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LIPOSOMIC NIFLUMIC ACID
NEW TRANSDERMAL ANTI-INFLAMMATORY MEDICINE

The encapsulation technology, of pharmaceutical molecules in liposomes, shows particular growth the last few years along with great scientific interest.

A variety of different molecules have been already encapsulated with success, resulting to a direct attenuation of toxicity, to an improvement of their pharmacokinetic properties and to an increase in their selectivity and specialization concerning the tissues and target organs.

In the international bibliography there is a wealth of references on liposomic pharmaceutical forms. Many of these are in the clinical study stage and some other have been already registered and marketed.

Among the other medicines, in liposomic form, we must mention econazole, amfotericin B, minoxidyl and some anticancer and antiviral medicines which are in the clinical study stage.

Concerning transdermal use, the major problem of the pharmaceutical substance transfer through the skin is owed to the unsatisfactory permeability of the substance or to the fast transfer into the blood and lymph resulting in decreased therapeutic concentration within the desired time limits. The liposomic forms are not only characterised by a satisfactory permeability but also by a great potential for controlled release of the active ingredient. These properties make liposomic forms ideal for transdermal use.

The present invention has as a main objective to prepare liposomes and to encapsulate into them the anti-inflammatory substance Niflumic Acid and its esters. The Niflumic Acid is a very well substantiated active ingredient which has been already released in various transdennal forms.

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It is aimed at the development of a new anti-inflammatory form with a leading liposomic form and advanced pharmacokinetic properties. The medicine will be applied to Arthropathies and similar situations in Traumatology and Reumatology.

The most important problem which is aimed to be solved by the present invention, is the selection of these lipids, for the liposomes preparation, that fulfils the following criteria :

- Biocompatibility with human skin
- Formation into liposomes with desirable physicochemical characteristics. (Structure, morphology, dimensions, number of lipid dual-layers, crystallisation and stability in connection to time temperature and concentration).
- Ability of liposomes to encapsulate the medicine by keeping its desirable physicochemical characteristics.

The selection of lipids, among these which fulfil the criteria, will be made according to the results of the pharmnacokinetic studies.

The lipid molecules which are going to be assessed are natural ones, like: HSPC (hydrogenated soy lipids), EPC (Mixture of saturated and unsaturated lipids from eggs) and some synthetic like : DSPC, DMPC and DPPC which are saturated esters of phosphatidylcholine. The techniques that are used for the preparation and the physicochemical characterisation of liposomes along with these for the system liposome- encapsulated medicine and also the methodologies for the pharmacokinetic studies are presented in the following section :

Scientific Methodology (full and analytical description)

I. Lipids and raw material supply, preparation of niflumic Acid Esters.

The lipids which are going to be used for the preparation of liposomes are either natural ones like the HSPC (hydrogenated soya lipid), EPC or synthetic lipids like phospholipids DSPC, DMPC, DPPC. The aliphatic chains of the synthetic lipids are stearic or myristate or palmitic acid which differs in the number of carbons

atoms, the length and the melting point of aliphatic chains, parameters crucial for the physicochemical characteristics of liposomes.

Liposomes will be prepared not only by combining different phospholipids but also by phospholipids with different levels of cholesterol. Cholesterol is regulating the stability of liposomes therefore essential for the controlled release of the medicine. Liposomes will also be prepared with composition similar to the skin's with ingredients like: ceramides, cholesterol, cholesterol sulfate and fatty acids. The aim is the preparation of products with greater possible biocompatibility.

II. Preparation of liposomes and Physicochemical characterisation.

Preparation methodology :

a) *Preparation of lipid film by hydration of lipid molecules.* The hydration will take place after the removal of the organic solvent (chloroform) in vacuum revolving distillation apparatus (flash evaporator)

The selection of the hydrating agent is important for pH adjustment in the internal environment of liposomes. TES (N-tris[hydroxymethyl] methyl 2-amino ethanesulfonic acid), MES (2-[N-morpholino] - ethanesulfonic acid) HEPES (N-[2-hydroxyethyl] piperazine -N-2-ethanesulfonic acid), Ammonium Sulphate will be used in which the pH, the osmotic and the ionic strength are monitored. The hydration of lipids is resulting to their self formation into multilayer liposomes.

b) *Preparation of single layer and oligo-layeral liposomes.* For transderrnal applications, the oligo-layeral liposomes have an advantage of the multilayer ones. (Better permeability due to smaller size). The methodology which will be used for the preparation liposomes comprises of the freezing of multilayer liposomes in a temperature of -70°C and immediate heating in a temperature higher than the melting point of the aliphatic chains (cis-trans). The procedure takes place ten times with final result the yield of oligo- layeral liposomes, situation that facilitates the size homogeneity at the next stage.

c) With an extrusion system among multicarbon filters of specified diameter, a homogeneous (dimensionally) sample of liposomes is received.

d) The separation of the buffer solution, which may not be encapsulated in the liposomes, is achieved by gel-chromatography.

For the implementation of the study, MLV (multilamellar vehicles), SUV (small unilamellar vehicles) and LUV (large unilamellar vehicles) liposomes will be prepared and assessed aiming the encapsulation, on one hand the hydrophilic part of the Niflumic acid into the hydratic part of the single layer liposomes and on the other hand the encapsulation of the lipophilic products of esters, into the lipid double-layers of the multilayer liposomes.

Physicochemical characterisation

The physicochemical parameters of liposomes that must be assessed at this stage are :

Dimensions - dimensions homogeneity - number of double layers - morphology, thermodynamic stability (in connection with the temperature and the lipid sample concentration) existence of interactions in the sample, possibility of phase separation.

The physicochemical techniques that will be used are of great resolution potential, specified as quantitative and qualitative optical-thermal techniques.

1. Quasi-elastic light scattering. Capable to study the full distribution of liposome dimensions.
   Study of possible interactions in the sample. Study of possible agglomeration of liposomes. Assessment of spherical morphology (Bi-reftactiveness)

2. Microscopy. Phase contrast microscopy and contrajunctional contrast microscopy (Nomarski-DIC), combined with image processing analysis This

technique has the ability to represent liposomes with a size greater than 0,5 μm.

3. Atomic force microscope (AFM) : Representation of liposomes with a size between 10 nm and 2 μm. Ability also to represent the lipid double-layers.

4. Differential scanning calorimetry (DSC) : Thermodynamic study of liposomes stability. Assessment and characterisation of phases and phase of transformations in lipid double-layers. Indirect purity test of liposomes.

III. Pharmaceutical molecules encapsulation, study of physicochemical characteristics of the medicine-liposome system.

Different kinds of liposomes will be used for the pharmaceutical molecules encapsulation.

a) Niflumic-acid encapsulation

The Niflumic-acid will be encapsulated in the internal part of liposomes following the method of active transfer due to different pH which is created inside and outside the liposomes. Because of the fact that the pharmaceutical molecule contains ionic groups, in the acidic internal environment of the liposome the molecule will receive protons and consequently it will be encapsulated. The encapsulation will take place at the transition temperature of the liposomes aliphatic chains. Percentage of encapsulation about 60%-80%. Ratio of niflumic acid to lipids 1:53 (Theoretical value 1:50). The separation of the non encapsulated Niflumic Acid will be succeeded by gel-chromatography, liposomes containing Niflumic acid will be received. The assay of the encapsulated Niflumic acid will be carried out with HPLC and UV-Vis.

The assessment of niflumic acid / lipids was carried out with UV/Vis. For the encapsulation of niflumic acid MLV (multilamellar vehicles), SUV (small unilamellar vehicles) and LUV (large unilamellar vehicles) liposomes were used, the greatest possible internal value.

The encapsulation into MLV liposome was greater than 90% whereas the ratio of niflumic acid to lipids was 1:55 (theoretical value 1:50).

b) Encapsulation of the lipophilic esters of the Niflumic Acid.

The lipophilic esters of the Niflumic acid will be encapsulated into the lipid double-layers of liposomes. Multilayer liposomes will be used in order to increase the available volume (of the double-layers) for the encapsulation. The addition of the lipophilic molecules will take place during the evaporation of the organic solvent from the lipids for the preparation of the lipid film (see 2a). The separation of the non encapsulated medicine will be managed with gel-Chromatography along with the test of the medicine/lipids ratio. The assay of the medicine will be carried out with HPLC.

c) Physicochemical characterization of the medicine-liposome system.

The physicochemical parameters are 1, 2, 3, 4 of pages 4, 5. In adition isothermic titration for the thermodynamic study of the encapsulation of the drug substances was applied.

At this point there is a great scientific interest especially concerning the physicochemical changes taking place in the liposome due to the encapsulation of the medicine.

The techniques which are used are similar to these on phase 2.

Additionally, the isothermal microcalorimetry titration technique will be applied for the thermodynamic study of the pharmaceutical molecules encapsulation. The suggested microscope upgrade will give the ability of a more detailed representation of the chemical composition of the liposomes surfaces under study. Especially for the case of the encapsulation into the lipid double layers it will be possible the <charting> of the active ingredients.

IV. Pharmacokinetic studies.

The kinetic study of medicines includes the testing of their release in buffer solutions (in vitro) together with the testing in 50% plasma of rat (in vivo).

The kinetic study will be carried out in connection with time in order to study the stability of the medicine (Refrigerator temperature 2°C, room temperature 25°C) and to study the release of the medicine at a temperature of 37°C.

Hyperthermal studies will be carried out at 45°C and 55°C in order to test the gradual release of the medicines and also to avoid possible toxicity phenomena. In all the cases, by using gel-chromatography, the free pharmaceutical agent, towards the encapsulated one, will be under control and the assay will be carried out with UV-Vis. The ratio medicine/lipid will be also determined.

The analytical methodology is the following:

a) Study of the release in a buffer solution.

TES (N-tris[hydroxymethyl] methyl 2-amino ethanesulfonic acid), MES (2-[N-morpholino] - ethanesulfonic acid) HEPES (N-[2-hydroxyethyl] piperazine -N-2-ethanesulfonic acid.

It is an indication of substance release from the liposome. After the encapsulation of the substance in the liposome, in specified intervals, the assay of the substance is determined with High Performance Liquid Chromatography (mobile phase ethanol-water) and UV-VIS.

b) In-vitro release of the medicine on the skin.

From hairless mice the dorsal skin is obtained, the adipose tissue is removed with a blade the fatty tissue and by the heating technique for 60 seconds the epidermis is separated from the chorium. The liposomic solution is applied and samples of serum are collected in standard time intervals which are analysed with the HPLC method.

c) In Vivo study of the medicine absorption from the skin.

Blood sample is also taken from the serum or the plasma from which, after centrifugation, the active ingredient is extracted and determined with HPLC and UV-Vis.

With this method the concentration of the medicine is specified topically on the skin, in the blood, in the body along with the total absorption.

V. Development of the final pharmaceutical form - Quality control

The liposomic medicine presenting the best results in absorption, is formed in cream, gel or lotion. For this reason product recipes are selected in which the dispersion or/and the dissolution of the liposomic product is possible.

From the prepared products, two recipes are selected, these fulfilling some criteria, like the application way, the appearance and the viscosity, in the best way.

The two products are set under stability test in order to specify the life time of them by studying the following parameters like : appearance, pH, viscosity, solid residue, active ingredient and conservatives determination etc. The study takes place at different temperatures e.g. 4, 25, 37, 45°C and under an alternative cycle scheme like freezing- defteezing.

The selected products are subject to microbiologic test for their concentration in microbes. A challenge test is also carried out to prove the efficiency of the conservation system.

VI. Efficacy of the liposomic medicines

a) Pharmacokinetic study.

It is carried out like in paragraph 4, that is to say in vitro for an indicative result and in vivo on hairless mice. The methodology followed is the same with this in paragraph 4 differing in the fact that instead of liposomic samples, a direct application, on the skin, of the final product is used. The studies carried out in comparison with the medicine in non liposomic form.

From the final products of the previous phase, it is selected the one which gives the best pharmacokinetic results, but it is also possible the study of a further improvement of the composition in connection with the release of the medicine.

b) Pharmacological study

The final selected products are tested for their pharmacological properties in vivo. For this reason an inflammation is caused on the skin of hairless mice with ultraviolet light. The inflammation is tested concerning the determination of prostaglandin formation and the causation of free radicals as this can be assessed by the reduction of the natural antioxidant skin protection. This is expressed by the reduction, in the skin of the antioxidant agents like vitamin E, vitamin C, ubiquinone and ubiquinole.

CLAIMS

1. Encapsulation of the anti-inflammatory medicine, Niflumic acid and all of its derivatives as synthetic esters, in liposomes prepared by lipids like EPC (Egg Phosphatidylcholine), DSPC (Distearoylphosphatidyl-choline), DMPC (Dimyristoylphosphatidylcholine), DPPC (Dipalmitoyl-phosphatidyl-choline), HSPC (Hydro-genated Soya Phosphatidyl-choline), and their mixtures as well as the mixtures of the above mentioned lipids and combinations thereof with Cholesterol and Ceramides.

2. Encapsulation according to claim 1, in the internal lipid layer and on the surface of the liposomes.

3. Encapsulation according to claim 1, characterized from the fact that the new liposomal products can be used topically, on the skin, eyes, ears and oral cavity, as anti-inflammatory, anti-rheumatic and analgesic drugs, as well as on arthropathies and similar disorders of traumatology concern.

4. Encapsulation of radiolabeled Niflumic acid and all of its derivatives as synthetic esters in liposomes prepared by lipids like EPC (Egg Phosphatidylcholine), DSPC (Distearoylphosphatidylcholine), DMPC (Dimyristoylphosphatidylcholine), DPPC (Dipalmitoylphosphatidyl-choline), HSPC (Hydrogenated Soya Phosphatidylcholine) and their mixtures as well as the mixtures of the above mentioned lipids and combinations thereof with Cholesterol and Ceramides.

5. Encapsulation of Niflumic acid and all of its synthetic esters according to claims 1, 2 and 4 as characterized from a methodology of encapsulation by which the active transfer of the medicine is carried out (pH gradient) to the interior of the liposomes.

6. The liposomal products and their pharmaceutical forms according to claims 1,2,3,4 as characterized from the reduction of the skin inflammation after the local treatment on the skin, eye, ear and oral cavity.

7. Encapsulation of Niflumic acid and all of its synthetic esters in liposomes according to claims 1,2 and 3 as characterized from the dissolution test of the medicine in pH = 1-10.

8. Encapsulation of Niflumic acid and all of its synthetic esters in liposomes according to claims 1,2,3 and 7 as characterized from the dissolution test of the medicine in pH = 4.5.

9. Encapsulation of Niflumic acid and all of its synthetic esters in liposomes according to claims 1,2,3,7 and 8 as characterized from the dissolution test of the medicine in pH = 7.1.

10. Niflumic acid and all of its synthetic esters after its encapsulation in liposomes according to claims 1,2,3,4,5 as characterized from the physicochemical parameters of the medicine-liposome system, using physicochemical techniques such as high resolution ability, qualitative, quantitative and optothermic techniques.

11. The liposomal products according to claim 1, characterized from the use of these products for in vitro / in vivo pharmacokinetic studies on mice, rats at temperatures 2-55°C.

12. Pharmaceutical form characterized from the fact that it contains encapsulated Niflumic Acid and all of its synthetic esters according to claims 1,2,3 and 4.

13. Pharmaceutical forms for dermal and transdermal use, according to claim 12, which contain encapsulated Niflumic Acid and may be cream, gel, ointment, cutaneous emulsion, cutaneous suspension, transdermal patch, impregnated dressing.

14. Pharmaceutical forms for oral use, according to claim 12, which contain encapsulated Niflumic Acid and may be gargle, gingival solution, oromucosal suspension, oromucosal drops, oromucosal gel, gingival gel, oromucosal solution, mouthwash.

15. Pharmaceutical forms for ocular use, according to claim 12, which contain encapsulated Niflumic Acid and may be eye cream, eye ointment, eye gel, eye drops solution, eye drops suspension, eye drops prolonged release.

16. Pharmaceutical forms for auricular use according to claim 12, which contain encapsulated Niflumic Acid and may be ear cream, ear gel, ear ointment, ear drops solution, ear drops suspension, ear drops emulsion, ear tampon.

17. Liposomal products according to claim 1, and pharmaceutical forms according to claim 12, 13, 14, 15, 16, which contain encapsulated Niflumic Acid.