Enrofloxacin Loaded Liposomes Obtained by High Speed Dispersion Method

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A method for the mass production of liposomes was developed: lipids and nonionic surfactant were dispersed in ethanol at 50°C, when ethanol evaporated, glycerin was added resulting in a homogeneous mixture. Parallely, enrofloxacin in a mixture of buffer solution and glycerin (1:1, v:v) was heated to 50°C. The drug solution was then added to the lipid mixture and homogenized at 3000 rpm for 2–3 min by a homogenizer. This method provided a suspension of multilamellar vesicles (high speed dispersed vesicles, HSDV) with an average diameter that ranged from 280 to 350 nm, their size distribution being bimodal. Such liposomes were characterized in comparison with other traditional liposomes, namely extrusion, reverse phase evaporation, ethanol injection and dehydration-rehydration vesicles. The entrapment efficiency of HSDV (20%) was never less than by others and they showed a high physical stability, since after 23 months both the size and the polydispersity retained the same values they had at the time of the preparation. Encapsulation efficiency also remained almost constant evidencing that the leakage from liposomes was apparently insignificant. Chemical stability of lipid and of the encapsulated drug showed that if liposomes are kept at 4°C and protected from natural light, they do not undergo any degradation.

Key words liposome; quinolone; enrofloxacin; stability; encapsulation

In recent years, fluoroquinolones, a group of drugs derived from nalidixic acid, have been developed as high potency antibacterial agents. These drugs are very useful in the treatment of diseases caused by intracellular bacteria, like *Mycobacterium*, *Mycoplasma*, *Chlamydia*, *Legionella* and *Brucella*. Nevertheless, such antibiotics do not concentrate effectively in cells that act as host for these pathogens. Some authors point out that encapsulation of quinolones in liposomes could improve their effectiveness.¹⁾ Because the liposomes themselves, as foreign particles, are absorbed by cells of the reticuloendothelial system (RES), they offer a unique opportunity to deliver the drug molecules directly into these cells, when the latter are infected by bacteria. One of these fluoroquinolones, enrofloxacin, has shown to be effective in the treatment of the main bacterial processes affecting farm animals. In this way the specific field of application of the drug determines the characteristics of the method of obtaining drug-loaded liposomes: it might allow a mass production of liposomes at a relatively low cost.

Classical methods of liposome production are normally expensive and are sometimes very difficult to scale up. Some of these methods, extrusion, freeze thaw sonication and dehydration-rehydration include as first step the formation of a thin phospholipid film, a procedure not suitable for any industrial process. Others like the surfactant solubilization method require a final dialysis step to eliminate the detergent used, which sometimes is very troublesome. Production by reverse phase evaporation necessitates the use of ether or a mixture of ether and chloroform, solvents that are expensive and must be removed. Microemulsification liposomes need the aid of a special device that represents an additional cost.

The ethanol injection method is very easy and it would seem to be a good candidate for scaling-up. However, its main problem is the presence of ethanol in the final suspension and the fact that only barely diluted liposome

solutions are obtained. In this method the encapsulation efficiencies are closely related with the ability of drugs to be solubilized in ethanol.²⁾

We describe a method to encapsulate enrofloxacin in liposomes that allows the economical production of a large amount of vesicles. This method, hereafter called high speed dispersion (HSD) is based on the fact that liposomes can be obtained by mixing water with a phospholipid and a hydrophilic nonionic surfactant in the presence of a dispersing agent, as has been pointed out by Matsumoto *et al.*³⁾ The amount of the hydrophilic surfactant used can range from 1 to 15% of phospholipid employed. Moreover, the formulation contains cholesterol and glycerin to increase the liposomal stability.

The aim of this work is the comparison of the HSD method with other classical methods, encapsulation percentage and size distribution being the parameters checked. The long term physical and chemical stability of these liposomes as well as the chemical stability of the drug were also studied.

