Università degli Studi di Firenze

Facoltà di Farmacia

Dipartimento di Scienze Farmaceutiche

TESI DI DOTTORATO IN
"CHIMICA E TECNOLOGIA DEL FARMACO"
XXI CICLO 2006-2008
Settore disciplinare: CHIM 09

SVILUPPO, CARATTERIZZAZIONE E VALUTAZIONE DI CARRIER COLLOIDALI O MICROPARTICELLARI PER LA VEICOLAZIONE DI FARMACI

Candidato
Gaetano Capasso

Docente Supervisore
Prof.ssa Paola Mura

Coordinatore del Corso
Prof. Fulvio Gualtieri

INDEX

INTRODUCTION

During the course of my doctorate, carried out in the research group directed by Prof. Paola Mura, I mainly dedicated my studies to the development, chemical-physical, characterization and technological and biopharmaceutical evaluation of colloidal carriers and cyclodextrin complexes for drug delivery.

In particular, I focused my research on the development of liposomes, as drug carriers, investigating various aspects of both their composition and the various techniques of preparation and characterization.

Liposomes are colloidal phospholipidic vesicles extensively investigated as safe and effective drug carrier systems. An increase in therapeutic efficacy has been demonstrated for liposomal formulations of several drugs with respect to administration of plain drugs.

The drugs that I examined for inclusion in various liposomal formulations have been different local anesthetics, such as benzocaine, butamben and prilocaine, and I also investigated the effect of their complexation with cyclodextrins.

Another area which may benefit of liposomal formulation is that of chemotherapeutic agents.

My research in this field was aimed at the development of a liposomal formulation for Irinotecan, a chemotherapeutic drug for colon cancer.

Parallely, I also devoted my studies to the preparation and characterization of drug complexes with cyclodextrins.

Cyclodextrins received an increasing interest in the pharmaceutical field due to their ability to favourably modify physical, chemical and biological properties of drug molecules through the formulation of inclusion complexes.

The drugs that I considered for their formulation as cyclodextrin complexes were metformin, an oral antihyperglycaemic agent and oxaprozin, an anti-inflammatory agent.

I turned particular attention to the study of the influence of the method used for the preparation of the drug-cyclodextrin complex on the performance of the final product.

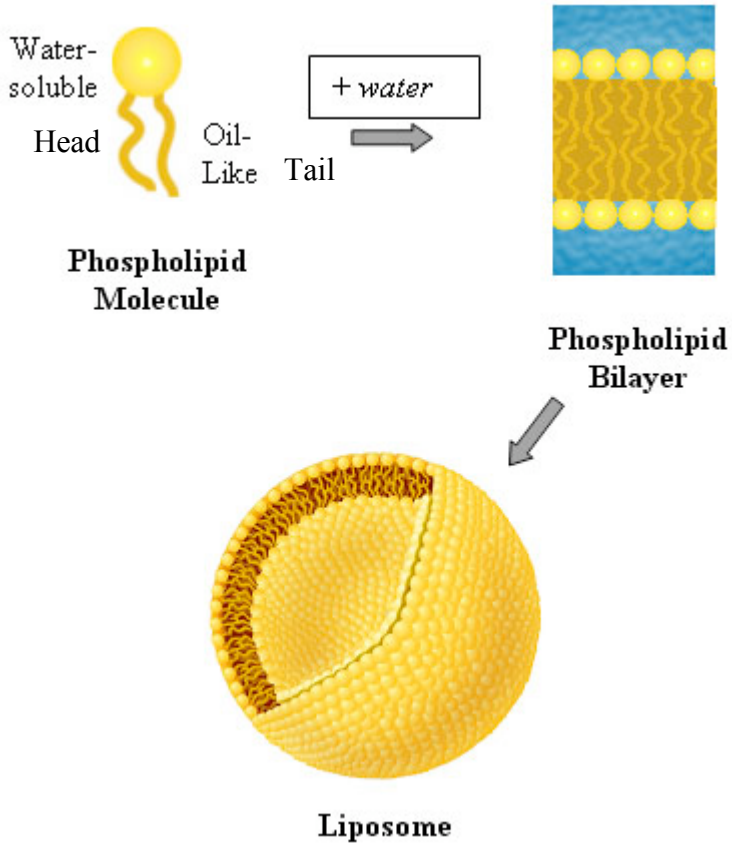
During the three years of my doctorate I carried out two research stages abroad, respectively at the Laboratory of Pharmaceutical Technology of the University of Seville, Spain, under the guidance of Prof. Antonio Maria Rabasco Alvarez and Prof.

Maria-Luisa Gonzalez Rodriguez, and at the “Department of Biopharmaceutical Sciences and Pharmaceutical Chemistry” of the University of California, San Francisco, U.S.A., under the guidance of Prof. Francis C. Szoka .

These internship periods allowed me to deepen and learn the use of new advanced techniques of microscopic investigation and of new methods of preparation of liposomes.

1. LIPOSOMES

The liposomes are colloidal particles consisting of lipid bilayers that surround one or more aqueous compartments. The lipids, which may be of natural or synthetic origin, orient their head toward the polar regions of the double-layer, so that to be in contact with the polar medium. Hydrophobic tails are turned instead towards the inside of the bilayer.



History and applications

The discovery of liposomes is attributed to A.D. Bangham, who in the early 60s studied the behavior of lecithin and other phospholipids in the hydration phase (Bangham et al., 1965). Quote the words of his article: "The liposomes are small vesicles of spherical shape that can be produced from natural non-toxic phospholipids and cholesterol. There are pockets of microscopic spherical shape, the walls are made from phospholipids identical to those that form cell membranes."

Since then numerous liposomal preparations were prepared to study biological processes and membrane proteins, exploiting their structural similarity with the animal cell. It was in the 70s that the liposomes were proposed as a carrier for drugs, but the early studies led to inadequate preparations with stability. In the early 90s the accumulated knowledge on polymorphism of lipids, the mechanisms of interaction between the liposome-membrane and drug-phospholipids made possible to overcome the initial difficulties and gain the first liposomal drugs (Lian et al., 2001).

Due to their biphasic nature, the liposomes can accommodate both lipophilic and hydrophilic substances, then, in principle, any type of drug. They are also used to carry DNA, proteins and peptides.

To ensure a sustained therapeutic action is necessary a sufficient stability over time, both in terms of shelf life and in vivo. Today liposomal formulations approved by the Food and Drug Administration U.S. are numerous, containing especially antifungals and anticancer drugs.

Most of these contain as the main constituent phosphatidylcholine, with chains of varying length and varying degrees of saturation. It is often also included cholesterol to adjust the stiffness and increase stability in vivo. The physical characteristics such as size, surface charge and the fluidity of membrane play a key role in the pharmacokinetics and activity of liposomal preparations.

The FDA in 2002 issued guidelines suggesting tests and controls that the industry should run for the development and marketing of liposomal preparations (Guidance for Industry: Liposome drug Products, FDA, 2002).

Advantages and Disadvantages

The liposomes are Drug Delivery Systems, able to direct and protect the drugs. They can also prolong the duration of the therapeutic effect, acting as a reservoir system. The main advantages of using these systems are:

- Biocompatibility with biological membranes
- Complete biodegradability
- Application versatility: being able to encapsulate both lipophilic and hydrophilic drugs
- Versatility of properties: the preparation method and the composition can modify many parameters, such as size, elasticity, etc..

Among the limitations and problems in their use, it should instead remember:

- Chemical Instability: the phospholipids exposed to oxygen, to light and high pH values, suffer reactions of oxidation and hydrolysis.
- Physical Instability: vesicles tend to settle, merge or join; these phenomena can be reduced by using charged particles, i.e. adding charged components, such as stearylamine (SA) and dicethylphosphate (DP).
- Loss of the active: this process may be slowed by increasing the rigidity of liposomes or proceeding to the lyophilization.

1.1 Preparation of liposomes

Classification

The classification of liposomes can be made according to procedures for the preparation or follow structural criteria (Torchilin et al., 2003).

Classification according to the preparation method

Thin Layer Evaporation (TLE): The phospholipids and other fat-soluble components are dissolved in a highly volatile organic solvent, such as dichloromethane or chloroform. The solvent is removed with the rotavapor until a thin lipid layer (thin layer) is obtained:

The film is then hydrated with water and subjected to agitation with whirling vortex. Depending on its solubility characteristics, the drug can be dissolved in the hydration phase (water) or in the organic solvent, together with the phospholipids.

The suspension must be heated above the transition temperature of the phospholipids. With this technique are obtained liposomes with high degree of lamellar (MLVs).

Sonicated Vesicles: in this case heating is not necessary. It can introduce the probe of sonicator within a suspension (costant temperature 0°C because the probe tends to dissipate energy and increase the temperature) or immerse the container in a sonication bath. It is a good method for producing SUVs (Small Unilamellar Vesicles) .

Reverse Phase Evaporation Vesicles (REV) the preparation of the lipid film is the same shown above. Then add the water and air, usually in the ratio of 1:3 v/v. Everything is then sonicated to form an A/O emulsion. The solvent is then evaporated to reach the reversal phase. We maintain the conditions of agitation and low pressure until the complete removal of the solvent.

Frozen and Thawed Multilamellar Vesicles (FATMLV): as starting material is used a suspension of MLV. This is frozen in liquid nitrogen and then thawed in thermostat bath at a temperature higher than that of transition. The operation is repeated 2 or 3 times at predetermined time intervals.

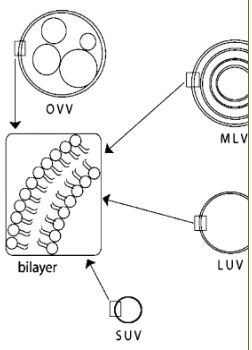
Dehydration-rehydration Vesicles (DRV): liposomes MLV are first prepared. These are then sonicated, freeze dried and put in buffered solution.

Vesicles by Extrusion Technique (VET): the liposomal suspension prepared by TLE or FATMLV is extruded through filters of polycarbonate (pores of about 400 nm). This method makes it possible to reduce the size.

Structural Classification

It is based on morphological characteristics and size of the vesicles

Abbreviation	Name	Diameter
MLVs	Multilamellar Vesicles	> 0,5 µm
OLVs	Oligolamellar Vesicles	0,1-1 µm
SUVs	Small Unilamellar Vesicles	20-100 nm
LUVs	Large Unilamellar Vesicles	>100 nm
GUVs	Gigant Unilamellar Vesicles	>1 µm
MVVs o OVVs	Multi (o Oligo)vesicular Vesicles	>1 µm



1.2 Characterization of liposomes

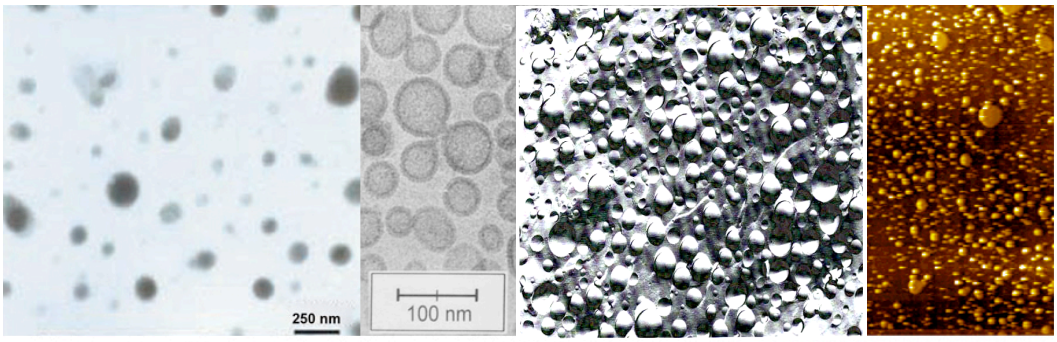
Several analytical techniques can be used to describe the characteristics of liposomes (Edwards et al., 2006).

Microscopy

Both the optical and the electronic microscopy are useful for the analysis of liposomes. Optical microscopy is easy to use, because it requires an ordinary optical microscope compound (MOC). The resolution is limited by the phenomenon of diffraction and is therefore relatively low (0.2µm). The transmission electron microscopy (TEM) allows magnification of 200,000 times, with a resolution of about 0.1 nm (10 Å).

This technique works with the attachment (staining) of the sample on a polycarbonate film, using a solution of uranyl molybdate or tungsten. It is also request the generation of vacuum. All this can lead to artifacts in the analysis. In recent years other techniques has been proposed, such as the atomic force microscopy (AFM). Extremely versatile, it requires no special treatment and allows the sample analysis in a variety of environmental conditions (in water, dry condition, at room temperature, hot condition, etc). It reach resolutions of 0.1 nm.

Images of liposomes obtained by a number of techniques (Nallamothu et al., 2006):



Number of lamellar

The number of lamellar of liposomes can be extremely variable: the fraction of phospholipids in the outer layer can range from 5% (LMV) to 70% (SUV) (Barenholz et al., 1977). A technique commonly used to determine the number of lamellar is the ^{31}P NMR and the addition of Mn^{2+} reduces the signal of phosphorus of the polar heads; the degree of lamellarity is derived from the ratio of the signal before and after the addition of Mn^{2+}. Other techniques are electron microscopy, the spread X-ray at small angles (SAXS) and methods based on changes in fluorescence signal, UV or visible of lipid marked after adding suitable reagents.

Size

The techniques available for the particle size determination are numerous. Among these one can remember the dynamic light scattering (DLS) or static (SLS),

microscopy, the size-exclusion chromatography (SEC), the field-flow fractionation (FFF), the analytical centrifugation and capillary electrophoresis.

Phospholipids

For this type of analysis, is usually employed a molybdate reactive-based, allowing the oxidation and coloring of phospholipids. Even some chromatographic techniques (HPLC, GC, TLC) may be used.

Encapsulation efficiency

The techniques for determining the amount of drug entrapped within liposomes are based on the measure of the concentration of active ingredient encapsulated in comparison with the total amount. The encapsulation efficiency percentage may be expressed as:

$$EE\% = \frac{C_{in}}{C_{tot}} \cdot 100 = \frac{C_{tot} - C_{out}}{C_{tot}} \cdot 100$$

Where C_{in} is the concentration of drug encapsulated, the C_{out} that of extra-liposomal drug and Ctot is the total concentration.

The methods used for separation of drug encapsulated and non-encapsulated are dialysis, filtration, centrifugation, chromatography, gel-permeation chromatography and ion exchange. The methods of quantification can be Spectrophotometric, enzymatic or electrochemical. The amount of total active ingredient is usually determined after having caused the complete lysis of liposomes, with the addition of alcohol, heating and / or use of surfactants (Grabielle-Madelmont et al., 2003).

Zeta potential

The charges exposed on the surface of liposomes play an important role on stability, the interaction with drugs and interaction with plasma proteins. The value of Z

potential can be achieved by measures the electrophoretical mobility of the particles, or using the spectroscopy correlation of photons (PCS).

Deformability

The use of ultra-deformable vesicles (increasing the elasticity of liposomes) facilitates the passage of the drug through the membranes, in some cases even through the skin intact (Schätzlein et al, 1997). There are various techniques for the measurement of vesicles deformability. Electron microscopy and atomic force (AFM) are applicable to particles above 10 microns and allow calculation of the elasticity constant of the generation of thermal fluctuations of the membrane (Lee et al., 2001).

Another interesting method, but only for large particles, may be to join the vesicles with a spherical surface and draw a portion of the membrane with a micropipette. In this way, the phospholipids form, between the surface and the vesicle, a thin cylinder defined tether (neck). Depending on the suction pressure is measured variations in the form of vesicles, the tether and the portion of membrane sucked, obtaining a measure of deformability defined *bending stiffness* (Waugh et al, 1987). A technique applicable to particles below 10 μm is the extrusion.

The liposomal suspension is introduced into a syringe and applying a positive pressure is forced to pass through a membrane, whose pore diameter is less than that of liposomes. The operation is repeated several times. The average diameter of liposomes is measured before and after extrusion (Van den Bergh et al., 2001).

When the liposomes are well deformable, can change shape and pass through the pores: the diameter does not undergo changes before and after extrusion. If they are not very deformable, decreased in size or fail to pass the membrane. With this technique the elasticity can be expressed quantitatively using the following formula (Cevc, 1995):

$$Elast. = J\left(\frac{R_v}{R_p}\right)^2$$

Where R_v is the radius of vesicles, R_p the pore, J the flow or rate of penetration. J is calculated measuring the flow of a constant volume of suspension through the membrane as a function of time.

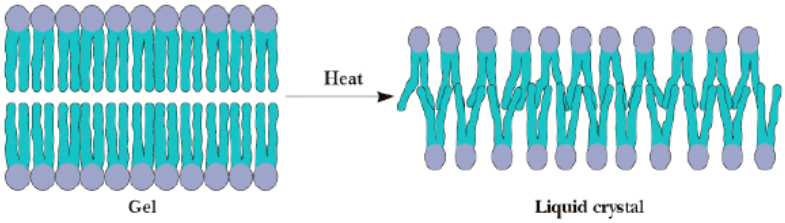
Thermometric Characteristics

The liposomes can be in three different thermometric states:

Gel: The alkyl chain fatty acids are tightly packed.

Ripple: is also called wavy phase; alkyl chains are in the bending. The temperature at which they move from the gel is called "ripple temperature".

Liquid crystal: the lipid matrix is less packed and is characterized by an increase in the disorder and the degrees of freedom. The temperature of transition phase is called transition temperature.



The differential scanning calorimetry (DSC) can reveal the phase transitions. Changes in Thermometric Characteristics provide useful information for the study of interactions molecule-model membrane.

Stability

One of the major limitations in the use of liposomes as carriers of drugs is their low stability. During the preparation and or storage can undergo many changes, both of chemical and physical nature (Zuidam et al, 1996).

Oxidation

Even in the absence of specific oxidants, the fatty acid chains of phospholipids tend to oxidize, especially if there are double bonds. These reactions are catalyzed by light, sonication or traces of metal ions. Besides oxidation reactions, can occur rearrangements of double bonds that lead to the formation of conjugated double bonds.

The main consequence of these degradation reactions is increased permeability of double layers and thus escape of drug. The degradation of phospholipids can be highlighted with thin-layer chromatography (TLC): The presence of a single blemish is a sign of good conservation. Another technique of analysis can be mass spectrometry.

The oxidation of liposomes can be reduced with certain precautions: use phospholipids very pure and free of oxidants, use of synthetic phospholipids, use of solvents and distillates without oxygen, avoid use of sonication and high temperatures, use inert atmosphere in preparation, use of antioxidants (α-tocopherol, vitamin C), low-temperature preservation and protection from light (Storm et al., 1993).

Hydrolysis

The phospholipids dispersed in water can be hydrolyzed to free fatty acids, following a kinetics of pseudo-first order. These reactions are catalyzed by acids and bases, may lead to increases in average size of particles and facilitate aggregation phenomena. The reactions of hydrolysis have the minimum speed when the pH is 6.5. Some cares to minimize the hydrolysis may be: maintain the pH near to neutrality, limit the concentration of tampons, avoid high temperatures, use for the double layer of molecules with ether connections rather than esters, resorting to freeze-drying.

Physical alterations

The stability of liposomes can also be affected by physical alterations. The vesicles can get closer and join one another to form large multi-liposomal systems (aggregation), merge their membranes and form larger vesicles (fusion) or lose the drug contained inside.

The aggregation is a reversible process, resorting to restlessness or mechanical changes in temperature. The membrane fusion is irreversible.

The loss of the active drug is less for small vesicles with amphiphilic character. The lipid composition is important: for the liposomes with great rigidity, the drug is more easily retained inside, but they are less used because drug delivery in vivo is too slow.

The suspensions of liposomes with lipophilic drugs may break up into two phases, especially if the solubility of the drug in lipid is reduced with the storage at low temperatures.

Physical alteration of liposomal dispersions can be minimized with the use of chelating ions (EDTA), cholesterol and other substances that increase the stiffness,

adding molecules which charge the lipid layer, using lyophilization with criprotector (Guidance for Industry: Liposome drug Products, FDA, 2002).

Lyophilization

The lyophilization or freeze-drying is used to improve the stability of liposomes. The removal of water can prevent the degradation reactions, especially those of hydrolysis. Obtaining a powder and thus reducing the vesicle mobility, reduces the molecular processes of chemical and physical degradation.

Unfortunately, the liposomes can also be damaged by freeze-drying process and this almost always requires the use of crioprotector agents. They are usually chosen among mono or disaccharides, whose mechanism of action has not yet been fully clarified.

Probably they form a coating in the amorphous matrix between the liposomes, preventing their aggregation and fusion. They could also form hydrogen bonds with the heads of ionic phospholipids, expelling water and replacing it. The rehydration always cause some leakage of the active principle and fusion of liposomes, even with the use of crioprotectors. These incidents may in part be reduced by proper choice of crioprotectors, using charged liposomes, slowly cooling the sample (Guidance for Industry: Liposome drug Products, FDA, 2002).

Preparation of sterile formulations

To ensure the sterility of liposomes, they can be prepared in aseptic conditions, but it is a costly and complex procedure. The treatment in autoclave can be used (15 min at 121 ° C), but the high temperature catalyzes the reactions of degradation. The technique is sometimes not usable, for example, when the suspension has a pH value very different from neutrality, phospholipids are partially oxidized, the drug is highly soluble in water (tends to leave the liposomes and go to the vehicle).

Sterilizing filtration with pores of about 200 nm is effective in removing bacteria and not destructive for small liposomes, but it does not allow the removal of viruses and small spores, and does not apply big and little deformable to liposomes.

Gamma radiation cannot be used because it is too destructive (Guidance for Industry: Liposome drug Products, FDA, 2002).

Cyclodextrins in liposomes

The idea of trapping drug-cyclodextrin complex in liposomes can combine the advantages of the use of cyclodextrins with that of the use of liposomes. These systems are called vesicles DCL (drug-in-cyclodextrin-in-liposomes). The advantages of such systems may be different (Cormack et al., 1994; Maestrelli et al., 2005; Cormack et al., 1996):

1) Increase the proportion of drugs not readily soluble in water which can be dispersed in the internal aqueous phase of liposomes.

2) Inclusion in the aqueous compartment of drugs that prevent the formation or stability of the liposome if introduced into the lipophilic phase.

3) When given intravenously, increased plasma half-life time of complex drug-cyclodextrin, for slowing of renal clearence.

4) Reduction of hemolytic and renal toxicity of cyclodextrins.

5) Increased flexibility in the development of liposomal formulations. One may be able to get vesicles where the drug is inserted in the hydrophilic phase and in the lipophilic phase (double loading).

6) Reduction of onset time of the drug and increased duration of action.

7) Increased stability of the drug. It was demonstrated that liposomes containing a multilamellar riboflavin/γCD complex protect the drug from degradation to the light better than cyclodextrin complexation alone (Loukas et al., 1995). Other good results were obtained with drugs easily hydrolysable (Loukas et al., 1998).

8) In some cases you may have even increased stability of the liposome. For example, the complexation with cyclodextrins has not only increased the encapsulation of nifedipine, but also improved the stability of the liposomes in plasma (Skalko et al., 1996).

It has already been successfully developed liposomes containing HPβCD complexs with dexamethasone, prednisolone, retinoic acid, ketoprofen and many other drugs. From the literature data HPβCD can be easily inserted into liposomes, but high doses can have a strong impact on vesicles (Fatouros et al., 2001).

It has been proven that the reduction of stability is dependent on the characteristics of both the liposome (type of phospholipids, preparative technique, size, etc) and the type of cyclodextrin. The MeβCD was found to be the most destabilizing.

Some studies suggested that cyclodextrins are detrimental to the stability not only for emulsifying action, but also for the extraction of various components from the lipid double layer (Hatzi et al., 2007). It has also been showed that liposomes with greater strength, better resist to the destabilizing effect of cyclodextrins. It may therefore be useful to use phospholipids saturated and / or enter cholesterol into the composition of liposomes DCL.

Liposomes with anticancer drugs

It has now been over 35 years since it was discovered that vigorous dispersal of purified phospholipids in water resulted in the formation of microscopic closed membrane spheres (Bangham, 1968).

These artificial membranes, referred to as liposomes, were found to consist of one or more lipid bilayers arranged concentrically around a central aqueous core. Studies on the membrane permeability of small molecules demonstrated that polar and charged molecules could be retained within liposomes, an observation that immediately suggested their potential as systems for the systemic delivery of drugs (Sessa and Weissmann, 1968).

Unfortunately, a significant amount of technological development was required before this potential could be realized.

In addition to a better understanding of the physical properties of membranes and their lipid components, techniques were required for the generation of unilamellar vesicles and encapsulation of drugs and macromolecules within them.

Although a wide variety of methods were developed for the formation of liposomes (Hope et al., 1986; Lichtenberg and Barenholz, 1988), many of them did not generate liposomes of optimal size and polydispersity and often were technically demanding and time consuming.

Furthermore, the drug-loading technology at the time was based on passive entrapment methods, which resulted in low encapsulation levels (<30%) and poor retention of drugs (Mayer et al., 1990a). Nevertheless, early animal studies using liposomal drug carriers were encouraging enough to warrant further development (see Mayer et al., 1990a and references therein).

The development of extrusion technology for the rapid generation of monodisperse populations of unilamellar vesicles (Hope et al., 1985; Mayer et al.,

1986b; Olson et al., 1979) allowed characterization of the physical properties and in vivo characteristics of a wide variety of liposomal systems.

This information revealed that optimized drug delivery systems would possess two key parameters: a small size (on the order of 100 nm) and long circulation lifetimes (half-life >5 h in mice).

The basic structural framework on which most delivery systems are based is the large unilamellar vesicle (LUV) with a diameter close to 100 nm. These systems possess internal volumes large enough to carry adequate quantities of encapsulated material but are small enough to circulate for a time sufficient to reach sites of disease, such as tumors or sites of inflammation. Vesicles that are much larger or smaller are rapidly cleared from the circulation. However, circulation lifetime is determined by factors other than size. Both circulation lifetimes and drug retention are dependent on lipid composition and were found to be greatly enhanced in systems made from phosphatidylcholine (or sphingomyelin) and cholesterol (Mayer et al., 1989, 1993; Webb et al., 1995, 1998a).

Further improvements in circulation longevity were achieved by the inclusion of ganglioside GM1 in the vesicle formulation (Boman et al., 1994; Gabizon and Papahadjopoulos, 1988; Woodle et al., 1994) or by grafting water-soluble polymers such as poly (ethylene glycol) (PEG) onto the vesicle surface, thereby generating vesicles known as ‘‘stealth’’ liposomes (Allen, 1994, 1998; Allen et al., 1991; Woodle et al., 1994).

A major advance in the design of the first generation of drug transport systems came with the development of methods for achieving the encapsulation and retention of large quantities of drug within liposomal systems.

Perhaps the most important insight in this area was the recognition that many chemotherapeutic drugs could be accumulated within vesicles in response to transmembrane pH gradients (Cullis et al., 1997; Madden et al., 1990; Mayer et al., 1986a).

The ability of ΔpH to influence transmembrane distributions of certain weak acids and bases has been recognized (see Cullis et al., 1997 and references therein). The fact that many chemotherapeutics were weak bases led to investigate the transport of these substances into liposomes in response to membrane potentials and ΔpH. Subsequent studies led to considerably broader applications involving the transport and

accumulation of a wide variety of drugs, biogenic amines, amino acids, peptides, lipids, and ions in LUVs exhibiting a pH (for a review, see Cullis et al., 1997).

Application of this technology led to the development of several liposomal anticancer systems that exhibit improved therapeutic properties over free drug. Early studies (see Mayer et al., 1990a and references therein) had shown that reduced side effects with equal or enhanced efficacy could be obtained in liposomal systems, despite low encapsulation levels and poor drug retention.

This led to initial efforts to develop a liposomal version of doxorubicin, the most commonly employed chemotherapeutic agent, which is active against a variety of ascitic and solid tumors, but yet exhibits a variety of toxic side effects.

The pH gradient approach (Mayer et al., 1989, 1990a–c, 1993) was expected to provide significant improvements in overall efficacy due to high drug-to-lipid ratios and excellent retention observed both in vitro and in vivo.

This has been realized in liposomal doxorubicin preparations that are currently either in advanced clinical trials (Cheung et al., 1999; Chonn and Cullis, 1995) or have been approved by the U.S. FDA for clinical use (Muggia, 2001).

Other liposomal doxorubicin formulations (Burstein et al., 1999; Campos et al., 2001; Coukell and Spencer, 1997; Gokhale et al., 1996; Gordon et al., 2000; Grunaug et al., 1998; Israel et al., 2000; Judson et al., 2001; Northfelt et al., 1998; Shields et al., 2001) are in various Phase I or II clinical trials, often with promising results.

A variety of other liposomal drugs are currently in preclinical or clinical development; these include vincristine (Gelmon et al., 1999; Millar et al., 1998; Tokudome et al., 1996; Webb et al., 1995, 1998a), mitoxantrone (Adlakha-Hutcheon et al., 1999; Chang et al., 1997; Lim et al., 1997, 2000; Madden et al., 1990), daunorubicin (Gill et al., 1996; Madden et al., 1990; Muggia, 2001; Pratt et al., 1998), ciprofloxacin (Bakker-Woudenberg et al., 2001; Webb et al., 1998b), topotecan (Tardi et al., 2000), and vinorelbine, to name a few.

Of these, the group of Prof. Szoka has been prominent in devising methods for the encapsulation of doxorubicin, vincristine, and ciprofloxacin (Szoka, 2004).

Liposomal delivery systems are finally reaching a stage of development where significant advances can reasonably be expected in short terms. The first of the conventional drug carriers are reaching the market while new liposomal drugs are being developed and entered into clinical trials.

These advances stem from the fact that the design features required of drug delivery systems that have systemic utility are becoming better defined.

Based on the studies indicated above, it is now known that liposomal systems that are small (diameter 100 nm) and that exhibit long circulation lifetimes (half-life 5 h in mice) following intravenous (iv) injection exhibit a remarkable property termed ‘‘disease site targeting’’ or ‘‘passive targeting’’ that results in large improvements in the amounts of drug arriving at the disease site.

For example, liposomal vincristine formulations can deliver 50- to 100-fold higher amounts of drug to a tumor site with respect to the free drug (Boman et al., 1994; Mayer et al., 1993; Webb et al., 1995, 1998a).

This can result in large increases in efficacy (Boman et al., 1994). These improvements stem from the increased permeability of the vasculature at tumor sites (Brown and Giaccia, 1998; Dvorak et al., 1988) or sites of inflammation, which results in preferential extravasation of small, long-circulating carriers in these regions.

The insights gleaned from conventional drug carriers have implications for the design of liposomal systems for the delivery of larger macromolecules.

There is currently much interest in developing systemic vectors for the delivery of the therapeutic genetic drugs such as antisense oligonucleotides or plasmid DNA.

To obtain appreciable amounts of a vector containing the antisense oligonucleotides or therapeutic gene to the site of disease, the vector must be stable, small, and long-circulating.

Of course, the vector must also be accumulated by target cells, escape the endocytotic pathway, and be delivered to the nucleus.

Over the past 20 years, the laboratory of Prof.Szoka has played a major role in the development of liposomal systems optimized for the delivery of both conventional drugs and, more recently, genetic drugs (Szoka, 2003).

Early studies on the production of LUVs by extrusion led to the characterization of several liposomal drug delivery systems (Bally et al., 1988; Boman et al., 1993, 1994; Chonn and Cullis, 1995; Cullis et al., 1997; Fenske et al., 1998; Hope and Wong, 1995; Madden et al., 1990; Maurer-Spurej et al., 1999; Mayer et al., 1986a), the development of new approaches for the loading of drugs via generation of ΔpH (Fenske et al., 1998; Maurer-Spurej et al., 1999) or other ion gradients (Cheung et al., 1998), and finally new methods for the encapsulation of antisense oligonucleotides (Maurer et al.,

2001; Semple et al., 2000, 2001) and plasmid DNA (Fenske et al., 2002; Maurer et al., 2001; Mok et al., 1999; Tam et al., 2000; Wheeler et al., 1999) within liposomes.

Encapsulation of Small, Weakly Basic Drugs within LUVs in Response to Transmembrane pH and Ion Gradients

The Formation of LUVs by Extrusion Methods

Many research questions in membrane science, specifically those involving the dynamic properties of lipid bilayers, can be addressed using very basic model membrane systems, such as the multilamellar vesicle (MLV) formed spontaneously upon vigorous agitation of lipid–water mixtures.

These large (1–10 μm) multilamellar liposomes are ideal for biophysical investigations of lipid dynamics and order using techniques such as fluorescence, electron spin resonance (ESR), or broadband (2H and 31P) nuclear magnetic resonance (NMR). However, many properties of biological membranes, such as the presence of pH or ion gradients, cannot be adequately modeled using large, multilamellar systems.

These kinds of studies require the use of unilamellar vesicles in the nanometer size range.

Investigations relating ion and pH gradients to lipid asymmetry (Cullis et al., 1997, 2000) were the driving force for the development of extrusion technology.

While it was clear that MLVs were not appropriate for such topics, it was also apparent that the methods available for the generation of unilamellar vesicles, which included dispersion of lipids from organic solvents (Batzri and Korn, 1973), sonication (Huang, 1969), detergent dialysis (Mimms et al., 1981), and reverse-phase evaporation (Szoka and Papahadjopoulos, 1978), had serious drawbacks (Cullis, 2000).

However, Papahadjopolous, Szoka, and co-workers (Olson et al., 1979) and had observed that sequential extrusion of MLVs through a series of filters of reducing pore size under low pressure gave rise to LUV systems. Further development of this method led to an approach involving direct extrusion of MLVs, at relatively high pressures (200–400 psi), through polycarbonate filters with a pore size ranging from 30 to 400 nm.

This allowed generation of narrow, monodisperse vesicle populations with a narrow size distribution and diameters close to the chosen pore size (Fig. 1) (Hope et al., 1985; Mayer et al., 1986).

The method is rapid and simple and can be performed for a wide variety of lipid compositions and temperatures. As it is necessary to extrude the lipid emulsions at temperatures 5–10°C above the gel-to-liquid crystalline phase transition temperature, the system is manufactured so that it may be attached to a variable-temperature circulating water bath.

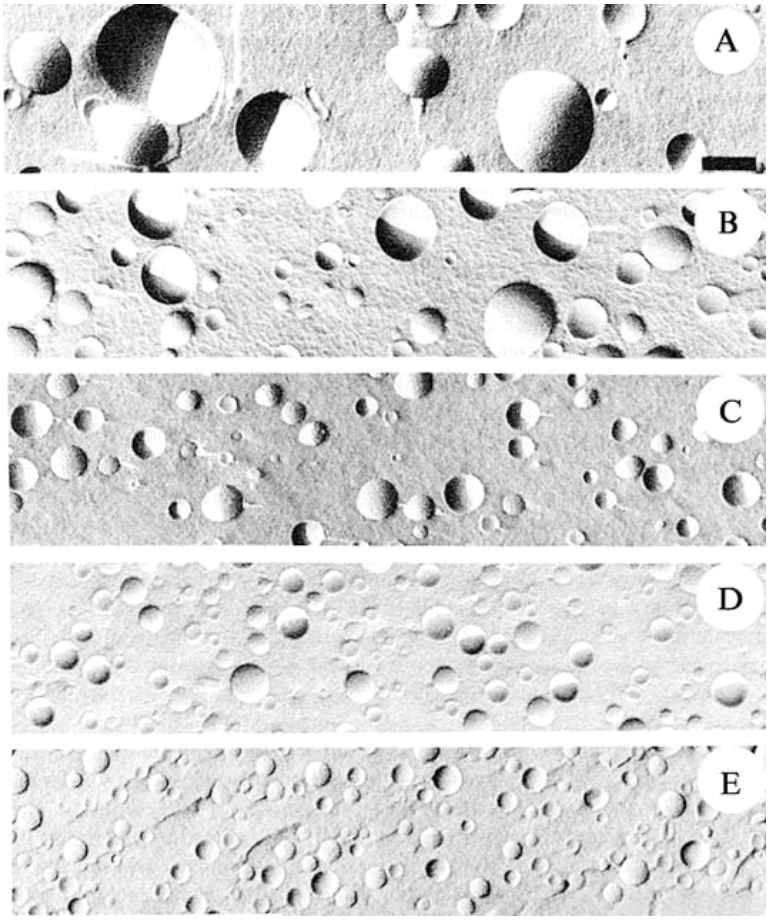
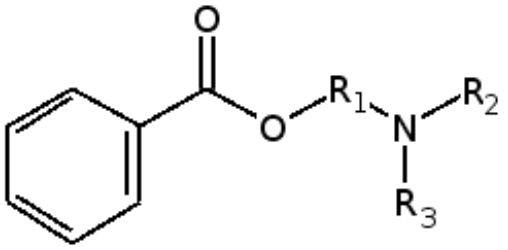


Fig. 1. Freeze-fracture electron micrographs of egg phosphatidylcholine LUVs prepared by extrusion through polycarbonate filters with pore sizes of (A) 400 nm, (B) 200 nm, (C) 100 nm, (D) 50 nm, and (E) 30 nm. The bar in (A) represents 150 nm. [Reprinted from Hope, M. J., Bally, M. B., Mayer, L. D., Janoff, A. S., and Cullis, P. R. (1986). Chem. Phys. Lipids 40, 89–107, with permission.]

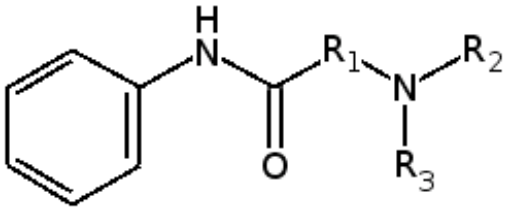
2. LOCAL ANESTHETICS: BENZOCAINE, BUTAMBEN, PRILOCAINE.

A local anesthetic is a drug that causes reversible local anesthesia and a loss of nociception.

When it is used on specific nerve pathways (nerve block), effects such as analgesia (loss of pain sensation) and paralysis (loss of muscle power) can be achieved.



Aminoester



Aminoamide

Clinical local anesthetics belong to one of two classes: aminoamide and aminoester local anesthetics.

Synthetic local anesthetics are structurally related to cocaine. They differ from cocaine mainly in that they have no abuse potential and do not act on the sympathoadrenergic system, i.e. they do not produce hypertension or local vasoconstriction, with the exception of Ropivacaine and Mepivacaine that produce weak vasoconstriction.

Local anesthetics vary in their pharmacological properties and they are used in various techniques of local anesthesia such as:

- Topical anesthesia (surface)
- Infiltration
- Plexus block
- Epidural (extradural) block

- Spinal anesthesia (subarachnoid block)

The local anesthetic lidocaine (lignocaine) is also used as a Class Ib antiarhythmic drug.

Mechanism of action

All local anesthetics are membrane stabilizing drugs; they reversibly decrease the rate of depolarization and repolarization of excitable membranes (like nociceptors). Though many other drugs also have membrane stabilizing properties, all are not used as local anesthetics, for example propranolol.

Local anesthetic drugs act mainly by inhibiting sodium influx through sodium-specific ion channels in the neuronal cell membrane, in particular the so-called voltage-gated sodium channels. When the influx of sodium is interrupted, an action potential cannot arise and signal conduction is inhibited.

The receptor site is thought to be located at the cytoplasmic (inner) portion of the sodium channel. Local anesthetic drugs bind more readily to sodium channels in inactivated state, thus onset of neuronal blockade is faster in neurons that are rapidly firing.

This is referred to as state dependent blockade. Local anesthetics are weak bases and are usually formulated as the hydrochloride salt to render them water-soluble. At the chemical's pKa the protonated (ionised) and unprotonated (unionised) forms of the molecule exist in an equilibrium but only the unprotonated molecule diffuses readily across cell membranes.

Once inside the cell the local anesthetic will be in equilibrium, with the formation of the protonated (ionised form), which does not readily pass back out of the cell. This is referred to as "ion-trapping". In the protonated form, the molecule binds to the local anaesthetic binding site on the inside of the ion channel near the cytoplasmic end. Acidosis such as caused by inflammation at a wound partly reduces the action of local anesthetics.

This is partly because most of the anaesthetic is ionised and therefore unable to cross the cell membrane to reach its cytoplasmic-facing site of action on the sodium channel. All nerve fibres are sensitive to local anesthetics, but generally, those with a smaller diameter tend to be more sensitive than larger fibres.

Local anesthetics block conduction in the following order: small myelinated axons (e.g. those carrying nociceptive impulses), non-myelinated axons, then large myelinated axons. Thus, a differential block can be achieved (i.e. pain sensation is blocked more readily than other sensory modalities).

Undesired Effects

Localized Adverse Effects

The local adverse effects of anesthetic agents include neurovascular manifestations such as prolonged anesthesia (numbness) and paresthesia (tingling, feeling of "pins and needles", or strange sensations). These are symptoms of localized nerve impairment or nerve damage.

Causes

Causes of localized symptoms include:

1. neurotoxicity due to allergenic reaction,
2. excessive fluid pressure in a confined space,
3. severing of nerve fibers or support tissue with the syringe/catheter,
4. injection-site hematoma that puts pressure on the nerve,
5. injection-site infection that produces inflammatory pressure on the nerve and/or necrosis.

General Adverse Effects

General systemic adverse affects are due to the pharmacological effects of the anesthetic agents used.

The conduction of electric impulses follows a similar mechanism in peripheral nerves, the central nervous system, and the heart. The effects of local anesthetics are therefore not specific for the signal conduction in peripheral nerves. Side effects on the central nervous system and the heart may be severe and potentially fatal.

However, toxicity usually occurs only at plasma levels which are rarely reached if proper anesthetic techniques are adhered to.

Additionally, persons may exhibit allergenic reactions to the anesthetic compounds and may also exhibit cyanosis due to methemoglobinemia.

Central nervous system

Depending on local tissue concentrations of local anesthetics, there may be excitatory or depressant effects on the central nervous system. At lower concentrations, a relatively selective depression of inhibitory neurons results in cerebral excitation, which may lead to generalized convulsions.

A profound depression of brain functions occurs at higher concentrations which may lead to coma, respiratory arrest and death.

Such tissue concentrations may be due to very high plasma levels after intravenous injection of a large dose. Another possibility is direct exposure of the central nervous system through the CSF, i.e. overdose in spinal anesthesia or accidental injection into the subarachnoid space in epidural anesthesia.

Cardiovascular system

The conductive system of the heart is quite sensitive to the action of local anesthetics.

Lidocaine is often used as an antiarrhythmic drug and has been studied extensively, but the effects of other local anesthetics are probably similar to those of Lidocaine. Lidocaine acts by blocking sodium channels, leading to slowed conduction of impulses.

This may obviously result in bradycardia, but tachyarrhythmia can also occur. With high plasma levels of lidocaine there may be higher-degree atrioventricular block and severe bradycardia, leading to coma and possibly death.

Hypersensitivity/Allergy

Adverse reactions to local anesthetics (especially the esters) are not uncommon, but true allergy is very rare. Allergic reactions to the esters is usually due to a sensitivity to their metabolite, para-aminobenzoic acid (PABA), and does not result in cross-allergy to amides.

Therefore, amides can be used as alternatives in those patients. Non-allergic reactions may resemble allergy in their manifestations.

In some cases, skin tests and provocative challenge may be necessary to establish a diagnosis of allergy. There are also cases of allergy to paraben derivatives, which are often added as preservatives to local anesthetic solutions.

Methemoglobinemia

The systemic toxicity of prilocaine is comparatively low, however its metabolite, o-toluidine, is known to cause methemoglobinemia. As methemoglobinemia reduces the amount of hemoglobin that is available for oxygen transport, this side effect is potentially life-threatening.

Therefore dose limits for prilocaine should be strictly observed. Prilocaine is not recommended for use in infants.

Local anesthetics in clinical use

Esters are prone to producing allergic reactions, which may necessitate the use of Amides.

The names of Amidic drugs contain an "i" somewhere before the ending-aine. Most ester local anesthetics are metabolized by pseudocholinesterases, while amidic local anesthetics are metabolized in the liver.

This can be a factor in choosing an agent in patients with liver failure (Stern, 2002).

Esters

- Benzocaine
- Butamben
- Chloroprocaine
- Cocaine
- Cyclomethycaine
- Dimethocaine/Larocaine
- Propoxycaine
- Procaine/Novocaine

- Proparacaine
- Tetracaine/Amethocaine

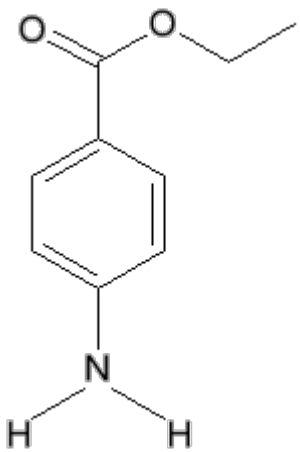
Amides

- Articaine
- Bupivacaine
- Carticaine
- Cinchocaine/Dibucaine
- Etidocaine
- Levobupivacaine
- Lidocaine/Lignocaine
- Mepivacaine
- Piperocaine
- Prilocaine
- Ropivacaine
- Trimecaine

Combinations

- Lidocaine/prilocaine (EMLA)

2.1 BENZOCAINE



Benzocaine is a local anesthetic commonly used as a topical pain reliever. It is the active ingredient in many over-the-counter anesthetic ointments. Benzocaine is an ester, and can be prepared from the organic acid PABA (para-aminobenzoic acid) and ethanol by Fischer esterification.

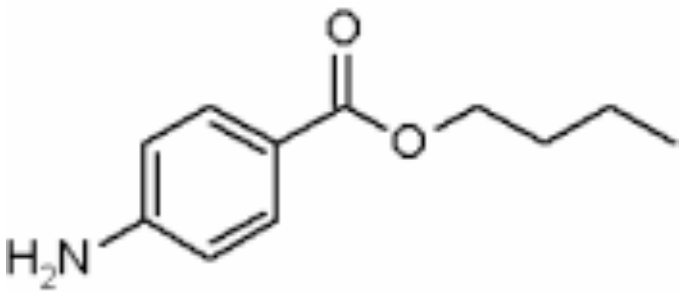
The melting point of benzocaine is 88-90 degrees Celsius, and the boiling point is 310 degrees Celsius. The density of benzocaine is 1.17 g/cm^3. Pain is caused by the stimulation of free nerve endings.

When the nerve endings are stimulated, sodium enters the neuron, which causes an electrical potential to build up in the nerve. Once the electrical potential becomes big enough the signal is propagated down the nerve toward the central nervous system, which interprets this as pain.

Esters of PABA work as a chemical barrier, stopping the sodium from being able to enter the nerve ending. Allergic reactions occur with ester local anaesthetics (like benzocaine) because of the PABA structure.

Benzocaine also is a well-known cause of methemoglobinemia. Since it may be used in topical creams with a concentration as much as 20%, it is not difficult to administer a dose sufficient to cause this problem.

2.2 BUTAMBEN



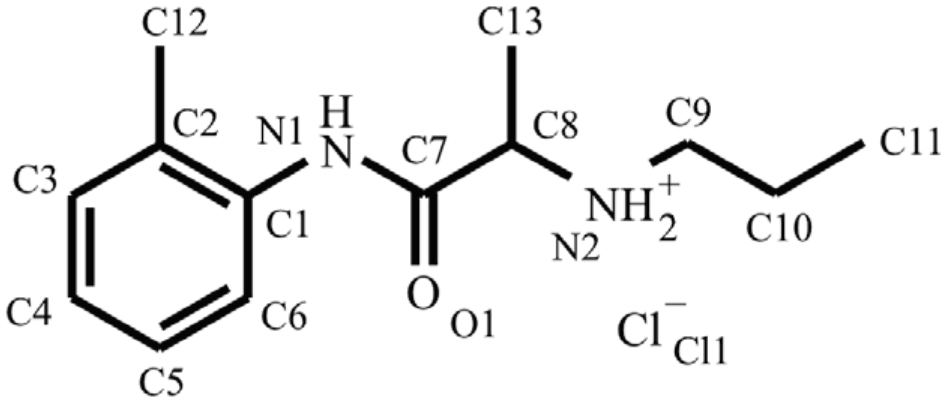
Butamben (butylamino-benzoate), is a local anesthetic of very limited water solubility (approximately 140 mg/L at room temperature).

Butamben is an ester, and can be prepared from the organic acid PABA (para-aminobenzoic acid) and ethanol by Fischer esterification. The melting point of butamben is 57-59 degrees Celsius.

