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STUDIES ON THE FORMULATION OF NANOSTRUCTURED CARRIERS FOR INCREASING THE BIOAVAILABILITY OF PRALIDOXIME CHLORIDE

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ABSTRACT: The life threatening intoxication with highly toxic organophosphorous nerve agents requires the immediate administration of antidotes such as oximes. For the standard treatment of this intoxication, pralidoxime chloride (PAM) proved to be one of the most important antidotes. This study focused on the formulation of modern colloidal vectors for the delivery of pralidoxime chloride, such as liposomes and cubosomes. Taking into consideration the fact that this drug owns a low bioavailability, due to its high hydrophilicity, the formulation aimed at improving this behaviour.

Keywords: nanotechnology, liposomes, cubosomes, oximes, pralidoxime chloride

INTRODUCTION:

The life threatening intoxication with highly toxic organophosphorous nerve agents requires the immediate administration of antidotes such as oximes.

For the standard treatment of this intoxication, pralidoxime chloride (PAM) proved to be one of the most important antidotes. It reactivates the human acethylcholinesterase (AchE) inhibited by a number of organophosphorous nerve agents. (Worek *et al.*, 2007)

This study focused on the formulation of modern colloidal vectors for the delivery of pralidoxime chloride. Taking into consideration the low bioavailability of this drug, due to its high hydrophilicity, the formulation aimed at improving this behaviour.

Recent researches in this field emphasize on making this type of drugs pass the blood brain barrier (Kufleitner *et al.*, 2010, Wagner *et al.*, 2010) and on proving the efficacy of pralidoxime in carbaryl poisoning.

Other studies go further on formulating sterically stabilized liposomes (SL*), which contain an organophosphorous hydrolyzing enzyme (Petrikovics *et al.*, 2000). These studies suggest that the protection of OP intoxication can be strikingly enhanced by adding OPA (organophosphorous antagonists) encapsulated within SL* to pralidoxime and atropine.

The present research practically focused on the formulation of colloidal vectors such as liposomes and cubosomes and encapsulation of the active substance in a manner that increases its bioavailability.

MATERIALS AND METHODS:

Chemicals and reagents

For the liposome preparation phosphatidylcholine and cholesterol were used as lipidic materials. The dissolution of these substances was performed using chloroform. The active substance, pralidoxime chloride, was dissolved in distilled water and used as aqueous phase in the preparation of liposomes.

For the cubosome formulation, the following substances have been used: glycerol monooleate and Pluronic F127. The drug pralidoxime was encapsulated during the preparation of cubosomes. In this case, chloroform and purified water have also been used as solvents.

Equipment

The optical properties of the systems (UV-VIS spectra) a UV-mini 1240 Shimadzu spectrophotometer was used.

The separation of liposomes from the unentrapped pralidoxime chloride was carried out by using a Nüve NF800R centrifuge with refrigeration.

For characterizing the size of liposomes, the DLS technique was used and the measurement was performed on a Zetasizer instrument.

Measuring methods

I. Taking into consideration that pralidoxime chloride has a maximum absorption at 294 nm (Klaus *et al.*, 1991), we used the UV-spectrophotometry analytical method for the determination of the unentrapped pralidoxime chloride and consequently for stating the liposomes' encapsulation capacity. After obtaining the samples' absorbance at 294 nm and making a calibration curve for PAM, it became possible to determine the concentration of this substance in solution.

II. The liposomes' dimensions depend on the method of preparation and on the equipment that is used. These are important features that influence the physical properties of liposomes, the encapsulation capacity of the active substance and their biological behavior. For determining the size distribution of liposomes, we applied the DLS method (Budai *et al.*, 2004, Woodle *et al.*, 1993)