Results

Comparison of HSD Liposomes with Traditional liposomes Table 1 shows the results with the different liposomes on the basis of encapsulation percentage. As can be observed, the encapsulation percentages of drug are strongly dependent on the method of purification employed. Methods involving a relatively long period of time are correlated with a poor, sometimes even null, encapsulation percentage, while a quicker method without dilution, as the minicolumn centrifugation one, always afforded higher values. In this way, when dialysis was used in the purification step, the lengthy period of the process involved that all the drug was released from the vesicles. It can be inferred that extrusion with incubation and dehydration-rehydration afforded similar encapsulation percentages (13–15%), whereas the HSD method achiev-

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Table 1. Characteristics of Liposomes Obtained by Several Methods

Methods of liposome production	Diameter of liposomes (nm) (mean $\pm$ S.D., $n=3$)	Purification process	Encapsulation percentage (mean $\pm$ S.D., $n=3$)
EXT	180 $\pm$ 15	Gel filtration	1—2
EXT	180 $\pm$ 15	Dialysis	<1
EXT	180 $\pm$ 15	Column centrif.	10 $\pm$ 2
EXT*	182 $\pm$ 14	Column centrif.	14 $\pm$ 3
EFT	190 $\pm$ 11	Column centrif.	10 $\pm$ 2
REV	192 $\pm$ 14	Column centrif.	1—2
ETI	85 $\pm$ 12	Column centrif.	15 $\pm$ 2
ETI	85 $\pm$ 12	Gel filtration	<1
DRV	>900	Ultracentrifugation	13 $\pm$ 6
HSD	315 $\pm$ 35	Gel filtration	<1
HSD	315 $\pm$ 35	Column centrif.	20 $\pm$ 5

Lipid composition: Lipoid-cholesterol 2:1 (mole ratio). EXT=liposomes obtained by extrusion method. EXT*=the same as EXT but including a lipid-drug incubation for 12 h prior to the extrusion. EFT=Extrusion with six freeze-thawing cycles. REV=reverse phase evaporation vesicles. ETI=liposomes obtained by ethanol injection method. DRV=dehydration-rehydration vesicles. HSD=vesicles obtained by high speed dispersion. Encapsulation percentage represents the ratio between the amount of drug found in purified liposomes and the drug found in an equal volume of unpurified liposome suspension.

ed a mean value of 20%. This represents an encapsulation efficiency of 0.140 mol of enrofloxacin encapsulated by mole of lipid. It is evident that no method has been shown to encapsulate the drug more efficiently than HSD. This method produces a vesicle population with a bimodal distribution (Fig. 1): some liposomes are centered around a number diameter of 200 nm, while others are distributed around a diameter of 500 nm. The value of its polydispersity is 0.4—0.5 (values of polydispersity can be in the range 0—1). Under the operating conditions described these results are fairly reproducible. The presence of cholesterol in lipid membrane does not affect significantly the entrapment of the drug (the following mole ratios of Lipoid:cholesterol have been checked: 1:0, 1:1, 1.6:1 and 2:1), although it is known that it may increase the structural resistance of the membrane.

Stability Liposomes Produced by HSD Method Stability has become one of the most important goals in liposome pharmaceutical formulations, since it determines the shelf-life of any colloidal preparation. From a chemical point of view, phospholipids can undergo a chemical degradation leading to build up of short-chain phospholipids and lyso-derivatives in the membrane.^{4,5} From a physical point of view, fusion and/or aggregation are the main phenomena which can affect the stability. Another important problem is the drug release from liposome that can yield a preparation with varying encapsulation efficiencies depending on the age of the preparation. In order to obtain information on the chemical drug stability and physical stability of liposomes, the suspension, placed in a topaz bottle (enrofloxacin as other quinolones is light-sensitive), was kept in a refrigerator at 4°C and over 23 months aliquots were drawn periodically and the encapsulation percentage, size distribution and structural properties of the drug were determined.

Size Distribution: Size and polydispersity values did not undergo significant changes. Number diameter was 300 $\pm$ 15 nm and intensity diameter was 350 $\pm$ 20 nm. Polydispersity ranged from 0.45 to 0.55 during the assay time (23 months). The slight differences observed are due

Fig. 1. Size Distribution of Vesicles Obtained by High Speed Dispersion Method Expressed as Number Diameter

to random variations in the measurement and do not suggest any kind of instability. The shape of size distribution was the same over this period, and remained bimodal and centered at 200 and at 500 nm. This result indicates that no apparent aggregation or fusion occurred and therefore the preparation is physically stable. The presence of glycerin and poloxamer in the composition is essential to ensure this stability. The high viscosity produced by glycerin limits the motion of vesicles, leading to difficulty in fusion and/or aggregation. Poloxamer aids in vesicle stabilization and, as observed in previous experiments, avoids the preparation creaming. A similar formulation without poloxamer underwent creaming in approximately 24 h.

Encapsulation Percentage: During the study only a slight variation of encapsulation percentage was observed. The initial value (20 $\pm$ 5%) decreased very moderately to a value of 17 $\pm$ 2% at the end of the 23 months. An equilibrium between the drug inside and outside the vesicle must be reached after the liposome formation and such equilibrium should avoid that the encapsulated drug went away.

Chemical Stability of Lipid: Chemical stability was checked by thiobarbituric acid (TBA) assay, which determines the presence of endoperoxides, and by TLC in order to visualize the products of lipid degradation. Endoperoxide analysis data remained practically unchanged during the time of the assay. At time 0 the value obtained was 0.037 $\pm$ 0.004, while after 23 months it was 0.038 $\pm$ 0.003 μ mol tetraethoxypropane (TEP)/ μ mol lipid, the difference being clearly not significant. It thus can be inferred that no endoperoxides were produced during the storage. This fact can be explained by the presence of a small amount of tocopherol in the composition of Lipoid S-100. Where TLC is concerned, liposome samples did not provide different spots than those of the standards (cholesterol, phosphatidylcholine, lyso-phosphatidylcholine and Lipoid-S100) and the liposome spots and *R_f* values coinciding with those of the standards (0.94 for cholesterol; 0.70 for phosphatidylcholine and 0.15 for lysophosphatidylcholine). It should be noticed that even unaltered Lipoid S-100 contains a small amount of lysophosphatidylcholine. In early stages of hydrolysis lysolipids alter the permeability of membranes. However

Fig. 2. HPLC Chromatogram of Enrofloxacin (A) after Exposition to White Light for Two Years and (B) Encapsulated in Liposomes and Kept at 4°C for 2 Years and Protected from Light

Peak (a) corresponds to enrofloxacin, peak (b) is the main degradation product, peak (c) corresponds to ciprofloxacin and peak (d) is the internal standard.

as it has been explained entrapment percentage remains practically constant. Thus, the possible increase in permeability produced by lysophosphatidylcholine present in the formulation does not seem to alter the final equilibrium between free and encapsulated drug.

Chemical Stability of Drug: Fluoroquinolones are relatively stable at different temperatures but are very sensitive to light.^{6,7} A sample of enrofloxacin exposed to the light produces a large number of degradation products. After a few days of exposure to white light, two degradation products appear, one of them has been identified as ciprofloxacin. After a longer period of time, the number of degradation products increases. En-

rofloxacin exposed for 2 years shows nine degradation products, as can be observed in the chromatogram in Fig. 2A. Peak (a) corresponds to enrofloxacin (retention time 12.5 ± 0.3 min), whereas peak (b) is the main product of degradation, and peak (c) corresponds to ciprofloxacin (retention time 9.5 ± 0.4 min), also a degradation product. Peak (d) corresponds to internal standard. Contrarily, enrofloxacin-containing liposomes kept at 4°C for 2 years and protected from light did not give any kind of degradation product. After separation of free drug, liposomes were disrupted by solubilization with ethanol and encapsulated drug was analyzed by HPLC. As can be seen in Fig. 2B the only peaks correspond to enrofloxacin

and internal standard. This confirms that enrofloxacin is very stable when it is protected from light and that encapsulation in liposomes does not affect this stability.

Conclusion

Based upon the above results we can conclude that the HSD method affords very stable liposomes. For enrofloxacin the entrapment efficiency is better than other classical methods. It has been confirmed that the concentration of drug inside liposomes remains practically unchanged, even if the drug is able to flow through the membrane. On the other hand, drug stability studies showed that enrofloxacin is, as are other fluoroquinolones, light-sensitive. When encapsulated in liposomes, however, if it is kept in the refrigerator and protected from light, no degradation products appear.

Experimental

Chemicals and Reagents Enrofloxacin was a gift from Cenavisa SA (Reus, Catalonia). It is a highly soluble drug in strong acid or basic solutions and poorly soluble at neutral pH. It was possible to solubilize enrofloxacin in 10 mM acetate buffer pH 4.7, 145 mM NaCl (mOsm/kg = 310 ± 5), but its solubility increased drastically up to 20 mg/ml when a 1.2 M acetate buffer was used (mOsm/kg = 1500 ± 30).

The lipid used was Lipoid S-100 (Lipoid KG, Ludwigshafen, Germany), a mixture of lipids whose main component is phosphatidylcholine (>94%).

Cholesterol was purchased from Sigma (St. Louis, U.S.A.) and Poloxamer 188 (Surfodid 7068) was a gift from Tenneco España SA (Barcelona, Catalonia). Other reagents were of analytical reagent grade. Water was double-distilled and deionized.

HPLC of Enrofloxacin HPLC was performed in a Waters 600 multisolvent delivery system connected to a Waters 486 tunable absorbance detector set to 276 nm (Waters-Millipore, Bedford MA, U.S.A.). The column was a reverse phase (C18) Spherisorb-ODS2 (150 mm, i.d. 0.46, 3 μ m). Mobile phase consisted of a variable mixture of acetonitrile (A) and a 0.75% solution of triethylamine adjusted at pH 3 with phosphoric acid (B). The flow was set at 1 ml/min and gradient was: 0 min 87% (A) 13% (B); 25 min 73% (A) 27% (B) with linear increase; 27 min 73% (A) 27% (B) fixed. Fifty μ l of each sample diluted to provide a concentration between 10 and 100 μ g/ml was injected. Quinine sulphate was used as internal standard.

Thin Layer Chromatography (TLC) TLC was performed on Silica gel 60 F₂₅₄ plates (Merck, Germany). Ten μ l aliquots (16 mg/ml) of lipids were spotted on a plate preactivated by heating. The chromatogram was developed with chloroform-methanol-water (65:25:4, v/v). In order to visualize the spots the dried plates were placed in an iodine tank for 10 min. Dark brown spots showed the presence of lipid.

Liposome Preparation High Speed Dispersion (HSD) Method: Two g of Lipoid S-100; 0.2 g of Poloxamer 188 and 0.3 g of cholesterol were dispersed in 10–15 ml of ethanol and warmed at 50°C, with stirring, until total dissolution. After solving the mixture, the temperature was kept at 50°C to evaporate the ethanol until the product looked like a gel. Then, 6 g of glycerin was added under the same conditions, obtaining, finally, an homogeneous mixture.

At the same time, 50 ml of 2% enrofloxacin in a mixture (1:1, v/v) of buffer solution and glycerin was heated to 50°C. After reaching this temperature, the aqueous solution was added to the lipidic mixture and homogenized without heating at 3000 rpm for 2–3 min by an electrical homogenizer. This method provided a very viscous multilamellar vesicles (MLVs) liposome suspension.

Extrusion: Extruded liposomes⁸⁾ were obtained in the sequential mode. Briefly, 40 μ mol of lipid was dissolved in chloroform and the solution dried in a rotary evaporator under reduced pressure. Lipid was dispersed in 4 ml of drug solution by vortex agitation and bath sonication. Multilamellar vesicles produced above were downsized to form oligolamellar vesicles by extrusion at 37°C in an extruder device (Lipex Biomembranes, Canada) through polycarbonate membrane filters of variable pore size (Nucleopore, U.S.A.) under nitrogen pressures of up to $55 \times 10^5 \text{ N} \cdot \text{m}^{-2}$. Liposomes were extruded in three steps: three con-

secutive extrusions through a 0.8 μ m pore diameter filter, followed by three consecutive extrusions through a 0.4 μ m pore diameter filter and, finally, the resultant liposome suspension was extruded four consecutive times through a 0.2 μ m filter.

Extrusion After Freezing-Thawing Cycles: Multilamellar liposomes obtained as in the extrusion method were frozen in liquid nitrogen and thawed in a 40°C water bath, where the freeze-thaw cycle was repeated five times. The resulting suspension of frozen and thawed liposomes was extruded as above.⁹⁾

Reverse Phase Evaporation: Liposomes were prepared by the REV method according to Szoka and Papahadjopoulos¹⁰⁾: after a thin film of 50 μ mol of lipid was formed in a rotary evaporator, 4 ml of ethyl ether was added and mixed gently. Then, 1.2 ml of drug solution was injected, and the preparation was sonicated at 4°C for 10–15 min in a bath sonicator until a stable emulsion was formed. The mixture was then placed on a rotary evaporator and the organic solvent was removed under reduced pressure (approx. 450 mmHg) at room temperature until a gel was formed. Two ml of drug solution was then injected into the gel and the mixture was again subjected to vacuum for 30 min. The liposome suspension obtained was extruded five times through a 0.2 μ m filter.

Ethanol Injection: Liposomes obtained by ethanol injection were prepared using an alcoholic solution of a 40 mg/ml lipid concentration. This lipid concentration afforded us a vesicle population with an average diameter in number of 50 nm and a polydispersity ranging from 0.30 to 0.36 as reported elsewhere.²⁾

Dehydration-Rehydration: Dehydration-rehydration liposomes¹¹⁾ were prepared from small unilamellar vesicles (SUV) obtained by sonication of multilamellar vesicles at 10 μ mol/ml lipid concentration, SUV were mixed with equal volume of the enrofloxacin solution, frozen and lyophilized on a Flexy-Dry (U.S.A.) freeze-dryer for 16–20 h. Controlled rehydration was carried out at room temperature by addition of distilled water followed by the corresponding buffer, with vigorous vortexing and incubation at room temperature for 30 min between additions.

Liposome Purification Four methods were checked: dialysis, ultracentrifugation, gel exclusion chromatography and minicolumn centrifugation.

Dialysis: A 4 ml sample was introduced in a cellulose acetate dialysis bag and was dialyzed against 200 ml of buffer solution under magnetic stirring for 24 h. At 2 h time intervals the dialysis solution was changed and an aliquot was taken to determine the drug concentration.

Ultracentrifugation: Dehydration-rehydration vesicles containing encapsulated drug were separated from free drug by centrifugation in a 50 Ti type rotor of a Beckmann L8-60 M ultracentrifuge. A 5 ml suspension volume was centrifuged for 30 min at 20000 rpm (= $35000 \times g$) at 7°C. This washing step was repeated twice.

Gel Exclusion Chromatography: A column of 160 $\times$ 10 mm was filled with acetate buffer hydrated Sephadex G-50. The eluent was acetate buffer at a flow rate of 0.1 ml/min. Samples of 0.25 ml were applied onto the column.

Minicolumn Centrifugation Method: A modification of the method of Fry *et al.*¹²⁾ was used. The barrel of a 2 ml plastic disposable syringe was plugged with a Whatman GF/B filter pad and then filled with acetate buffer hydrated Sephadex G-50. The barrel was suspended in a centrifuge tube and spun at 2000 rpm for 5 min in a bench centrifuge to remove excess buffer solution. After spinning, the gel column became dry and detached from the sides of the barrel. Exactly 0.4 ml of liposome suspension was applied dropwise to the top of the gel bed with the column suspended in an empty centrifuge tube. The column was again spun at 2000 rpm for 5 min. Then 0.2 ml of acetate buffer was applied to the gel bed and the barrel was again spun under the same conditions but without changing the centrifuge tube. At last, 0.6 ml of pure liposome suspension was obtained, while free drug remained in the gel bed.

Lipid Determination: One of the easiest methods to determine phospholipids is the Stewart-Marshall method.¹³⁾ This assay is more suitable than others¹⁴⁾ for an industrial preparation. As a preliminary assay we observed that enrofloxacin interferes with the lipid determination by Stewart-Marshall method. It has been reported that a related fluoroquinolone, called ofloxacin, reacts with ferrous salts forming an insoluble chelate between the keto and adjacent carboxyl groups and the metal cation.¹⁵⁾ In the same way, enrofloxacin reacts with the reagent producing a dark precipitate, and the measured absorbance is bigger than the theoretical one. This made it necessary prior to performance

of the assay, to separate the drug from the lipid; this separation allowed an easy determination of the drug. In order to perform it C18 Sep-Pak cartridges (Waters-Millipore, U.S.A.) were used.¹⁶⁾ After solvating the cartridge with methanol and methanol-acetate buffer (2:1, v/v), 0.1 ml of liposome suspension was applied to the cartridge. Disruption of the membrane structure was achieved by eluting with methanol-acetate buffer (2:1, v/v). In this aqueous eluate the drug was contained. Then, the column was flushed with chloroform, so that the organic eluate contained the lipid and the lipid determination according the Stewart-Marshall method could be carried out without interference. This method was confirmed to produce a complete separation of lipid and drug.

Drug Assay Enrofloxacin present in the aqueous eluate obtained from the Sep-Pak cartridge was determined spectrophotometrically at 318 nm, after a 10000 fold dilution.

Size and Distribution Size Determination Diameter and polydispersity of vesicles were determined by photon correlation spectroscopy which analyzes the fluctuations in scattered light intensity generated by diffusion of vesicles in solution. An Autosizer IIc (Malvern Instruments, U.K.) consisting of a 5 mW, 623.8 nm, helium-neon laser irradiating the scattering cell placed inside a temperature regulated housing was used. Data acquisition was via a Malvern 7032-N, 72 channel multibit correlator. Experimental conditions were: temperature 25.0°C; reference angle 90°; viscosity 0.899×10^{-3} Pa·s; refractive index 1.330. The exponential sampling method¹⁷⁾ was used for data analysis.

Phospholipid Oxidation Determination Endoperoxides were detected by the thiobarbituric method.¹⁸⁾ The method is based on the reaction of malondialdehyde (an endoperoxide breakdown product at elevated temperatures) with thiobarbituric acid which give a red chromophore that absorbs at 532 nm.

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