Esters of PABA work as a chemical barrier, stopping the sodium from being able to enter the nerve ending. Allergic reactions occur with ester local anaesthetics (like benzocaine) because of the PABA structure.

2.3 PRILOCAINE

Prilocaine hydrochloride (PRLHCl) is a local anaesthetic drug of the amide type. The compound is official in the United States Pharmacopoeia (USP) and the Pharmacopoeia Europaea and is therapeutically used for intravenous regional anaesthesia and in dentistry where it shows a medium duration of action compared to other local anaesthetic drug compounds (Saia Cereda et al., 2004).



Molecular structure of PRLHCl with atom numbers

In none of the previous analytical studies dealing with the solid-state properties of **PRLHCl** the existence of different solid-state forms has been mentioned.

Moreover, in the Cambridge Structural Database the crystal structures of all frequently used LA of the amide type can be found, such as Lidocaine , Lidocaine hydrochloride monohydrate, Dibucaine hydrochloride monohydrate, Mepivacaine hydrochloride, Bupivacaine hydrochloride, Bupivacaine hydrochloride ethanol solvate, Ropivacaine hydrochloride monohydrate, Phenacaine hydrochloride monohydrate (an amidine) but not that of **PRLHCl**.

The hydrochloride salts of these compounds mostly crystallize in the space group *P*21 and the organization or molecular packing in the crystals show pronounced

similarities. Moreover, the protonated drug molecules in all cases (amide type and ester type LA) are linked by hydrogen bonds via the chloride anions forming parallel orientated, infinitive hydrogen-bonded chains.

The multiple occurrence of solvated forms within the group of the amide type LA is likely due to the ability to form intermolecular hydrogen bonds with the N1 amine group.

The alkyl side chain of the **PRLHCl** molecule may adopt different conformations. Such flexible aliphatic side chains are relevant for the formation of different (conformational) polymorphs and this is a common structural feature of local anaesthetics.

3. NON-STEROIDAL ANTI-INFLAMMATORY DRUGS: OXAPROZIN

Non-steroidal anti-inflammatory drugs, usually abbreviated as NSAIDs or NAIDs , are drugs with analgesic, antipyretic and, in higher doses, anti-inflammatory effects - they reduce pain, fever and inflammation.

The term "non-steroidal" is used to distinguish these drugs from steroids, which (among a broad range of other effects) have a similar eicosanoid-depressing, anti-inflammatory action. As analgesics, NSAIDs are unusual in that they are non-narcotic drugs. NSAIDs are sometimes also referred to as non-steroidal anti-inflammatory agents/analgesics (NSAIAs) or non-steroidal anti-inflammatory medicines (NSAIMs). The most prominent members of this group of drugs are aspirin, ibuprofen, and naproxen. Paracetamol is not an NSAID because, although it is an antipyretic and analgesic, it is not an anti-inflammatory drugs. Beginning in 1829, with the isolation of salicin from the folk remedy white willow bark, NSAIDs are an important part of the pharmaceutical treatment of pain (at low doses) and inflammation (at higher doses).

Part of the popularity of NSAIDs is that, unlike opioids, they do not produce sedation or respiratory depression and have a very low addiction rate. NSAIDs, however, are not without their own problems.

Mode of action

Most NSAIDs act as non-selective inhibitors of the enzyme cyclooxygenase, inhibiting both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoenzymes.

Cyclooxygenase catalyzes the formation of prostaglandins and thromboxane from arachidonic acid (itself derived from the cellular phospholipid bilayer by phospholipase A_2).

Prostaglandins act (among other things) as messenger molecules in the process of inflammation. A newly discovered COX-3 may also have some role.

Uses

NSAIDs are usually indicated for the treatment of acute or chronic conditions where pain and inflammation are present.

Recently research evidenced their potential for prevention of colorectal cancer, and treatment of other conditions, such as cancer and cardiovascular diseases. NSAIDs are generally indicated for the symptomatic relief of the following conditions:

- Rheumatoid arthritis
- Osteoarthritis
- Inflammatory arthropathies (e.g. ankylosing spondylitis, psoriatic arthritis, Reiter's syndrome)
- Acute gout
- Dysmenorrhoea (menstrual pain)
- Metastatic bone pain
- Headache and migraine
- Postoperative pain
- Mild-to-moderate pain due to inflammation and tissue injury
- Pyrexia (fever)
- Ileus
- Renal colic
- They are also given to neonate infants whose ductus arteriosus is not closed within 24 hours of birth .

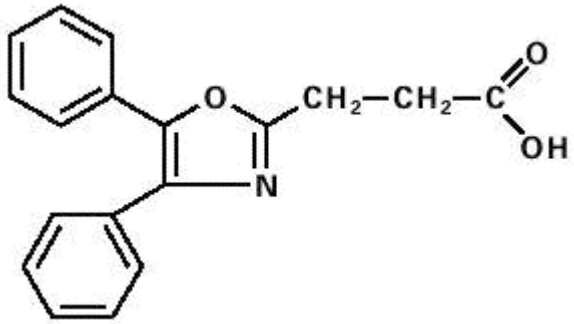
Aspirin, the only NSAID able to irreversibly inhibit COX-1, is also indicated for inhibition of platelet aggregation.

This is useful in the management of arterial thrombosis and prevention of adverse cardiovascular events. Aspirin inhibits platelet aggregation by inhibiting the action of thromboxane -A. In 2001 NSAIDs accounted for 70,000,000 prescriptions and 30 billion over-the-counter doses sold annually in the United States (Green, 2001).

With the aging of the Baby Boomer generation and the associated rise in the incidence of osteoarthritis and other such conditions for which NSAIDs are indicated, the use of NSAIDs may increase further still.

One study has suggested that taking NSAIDs (or COX inhibitors in general) while smoking marijuana may prevent the death of brain cells resulting from THC intoxication. However, neurotoxicity of marijuana is still a matter of dispute.

3.1 OXAPROZIN



Oxaprozin (3-(4,5-diphenyl-1,3-oxazol-2-yl) propionic acid) is a Non-Steroidal Anti-Inflammatory drug, mainly used for the treatment of pain, pyrexia and various inflammatory disorders and joint pain associated with osteoarthritis and rheumatoid arthritis.

Oxaprozin has become one of the leading NSAIDS on the US market, however its low aqueous solubility and poor stability may reduce its therapeutic effectiveness and enhance the appearance of some adverse events such as gastro-duodenal mucosal injury. Normal adult dosage is 1200 mg daily, not to exceed 1800 mg per day.

4. ORAL ANTI-HYPERGLYCAEMIC DRUGS: METFORMIN.

Anti-diabetic drugs treat diabetes mellitus by lowering glucose levels in the blood. With the exceptions of insulin, exenatide, and pramlintide, all are administered orally and are thus also called oral hypoglycemic agents or oral antihyperglycemic agents.

There are different classes of anti-diabetic drugs, and their selection depends on the nature of the diabetes, age and situation of the person, as well as other factors.

Diabetes mellitus type 1 is a disease caused by the lack of insulin. For the treatment of type 1 diabetes, insulin must be used, which must be injected or inhaled.

Diabetes mellitus type 2 is a disease of insulin resistance by cells. Treatments include (1) agents which increase the amount of insulin secreted by the pancreas, (2) agents which increase the sensitivity of target organs to insulin, and (3) agents which decrease the rate at which glucose is absorbed from the gastrointestinal tract.

Several groups of drugs, mostly given by mouth, are effective in Type II diabetes, often used in combination.

The therapeutic drug combination may include insulin, not necessarily because oral agents have failed completely, but in search of a desired combination of effects. The great advantage of injected insulin in Type II diabetes is that a well-educated patient can adjust the dose, or even take additional doses, when requested by blood glucose levels measured by the patient himself.

Insulin

Insulin is usually given subcutaneously, either by injections or by an insulin pump. Research is underway for other routes of administration. In acute care settings, insulin may also be given intravenously. There are several types of insulin, characterized by the rate by which they are metabolized by the body.

Secretagogues

Sulfonylureas

Sulfonylureas were the first widely used oral hypoglycaemic medications. They are *insulin secretagogues*, triggering insulin release by direct action on the K_{ATP} channel of the pancreatic beta cells. Eight types of these pills have been marketed in North America, but not all remain available.

The "second-generation" drugs are now more commonly used. They are more effective than first-generation drugs and have fewer side effects. All may cause weight gain. Sulfonylureas bind strongly to plasma proteins.

Sulfonylureas are only useful in Type II diabetes, as they work by stimulating endogenous release of insulin. They work best with patients over 40 years old, who have had diabetes mellitus for less than ten years.

They can not be used with Type I diabetes, or pregnancy diabetes. They can be safely used in association with metformin or glitazones. The primary side effect is hypoglycemia.

- First-generation agents
  - tolbutamide
  - acetohexamide
  - tolazamide
  - chlorpropamide

- Second-generation agents
  - glipizide
  - glyburid
  - glimepiride
  - gliclazide

Meglitinides

Meglitinides help the pancreas to produce insulin and are often called "short-acting secretagogues." Their mode of action is original, affecting potassium channels (Rendell, 2004).

By closing the potassium channels of the pancreatic beta cells, they open the calcium channels, hence enhancing insulin secretion (Healthvalue, 2007).

They are taken with meals to boost the insulin response to each meal.

- repaglinide- The maximum dosage is 16 mg/day, taken 0 to 30 minutes before meals. If a meal is skipped, the medication is also skipped.
- nateglinide- The maximum dosage is 360 mg/day, usually 120 mg three times a day (TID). It also follows the same recommendations as repaglinide.

Adverse reactions include weight gain and hypoglycemia.

Sensitizers

Biguanides

Biguanides reduce hepatic glucose output and increase uptake of glucose by the periphery, including skeletal muscle.

Although it must be used with caution in patients with impaired liver or kidney function, metformin has become the most commonly used agent for type 2 diabetes in children and teenagers.

Among the most common anti-diabetic drugs, metformin, a biguanide, is the only widely used oral drug that does not cause weight gain.

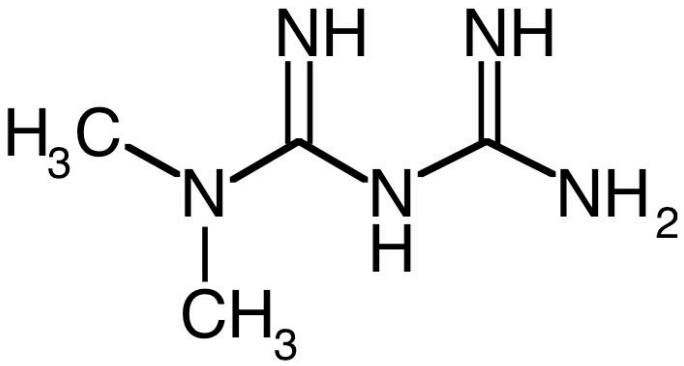
- Metformin may be the best choice for patients who also have heart failure (Eurich et al., 2007).
- phenformin used from 1960s through 1980s, withdrawn due to lactic acidosis risk.
- Buformin also withdrawn due to lactic acidosis risk.

Metformin should be temporarily discontinued before any radiographic procedure involving intravenous iodinated contrast as patients are subjected to an increased risk of lactic acidosis.

Metformin is usually the first-line medication used for treatment of Type-2 diabetes. Initial dosing is 500 mg twice daily, but it can be increased up to 1000 mg twice daily.

It is also available in combination with other oral anti-diabetic medications.

4.1 METFORMIN



Metformin is an oral anti-diabetic drug bolonging to the biguanide class. It is the first-line drug for the treatment of Type 2 diabetes, particularly in overweight and obese people and those with normal kidney function, and evidence suggests it may be the best choice for people with heart failure.

Metformin is the most popular anti-diabetic drug in the United States and one of the most prescribed drugs in the country overall, with nearly 35 million prescriptions filled in 2006 for generic metformin alone.

It is also used in the treatment of polycystic ovary syndrome. When prescribed appropriately, metformin causes few adverse effects—the most common is gastrointestinal upset—and, unlike many other anti-diabetic drugs, does not cause hypoglycemia if used alone. It also helps to reduce LDL cholesterol and triglyceride levels, and may aid weight loss.

As for 2008, metformin is one of only two oral anti-diabetics in the World Health Organization Model List of Essential Medicines (the other being glibenclamide).

Indications

The main use for metformin is in the treatment of diabetes mellitus Type 2, especially when this accompanies obesity and insulin resistance. Metformin is the only anti-diabetic drug that has been proven to protect against the cardiovascular complications of diabetes.

This was first shown in the United Kingdom Prospective Diabetes Study, a large study of overweight patients with diabetes.

Unlike the other most-commonly prescribed class of oral anti-diabetic drugs, the sulfonylureas, metformin (taken alone) does not induce hypoglycemia. Hypoglycemia during intense exercise has been documented, but it is extremely rare.

It also does not cause weight gain, and may indeed produce minor weight loss. Metformin also modestly reduces LDL and triglyceride levels.

It is also being used increasingly in polycystic ovary syndrome (PCOS), non-alcoholic fatty liver disease (NAFLD) and premature puberty, three other diseases that feature insulin resistance; these indications are still considered experimental.

Although metformin is not licensed for use in PCOS, the United Kingdom's National Institute for Health and Clinical Excellence recommends that women with PCOS and a body mass index above 25 be given metformin when other therapies have failed to produce results.

The benefit of metformin in NAFLD has not been extensively studied and may be only temporary. It may reduce weight gain in patients taking atypical antipsychotics.

Mechanism of action

Metformin improves hyperglycemia primarily through suppression of hepatic glucose production (hepatic gluconeogenesis).

The "average" person with Type 2 diabetes has three times the normal rate of gluconeogenesis; metformin treatment reduces this by over one third. Metformin activates AMP-activated protein kinase (AMPK), a liver enzyme that plays an important role in insulin signaling, whole body energy balance, and the metabolism of glucose and fats; activation of AMPK is required for metformin's inhibitory effect on the production of glucose by liver cells.

Research published in 2008 further elucidated metformin's mechanism of action, showing that activation of AMPK is required for an increase in the expression of SHP, which in turn inhibits the expression of the hepatic gluconeogenic genes PEPCK and Glc-6-Pase.

Metformin is frequently used in research along with AICAR as an AMPK agonist. The mechanism by which biguanides increase the activity of AMPK remains

uncertain; however, research suggests that metformin increases the amount of cytosolic AMP (as opposed to a change in total AMP or total AMP/ATP).

In addition to suppressing hepatic glucose production, metformin increases insulin sensitivity, enhances peripheral glucose uptake, decreases fatty acid oxidation, and decreases absorption of glucose from the gastrointestinal tract.

Increased peripheral utilization of glucose may be due to improved insulin binding to insulin receptors. AMPK probably also plays a role, as metformin administration increases AMPK activity in skeletal muscle.

AMPK is known to cause GLUT4 translocation, resulting in insulin-independent glucose uptake.

Some metabolic actions of metformin appear to occur by AMPK-independent mechanisms; a recent study found that "the metabolic actions of metformin in the heart muscle can occur independent of changes in AMPK activity and may be mediated by p38 MAPK- and PKC-dependent mechanisms".

Metformin is not metabolized, but it is primarily excreted in the urine with an elimination half-life of 6.2 hours.

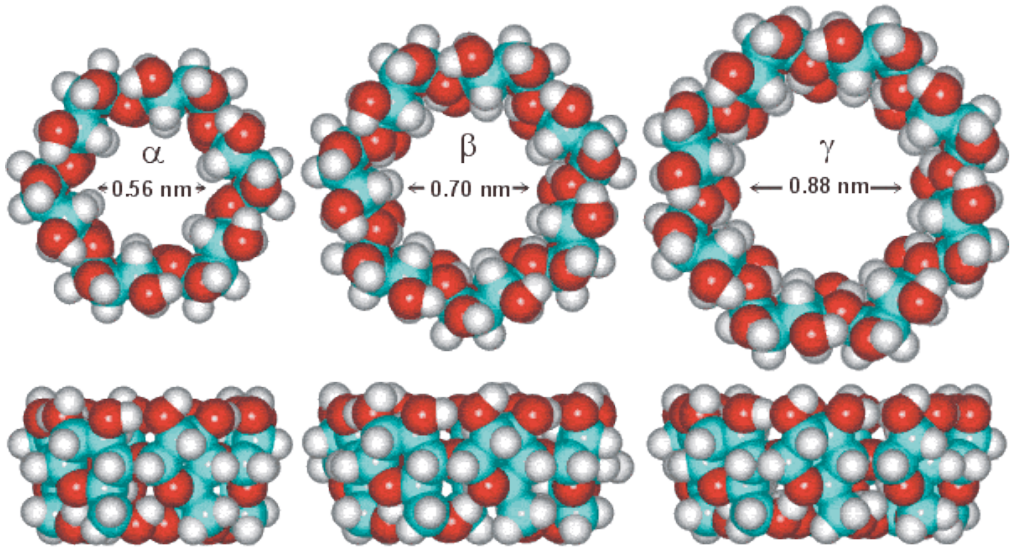
5. CYCLODEXTRINS

Cyclodextrins (sometimes called cycloamyloses) are a family of cyclic oligosaccharides, composed of 5 or more α-D-glucopyranoside units linked 1->4, as in amylose (a fragment of starch).

The 5-membered macrocycle is not natural. Recently, the largest well-characterized cyclodextrin contains 32 1,4-anhydroglucopyranoside units, while as a poorly characterized mixture, even at least 150-membered cyclic oligosaccharides are also known.

The most common cyclodextrins contain a number of glucose monomers ranging from six to eight units in a ring, creating a cone shape, thus denoting:

- α-cyclodextrin: six membered sugar ring molecule
- β-cyclodextrin: seven sugar ring molecule
- γ-cyclodextrin: eight sugar ring molecule



Cyclodextrins are produced from starch by means of enzymatic reaction.

Over the last few years they have found a wide range of applications in food, pharmaceutical and chemical industries as well as agriculture and environmental engineering.

Cyclodextrins, as they are known today, were called "cellulosine" when first described by A. Villiers in 1891. Soon after, F. Schardinger identified the three naturally occurring cyclodextrins -α, -β, and -γ. These compounds were therefore referred to as "Schardinger sugars".

For 25 years, between 1911 and 1935, Pringsheim in Germany was the leading researcher in this area, demonstrating that cyclodextrins formed stable aqueous complexes with many other chemicals.

By the mid 1970's, each of the natural cyclodextrins had been structurally and chemically characterized and many more complexes had been studied. Since the 1970s, extensive work has been conducted by Szejtli and others exploring encapsulation by cyclodextrins and their derivatives for industrial and pharmacologic applications.

Structure

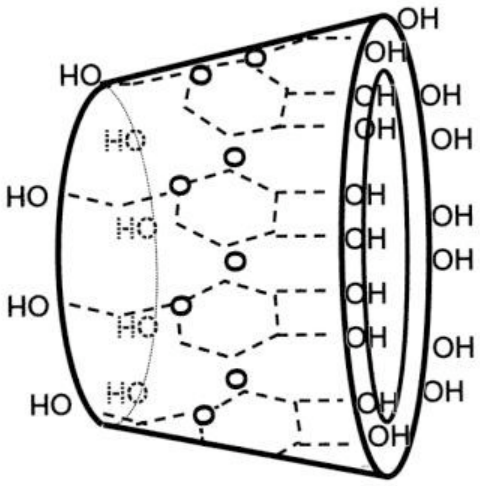
Typical cyclodextrins are constituted by 6-8 glucopyranoside units, and can be topologically represented as toroids with the larger and the smaller openings of the toroid exposing to the solvent secondary and primary hydroxyl groups respectively. Because of this arrangement, the interior of the toroids is not hydrophobic, but considerably less hydrophilic than the aqueous environment and thus able to host hydrophobic molecules.

In contrast, the exterior is sufficiently hydrophilic to impart cyclodextrins (or their complexes) water solubility.

The formation of the inclusion compounds greatly modifies the physical and chemical properties of the guest molecule, mostly in terms of water solubility.

This is the reason why cyclodextrins have attracted much interest in many fields, especially for pharmaceutical applications: since inclusion compounds of cyclodextrins with hydrophobic molecules are able to penetrate body tissues, they can be used to release biologically active compounds under specific conditions.

In most cases the mechanism of controlled dissociation of such complexes is based on pH change of water solutions, leading to the cleavage of hydrogen or ionic bonds between the host and the guest molecules.



Synthesis

The production of cyclodextrins is relatively simple and involves treatment of ordinary starch with a set of easily available enzymes.

Commonly cyclodextrin glycosyltransferase (CGTase) is employed along with α-amylase. First starch is liquified either by heat treatment or using α-amylase, then CGTase is added for the enzymatic conversion.

CGTases can synthesize all forms of cyclodextrins, thus the product of the conversion results in a mixture of the three main types of cyclic molecules, in ratios that are strictly dependent on the enzyme used: each CGTase has its own characteristic α:β:γ synthesis ratio.

Purification of the three types of cyclodextrins takes advantage of the different water solubility of the molecules: β-CD which is very poorly water soluble (18.5 g/l) (at 25°C) can be easily retrieved through crystallization while the more soluble α- and γ-CDs (145 and 232 g/l respectively) are usually purified by means of expensive and time consuming chromatography techniques.

As an alternative, a "complexing agent" can be added during the enzymatic conversion step: such agents (usually organic solvents like toluene, acetone or ethanol) form a complex with the desired cyclodextrin which subsequently precipitates.

The complex formation drives the conversion of starch towards the synthesis of the precipitated cyclodextrin, thus enriching its content in the final mixture of products.

Uses

Cyclodextrins are able to form host-guest complexes with hydrophobic molecules given the unique nature imparted by their structure.

As a result, these molecules have found a number of applications in a wide range of fields.

In addition to the above mentioned pharmaceutical applications for drug release, cyclodextrins can be employed in environmental protection: these molecules can effectively immobilise inside their rings toxic compounds, like trichloroethane or heavy metals, or can form complexes with stable substances, like trichlorfon (an organophosphorus insecticide) or sewage sludge, enhancing their decomposition.

In the food industry cyclodextrins are employed for the preparation of cholesterol free products: the bulky and hydrophobic cholesterol molecule is easily lodged inside cyclodextrin rings that are then removed.

Weight loss supplements are marketed from alpha-cyclodextrin which claim to bind to fat and be an alternative to anti-obesity medications which avoids their possible unpleasant side effects.

Other food applications include the ability to stabilize volatile or unstable compounds and the reduction of unwanted tastes and odour. Cyclodextrins are used in alcohol powder, a powder for mixing alcoholic drinks.

The strong ability of complexing fragrances can also be used for another purpose: first dry, solid cyclodextrin microparticles are exposed to a controlled contact with fumes of active compounds, then they are added to fabric or paper products. Such devices are capable of releasing fragrances during ironing or when heated by human body. Such a device commonly used is a typical 'dryer sheet'. The heat from a clothes dryer releases the fragrance into the clothing.

The ability of cyclodextrins to form complexes with hydrophobic molecules has led to their usage in supramolecular chemistry. In particular they have been used to synthesize certain mechanically-interlocked molecular architectures, such as rotaxanes and catenanes, by reacting the ends of the threaded guest.

The application of cyclodextrin as supramolecular carrier is also possible in organometallic reactions. The mechanism of action probably take place in the interfacial region (Leclercq L. et al.,2007).

Wipff also demonstrated by computational study that the reaction occurs in the interfacial layer. The application of cyclodextrins as supramolecular carrier is possible in various organometallic catalysis.

Both β-cyclodextrin and MβCD remove cholesterol from cultured cells. The methylated form MβCD was found to be more efficient than β-cyclodextrin.

The water-soluble MβCD is known to form soluble inclusion complexes with cholesterol, thereby enhancing its solubility in aqueous solution.

Methyl-β-cyclodextrin is employed for the preparation of cholesterol-free products: the bulky and hydrophobic cholesterol molecule is easily lodged inside cyclodextrin rings that are then removed.

It is also employed to disrupt lipid rafts by removing cholesterol from membranes.

Study of inclusion complex

There is a direct correlation between the stability of the complex and the improvement of the solubility of the "guest" molecule.

To express quantitatively the increase in solubility, may therefore be useful to calculate the Association Constant Kc of the complex.

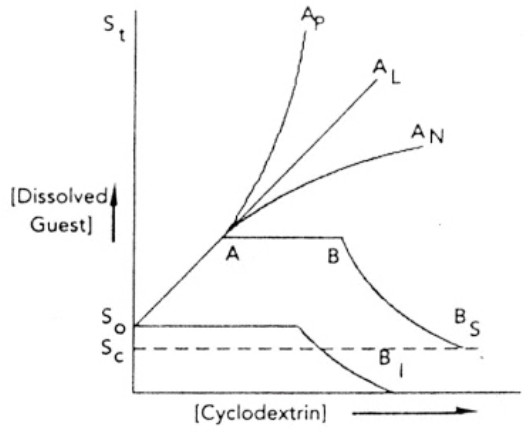
This is calculated at constant temperature and pH, considering the following equilibrium:

$$CD + G \rightleftharpoons CDG \qquad (M^{-1})$$

The method most used to study the formation of the complex of inclusion and to get the Kc is that of Higuchi and Connors (Higuchi et al., 1965), with whom is studied the change in solubility of the drug with increasing concentration of cyclodextrin.

The diagrams obtained are of two types, called A and B.

The curves of type A indicate the formation of inclusion complexes soluble, while those of type B are related to complexes with low solubility (Bs) or insoluble (Bi).



In the case of complexes of drug-cyclodextrin 1:1 molar ratio, can be calculated the costant association from the slope of the linear portion of the curve:

$$K = \text{slope} \times S_0 \, (1\text{-slope})$$

Where S_0 is the solubility of the drug without cyclodextrin.

Important factors that affect the formation and stability of the complex in solution are the temperature, pH, the presence of other solutes and other solvent.

The choice of the method of solid complex preparation (grinding, kneading, etc.) can greatly influence the formation and the properties of the final product.

The most effective method depends on the type of drug and cyclodextrin and must be selected case by case.

Methods of formation of drug – cyclodextrin complex

The preparation of drug–cyclodextrin binary systems in solid form can be carried out in various ways.

Although the various techniques are not particularly difficult, it should be considered that there is not an effective method for any type of drug and cyclodextrin. Moreover, the product is always a mixture, i.e. a combination of complex and the molecule "guest" not included and empty cyclodextrin.

The most effective techniques are generally those that provide a solid-state conversion of a solution containing the drug and cyclodextrins; in this case the main problem is often the identification of a effective common solvent.

1) The spray drying technique (SP) plans to separately solubilise the drug and cyclodextrin in the minimum amount of solvent, join the two solutions and remove the solvent by spray-drying.

2) The coevaporation technique (COE) consists of preparing the solution of two components in the same way and the subsequent evaporation of the solvent, performed by using a rotavapor.

3) The co-lyophilization technique (COL) has the fundamental requisite of an adequate water solubility of both the drug and the cyclodextrin. Again the two substances are dissolved separately and the two solutions are then combined, and freezed to about -20 ° C, then the solvent is sublimated by lyophilization.

A reference is always prepared, constituted by the simple physical mixture (PM) of the two powders in the stoichiometric ratio as defined by solubility phase studies. Other preparation procedures used are cogrinding (GR) in ball mill, or kneading (KN) in a mortar, or heating in sealed container (SH).

All methods lead to partial or total inclusion of the drug in cyclodextrin and can reduce the degree of crystallinity of the drug to obtain an amorphous product, stabilized by the presence of cyclodextrins, with better characteristics of dissolution rate.

The most effective method depends on the type of drug and cyclodextrin, and must be experimentally selected.

Advantages and disadvantages in the use of cyclodextrins

Increased solubility

The most obvious advantage in the use of cyclodextrins is to increase the solubility of drugs not readily soluble in water, and thereby increase their bioavailability.

The cyclodextrin complexation can be useful even for drugs soluble in water: it can improves the absorption of these substances for through skin or mucous membranes.

To explain this action different theories, have been developed (Loftsson et al., 1997).

Firstly the increase in solubility of the drug improves its availability on the surface of the absorption barrier (skin, mucous membranes, eye, etc).

It was noted that the choice of solvent, for its importance on the partition coefficient between vehicle and membrane, can markedly influence the improvement of bioavailability obtained with the cyclodextrins.

Another important mechanism is involved. It has been described the ability of cyclodextrins to remove cholesterol from biological membranes, thus increasing the fluidity of membranes and facilitating the permeation of drugs, especially water-soluble (Matsuda et al., 1999). Unlike other enhancers, the cyclodextrins seem solubilise components of the membrane without entering inside, thus demonstrating a moderate and reversible effect.

Reduced toxic effects

The cyclodextrins were also employed to reduce the irritation caused by certain drugs.

The increased efficiency and improved therapeutic activity can help to reduce the doses and therefore the toxicity of different active ingredients.

In addition, the increase in solubility achieved by the inclusion in cyclodextrins can reduce the risks in the parenteral administration of compounds not readily soluble in water.

Increased stability

The cyclodextrins can improve the stability of many drugs, protecting them from dehydration, oxidation, hydrolysis.

This sort of "molecular shield" offers protection against both interactions with the solvent and metabolic reactions. In some cases, the reactions are reduced degradation rate of more than 100 times (Nicolazzi et al., 2002).

Checking the time of action

The cyclodextrins are widely used in pharmaceutical formulations for a controlled release over time.

The hydrophilic derivatives (idrossipropilβ, dimetilβ, solfobutilβ, etc.) are particularly useful to shorten the on-set time of drugs not readily soluble in water; the hydrophobic ones (triacetilβ, etc.) may prolong the action of well-soluble drugs in their aqueous phase.

Combining different cyclodextrins or integrating them with other drug delivery systems (liposomes, microspheres, etc) it is possible to adjust in various ways the drug bioavailability, for example simultaneously exploiting both effects: longer duration of action and shortening of the onset time (Ma et al., 2000).

Limitations and problems

In the use of cyclodextrins there are some difficulties to be taken into account. One of these is obtaining high efficiency of encapsulation, which depends on several factors: the size of the drug, the type of cyclodextrins, the technique of preparation of the complex, the choice of solvent.

Another problem is the high molecular weight of cyclodextrins, which requires the use of large quantities of the substance and this could lead to pharmaceutical formulations of excessive size or volume.

Further limits may be toxicity, the high cost, low solubility of natural cyclodextrins (Hirayama et al., 1999).

Other types of cyclodextrins

The natural cyclodextrins contain 18 (αCD), 21 (βCD) or 24 (γCD) hydroxyl groups that can be chemically modified. Many derivatives of natural cyclodextrins have been prepared to suitably modify their physical chemical characteristics, reduce the toxicity or increase the capacity of inclusion (Szente et al., 1999).

There are several ways to quantitatively express the degree of substitution..

The degree of molar substitution (MS) is the average value of moles of substituent agent, such as an acetyl group, for each mole of glucopyranose. In some cases, the group added contains points of attachment for more groups, which can also happen to form new binding reactions during the changeover.

Then you can also find MS values higher than 3 per unit glucopyranose.

The degree of substitution (DS) is the average number of Hydroxyl groups replaced in each unit glucopyranose. Since each unit contains three Hydroxyl groups, this is the maximum possible value of DS.

Hydroxypropyl β cyclodextrin (HPβCD)

The HPβCD can be used for parenteral administration. The functionalization of hydroxyl has significantly reduced the haemolytic and nephrotoxicity effects of natural βCD.

From in vitro studies HPβCD is not teratogenic; in acute toxicity studies on monkeys, a dose of 1 g / kg e.v. was not lethal (Brewster et al., 1990).

The HPβCD does not accumulate in the body, the clearance is complete in a short time: in rats and dogs as long plasma half-life after intravenous administration is respectively 0.4 and 0.8 hours (Monbaliu et al., 1990).

The chronic toxicity was studied in rats and no effect was observed after treatment of 50 mg /kg. A dose of 400 mg / kg was found to give blood renal and metabolic toxicity (changes in weight).

Many of these effects are reversible after treatment.

At these doses, although high, were not detected effects of embryotoxicity or teratogenicity in rabbits. Fertility problems were found only in rats (Coussement et al., 1990).

Triacetyl β cyclodextrin (TAβCD)

In literature is reported the use of TAβ derivatives as carriers of drugs very soluble in water, in order to slow down the dissolution in aqueous media and gain extended-release forms.

Even if some research about the subcutaneously administration of this cyclodextrin (Matsubara et al., 1994), has been conducted there are no sufficient data to ensure the safety of parenteral use.

6. CHEMOTHERAPEUTIC DRUGS: IRINOTECAN

Chemotherapy, in its most general sense (Chemotherapy, Dorland's Medical Dictionary), refers to treatment of disease by chemicals that kill cells, specifically those of micro-organisms or cancer. In popular usage, it will usually refer to antineoplastic drugs used to treat cancer or the combination of these drugs into a cytotoxic standardized treatment regimen as opposed to a targeted therapy.

In its non-oncological use, the term may also refer to antibiotics (*antibacterial chemotherapy*). In that sense, the first modern chemotherapeutic agent was Paul Ehrlich's arsphenamine, an arsenic compound discovered in 1909 and used to treat syphilis.

This was later followed by sulfonamides discovered by Domagk and penicillin discovered by Alexander Fleming. Other uses of cytostatic chemotherapeutic agents (including the ones mentioned below) are the treatment of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis and the suppression of transplant rejections (see immunosuppression and DMARDs).

The use of chemical substances and drugs as medication can be traced back to the ancient Indian system of medicine called Ayurveda, which uses many metals besides herbs for treatment of a large number of ailments.

More recently, Persian physician, Muhammad ibn Zakarīya Rāzi (Rhazes), in the 10th century, introduced the use of chemicals such as vitriol, copper, mercuric and arsenic salts, sal ammoniac, gold scoria, chalk, clay, coral, pearl, tar, bitumen and alcohol for medical purposes.

The first drug used for cancer chemotherapy, however, dates back to the early 20th century, though it was not originally intended for that purpose. Mustard gas was used as a chemical warfare agent during World War I and was studied further during World War II.

During a military operation in World War II, a group of people were accidentally exposed to mustard gas and were later found to have very low white blood cell counts (Hirsch, 2006). It was reasoned that an agent that damaged the rapidly-growing white blood cells might have a similar effect on cancer.

Therefore, in the 1940s, several patients with advanced lymphomas (cancers of certain white blood cells) were given the drug by vein, rather than by breathing the

irritating gas. Their improvement, although temporary, was remarkable (Goodman et al., 1946,1984).

That experience led researchers to look for other substances that might have similar effects against cancer.

As a result, many other drugs have been developed to treat cancer, and drug development since then has exploded into a multibillion-dollar industry.

The targeted-therapy revolution has arrived, but the principles and limitations of chemotherapy discovered by the early researchers still apply (Joensuu, 2008).

Cancer is the uncontrolled growth of cells coupled with malignant behavior: invasion and metastasis. Cancer is thought to be caused by the interaction between genetic susceptibility and environmental toxins.

In the broad sense, most *chemotherapeutic* drugs work by impairing mitosis (cell division), effectively targeting fast-dividing cells. As these drugs cause damage to cells they are termed *cytotoxic*. Some drugs cause cells to undergo apoptosis (so-called "programmed cell death").Scientists have yet to identify specific features of malignant and immune cells that would make them uniquely targetable (barring some recent examples, such as the Philadelphia chromosome as targeted by imatinib).

This means that other fast-dividing cells, such as those responsible for hair growth and for replacement of the intestinal epithelium (lining), are also often affected. However, some drugs have a better side-effect profile than others, enabling doctors to adjust treatment regimens to the advantage of patients in certain situations.

As chemotherapy affects cell division, tumors with high *growth fractions* (such as acute myelogenous leukemia and the aggressive lymphomas, including Hodgkin's disease) are more sensitive to chemotherapy, as a larger proportion of the targeted cells are undergoing cell division at any time. Malignancies with slower growth rates, such as indolent lymphomas, tend to respond to chemotherapy much more modestly.

Drugs affect "younger" tumors (i.e., more differentiated) more effectively, because mechanisms regulating cell growth are usually still preserved. With succeeding generations of tumor cells, differentiation is typically lost, growth becomes less regulated, and tumors become less responsive to most chemotherapeutic agents.

Near the center of some solid tumors, cell division has effectively ceased, making them insensitive to chemotherapy. Another problem with solid tumors is the fact that the chemotherapeutic agent often does not reach the core of the tumor. Solutions to this problem include radiation therapy (both brachytherapy and teletherapy)

and surgery. Over time, cancer cells become more resistant to chemotherapy treatments. Recently, scientists have identified small pumps on the surface of cancer cells that actively move chemotherapy from inside the cell to the outside. Research on p-glycoprotein and other such chemotherapy efflux pumps, is currently ongoing. Medications to inhibit the function of p-glycoprotein are undergoing testing as of June, 2007 to enhance the efficacy of chemotherapy.

There are a number of strategies in the administration of chemotherapeutic drugs used today. Chemotherapy may be given with a curative intent or it may aim to prolong life or to palliate symptoms. *Combined modality chemotherapy* is the use of drugs with other cancer treatments, such as radiation therapy or surgery.

Most cancers are now treated in this way. *Combination chemotherapy* is a similar practice that involves treating a patient with a number of different drugs simultaneously. The drugs differ in their mechanism and side-effects.

The biggest advantage is minimising the chances of resistance developed by any one agent. In *neoadjuvant chemotherapy* (*preoperative* treatment) initial chemotherapy is aimed at shrinking the primary tumour, thereby rendering local therapy (surgery or radiotherapy) less destructive or more effective. *Adjuvant chemotherapy* (*post*operative treatment) can be used when there is little evidence of cancer present, but there is risk of recurrence. This can help reduce chances of developing resistance.

It is also useful in killing any cancerous cells which have spread to other parts of the body. This is often effective as the newly growing tumours are fast-dividing, and therefore very susceptible.

Palliative chemotherapy is given without curative intent, but simply to decrease tumor load and increase life expectancy. For these regimens, a better toxicity profile is generally expected.

All chemotherapy regimens require that the patient be capable of undergoing the treatment. Performance status is often used as a measure to determine whether a patient can receive chemotherapy, or whether dose reduction is required.

Types of chemotherapeutic drugs

The majority of chemotherapeutic drugs can be divided in alkylating agents, antimetabolites, anthracyclines, plant alkaloids, topoisomerase inhibitors, and other antitumour agents.

All of these drugs affect cell division or DNA synthesis and function in some way. Some newer agents do not directly interfere with DNA.

These include monoclonal antibodies and the new tyrosine kinase inhibitors e.g. *imatinib mesylate* (*Gleevec* or *Glivec*), which directly targets a molecular abnormality in certain types of cancer (chronic myelogenous leukemia, gastrointestinal stromal tumors). These are examples of targeted therapies.

In addition, some drugs that modulate tumor cell behaviour without directly attacking those cells may be used. Hormone treatments fall into this category of adjuvant therapies.

Where available, Anatomical Therapeutic Chemical Classification System codes are provided for the major categories.

Alkylating agents

Alkylating agents are so named because of their ability to add alkyl groups to many electronegative groups under conditions present in cells.

Cisplatin and carboplatin, as well as oxaloplatin, are alkylating agents. Other agents are mechlorethamine, cyclophosphamide, chlorambucil.

They work by chemically modifying a cell's DNA.

Anti-metabolites

Anti-metabolites masquerade as purine (azathioprine, mercaptopurine) or pyrimidine - which become the building blocks of DNA.

They prevent these substances from becoming incorporated in to DNA during the "S" phase (of the cell cycle), stopping normal development and division.

They also affect RNA synthesis. Due to their efficiency, these drugs are the most widely used cytostatics.

Plant alkaloids and terpenoids

These alkaloids are derived from plants and block cell division by preventing microtubule function.

Microtubules are vital for cell division, and, without them, cell division cannot occur. The main examples are vinca alkaloids and taxanes.

Vinca alkaloids

Vinca alkaloids bind to specific sites on tubulin, inhibiting the assembly of tubulin into microtubules (M phase of the cell cycle).

They are derived from the Madagascar periwinkle, *Catharanthus roseus* (formerly known as *Vinca rosea*).

The vinca alkaloids include:

- Vincristine
- Vinblastine
- Vinorelbine
- Vindesine

Podophyllotoxin

Podophyllotoxin is a plant-derived compound which is said to help with digestion as well as used to produce two other cytostatic drugs, etoposide and teniposide.

They prevent the cell from entering the G1 phase (the start of DNA replication) and the replication of DNA (the S phase). The exact mechanism of its action is not yet known.

The substance has been primarily obtained from the American Mayapple (*Podophyllum peltatum*).

Recently it has been discovered that a rare Himalayan Mayapple (*Podophyllum hexandrum*) contains it in a much greater quantity, but, as the plant is endangered, its supply is limited.

Studies have been conducted to isolate the genes involved in the substance's production, so that it could be obtained recombinantively.

Taxanes

The prototype taxane is the natural product paclitaxel, originally known as Taxol and first derived from the bark of the Pacific Yew tree.

Docetaxel is a semi-synthetic analogue of paclitaxel. Taxanes enhance stability of microtubules, preventing the separation of chromosomes during anaphase.

Topoisomerase inhibitors

Topoisomerases are essential enzymes that maintain the topology of DNA. Inhibition of type I or type II topoisomerases interferes with both transcription and replication of DNA by upsetting proper DNA supercoiling.

- Some type I topoisomerase inhibitors include *camptothecins*: irinotecan and topotecan.
- Examples of type II inhibitors include amsacrine, etoposide, etoposide phosphate, and teniposide. These are semisynthetic derivatives of epipodophyllotoxins, alkaloids naturally occurring in the root of American Mayapple (*Podophyllum peltatum*)

Antitumour antibiotics

These include the immunosuppressant dactinomycin (which is used in kidney transplantations), doxorubicin, epirubicin, bleomycin and others.

Monoclonal antibodies

Monoclonal antibodies work by targeting tumour specific antigens, thus enhancing the host's immune response to tumour cells to which the agent attaches itself. Examples are trastuzumab (Herceptin), cetuximab, and rituximab (Rituxan or Mabthera).

Bevacizumab (Avastin) is a monoclonal antibody that does not directly attack tumor cells but instead blocks the formation of new tumor vessels.

Hormonal therapy

Several malignancies respond to hormonal therapy, which, in the strict sense, is not chemotherapy.

Cancer arising from certain tissues, including the mammary and prostate glands, may be inhibited or stimulated by appropriate changes in hormone balance.

- Steroids (often dexamethasone) can inhibit tumour growth or the associated edema (tissue swelling), and may cause regression of lymph node malignancies. Dexamethasone is also an antiemetic, so it may be used with cytotoxic chemotherapy even if it has no direct effect on the cancer.

- Prostate cancer is often sensitive to finasteride, an agent that blocks the peripheral conversion of testosterone to dihydrotestosterone.
- Breast cancer cells often highly express the estrogen and/or progesterone receptor. Inhibiting the production (with aromatase inhibitors) or action (with tamoxifen) of these hormones can often be used as an adjunct to therapy.
- Gonadotropin-releasing hormone agonists (GnRH), such as goserelin possess a paradoxical negative feedback effect followed by inhibition of the release of FSH (follicle-stimulating hormone) and LH (luteinizing hormone), when given continuously.

Some other tumours are also hormone-dependent, although the specific mechanism is still unclear.

Side-effects

The treatment can be physically exhausting for the patient. Current chemotherapeutic techniques have a range of side effects mainly affecting the fast-dividing cells of the body.

Important common side-effects include (dependent on the agent):

- Pain
- Nausea and vomiting
- Diarrhea or constipation
- Anemia
- Malnutrition
- Hair loss
- Memory loss
- Depression of the immune system, hence (potentially lethal) infections and sepsis
- Weight loss or gain
- Hemorrhage
- Secondary neoplasms
- Cardiotoxicity
- Hepatotoxicity
- Nephrotoxicity
- Ototoxicity

Immunosuppression and myelosuppression

Virtually all chemotherapeutic regimens can cause depression of the immune system, often by paralysing the bone marrow and leading to a decrease of white blood cells, red blood cells, and platelets.

The latter two, when they occur, are improved with blood transfusion. Neutropenia (a decrease of the neutrophil granulocyte count below 0.5 x 10^9/litre) can be improved with synthetic G-CSF (granulocyte-colony stimulating factor, e.g., filgrastim, lenograstim, Neupogen, Neulasta).

In very severe myelosuppression, which occurs in some regimens, almost all the bone marrow stem cells (cells that produce white and red blood cells) are destroyed, meaning *allogenic* or *autologous* bone marrow cell transplants are necessary. (In autologous BMTs, cells are removed from the patient before the treatment, multiplied and then re-injected afterwards; in *allogenic* BMTs the source is a donor).

However, some patients still develop diseases because of this interference with bone marrow.

Nausea and vomiting

Nausea and vomiting caused by chemotherapy; stomach upset may trigger a strong urge to vomit, or forcefully eliminate what is in the stomach.

Stimulation of the vomiting center results in the coordination of responses from the diaphragm, salivary glands, cranial nerves, and gastrointestinal muscles to produce the interruption of respiration and forced expulsion of stomach contents known as retching and vomiting.

The vomiting center is stimulated directly by afferent input from the vagal and splanchnic nerves, the pharynx, the cerebral cortex, cholinergic and histamine stimulation from the vestibular system, and efferent input from the chemoreceptor trigger zone (CTZ). The CTZ is in the area postrema, outside the blood-brain barrier, and is thus susceptible to stimulation by substances present in the blood or cerebral spinal fluid.

The neurotransmitters dopamine and serotonin stimulate the vomiting center indirectly via stimulation of the CTZ. The 5-HT_3 inhibitors are the most effective antiemetics and constitute the single greatest advance in the management of nausea and vomiting in patients with cancer.

These drugs are designed to block one or more of the signals that cause nausea and vomiting. The most sensitive signal during the first 24 hours after chemotherapy appears to be 5-HT_3. Blocking the 5-HT_3 signal is one approach to preventing acute emesis (vomiting), or emesis that is severe, but relatively short-lived. Approved 5-HT_3 inhibitors include Dolasetron (Anzemet), Granisetron (Kytril), and Ondansetron (Zofran).

The newest 5-HT_3 inhibitor, palonosetron (Aloxi), also preventing delayed nausea and vomiting, which occurs during the 2-5 days after treatment. Another drug to control nausea in cancer patients became available in 2005.

The substance P inhibitor aprepitant (marketed as Emend) has been shown to be effective in controlling the nausea of cancer chemotherapy.

The results of two large controlled trials were published in 2005, describing the efficacy of this medication in over 1,000 patients (Gralla et al., 2005).

Some studies (Tramer et al., 2001) and patient groups claim that the use of cannabinoids derived from marijuana during chemotherapy greatly reduces the associated nausea and vomiting, and enables the patient to eat. Some synthetic derivatives of the active substance in marijuana (Tetrahydrocannabinol or THC) such as Marinol may be practical for this application.

Natural marijuana, known as medical cannabis is also used and recommended by some oncologists, though its use is regulated and not legal everywhere.

Other side-effects

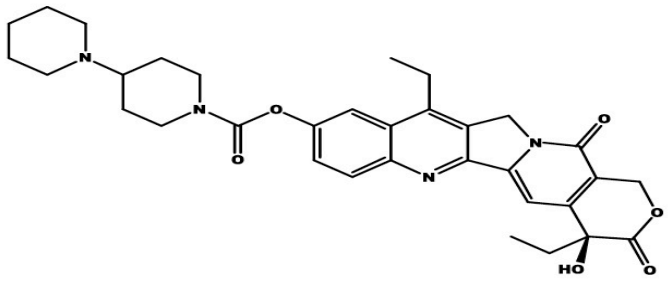
In particularly, large tumors, such as large lymphomas, some patients develop tumor lysis syndrome from the rapid breakdown of malignant cells.

Although prophylaxis is available and is often initiated in patients with large tumors, this is a dangerous side-effect that can lead to death if left untreated.

Some patients report fatigue or non-specific neurocognitive problems, such as an inability to concentrate; this is sometimes called post-chemotherapy cognitive impairment, referred to as "chemo brain" by patients' groups (Tannock et al., 2004).

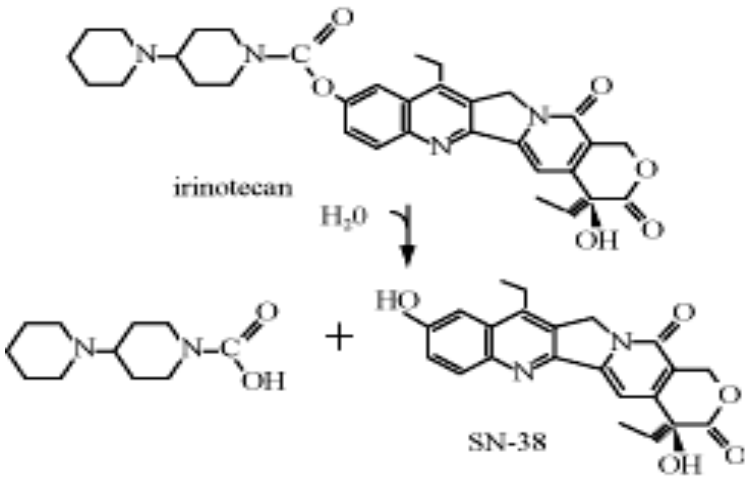
Specific chemotherapeutic agents are associated with organ-specific toxicities, including cardiovascular disease (e.g., doxorubicin), interstitial lung disease (e.g., bleomycin) and occasionally secondary neoplasm (e.g., MOPP therapy for Hodgkin's disease).

6.1 IRINOTECAN



Irinotecan

Irinotecan (7-ethyl- 1 O-(4-[1 -piperidino]- I-piperidino) carboxylcamptothecin, CPT- 11) is a water-soluble prodrug that can be converted to SN-38, an active metabolite that exhibits antitumor activity via the inhibition of topoisomerase I activity.



This camptothecin-based drug has passed clinical trials and is currently approved for the treatment of colonic, ovarian, and small cell lung cancer, and is increasingly used in combination with other standard chemotherapeutic agents for enhanced therapy. However, irinotecan was discovered to have serious side effects such as myelosuppression and gastrointestinal disorders (mainly diarrhea), which are recognized as constituting dose-limiting toxicity for this drug.

The basic labile characteristic of the lactone E ring in irinotecan is reversible and pH-dependent hydrolysis yields the inactive carboxylate species. Only lactone species can inhibit topoisomerase 1, but this active molecule is very rarely found under physiological conditions.

Consequently, finding an effective drug delivery system to reduce toxicity and preserve the active form of the drug is very important.

7. EXPERIMENTAL DESIGN

Experimentation is generally carried out to determine the relationship between factors acting on the system and the response or properties of the system. The information is then used to achieve the aims of the project (Goupy, 1993).

One way of finding out which factors have an effect would be to change them one at a time (Lewis et al., 1999). The response y is then measured as a function of several values of the unfixed variable x_1. At the end of the experiment on this first variable, a curve is drawn of $y= f(x_1)$. It the experimenter wishes to study all the variables, the whole experiment must be repeated for each one (Goupy, 1993). This one-factor-at-a-time method (OVAT) is inefficient, can give misleading results, and in general should be avoided (Lewis et al., 1999).

In the vast majority of cases the preferred approach is to vary all factors together in a programmed and rational way, thus a maximum of information is gained from a minimum of experiments (Lewis et al., 1999). This strategy is called experimental design and can be defined as setting up experiments in such a manner that the information required is obtained as efficiently and precisely as possible (Lewis et al., 1999). This chemometric tool offers several advantages and some of these are (Goupy, 1993):

- Fewer trials
- Large number of factors studied
- Detection of interaction between factors
- Detection of optima
- Best result precision
- Optimisation of results
- Model-building from the results.

Common terms used in experimental design are here introduced and defined (Lundstedt et al., 1998):

Experimental domain: the experimental range that is investigated (defined by the variation of the
experimental variables);

Factors: experimental variables that can be changed independently of each other;

Independent variables: same as factors;

Continuous variables: independent variables that can be changed continuously*;*
Discrete variables: independent variables that are changed step-wise*;*
Responses: the measured value of the result(s) from experiments;
Residual: the difference between the calculated and the experimental result.

The response is a measurable manifestation of the results obtained varying the studied factors.

If k factors are considered, the response y can be written as $y = f(x_1, x_2, \ldots . x_k)$, where f is a polynomial function (Peissik, 1995). The objective of the experimenter is to describe the dependence existing between an experimental response and the chosen factors by a mathematical model.

There are different kinds of models to apply (Lundstedt et al., 1998*)*:

- Linear model (ε is the experimental error)

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_k x_k + \varepsilon$$

- Linear model with an interaction between the different experimental variables

($j \neq k$)

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_k x_k + \beta_{12} x_1 x_2 + \ldots + \beta_{1k} x_1 x_k + \beta_{23} x_2 x_3 + \ldots$$
$$\ldots + \beta_{2k} x_2 x_k + \ldots + \beta_{1jk} x_1 x_j x_k + \ldots + \varepsilon$$

- Quadratic model

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_k x_k + \beta_{12} x_1 x_2 + \ldots + \beta_{1k} x_1 x_k + \beta_{11} x_1^2 + \ldots + \beta_{kk} x_k^2 + \varepsilon$$

Independently from the kind of postulated model, the researcher is required to supply minimum and maximum values for each factor that define the experimental domain to be investigated during the optimization procedure.

The combination of the different factor levels used to perform the actual experiments are then decided by which statistical design is chosen to estimate the coefficients of the model (Costa Ferreira et al., 2007).

The experiments are then run in random chronological order, so that it is possible to prevent an uncontrolled factor causing a biased result.

After estimating the model, analysis of variance (ANOVA) is performed on the regression results so that the most appropriate model with no evidence of lack of fit can be used to represent the data.

Statistical designs can be in outline classified in screening designs and response surface designs.

Screening designs present two levels for each factor and provide simple models with information about dominating variables and information about ranges; linear models and interaction models are sufficient. In addition, screening designs provide few experiments/factors ratio which means that relevant information is gained in only a few experiments (Trygg & Wold, 2002).

In full factorial design every level of a factor is investigated at both levels of all the other factors and this design allows the estimation of a factor independent of all the other effects. However, investigating more than 5 factors with the full fractional design becomes time consuming.

In this case it is preferable to use a fractional factorial design, which reduces the experimental runs without the loss of too much information regarding the estimated factors involved.

This type of design is the most used for screening phase and takes advantage of the fact that 3-way and higher interactions are seldom significant. The downside is the loss of information for not performing all experiments (Trygg & Wold, 2002). Finally, if the effect of many factors is to be studied, Plackett-Burman designs can be efficiently used for estimating only the main effects (Lewis et al., 1999).

After screening, the goal of the investigation is usually to create a valid map of the experimental domain given by the significant factors and their ranges. This is done performing response surface methodology (RSM), where a quadratic polynomial model is estimated.

The higher order model has an increased complexity and therefore also requires more experiments than screening designs (Trygg & Wold, 2002). The most common used design in RSM are Central Composite, Box-Behnken, Doehlert and mixture designs, where factors are investigated at least at three levels and thus curved dependences can be modelled (Costa Ferreira et al., 2007).

After running the experiments according to the experimental plan, the coefficients of the model are estimated and the obtained model is then examined by ANOVA to evaluate regression significance and lack of fit.

SS_T is the total sum of squares of differences between the experimental values and the grand average of the data set. This sum has $n-1$ degrees of freedom since it represents the total variance in the data (Costa Ferreira et al., 2007). The total data

variance SS_T is divided into two main contributions, the sum of squares explained by the regression, SS_{regr} , and the residual sum of squares, SS_{resid}.

Both summations are taken over all the experimental design levels, i= 1,2,…m and all the replicates performed at each level, j=1,2,…n_i. SS_{regr} is a sum of squares of differences between values predicted by the regression and the average of all the response values and has p-1 degrees of freedom, where p is the number of coefficients in the model. SS_{resid} is a sum of squares of differences or residuals between all the experimental values and the predicted values from the model.

It has n-p degrees of freedom, where n is the total number of experimental data used to determine the model. Regression significance can be tested by comparing the calculated variance ratio between regression and residuals with the tabled F-distribution value for p-1 and n-p degrees of freedom at the desired confidence level, usually 95%. The regression is significant if the calculated value is greater than the tabled one (Costa Ferreira et al., 2007).

If there are replicates among the experiments, model quality can be judged by decomposing the SS_{resid} into two contributions, the lack-of-fit and the pure error sums of squares, SS_{lof} and SS_{pe}.

The SS_{lof} is a sum of squares of differences between the values predicted at each level and the average experimental value at that level and has m-p degrees of freedom, where m is the number of distinct levels in the experimental design. The SS_{pe} is a sum of squares of differences between all the individual experimental values and the average of the experimental values at the same level.

It has n-m degrees of freedom. Regression lack of fit is determined performing an F-test by comparing the calculated variance ratio between lack of fit and pure experimental error with the tabled F-value for m-p and n-m degrees of freedom at the desired confidence level, usually 95%.

If the calculated quotient is greater than the tabled value there is evidence of model lack of fit and the model must be discarded. If not, the model can be accepted at this confidence level as providing an adequate representation of the data (Costa Ferreira et al., 2007).

Table 1: ANOVA table

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F* ratio
Regression	$SS_{regr} = \Sigma(\hat{y}_i - \bar{y})^2$	*p-1*	$SS_{regr} / p-1$	$\frac{SS_{regr}}{p-1} \Big/ \frac{SS_{resid}}{n-p}$
Residuals	$SS_{resid} = \Sigma(y_i - \hat{y}_i)^2$	*n-p*	$SS_{resid} / n-p$	
Lack of fit	$SS_{lof} = SS_{resid} - SS_{pe}$	*m-p*	$SS_{lof} / f-p$	$\frac{SS_{lof}}{f-p} \Big/ \frac{SS_{pe}}{n-f}$
Pure error	$SS_{pe} = \Sigma(y_i - \bar{y}_i)^2$	*n-m*	$SS_{pe} / n-f$	

p is the number of coefficients in the model; *n* is the total number of experimental data used to determine the model; *m* is the number of distinct levels in the experimental design.

Doehlert designs

The designs we have discussed so far are all symmetrical—that is, they have the same number of levels for each factor under study.

Sometimes, however, it is advantageous to use designs where different factors are studied at different numbers of levels. A simple example is a 2 × 3 factorial design.

Modern statistical design programs provide a wide variety of mixed *k*-factorial designs of the type 2^p3^{k-p}, where $p < k$ and p factors are studied at two levels and the other $k - p$ factors at three levels.

Doehlert designs comprise another class of experimental designs, with which different factors can be studied at different numbers of levels. They are attractive for treating problems where specific information about the system indicates that some factors deserve more attention than others, but they also have other desirable characteristics.

All Doehlert designs are generated from a regular simplex, a geometrical figure containing $k + 1$ points, where k is the number of factors. For two factors, the regular simplex is an equilateral triangle.

For Doehlert designs of type D-1, which are the most popular, the coordinates of this triangle are those given in the first three lines of table 2, n coded units. The other

runs of the design are obtained by subtracting every run in the triangle from each other, as shown in the table. This corresponds to moving the simplex around the design center.

Table 2. Coded factor levels for the two-factor Doehlert D-1 design

Run	x_1	x_2	Subtraction
1[a]	**0.0**	**0.0**	
2[a]	**1.0**	**0.0**	
3[a]	**0.5**	**0.866**	
4[b]	−1.0	0.0	**1–2**
5[b]	−0.5	−0.866	**1–3**
6[b]	−0.5	0.866	**3–2**
7[b]	0.5	−0.866	**2–3**

[a] The runs in bold face are those defining the initial simplex. [b] The other runs are obtained by subtracting every run from each other.

For any number of factors k, one of the points of the simplex is the origin, and the other k points lie on the surface of a sphere with radius 1.0 centered on the origin, in such a way that the distances between neighboring points are all the same.

Each of these points subtracted from the other k points forms k new points, so the design matrix has a total of $k^2 + k + 1$ points.

Since the points are uniformly distributed on a spherical shell, Doehlert suggested that these designs be called uniform shell designs.

The coordinates of the D-1 designs for three and four factors are presented in table 3. Note that the design for $k = 3$ is the same as the corresponding Box–Behnken design.

Table 3. Coded factor levels for the three- and four-factor Doehlert D-1 designs

Run	Three-factor			Four factor			
	x_1	x_2	x_3	x_1	x_2	x_3	x_4
1	0	0	0	0	0	0	0
2	1	0	0	1	0	0	0
3	0.5	0.866	0	0.5	0.866	0	0
4	0.5	0.289	0.817	0.5	0.289	0.817	0
5	−1	0	0	0.5	0.289	0.204	0.791
6	−0.5	−0.866	0	−1	0	0	0
7	−0.5	−0.289	−0.817	−0.5	−0.866	0	0
8	0.5	−0.866	0	−0.5	−0.289	−0.817	0
9	0.5	−0.289	−0.817	−0.5	−0.289	−0.204	−0.791
10	−0.5	0.866	0	0.5	−0.866	0	0
11	0	0.577	−0.817	0.5	−0.289	−0.817	0
12	−0.5	0.289	0.817	0.5	−0.289	−0.204	−0.791
13	0	−0.577	0.817	−0.5	0.866	0	0
14				0	0.577	−0.817	0
15				0	0.577	−0.204	−0.791
16				−0.5	0.289	0.817	0
17				0	−0.577	0.817	0
18				0	0	0.613	−0.791

Run	Three-factor			Four factor			
	x_1	x_2	x_3	x_1	x_2	x_3	x_4
19				−0.5	0.289	0.204	0.791
20				0	−0.577	0.204	0.791
21				0	0	−0.613	0.791

Despite being spherical, Doehlert designs have none of the classical properties of response surface designs.

They are neither orthogonal nor rotatable, and the variance of the predicted values is not uniform over the experimental range. However, they have other interesting features that make them advantageous in some scenarios.

Perhaps their most important property is the ability for uniform space-filling, which is unaffected by rotation. This is very convenient when one wishes to cover an experimental range, no matter how irregular, with a uniform grid of points.

Doehlert designs then can be displaced to more promising regions while preserving some of the runs already carried out.

This property is illustrated in figure 1. The initial two-factor design is a hexagon, where the letters **BCDEFG** denote its vertices. These points, along with the **A** center point, define five levels for the x_1 variable but only three levels for x_2. If the researchers decide to shift the initial design to higher values of x_1 and lower values of x_2 the **PONLGFM** hexagon can be used, which includes vertices **F** and **G** from the initial configuration.

If experimentation is very costly or lengthy, the values already observed for the **F** and **G** vertices can be retained. In case the researchers wish to raise the levels of both x_1 and x_2, displacement to the **BGLKJIH** hexagon is warranted.

This time, vertices **B** and **G** belong to the initial hexagon. Finally, if it is decided to shift the levels of only one variable, say x_1, the experimenters might perform new runs only at the **Q**, **R** and **S** vertices to complete the new Doehlert design **AESRQCD**, which has two lower levels along x_1 while keeping the same levels for x_2.

Note that the space-filling property of Doehlert designs is clearly illustrated in Figure 3. No gaps in the experimental region are left as one hexagon substitutes another.

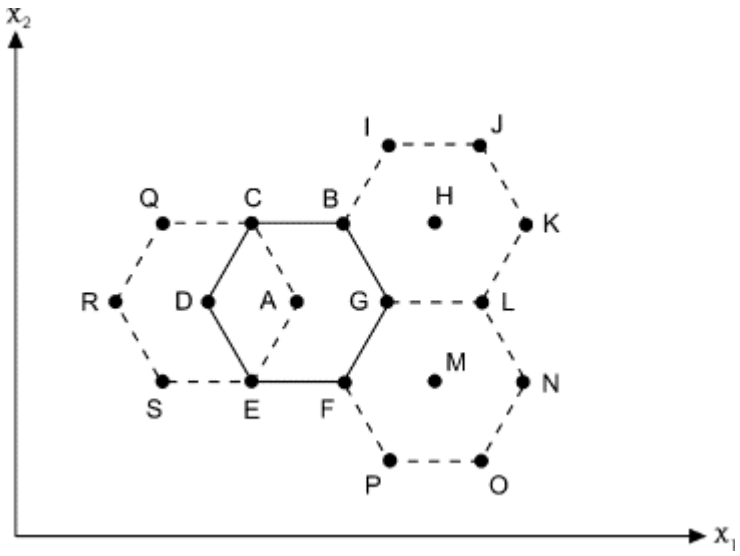


Fig. 1. Hexagonal Doehlert two-factorial design with three possible displacements in the experimental space.

Compared to central composite or Box–Behnken designs, Doehlert designs are more economical, especially as the number of factors increase.

The basic hexagon in Figure 1 has six points lying on a circumference around the center point, whereas the two-factor central composite design has eight points, also lying on a circumference surrounding its center point.

Likewise, the three-factor Doehlert design has 13 points, but the central composite design requires 15. On the other hand, central composite designs are rotatable, a general property that Doehlert designs do not have.

Furthermore, since central composite designs consist of factorial and axial blocks, they provide the basis for an efficient sequential strategy.

Linear models can be fitted in a first stage, after which the design can be augmented with complementary points, should quadratic models prove necessary.

Finally, using full designs, Doehlert or otherwise, to fit second-order models is hardly practicable for more than four factors, since a five factor quadratic model has twenty coefficients to be determined and it is unlikely that all factors will be relevant. Fractional factorial screening designs to discriminate between inert and relevant factors should always be applied before higher order designs when many factors are being investigated.

Another very interesting feature of Doehlert designs is the possibility of introducing variations in new factors during the course of an experimental study, without losing the runs already performed.

Sometimes we might wish to study first – say – the two factors that seem more promising, analyze the results, and only then introduce variation in a third factor, then in a fourth, and so on.

With Doehlert D-1 designs this is possible, provided that all potential factors of interest are introduced in the experiments right from start, set at their average levels (that is, zero in coded units).

For example, let us say that there are four factors of potential interest. We can begin with the two-factor design defined in Table 3, taking care to keep the levels of factors 3 and 4 fixed at zero in all runs.

Then, when we wish to study the influence of the two factors that have been kept fixed, we only have to add to the initial design the runs corresponding to the rest of the rows in the four-factor design in the Table 3.

As can be seen in Table 3, a Doehlert design of type D-1 with three or more factors always has one factor at five levels (the first one), one factor at three levels (the last), and the others all at seven levels.

Two other Doehlert design types, D-2 and D-3, can be generated by different simplexes and result in different level distributions.

EXPERIMENTAL PART

8. Development of Benzocaine liposomes. Optimization of Formulation Variables using Experimental Design methodologies.

Benzocaine is an ester-type local anaesthetic agent widely used in topical, dermal, and mucous formulations.

It is characterized by a rapid but short-lived effect compared with the potential duration of pain (Covino and Vassallo, 1976). Moreover, toxic effects of benzocaine, due to systemic absorption, have been reported (Barclay and Vega, 2004).

Therefore, it could be very helpful to develop a new delivery system aimed at both modulating drug release rate to prolong its therapeutic effect, and improving its localization in the skin to decrease systemic toxicity.

Liposomes have been widely used as safe vehicles for topical drug delivery systems due to their ability to entrap drugs and deliver them to the skin, thus enhancing their therapeutic effectiveness (Margalit, 1995; Verma et al., 2003).

Their delivery mechanism is associated with accumulation in the stratum corneum and upper skin layers of the liposomes, which act as a local drug reservoir, reducing side effects in virtue of a decreased systemic absorption (Fresta and Puglisi, 1996; Mezei and Gulasekharam, 1982).

In particular, it has been reported that liposomal formulations of some local anaesthetics exhibited improved clinical efficacy with respect to administration of the corresponding plain drugs (Bucalo et al., 1998; Fisher et al., 1998; Grant and Bansinath, 2001; Lim et al., 2005).

In a previous study carried out in our laboratory, it was been demonstrated the significant ($P < 0.01$) improvement of both intensity of anaesthetic effect and duration of action of benzocaine entrapped in multilamellar liposomes with respect to the plain drug (Mura et al., 2007). On the other hand, it has been demonstrated that the effectiveness of a liposomal formulation is clearly influenced by the composition and structure of the vesicles (Anderson and Omri, 2004; Kirjavainen et al., 1996; López-Pinto et al., 2005).

Therefore, we considered it worthy of interest to continue this study and evaluate in depth the effects of modifications of the composition of the liposomal vesicles, with the aim of optimizing the effectiveness of the benzocaine liposomal formulation.

However, due to the several possible variables that should be simultaneously considered in such a study, it was considered very difficult to assess the effects of their variations by using a conventional mono-varied approach; i.e. by varying one parameter at a time and keeping the others constant.

Moreover, although an incremental improvement can be achieved through successive approximation experiments, it is not possible to establish when and whether the optimal formulation has actually been obtained, nor to identify and quantify possible interaction effects among the variables.

Statistical experimental design methodologies are a powerful tool in the design of pharmaceutical dosage forms, allowing a rational and effective investigation of the influence of formulation parameters on the selected responses with a shortening of the experiment time (Gabrielson et al., 2002; Lewis et al., 1999).

Moreover, multivaried strategies enable simultaneous evaluation of the influence of the different variables involved, being therefore particularly useful when, as in the present case, different factors have to be evaluated contemporaneously.

In particular, the usefulness of statistical experimental design strategies in the development of different kinds of liposomes has been recently demonstrated, allowing a quick and efficient evaluation and prediction of the effects of formulation changes on the considered responses (El Samaligy et al., 2006; Seth and Misra, 2002; Sun and Zhang, 2004).

Thus in the present work, an experimental design methodology was used to evaluate the effects of varying the composition of the liposomal vesicles and to optimize the benzocaine liposomal formulation in terms of percent of permeated drug.

We started from the composition that was previously found to be the most effective; i.e., 50:50 w/w phosphatidylcholine/cholesterol mixture as lipid phase, and 50:50 v/v ethanol/water mixture as hydrophilic phase (Mura et al., 2007).

The considered variables for the lipid phase were the use of potassium glycyrrhizinate, as an alternative to cholesterol, and the presence of a cationic (stearylamine) or anionic (dicethylphosphate) surfactant, since it has been reported that all these components should increase the bilayer fluidity and/or the drug skin permeability (Fang et al., 2006; Mohammed et al., 2004; Trotta et al., 2004).

As for the hydrophilic phase, the hydration volume and the percent of ethanol in the hydroalcoholic mixture were considered as other important formulation variables,

due to the solubilizing effect and the possible enhancer role shown by this solvent (Fang et al., 2006; Kirjavainen et al., 1999; Touitou et al., 1997).

The first step of the study was a preliminary screening phase, according to a D-optimal design strategy, aimed at identifying the most important factors influencing the selected response.

Doehlert design was then applied for the response-surface study of the factors selected in the preliminary phase.

8.1 Materials and Methods

Materials

Benzocaine base (BZC) (ethyl-4-aminobenzoate) (pKa = 2.8, log P = 1.9) was from Fluka-Sigma-Aldrich (Italy). Cholesterol (CH), l-α-phosphatidylcholine (PC), potassium glycyrrhizinate (KG), stearylamine (SA), and dicethylphosphate (DP) were provided by Sigma-Aldrich (Italy).

All other reagents were of analytical grade.

Software for Experimental Design

The software NEMRODW was used for generation and evaluation of the statistical experimental design (Mathieu et al., 2000).

Liposome Preparation

Multilamellar vesicles (MLV) were prepared by thin-layer evaporation (TLE). According to this technique (Mura et al., 2007), the lipid phase (consisting of a mixture of 50 mg PC 40 mg of CH or KG, and 10 mg of DP or SA), was dissolved in chloroform, which was then removed under reduced pressure in a rotary evaporator at 58°C, thus obtaining a thin film of dry lipid on the flask wall.

Evaporation was continued for 1h after the dry residue appeared, to completely remove all the traces of the solvent.

The film was then hydrated by adding a variable volume of an ethanol/water mixture (at different v/v ratios) under vigorous mechanical shaking with a vortex mixer

until vesicle formation. The BZC was dissolved at 1.0% w/v in hydrophilic phase during liposome preparation.

All products were sealed in glass containers and stored in the dark at 4°C.

Liposome Characterization

Determination of Liposomal Size. The average particle size of the vesicles was determined by quasielastic light scattering (QELS) using a Brookhaven Instrument (New York, NY) endowed with a BI-9000AT correlator card and a BI-200 SM goniometer. The light source was the double frequency of a Coherent Innova diode pumped Nd-YAG laser, (λ= 532 nm, 20 mW).

The laser long-term power stability was ±0.5%. Self-beating detection was recorded using decahydronaphthalene (thermostated by a water circulating system) as index matching liquid.

A probe was inserted in the sample to monitor temperature while simultaneously recording autocorrelation functions; the temperature was set at 20°C. The intensity of the laser light scattered by the samples was detected at an angle of 90°with an EMI 9863B/350 photomultiplier.

Liposome suspensions were suitably diluted with distilled water in order to avoid multiscattering phenomena. At least three independent samples were taken, each of which was measured at least twice, up to four times.

For each specimen, 10 autocorrelation functions were analyzed using a cumulative analysis. Samples were analyzed 24h after their preparation.

Determination of Encapsulation Efficiency. Liposome encapsulation efficiency was determined using the dialysis technique for separating the nonentrapped drug from liposomes (Maestrelli et al., 2005).

The suitability of this method (which gave results comparable to those obtained by the ultracentrifugation technique) has been previously demonstrated (Maestrelli et al., 2005; Mura et al., 2007).

According to this method, 3 mL of drug-loaded liposomal dispersion were put into a cellulose acetate dialysis bag (Spectra/Por®, MW cutoff 12000, Spectrum, Canada) immersed in a closed vessel containing 150 mL of a 50:50 v/v ethanol/water solution at 20°C, magnetically stirred at 30 rpm. Samples, withdrawn at given time

intervals from the receiver solution, were replaced with equal volumes of fresh solvent. The BZC was spectrometrically assayed at 282 nm (UV-1601 Shimadzu).

The experiment was stopped when constant drug concentration values were obtained in subsequent withdrawals from the receiver phase (taking into account the progressive dilution of the medium).

The percent of encapsulation efficiency (EE%) was calculated according to the following equation:

EE%= [total drug]-[diffused drug]/[total drug]*100

Each result is the mean of at least three separate experiments.

In Vitro Permeation Studies Through Artificial Membrane

Permeation studies of BZC from the different formulations through artificial membranes were performed for 24h at 37 ± 1°C using a Sartorius Model SM 16750 apparatus (Sartorius Membranfilter GmbH, Gottingen, Germany).

A cellulose nitrate membrane (effective permeation area 40 cm2) impregnated with lauryl alcohol (membrane weight increase 90–110%) as lipid phase was used as artificial lipophilic membrane simulating the epidermal barrier (Maestrelli et al., 2005; Mura et al. 1993).

The receiver phase consisted of 100 mL degassed pH 7.4 phosphate buffer solution continuously circulating on the lower side of the diffusion cell by means of a peristaltic pump. Permeation experiments were performed in non-occlusive mode.

The upper side of the diffusion cell (donor compartment) was filled with 1.5 g of the liposome suspension.

Sink conditions were maintained for the duration of diffusion experiments.

At given intervals, 3 mL samples were withdrawn from the receiver phase and spectrometrically assayed for drug content at 285 nm. No interference was found for other components. Samples were replaced with equal volumes of fresh receptor medium and the correction for the cumulative dilution was calculated.

Experiments were repeated three times. The percent of drug permeated into the receptor compartment as a function of time was calculated and the results were averaged.

Gel Preparation

A 0.5 % w/v Carbopol gel base was prepared by suspending 0.5 g Carbopol 940 in 99.5 mL of bidistilled water, stirring for 24h at room temperature and then adding triethanolamine up to pH 7.0 for gelification.

The resulting gel was stored in capped glass containers, at 4°C, in the dark.

Gels loaded with the drug were prepared by mixing (50/50 w/w) Carbopol gel with a 1% w/v BZC aqueous solution or the different 1% w/v BZC liposomal suspensions, thus obtaining the final drug concentration of 0.5 % w/w.

In Vivo Studies

The anaesthetic activity of BZC formulated in aqueous Carbopol gel, as such or entrapped in liposomes, was assayed in vivo in albino rabbits according to the conjunctival reflex test (Ghelardini et al., 2001; Mura et al., 2007).

Male albino rabbits (2.5–3.0 kg body weight) from Morini (San Polo d'Enza, Italy) were used. One rabbit was housed per cage and placed in the experimental room 24h before the test for acclimatization. The animals were kept at 23 ± 1°C with a 12h light/dark cycle, fed with a standard diet and tap water ad libitum.

All experiments were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, by making all efforts to minimize animal suffering and to limit the number of animals used.

Rabbits were divided into as many groups (each formed by six rabbits) as the number of formulations to test. A fixed amount of each sample was instilled in the conjunctival sac of the right eye of the rabbit, whereas a corresponding blank formulation (without drug) was simultaneously instilled in the left eye as control.

The external sides of rabbit eyes were then stimulated at interval times with a cat whisker to induce the conjunctival reflex and, consequently, the palpebral closure.

The local anaesthetic activity of the drug is indicated by the necessity of a higher number of stimuli to provoke the reflex.

8.2 Results and Discussion

In the present study, a multivariate strategy (Lewis et al., 1999) was utilized for systematically evaluating the effects of variations of the composition of a liposomal-BZC formulation on encapsulation efficiency (EE%) and percent drug permeated after 180 min (*P*%), chosen as the responses to be optimized.

We started from the liposomal formulation that resulted in the best outcome in our previous study (Mura et al., 2007), consisting of a 50:50 w/w phosphatidylcholine/cholesterol mixture as lipid phase and of 10 mL of a 50:50 v/v ethanol/water mixture as hydrophilic phase.

As for the lipophilic phase, we considered it worthy of interest to evaluate the effects of modifying the bilayer composition by replacing cholesterol with potassium glycyrrhizinate and adding a cationic (stearylamine) or anionic (dicethylphosphate) surfactant.

These additives were selected based on previous studies, in which their presence in the lipid bilayers of the vesicles gave rise to higher drug skin-delivery (Fang et al., 2006; Mohammed et al., 2004; Trotta et al., 2004).

On the other hand, with regard to the hydrophilic phase, the effects of varying the percent (v/v) of ethanol in the hydroalcoholic mixture and the volume of the hydration phase were judged as important factors to be investigated, considering both the solubilizing power towards BZC and the potential permeation enhancer role of ethanol. Experimental design (Lewis et al., 1999) was the selected multivariate strategy for the optimization of the drug permeation, and the independent variables and their respective levels were:

U1: cholesterol (CH) or potassium glycyrrhizinate (KG)

U2: stearylamine (SA) (cationic surfactant) or dicethylphosphate (DP) (anionic surfactant)

U3: % ethanol (40, 50, or 70 % (v/v))

U4: hydration phase volume (4, 7, or 10 mL)

The following qualitative model was hypothesized among responses and factors under study that contained one constant plus, for each factor, a number of terms equal to its number of levels minus one (Broudiscou et al., 1996):

$$y = b_0 + b_1 A(x_1 A) + b_2 A(x_2 A) + b_3 A(x_3 A) + b_3 B(x_3 B) + b_4 A(x_4 A) + b_4 B(x_4 B)$$

where *y* represents the response; *b*1*A* and *b*2*A* are the coefficients relative to the effect on the response of the level changes of the factors U1 and U2, respectively; whereas *b*3*A, b*3*B, b*4*A,* and *b*4*B* are the coefficients relative to the changes of level of the factors U3 and U4, respectively.

The effects of variations in the factor levels on the responses was evaluated by a ninerun D-optimal matrix (Table 1). This matrix was obtained by applying a D-optimal algorithm to an asymmetric screening design, which enables a rapid examination of factors at different numbers of levels (Lewis et al., 1999).

In compliance with this experimental plan, nine different liposome batches were then prepared (Table 2).

Table 1
Nine-run asymmetric screening matrix

Trials	Variable U_1	Variable U_2	Variable U_3	Variable U_4
1	1	1	1	1
2	1	2	2	2
3	1	1	3	3
4	2	1	2	3
5	2	2	3	1
6	2	1	1	2
7	1	1	3	2
8	1	2	1	3
9	1	1	2	1

Table 2
Experimental plan and observed responses (encapsulation efficiency (EE%) and percent drug permeated at 180 min (P%)) for liposomal formulations containing cholesterol (CH) or potassium glycyrrhizinate (KG), stearylamine (SA) or dicethylphosphate (DP) and different % of ethanol and volumes of hydration phase

Sample	Variable U_1	Variable U_2	Variable U_3 (% ethanol)	Variable U_4 (mL hydr. phase)	EE%	P%
1	CH	SA	40	4	32.93	8.07
2	CH	DP	50	7	25.76	37.47
3	CH	SA	70	10	32.89	16.80
4	KG	SA	50	10	43.85	17.73
5	KG	DP	70	4	57.11	8.28
6	KG	SA	40	7	21.02	13.73
7	CH	SA	70	7	30.36	11.33
8	CH	DP	40	10	9.97	23.01
9	CH	SA	50	4	51.84	4.71

Light scattering analysis of the obtained batches showed that they had substantially homogeneous dimensions.

In fact, mean particle size of the vesicles was not greatly influenced by variations in the lipid bilayer composition, and it ranged from a minimum value of 490 ± 70 nm to a maximum of 600 ± 90 nm for the liposomal dispersions corresponding, respectively, to samples 5 and 9 in Table 2.

These batches were then evaluated in random order for both encapsulation efficiency and percent drug permeated after 180 min.

Statistical evaluation of the experimental results showed that, for both responses, the regression model explained the response variation due to the change in level factors.

The graphic analysis of the effects was used to evaluate the different effect of factor levels and determine the most suitable level of each variable to be selected for optimizing the considered response (Furlanetto et al., 2006).

The results of the graphic analysis are presented in Fig. 1.

In particular, coefficients A, B, C, and D indicate the effects on the response due to the change of level of the factors U1, U2, U3, and U4, respectively.

The lengths of the bars are related to the effects of the level on the response.

The bars with maximum lengths are those relative to the levels that determine a maximization of the response.

On the other hand, bars of similar length are indicative of factors whose level change has similar effects on the response.

The obtained results point out that maximization of the two selected responses requested opposite levels of the considered factors.

In fact, for example, in the case of factor U1, we would have to use the level 2 (KG) for maximizing EE%, and instead the level 1 (CH) for *P*%.

This finding was in disagreement with literature data (Trotta et al., 2004), where the presence of KG, rather than CH, resulted in more elastic liposomes, which were able to produce an increase in the amount of permeated drug.

Possible interactions of KG with the ionic surfactant present in the bilayer structure could reduce its “elastic” properties and thus be responsible for this unexpected result.

On the other hand, in the case of factor U2 (the ionic surfactant), the level 1 (SA) was better for EE% and, on the contrary, the level 2 (DP) for *P*%.

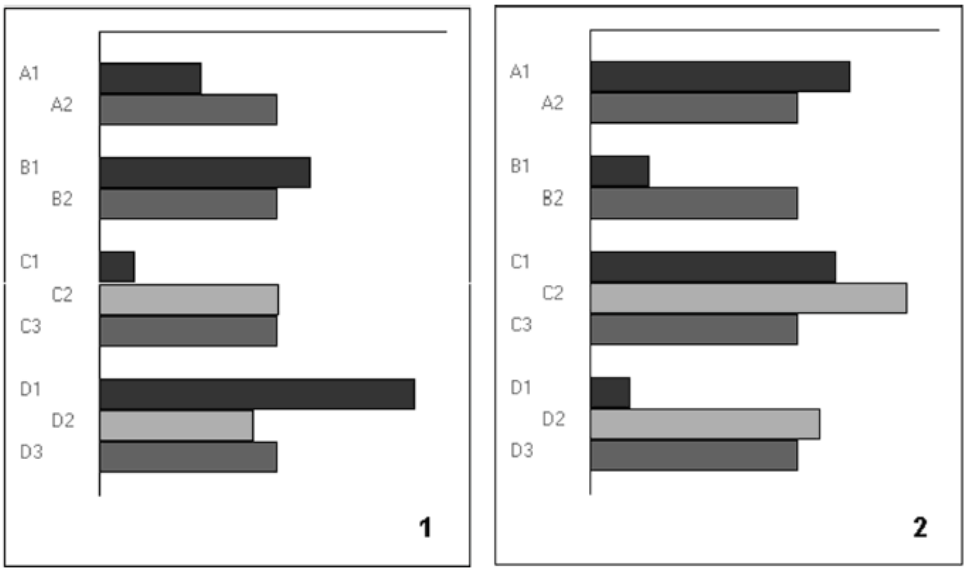


Figure 1. Graphic analysis of effects of factor levels on (1) encapsulation efficiency and (2) percent drug permeated at 180 min. A: Cholesterol (A1) or potassium glycyrrhizinate (A2); B stearylamine (B1) or dicethylphosphate (B2); C: % EtOH (C1 = 40, C2 = 50, C3 = 70); D: hydration phase volume (D1 = 4, D2 = 7, D3 = 10 mL). The length of each bar indicates the relative weight of the level of each considered factor.

The higher effectiveness of SA vs. DP in improving drug loading into the vesicles, and at the same time its lower efficacy in promoting drug permeation, could presumably be due to electrostatic repulsive forces between the amino groups of BZC and SA molecules, as has been previously observed for other cationic drugs (Webb et al., 1995).

In fact, considering that the drug is dissolved in the inner hydro-alcoholic phase of the vesicles, these repulsion forces could decrease the drug's ability to cross the phospholipid bilayer.

These results, as well as those related to the other considered variables (percent of ethanol and mL of hydration phase) all indicate that the factors favorable for improving the affinity of the drug for the liposomal structure and thus its incorporation into the vesicles (expressed by EE%) gave rise instead to a decrease of its permeation rate, and vice versa.

Therefore, considering that drug permeation properties play a more important role than the encapsulation efficiency in determining the therapeutic efficacy of the liposomal BZC formulation, it was decided to favor *P*% as the most important response to be maximized.

For this reason, the second step of the statistical optimization involved only this response.

Consequently, we fixed the qualitative factors, choosing CH as stabilizing agent and DP as ionic surfactant, as indicated by the graphic analysis of the effects for maximizing *P*%, and we continued the study of the quantitative factors (percent ethanol in the hydroalcoholic mixture and volume of the hydration phase) in a wider experimental domain, in order to investigate their effects on *P*% in greater detail.

A response surface study was then carried out by means of a 9-run Doehlert design for two factors.

According to this plan, seven distinct liposomal formulations were produced in random order and a total of nine permeation experiments were performed, including a threefold repetition of the central point.

The experimental plan and the obtained responses are reported in Table 3.

Figure 2 shows some representative drug permeation profiles obtained from these experiments. Analysis of variance (ANOVA) indicated that the assumed regression model was significant and valid (Lewis et al., 1999).

From the response surface obtained (Fig. 3) it is possible to point out that poor results are obtained using low levels of both factors, thus revealing a negative interaction between the two factors.

Table 3
Experimental plan of Doehlert design and obtained response (percent drug permeated at 180 min, P%)

Trials	Variable U_1 (% ethanol)	Variable U_2 (mL hydr. phase)	P%
1	70	15	16.24
2	70	3	15.33
3	96	12	21.15
4	44	6	13.71
5	44	12	17.00
6	96	6	22.43
7	70	9	12.47
8	70	9	14.94
9	70	9	14.03

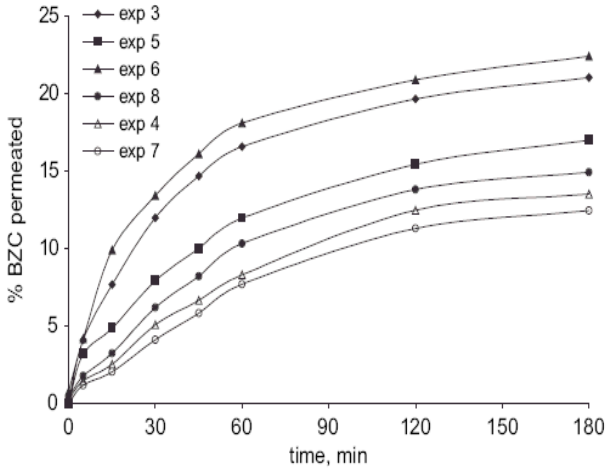


Figure 2. Permeation profiles of benzocaine (BZC) from some representative liposomal formulations prepared according to the Doehlert design (see Table 3).

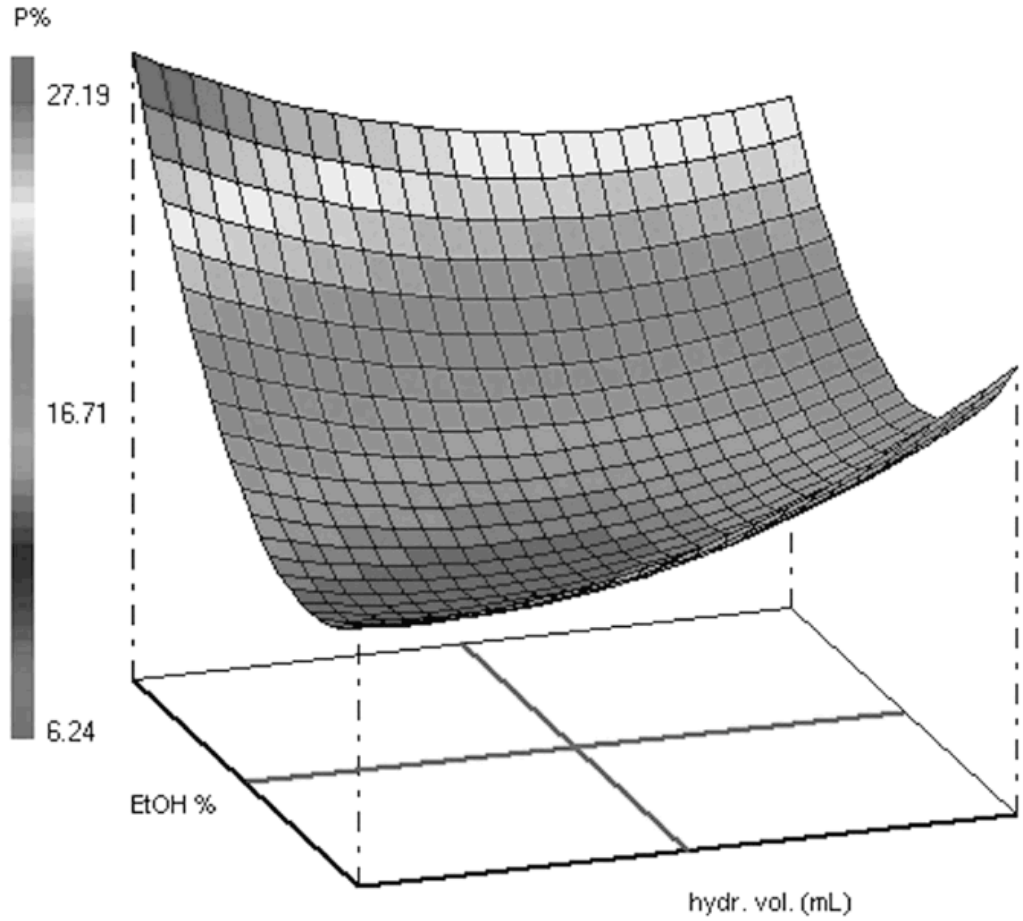


Figure 3. Response-surface plot of percent drug permeated at 180 min (*P*%) obtained by plotting % EtOH (v/v) against hydration phase volume (mL).

However, the positive principal effect of the factor percent ethanol is clearly the most important one.

In fact, when this factor is at a high level, the response is always high, whatever the value of the hydration phase volume.

Obviously, due to the negative interaction between the variables, the best results can be obtained using high values of percent ethanol and low values of hydration phase volume. In particular, the maximum response is obtained with 96% ethanol and 3 mL of hydration phase volume.

Therefore, a BZC liposomal formulation was prepared according to the results of the statistical experimental design (lipid phase: PC-CH-DP 50/40/10 w/w; hydration phase: 3 mL of 96% ethanol solution) and evaluated for drug permeation properties in comparison with the best formulation of our previous study (Mura et al., 2007) tested in the same conditions (Fig. 4).

The experiment was repeated three times and the experimentally obtained mean *P*% value at 180 min (23.8 ± 1.3) was found inside the confidence interval ($P < 0.05$), calculated around the predicted value (24.8%) of the response (Lewis et al., 1999). Moreover, the drug permeation profile from the optimized formulation was clearly better, not only than all the previously examined liposomal formulations (Fig. 2), but also than that of the starting reference formulation one (Fig. 4).

The optimized BZC liposomal dispersion, together with the starting reference preparation, was then formulated as Carbopol aqueous gel to test its anaesthetic activity in the rabbit model in comparison with a gel containing a solution of the plain drug at the same concentration.

It was necessary to use gel formulations, since they allowed deposition of a more constant and reproducible amount of the drug with respect to liquid formulations, and therefore reduced the variability of the in vivo experimental conditions.

Results of in vivo studies, summarized in Table 4, confirmed our previous findings (Mura et al., 2007), showing that the entrapment of BZC in liposomes allowed a statistically significant extension ($P < 0.01$) of its duration of activity (from 30 to 60 min) and also a significantly stronger anaesthetic power with respect to the formulation containing the plain drug.

Moreover, the optimized formulation exhibited a further significant ($P < 0.05$) improvement of the intensity of the therapeutic effect during the first 15 min in comparison with the starting BZC liposomal reference preparation.

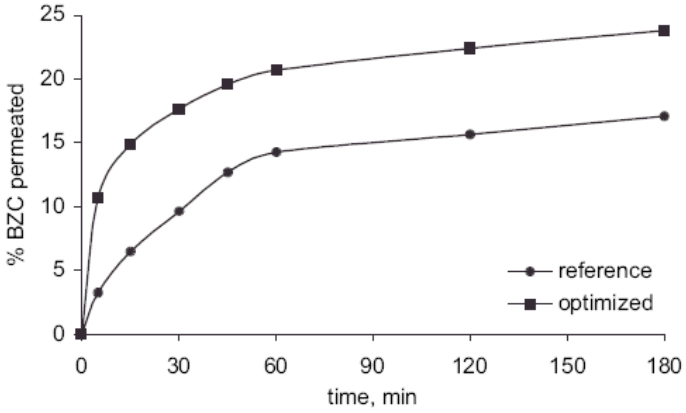


Figure 4. Comparison of the permeation profile of benzocaine (BZC) from the optimized liposomal formulation and from the reference one.

Table 4
Effect induced by the benzocaine (BZC) liposomal formulations on the rabbit conjunctival reflex test

		Number of stimuli to induce conjunctival reflex							
		Time after treatment							
Sample	Eye	*5 min*	*10 min*	*15 min*	*20 min*	*25 min*	*30 min*	*40 min*	*60 min*
Control (empty gel)	left	1.0 ±0.0	1.0 ± 0.0	1.1 ± 0.2	1.5 ± 0.3	1.4 ± 0.2	1.2 ± 0.2	1.0 ± 0.0	1.0 ± 0.0
Control (empty gel)	right	1.0 ± 0.0	1.0 ± 0.0	1.2 ± 0.3	1.2 ± 0.2	1.3 ± 0.3	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Gel + aqueous solution	left	1.1 ± 0.1	1.3 ± 0.3	1.4 ± 0.4	1.2 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Gel + 0.5%BZC aqueous sol.	right	25.5 ± 2.8*	21.4 ± 3.3*	19.8 ± 2.6*	17.8 ± 2.5*	16.1 ± 1.7*	14.1 ± 1.6*	2.3 ± 0.2	1.0 ± 0.0
Gel + liposomal reference form.	left	1.1 ± 0.1	1.8 ± 0.4	1.6 ± 0.3	1.5 ± 0.3	1.7 ± 0.2	1.9 ± 0.5	1.0 ± 0.0	1.0 ± 0.0
Gel + BZC lipos. ref. form	right	37.5 ± 2.6*^	35.8 ± 4.2*^	32.4 ± 3.4*^	28.6 ± 3.3*^	26.2 ± 3.2*^	23.5 ± 2.7*^	11.6 ± 3.5*^	5.8 ± 1.9*^
Gel + liposomal optimised form. solution	left	1.1 ± 0.1	1.3 ± 0.5	1.2 ± 0.2	1.6 ± 0.2	1.4 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Gel + BZC lipos. optimised form.	right	46.5 ± 2.2*^°	42.4 ± 3.5*^°	39.2 ± 3.2*^°	29.4 ± 3.2*^	25.5 ± 3.0*^	22.4 ± 3.1*^	12.3 ± 2.9*^	4.7 ± 1.7*^

There were 6 rabbits per group. Each value represents the mean of six separate experiments. *P< 0.01 in comparison with control; ^P<0.01 in comparison with gel+BZC solution; ^P < 0.05 in comparison with the ref. liposomal gel formulation.

In conclusion, experimental design strategy was confirmed as a very useful tool in preformulation studies, allowing a rational approach to the development of effective liposomal formulations.

In particular, graphic analysis of the effects enabled identification of the formulation factors active on the considered responses, and determination of their best level for response optimization.

Moreover, the subsequent response-surface study pointed out a negative interaction between percent ethanol and volume of the liposome hydration phase, and

allowed prediction of the best formulation conditions for maximizing BZC permeation rate.

A good correspondence ($P < 0.05$) has been found between the predicted and the experimental checkpoint, thus demonstrating the suitability of the proposed approach to optimize the composition of liposomal formulations and to predict the effect of formulation variables on the considered experimental response.

Moreover, in vivo studies showed that the optimized formulation gave rise to a further significant ($P < 0.05$) improvement of BZC therapeutic efficacy with respect to the previously found best liposomal BZC formulation (Mura et al., 2007), thus confirming the relationship between the in vitro drug permeation rate and the intensity of its anaesthetic effect, and demonstrating the actual effectiveness of the proposed approach for the liposomal formulation optimization.

9. Development of benzocaine ethosomes. Influence of the preparation method on the properties and in vivo efficacy of drug-loaded ethosomes.

In my previous study (Mura et al. J. Lipos. Res. 2008), I found that vesicles containing ethanol in relatively high concentrations, therefore named "ethosomes", revealed to be more efficient than classic liposomes (Mura et al., 2007) at delivering benzocaine through the skin in terms of quantity and depth according to the results obtained for other drugs (Touitou et al., 2000; Esposito et al., 2004; Godin and Touitou, 2003; López-Pinto et al., 2005).

Several authors evidenced that ethosomal formulations were able to enhance permeation through the stratum corneum barrier, improve drug skin accumulation and assure sustained drug release (Dayan and Touitou, 2000, Paolino et al., 2005).

These effects are attributable not only to the solvent action of ethanol on the stratum corneum of the skin but also to the higher deformability and malleability of these vesicles that can better penetrate intact through the skin (Elsayed et al., 2006; Elsayed et al., 2007; Dubey et al., 2007).

We previously demonstrated that classic liposomal formulations of BZC allowed a significant improvement of its therapeutic efficacy in terms of both intensity of anaesthetic effect and duration of action with respect to plain drug (Mura et al., 2007). Optimization, by means of an experimental design methodology, of the composition of BZC liposomal formulation enabled a further significant improvement of the drug anaesthetic efficacy with respect to the initial formulation (Mura et al., 2008).

The hydrophilic phase of the optimized composition was composed of a 96% v/v ethanol-water solution (Mura et al., 2008), and thus the obtained vesicles can be considered as ethosomes (Touitou et al., 2000).

The lipophilic phase consisted instead of a cholesterol-phosphatidylcoline-dicethylphosphate (50:40:10 w/w) mixture (Mura et al., 2008); the presence of the anionic surfactant dicethylphosphate improved the flexibility of the vesicle membrane 7 n and then its ability to penetrate into the skin (Trotta et al., 2004, Fang et al., 2006).

However, it should be considered that the effectiveness of a liposomal formulation is clearly influenced not only by the composition of the vesicles, but also by their structure and dimensions, which are both closely connected with the technique

used for their preparation (Walde and Ichikawa, 2001; Maestrelli et al., 2006). Therefore we considered it worth of interest to extend our previous studies and, by keeping constant the vesicle composition, to investigate in depth the role of the preparation method on both the vesicle properties and the drug anaesthetic efficacy. Toward this purpose, ethosomes consisting of the optimized composition (Mura et al., 2008) were prepared by different methods, namely thin layer evaporation, freezing and thawing, reverse phase evaporation, extrusion and sonication techniques, thus obtaining, respectively, multi-layer vesicles (MLV), frozen and thawed MLV (FATMLV), large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV).

The aim of this work was to find the most effective operative conditions to improve the effectiveness of BZC-loaded ethosomes in terms of both encapsulation efficiency and vesicle stability, as well of drug therapeutic efficacy.

All ethosomal systems were characterized for drug encapsulation efficiency, particle size, Zeta-potential and morphology by using, respectively, dialysis, light scattering, Transmission Electron Microscopy and Confocal Laser Scanning Microscopy techniques, and checked for stability during storage at 4°C.

Drug permeation behaviour from ethosomal gel formulations was evaluated *in vitro* by using artificial lipophilic membranes simulating skin behaviour, whereas the BZC anaesthetic efficacy was tested *in vivo* on rabbits.

9.1 Materials and methods

Materials

Benzocaine base (BZC) (ethyl-4-aminobenzoate) (pKa=2.8, log P=1.9 (Clarke's, 2004), cholesterol (CH), dicethylphosphate (DP), and 1-α-phosphatidylcholine (PC) were provided by Fluka-Sigma-Aldrich (Italy).

All other reagents were of analytical grade.

Preparation of ethosomes

Ethosomes consisting of mixtures of PC, CH and DP (50/40/10 w/w) as lipid phase and 96% v/v ethanol-water as hydration phase (Mura et al. 2008) were prepared by different techniques.

Multi-lamellar Vesicles (MLV) were obtained by thin layer evaporation (TLE) (Bangham et al., 1965).

Briefly, 100 mg of lipid mixture were dissolved in chloroform and evaporated under vacuum until solvent removal, thus obtaining a thin film of dry lipid on the flask wall. Evaporation was continued for 2 h after the dry residue appeared, to completely remove all traces of solvent.

The film was then hydrated by adding the hydrophilic phase, containing 1% w/v of dissolved BZC, under vigorous stirring with a vortex mixer until vesicle formation.

Frozen and thawed MLV (FATMLV) were obtained by submitting a given volume of MLV suspension to nine alternate cycles of freezing in liquid nitrogen for 30 s and thawing in a bath at 58°C for 30 s (Mayer et al., 1985).

Large Unilamellar Vesicles (LUV) were realized by reverse phase evaporation method (Szoka, 1978).

In brief the lipid phase was dissolved in diethylether and then mixed with the hydrophilic phase containing 1% w/v of BZC (organic phase: hydrophilic phase 3:1 v/v) in a ultrasound bath (Transonic 460 H, Singen) at 0°C for 5 min at 355W, to obtain a water-in-oil emulsion.

After removal of organic solvent under reduced pressure, a gel was formed that, upon vigorous mechanical agitation, evolved into a colloidal dispersion of large vesicles.

Small Unilamellar vesicles (SUV) were prepared by extrusion or sonication. Extruded SUV (SUV ext) were obtained by forcing 2 mL of FATMLV for 5 times through a 0.2 nm polycarbonate membrane in a mini-extruder (Avanti Polar Lipid, Birmingham, AL).

Sonicated SUV (SUV son) were realized by submitting MLV suspensions to ultrasonication (58 °C, 355 W energy, input time 60 s, idle time 60 s, with 10 cycle iterations (Mura et al., 2007). All batches were stored at 4°C.

All products were quickly sealed in glass containers and stored in the dark at 4°C.

Determination of entrapment efficiency (EE%) of ethosomes

Ethosomes entrapment efficiency (EE%) was indirectly measured by determining the amount of the non-entrapped drug using the dialysis method, according to a previously developed procedure, as reported in the chapter 8.1.

Determination of particle size and Zeta-potential of ethosomes

The average diameter and Zeta-potential of the ethosomes were determined with a Zetamaster apparatus (Malvern Instruments, Malvern, UK) at a temperature of 25±0.1 °C. Samples were analyzed 24 h after preparation.

For the particle size measurement, ethosome dispersions were properly diluted with distilled water, in order to avoid multiscattering phenomena.

The intensity of the laser light scattered by the samples was detected at an angle of 90° with a photomultiplier.

At least three independent samples were taken, each of which was measured at least twice, up to four times. For each specimen, 10 autocorrelation functions were analysed using a cumulant analysis.

From this analysis, the *z*-average value was obtained, which approximates the diameter of the liposomes.

The particle size distribution was characterised by means of the polydispersity index (P.I.), which is indicative of the width of the size distribution.

As for surface charge determination, ethosome dispersions, suitably diluted with distilled water, were dropped into the Zetamaster electrophoretic cell and the Zeta-potential determined by electrophoretic mobility (μ) measurements.

The mobility μ was converted into Zeta-potential by the Smoluchowski equation $Z=\mu\eta/\varepsilon$, where η is the viscosity and ε the permittivity of the solution.

For each batch were taken at least 5 independent samples, each of which was analyzed at least 3 times.

Stability studies

In order to investigate and compare the stability during the time of the different kinds of vesicles, each batch of ethosomes was stored at 4°C during 3 months.

At prefixed time intervals, samples were withdrawn and characterized for their mean particle size and EE% values.

Transmission Electron Microscopy (TEM)

The morphology of ethosomes was investigated by Transmission Electron Microscopy (TEM) (Philips CM 10, Philips, USA).

Copper grids were coated with a collodion solution and then a drop of ethosomal dispersion was applied and kept in contact for 15 min.

Finally, grids were picked up, blotted with filter paper, left dry for 3 min and then examined with TEM (Manosroi et al., 2004).

Confocal Laser Scanning Microscopy (CLSM)

CLSM studies were carried out to investigate the ethosome morphology and lamellarity directly in solution.

Towards this aim, ethosomal dispersions were prepared by adding a hydrophobic fluorescent probe, i.e. rhodamine 6G, in the hydration phase.

Analyses were carried out on a drop of suitably diluted, freshly prepared ethosomal dispersion using a Leica TCS SP II Laser Scanning Confocal Imaging System (Leica, Heidelberg, Germany).

The apparatus was equipped with a Kr-Ar-He-Ne ion laser and a Leica DM IRE 2 inverted microscope endowed with HC PL Fluotar Leica X10 and X20 dry objectives and HCX PLAN APO Leica X40 multi-immersion objective on its oil position (numeric aperture 0.85). Samples were analyzed by using the transmitted light and fluorescence (λ_{exc}= 488 nm, λ_{em}= 520 nm) (López-Pinto et al., 2005).

Gel preparation

A 0.5 % w/v Carbopol gel base was prepared according to previously developed procedure has reported in the chapter 8.1.

In vitro Permeation Studies

In vitro permeation studies from Carbopol gel formulations containing the drug, as such or entrapped in the different types of ethosomal vesicles, were performed for 3 h at 37±1°C using Franz diffusion cells (Vidrafoc, Barcelona, Spain), with an effective diffusion area of 2.54 cm^2 and a receiver compartment of 14.5 ml volume (degassed pH 7.4 phosphate buffer solution).

The donor compartment was filled with 3 ml of gel. Cellulose nitrate membranes impregnated with lauryl alcohol (membrane weight increase 90-110 %) as lipid phase were used as artificial diffusion membranes simulating the epidermal barrier (Maestrelli et al., 2005).

At appropriate intervals, 0.5 mL samples were withdrawn from the receiver phase and spectrometrically assayed for drug content at 282 nm.

No interference was found for other components. Samples were replaced with equal volumes of fresh receptor medium and the correction for the cumulative dilution was calculated. Experiments were repeated three times.

The % of drug permeated into the receptor compartment as a function of time was calculated and the results were averaged (C.V. < 1.5 %).

In vivo studies

The anaesthetic activity of BZC formulated in aqueous Carbopol gel, as such or entrapped in the different kinds of ethosomal vesicles, was assayed *in vivo* in albino rabbits according to the conjunctival reflex test (Ghelardini et al., 2001), using the same procedure as reported in the chapter 8.1.

9.2 Results and discussion

Characterization of ethosomes

The mean entrapment efficiency (EE%), particle size and Zeta-potential values of freshly-prepared vesicles obtained with the different examined procedures are collected in Table 1.

Table 1: Drug entrapment efficiency (EE%), particle size, polydispersity index (P.I.) and Zeta-potential of the different kinds of ethosome vesicles

SAMPLE	EE ± s.d. (%)	diameter ± s.d. (nm)	P.I.	Zeta-potential ± s.d. (mV)
MLV	94.8±2.1	392.7±0.5	0.2	-24.7±0.8
LUV	90.9±2.3	484.3±10.0	0.3	-43.5±0.5
FATMLV	79.2±4.9	586.9±30.0	0.3	-41.5±0.9
SUV (by sonication)	60.7±7.2	189.3±13.7	0.3	-20.4±7.4
SUV (by extrusion)	86.8±6.5	295.8±33.2	0.4	-21.5±1.1

As it is evident, MLV and LUV vesicles showed the highest EE% values, followed by SUV vesicles obtained by extrusion, then by FATMLV and, finally, by SUV obtained by sonication.

These results were found to be dependent not only by the vesicles characteristics, such as in particular their dimensions, but also by the method used for their preparation. In fact, for example, the lower EE% value exhibited by FATMLV with respect to MLV vesicles, in spite of their higher particle size, can be attributed to the particular conditions used for their manufacture procedure.

In fact, the alternating cycles of freezing and thawing of MLV vesicles required by FATMLV preparation method give rise to larger vesicles than the initial ones, but can bring about to some loss of the entrapped drug (Maestrelli et al., 2006).

On the other hand, SUV vesicles obtained by sonication showed the lowest EE% value.

The reduced volume of the inner compartment with respect to the other formulations, due to the smallest dimensions of these uni-lamellar vesicles, can be considered the main factor responsible for this finding.

In agreement with this consideration, the better EE% of SUV vesicles obtained by extrusion with respect to those prepared by sonication is attributable to their greater dimensions.

At this regards, it should be pointed out that SUV extruded vesicles showed a mean particle size clearly higher (+47.5%) with respect to the pores of the membrane used for their extrusion.

This phenomenon confirms the flexibility and deformability of these vesicles, which is attributable to the presence of both the anionic surfactant and ethanol in their structure (Trotta et al., 2004; Fang et al., 2006).

However, a reduction in homogeneity in this batch can be evidenced, as indicated by the increase in polydispersity index.

As for the surface charge, measurements of Zeta-potential values indicated that all the kinds of vesicles showed a sharp negative charge, which is mainly attributable to the presence of the negatively charged lipid dicethylphosphate.

Stability studies

As well known, the poor stability during storage represents a critical point in the development of effective vesicular systems, even though ethosomes seem to be more stable with respect to conventional liposomes (Dubey et al., 2007).

Therefore, in order to investigate their stability on ageing, each lot of vesicles was stored at 4°C for 3 months and, at prefixed time intervals, samples were collected and analysed to determinate EE% (Figure 1) and particle size (Figure 2).

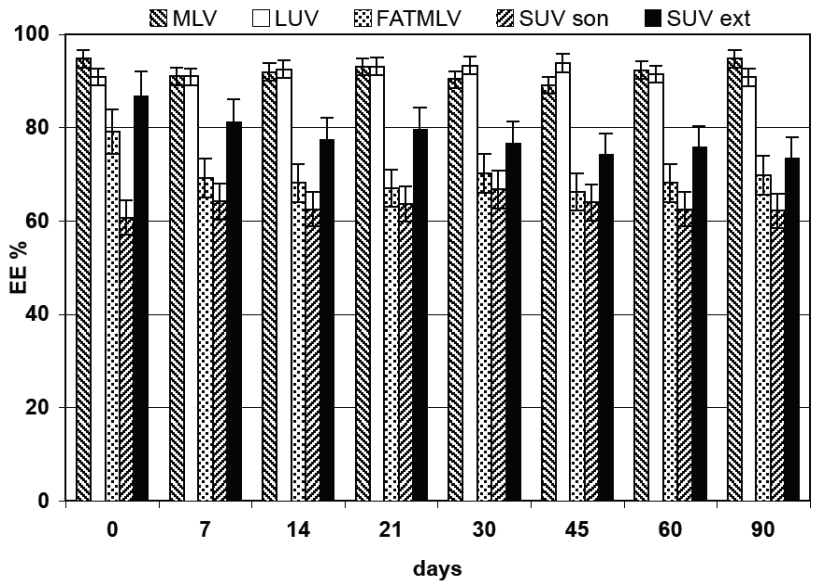


Figure 1

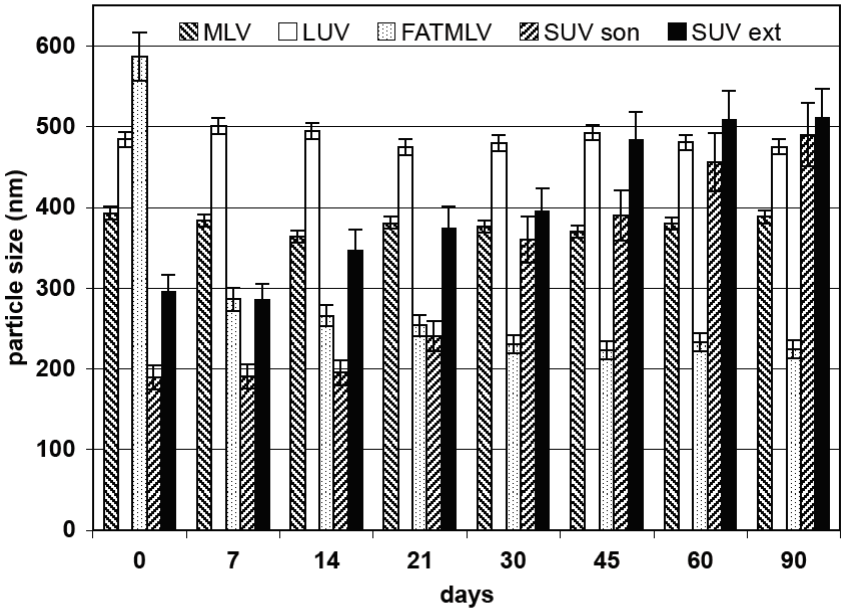


Figure 2

MLV and LUV vesicles resulted substantially stable in terms of both drug entrapping yield and particle dimensions, which remained almost unchanged during the considered storage period.

Instead, FATMLV vesicles revealed a rapid decrease in particle size during the storage, probably due to the untidy and weak structure of their membranes that is easily

disrupted, and undergo rearrangements in vesicles of reduced dimensions and irregular morphology (Maestrelli et al., 2006).

On the contrary, SUV vesicles showed a marked tendency to form aggregates, which was attributable to the very high surface area exposed to the dispersion medium, due to their smaller dimensions.

This phenomenon was particularly evident for vesicles obtained by extrusion. Therefore, also considering their less homogeneous dimensional distribution, SUV vesicles obtained by extrusion were excluded from further studies.

Morphological studies

Morphological analysis of vesicles performed by TEM and CLSM revealed that all kinds of ethosomal dispersions consisted of homogeneous, regular, spherical-shaped vesicles, with the exception of FATMLV ones, which showed a less regular morphology due to the "traumatic" preparation method, as reported also by other authors (Mayer et al., 1985; Maestrelli et al., 2006).

The size distribution of all ethosomal dispersions was in good agreement with findings of previous light scattering measurements.

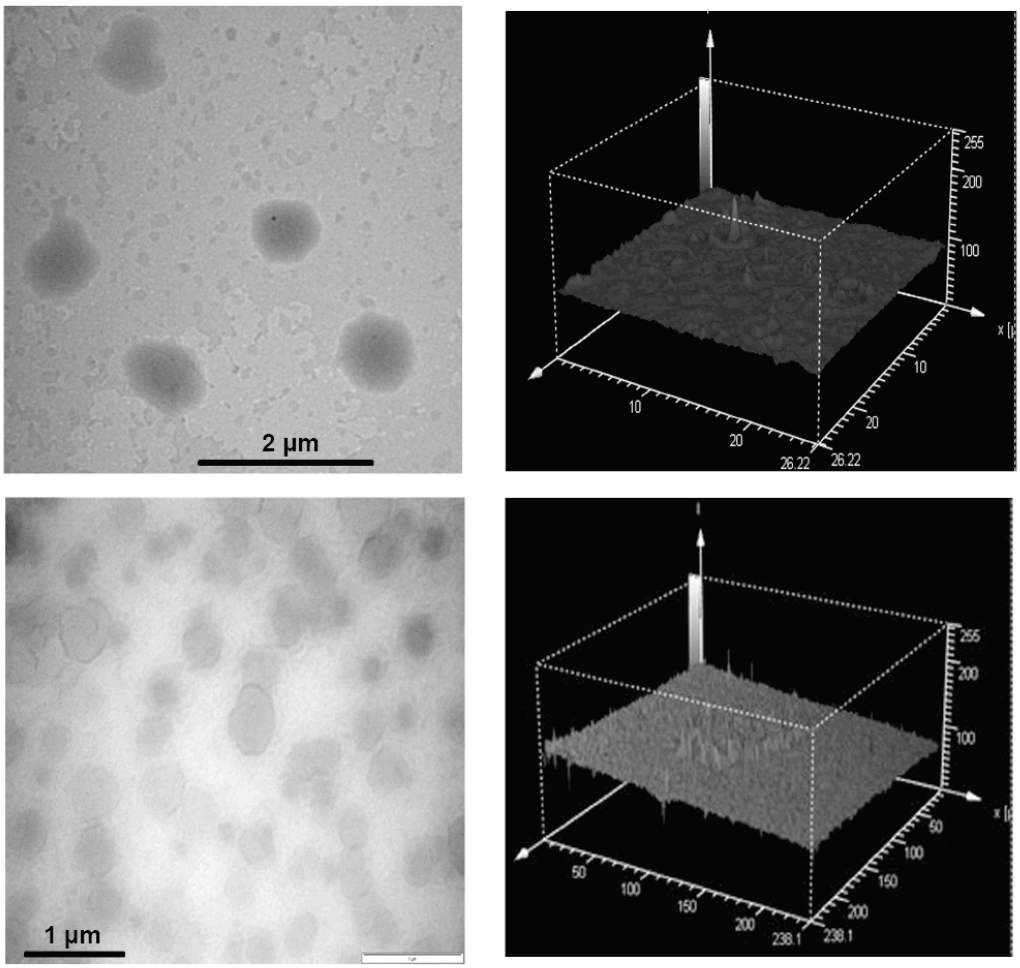


Figure 3: presents, as an example, the TEM and CSLM micrographs of samples of MLV and LUV dispersions. TEM images show in both cases the presence of almost uniform, round-shaped vesicles and the substantial absence of aggregates. CSLM analysis confirmed, respectively, the actual multi- and uni-lamellarity of MLV and LUV vesicles.

In vitro drug permeation studies

Topical liposomal gel formulations were prepared by incorporation of drug ethosomal dispersions in a structured vehicle of Carbopol.

This polymer was chosen due to its bioadhesive properties and its proved compatibility with both liposomes and ethosomes (Mura et al., 2007, 2008).

A Carbopol gel formulation containing a drug aqueous solution was also prepared as a reference.

In vitro drug permeation studies from these formulations were performed by using an appositely developed artificial lipophilic membrane, which previously showed a satisfactory correspondence with permeation data obtained by using excised rat skin (Mura et al., 2007), thus allowing overcoming of problems of longer experimental times, higher costs and lower reproducibility related to the use of animal membranes. Results of permeation studies are shown in Figure 4.

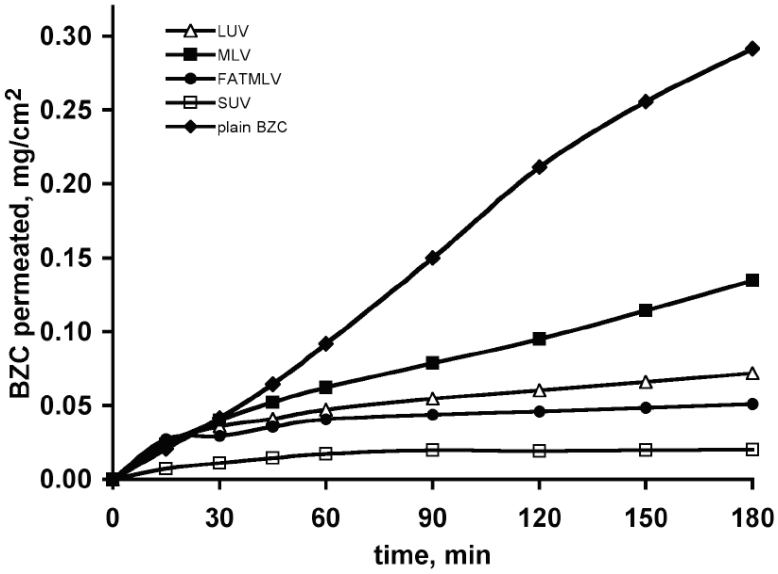


Figure 4

The gel formulation containing the drug solution exhibited the highest permeation rate, since the drug is free to directly diffuse through the artificial membrane. Slower release rates were instead observed from gels containing ethosomal drug dispersions, due to the lower diffusion rate of the drug entrapped into the vesicles. Interestingly, different permeation profiles were observed, depending on the vesicle characteristics. In particular, the drug diffusion rate was in the order MLV>LUV≈FATMLV>SUVson. It could be hypothesized that, in agreement with our previous results (Mura et al., 2007), the drug permeation rate was directly related to the vesicle EE% and inversely related to the vesicle dimensions. In fact, the faster drug permeation shown by gel with MLV vesicles can be attributed to the favourable combination between the vesicle dimensions (lesser than FATMLV and LUV vesicles) and the optimal EE% (94.8 %). The apparent exception of sonicated SUV vesicles, which showed the lowest drug permeation from gel, in spite of their smallest dimensions, could be attributed to their lowest EE% (60.7%).

In vivo studies

Results of *in vivo* experiments obtained from Carbopol gel formulations containing the plain drug or the drug entrapped in the different kinds of ethosomal vesicles are reported in Table 2.

Table 2: Effect induced by different Carbopol gel formulations of benzocaine (BZC) on the rabbit conjunctival reflex test

	Number of stimuli to induce conjunctival reflex					
SAMPLE	*eye*	*Time After Treatment*				
		10 min	*20 min*	*30 min*	*40 min*	*60 min*
Control (empty gel)	left	1.0 ±0.0	1.5 ±0.3	1.2 ±0.2	1.0±0.0	1.0±0.0
Control (empty gel)	right	1.0 ±0.0	1.2 ±0.2	1.0 ±0.0	1.0±0.0	1.0±0.0
Gel+aqueous solution	left	1.3 ±0.3	1.2 ±0.2	1.0 ±0.0	.0±0.0	1.0±0.0
Gel+ 0.5%BZC aqueous sol.	right	21.4±3.3*	17.8±2.5*	14.1±1.6*	.3±0.2	1.0±0.0
Gel + MLV	left	1.8 ± 0.4	1.5 ± 0.3	1.9 ± 0.5	1.0±0.0	1.0±0.0
Gel +0.5%BZC-loaded MLV	right	42.4±3.5*^°	36.4±3.2*^°	22.4±3.1*^	12.3±2.9*^	4.7±1.7*^
Gel + FATMLV solution	left	1.3 ± 0.5	1.6 ± 0.2	1.0 ± 0.0	1.0± 0.0	1.0±0.0
Gel +0.5%BZC-loaded FATMLV.	right	29.8 ± 5.5*^	29.8 ± 4.5*^	19.7 ± 4.1*^	10.2 ± 2.5*^	3.4 ± 1.2*
Gel + LUV	left	1.1 ±0.0	1.2 ±0.2	1.1 ±0.0	1.3±0.0	1.2±0.0
Gel +0.5%BZC-loaded LUV	right	40.2 ± 4.6*^°	34.2 ± 3.7*^°	21.9 ± 4.3*	9.4 ± 3.1*^	2.0 ± 1.5
Gel + SUV	left	1.2 ±0.0	1.1 ±0.2	1.3 ±0.0	1.1±0.0	1.1±0.0
Gel +0.5%BZC-loaded SUV	right	27.1 ± 5.2*	28.9 ± 5.2*^	22.7 ± 3.4*	18.5 ± 2.2*^°	11.9 ± 1.8*^°

There were 6 rabbits per group. Each value is the mean of 6 separate experiments. *P< 0.01 in comparison with control; ^P<0.01 in comparison with gel+BZC solution; °P<0.05 in comparison with the other preparations

It is evident that BZC ethosomal formulations were successful in significantly improving both intensity and duration of the drug anaesthetic effect with respect to the corresponding BZC solution at the same concentration.

Such findings can be attributed to the favourable effect of the ethosomal carriers that, in virtue of their great skin affinity and high deformability, undergo a preferential uptake and enable a better skin penetration of the entrapped drug. Interestingly, some important differences were observed, depending on the kind of ethosomal vesicles.

In fact, the best results in terms of improved intensity of anaesthetic effect during the first 20 min after gel application were given by the formulation containing MLV vesicles, immediately followed by that with LUV vesicles, and then by those with FATMLV and sonicated SUV vesicles. The order of effectiveness was MLV≈LUV>FATMLV≈SUV, similar to that observed in permeation studies.

However, as for the improvement in duration of drug action, the most effective formulations were those with SUV and MLV vesicles, and the effectiveness order was SUV≥MLV>LUV≈FATMLV. In particular, unexpectedly, SUV vesicles, which initially exhibited the lowest improvement in intensity of drug anaesthetic effect, showed instead the greatest prolongation of this effect, statistically higher ($P<0.05$) than that obtained with MLV vesicles. The initial behaviour can be attributed to the lowest EE% of SUV vesicles (as in *in vitro* experiments), while the final one is probably due to the reduced particle size and then to the very high surface area of these vesicles, that allows a more intimate and extended contact with the skin during the time (Verma et al., 2003b).

In conclusion, ethosomal formulations of BZC enabled a significant improvement of its therapeutic effectiveness in terms of both intensity and duration of anaesthetic effect. The study pointed out the influence of the structure and size of the ethosomal vesicles, and, consequently, of the technique used for their preparation, on the vesicle EE% as well on the permeation rate and *in vivo* anaesthetic efficacy of the entrapped drug. MLV vesicles obtained by thin layer evaporation can be considered as the best ones, by combining the highest EE% with the strongest initial improvement of intensity of the drug anaesthetic effect, and allowing a 100% increase of its duration.

On the other hand, SUV vesicles, even if less effective than MLV in the initial phase, due to their low EE%, showed instead the highest drug-reservoir effect, due to their closer and more widen contact with the skin in virtue of their greatest surface area, thus allowing the longest extension of the drug therapeutic action.

10. Development of liposomes loaded with Benzocaine and Butamben as cyclodextrin complexes. Pre-formulation and characterization studies

This part of my thesis work has been carried out in collaboration with the Laboratory of Pharmaceutical Technology, University of Seville, Spain, under the guidance of Prof. Antonio Maria Rabasco Alvarez and Prof. Maria-Luisa Gonzalez Rodriguez.

In our previous studies we have demonstrated the effectiveness of liposomal formulations to control the topical delivery of BZC, prolonging its effect and reducing its toxicity.

In particular, in our previous works we developed liposome formulations able to improve BZC anaesthetic effect in vivo by entrapping the drug in the hydrophilic phase of liposomes or in ethosomes (Mura et al., 2007, 2008).

On the other hand, also cyclodextrin (CD) approach has been used to increase drug bioavailability by topical route.

In our laboratory it has been recently investigated the possibility of using a combined strategy aimed at simultaneously exploiting the cyclodextrin solubilizing power towards drugs and the liposome carrier function through the skin (Maestrelli et al, 2006) and it has been developed a liposome formulation loaded with ketoprofen-cyclodextrin complex.

Therefore, in this work, we thought it worthy of interest to use such a combined approach to prepare liposomes loaded with cyclodextrin complex of two different local aneesthetics i.e. benzocaine (BZC) or butamben (BTM).

Moreover, since the corneal epithelium, at physiological pH, is negatively charged, adsorption through the skin may be improved by using positively charged liposomes that can enhance the bioavailability of entrapped drugs over neutral or negatively charged vesicles.

For these reasons we considered interesting to add in the liposome formulation a positive cationic phospholipid such as stearylamine.

10.1 Materials and methods

Materials

Benzocaine base (BZC), Butamben (BTM), cholesterol (CH), stearylamine (SA), and l-α-phosphatidylcholine (PC) were provided by Fluka-Sigma-Aldrich (Italy), hydroxypropyl β-cyclodextrin (HPβCD) (MS 0.65) and βCD were a gift of Roquette. All other reagents were of analytical grade.

Binary system preparation method.

BZC-CD, BTM-CD equimolar systems with both HPβCD and natural βCD were obtained according to three different methods:

Physical mixing: Physical mixtures (P.M.) were obtained by 15 min tumble mixing equimolar amounts of the respective simple components previously sieved (75-150 μm sieve granulometric fraction).

Co-grinding: co-ground products (GR) were prepared by ball-milling physical mixtures in a high-energy vibrational micro-mill (Mixer Mill MM 200 Retsch, GmbH, Düsseldorf, Germany) at a frequency of 24 hertz for 30 min.

Coevaporation: Coevaporated products (COE) were prepared by coevaporation of equimolar drug-CD ethanol-water (5:5 v/v) solutions in a rotary evaporator (Heidolph Laborota 4000) at 85°C. The resulting products were then dried in a vacuum desiccator for 48 h to remove traces of solvents.

Each solid product was sieved and the 75-150 μm granulometric sieve fraction used for the following tests.

Phase Solubility Studies

Phase solubility studies were performed at 25°C for 72 h by adding an excess of drug to 10 mL of pH 4.5 phosphate buffer solution containing increasing amount of CD. BZC was determined spectrophotometrically at 280 nm and BTM at 287.2 nm .

Dissolution rate studies

Dissolution rate of both drugs, alone and from the different drug-CD binary systems, was determined during 60 min at 37°C according to the dispersed amount method, by adding 500 mg of drug or drug-equivalent in 100 ml of water in a 300-ml beaker, which was stirred with a three-blade glass propeller centrally immersed at 25 mm from the bottom and rotating at a frequency of 100 rpm.

At time intervals, 3 ml samples were withdrawn and spectrophotometrically analyzed for drug content, as described above.

The same volume of fresh medium was added and the correction for the cumulative dilution was calculated.

Each test was repeated four times (coefficient of variation < 1.5 %).

X-ray powder diffraction (XRPD)

X-ray powder diffraction patterns were taken at ambient temperature with a Brucker D8. The samples were analysed in the 5–36° 2θ range at a scan rate of $0.05°s^{-1}$.

Differential Scanning Calorimetry (DSC)

DSC analyses were performed with a Mettler TA4000 Star^e system equipped with a DSC 2 cell. Samples were sealed in pierced Al pans and scanned at 10 $Kmin^{-1}$ in the 30–120°C temperature range under static air.

Fourier transform infrared spectroscopy (FTIR)

FT-IR spectra (Perkin-Elmer Mod. 1600) of individual BZC, BTM, HPβCD, βCD, and drug–CD binary systems were obtained as Nujol dispersion in the 4000–600 cm^{-1} region.

Liposomes preparation method

MLV (Multilayer vesicles) liposomes containing 1% w/v of drug were obtained by TLE (thin layer evaporation) using a phosphatidylcoline-cholesterol-sterylamine mixture in the molar ratio of 5.5:1:1.5.

The lipid phase was dissolved in the minimum amount of an organic solvent (chloroform); it was then removed under reduced pressure using a rotary evaporator (Büchi R 200/205) at 55°C, thus obtaining a thin film of dry lipid on the wall of the flask. Evaporation was continued for 2 h after the dry residue appeared to remove the traces of organic solvent.

Finally, the film was hydrated by adding the hydration phase under vigorous stirring in order to favour the formation of vesicles.

In the absence of CD, the hydrophilic phase was a mixture of water/ethanol 60:40 v/v, where the drug was dissolved.

On the contrary, in the presence of CD, the hydrophilic phase was water, where the maximum drug solubility was 0.5 % w/v for BZC and 0.3% w/v for BTM. Therefore, to reach the final concentration of 1%, the remaining drug amount was dissolved in the lipophilic phase.

Liposome characterization

Particle size and charge (ζ potential), encapsulation efficiency (EE%) and liposome morphology (Confocal Laser Scanning Microscopy-CSLM) analyses were performed according to the previously developed procedures, as reported in the chapter 8 and 9.

In Vitro Permeation Studies Through Artificial Membrane

Permeation studies of BZC and BTM from the different formulations through artificial membranes were performed for 24h at 37 ± 1°C using a Sartorius Model SM 16750 apparatus (Sartorius Membranfilter GmbH, Gottingen, Germany).

The procedure was the same as described in the chapter 8.

In Vitro Permeation Studies Through Artificial Membrane

Permeation studies of BZC and BTM from the different formulations through artificial membranes were performed for 24h at 37 ± 1°C using a Sartorius Model SM 16750 apparatus (Sartorius Membranfilter GmbH, Gottingen, Germany).

The procedure was the same as described in the chapter 8.

In vivo studies

The anaesthetic activity of drugs formulated in aqueous Carbopol gel, as such or entrapped in the liposomes, was assayed *in vivo* in albino rabbits according to the conjunctival reflex test (Ghelardini et al., 2001) following the same procedure as described in the chapter 8.

10.2 Results and discussion

Studies of Binary systems of BZC-CD and BTM-CD

Phase solubility studies revealed the formation of 1:1 BZC-CD complex with both the examined CDs.

Both the CD demonstrated a good solubilizing power toward BZC; $K_{1:1}$ values were 352 and 1315 M^{-1} for the complexes with HPβCD and βCD, respectively (Fig.1)

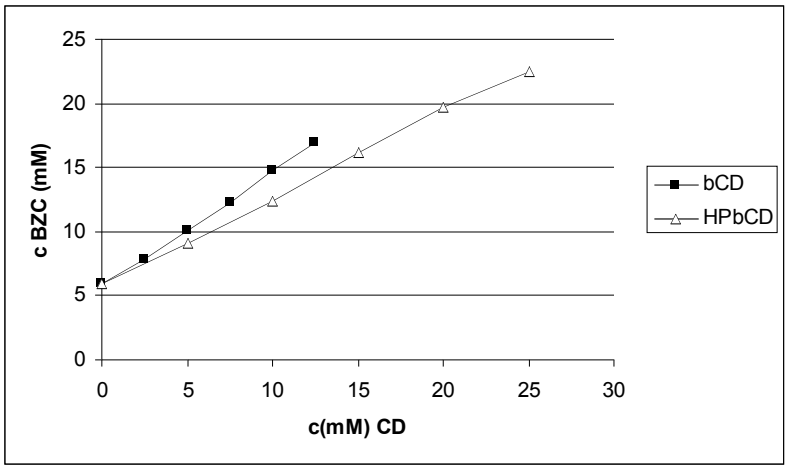


Figure 1: Phase solubility studies of BZC in the presence of βCD and HPβCD.

Also for BTM phase solubility studies revealed the formation of 1:1 complexes with both CDs, which shewed a very similar solubilizing power towards the drug.

In this case, the $K_{1:1}$ values were 273 and 290 M^{-1} for the complexes with HPβCD and βCD, respectively (Fig.2)

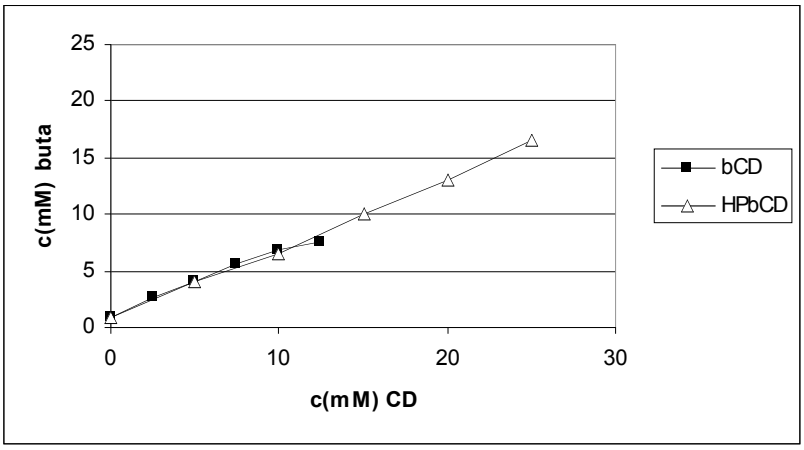


Figure 2: Phase solubility studies of BTM in the presence of βCD and HPβCD.

Solid state studies

DSC

Thermograms obtained from the different binary systems are reported in figure 3A and 3B.

The thermal curve of pure BZC and BTM showed a typical sharp melting endotherm at about 94°C and 60°C, respectively, indicative of their anhydrous and crystalline state, while pure CD exhibited a broad endothermal effect corresponding to dehydration.

The DSC curve for the untreated physical mixture consisted of the superimposition of the thermal profiles of drug with both CDs with no significant changes in the drug melting peak parameters (except for a shift to lower temperature), suggesting no drug–CD interactions.

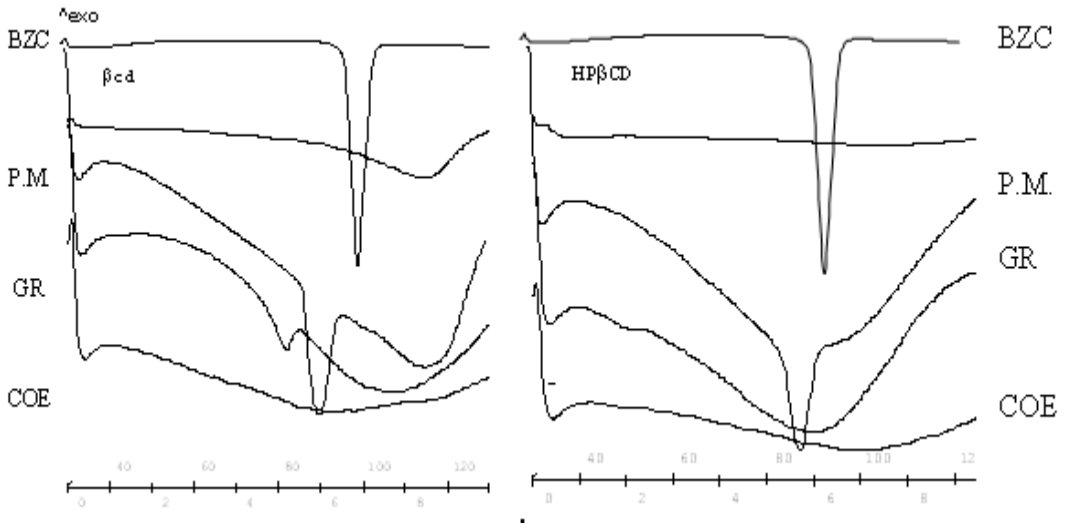


Figure 3A: Thermograms of BZC, βCD, HPβCD and their PM, GR and COE products.

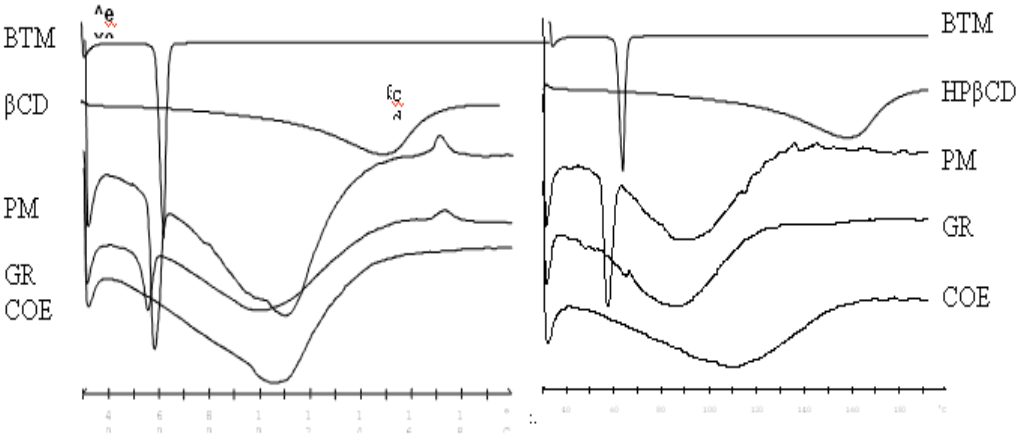


Figure 3B: Thermograms of BTM, βCD, HPβCD and their PM, GR and COE products.

Also for BTM series was observed a similar behaviour as shown in figure 3B. The loss of drug melting peak seems indicate that COE product with both CD and GR system with HPβCD allowed a complete drug amorphization and or complexation.

XRPD

X-ray Powder diffractometry was used to investigate more in depth the differences in the solid-state between drug-CD products prepared by the different methods.

Fig. 4A and 4B show the X-ray powder diffraction patterns of pure BZC, BTM, βCD, and their 1:1 mol–mol systems obtained by physical mixing, co-grinding and co-evaporation.

The diffraction patterns of both drugs and βCD displayed several sharp peaks, indicative of their crystalline nature.

A crystalline pattern, given by the sum of the spectra of pure components, was obtained for the corresponding untreated physical mixtures.

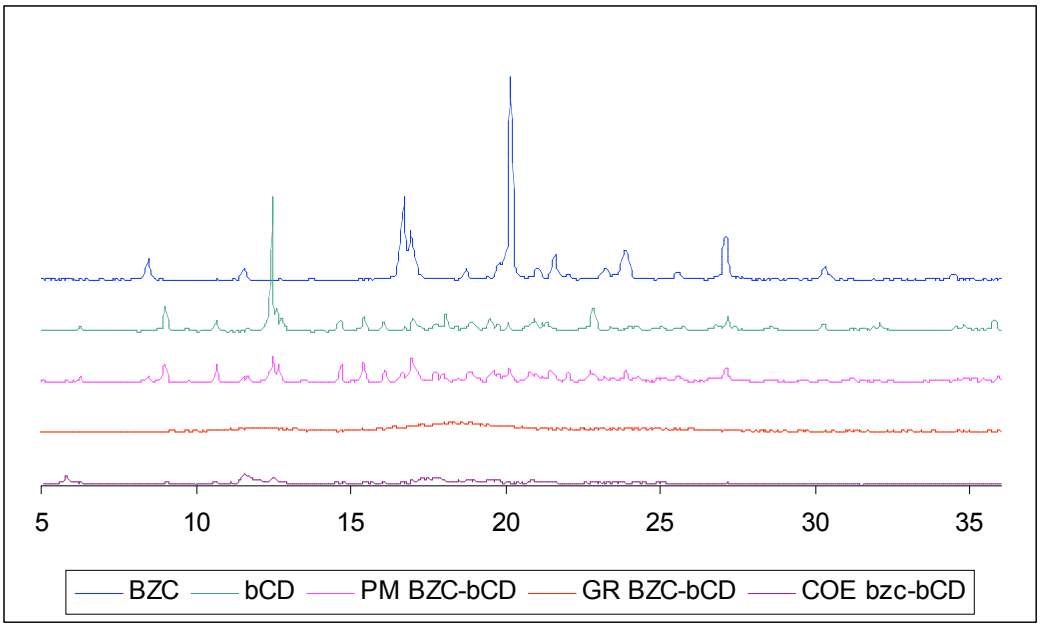


Figure 4A: XRPD patterns of pure benzocaine (BZC), pure βCD and BZC/βCD binary systems prepared by physical mixing, co-evaporation, and co-grinding.

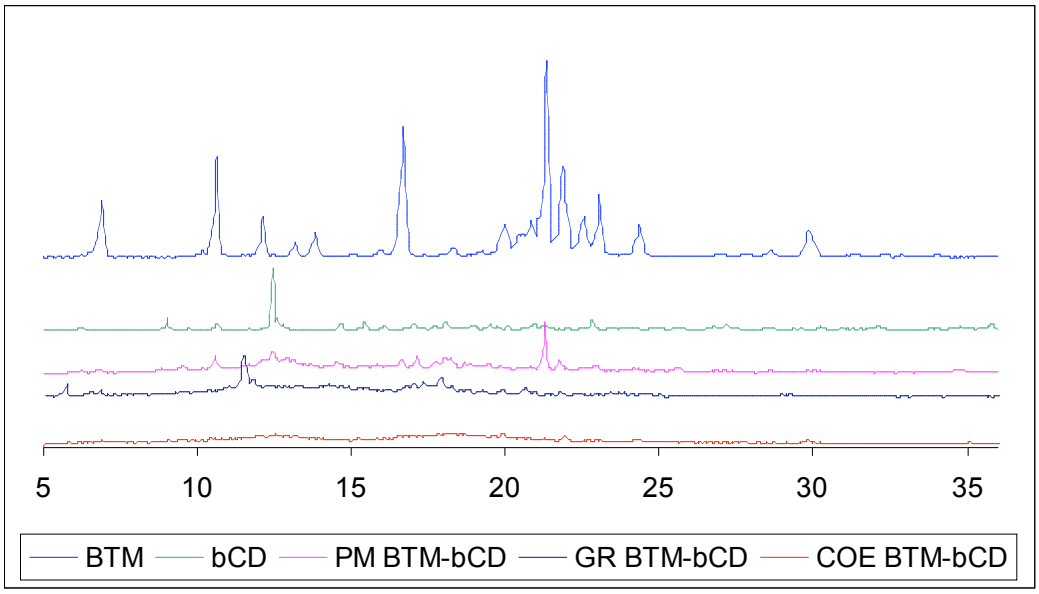


Figure 4B: XRPD patterns of pure butamben (BTM), pure βCD and BTM/βCD binary systems prepared by physical mixing, co-evaporation, and co-grinding.

The characteristic peaks of drug and βCD, even though markedly reduced in intensity, were still detectable in the products obtained by co-grinding.

On the contrary, a diffuse pattern, with very few low-intensity peaks, was obtained for the samples prepared by coevaporation, suggesting an almost complete drug amorphization and/or complexation.

Diffractograms of drug-HPβCD complexes, reported in figures 4C and 4D, definitely confirmed the DSC findings, showing the complete loss of drug crystallinity in COE products obtained with βCD and both GR and COE systems prepared with HPβCD.

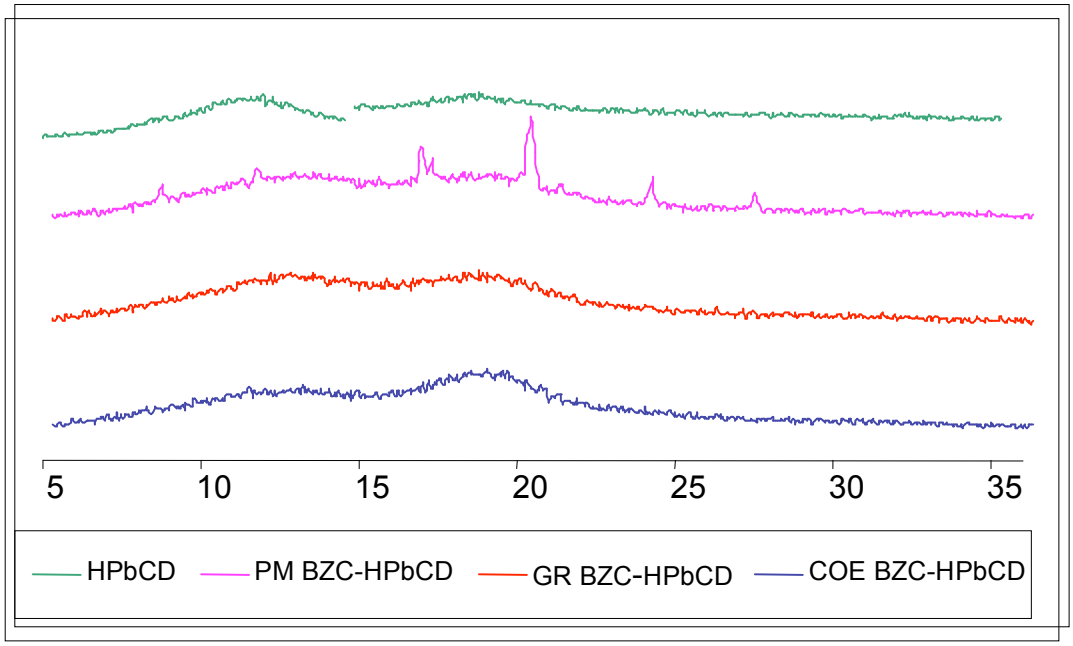


Figure 4C: XRPD patterns of pure HPβCD and BZC/HPβCD binary systems prepared by physical mixing, co-evaporation, and co-grinding.

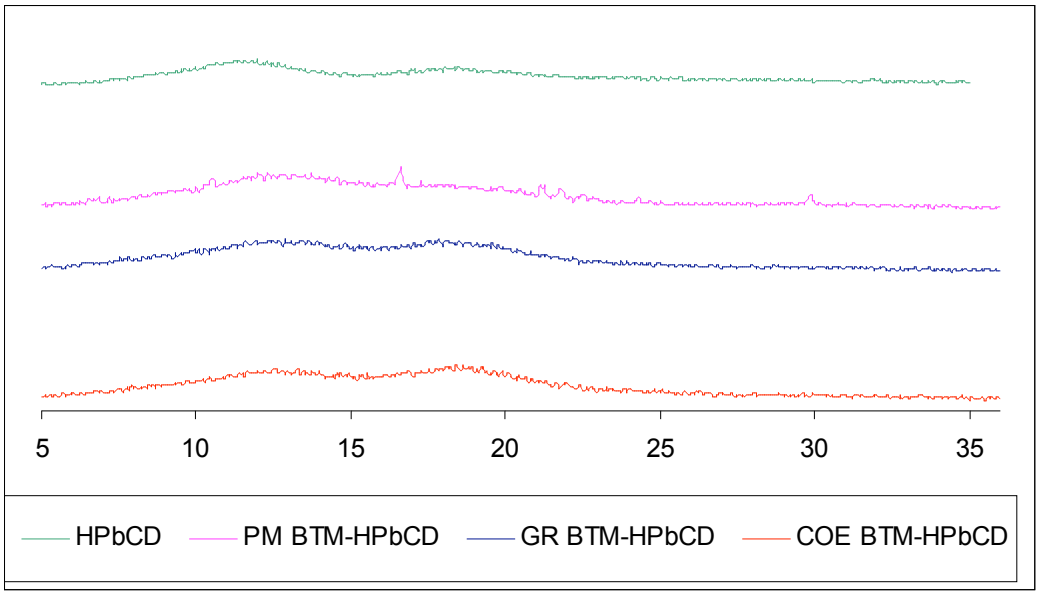


Figure 4D: XRPD patterns of pure HPβCD and BTM/HBβCD binary systems prepared by physical mixing, co-evaporation, and co-grinding.

FTIR analysis

FTIR spectra of pure components and the respective drug–CD (1:1 mol/mol) products obtained by physical mixing, co-grinding and co-evaporation are presented, respectively, in Figures 5A and 5B .

The reported section, between 1800 and 1600 cm^{-1}, is indicative of C=O stretching bands characteristic of the two pure drugs.

The FTIR spectrum of drug–CD physical mixture freshly prepared at 25 °C can be considered as the result of the sum of drug and CD spectra, thus confirming the absence of solid-state interactions between the components, as indicated by DSC results. No modifications were observed for GR systems with βCD.

On the contrary, the FTIR spectra of drug–βCD products obtained by co-evaporation and drug-HPβCD products obtained by co-evaporation and co-grinding showed some differences with respect to those of the corresponding physical mixtures, revealing a modification of the environment of drug and thus indicating some drug–CD interactions, in accordance with the results obtained by DSC analysis.

The changes observed in the FTIR spectra of the various samples, such as shift of peaks, or their reduction in intensity up to almost complete disappearance, depended on their preparation method, suggesting different degrees of interaction and/or amorphization in the different products, thus confirming that coevaporated products obtained with both drugs and HPβCD allowed the better drug-CD interaction and a complete drug amorphization, as observed also by XRPD analysis.

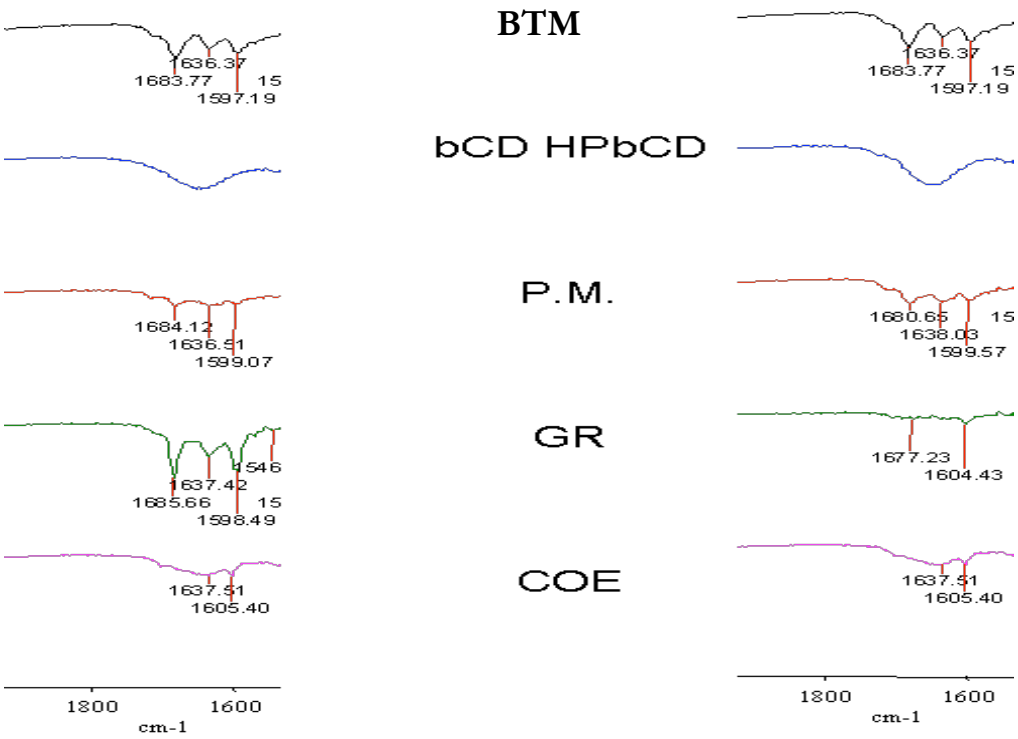


Figure 5A: FTIR profiles of pure BTM, βCD, HPβCD and BTM/CD binary systems prepared by physical mixing, co-evaporation, co-grinding.

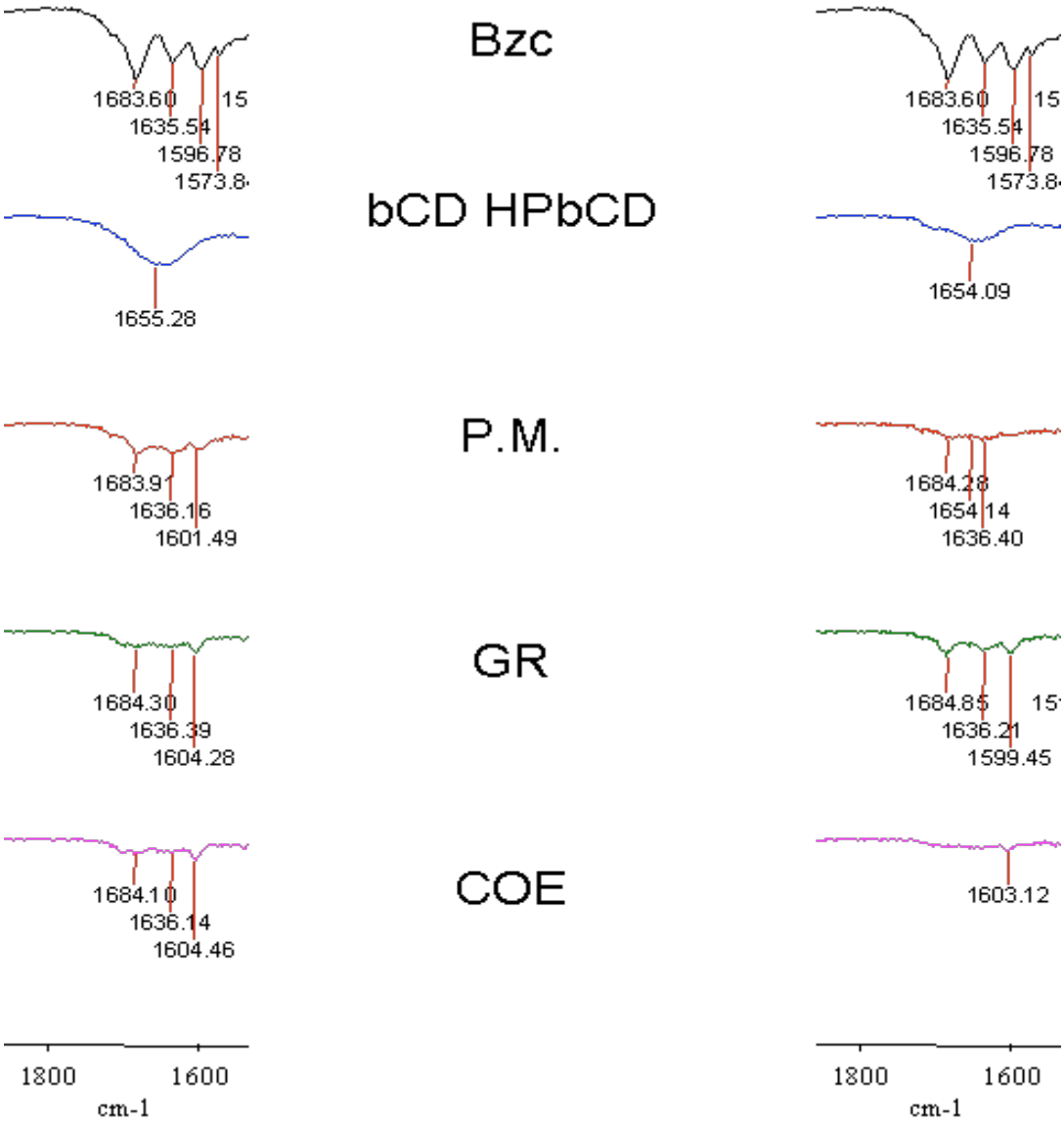


Figure 5B: FTIR profiles of pure BZC, βCD, HPβCD and BZC/CD binary systems prepared by physical mixing, co-evaporation, co-grinding.

Dissolution studies

The results of dissolution studies are shown in Figure 6 were only the data obtained with the BZC series are reported, since a very similar trend has been obtained also with BTM.

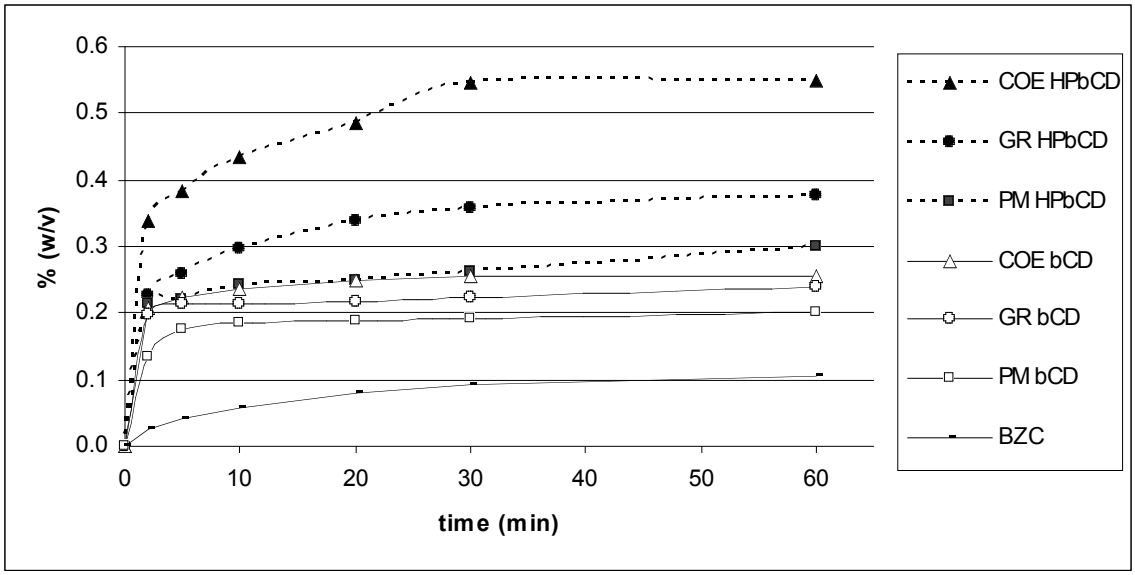


Figure 6: Dissolution rate of BZC from drug-CD systems

The dissolution profiles evidenced that the binary systems with HPβCD had a better performance with respect to those with native CD. That's probably due to a combined effect of both preparation technique and CD type.

In fact coevaporation technique is probable able to increase the surface area exposed to the dissolution medium and reduce drug crystallinity, and HPβCD, due to its amorphous nature, is more effective than βCD to stabilize this amorphous form.

Coevaporation resulted the best preparative technique, giving rise to the most effective dissolution profile and allowing achievement of a final drug solubility of 0.5 % w/v for BZC and 0.3% w/v for BTM.

Therefore, liposomes were prepared by adding this drug concentration, as CD complex, to the aqueous phase, and the remaining amount as plain drug to the lipophilic phase, in order to raise 1% w/v of final drug concentration in the liposomal formulation.

Development of Liposomal formulation

In order to select the most suitable amount of stearylamine (SA) to use for the liposome formulation, we prepared liposomes containing a fixed concentration of BZC at 1% w/v in the hydrophilic phase (H_2O/EtOH 60/40 v/v), and different stearylamine amounts in the lipophilic phase (1:1:0; 6:1:1; 5,5:1:1,5 phosphatidylcholine (PC):cholesterol (CH):stearylamine (SA) molar ratio).

The obtained liposomes were characterized through the determination of encapsulation efficiency, particle size, ζ-potential and in vitro permeation rate, while therapeutic efficacy was assessed by in vivo studies. The results of these studies are summarized in Tables 1 and 2, respectively.

PC:CH:SA molar ratio	EE%	Particle size (nm)	ζ potential (mV) ± s.d.	Permeation % (3h)	Polidispersion Index
1:1:0	47.73	627.0	-60.84 ± 1.02	5.2	0.2
6:1:1	40.63	392.8	+30.38 ± 1.04	5.1	0.3
5.5:1:1.5	50.47	390.2	+ 33.27 ± 0.32	8.3	0.1

Table 1: Liposome containing 1%BZC in the aqueous phase (H_20/EtOH).

The liposome formulation with the molar ratio 5.5:1:1.5 showed the best EE% and the highest permeation %, probably due to the greater amount of stearylamine in this formulation.

It can be observed that the presence of stearylamine influenced not only, as expected, the ζ potential, but also the size of liposomes.

In fact, ζ potential changed sign from negative (-60.84 mV), in the absence of the cationic additive, to positive (+33.27 mV), since SA is positively charged.

Moreover, the particle size was reduced from 627 to about 390 nm for liposomes without and with SA.

This finding could be attributed to a rearrangement or the liposomal bilayer as a consequence of the insertion of SA molecules.

In vivo studies, reported in Table 2, showed that this formulation also had the greatest therapeutic effect, thus confirming that the positive charge of stearylamine could favourably modify the carrier properties of liposomes and increase drug permeation thorough the skin .

EFFECT INDUCED BY THREE DIFFERENT PREPARATIONS								
	N° of stimulations to induce conjunctival reflex							
OCULAR	After Treatment							
PC/CH/SA molar ratio	5 min	10 min	15 min	20 min	25 min	30 min	40 min	60 min
BZC 1% gel								
1:1:0	35.9 ± 2.7	36.7 ± 4.3	35.2 ± 5.1	26.3 ± 4.4	24.1 ± 3.0	12.2 ± 2.3	6.6 ± 3.1	2.2 ± 1.0
6:1:1	42.3 ± 2.0	31.6 ± 3.6	29.5 ± 3.2	21.6 ± 2.4	18.4 ± 3.1	13.4 ± 2.0	2.4 ± 1.2	1.5 ± 0.0
5.5:1:1.5	48.8 ± 2.1*	44.1 ± 4.2*	41.6 ± 4.5*	35.9 ± 3.1^	27.2 ± 2.0^	23.6 ± 2.5*	18.9 ± 2.9*	11.5 ± 2.8*
There were 3 rabbits per group. Each value represents the mean of six separate experiments.. *^P< 0.05 in comparison with the other preparation.								

Table 2: Effect induced by BZC liposomal preparations in the rabbit cojunctival reflex test.

Therefore, we choose the formulation (PC:CH:SA 5.5:1:1.5) for the lipophilic phase of the vesicles, and liposomes were prepared with a double-loading technique by adding BZC or BTM as plain drug in the lipophilic phase and the corresponding drug-CD complex in the hydrophilic phase. In particular, we added the drug-Cd complex until its maximum solubility in the aqueous phase (water) i.e., 0.5% and 0.3% w/v for BZC and BTM respectively, and the remaining amount of the drug until 1% w/v was added in the lipophilic phase.

Liposomes were successfully prepared with drug alone or as COE drug-HPβCD, as shown in figure 7.

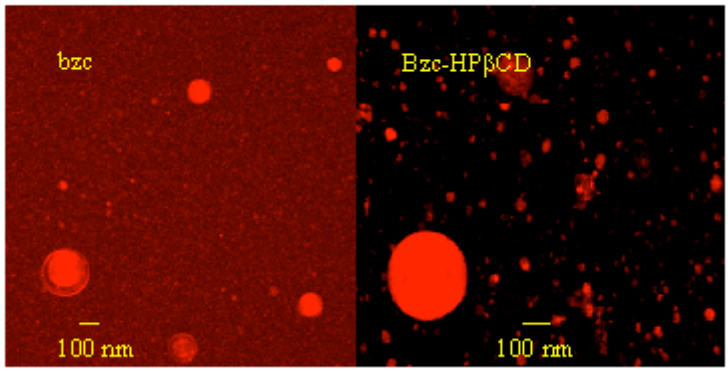


Figure 7: CSLM micrographs

The liposomes were characterized through the determination of encapsulation efficiency, liposome size and charge, and in vitro drug permeation rate, while the drug therapeutic efficacy was evaluated by in vivo studies.

The results are summarized in Tables 3 and 4.

batch	EE%	Particle size (nm)	ζ potential (mV) ± s.d.	Permeation % (3h)	Polidispersion Index
BZC	83.94	720.2	+25.72 ± 2.26	3.19	0.2
BZC-HPbCD+BZC	59.16	661.2	+31.18 ± 1.44	2.27	0.3
BTM	94.40	703.6	+22.47 ± 4.14	4.88	0.2
BTM-HPbCD+BTM	82.23	680.8	+27.33 ± 1.53	1.11	0.2

Table 3: Liposome (PC:CH:SA 5.5:1:1.5 molar ratio) containing 1% drug, loaded in part in the aqueous phase, as CD complex, and in part as plain drug, in the lipophilic one.

The double-charge technique, did not influence the liposomal size, with respect to the corresponding liposomes loaded with the plain drug dissolved in the lipophilic phase. Also the ζ potential remained almost constant, changing from 25 to 31 mV and from 22 to 27 mV respectively, for BZC and BTM, passing from the single to the double-loading.

A significant difference in size was instead observed between liposomes containing the drug all loaded the in the internal aqueous phase (see Table 1), and those containing the drug all loaded in the bilayer phase or, in the case of double-changed liposome, in part loaded in the aqueous phase, as CD complex, and in part as plain in lipophilic phase.

This effect can be attributed to a bilayer rearrangement of the liposome, as a consequence of the presence of the drug in the bilayer, which give rise to an increase in the vesicle dimensions, raising from about 390 to around 700 nm.

A reduction of EE% was observed with the double-charge technique.

The obtained results can be explained by the different preparation method of liposomes used in the two cases.

In fact, the vesicles containing drug alone, due to the very low water solubility of the drug, were prepared by dissolving it in the lipophilic phase, and, due to the high lipophilicity of these drugs, they have a great affinity for the lipophilic phase of liposomes.

By contrast, the vesicles containing the drug as hydro-soluble complex were prepared by dissolving it in the water used for hydration of the lipidic film.

Therefore, since in the case of MLV liposomes the volume occupied by the aqueous phase is smaller than that occupied by the lipidic phase, when the drug is added to the organic phase, it is entrapped in greater amount in the multi-layer liposomal membrane, with respect to the hydrophilic complex into the aqueous core.

In vivo studies showed that CD presence could improve the drug anaesthetic effect for the first 15 min compared with liposomes realized with drug alone.

EFFECT INDUCED BY FOUR DIFFERENT PREPARATIONS								
	N° of stimulations to induce conjunctival reflex							
OCULAR	After Treatment							
	5 min	10 min	15 min	20 min	25 min	30 min	40 min	60 min
Lipo BZC	37.2 ± 3.0	38.4 ± 2.1	34.1 ± 2.5	24.6 ± 3.8	22.2 ± 3.2	10.7 ± 2.4	5.4 ± 3.0	0.4 ± 0.1
Lipo BZC HPCD	49.2 ± 3.1*	47.5 ± 3.7*	41.8 ± 2.3^	25.3 ± 3.5	16.6 ± 3.3	14.7 ± 2.2	1.0 ± 0.0	
Lipo butamben	40.6 ± 2.5	35.8 ± 4.0	31.5 ± 3.5	25.3 ± 3.0	21.3 ± 3.1	17.6 ± 2.5*	8.3 ± 2.6	1.0 ± 0.0
Lipo Butamben HPCD	49.3 ± 3.1*	46.1 ± 2.9*	40.2 ± 3.5*	33.6 ± 2.7^	29.3 ± 2.0^	19.5 ± 2.3	10.2 ± 2.3	3.7. ± 2.2
There were 3 rabbits per group. Each value represents the mean of five separate experiments.. *P< 0.01 ;^P< 0.05 versus the corresponding lipo BZC/Lipo buta								

Table 4: Effect induced by liposomal preparations of plain Benzocaine (BZC), plain Butamben (BTM), or, respectively combined with BZC/HPβCD, and BTM/HPβCD on the rabbit conjunctival reflex test.

In conclusion, the addition of stearylamine in the lipophilic phase of liposome allowed an improvement of the drug anaesthetic effect, allowing a better drug permeation through the skin and a more easy penetration of drug inside the rabbit eye conjunctiva.

Moreover, even if drug-cyclodextrin complexation reduced the encapsulation efficiency, the CD presence can further improve the therapeutic action of the drug significantly enhancing its effect during the first 15 min.

11. Prilocaine-HPβCD complexes encapsulated in liposomes: pre-formulation and characterization studies.

Considering the interesting and promising results obtained by the previous study (Chapter 10), we carried out further studies to evaluate the effect of the combined approach of liposomal formulation and cyclodextrin complexation applied to another anaesthetic drug, i.e. prilocaine.

Prilocaine hydrochloride (PRL.HCl) is therapeutically used for intravenous regional anaesthesia and in dentistry, where it shows a medium duration action in comparison with to other local anaesthetic drug compounds.

The base form has the same activity but it has a very low melting point, so it is liquid at ambient temperature and it is insoluble in water. Therefore, we considered interesting to complex it with cyclodextrin, in order to increase its water solubility and then can insert it in the aqueous phase of the liposomes.

Moreover, in our previous work, we successfully obtained double-charged liposomes that revealed the best performance in terms of therapeutic activity of the formulation. So, also in this case, we prepared the liposomes by using the same lipophilic phase developed in the previous work (PC: CH: SA 5.5:1:1.5 molar ratio) and adding the drug in its lipophilic form to the lipid phase and in its water-soluble form as complex with CD, in the aqueous phase.

The liposomes loaded with prilocaine and its complex with cyclodextrin were characterized for their physical-chemical properties and subsequently evaluated in vivo as for the drug anaesthetic efficacy.

11.1 Materials and methods

Materials

Prilocaine hydrochloride (PRL HCl), cholesterol (CH), stearylamine (SA), and l-α-phosphatidylcholine (PC) were provided by Fluka-Sigma-Aldrich (Italy), HPβCD(MS 0.65) was a gift of Roquette. All other reagents were of analytical grade.

Preparation of Prilocaine base

To prepare prilocaine base (PRL) 100 mg of PRL HCl were dissolved in 20 ml of water and NaOH 1M was added in equimolar amount. $CHCl_3$ was added to extract PRL then the solution was filtered and the organic solvent removed through evaporation by a rotary evaporator. PRL was obtained as an oil.

Preparation of PRL-CD complex and its characterization

PRL-CD equimolar systems with HPβCD were obtained according to the coevaporation technique (COE) by adding an aqueous solution of HPβCD to PRL. The system was stirred 3 h and then the solvent was removed by evaporation. It was obtained a white powder.

X-ray powder diffraction (XRPD)

The X-ray powder diffraction patterns were taken, for all solid samples, at ambient temperature with a Brucker D8. The samples were analysed in the 5–30° 2θ range at a scan rate of $0.05°s^{-1}$.

Differential Scanning Calorimetry (DSC)

DSC analysis was performed with a Mettler TA4000 Star® system equipped with a DSC 25cell. Samples were sealed in pierced Al pans and scanned at 10K min^{-1} in 30–300°C Temperature range under static air.

Fourier transform infrared spectroscopy (FTIR)

FT-IR spectra (Perkin-Elmer Mod. 1600) of individual PRL HCl, PRL, HPβCD and PRL-HPβCD binary systems were obtained as Nujol dispersion in the 4000–600 cm^{-1} region.

Liposome preparation method

MLV (Multilayer vesicles) liposomes containing 1% w/v of drug were obtained by TLE (thin layer evaporation) with a phosphatidylcoline-cholesterol-stearylamine molar ratio of 5.5:1:1.5. The hydrophilic phase was water.

To obtain loaded MLV liposomes, PRL was added in the lipophilic phase, while PRL.HCl and PRL-HPβCD complex, were added to the hydrophilic phase at different concentrations. In order to reach the suitable drug concentration, a double charge was performed.

Liposome characterization

Particle size and charge (ζ potential) and encapsulation efficiency (EE%) were determined according to the previously described procedures, as reported in the chapter 10. Drug was assayed according to European Pharmacopoeia 6th edition by HPLC analysis using a Merck Hitachi Elite Lachrom chromatograph with UV-VIS detector at λ=240nm .The column was a C_{18} (Merck Hibar RT 150 x 4.6mm, 5µm).

The mobile phase was a mixture 40:60 acetonitrile /phosphate buffer pH 8.0, T=40°C, flux 1ml/min and the volume injected= 20µl. The Standard curve was realized in a concentration range between 2 and 20 µg /ml (LOQ=0,492 µg/ml, LOD= 0,14775 µg/ml)

The liposome elasticity was determined by measuring the size of the liposomes before and after filtration through a microporous filter with pore diameter of 100 nm (Isopore, Millipore, Bedford, MA, USA) using a LiposoFast-Basic (Avestin Europe GmbH) connected to a 3 Atm pressure source.

In vivo studies

The anaesthetic activity of PRL, as such or entrapped in the different kinds of MLV vesicles, was assayed *in vivo* in Wistar mice according to the backbone muscle test.

For the tests were used twelve Wistar mice. Animal rooms were kept at a temperature of about 23 °C with a light/dark cycle of 12 h.

The animals were fed with a standard diet and tap water ad libitum. All experiments were carried out in accordance with the NIH guide for the Care and Use of Laboratory animals. All efforts were made to minimize animal suffering and to limit the number of animal used.

The day before the test, the back of each animal was shaved using a depilatory cream and 4/6 circles with a diameter of 0.7 cm were drawn in ink on the skin of the animal, at a distance of about 1 cm apart. The test was carried out by injecting in the centre of each circle a different sample. In one of the circles was injected a suspension of liposomes without the drug as control. The volume injected was 0.1 mL.

The painful stimulation was induced with the penetration subcutaneously (about 1 mm in depth) with a thin syringe at the centre of each circle.

It was recorded a negative response to the test, or lack of local anaesthetic effect, each time the stimulus was followed by contraction of the muscle ridge, forming obvious folds of skin on the back. The absence of this response was instead recorded as a positive test, or presence of a local anaesthetic effect.

The experiment does not provide quantitative assessments, but only information on the timing of action of the drug. Each sample was tested at least four times, including the control.

11.2 Results and discussion

Solid state studies

DSC

The thermal curves obtained for pure components PRL.HCl, HPβCD and for the PRL-HPβCD complex are reported in Figure 1.

The DSC curve of pure PRL HCl showed a typical sharp melting endotherm at 169,0 °C (ΔH=118,69 J/g) indicative of its anhydrous and crystalline state, while pure HPβCD exhibited a broad endothermal effect corresponding to its dehydration. It was not possible to obtain a DSC profile of PRL because it's an oil at ambient temperature.

The DSC curve of PRL-HPβCD COE product was typical of an amorphous substance.

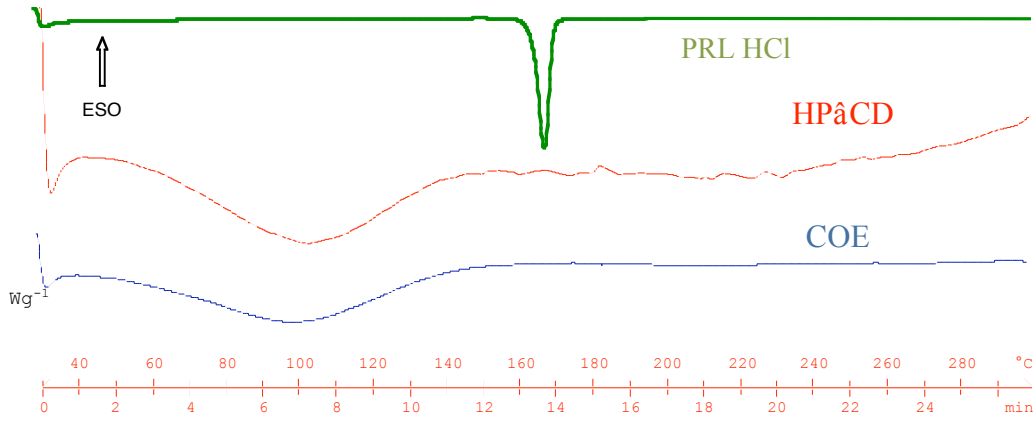


Figure1: Thermograms of PRL.HCl, HPβCD and COE PRL-HPβCD.

XRPD

X-ray powder diffractometry was used to investigate more in depth the solid-state properties of the products. Figure 2 shows the X-ray powder diffraction patterns of PRL.HCl, HPβCD and COE PRL-HPβCD.

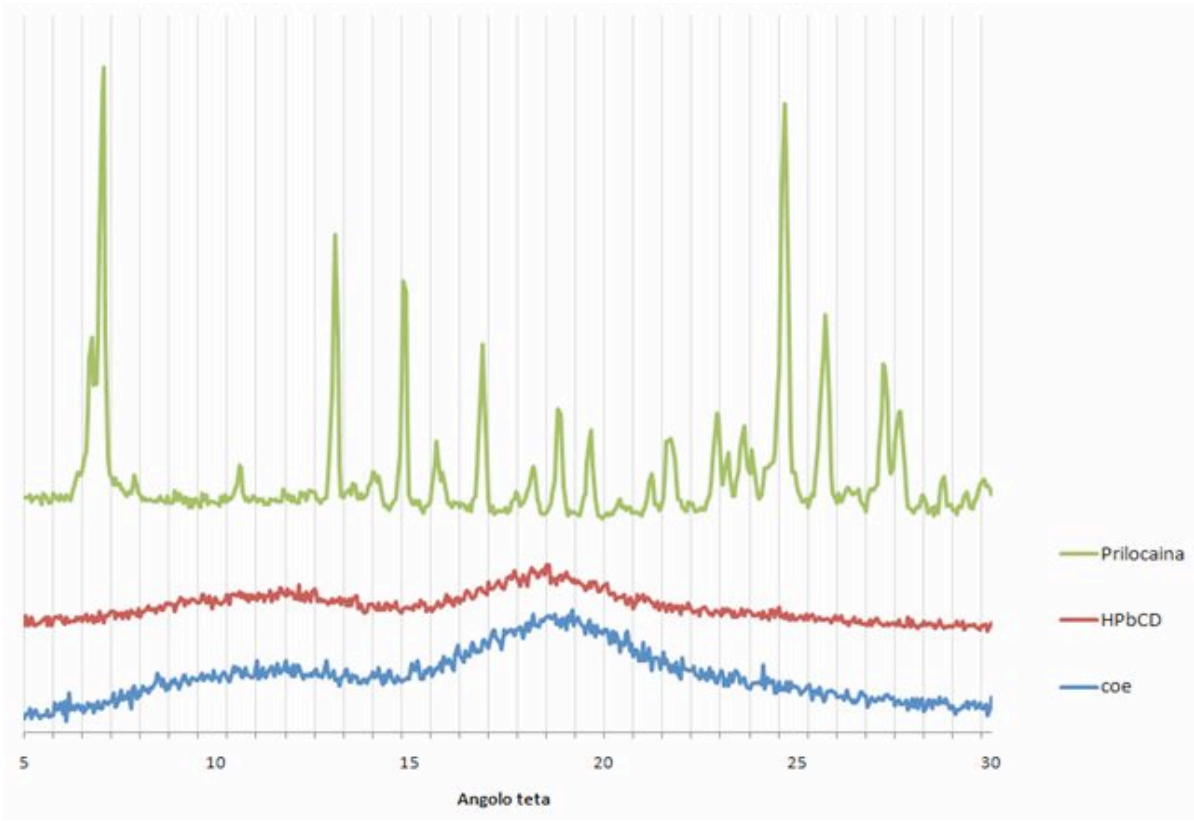


Figure 2: XRPD patterns of PRL HCl, HPβCD and COE PRL-HPβCD

The diffraction pattern of PRL.HCl, displayed several sharp peaks, indicative of its crystalline nature.

A diffuse pattern was obtained for HPβCD and also for the complex with PRL prepared by coevaporation suggesting the formation of an amorphous complex product, confirming DSC results.

FTIR

FTIR spectra of pure components, PRL.HCl, PRL, HPβCD, PRL-HPβCD COE are presented respectively in Figures 3 and 4.

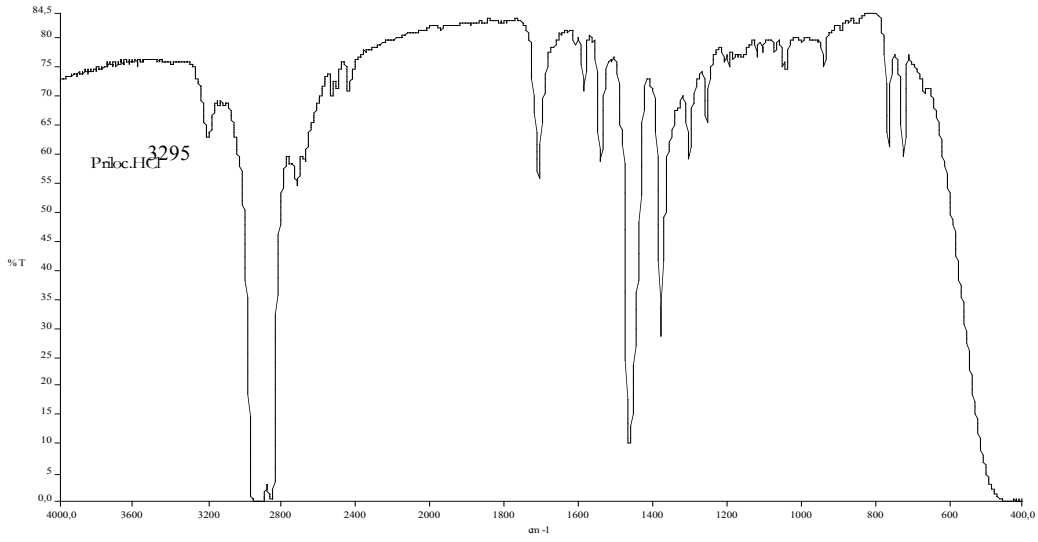


Figure 3: FTIR spectra of Prilocaine chlorohydrate (PRL.HCl)

In the FTIR spectrum of the PRL.HCl, the most intense and characteristic signal is that of the carbonyl stretching at around 1700 cm^{-1}.

Other characteristic peaks are or of very low intensity or are covered by the nujol dispersion, that causes the intense CH stretching band at around 2900 cm^{-1} and two intense peaks due to CH bending at 1376 cm^{-1} and 1461 cm^{-1}.

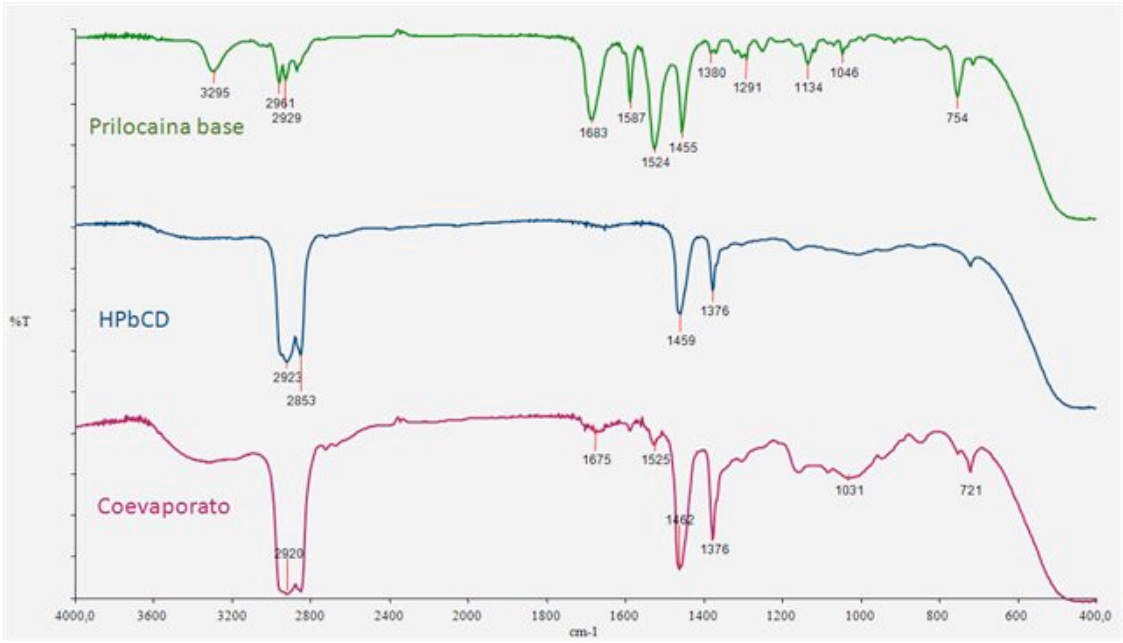


Figure 4: FTIR spectra of PRL base, HPβCD and PRL-HPβCD binary system prepared by co-evaporation.

The FTIR spectrum of PRL base showed a similar pattern to that of PRL:HCL characterized by a sharp peak at about 1700 cm^{-1} indicative of C=O streching.

The spectrum of oleous liquid PRL base was recorded on the pure sample, not dispersed in nujol but directly applied to the tablets of NaCl.

This led to obtain a "cleaner" spectrum, without the strong peaks of stretching at around 2900 cm^{-1} and of bending at 1376 cm^{-1} and 1459 cm^{-1}, typical of nujol.

It should be noted, however, a particularly broad band with maximum at 3295 cm^{-1} associated to NH stretching.

This enlargement is likely due to the presence of hydrogen bonds and can suggest the presence of residual water, used to dissolve the PRL.HCL for the following obtaining of PRL base by NaOH addition.

The HPβCD pattern did not show characteristic peaks that can be distinguished from those of nujol.

The spectrum of PRL-HPßCD coevaporated product showed that the signals of the drug were significantly flattened as a consequence of the interaction with the cyclodextrin molecules.

We note, however, a very wide band between 3200 cm^{-1} and 3500 cm^{-1} attributable to NH stretching.

The presence of residual water seems even greater than in the case of PRL base.

The C=O stretching band, that in the spectrum of the drug exhibits an intense signal peaked at 1683 cm^{-1}, in the coevaporated product is very weak and shifted to a slightly lower frequency (1675 cm^{-1}).

This could suggest the presence of solid-state interactions between the drug and cyclodextrin.

In the spectrum of the coevaporated product is also present the bending band at around 1525 cm^{-1}, even though strongly reduced in intensity in comparison to that of the drug.

The bending peak at 1462 cm^{-1}, present in the spectrum of COE, is more intense than that of pure drug at 1455 cm^{-1}, due to the superimposition of the bending band of nujol at 1459 cm^{-1}.

Loaded-liposomes

The liposomes were loaded with different drug concentrations by adding the PRL base to the lipophilic phase and the chlorohydrate form and the complex of the base form with cyclodextrin to the aqueous phase.

It was possible to add the chlorohydrate form to all the different tested concentrations (1%, 2.5% , 5% w/v), while for PRL base we cannot overcome the 2.5% w/v because we have instability problems.

We also prepared double-charged liposomes, by adding PRL in the lipophilic phase and PRL-HPβCD complex in aqueous phase.

The results obtained in terms of particle size, ζ-potential and deformability of the vesicles, expressed as ratio diameter size, and poly-dispersion index are reported in Table 1.

LIPOSOMES	D	P.S. (nm) n=5	ζpotential (mV) n=5	P.I. n=5
empty	1.09	431	+34.6	0.202
PRL HCl 1% in aqueous phase	1.04	331	+59.6	0.185
PRL HCl 2.5% in aqueous phase	1.43	659	+55.4	0.158
PRL HCl 5% in aqueous phase	1.22	506	+55.4	0.130
PRL base 1% + HPβCD in aqueous phase	1.04	479	+36	0.190
PRL base 1 in lipophilic phase	1.07	433	+32.5	0.270
PRL base 2.5% in lipophilic phase	1.09	367	+25.1	0.330
PRL base + HPβCD Double charging 0.5 + 0.5 %	1.08	375	+28.0	0.180
PRL base + HPβCD Double charging 1 + 1 %	1.11	324	+28.7	0.280

Table 1: Ratio diameter size (D), particle size (P.S), ζ potential and poly-dispersion Index (P.I)

From Table 1, we can see that there is not a close relationship between the size of vesicles (P.S.) and concentration of loaded drug.

Moreover, it can be observed that the loading of large amounts of PRL.HCl leads to particles with bigger size.

It is also interesting to note that liposomal suspensions loaded with the hydrochloride form of the drug are more homogeneous, with Polidispersion Index values lower than those of vesicles containing the base form.

As for the ratio between the diameters of the vesicles before and after extrusion (D), it is very close to 1 when the vesicles have sufficiently small dimensions. For liposomal dispersions with the average diameter greater than 500 nm extrusion causes a quite marked reduction in size.

These results agree with those published by some authors who carried out an analogous study (Trotta et al. 2004).

The values of ζ–potential obtained are all positive, especially for the presence of stearylamine. The liposomes containing the drug in the hydrochloride form gave the higher values of potential, and thus these preparations should have less problems of physical stability. For liposomal suspensions containing large amounts of the drug in the base form, phenomena of merge and aggregation are more likely.

In vivo studies

Results of in vivo studies are reported in Figures 5, 6 and 7.

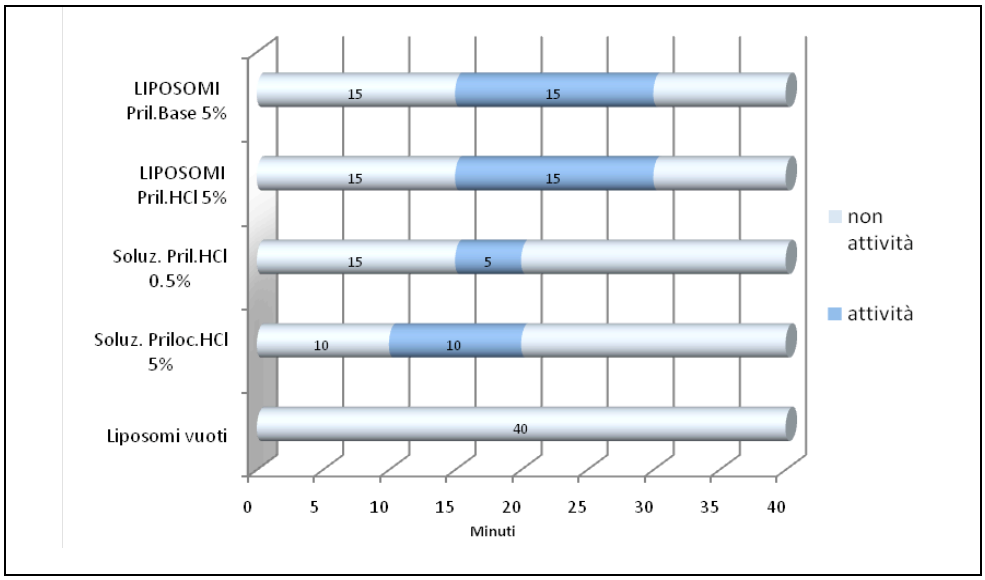


Figure 5: Liposomes with PRL base 5%, PRL HCl 5%, PRL HCl solution 0.5%, PRL HCl 5% solution and empty liposomes.

As shown in Figure 5, liposomes containing PRL and PRL.HCl have both the same effect, showing a more prolonged *anaesthetic* effect and a delayed start of action with respect to the PRL solution, thus confirming the potential of liposomal formulation as effective delivery systems for this kind of drug.

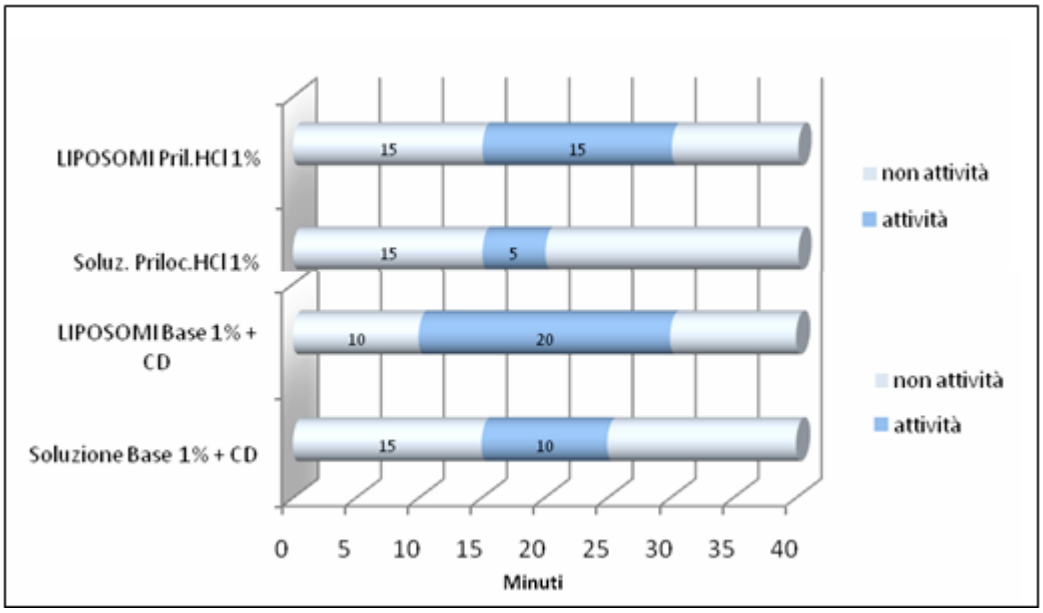


Figure 6: Liposomes with PRL.HCl 1%, PRL.HCl 1% solution, liposomes with PRL base 1% plus CD, PRL base solution 1% plus CD solution.

In Figure 6 we can see that liposomes containing PRL.HCl 1% have an activity three-times superior in terms of duration of drug anaesthetic effect with respect to the simple solution with PRL.HCl 1%.

Liposomes containing the drug as complex with cyclodextrin showed a reduced lag time before the appearance of the drug anaestethic effect with respect to other liposomal formulation and, moreover, they showed the longest duration of effect.

This behaviour can be attribute to the cyclodextrin enhancer properties that improve the drug effectiveness.

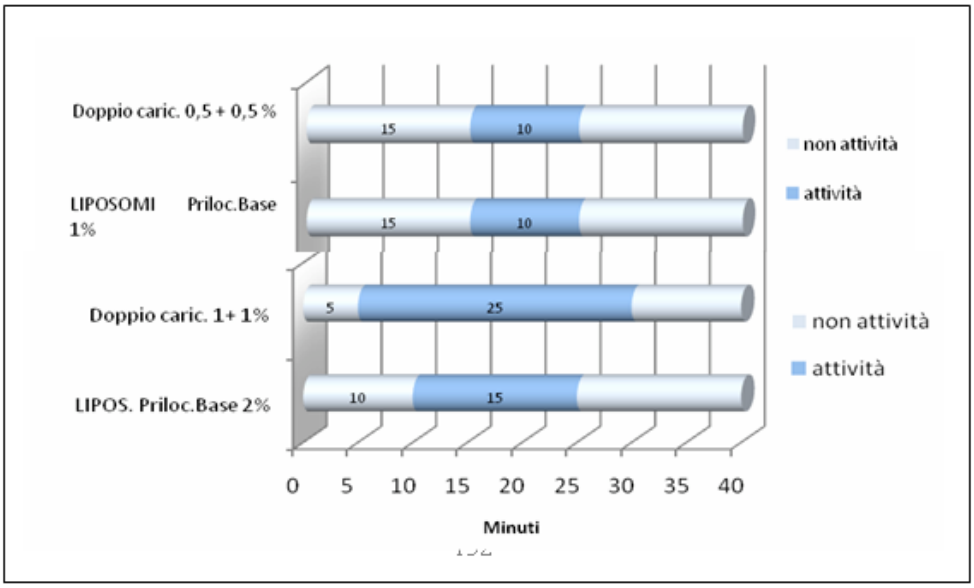


Figura 7: Liposomes with double charge (0.5+0.5%), PRL base 1%, Double charge 1+1% and PRL base 2%.

In figure 7, the double-charged liposomes with the total drug dose of 2% w/v showed the best therapeutic activity, exhibiting the longest duration of effect and a beginning of the effect in a shorter time than the other liposomes preparations.

It was supposed a synergic effect between liposomal carrier and cyclodextrin on drug activity.

In conclusions, we developed and tested ten liposomal preparations containing the drug as free base or as hydrochloride salt varying drug concentrations from 1 to 5 %, and using it complexed or not with HPβCD.

These tests allowed to verify the actual formation of the vesicles and to accurately characterize the liposomes.

The best formulations have been then selected to evaluate their effectiveness in vivo. Liposomal suspensions have shown greater effectiveness, especially in terms of occurrence and duration of action compared to the corresponding non-liposomal preparations.

Moreover, the use of cyclodextrin complexation has proved to be useful to further increase the duration of the drug anaesthetic effect and reduce the time for the appearance of the effect.

The liposomes that have provided the best results were those with PRL base in the aqueous phase complexed with hydroxypropyl-β-cyclodextrin and those "double-charged", with prilocaine base in the aqueous phase, complexed with cyclodextrin, and in the lipophilic phase as free base.

The results obtained have led to a marked improvement in the drug therapeutic activity.

The use of vesicles type DCL (drug-in-cyclodextrins-in-liposomes) represents a good approach for controlling the release of prilocaine and avoid the use of vasoconstrictors for the prolongation of the anaesthetic effect.

12. Comparative study of oxaprozin complexation with natural and chemically-modified cyclodextrins in solution and in the solid state.

Cyclodextrin complexation of anti-inflammatory drugs generally allowed obtainment not only of improvement of solubility, but also of additional advantages such as masking of taste, lowering of dose, reduction of side effects (particularly gastric irritation) (Otero-Espinar et al, 1991; Elkheshen et al., 2002; Imai et al., 1984).

In the present work we investigated the possibility of improving the unfavourable chemical-physical properties of oxaprozin, a very poorly water soluble NSAID, by cyclodextrin complexation.

With this aim we carefully examined the performance of a series of cyclodextrins, both natural (α–, β–, γ– Cd) and derivative (hydroxypropyl-βCd, heptakis-2,6-di-O-methyl-βCd, amorphous randomly substituted methyl-βCD and semi-crystalline methyl-βCD), in order to evaluate the role of both the cyclodextrin cavity size, their amorphous or crystalline state and the presence and type of substituent on their ability to establish effective interactions with the drug.

Moreover, several methods have been proposed for complex preparation, and the best process must be chosen for each guest to be complexed with each cyclodextrin (Mura et al., 1999; Al-Marzoqi et al., 2007). Therefore, equimolar solid systems of the drug with the selected cyclodextrins were prepared by different techniques (physical mixing, kneading, co-grinding, coevaporation, sealed-heating and colyophilization), in order to investigate the influence of the preparation method on the physical-chemical properties of the end product and to arrive to a rational and careful selection of the most successful system for improving the oxaprozin dissolution properties.

Drug-cyclodextrin interactions in solution and in the solid state were investigated by phase-solubility analysis, Differential Scanning Calorimetry (DSC), Powder X-ray Diffractometry, Fourier Transform Infrared Spectroscopy and Scanning Electron Microscopy.

The dissolution rate of the different solid-systems was determined according to the dispersed amount method.

12.1 Materials and methods

Materials

Oxaprozin (OXA) was a gift from S.I.M.S. (Incisa Valdarno, Firenze, Italy) and was used as received. Crystalline α-cyclodextrin (αCd), γ-cyclodextrin (γCd) and 2,6 di-O-methyl ß-cyclodextrin (DIMEB) were purchased from Sigma Chemical Co. (Saint Louis U.S.A.).

Amorphous methyl-ß-cyclodextrin (RAMEB), with an average molar substitution degree per anhydroglucose unit of 1.8 was a gift from Wacker-Chemie GmbH (München, Germany).

Crystalline β-cyclodextrin (βCd), partially crystalline methyl-ß-cyclodextrin (CRYSMEB), and amorphous hydroxypropyl-ß-cyclodextrin (HPβCd) with an average substitution degree per anhydroglucose unit of 0.65 was kindly donated by Roquette.

Phase-solubility studies

An excess amount of drug (60 mg) was added to 10 mL of pH 5.5 phosphate buffer solutions containing increasing concentrations of Cd in sealed glass containers preserved from the light and electromagnetically stirred (500 rpm) at constant temperature (25 °C) until equilibrium (3 d).

Aliquots were withdrawn, filtered (0.45 μm pore size) and spectrometrically assayed for drug concentration at 285.2 nm (UV/VIS 1600 Shimadzu spectrophotometer, Tokyo, Japan).

The presence of Cd did not interfere with the spectrophotometric assay of OXA. In fact it has been verified that the UV absorbance (at the selected λ_{max}) of a 4.0 μg/mL OXA solution did not change in the presence or not of different concentrations of any of the examined Cds.

Each test was performed in triplicate (coefficient of variation (C.V.) <3%).

The apparent 1:1 binding constants of the different OXA-Cd complexes were calculated from the slope of the straight lines of the phase-solubility diagrams (Higuchi et al., 1965).

Preparation of solid systems

Six different methods were used for the preparation of equimolar drug-cyclodextrin solid systems.

Physical mixing: Physical mixtures (PM) were obtained by 15 min tumble mixing equimolar amounts of the respective simple components (75-150 µm sieve granulometric fraction).

Kneading: Kneaded products (KN) were prepared by adding a small volume of ethanol to a known amount of the physical mixture. The resultant mixture was kneaded thoroughly with a pestle to obtain homogeneous slurry and continued until the solvent was completely removed. The sample was kept in an oven at 40°C for 24 h to remove traces of solvent.

Co-grinding: co-ground products (GR) were prepared by ball-milling physical mixtures in a high-energy vibrational micro-mill (Mixer Mill MM 200 Retsch, GmbH, Düsseldorf, Germany) at a frequency of 24 hertz for 30 min.

Coevaporation: Coevaporated products (COE) were prepared by coevaporation of equimolar drug-Cd ethanol-water (5:5 v/v) solutions in a rotary evaporator (Heidolph Laborota 4000) at 85°C. The resulting products were then dried in a vacuum desiccator for 48 h to remove traces of solvents.

Sealed-heating: Sealed-heated products (SH) were prepared by heating in sealed glass containers at 90°C for 3 h known amounts of drug-Cd physical mixtures, added of 10 µl bidistilled water. Then the samples were removed and kept in a desiccator overnight to remove traces of water.

Colyophilization: Colyophilized products (COL) were prepared by freeze-drying (Lyovac GT2, Leybold-Heraeus) at –50 °C and $1.3.10^{-2}$ mm Hg 100 mL of equimolar drug-Cd water-ethanol solutions at suitable concentrations on pre-chilled shelves of 20 cm diameter and 18 mm height. Samples were kept in a desiccator, due to their high hygroscopicity.

Each solid product was sieved and the 75-150 µm granulometric sieve fraction used for the following tests.

Differential scanning calorimetry (DSC)

DSC analyses of the individual components or the different OXA-Cd combinations were performed using a Mettler TA4000 Star^e system equipped with a DSC 25 cell. Weighed samples (5-10 mg, Mettler M3 Microbalance) were scanned in Al pans pierced with a perforated lid at 10 °C/min from 30 to 200°C temperature range under static air. The instrument was calibrated using Indium as a standard (99.98% purity; melting point 156.61°C; fusion enthalpy 28.71 $J.g^{-1}$).

Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra (Perkin-Elmer Mod. 1600) of the individual components and of the different OXA-Cd solid systems were obtained as Nujol dispersion in the 4000-600 cm^{-1} region.

X-ray Powder Diffractometry (XRPD)

The X-ray powder diffraction patterns of the individual components or the different OXA-Cd combinations were taken at ambient temperature with a Bruker D8-advance apparatus (θ/θ geometry) using a Cu Kα radiation and a graphite monochromator, at a 40 mV voltage and 55 mA current, over a 5-40° 2θ range at a scan rate of 0.05 $°s^{-1}$.

Scanning Electron Microscopy (SEM)

Surface morphology of pure components and the different drug-Cd equimolar systems obtained by different techniques was examined using a Philips XL-30 Scanning Electron Microscope equipped with an image analysis system.

Prior to examination, samples were sputter coated with gold–palladium under argon atmosphere (to render them electrically conductive) using a Fine Coat Ion Sputter (JFC-1100 JEOL).

Dissolution studies

Dissolution rates of OXA, both alone and from the different drug-Cd systems, were determined in pH 5.5 phosphate buffer at 37±0.5°C according to the dispersed amount method, by adding 30 mg of drug or drug-equivalent to 300 mL of pH 5.5 phosphate buffer, in a 400 mL beaker.

A glass three-blade propeller (19 mm diameter) was immersed in the beaker 25 mm from the bottom and rotated (f = 100 min^{-1}). Suitable aliquots were withdrawn with a filter-syringe (pore size 0.45 μm) at the specified times and the drug concentration was spectrometrically assayed (UV/VIS 1601 Shimadzu).

The same volume of fresh medium was added to the beaker and the correction for the cumulative dilution was calculated. Each test was repeated three times (coefficient of variation <5%).

Dissolution was characterised through the percent of drug dissolved after 10 min, as index of the rate of dissolution, and the Dissolution Efficiency at 60 min, as index of the totality of the process.

Dissolution efficiency (D.E.) was calculated from the area under the dissolution curve at time t (measured using the trapezoidal rule) and expressed as a percentage of the area of the rectangle described by 100 % dissolution in the same time (Khan et al., 1975).

12.2 Results and discussion

Phase-solubility studies

The solubility of OXA increased linearly with increasing cyclodextrin concentration, giving in all cases A_L-type phase-solubility diagrams, indicative of the formation of soluble complexes of probable 1:1 mol:mol stoichiometry (Fig. 1) (Higuchi et al., 1965).

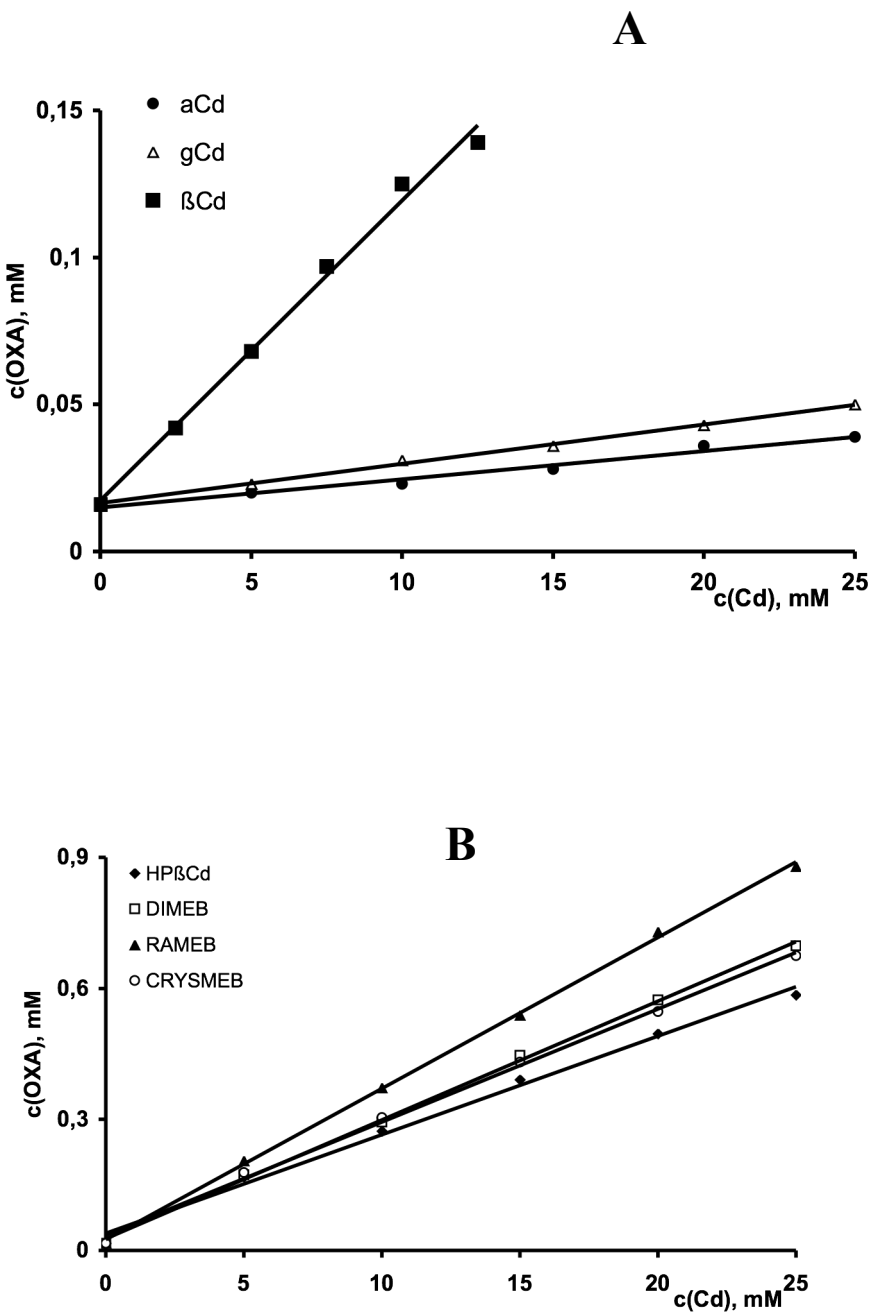


Figure 1. Phase-solubility studies of oxaprozin (OXA) and natural (A) or derivative (B) cyclodextrins in pH 5.5 buffered water at 25 °C.

The apparent 1:1 stability constants, calculated from the straight lines of the diagrams, and the relative solubilizing efficiency values are collected in Table 1.

Table 1 Apparent stability constants of 1:1 complexes of oxaprozin with the different examined Cds, and related solubilizing efficiency values

Cd type	$K_{1:1}$ M^{-1}	Solubilizing efficiency*
αCd	60	2.25
βCd	350	8.70
γCd	80	2.69
HPßCd	1445	31.0
DIMEB	1750	35.9
RAMEB	2240	45.6
CRYSMEB	1660	34.2

*ratio between solubility of drug in the presence of 20 mM Cd (or 12.5 mM ßCd) and drug alone in pH 5.5 phosphate buffer at 25 °C.

Among the natural Cds, βCd was clearly the most effective partner, indicating that its cavity has the most suitable dimensions to accommodate the OXA molecule. However, the stability constants of the complexes with all the examined βCd-derivatives were distinctly higher than that of the parent βCd.

Analogous results have previously been obtained with other NSAIDs derivative of propionic acid such as naproxen (Bettinetti et al., 1989), ketoprofen (Mura et al., 1998) and flurbiprofen (Cirri et al., 2005).

The better performance of these derivatives has been attributed to the presence of hydroxypropyl and even more of methyl substituents that expanded the hydrophobic region of the macromolecule, by capping the edge of the cavity, and increased substrate binding via a hydrophobic effect.

The stability constant values of the complexes with OXA were in the order RAMEB>DIMEB≈CRYSMEB≥ HPβCd>>βCd>>γCd≈αCd. The same rank order was observed also as for their solubilizing efficiency towards OXA (Table 1).

Based on these results, βCd, among the natural Cds, and RAMEB and DIMEB, among its derivatives, were selected for further studies as the best potential carriers for OXA. Solid drug-Cd binary systems with selected Cds were then prepared at 1:1 molar

ratio (as indicated by phase-solubility studies) by using different techniques (physical mixing, kneading, coevaporation, co-grinding, sealed-heating, and freeze-drying), in order to select the most suitable one for the preparation of solid inclusion complexes (Mura et al., 1999). The solid-state properties of the obtained products were then examined by DSC, FT-IR, XRPD and SEM analyses.

Solid-state studies

The DSC curves of pure components and of the various drug-Cd equimolar systems obtained with the different techniques are shown in Figure 2, whereas the relevant thermal parameters are collected in Table 2.

Table 2 Thermal parametres of oxaprozin (OXA), alone and in its equimolar physical mixtures (P.M.),sealed-heated (S.H.), kneaded (KN), coground (GR), coevaporated (COE) and colyophilized (COL) products with the examined Cds.

sample	T_{onset} (°C)	T_{peak} (°C)	ΔH_{fus} (J/g)
OXA	161.3	161.7	121.6
OXA-βCd P.M.	160.2	161.8	124.8
OXA-DIMEB P.M.	133.4	146.1	88.6
OXA-RAMEB P.M.	135.2	140.2	21.6
OXA-βCd S.H.	157.5	160.0	94.2
OXA-DIMEB S.H.	136.4	145.5	55.6
OXA-RAMEB S.H.	112.0	118.0	10.3
OXA-βCd KN	159.6	161.2	120.1
OXA-DIMEB KN	--	--	--
OXA-RAMEB KN	--	--	--
OXA-βCd GR.	140.4	156.4	10.8
OXA-DIMEB GR	--	--	--
OXA-RAMEB GR	--	--	--
OXA-βCd COE	159.7	161.8	40.8
OXA-DIMEB COE	110.6	124.9	12.5
OXA-RAMEB COE	--	--	--
OXA-βCd COL	160.8	162.0	116.1
OXA-DIMEB COL	117.6	133.5	10.3
OXA-RAMEB COL	119.7	130.5	11.6

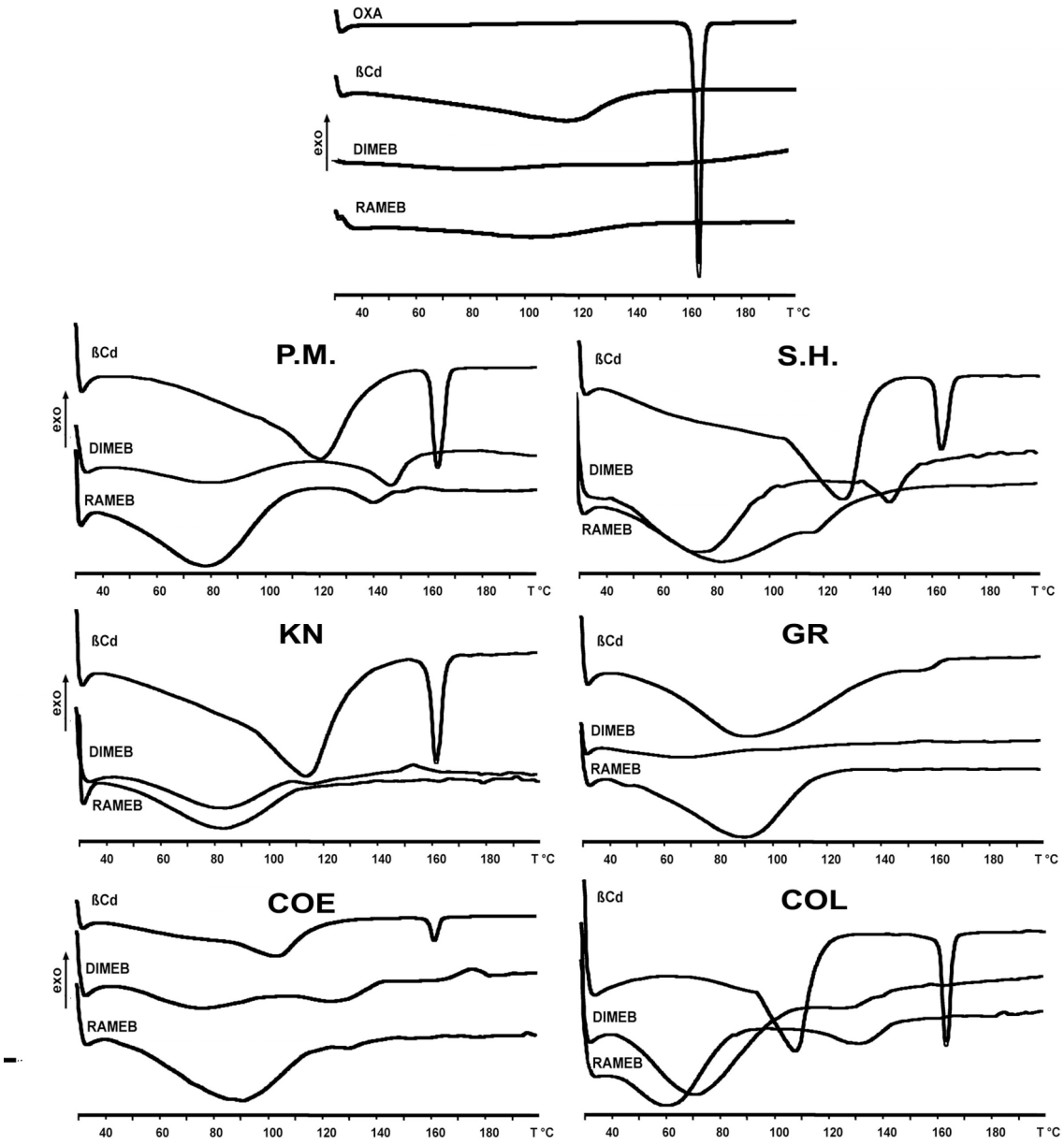


Figure 2. DSC curves of pure oxaprozin (OXA), ßCd, DIMEB and RAMEB and of equimolar drug-Cd physical mixtures (P.M.), sealed-heated (S.H.), kneaded (KN), co-ground (GR), coevaporated (COE) and colyophilized (COL) products.

The DSC curve of OXA was typical of a crystalline anhydrous substance, with a sharp fusion endotherm peaked at 161.3 °C.

Liberation of crystal water from βCd (14.5 % as mass fraction) was observed as an intense endothermic effect peaked at about 119 °C. Broader endotherms were instead associated with water losses from βCd-derivatives, respectively of 5.7% and 16% as mass fraction for DIMEB and RAMEB, respectively.

The thermal profile of the drug maintained its shape in its PM with βCd, while a marked broadening with a concomitant shift to lower temperatures was evident in its blend with DIMEB and even more with RAMEB. The characteristic drug melting peak appeared practically unchanged in all the examined binary systems with ßCd, except in COE products, where it appeared to be strongly reduced in intensity, and in GR products, where it almost totally disappeared.

This modification of the DSC melting peak can taken as a proof of interactions between the components and/or of consequent progressive drug amorphization. As previously observed with other NSAIDs, (Mura et al., 1998; Mura et al., 1995; Mura et al., 2002) hydrophilic βCd-derivatives were more effective than natural βCd in establishing solid-state interactions with the drug.

In fact, OXA fusion endotherm was markedly broadened and reduced in intensity in all the systems with DIMEB and RAMEB, up to its complete disappearance in KN and GR products.

Moreover, it was confirmed that also the crystalline or amorphous state of the carrier plays a role in inducing drug amorphization, mediated by a highly dispersed physical state of the drug within the carrier matrix (Mura et al., 2002).

In particular the amorphous derivative, i.e. RAMEB, demonstrated the highest amorphizing power toward the drug with all the preparation techniques used, as can be observed by comparing the corresponding ΔH_{fus} values of OXA in the different systems (Table 2). On the other hand, as for the influence of the preparation method, co-grinding technique appeared as the most powerful in inducing drug-Cd interactions as well as drug amorphization with all the examined Cds.

This was a rather unexpected result, since colyophilization technique often showed to be more efficacious in this regard than simple co-grinding (Bettinetti et al., 1992; Blanco et al., 1991; Junco et all., 2002).

However, the suitability of Cd co-grinding technique to obtain and stabilize drugs in the amorphous form has been well demonstrated (Mura et al., 2001;Mura et al., 2002; Cirri et al., 2004).

X-ray diffraction patterns (Fig. 3) substantially confirmed the results of DSC analysis. In fact, only the co-grinding technique allowed total or almost total sample amorphization of products with derivatives and natural βCd, respectively.

On the contrary, typical drug crystallinity peaks were present in both COE and COL systems with βCd and some slight residual drug crystallinity, as revealed by the characteristic peak of OXA at 9 °2 Θ, was still detectable in the corresponding systems with RAMEB and DIMEB.

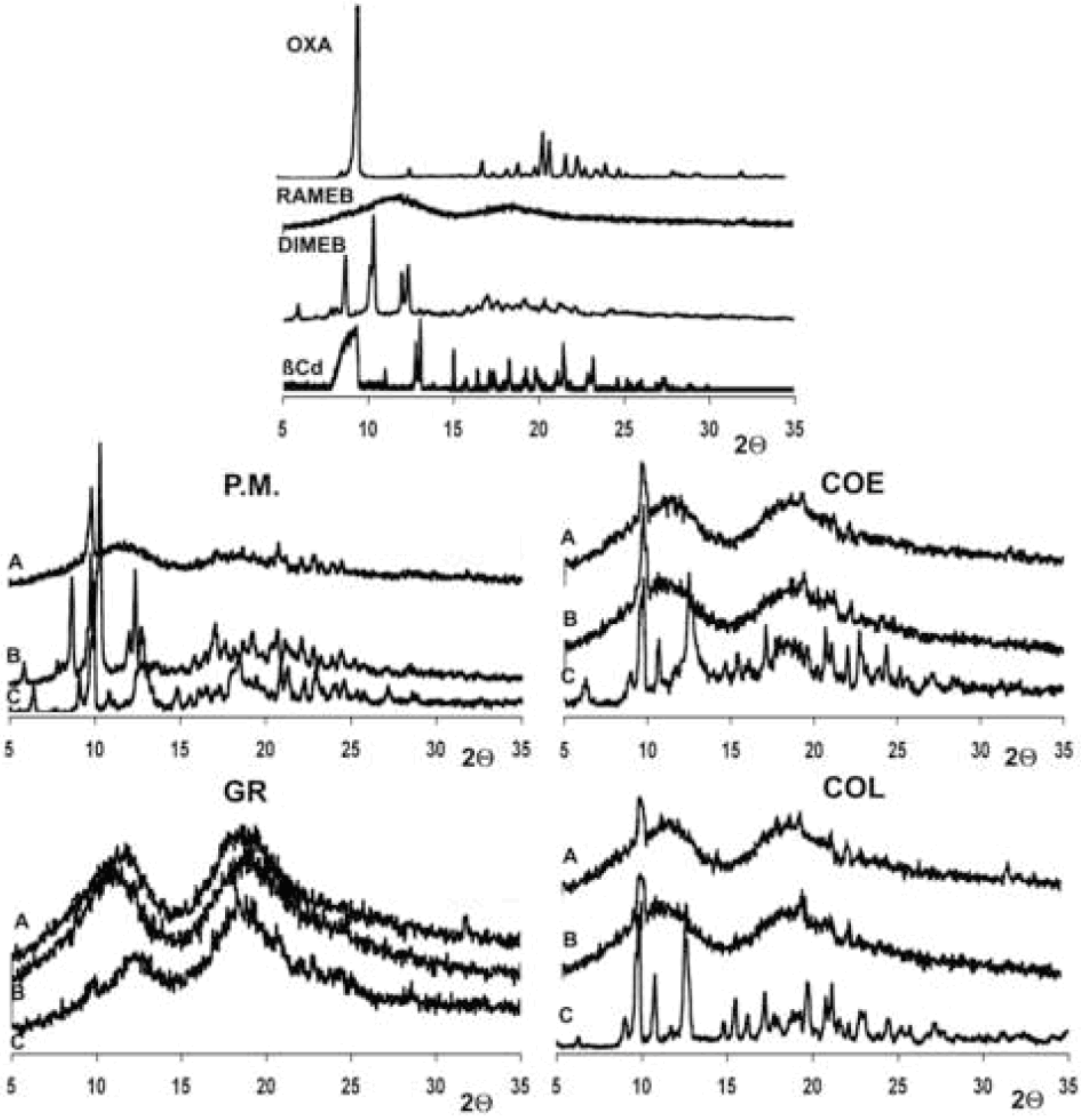


Figure 3. X-ray diffraction patterns of pure oxaprozin (OXA), ßCd, DIMEB and RAMEB and of equimolar drug-Cd physical mixtures (P.M.), coevaporated (COE), co-ground (GR), and colyophilized (COL) products.

FTIR analysis further evidenced the greatest effectiveness of the co-grinding technique in producing effective drug-carrier solid-state interactions.

In fact, no important variations with respect to the corresponding physical mixture were observed in the patterns of the products obtained with the different techniques, except the co-ground systems, where a broadening of the characteristic acid carbonyl stretching band of OXA accompanied by a significant shift from 1718 to 1700 cm^{-1} was observed, as is shown in Fig. 4 for the series of OXA-RAMEB binary systems.

This effect can be attributed to the breakdown of the intermolecular hydrogen bonds and formation of a monomeric dispersion of drug as a consequence of the interaction with the Cd (Mura et al., 1998).

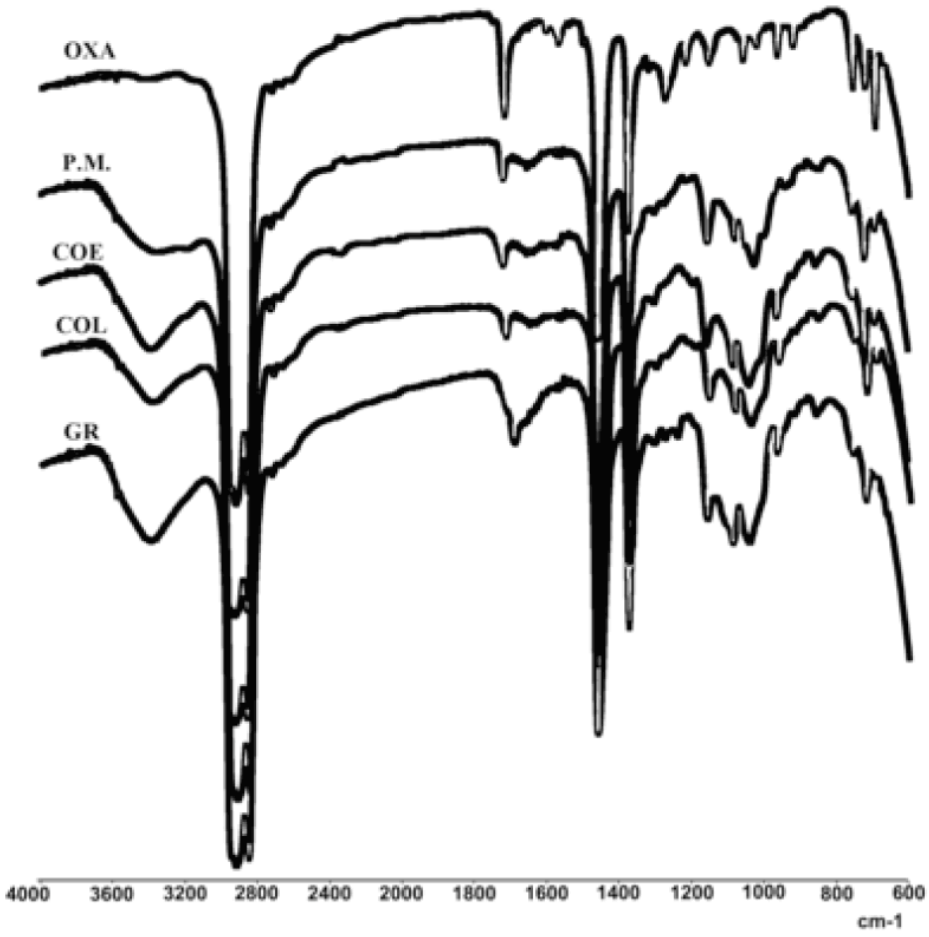


Figure 4. FT-IR spectra of pure oxaprozin (OXA), ßCd, and RAMEB and of equimolar drug-Cd physical mixtures (P.M.), coevaporated (COE), colyophilized (COL), and co-ground (GR) products.

Selected micrographs obtained from SEM analysis are shown in Fig. 5.

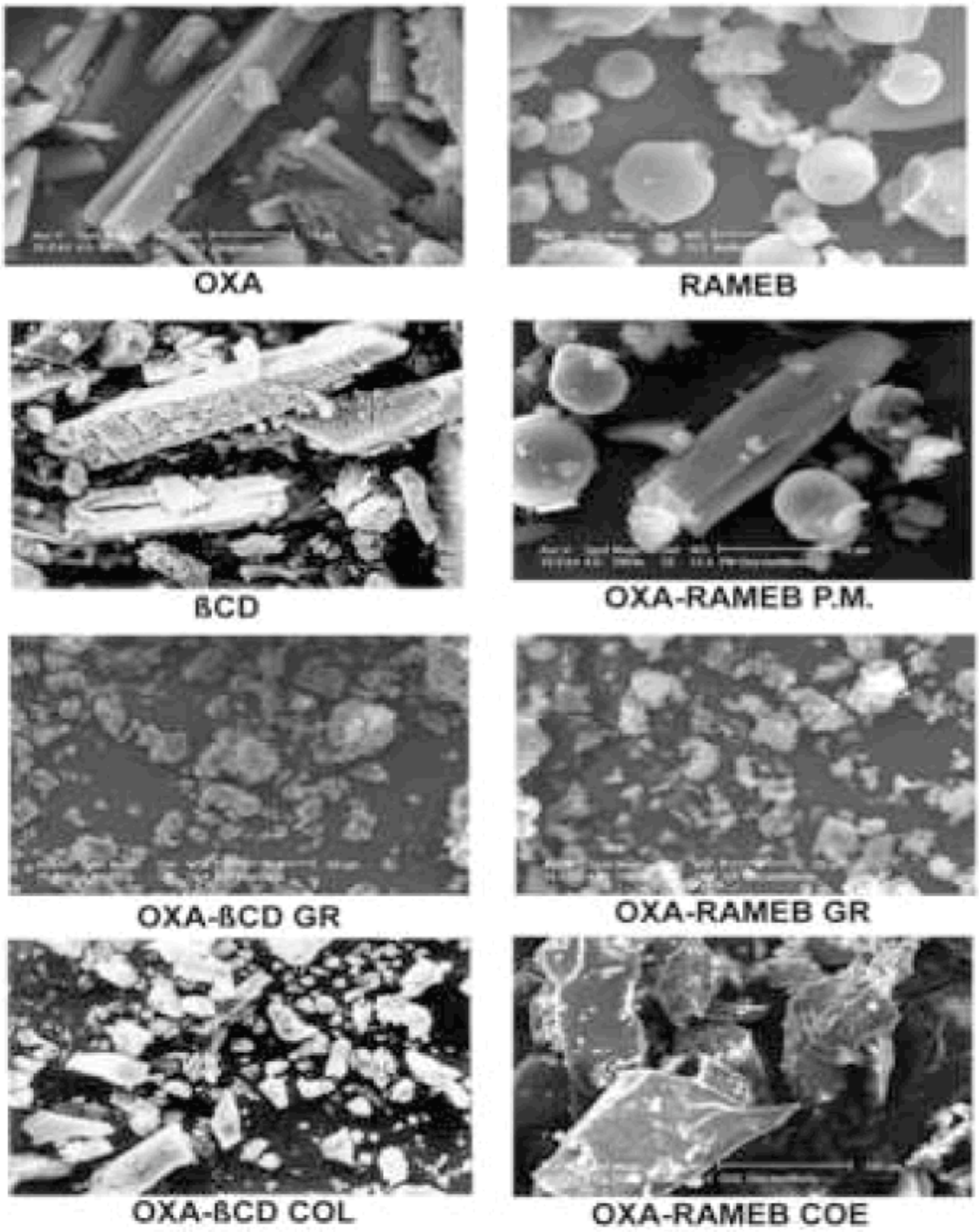


Figure 5. SEM micrographs of of equimolar drug-Cd physical mixtures (P.M.), co-ground (GR), coevaporated (COE) and colyophilized (COL) products.

OXA particles appeared under scanning electron microscopy as polyhedric crystals with smooth surfaces, partially agglomerated in bundles.

ßCd and DIMEB consisted of large crystalline particles of rather irregular shape and size, whereas RAMEB appeared as amorphous spherical particles.

In keeping with the DSC and X-ray analyses findings, the characteristic drug crystals, mixed with Cd particles, were clearly evident in all physical mixtures.

Distinctive drug crystals, dispersed or adhered to the surface of the carrier, were well detectable in all the products with ßCd, except the GR one.

In fact, in this case, the original morphology of both drug and ßCd disappeared, and only amorphous pieces of irregular size were present, making it no longer possible to differentiate the two components.

A similar aspect was found for GR products with DIMEB and RAMEB, while some residual drug crystals were still noticed in the corresponding COE and COL systems.

Dissolution studies

The most significant dissolution parameters obtained from the different OXA-Cd systems examined are collected in Table 3, while the drug dissolution profiles from selected binary products are shown in Figures 6 and 7.

As for the influence of the preparation technique (Fig. 6), dissolution tests revealed that co-grinding was clearly the most effective one in improving the drug dissolution behaviour, followed by colyophilization, and then by coevaporation while sealed-heating (curve not shown) was the worst one, giving results not significantly different from the simple physical mixture.

These results were in full agreement with those of solid-state studies.

In fact, the best dissolution profiles shown by co-ground products can be attributed to the higher amorphization degree and stronger drug-Cd solid-state interactions obtained with the co-grinding technique, as revealed from DSC, X-ray diffractometry and FT-IR analyses.

Table 3 Percent dissoved at 10 min (P.D.10) and Dissolution Efficiency (D.E.60) at 60 min of oxaprozin (OXA), alone and from its equimolar physical mixtures (P.M.), sealed-heated (S.H.), kneaded (KN), coground (GR), coevaporated (COE) and colyophilized (COL) products with the examined Cds.

sample	P.D.10	D.E.60
OXA	6.5	6.9
OXA-ßCd P.M.	9.8	10.3
OXA-DIMEB P.M.	11.3	12.6
OXA-RAMEB P.M.	12.4	13.0
OXA-ßCd S.H.	10.2	10.8
OXA-DIMEB S.H.	11.6	12.9
OXA-RAMEB S.H.	12.7	13.3
OXA-ßCd KN	12.4	13.1
OXA-DIMEB KN	13.5	14.0
OXA-RAMEB KN	14.1	14.7
OXA-ßCd GR.	16.4	18.4
OXA-DIMEB GR	28.6	29.7
OXA-RAMEB GR	46.6	46.9
OXA-ßCd COE	13.0	13.7
OXA-DIMEB COE	16.3	17.0
OXA-RAMEB COE	17.3	18.1
OXA-ßCd COL	14.1	14.8
OXA-DIMEB COL	17.4	18.7
OXA-RAMEB COL	18.1	19.7

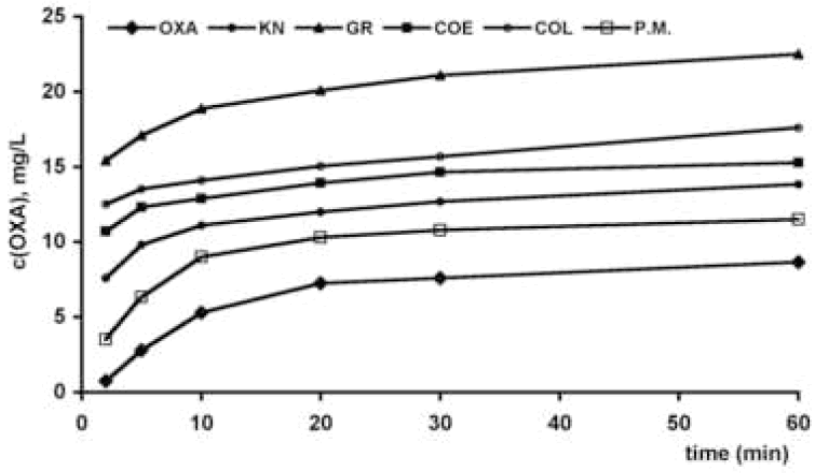


Figure 6. Dissolution curves of oxaprozin (OXA) alone and from equimolar physical mixtures (P.M.), kneaded (KN), co-ground (GR), coevaporated (COE) and colyophilized (COL) products with ßCd.

On the other hand, a comparison of the performance of the three different carriers (Fig. 7) evidenced the same trend observed in previous phase solubility studies.

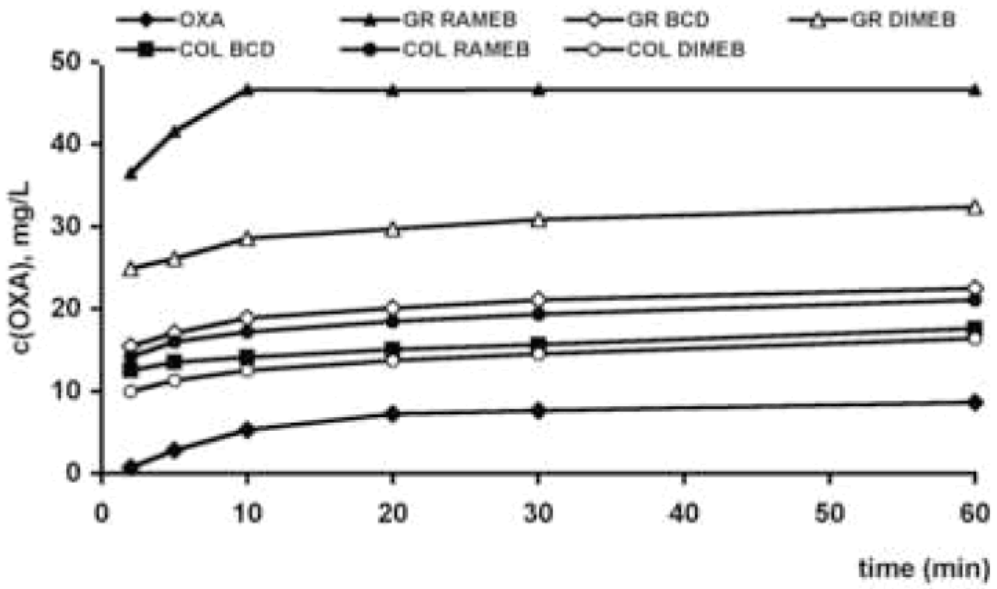


Figure 7. Dissolution curves of oxaprozin (OXA) alone and from equimolar co-ground (GR) and colyophilized (COL) products with ßCd, DIMEB and RAMEB.

In particular, RAMEB confirmed to be the best partner for OXA, exhibiting the highest complexing and solubilizing power, and giving rise to the product with the best dissolution profile.

Moreover, over-saturation levels were not achieved with respect to the drug solubility values obtained in phase-solubility studies, and therefore high stability of the obtained solutions is expected.

In conclusion, cyclodextrin complexation was successful in improving OXA dissolution properties. βCd showed the best performance among the natural Cds, indicating that its cavity was the most suitable for accommodating the drug molecule.

The presence of substituents on the rim of the βCd cavity significantly improved its complexing and solubilizing effectiveness towards the drug, and methylated derivatives were better than the hydroxy-propylated ones.

Moreover, also the amorphous nature of the partner was important.

In fact, among the examined methyl-derivatives, RAMEB proved to be the most effective in performing solid-state interactions and in improving drug wettability and dissolution properties.

Therefore the choice in pharmaceutical formulations of the amorphous RAMEB rather than the crystalline DIMEB can be recommended, also taking into account economic considerations.

However, the anhydrous and nonhygroscopic nature of crystalline DIMEB could be particularly advantageous in case of moisture-sensitive formulations (Mura et al., 2001).

13. Physical–chemical characterization of binary systems of metformin hydrochloride with triacetyl-β-cyclodextrin.

In the last years cyclodextrins (CyDs) received an increasing interest in the pharmaceutical field due to their ability to favourably modify physical, chemical and biological properties of drug molecules through the formation of inclusion complexes (Hirayama et al., 1999). Recently, several kinds of chemically modified CyDs have been prepared in order to improve the physicochemical properties and inclusion abilities and extend the spectrum of the pharmaceutical applications of the parent molecules (Uekama et al., 1998; Loftsson et al., 2007) .

Among these, the hydrophilic CyDs have been extensively employed as helpful carriers to improve dissolution rate and bioavailability of poorly water-soluble drugs (Loftsson et al., 2005; Mura et al., 2005; pinto et al., 2005; Liu et al., 2006). On the contrary, there are less data about the use of the hydrophobic CyD derivatives, such as the peracylated ones, which have been proposed as sustained-release carriers for highly soluble drugs with short biological half-lives, in virtue of the formation of poorly water-soluble complexes (Nakanishi et al., 1997; Fernandes et al., 2002; Fernandes et al., 2003).

Metformin hydrochloride is an oral anti-hyperglycaemic agent highly water-soluble, whose low bioavailability and short and variable biological half-life (1.5–4.5 h) needs frequent administrations to maintain effective plasma concentrations, thus making the development of sustained-release forms desirable (Marchetti et al., 1989).

Moreover, the oral absorption of metformin is mainly confined to the upper part of the gastrointestinal tract, thus requiring the development of suitable delivery systems with a timely modulation of the drug release rate (Sheen et al., 1996; Vidon et al., 1988; Marathe et al., 2000).

Thus, we considered it worthy of interest to evaluate the effectiveness of triacetyl-β-cyclodextrin (TAβCyD), a hydrophobic CyD derivative practically insoluble in water, as a carrier for obtaining a slow-dissolving complex of the drug, to be used for

the subsequent development of a well-timed sustained-release oral dosage form of metformin.

It is known that different methods can be employed for preparing solid drug–cyclodextrin complexes, and the choice of the most efficacious one should be carefully evaluated case by case (Mura et al., 1999; Juno et al., 2002).

In particular, an in depth characterization of the solid-state properties of the obtained products is strongly advisable, since they can affect the drug–carrier interactions, which in turn influence the dissolution rate and drug stability (Bettinetti et al., 2002).

Therefore, in the present work, equimolar drug–TAβCyD solid compounds were prepared by different methods, i.e., physical mixing, kneading, co-grinding, sealed-heating, and spray drying and characterized by differential scanning calorimetry, X-ray powder diffractometry, Fourier transform infrared spectroscopy and scanning electron microscopy, in order to carefully investigate and compare the physical–chemical properties of the obtained products, for a rational selection of the best one.

In addition, the *in vitro* dissolution behaviour of the different products was determined according to the dispersed amount method, with the aim of studying possible implications of the system preparation method on the dissolution properties of the drug.

13.1 Materials and methods

Materials

Metformin hydrochloride (MF·HCl) was kindly supplied by Menarini (Firenze, Italy). Triacetyl-β-cyclodextrin (TAβCyD) (Cavasol® W7 TA) was a kind gift of Wacker-Chemie (GmbH, Germany). All other chemicals and solvents were of analytical reagent grade.

Preparation of solid binary systems

MF·HCl–TAβCyD equimolar systems were obtained from the individual components previously sieved (75–150 μm): (a) by tumble mixing for 20 min with a turbula mixer (physical mixtures, PM); (b) by ball-milling physical mixtures in a high

vibrational micro-mill for 30 min at 24 Hz (co-ground systems, GR); (c) by wetting physical mixtures in a mortar with the minimum volume of an ethanol–water 1:1 (v/v) solution and grinding thoroughly the slurry with a pestle to obtain a paste which was then dried under vacuum at 40 ◦Cup to constant weight (kneaded systems, KN); (d) by heating physical mixtures in sealed containers at 90°C for 2 h (sealed-heated systems, SH); (e) by dissolving physical mixtures in an ethanol: water 8:2 (v/v) solution and then spray-drying (IRA Mini Spray Ho, Italy) under the following conditions: inlet temperature, 120 °C; outlet temperature, 70 °C; flow rate of the solution, 13mLmin^{-1}; atomising air pressure, 3 kg/m^2; vacuum conditions of 70mm H_2O (spray dried systems, SP).

To exclude any effect of sample preparation method on the drug and carrier physicochemical characteristics, samples of pure MF·HCl and TAβCyD have been treated with the same techniques used for preparation of equimolar binary systems.

Differential scanning calorimetry (DSC)

DSC analysis was performed with a Mettler TA4000 Stare system (Mettler Toledo, Switzerland) equipped with a DSC 25 cell.

Samples of about 5–10 mg were accurately weighed (Mettler MX5 microbalance) in sealed aluminium pans with pierced lid and scanned at 10 Kmin^{-1}, under static air atmosphere, in the 30–200°C temperature range. Measurements were carried out at least in triplicate.

The instrument was calibrated using Indium as a standard (99.98 % purity; melting point 156.61°C; fusion enthalpy 28.71 J g^{-1}).

X-ray powder diffractometry (XRPD)

The powder X-ray diffraction patterns were taken at ambient temperature with a Brucker D8 apparatus (θ/θ geometry) using a Cu K αradiation and a graphite monochromator. The samples were analysed in the 5–30◦ 2θ range at a scan rate of 0.05◦s^{-1}.

Fourier transform infrared spectroscopy (FTIR)

Infrared spectra were recorded using a Perkin-Elmer Model 1600 spectrophotometer on KBr disks in the range between 4000 and 400 cm^{-1}.

Scanning electron microscopy (SEM)

Surface morphology of pure components and their equimolar binary systems obtained by different techniques was examined using a Philips XL-30 scanning electron microscope equipped with an image analysis system.

Prior to examination, samples were sputter coated with gold–palladium under argon atmosphere (to render them electrically conductive) using a gold sputter module in a high vacuum evaporator.

Dissolution rate studies

In vitro dissolution rate studies of MF·HCl alone and from all the drug–carrier binary systems obtained with the different techniques were performed according to the dispersed amount method.

Samples containing 50 mg of drug or its equivalent as binary system with TAβCyD were added in a 400mL beaker containing 300mL of intestinal artificial fluid (phosphate buffer at pH 6.5) at 37±0.5 °C, and stirred at 100 rpm with a glass three-blade propeller (19mm diameter) immersed in the beaker 25mm from the bottom. At settled time intervals, samples were withdrawn with a syringe-filter (pore size 0.45 μm) and replaced with an equal volume of fresh medium.

The drug concentration was spectrometrically determined (UV–vis 1600 Shimadzu spectrophotometer, Tokyo, Japan) at 232.2 nm. Each test was repeated three times (coefficient of variation < 5%).

Dissolution efficiency (DE) was calculated from the area under the dissolution curve at time *t* and expressed as a percentage of the area of the rectangle described by 100% dissolution in the same time (Khan et al., 1975).

13.2 Results and discussion

Solid-state studies

In order to correctly and accurately investigate drug–carrier solid-state interactions and exclude possible solid-state modifications due to the sample treatment, solid state studies were performed not only on the various MF·HCl–TAβCyD binary systems obtained with the different preparation techniques, but also on the pure components subjected to these same processes.

Differential scanning calorimetry (DSC)

The thermal curve of pure MF·HCl (Fig. 1A, curve a) indicated its crystalline anhydrous state and was characterized by a sharp endothermic fusion peak at 231.0±0.6 ◦C with an associated fusion enthalpy of 292±12 J/g.

The thermal behaviour of TAβCyD (Fig. 1A, curve b) was instead more complex. The sample immediately started losing the weakly hydrogen-bonded water (as shown by the broad initial endothermic band), transforming into a lower melting anhydrous polymorph II which fuses at 191.8±1.9°C and then recrystallizes into a higher melting form, whose fusion endotherm peaked at 219.8±2.0°C.

An analogous thermal behaviour has been described by Bettinetti et al. for commercial TAβCyD. The thermal profile of the drug was almost unaffected by the different treatments, including spray drying (DSC curves not shown); on the contrary, in the case of TAβCyD this happened only for the sealed-heated product (Fig. 1B, curve b1).

In fact, the DSC profiles of TAβCyD treated with both the kneading and co-grinding techniques were different from that of the original sample (Fig. 1B, curves b2 and b3). In particular, after the initial dehydration band, the appearance of a glass transition at about 135°C was observed followed by an exothermic effect, peaking at 164.9°C. This can be attributed to the recrystallization of an amorphous form, obtained during the mechanical treatment of the sample, into the higher melting crystalline form, characterized by a sharp fusion peak at 219.8°C.

A similar thermal behaviour was observed for X-ray amorphous TAβCyD and TAβCyD obtained, respectively, by microwave drying of a propanol–water solution or by spray drying of a water–acetone solution (Bettinetti et al., 2006).

Finally, the spray-dried sample (Fig. 1B, curve b4) exhibited a flat profile with the complete disappearance of both exothermic and endothermic phenomena, suggesting the formation of a more stable amorphous form of the CyD.

The thermal curve of the physical mixture (Fig. 1A, curve c) was practically the sum of those of pure components, showing an initial broad endothermic band, due to water evaporation, followed by three sharp endothermic peaks, due, respectively, to the melting of the two polymorphic forms of TAβCyD and then of the drug.

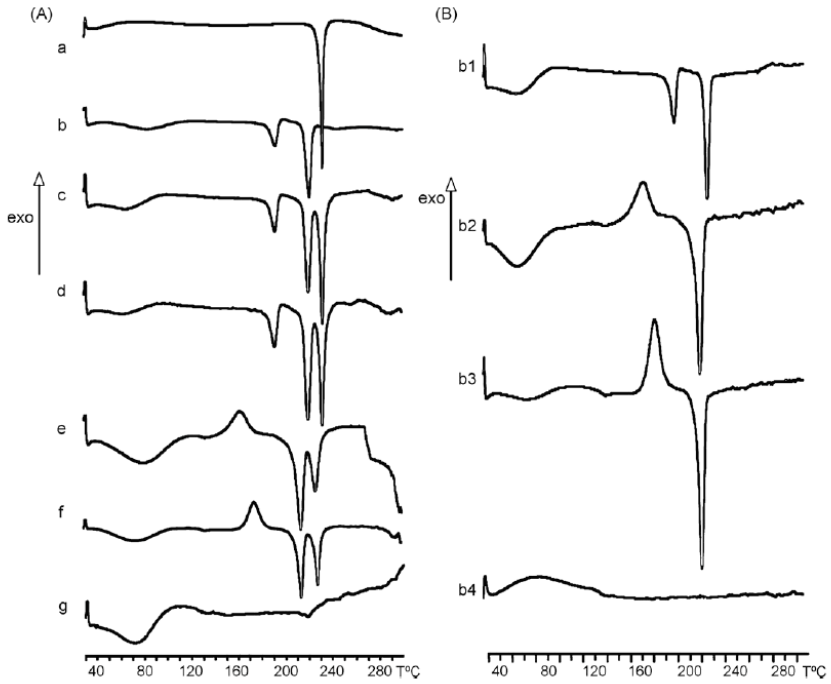


Fig. 1. DSC curves of (A) pure components and their equimolar systems obtained with the different techniques and (B) of TAβCyD after treatment with the same techniques. *Key*—A: (a) MF·HCl, (b) TAβCyD, (c) physical mixture, (d) sealed-heated, (e) kneaded, (f) co-ground, and (g) spray-dried products; B: TAβCyD after (b1) sealed-heating, (b2) kneading, (b3) grinding, or (b4) spray drying.

The binary product obtained by sealed-heating (Fig. 1A, curve d) displayed a very similar behaviour to that of the physical mixture, accounting for the absence of apparent solid-state interactions between drug and CyD.

On the other hand, the thermal profiles of both the binary kneaded and coground products (Fig. 1A, curves e and f) showed the presence of an additional exothermal effect, followed by the fusion peak of the higher melting polymorphic form of TAβCyD and then of the drug.

DSC analysis of pure components made it possible to exclude drug–carrier interactions as being responsible for such exothermal phenomenon and to correctly attribute it to the presence of a TAβCyD unstable amorphous form, obtained during kneading or grinding process, which, during the DSC heating, recrystallizes into the more stable higher melting crystalline form (Fig. 1B, curves b2 and b3).

Some reduction of fusion enthalpy and lowering of melting temperature of MF·HCl, observed in the binary kneaded and, particularly, in the coground products,

can be ascribed to some drug–CyD interactions occurring during sample preparation (Mura et al., 1999). The DSC curve of the spray-dried binary product (Fig. 1A, curve g) showed the complete disappearance of all melting peaks corresponding to both components, indicating total system amorphization as a consequence of strong drug–carrier interactions and/or drug inclusion complexation.

In fact, the absence of the drug melting peak in this system is not attributable to the spray-drying process, which does not substantially affect the solid-state properties of MF·HCl, since the thermal behaviour of the spray-dried drug alone was very similar to that of the untreated sample (curve not shown).

X-ray powder diffractometry (XRPD)

The X-ray diffraction patterns of MF·HCl, TAβCyD, and their respective equimolar binary systems obtained with the different techniques are shown in Fig. 2A, whereas representative spectra of pure components after the different treatments are presented in Fig. 2B.

A series of sharp and intense typical diffraction peaks indicated the crystalline state of pure MF·HCl. Also the TAβCyD diffraction pattern was characterized by the presence of several sharp peaks indicative of its crystallinity.

The diffraction pattern of the physical mixture was simply the superimposition of those of pure components (Fig. 2A, curve c), indicating the presence of both MF·HCl and TAβCyD in the crystalline state.

The diffraction characteristics of the individual components were maintained also in the binary product obtained by sealed-heating (Fig. 2A, curve d), confirming the ineffectiveness of this technique in establishing solid-state drug–CyD interactions, in agreement with the results of DSC analysis.

The loss of crystallinity observed in the kneaded product (Fig. 2A, curve e), and even more in the co-ground product (Fig. 2A, curve f), can be considered as a consequence of drug–carrier interactions brought about by the mechanical treatment. In fact, the kneading and co-grinding processes caused almost complete amorphization of pure TAβCyD (Fig. 2B, curves b2 and b3), whereas it did not markedly reduced drug crystallinity (Fig. 2B, curves a1 and a2).

On the other hand, the spray-dried compound, according to DSC analysis results, presented a completely amorphous diffraction pattern, with the disappearance of the characteristic crystallinity peaks of both MF·HCl and TA(CyD (Fig. 2A, curve g).

Considering that the spray-drying process caused amorphization of pure carrier (Fig. 2B, curve b4) but, in agreement with DSC results, it did not cause an appreciable reduction of crystallinity of pure MF·HCl (Fig. 2B, curve a3), the result obtained for the binary spray-dried product could be imputable to the formation of strong interactions between drug and TAβCyD and/or to the possible drug inclusion complexation.

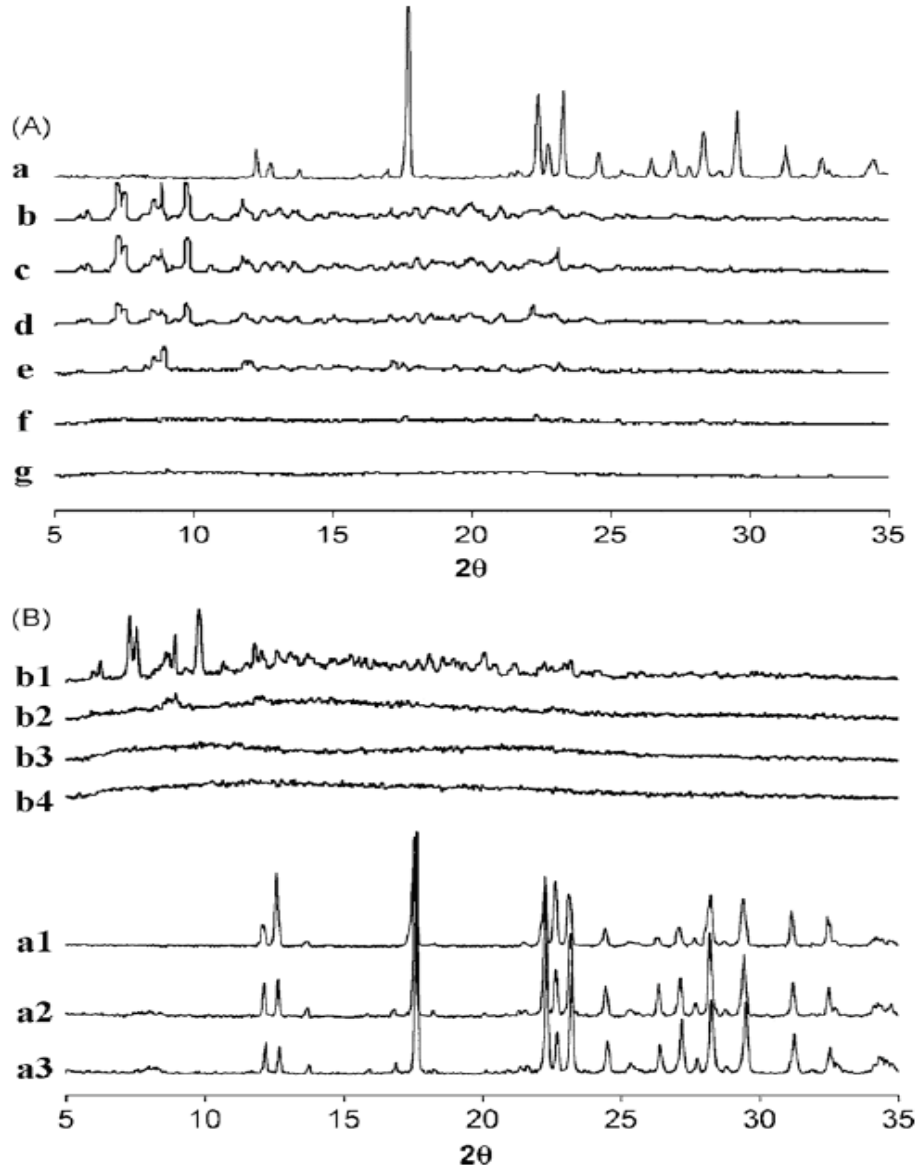


Fig. 2. X-ray powder diffraction patterns of (A) pure components and their equimolar systems obtained with the different techniques and (B) of pure components after treatment with the same techniques. *Key*—A: (a) MF·HCl; (b) TAβCyD, (c) physical mixture, (d) sealed-heated, (e) kneaded, (f) co-ground, (g) spray-dried product; B: MF·HCl after (a1) kneading, (a2) grinding or (a3) spray drying and TAβCyD after (b1) sealed-heating, (b2) kneading, (b3) grinding or (b4) spray drying.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of MF·HCl, TAβCyD, and their respective equimolar binary systems in the 4000–3000 and 2000–1500 cm^{-1} regions (selected as the most interesting ones to point out eventual drug–carrier solid-state interactions) are shown in Fig. 3.

The FTIR spectrum of pure MF·HCl showed two typical bands at 3369 and 3294 cm^{-1} (Fig. 3A, a) relative to the N–H primary stretching vibration and a band at 3155 cm^{-1} due to the N–H secondary stretching, and characteristic bands at 1626 and 1567 cm^{-1} (Fig. 3B, a) assigned to C N stretching. TAβCyD displayed a very strong band at 1741 cm^{-1} due to the C O vibration of the acetyl group (Fig. 3B, b).

The physical mixture spectrum (Fig. 3A and B, c) can be considered as the sum of pure MF·HCl and TAβCyD spectra.

No significant shifts or reduction in intensity of the FTIR bands of MF·HCl were observed in the binary sealed-heated product (Fig. 3A and B, d).

On the contrary, the FTIR spectra of the binary kneaded (Fig. 3A and B, e) and even more so of the co-ground (Fig. 3A and B, f) products presented appreciable shifts and reduction in intensity of the characteristic MF·HCl bands, evidencing the presence of more or less intense solid-state interactions between the components.

The FTIR spectrum of the spray-dried compound, on the other hand, showed a strong reduction (Fig. 3B, g) or the complete disappearance (Fig. 3A, g) of the characteristic MF·HCl bands, indicative of strong drug–carrier interactions and, possibly, inclusion complexation of the drug, thus substantially confirming the results previously obtained by DSC and X-ray diffraction analysis.

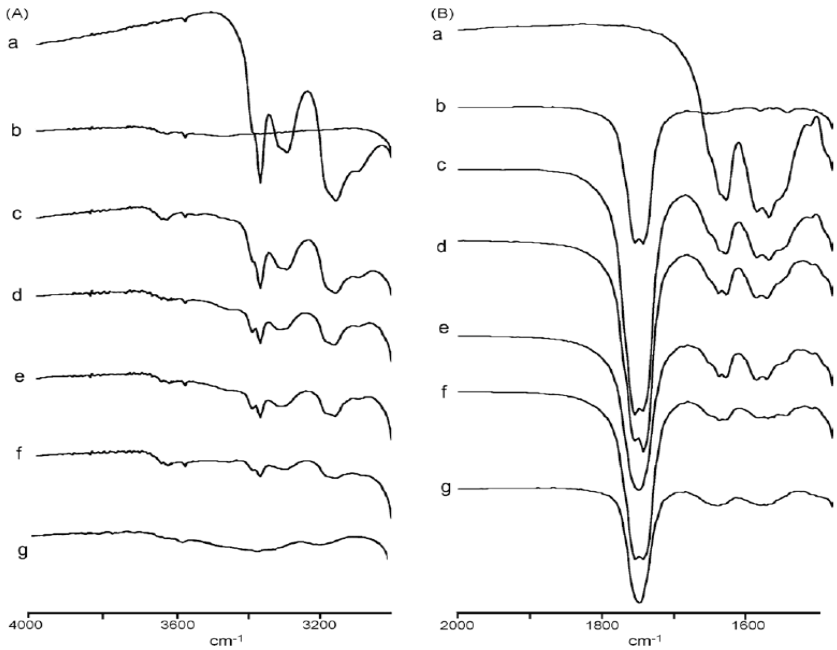


Fig. 3. FTIR spectra of pure components and their equimolar systems obtained with the different techniques in the 3000–4000 cm^{-1} (A) and 1500–2000 cm^{-1} (B) regions. Key: (a) MF·HCl, (b) TAβCyD, (c) physical mixture, (d) sealed-heated, (e) kneaded, (f) co-ground and (g) spray-dried products.

Scanning electron microscopy (SEM) studies

SEM analyses were performed on pure MF·HCl and TAβCyD samples and on their equimolar combinations obtained by different preparation methods, in order to gain insight about the possible morphological changes caused by the different treatments. MF·HCl particles appeared as lamellar, rather irregular-sized, crystals, with a tendency to self-agglomerate (Fig. 4A); on the contrary TAβCyD consisted of homogeneous small crystals (Fig. 4B).

The micrographs of the drug–carrier equimolar physical mixture and sealed-heated product (not shown) clearly displayed MF·HCl crystals dispersed on the surface of the almost unmodified carrier particles.

The kneaded and co-ground products presented instead a different morphology, showing a uniform, finely dispersed, powder with an evident particle size reduction and loss of crystallinity with respect to the original components (Fig. 4C).

However, the most marked change in morphology was undoubtedly observed for the spraydried product, which appeared formed by amorphous round particles of very homogeneous and small dimensions (2–5 μm) (Fig. 4D).

These findings were consistent with the above results of solid-state studies, confirming complete system amorphization and very intimate interaction between the components brought about by the spray-drying process of the drug–carrier mixture.

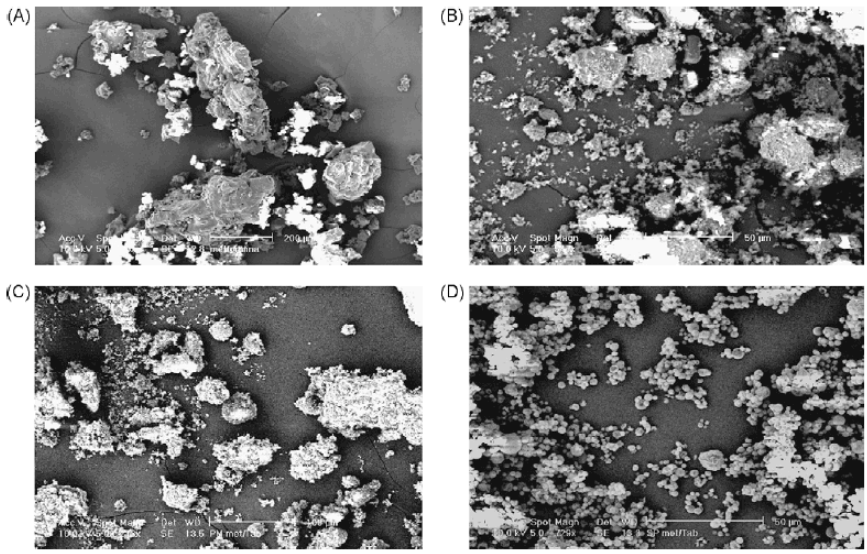


Fig. 4. Scanning electron micrographs of pure metformin·HCl (A) and TAβCyD (B) and of their equimolar co-ground (C) and spray-dried (D) products.

Dissolution studies

The dissolution profiles of MF·HCl alone and from its different binary systems with TAβCyD in simulated intestinal fluid (pH 6.5) are shown in Fig. 5, whereas the related dissolution parameters, expressed as percent drug dissolved, and dissolution efficiency values at various times are presented in Table 1. MF·HCl completely dissolved within a few minutes, reflecting its high aqueous solubility.

The dissolution from the physical mixture showed approximately the same behaviour of pure MF·HCl, with only a very slight initial slowing down of the drug dissolution rate, due to the presence of the hydrophobic cyclodextrin, which reduces the drug wettability.

The sealed heated product presented a dissolution profile similar to that of the physical mixture, reaching 100% dissolved drug within less than 10 min, thus further confirming the incapability of this technique to promote formation of effective drug–carrier interactions.

Table 1
Percent dissolved (PD) and dissolution efficiency (DE) values at different times (min) of metformin hydrochloride (MF·HCl) alone and from its equimolar binary systems with TAβCyD obtained by physical mixing (PM), sealed-heating (SH), kneading (KN), co-grinding (GR), and spray-drying (SP)

Sample	PD (10 min)	PD (40 min)	PD (120 min)	PD (420 min)	DE (10 min)	DE (40 min)	DE (120 min)	DE (420 min)
MF·HCl	100				90			
PM	100				83			
SH	100				79			
KN	60	97			38	70		
GR	45	65	100		23	44	74	
SP	8	13	33	100	5	9	17	68

On the contrary, the MF·HCl dissolution rate from kneaded and even more from co-ground products was significantly retarded, reaching 100% of dissolved drug after about 40 min and 2 h, respectively.

The observed significant slowing of drug dissolution rate can be attributed to the interactions between the drug and the hydrophobic carrier established during the sample treatment, which were more or less intense, depending on the different conditions used for the kneading and co-grinding methods, respectively. Finally, the clearly greater effectiveness of the spray-drying method in inducing powerful drug–CyD interactions, which has already emerged from solid-state studies, was further confirmed from the results of dissolution tests.

In fact, the spray-dried systems showed the greatest retarding effect on the dissolution rate of MF·HCl, and allowed obtainment of an almost linear slow-dissolving profile, reaching 100% of dissolved drug after only about 7 h.

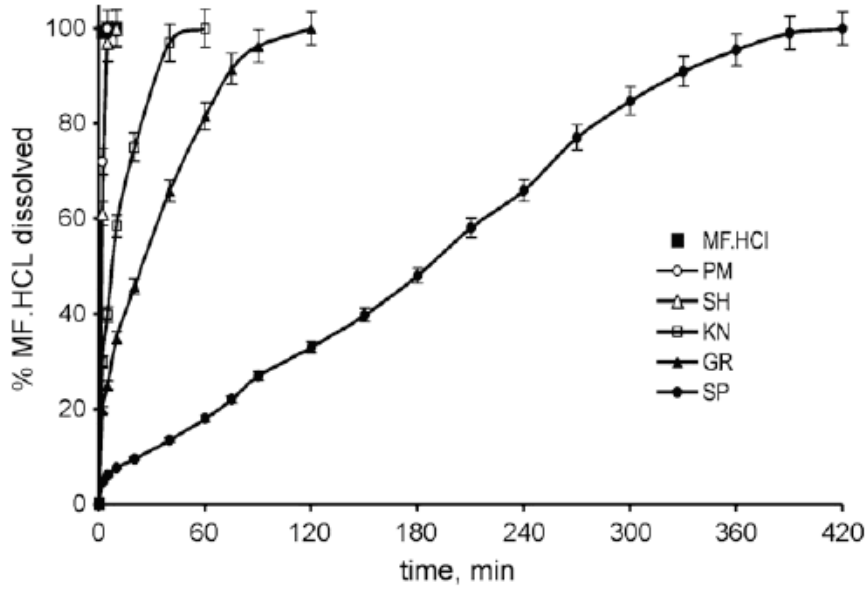


Fig. 5. Dissolution curves of metformin HCl (MF·HCl) alone or from its equimolar physical mixture (PM), sealed-heated (SH), kneaded (KN), co-ground (GR), and spray-dried (SP) products with TAβCyD.

In conclusion, this work has demonstrated the actual effectiveness of the hydrophobic cyclodextrin-derivative TAβCyD as a carrier for obtaining a slow-dissolving form of MF·HCl, but it has pointed out that it is strongly dependent on the preparation technique used for obtaining the drug–carrier product.

In fact, the results have pointed out the fundamental role played by the preparation method in promoting efficacious interactions between the components, able to adequately modify the drug dissolution behaviour.

In particular, results of solid state studies were all consistent in indicating that the most evident drug–carrier solid-state interactions occurred in the MF·HCl–TAβCyD system obtained by spray-drying, followed by those prepared by co-grinding and then by kneading.

The spray-dried product also gave rise to the most intense effect on the drug dissolution rate, as clearly indicated by the time to dissolve 100% MF·HCl, which varied from less than 10 min for sealed-heated systems, to about 40, 120 and 420 min for kneaded, co-ground and spray-dried products, respectively.

Therefore, the MF·HCl–TAβCyD spray-dried and co-ground products were selected as the most effective candidates for the subsequent development of a well-timed sustained-release dosage form of the drug.

14. THE LIPOSOMAL FORMULATION OF IRINOTECAN

In the last year of my PhD, I joined 6 months to the group of Francis Szoka, Professor of Biopharmaceutical Sciences and Pharmaceutical Chemistry at the University of California, San Francisco. Prof. Szoka is well known in the scientific world for his studies in the liposome field, particularly for the development of liposomal structures specific for cancer chemotherapy and for gene delivery.

During this period, a study has been undertaken aimed at finding the best formulation and the most suitable preparative conditions for the development of an effective liposomal formulation of the anticancer drug Irinotecan.

With this aim, two types of formulations, i.e. 1 (DSPC:CHOL:DSPE-mPEG2000-55:40:5) and 2 (DSPC:CHOL-55:45) have been investigated.

We chose distearoylphosphatidylcholine (DSPC) together with cholesterol (CHOL) as basis liposomal formulation, since this combination showed to be particularly effective for the encapsulation of anticancer drugs (Ramsay et al., 2007); we then carried out a modified formulation by replacing a part of CHOL with a corresponding part of distearoylphosphatidylethanolamine-m PEG2000, which was used to prevent the attack by the immune system and increase the circulation lifetime of liposomes in the blood circle, and thus increase the chance for the drug to enter target sites so as to improve the efficiency of drug delivery (Chou et al., 2002). Eight different experimental protocols for the production of such liposomal formulations have been then investigated.

The drug, which is a weak base, has been encapsulated using the “remote-loading” technique. This technique, is based on the drug loading on preformed liposomes and it exploits the permeability of the liposomal membrane to the neutral form of the basic drug (Fenske et al., 2005).

The drug diffuse within the liposomal core according to the concentration gradient and after it is protonated; thus it remains entrapped within the liposomal vesicle, being the membrane impermeable to the charged form. The method involves the formation of a trans-membrane pH-gradient, which can be obtained through the use of

different buffers. This method allows efficient drug encapsulation, generally greater than 80%, but also presents some disadvantages.

For example, several clinical formulations of such liposomal drugs require the generation of the pH gradient just prior to drug loading, due to gradient and/or drug instability.

A second disadvantage is the potential hydrolysis of lipids at acidic pH, which can introduce liposome instability during long-term storage. The ideal loading method would allow an efficient encapsulation at neutral pH, to prevent drug and lipid degradation (Dicko et al., 2007). For each evaluated liposomal formulation and experimental protocol, the encapsulation efficiency (EE%) has been determined and *in vitro* drug release studies have been performed. The study will continue with *in vivo* studies to evaluate the antitumoral activity of the selected formulation prepared according to the most effective experimental protocol.

14.1 Materials and methods

Materials

Irinotecan was purchased from Ivy Fine Chemicals (Cherry Hill, NJ, USA). Distearoylphosphatidylcholine (DSPC) and 1,2-distearoylphosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-mPEG2000) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Cholesterol (CHOL) was purchased from Sigma-Aldrich. Sephadex G25 Column was purchased from GE Healthcare. All other reagents were of analytical grade.

Protocols investigated

Five different protocols to encapsulate Irinotecan in the liposome according to the remote-loading methodology were selected by literature data, while three new protocols were carried out by myself. During the study I devoted particular attention to the following experimental variables:

- pH and composition of internal buffer
- pH and composition of external buffer
- drug/lipid molar ratio

- type of solvent for drug solution
- incubation time.

The 8 different protocols investigated are summarized in Table 1.

Table 1: **Investigated Protocols**

PROTOCOL	***INTERNAL BUFFER***	***EXTERNAL BUFFER***	***DRUG/LIPID molar ratio***	***DRUG SOLUTION***	***INCUBATION***	***EE% FROM PAPER***
1 Drummond et al., Cancer Res 2006	*650mM TEA-SOS pH=6*	*5mM HEPES,5% Glucose pH=6.5* *5mM Hepes, 140mM NaCl pH=6.5*	*0.75:1*	*5mM HEPES, 5% Glucose pH=6.5*	*30min al 60°C and then quench on ice for 15min*	*100%*
2 Ramsay et al., Clin Cancer Res 2008	*300 mM Copper sulphate*	*300 mM sucrose, 20 mM HEPES, 150 mM EDTA pH=7.5* *20mM Hepes, 150mM NaCl pH=7.5*	*0.2:1*	*ddWater*	*1 hour at 50°C*	*98%*
3 Dicko et all., Int. J. Pharm. 2007	*100 mM Copper gluconate, 180 mM TEA pH=7*	*300mM sucrose, 40mM Phosphate, 10mM EDTA pH=7* *20mM Hepes, 150mM NaCl pH=7.5*	*0.2:1*	*300mM Sucrose, 40mM phosphate pH=7 or ddWater*	*1 hour at 50°C*	*>95%*
4 Tardi et al., Biochim Biophys. 2007	*100mM Copper gluconate, 220mM TEA pH=7.4*	*300mM sucrose, 20mM Hepes, 30mM EDTA pH7.4* *20mM Hepes, 150 mM NaCl pH=7.5*	*0.1:1*	*ddWater*	*1 hour at 50°C*	*>95%*
5 Chou et al., J. Biosci. Bioeng. 2003	*500mM Citrate buffer pH=3*	*500mM Sodium Citrate buffer pH=7*	*0.3:1*	*ddWater*	*10 min at 60°C*	*97-99%*
6	***300mM 1,2,3,4 butane tetracarboxylic acid pH=6(w/NH_4OH)***	***5mM Hepes, 5% Glucose pH=6.5*** ***5mM Hepes,140mM NaCl pH=6.5***	***0.2/1***	*ddWater*	*1 hour at 50°C*	
7	***650 TEA-phytic acid pH=6***	***5mM Hepes, 5% Glucose pH=6.5*** ***5mM Hepes,140mM NaCl pH=6.5***	***0.2/1***	*ddWater*	*1 hour at 50°C*	

8	***250mM ammonium sulphate***	***5mM Hepes, 5% Glucose pH=6.5*** / ***5mM Hepes,140mM NaCl pH=6.5***	***0.2/1***	*ddWater*	*1 hour at 50°C*	

Liposome preparation

The liposomes were first prepared by thin layer evaporation. Liposomal Irinotecan was then obtained according to the pH-gradient loading technique.

The influence of the main parameters that govern this process, including drug loading time, incubation temperature, buffer composition for hydration, and pH, was investigated.

The uptake of Irinotecan into liposomal systems in response to the magnitude of the pH gradient was also examined.

The phospholipids and cholesterol were dissolved in chloroform according to two different formulations: 1) DSPC:CHOL:DSPE-mPEG(2000), 55:40:5 (where DSPE-mPEG2000 was used to obtain stealth liposomes and avoid they are recognized by the immune system), and 2) DSPC:CHOL, 55:45.

The solvent was evaporated under a stream of nitrogen and dried under vacuum for at least 2 h. The thin lipid layer thus obtained was hydrated with an internal buffer, according to the related protocol.

Then the sample was sonicated at 70°C for 10 min. The resulting lipid suspension was extruded 11 times at 70 °C through two polycarbonate filters with 200 nm diameter and 11 times at 70°C through two polycarbonate filters with 100 nm diameter at moderate pressure using a liposome extruder (Lipex Inc., Vancouver, BC).

The resultant LUVs typically possessed a mean vesicular diameter of about 110 ± 30 nm as determined using Phase Analysis Light Scattering (ZetaPALS, Brookhaven Instruments Corp., Holtsville, NY). The LUVs external buffer was exchanged, using Sephadex G-25 size exclusion chromatography, with the first internal buffer. After, the vesicular diameter was determined using the same Light Scattering.

Then Irinotecan was incubated in the presence of preformed liposomes, which were maintained at 50°C for 10 min prior to drug addition.

Drug uptake was determined at indicated time points by sampling different aliquots and separating encapsulated from free drug using Sephadex G-25 spin columns equilibrated with the appropriate buffer.

Liposome Characterization

Determination of Liposomal Size.

The average particle size of the vesicles was determined by light scattering using Phase Analysis Light Scattering (ZetaPALS, Brookhaven Instruments Corp., Holtsville, NY).

Determination of Encapsulation Efficiency (EE%).

Irinotecan EE% was determined by UV spectrophotometric assay of drug concentration in solution at 370 nm.

Briefly, a portion of the samples collected from the spin Sephadex G-25 columns was adjusted to a suitable final volume with the buffer. Subsequently, Triton X-100 1% was added to lyse the liposomes and the samples were heated in a water bath at >90°C until the cloud point of the surfactant was observed.

The samples were then cooled to room temperature and the absorbance was determined; drug concentration was then determined using a freshly prepared Irinotecan standard curve. (Agilent/Hewlett Packard UV–Vis spectrophotometer (model 8453), Agilent Technologies, Mississauga, ON, Canada). The experiments were performed in duplicate.

The EE% was then calculated according to the following equation :

$$[\text{Encapsulated drug}] \ / \ [\text{Total drug}] \ \text{x} \ 100 \ = \ \% \ \text{EE}$$

In vitro studies: Leakage assay

The liposomes were added to fetal bovine serum. The release experiments were carried out at 37°C. Aliquots collected at selected time points over 24 h for 5 days, were centrifuged using columns bio-spin 6 and Sepharose CL-2B at 2,250 rpm for 2

min at 10°C using Microcon YM-100 centrifugal filters units (Millipore, Billerica, MA) to separate the encapsulated drug from the released one.

All samples were analyzed by UV spectrometry at 370 nm to determine the concentration of Irinotecan. (Agilent/Hewlett Packard UV–Vis spectrophotometer (model 8453), Agilent Technologies, Mississauga, ON, Canada).

The percent of drug release was calculated as the ratio between the free drug at a given time and total encapsulated drug (Watanabe et al., 2008).

The percent of drug retained was obtained by the ratio between the drug still encapsulated after a given time and total encapsulated drug (at time = 0):

[Encapsulated] t=n / [Encapsulated] t=0 x 100 = % retained

14.2 Results and discussion

Drug encapsulation efficiency

Table 2 shows the EE% values obtained from the two Irinotecan liposomal formulations prepared according to the eight different experimental protocols investigated.

The formulation 1 (DSPC:DSPE-mPEG(2000):CHOL;55:5:40) made according to the protocol n. 5 (internal buffer: 500mM Citrate buffer pH=3, external buffer: 500mM Sodium Citrate buffer pH=7) and the protocol n. 7 (internal buffer: 650 TEA-phytic acid pH=6, external buffer: 5mM Hepes, 5% Glucose pH=6.5, 5mM Hepes, 140mM NaCl pH=6.5), and the formulation 2 (DSPC:CHOL; 55:45) prepared according to the protocol n.1 (internal buffer: 650mM TEA-SOS pH=6, external buffer: 5mM Hepes, 5% Glucose pH=6.5, 5mm Hepes, 140mM NaCl pH=6.5) didn't work well, because during the liposome preparation, the size of the vesicles began too big, more than 1000 nm.

Formulation 1 (DSPC:DSPE-mPEG(2000):CHOL;55:5:40) made according to the protocol n. 2 (internal buffer: 300 mM Copper sulphate, external buffers: 300 mM sucrose / 20 mM HEPES/15 mM EDTA pH=7.5 and 20 mM Hepes, 150mM NaCl pH=7.5) exhibited the maximum encapsulation efficiency (EE%=78.07%).

In the case of formulation 2 (DSPC:CHOL; 55:45) the best results were obtained by using the Protocol n. 5 (internal buffer: 500mM Citrate buffer pH=3,

external buffer: 500 mM Sodium Citrate buffer pH=7), which allowed obtainment of 98.64% of encapsulation efficiency (EE%).

Table 2: EE% values obtained with formulations 1(DSPC:DSPE-mPEG(2000):CHOL;55:5:40) and 2 (DSPC:CHOL; 55:45) prepared according to the eight investigated protocols

	Formulation 1	**Formulation 1 R**	**Formulation 2**	**Formulation 2 R**
Prot 1	EE%= 19.28%	EE%= 19.49%		
Prot 2	EE%= 78.07%	EE%= 55.87%	EE%= 43.47%	EE%= 39.70%
Prot 3	EE%= 9.87%		EE%= 8.98%	EE%= 12.42%
Prot 4	EE%= 33.93%		EE%= 28.84%	EE%= 34.52%
Prot 5			EE%=98.64%	EE%= 65.03%
Prot 6	EE%= 48.86%	EE%= 37.86%	EE%= 53.83%	EE%= 39.03%
Prot 7			EE%= 45.18%	
Prot 8	EE%= 54.95%	EE%= 38.55%	EE%= 17.74%	

In vitro studies

Leakage assay

The % drug retained after 5 days, for the two liposomal Irinotecan formulations prepared according to the eight protocols, (shown in the Table 3) were determined to evaluate whether the rate of Irinotecan release from the liposomes was influenced by the drug loading method used.

The results of % Irinotecan retained as a function of time from the two liposomal formulations prepared according to the different experimental protocols (see Table 1) are shown in the following graphs (from Fig. 1 to Fig. 13).

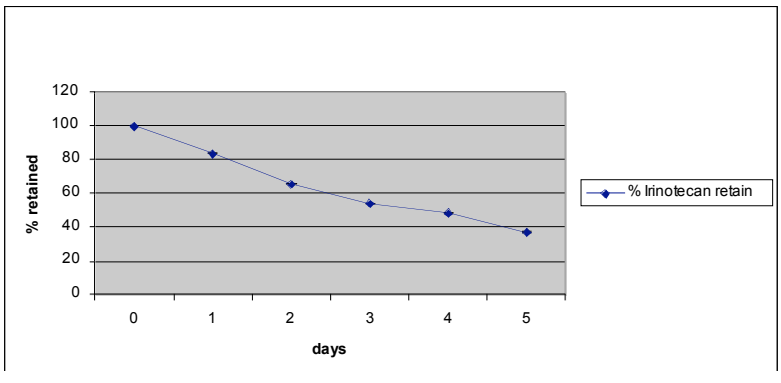


Figure 1: Formulation1 protocol 1: Internal buffer: 650mM TEA-SOS pH 6.0; External buffer 1: 5 mM Hepes, 5% Glucose pH 6.5;External Buffer 2: 5 mM Hepes, 140 mM NaCl pH=6.5.

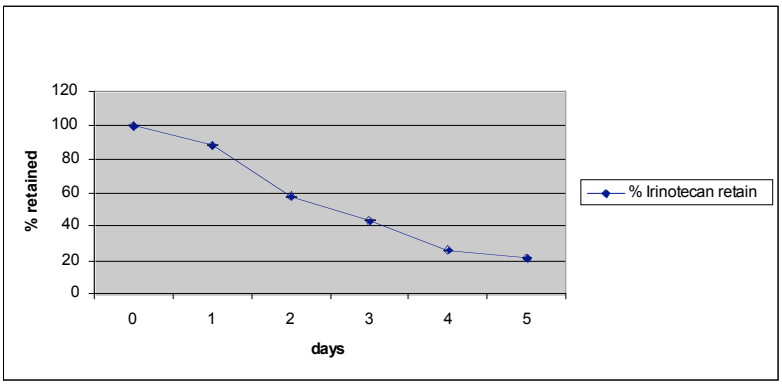


Figure 2: Formulation1 protocol 2:Internal buffer: 300 mM Copper sulfate; External buffer 1: SHE Buffer:300 mM sucrose / 20 mM HEPES/15 mM EDTA pH=7.5; External Buffer 2: HEPES-buffered saline:20mM Hepes,150mM NaCl pH=7.5.

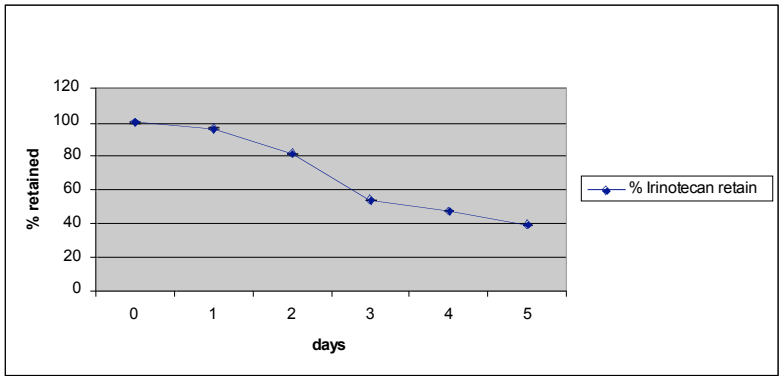


Figure 3: Formulation 1 Protocol 3：Internal buffer:100 mM copper gluconate/180 mM TEA pH=7; External buffer 1:SHE Buffer: 300 mM sucrose/ 40 mM Phosphate/10mM EDTA pH=7; External Buffer 2: HBS buffer: 20 mM HEPES, 150 mM NaCl, pH=7.4.

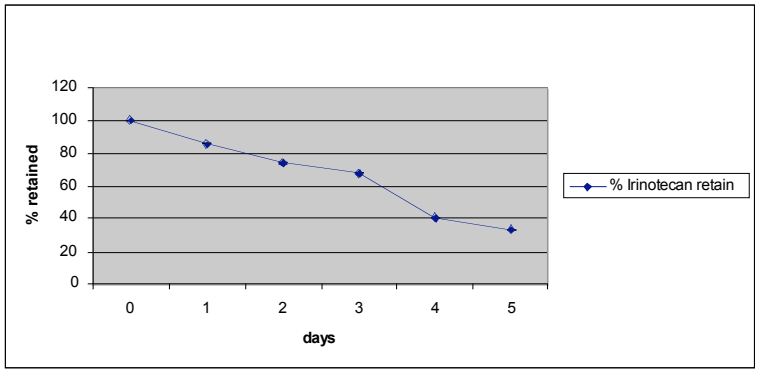


Figure 4: formulation 1 protocol 4: Internal buffer: 100mM copper gluconate/220mM TEA pH=7.4; External buffer 1: SHE Buffer: 300mM sucrose/20mM HEPES/30mM EDTA pH=7.4; External Buffer 2:HBS Buffer: 20mM HEPES/150 mM NaCl pH=7.4%.

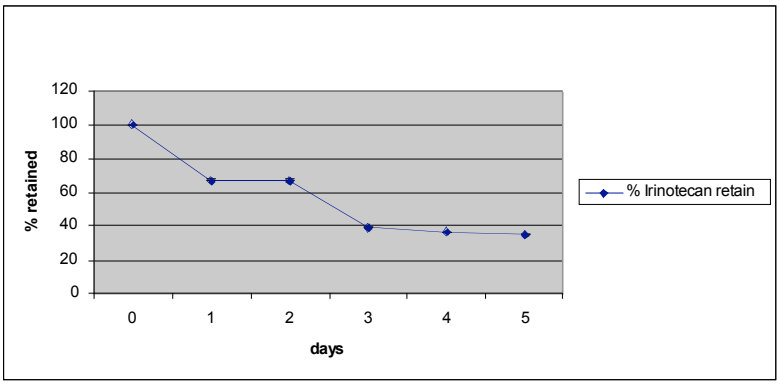


Figure 5: Formulation 1 protocol 6: Internal buffer:300mM 1,2,3,4 butane tetracarboxylic acid pH=6 (water/NH_4OH); External buffer 1: 5mM Hepes, 5% Glucose pH=6.5; External Buffer: 2 5mM Hepes,140 mM NaCl pH=6.5.

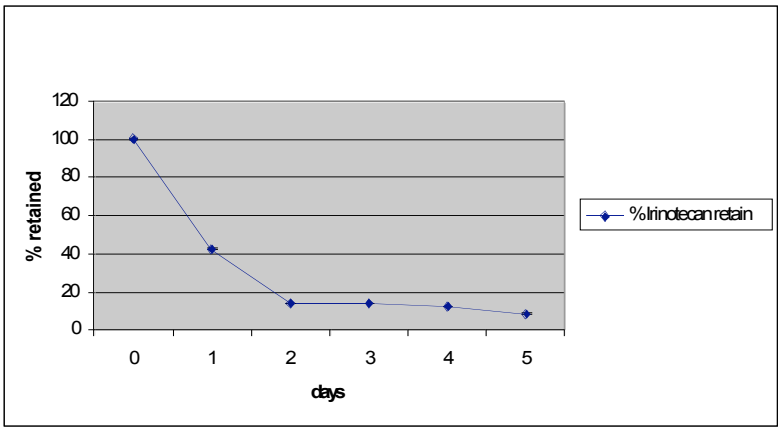


Figure 6: Formulation 1 Protocol 8: Internal buffer: 250 mM ammonium sulphate; External buffer 1: 5 mM Hepes, 5% Glucose pH=6.5; External Buffer 2: 5 mM Hepes,140mM NaCl pH=6.5.

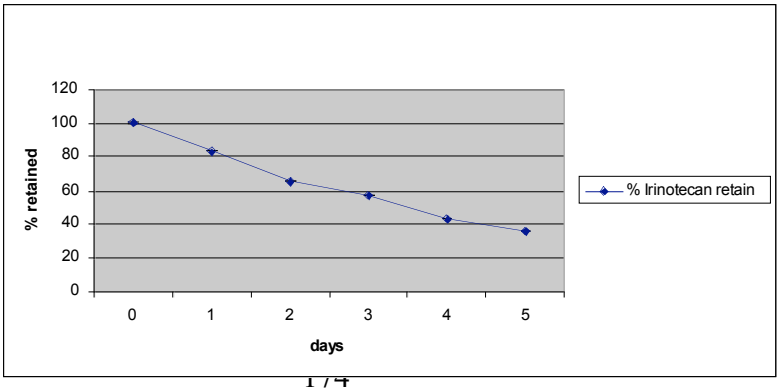


Figure 7: Formulation 2 Protocol 2: Internal buffer: 300 mM Copper sulfate; External buffer 1: SHE Buffer:300 mM sucrose / 20 mM HEPES/15 mM EDTA pH=7.5 ;External Buffer 2: HEPES-buffered saline:20mM Hepes,150mM NaCl pH=7.5.

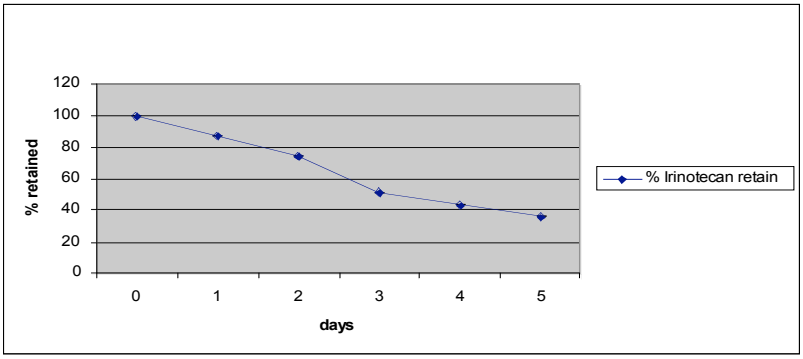


Figure 8: Formulation 2 Protocol 3: Internal buffer:100 mM copper gluconate/180 mM TEA pH=7; External buffer 1:SHE Buffer: 300 mM sucrose/ 40 mM Phosphate/10mM EDTA pH=7; External Buffer 2: HBS buffer: 20 mM HEPES, 150 mM NaCl, pH=7.4.

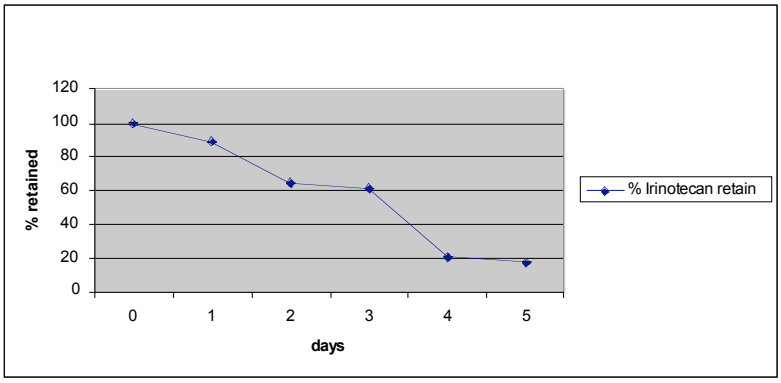


Figure 9: Formulation 2 Protocol 4: Internal buffer: 100mM copper gluconate/220mM TEA pH=7.4; External buffer 1: SHE Buffer: 300mM sucrose / 20mM HEPES/30mM EDTA pH=7.4; External Buffer 2:HBS Buffer: 20 mM HEPES/150 mM NaCl pH=7.4.

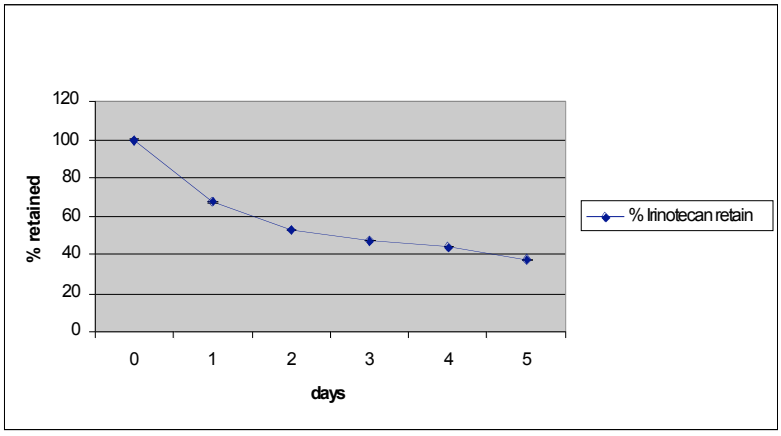


Figure 10: Formulation 2 Protocol 5: Internal buffer:500mM Citrate buffer Ph=3; External buffer: 500mM Sodium Citrate buffer pH=7.

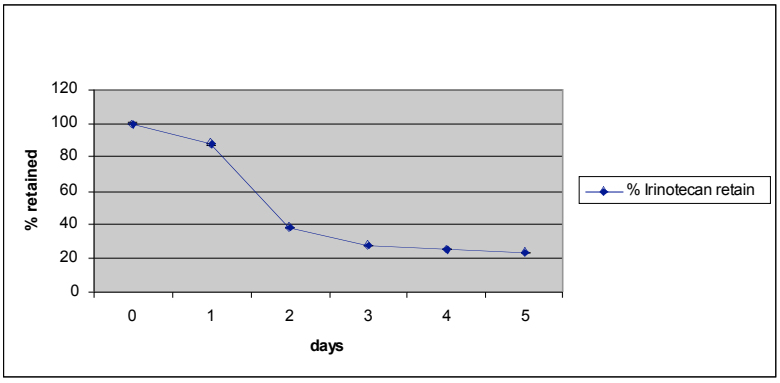


Figure 11: Formulation 2 Protocol 6: Internal buffer:300mM 1,2,3,4 butane tetracarboxylic acid pH=6 (water/NH_4OH); External buffer 1: 5mM Hepes, 5% Glucose pH=6.5; External Buffer: 2 5mM Hepes,140 mM NaCl pH=6.5.

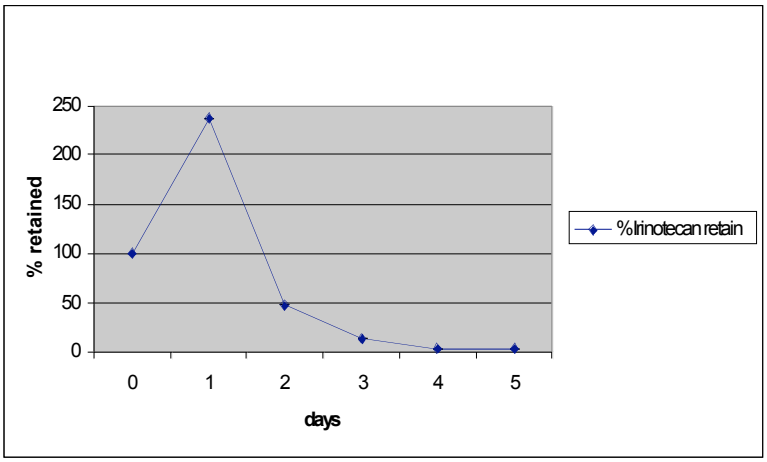


Figure 12: Formulation 2 Protocol 7: Internal buffer: 650 TEA-phytic acid pH=6; External buffer 1: 5mM Hepes, 5% Glucose pH=6.5; External Buffer 2: 5mM Hepes,140mM NaCl pH=6.5.

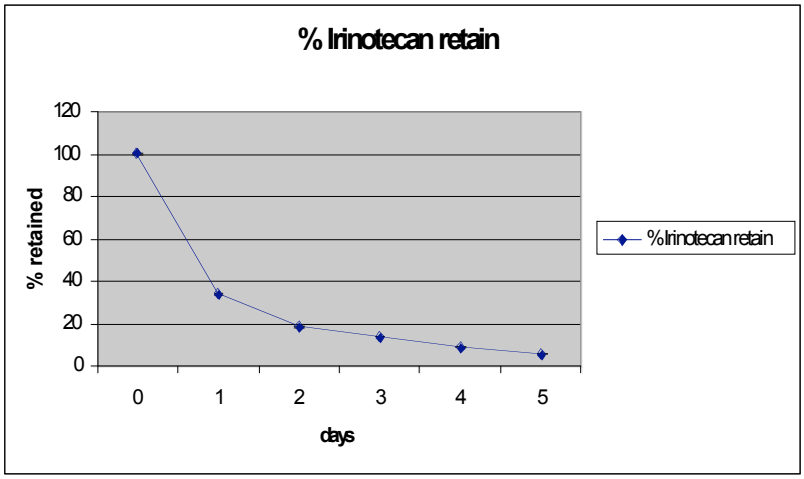


Figure 13: Formulation 2 Protocol 8: Internal buffer: 250mM ammonium sulphate; External buffer 1: 5mM Hepes, 5% Glucose pH=6.5; External Buffer 2: 5mM Hepes,140mM NaCl pH=6.5.

Table 3: % Irinotecan retained after 5 days inside liposomal formulations 1 (DSPC:DSPE-mPEG(2000):CHOL; 55:5:40) and 2 (DSPC:CHOL; 55:45) prepared according to the eight investigated protocols

type of Protocol	**Formulation 1**	**Formulation 2**
Prot 1	%retained=37.14%	
Prot 2	%retained=21.04%	%retained=35.62%
Prot 3	%retained=39.47%	%retained=35.71%
Prot 4	%retained=33.65%	%retained=17.27%
Prot 5		%retained=37.18%
Prot 6	%retained=31.19%	%retained=23.85%
Prot 7		%retained=2.97%
Prot 8	%retained=8.55%	%retained=5.62%

As shown in Figure 14, in the case of the liposomal Formulation 1 (DSPC:DSPE-mPEG(2000):CHOL; 55:5:40) the highest % of Irinotecan retained inside the liposomes after 5 days was obtained with the Protocol n. 3 (internal buffer: 100 mM Copper Gluconate, 180 mM TEA pH=7, external buffer: 300mm Sucrose, 40 mM phosphate, 10 mM EDTA pH=7 and 20 mM Hepes, 150 mM NaCl pH=7.5). The result obtained was 39.47%.

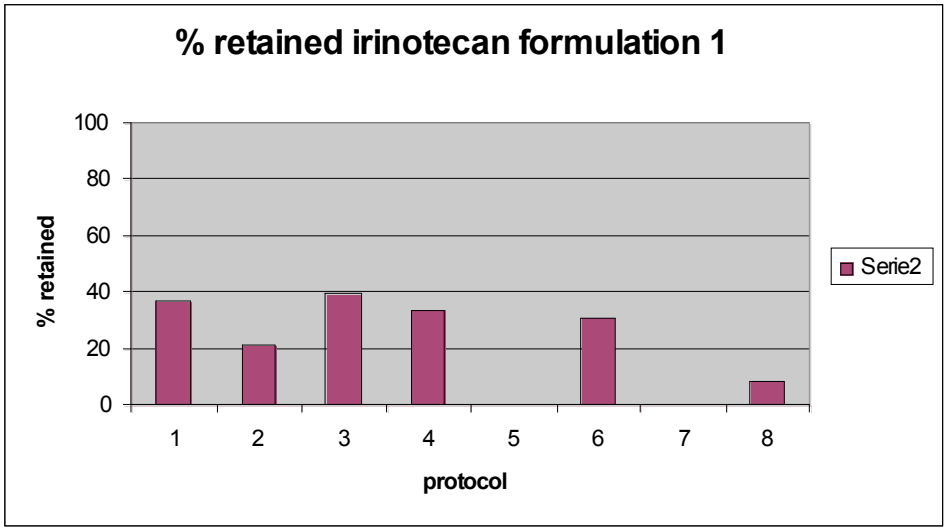


Figure 14:% Irinotecan retained after five days in liposomal formulation 1 (DSPC/CHOL/DSPE-mPEG2000 (55:40:5)) prepared according to the different eight experimental protocols

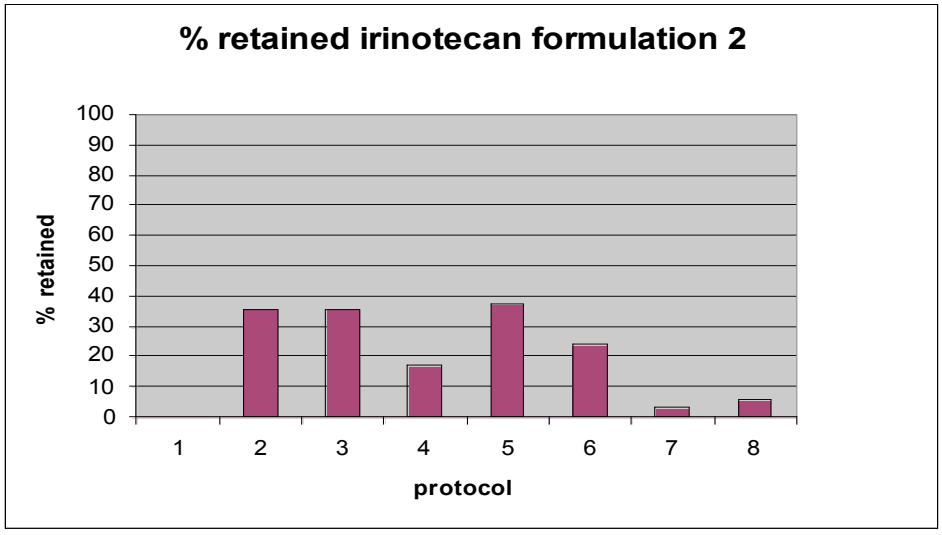


Figure 15: % Irinotecan retained after five days in liposomal formulation 2 (DSPC/CHOL (55:45)) prepared according to the different eight experimental protocols

On the contrary, as shown in Figure 15, in the case of the liposomal Formulation 2 (DSPC:CHOL;55:45) the highest % of Irinotecan retained after 5 days was observed for that prepared according to the Protocol n. 5 (internal buffer: 500 mM Citrate buffer

pH=3, external buffer: 500 mM Sodium Citrate buffer pH=7). The result obtained was 37.18%.

In conclusion, the best liposome formulation was Formulation 2 (DSPC:CHOL; 55:45) prepared according to the Protocol n. 5 (obtained from the paper of Chou et al., 2003) where the internal buffer was 500 mM Citrate buffer pH=3, the external buffer was 500 mM Sodium Citrate buffer pH=7, the molar ratio was 0.3:1 and the incubation time was 10 min at 60°C. It gave the EE%=98.64% and % retained drug (after 5days)=37.18%.

This Protocol allowed obtainment of the best results in terms of both EE% and % retained drug over all the tested protocols for the formulation 2 and this result is probably due to the wide difference of pH between internal and external buffer.

Instead, for the formulation 1 (DSPC:DSPE-mPEG(2000):CHOL;55:5:40) the best preparation protocols were the Protocol n. 2 (obtained from the paper of Ramsay et al., 2008) where the internal buffer was 300 mM Copper sulphate, the external buffers were SHE buffer: 300 mM sucrose / 20 mM HEPES/15 mM EDTA pH=7.5 and HEPES-buffered saline: 20mM Hepes,150 mM NaCl pH=7.5, the molar ratio was 0.2:1 and the incubation time was 1 hour at 50°C, and the Protocol n. 6, where the internal buffer was 300 mM 1,2,3,4 butane tetracarboxylic acid pH=6 (water/NH_4OH), the external buffers were 5 mM Hepes, 5% Glucose pH=6.5 and 5 mM Hepes,140 mM NaCl pH=6.5, the molar ratio was 0.2:1 and the incubation time was 1 hour at 50°C.

The results were for Protocol n. 2, EE%= 78.07% and % drug retained (after 5 days)=21.04%, and for Protocol n. 6, EE%= 48.86% and % drug retained (after 5 days)=31.19%. This kind of formulation is very important for the aim of the work, because it contains DSPE-mPEG(2000) that gives the stealth effect and improves the long term circulation of the liposomes. This effect is particularly advisable in liposomal formulations of anticancer drugs, since longer blood residence time will result in repeated passages through the tumor microvascular bed of high concentrations of vesicles and, consequently, improved uptake of liposomes by tumors.

The protocol n. 2 didn't had the maximum results in terms of % Irinotecan retained, however it represents the best compromise for this formulation, due to its highest value of EE%.

CONCLUSIONS

The work performed in this doctoral thesis has highlighted the importance of liposomal formulations and of cyclodextrin complexation in pharmaceutical field.

Liposomal formulation of local anaesthetics allowed an improvement of their therapeutic effectiveness in terms of intensity and/or duration of action.

It has been demonstrated that the drug release rate and skin penetration ability are dependent by the liposomal carrier composition and by the vesicle characteristicss (such as size, lamellarity, etc.), which in their turn are strictly related to their preparation method.

Regarding the composition of liposomes, we started from the classical composition of the lipidic phase consisting in a mixture of phosphatidylcholine and cholesterol, and we evaluated the effect of variations in their relative amount, and of the addition of other compounds on the drug encapsulation efficiency, Zeta-potential and vesicle stability, drug release rate and permeability and carrier guidance on the target.

With this purpose, we investigated the effect of the addition of cationic (stearylamine) or anionic (dicethylphosphate) surfactants.

In particular we demonstrated the favourable effect of the presence of dicethylphosphate, which improved the flexibility of the vesicle membranes, thus increasing the permeability of liposomes from gel formulation through the skin.

As for the composition of the hydration phase, we pointed out the importance to use ethanol-water mixtures, rather than water alone.

In fact, the greater the amount of ethanol in the mixture, the higher was the drug permeation rate. The use of experimental design was very useful in this study, since it enabled to reduce the number of experiments to obtain the optimized composition.

The experimental design allowed variation of several parameters simultaneously, and examination of possible interaction among the variables, thus confirming as a valuable investigative tool in the pharmaceutical field.

A following study performed with the optimized liposomal composition, allowed to demonstrate the importance of the liposome preparation method on the performance of the final product.

With this aim, the liposomes were prepared by different techniques, and characterized for their physicochemical properties, encapsulation efficiency and drug permeation. The work enabled a rational selection of the most suitable preparation technique of the liposomal dispersion as a function of the desired effect for the carried anaesthetic drug in terms of improvement of intensity of action or prolongation of its duration.

Cyclodextrin complexation using a highly soluble derivative of native β-cyclodextrin (i.e. amorphous randomly substituted methyl-β-cyclodextrin (RAMEB)) was successful in improving the wettability and dissolution properties of an anti-inflammatory drug such as oxaprozin, thus allowing a reduction of the dose, and, consequently, of the appearance of side-effects.

On the other hand, cyclodextrin complexation using a hydrophobic derivative of native β-cyclodextrin practically insoluble in water (i.e. triacetyl-β-cyclodextrin) was effective in properly reducing the dissolution rate of metformin hydrochloride (MF·HCl), an oral anti-hyperglycaemic agent very highly soluble in water.

The obtained slow-dissolution complex is suitable for the development of a sustained-release formulation of the drug, able to prolong its duration of action and reduce the frequency of administration, thus bettering the patient compliance.

Subsequent work has also demonstrated the efficacy of cyclodextrin complexation for the development of "drug - in cyclodextrin - in liposome" delivery systems, which showed a further improvement of the therapeutic effect of the carried drug compared with liposomes containing the plain drug.

This new technique has been successfully applied for the development of liposomal formulations of butamben of prilocaine, both as complexes with a highly soluble derivative of native β-cyclodextrin, i.e. hydroxypropyl-β-cyclodextrin (HP-β-cyclodextrin). It was also assessed the effectiveness of a double-loading methodology able to increase both liposome encapsulation efficiency and therapeutic effectiveness of the carried hydrophobic drug.

It was possible to add the drug both in the liposomal lipid phase, by dissolving it in the organic solvent together with phospholipids, and in the hydration phase, by dissolving it as cyclodextrin complex.

Finally, a study has been undertaken aimed at developing an effective liposomal formulation of the anticancer drug Irinotecan. Two types of liposomal lipid phase formulations (i.e. distearoyl-phosphatidylcholine – cholesterol – distearoylphosphatidyl-

ethanolamine-mPEG2000, DSPC:CHOL:DSPE-mPEG2000 55:40:5 and distearoyl-phosphatidylcholine – cholesterol, DSPC:CHOL 55:45) were tested. The drug loading was carried out according to eight different experimental protocols on preformed liposomes (obtained by thin layer evaporation) using a passive remote-loading technique based on the presence of a pH gradient between outside and inside of liposomal vesicles.

The study allowed identification, for each examined formulation, of the best experimental protocol in terms of encapsulation efficiency and drug release. In particular, the formulation containing DSPE-mPEG2000 was selected, since it gives rise to stealth-liposome, with a long plasma circulation time.

A longer blood residence time will result in repeated passages through the tumor microvascular bed of high concentrations of vesicles and, consequently, in a greater efficiency of their extravasation process.

REFERENCES

Abugo O. *et al*, "Direct measurements of hemoglobin interactions with liposomes using EPR spectroscopy", Artif Cells Blood Substit Immobil Biotechnol, 29 (2001) 5-18

Adlakha-Hutcheon, G., Bally, M. B., Shew, C. R., and Madden, T. D. (1999). Controlled destabilization of a liposomal drug delivery system enhances mitoxantrone antitumor activity. Nat. Biotechnol. 17, 775–779.

Allen, T. M. (1994). Long-circulating (sterically stabilized) liposomes for targeted drug delivery. Trends Pharmacol. Sci. 15, 215–220.

Allen, T. M. (1998). Liposomal drug formulations. Rationale for development and what we can expect for the future. Drugs 56, 747–756.

Allen, T. M., Hansen, C., Martin, F., RedemAnn, C., and Yau-Young, A. (1991). Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochim. Biophys. Acta 1066, 29–36.

Al-Marzouqi, A., Jobe, B., Dowaidar, A., Corti, G., Cirri, M., Mura, P.: Physicochemical characterization of drug-cyclodextrin complexes prepared by

supercritical carbon dioxide and by conventional techniques. J. Incl. Phenom. Macrocycl. Chem. 57, 223-231 (2007).

Anderson, M., Omri, A. (2004). The effect of different lipid components on the in vitro stability and release kinetics of liposome formulations. *Drug Delivery* 11:33–39.

Anurukvorakun O., Suntornsuk W., Suntornsuk L., Yekkala R., Vandenwayenberg S., Hoogmartens J. and Adams E., *J. Chromatogr. A* 1134 (2006), p. 326.

Arambiri I., Lasa M., Garcia R. and Millan E., J. Chromatogr. A 1033 (2004), p. 193.
Araujo P. and Froyland L., *J. Choromatogr. B* 830 (2006), p. 212.

Araujo P., Couillard F., Leirnes E., Ask K., Bøkevoll A. and Frøyland L., J. Chromatogr. A A1121 (2006), p. 99

Araujo P., Trends Anal. Chem. 19 (2000), p. 524.

Artiss, J.D.; Brogan, K.; Brucal, M.; Moghaddam, M.; Jen, K.L.C. (2006), "The effects of a new soluble dietary fiber on weight gain and selected blood parameters in rats", Metabolism 55 (2): 195–202

Asayama , Aramak, Yoshida, Tasuchya(1992) “liposome res” 2 -275

Bakker-Woudenberg, I. A., Ten Kate, M. T., Guo, L., Working, P., and Mouton, J. W. (2001). Improved efficacy of ciprofloxacin administered in polyethylene glycol-coated liposomes for treatment of Klebsiella pneumoniae pneumonia in rats. Antimicrob. Agents Chemother. 45, 1487–1492.
Bally, M. B., Mayer, L. D., Loughrey, H., Redelmeier, T., Madden, T. D., Wong, K., Harrigan, P. R., Hope, M. J., and Cullis, P. R. (1988). Dopamine accumulation in large unilamellar vesicle systems induced by transmembrane ion gradients. Chem. Phys. Lipids 47, 97–107.

Bangham A.D. *et al*, “Diffusion of Univalent ions across the lamellae of swollen phospholipids” J Mol Biol, 13 (1965) 238-252

Bangham A.D., Standish M.M., Watldm J.C.. Journal of Molecular Biology. 1965; 13: 238.

Bangham, A. D. (1968). Membrane models with phospholipids. Prog. Biophys. Mol. Biol. 18, 29–95.

Bangham, A.D., Standish, M.M., Watkins, J.C., Diffusion of univalent ions across the lamellae of swollen phospholipid. J. Mol. Biol. 13, 238-252

Baranda A.B., Etexbarria N., Jimenez R.M. and Alonso R.M, *Talanta* 67 (2005), p. 933.

Barclay, L., Vega, C. (2004). Methaemoglobinaemia linked to topical benzocaine use. *Arch Intern Med* 164:1192–1195.

Barenholz Y., Gibbs D., Litman B., Goll J., Thompson T., Carlson F.. Biochemistry. 1977; 16: 2806-2810.

Bartfield J.M., Homer P.J., Ford D.T., Sternklar P. Buffered lidocaine as a local anesthetic: an investigation of shelf life. *Annals of Emergency Medicine*. 1992; 21: 16-9.

Basomba A. *et al*, "Allergen vaccination with liposome-encapsulated extract of *Dermatophagoides pteronyssinus*: a randomized, double blind, placebo controlled trial in asthmatic patients" J Allergy Clin Immun, 109 (6) (2002) 943-948

Batzri, S., and Korn, E. D. (1973). Single bilayer liposomes prepared without sonication. Biochim. Biophys. Acta 298, 1015–1019.

Benedicte A. I. van den Bergh, Philip W. Wertz, Hans E. Junginger, Joke A. Bouwstra Elasticity of vesicles assessed by electron spin resonance, electron microscopy and extrusion measurements International Journal of Pharmaceutics, Volume 217, Issues 1-2, 17 April 2001, Pages 13-24

Bettinetti G.P., Mura P., Faucci M.T., Sorrenti M., Setti M., Eur. J. Pharm. Sci. 15 (2002) 21-29.

Bettinetti G.P., Sorrenti M., Catenacci L., Ferrari F., Rossi S., J. Pharm. Biomed. Anal. 41 (2006) 1205-1121.

Bettinetti G.P., Sorrenti M., Rossi S., Ferrari F., Mura P., Faucci M.T., J. Pharm. Biomed. Anal., 30 (2002) 1173-1179.
Bettinetti, G.P., Gazzaniga, A., Mura, P. Giordano, F., Setti, M.: Thermal behaviour and dissolution properties of naproxen in combinations with chemically modified □-cyclodextrins. Drug Dev. Ind. Pharm. 18 39-53 (1992).

Bettinetti, G.P., Mura, P., Liguori, A. Bramanti, G., Giordano, F.: Solubilization and interaction of naproxen with cyclodextrins in aqueous solution and in the solid state. Farmaco 44 195-213 (1989).

Biwer A, Antranikian G, Heinzle E. Enzymatic production of cyclodextrins. Appl Microbiol Biotechnol 2002;59:609-17.

Blanco, J., Vila-Jato J.L., Otero, F., Anguiano, S.: Influence of preparation of inclusion complexes of naproxen with different cyclodextrins. Drug Dev. Ind. Pharm. 17 943-957 (1991).

Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Boman, N. L., Masin, D., Mayer, L. D., Cullis, P. R., and Bally, M. B. (1994). Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cuRes. mice bearing P388 tumors. Cancer Res. 54, 2830–2833.

Boman, N. L., Mayer, L. D., and Cullis, P. R. (1993). Optimization of the retention properties of vincristine in liposomal systems. Biochim. Biophys. Acta 1152, 253–258.

Bombardier C, Laine L, Reicin A, Shapiro D, Burgos-Vargas R, Davis B, et al. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. N Engl J Med 2000;343(21):1520-8.

Box G.E.P. and Wilson K.B., J. R. Statist. Soc. B13 (1951), p. 1.

Box G.E.P., Hunter J.S.and Hunter W.G., Statistics for Experimenters (second ed.), Wiley-Interscience, New York (2005).

Box G.E.P.and Behnken D.W., Technometrics 2 (1960), p. 195.

Box G.E.P.and Draper N.R., Empirical Model-Building and Response Surfaces, Wiley, New York (1987).

Box G.E.P.and Hunter J.S., Ann. Math. Stat. 28 (1957), p. 195.

Brachet A., Rudaz S., Mateua L., Christen P. and Veuthey J.L., J. Sep. Sci. 24 (2001), p. 865.

Breitkreitz M.C., Jardim I.C.S.F., Bruns R.E., Presented at the 10th International Conference on Chemometrics in Analytical Chemistry, Aguas de Lindoia, 10–15 September 2006, abstract P083.

Brewster M. E., Estes K. S. Bodor N. An intravenous toxicity study of 2 hydroxypropyl-B-cyclodextrin, a useful drug solubilizer in rats and monkeys. International Journal of Pharmaceutics. 1990; 59: 231-243.
Broudiscou, A., Leardi, R., Phan-Tan-Luu, R. (1996). Genetic algorithm as a tool for selection of D-optimal design. *Chem Intell Lab Sys* 35:105–116.

Brown, J. M., and Giaccia, A. J. (1998). The unique physiology of solid tumors: Opportunities (and problems) for cancer therapy. Cancer Res. 58, 1408–1416.

Bruns R.E., Scarminio I.S. and Neto B.B., Statistical Design—Chemometrics, Elsevier, Amsterdam (2006).

Bucalo, B.D., Mirikitani, E.J., Moy, R.L. (1998). Comparison of skin anaesthetic effect of liposomal lidocaine, nonliposomal lidocaine and EMLA using 30-minute application time. *Dermatol Surg* 24:537–541.

Bue J. *et al*, "Co-delivery of IL-2 or liposomes augment the responses of mice to a DNA vaccine for pseudorabies virus IE180", Comp Immun Microbio Infect Dis 26 (3) (2003) 175-187

Burstein, H. J., Ramirez, M. J., Petros, W. P., Clarke, K. D., Warmuth, M. A., Marcom, P. K., Matulonis, U. A., Parker, L. M., Harris, L. N., and Winer, E. P. (1999). Phase I study of Doxil and vinorelbine in metastatic breast cancer. Ann. Oncol. 10, 1113–1116.

Campbell R.B. *et al*, "Phospholipid-cationic lipid interactions: influences on membrane and vesicle properties", BBA: Biomembr 1512 (2001) 27-39

Campos, S. M., Penson, R. T., Mays, A. R., Berkowitz, R. S., Fuller, A. F., Goodman, A., Matulonis, U. A., Muzikansky, A., and Seiden, M. V. (2001). The clinical utility of liposomal doxorubicin in recurrent ovarian cancer. Gynecol. Oncol. 81, 206–212.

Cantu M.D., Toso D.R., Lacerda C.A., Lancas F.M., Carrilho E. and Queiroz M.E., Anal. Bioanal. Chem. 386 (2006), p. 256.

Carasek E., Cudjoe E. and Pawliszyn J., J. Chromatogr. A 1138 (2007), p. 10.

Cardenes L., Martin-Calero A., Ayala J., Gonzalez V. and Afonso A., Anal. Lett. 39 (2006), p. 405.

Casas V., Llompart M., Garcia-Jares C., Cela R. and Dagnac T., J. Chromatogr. A 1124 (2006), p. 148.

Castro Mejias R., Natera Marin R., Garcia Moreno M.D. and Garcia Barroso C., J. Chromatogr. A 995 (2003), p. 11.

Cavalli S., Polosello S. and Sacan G., J. Chromatogr. A 1039 (2004), p. 155.

Ceruti M. *al*, "Preparation, characterization, cytotoxicity and pharmacokinetics of liposomes containing water-soluble prodrugs of paclitaxel" J Contr Rel 63 (2000) 141-153

Cervera R, Balasch J. The management of pregnant patients with antiphospholipid syndrome. Lupus 2004;13(9):683-7.

Cevc G. *et al*, "Ultradeformable lipid vesicles can penetrate the skin and other semi-permeable barriers unfragmented. Evidence from double label CLSM experiments and direct size measurements" Bioch Biophys Acta, 1564 (2002) 21-30

Cevc G. Handbook of Biological Physics. Lipowsky Editor. 1995; pp. 465–490.

Chaicumpa W. *et al*, "Immunogenicity of liposome-associated and refined antigen oral cholera vaccines in Thai volunteers" Vaccine, 16 (7) (1998) 678-684

Chandrasekharan N.V., Dai H., Roos K.L., Evanson N.K., Tomsik J., Elton T.S., Simmons D.L. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. Proc Natl Acad Sci U S A 2002;99:13926-31.

Chang, C. W., Barber, L., Ouyang, C., Masin, D., Bally, M. B., and Madden, T. D. (1997). Plasma clearance, biodistribution and therapeutic properties of mitoxantrone encapsulated in conventional and sterically stabilized liposomes after intravenous administration in BDF1 mice. Br. J. Cancer 75, 169–177.

Chemotherapy at Dorland's Medical Dictionary

Chen S., Lostritto R. "Diffusion of benzocaine in poly(ethylene-vinyl acetate) membranes: Effects of vehicle ethanol concentration and membrane vinyl acetate content" J Controlled Release 38 (1996) 185-191

Cheung, B. C. L., Sun, T. H. T., Leenhouts, J. M., and Cullis, P. R. (1998). Loading of doxorubicin into liposomes by forming Mn2þ-drug complexes. Biochim. Biophys. Acta 1414, 205–216.

Cheung, T. W., Remick, S. C., Azarnia, N., Proper, J. A., Barrueco, J. R., and Dezube, B. J. (1999). AIDS-related Kaposi's sarcoma: A phase II study of liposomal doxorubicin. The TLC D-99 Study Group. Clin. Cancer Res. 5, 3432–3437.

Chonn, A., and Cullis, P. R. (1995). Recent advances in liposomal drug-delivery systems. Curr. Opin. Biotechnol. 6, 698–708.

Chou T., Chen S., Chu I., Effect of composition on the stability of liposomal irinotecan prepared by a pH Gradient method. Journal of Bioscience and bioengineering vol.95, No. 4 (2003), 405-408.

Cirri, M., Maestrelli, F., Furlanetto, S., Mura, P.: Solid state characterization of glyburide-cyclodextrin co-ground products. J. Therm Anal. Calorim. 77 413-422 (2004).

Cirri, M., Maestrelli, F., Orlandini, S., Furlanetto, S., Pinzauti, S., Mura, P.: Determination of stability constant values of flurbiprofen-cyclodextrin complexes using different techniques. J. Pharm. Biomed. Anal. 37 995-1002 (2005).

Clarke's Analysis of Drugs and Poisons, 3rd Ed., A.C. Moffat, M.D. Osselton, B. Widdop (Eds.), London, Pharmaceutical Press, 2004.

Coderch L. , "Influence of cholesterol on liposome fluidity by EPR .Relationship with percutaneous absorption" J Contr Rel 68 (2000) 85-95

Conde F.J., Afonso A.M., Gonzalez V. and Ayala J.H., Anal. Bioanal. Chem. 385 (2006), p. 1162.

Cornell J.A., Experiments with Mixtures: Designs, Models and the Analysis of Mixture Data, Wiley, New York (1990).

Corti G., Capasso G., Maestrelli F., Cirri M., Mura P. - Physical-chemical characterization of binary systems of metformin hydrochloride with triacetyl-βcyclodextrin - Journal of Pharmaceutical and Biomedical Analysis. 2007; 45: 480-486.

Costanzo R. *et al*, "ESR study of order ad dynamics in lecithin liposomes with high cholesterol content" Spectrochim Acta 50A (2) (1994) 203-208

Coukell, A. J., and Spencer, C. M. (1997). Polyethylene glycol-liposomal doxorubicin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy in the management of AIDS-related Kaposi's sarcoma. Drugs 53, 520–538.

Coussement W., Van Cauteren H., Vandenberghe J., Vanparys P., Teuns G., Lampo A. and Marsboom R.. Toxicological profile of hydroxypropyl B-cyclodextrin (HP B-CD) in laboratory animals. D. Duchene (ed.) 5th International Symposium on Cyclodextrins. 1990. pp. 552-524.

Covino, B.G., Vassallo, H. (1976): *Local anaesthetics: mechanisms of action and clinical use*. New York: Grune and Stratton (Eds.)

Cullis, P. R. (2000). Liposomes by accident. J. Liposome Res. 10, ix–xxiv.

Cullis, P. R., Hope, M. J., Bally, M. B., Madden, T. D., Mayer, L. D., and Fenske, D. B. (1997). Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles. Biochim. Biophys. Acta 1331, 187–211.

Da Silva G.A., Augusto F. and Poppi R.J., J. Chromatogr. A 1138 (2007), p. 251.

Dayan, N. Touitou E. Carriers for skin delivery of trihexyphenidyl HCl: ethosomes vs. liposomes Biomaterials 21 (2000) 1879-1885

De Andrade J.B., Souza E.T., Rodrigues F.M., Martins C.C., Oliveira Santos F.and Pereira P.A.P., Microchem. J. 82 (2006), p. 142.

De Aragao N.M., Veloso M.C.C., Bispo M.S., Ferreira S.L.C.and De Andrade J.B., Talanta 67 (2005), p. 1007.

Deming S.N.In : B.R. Kowalski, Editor, Chemometrics, Mathematics and Statistics in Chemistry, Reidel, Dordrecht (1981).

Derringer G. and Suich R. , J. Qual. Technol. 12 (1980), p. 214.

Destandau E., Vial J., Jardy A., Hennion M.C., Bonnet D. and Lancelin P., *Anal. Chim. Acta* 572 (2006), p. 102.

Diana J., Ping G., Roets E. and Hoogmartens J., Chromatographia 56 (2002), p. 313.

Dicko A., Tardi P., Xie X., Mayer L., Role of copper gluconate/triethanolamine in irinotecan encapsulation inside the liposomes. International journal of Pharmaceutcs 337 (2007) 219-228.

Ding L., Ni K., Chen Y., Hu Y., Qiu S. and Yu R., Yaowu Fenxi Zazhi 15 (1995), p. 3.

Ding L., Ni K., Chen Y., Hu Y.and Yu R., *SEPU* 11 (1993), p. 293.

Do B., Robinet S., Pradeau D. and Guyon F., Analyst 126 (2001), p. 594.

Do B., Robinet S., Pradeau D.and Guyon F., J. Chromatogr. A 918 (2001), p. 87.

Doehlert D.H. and Klee V.L., Discrete Math. 2 (1972), p. 309.

Doehlert D.H., Appl. Statist. 19 (1970), p. 231.

Dollo G., Le Corre P., Chevanne F., Le Verge R.. Inclusion complexation of amide-typed local anesthetics with β-cyclodextrin and its derivatives. *International Journal of Pharmaceutics*. 1996; 131: 219-228.

Dos Santos W.N.L., Dias F.D.S., Fernandes M.S., Rebouças M.V., Vale M.G.R., Welz B. and Ferreira S.L.C., J. Anal. At. Spectrom. 20 (2005), p. 127.

Draper N.R. and Smith H., Applied Regression Analysis, Wiley, New York (1981).

Dripps R. D., Eckenhoff J.E.,Vandam L.D., Longnecker D. E., F. L. Murphy F.L.. *Introduction to anesthesia*. 8th ed. 1992.

Dron J., Garcia R. and Millan E., J. Chromatogr. A 963 (2002), p. 259.

Drummond D. C., Noble C. O., Guo Z., Hong K., Park J. W., Kirpotin D. B. Development of a highly active nanoliposomal irinotecan using a novel intraliposomal stabilization strategy. Cancer Research 2006; 66: (6), 3271-3277

Dubey V, Mishra D, Dutta T, Nahar M, Saraf DK, Jain NK. Dermal and transdermal delivery of an anti-psoriatic agent via ethanolic liposomes. J Control Release. 2007 Nov 6;123(2):148-54

Durand J.P., Gautier S., Robert E., Guilhem M.C. and Phan-Tan-Luu R., J. High Resolut. Chromatogr. 20 (1997), p. 289.

Dutta A.K., Avery B.A. and Wyandt C.M., *J. Chromatogr. A* 1110 (2006), p. 35.

Düzgünes N. *et al*, "Physicochemical characterization of large unilamellar phospholipids vesicles prepared by reverse-phase evaporation", Biochim Biophys Acta, 732 (1983) 289-299

Dvorak, H. F., Nagy, J. A., Dvorak, J. T., and Dvorak, A. M. (1988). Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. Am. J. Pathol. 133, 95–109.

Edwards K.A., Baeumner A.J. Analysis of liposomes. 2006; 68: 1432–1441.

El Maghraby *et al*, "Skin delivery of 5-fluorouracil from ultradeformable and standard liposomes in-vitro", J Pharm Pharmac 53 (2001) 1069-1077

El Samaligy, M.S., Afifin, N., Mahmoude, E.A. (2006). Increasing bioavailability of silymarin Using a buccal liposomal delivery system: preparation and experimental design investigation. *Int J Pharm* 308:140–148.

Elkheshen, S.A. , Ahmed, S. M., Al-Quadeib B.T.: Inclusion complexes of piroxicam
Elorza B.*et al*, "Characterization of 5-fluorouracil loaded liposomes prepared by reverse-phase evaporation or freezing-thawing extrusion methods: study of drug release" Biochim Biophys Acta 1153 (1993) 135-142

Elsa C. Y. Yan and Kenneth B. Eisenthal"Effect of cholesterol on Molecular Transport of Organic Cations across Liposome Bilayers Probed by Second Harmonic generation" Biophisical Journal 79 (2000) 898-903

Elsayed M.M., Abdallah O.Y., Naggar V.F., Khalafallah N.M. Deformable liposomes and ethosomes: mechanism of enhanced skin delivery. Int J Pharm. 2006 Sep 28;322(1-2):60-6

Elsayed M.M., Abdallah O.Y., Naggar V.F., Khalafallah N.M.. 2007 Deformable liposomes and ethosomes as carriers for skin delivery of ketotifen. Pharmazie, 62(2):133-7.

Esposito, E., Menegatti, E., Cortesi, R., 2004 Ethosomes and liposomes as topical vehicles for azelaic acid: a preformulation study. J. Cosmetic. Sci. 53(3) 253-264

Eurich D.T., McAlister F.A., Blackburn D.F., et al (2007). "Benefits and harms of antidiabetic agents in patients with diabetes and heart failure: systematic review". BMJ 335 (7618): 497.

Ezquerro O., Garrido-Lopez A.and Tena M.T., J. Chromatogr. A 1102 (2006), p. 18.
Fang J.Y. *et al*, "Effect of liposomes and niosomes on skin permeation of enoxacin" Int J Pharm 219 (2001) 61-72

Fang N. *et al*, "BHEM-Chol/DOPE liposome induced perturbation of phospholipid bilayer" Coll Surf B: Biointerf 29 (2003) 233-245

Fang, J., Hwang, T., Huang, Y., Fang, C. (2006). Enhancement of transdermal delivery of catechins by liposomes incorporating anionic surfactants and ethanol. *Int J Pharm* 310:131 138.

Faria A.M., Collins K.E. and Collins C.H., *J. Chromatogr. A* 1122 (2006), p. 114.

Faria A.M., Magalhães D.R., Collins K.E. and Collins C.H., Anal. Chim. Acta 550 (2005), p. 137.

Fatouros D.G. *et al*, "Liposome encapsulating prednisolone and prednisolone-cyclodextrin complexes: comparison of membrane integrity and drug release" Europ J Pharm Sci, 13 (2001) 287-296

Fatouros D.G., Hatzidimitriu K., Antimisiaris S.G. Liposomes encapsulating prednisolone- cyclodextrin complexes: comparision of membrane integrity and drug release. European Journal of Pharmaceutical Science. 2001; 13: 287-296.

Fenske D.B., Cullis P.R., Entrapment of Small Molecules and Nucleic Acid–Based Drugs in Liposomes Methodology of liposome drug delivery systems, Methods in enzymology, Vol. 391 , 7 40

Fenske, D. B., MacLachlan, I., and Cullis, P. R. (2001). Long-circulating vectors for the systemic delivery of genes. Curr. Opin. Mol. Ther. 3, 153–158.

Fenske, D. B., MacLachlan, I., and Cullis, P. R. (2002). Stabilized plasmid-lipid particles: A systemic gene therapy vector. Methods Enzymol. 346, 36–71.

Fenske, D. B., Maurer, N., and Cullis, P. R. (2003). Encapsulation of weakly-basic drugs, antisense oligonucleotides and plasmid DNA within large unilamellar vesicles for drug delivery applications. In ‘‘Liposomes: A Practical Approach’’ (V. P. Torchilin and V. Weissig, eds.), pp. 167–191. Oxford University Press, Oxford.

Fenske, D. B., Wong, K. F., Maurer, E., Maurer, N., Leenhouts, J. M., Boman, N., Amankwa, L., and Cullis, P. R. (1998). Ionophore-mediated uptake of ciprofloxacin and vincristine into large unilamellar vesicles exhibiting transmembrane ion gradients. Biochim. Biophys. Acta 1414, 188–204.

Fernandes C.M., Veiga F.J.B., Chem. Pharm.Bull. 50 (2002) 1597-1602.

Ferreira S.L.C., Bruns R.E., Da Silva E.G.P., Dos Santos W.N.L., Quintella C.M., David J.M., De Andrade J.B., Breitkreitz M.C., Jardim I.C.S.F., Neto B.B. Statistical designs and response surface techniques for the optimization of chromatographic systems. Journal of chromatography A, 1158 (2007) 2-14.

Ferriera S.L.C., dos Santos W.N.L., Quintella C.M., Neto B.B. and Bosque-Sendra J.M., Talanta 63 (2004), p. 1061.

Fisher, R., Hung, O., Mezei, R., Stewart, M. (1998). Topical anaesthesia of intact skin: liposomeencapsulated tetracaine vs EMLA. *Br J Anaest* 81:972–973.

Fiske, C. H., and Subbarow, Y. (1925). The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375–400.

Fraile R.and Sanchez V., J. High Resolut. Chromatogr. 16 (1993), p. 169.

Fresta M., Panico A.M., Bucolo C., Giannavola C., Puglisi G. Characterization and in-vivo ocular absorption of liposome-encapsulated acyclovir. J Pharm Pharmacol. 1999 May;51(5):565-76.

Fresta M.“Caratterizzazione chimico fisica e tecnologica dei liposomi ” 1° corso della scuola dottorale per la formazione avanzata in discipline tecnologico-farmaceutiche (2001) 50-89

Fresta, M., Puglisi G. (1996). