrifugation × g				
	1500	3300	5900	9200	37000
Fat emulsion	no creaming	little creaming	creaming	creaming/ no coalescence	separation of phase/ no coalescence
MTP-Chol emulsion	no creaming	little creaming	little creaming	creaming/ no coalescence	separation of phase/ no coalescence
Diazepam emulsion	no creaming	little creaming	creaming	creaming/ no coalescence	separation of phase/ no coalescence
Amphotericin B emulsion	no creaming	little creaming	creaming	creaming	separation of phase

TABLE 4

Effect of pH on emulsion stability

Emulsion	Size (nm)			
	pH 4	pH 6	pH 8	pH 10
Fat emulsion	305 ± 20	300 ± 15	310 ± 31	396 ± 22
MTP-Chol emulsion	170 ± 18	182 ± 28	190 ± 25	184 ± 33
Diazepam emulsion	250 ± 35	240 ± 23	243 ± 31	254 ± 29
Amphotericin B emulsion	292 ± 38 (78%) 949 ± 79 (22%)	374 ± 130	309 ± 37 (90%) 1950 ± 440 (10%)	261 ± 48 (70%) 710 ± 59 (30%)

Sterilization

This emulsion can be sterilized by heat (121°C, 20 min). The particle size does not change during the heat sterilization process (Table 2), except in the case of amphotericin B emulsion, since amphotericin B was degraded by heat (Asher et al., 1977; Davis and Washington, 1988). It was noted that some of the amphotericin B was precipitated from the emulsion during the sterilisation procedure in these experiments. Such precipitation may result in an overestimate of the amount of amphotericin B lost in the sterilization procedure and the change in the particle size.

It is also possible to sterilize the emulsions of smaller particle size by filtration. No change was found after filtration.

Stability

The stability of the emulsion was evaluated by ultracentrifugation. Four emulsions (fat emulsion, MTP-Chol, diazepam and amphotericin B emulsions) were centrifuged up to $37000 \times g$; phase separation occurred, but no coalescence, and the two phases were readily dispersed by stirring (Table 3).

There was no marked influence of pH on the stability of the emulsion. Changing the pH (from 4 to 10) did not alter the particle size (Table 4).

A stability study for 6 months of the MTP-Chol emulsion indicated that no marked difference was observed in the mean particle size of the emulsion during this period (Table 5).

Biological activity

The immunomodulating activity of MTP-Chol emulsions was tested. The oil used was ethyl oleate, with 10 mg of drug per ml. Fig. 2 shows that the MTP-Chol emulsion could activate macrophages for a cytostatic activity against tumour cells and induce the L-arginine-dependent pathway of nitroxide production, believed to be an effector mechanism (Drapier et al., 1988). In contrast, the empty emulsion had no effect. The MTP-Chol emulsion induced maximal activation at a dose one-tenth of that necessary when a soluble form (MDP) was used.

TABLE 5

Effect of the storage at 4°C on emulsion stability

Emulsion	Size (nm)				
	0 months	0.5 month	1 month	3 month	6 month
Fat emulsion	298 ± 25	301 ± 30	290 ± 28	299 ± 24	310 ± 32
MTP-Chol emulsion	180 ± 37	174 ± 28	184 ± 23	188 ± 34	196 ± 29
Diazepam emulsion	246 ± 59	250 ± 33	265 ± 40	257 ± 31	263 ± 42
Amphotericin B emulsion	315 ± 83 (81%) 908 ± 210 (19%)	268 ± 70 (78%) 1050 ± 270 (22%)	305 ± 110 (74%) 760 ± 140 (26%)	ND	ND

ND, not determined.

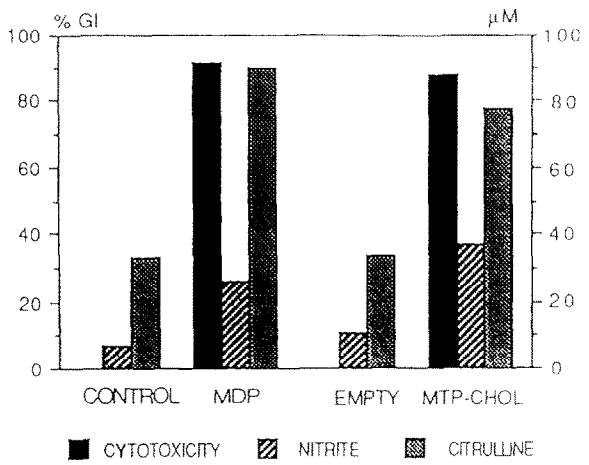

Fig. 2. Macrophage activation in vitro by MTP-Chol emulsion. Rat alveolar macrophages were incubated in the presence of immunomodulators for 21 h as described in Barratt et al. (1991). Their ability to inhibit the growth of syngeneic tumour cells (CYTOTOXICITY) and to metabolize arginine to nitrite and citrulline was then determined. Cytotoxicity is expressed as %GI (left-hand scale): Percentage growth inhibition of tumour cells by treated macrophages as compared with control macrophages (CONTROL); nitrite and citrulline are expressed as concentration in culture medium in μM (right-hand scale). MDP, solutions of MDP at 10^{-6} M; MTP-Chol, emulsion containing MTP-CHOL at 10^{-7} M; E, empty emulsion at the same concentration.

Table 6 shows the anti-metastatic potential of the emulsion in a mouse model. A significant reduction in the number of liver metastases was seen after administration of the MTP-Chol emulsion both intravenously and intragastrically; however, by the oral route larger doses were necessary to produce a smaller effect. The empty emulsion had no significant effect.

TABLE 6

Percentage inhibition of metastases

Emulsion	Route of administration [a]	
	I.V.	Oral (intragastric)
Empty emulsion	31 [b]	2
MTP-Chol emulsion [c]	75 [d]	25 [d]

[a] Mice were treated on days −2, 2, 6, 10 with respect to tumour cell inoculation. Each dose was given in a volume of 0.2 ml (i.v.) or 1 ml (oral).

[b] Calculated according to the formula $\%I = (C - T)/C \times 100$ where C is the mean number of metastases in control mice and T the mean number in treated mice;

[c] Each dose contained 5 μg (i.v.) or 100 μg (oral) of MTP-Chol.

[d] The number of metastases was significantly smaller than that in control mice; $P < 0.05$, Student's two-tailed t-test.

Discussion

Injectable emulsions are formed easily by this new procedure which is a development of the 'diffusion and stranding' method for nanocapsule preparation (Fessi et al., 1987). The mechanism probably involves complex interfacial hydrodynamic phenomena. Addition of the alcoholic-oily solution results in spontaneous emulsification of the oily solution in the form of very small particles, probably due to interface instability arising from rapid diffusion of the ethanol or methanol across the interface and marked decrease in the interfacial tension. The presence of surfactants (lecithin + Synperonic) is needed for the physical stability of the emulsion. Acetone can also be used in this method to substitute for ethanol or methanol.

In addition, other oils were successfully used as core materials, such as benzylbenzoate, Labrafil, etc. The emulsion size was influenced by core materials. The particle size of the emulsion with soybean oil is larger than that with ethyl oleate. The possible reason is that viscosity of soyabean oil is greater than that of ethyl oleate, and so this oil is more difficult to disperse during the emulsification.

The emulsion proposed is very stable. After 6 months, no marked difference was observed in the mean particle size of the emulsion. They can also be sterilized by heat sterilization or filtration. The results obtained with MTP-Chol emulsion show that this form retained biological activity. Since the target cell for this drug is phagocytic cells, the colloidal form is particularly appropriate. Other potential uses of injectable emulsions as drug carriers are: diazepam (to reduce pain upon injection); amphotericin B (to reduce toxic side-effects and to improve uptake by infected macrophages).

The double population observed in the amphotericin B emulsion is not ideal, but the system is very stable after 6 months. We now need to develop a new surfactant system for this drug with a higher oil concentration, in order to obtain a single, homogeneous population.

The method described in this article provides a simple and convenient way of preparing stable homogeneous emulsions suitable for intravenous applications. The procedure can be scaled-up easily and can be used with a variety of different core materials and active ingredients.

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