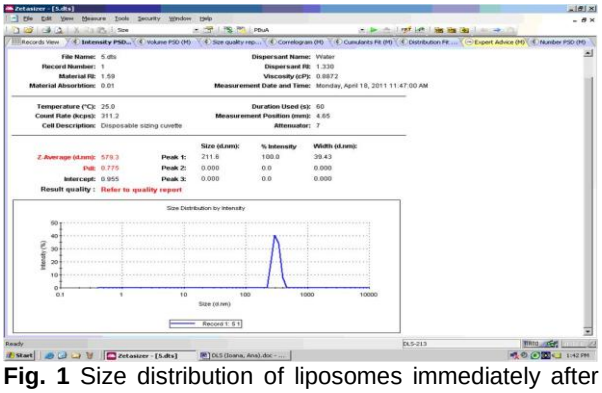

Fig. 1 Size distribution of liposomes immediately after preparation

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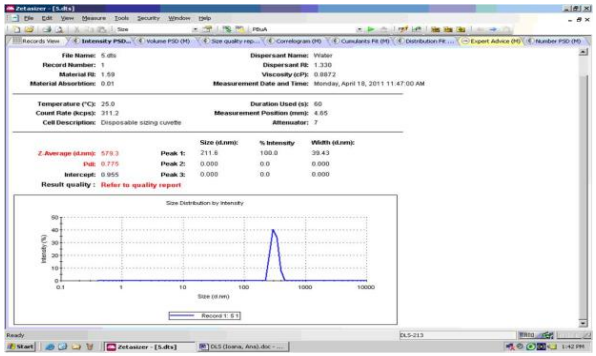


Fig. 2 Size distribution of liposomes after 3 months of storage

Formulation of liposomes

Liposomes are mainly used as nanocarriers in drug delivery protocols. Such use relies on the possibility of loading them either with hydrophilic drugs into their aqueous inner space or lipophilic drugs into the phospholipidic bilayer (Torchilin *et al.*, 2006).

For our study, we used the lipid film hydration as a method of formulation, described by Bangham (Bangham *et al.*, 1965). Following this method, lipids are dissolved in organic solvents (chloroform or mixtures with methanol) and the solvent is then removed in a vacuum rotaevaporator, forming a thin film on the walls of a round-bottomed flask. Then the film is rehydrated with the required aqueous phase, preheated at a temperature situated above the phase transition of the lipids used at preparation. The thin film is detached from the walls by stirring, resulting multilamellar vesicles, different in size.

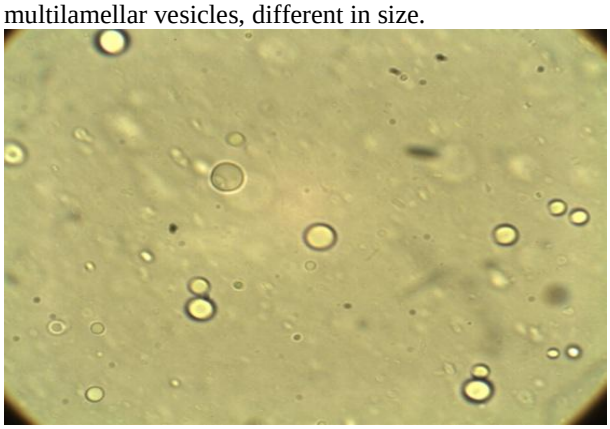


Fig. 3 Microscope 100x-liposomes, manual stirring

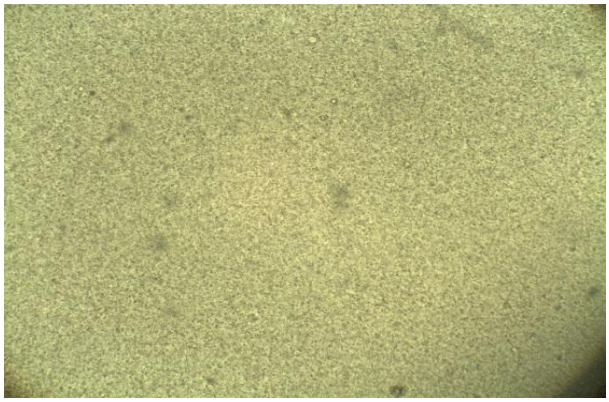


Fig. 4 Microscope 10x-liposomes after filtration with an 800 nm filter

Simultaneously, the active substance can be encapsulated, dependently on its physicochemical properties, by introduction in the film with lipids or in the aqueous solution used for rehydration, in the case of hydrophilic substances.

In order to homogenize the dispersion, so as to reduce the size of the resulting multilamellar liposomes at a nanoscale, the dispersion is subject to ultrasonication. In this way, unilamellar small vesicles are obtained.

Formulation of cubosomes

Cubosomes are discrete, sub-micron, nanostructured particles of bicontinuous cubic liquid crystalline phase. Cubosomes possess the same microstructure as the parent cubic phase but have much larger specific surface area and their dispersions have much lower viscosity than the bulk cubic phase (Gustaffson *et al.*, 1996).

The preparation of cubosomes mostly involves simple emulsification of monoglycerideand a polymer, accompanied by sonication and homogenization. The preparation methods fall into two categories, including top-down and bottom-up techniques(Garg *et al.*, 2006).

In the top-down technique, a coarse dispersion of cubosomes is usually formed first, which is then tailored into more uniform and finer particle dispersions with the help of high energy input devices (e.g. homogenization and sonication). In other words, it implies the fragmentation of a massive LC phase in an aqueous solution of the emulsifier. The top-down approach allows, in principle, for the entrapment of hydrophilic drugs.

In the bottom-up technique, cubosomes are formed instead by assembling nanomaterials into the final cubosome dispersion. Practically, the bottom-up approach is accomplished by diluting and then homogenizing a liquid mixture of the emulsifier and lipid in water (nanoparticles form by nucleation in the aqueous solution).

In general, a hydrophilic polymeric stabilizer is used to efficiently cover the outer surface of the dispersed particles and to retain the structures of these.

RESULTS AND DISSCUSSION:

Formulation of liposomes encapsulating Pralidoxime Chloride

Method I: 80 mg of phosphatidylcholine were weighed in an appropriate vessel, and then dissolved in 2 mL of chloroform. The solvent was then evaporated with the help of a rotary evaporator and in this way, a lipidic film of PC (phosphatidylcholine) has been obtained. The lipidic film was hydrated with 2 mL of hot water (60˚C). In order to facilitate the visualization at the microscope, a drop of methylene blue was added. The dispersion thus obtained was manually stirred for 2 minutes. Consequently, the liposomes were ultrasonicated, then passed through a 800 nm filter. Eventually, they were centrifuged 6 minutes at 10.000 rpm.

Method II:20 mg of PC (for obtaining a concentration of 10 mg/mL PC) were weighed in an

appropriate vessel. The PC was then solubilized in 2 mL of chloroform, which was evaporated with the help of a rotary evaporator. A lipidic film of phosphatidylcholine was obtained. Separately, 5 mL of PAM solution was prepared by dissolving 5 mg of PAM (analytical balance) in 5 mL of water (1 mg/mL PAM). 2 mL of this solution was heated at 60˚C and after that, it was used for hydrating the lipidic film. The liposomal dispersion thus obtained was manually stirred then centrifuged 10 minutes at 15.000 rpm.

Formulation of cubosomes encapsulating Pralidoxime Chloride

Formulation I. 300 mg of glycerol monooleate were brought in a 5 mL vessel and heated at 45°C. 75 µL of 1 mg/mL PAM solution are easily brought over GMO. The system thus obtained is kept with a tight lid 3 days, until cubic phase is formed. After that, the cubic phase must be dispersed in a solution of Pluronic F127 (9:1 ratio lipid:Pluronic). For instability reasons, this formulation could not be further used for the release studies of the active substance. Therefore, we developed a second version of the cubosomes.

Formulation II**.** 100 mg GMO are weighed on the analytical balance. 5 mL of Pluronic F127 are then added together with 5 mL of chloroform. The solvent is evaporated using a rotary evaporator and over the lipidic film, 10 µL of PAM water solution (1 mg/mL) is added. The system thus obtained is then dispersed in 5 mL of distilled water, by 10 minutes magnetic stirring, followed by 10 minutes ultrasonication.

Determination of the liposomes encapsulation capacity for pralidoxime chloride

In order to determine the encapsulation capacity, we prepared two types of liposomes. The first type is represented by liposomes formed with phosphatidylcholine at a concentration of 10 mg/mL and pralidoxime chloride 1 mg/mL. The second type consists of liposomes prepared with both phosphatidylcholine and cholesterol. In this latter type, PC and cholesterol are combined in a ratio of 4:1 (8 mg/mL PC and 2 mg/mL Chol).

Initially, the liposomes must be separated from the surrounding solution (containing the unentrapped drug) using an appropriate method. In this solution, we determine the concentration of the active substance, which could not be entrapped in the liposomes. In addition, we can calculate by difference the quantity of PAM which could be encapsulated.

The separation method consisted of using a Millipore filter for centrifugation. 1mL of sample 2 was introduced in the Amicon Ultra-4 Centrifugal Filter and centrifuged 10 minutes at 4500 rpm at the Nuve NF800R centrifuge (using 1 mL of water for balancing). The liposomes were retained on the filter and the solution of the unentrapped PAM was separated.

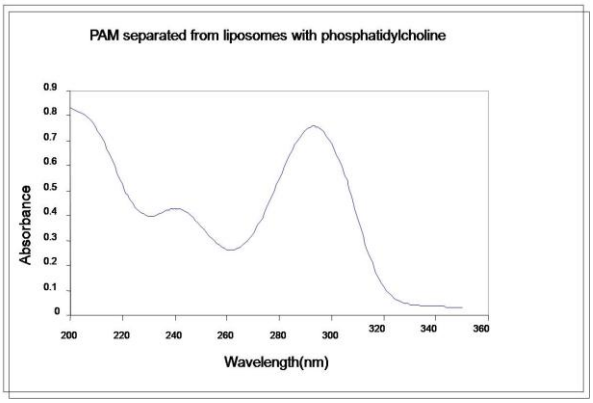
Determinationof the unentrapped PAMwas performed as follows: 100 µL from the separated solution were diluted with 4 mL of water, and 1mL of this solution was further diluted with 3 mL of water. The sample was then analyzed at the spectrophotometer, using a solution of blank liposomes as witness sample. Figure 14 shows the UV spectrum of the diluted solution of unentrapped PAM. Using the absorbance of this sample at 294 nm (PAM maximum absorbance) and the PAM calibration curve, it was possible to determine the concentration of the active substance in the solution.

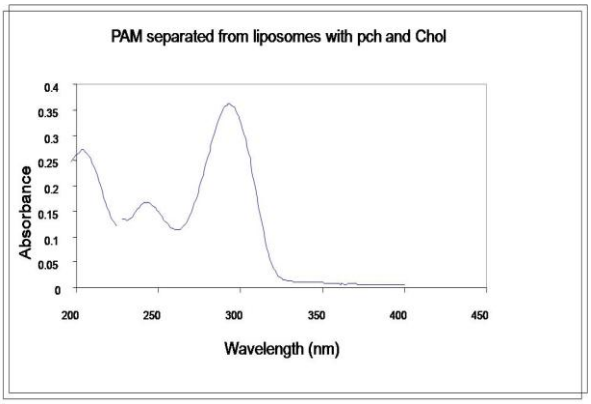
Considering the first liposomal formulation and the fact that the initial concentration in the liposomal dispersion was that of 1 mg/mL PAM we can, by difference, deduce the encapsulation yield. Thus, we have a quantity of 0,0530 mg of encapsulated PAM, which in matter of percent becomes 5,3%. In the same manner, we obtain a percentage of 11,99% for the second formulation.

We can therefore conclude that a small amount of cholesterol may increase the stiffness of the liposomal membrane. In this way, we can obtain an encouraging quantity of a hydrophilic substance encapsulated in liposomes.

Release profile of PAM from cubosomes

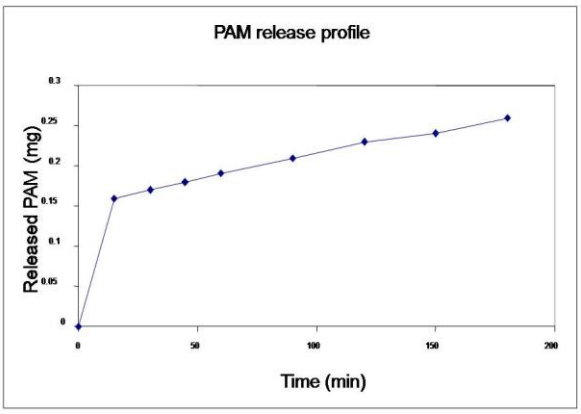
The release of pralidoxime chloride from cubosomes formulation was investigated following a dialysis method. A regenerated cellulose membrane was used for the release experiment.





The membrane was hydrated before experiment for 40 minutes with sterile isotonic saline solution to remove any trace of sodium-azide storage solvent. Experiment was carried out at room temperature for 24 h and sink conditions were maintained throughout experiments. At different times, 3 mL of the first solution were withdrawn, replaced with the same

volume of fresh distilled water. The graphic shows the release profile of pralidoxime chloride.



The mathematical models are used to evaluate the kinetics and mechanism of drug release from their formulations. The model that best fits the release data is selected based on the correlation coefficient (r) value in various models. The model that gives high 'r' value is considered as the best fit of the release data.

The Higuchi release equation

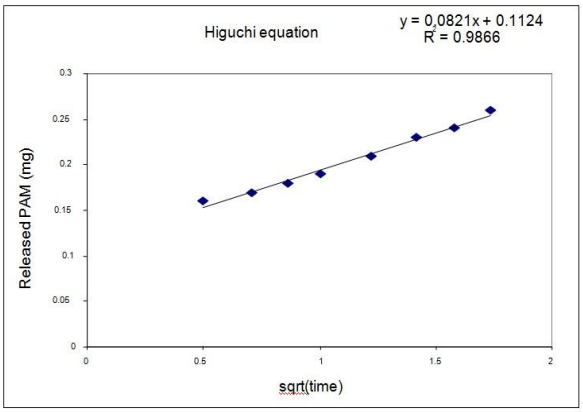
Q=KH*t1/2 , where:

Q=cumulative amount of drug release at time t

KH=Higuchi constant

t=time in hours

The Higuchi equation suggests that the drug releases by diffusion. A graph is plotted between the square root of time taken on x-axis and the cumulative percentage of drug release on y-axis.



Noyes-Withney equation $-\ln(1-\frac{m(t)}{m_{\infty}}) = kt$

Where

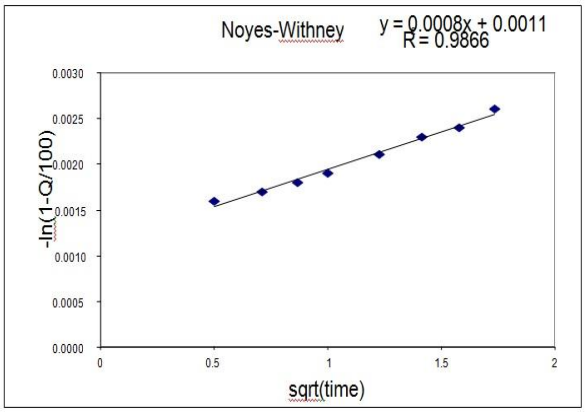
m(t)=amount of drug released at time t

m=total amount of drug in dosage form

k=kinetic constant

t=time in hours

The Noyes-Withney equation corresponds to Fick's law of diffusion, according to which the diffusive flux goes from regions of high concentration to regions of low concentration, with a magnitude that is proportional to the concentration gradient.



The Weibull model

- $M=M_0(1-e^{-k(t-T)})$

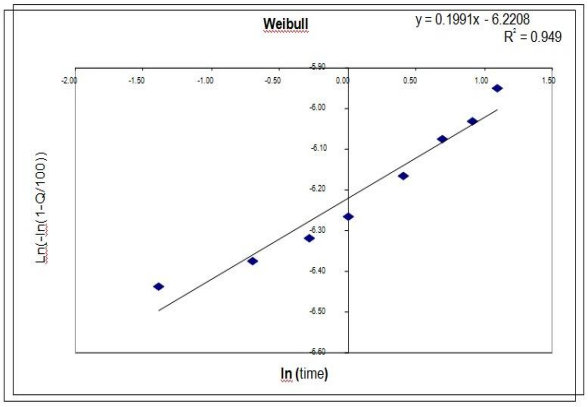
Where

m(t)=amount of drug released at time t

m=total amount of drug in dosage form

k=kinetic constant

t=time in hours



The Peppas equation

The release profile of various formulations was analyzed using a power law equation proposed by Peppas, which combines the effects of the mechanisms of diffusion and erosion of drug release from colloidal systems.

$F=(Mt/M)=K_m t^n$

where:

F=fraction of drug released at time t

Mt=amount of drug released at time t

M=total amount of drug in dosage form

Km=kinetic constant

n=diffusion or release exponent

t=time

'n' is estimated from linear regression of log (Mt/M) versus log t.

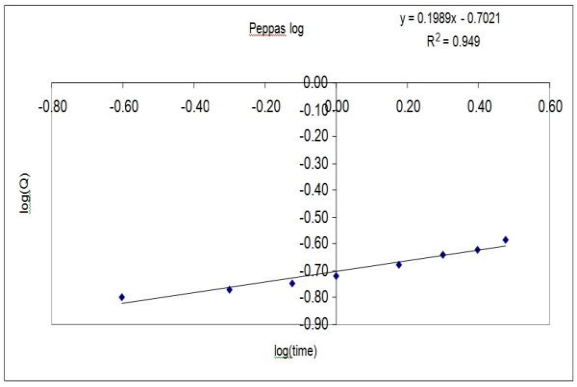
Discussion:

-if n<0,5 indicates fickian diffusion

-if 0,45<n<0,89 indicates anomalous diffusion or non fickian diffusion

-if n=0,89 and above indicates case 2 relaxation or super case transport 2, which refer to the erosion of a polymeric chain

Anomalous diffusion or non fickian diffusion refers to the combination of both diffusion and erosion controlled rate release.



The 'n' value lies below 0,5, demonstrating that the mechanism controlling the drug release was the quasi Fickian. At the same time, the highest correlation coefficient, 'r' is obtained for the Higuchi and Noyes-Withney equation, which drives us to the conclusion that the most probable release mechanism of pralidoxime chloride is the diffusion.

CONCLUSIONS:

This study shows that it is possible to incorporate pralidoxime chloride into modern colloidal vectors such as liposomes and cubic liquid crystals. It has been proved that, by adjusting the method of formulation for these carriers, various entrapment efficacies can be obtained. Thus, liposomes formed only with a lecithin lipidic film can encapsulate 5,3% of pralidoxime chloride, while by increasing the rigidity of the liposomes' membrane, a higher percent can be achieved (11,99%). This stiffness was obtained by adding cholesterol in a suitable proportion.

For further studies, we state that in the formulation of liposomes with an acethycholinesterase reactivator, other substances can be used. For example, a continuation would be that of using synthetic lipids with functional groups for drug targeting. This is a method of delivering medication to a patient in a manner that increases the concentration of the medication in some parts of the body relative to others. The goal of a targeted drug delivery system is to prolong, localize, target and have a protected drug interaction with the diseased tissue.

Moreover, another modern type of nanostructured carrier was formulated: cubosomes. The release kinetics from the cubic phase also showed optimistic results. We could apply a mathematical model to the release data that resulted in a mechanism of diffusion for our formulation.

Furthermore, taking into consideration the advantages of these modern drug carriers we can state that their use in increasing the bioavailability of hydrophilic drugs is of great importance for the future of the pharmaceutical research and industry.

ACKNOWLEDGEMENT:

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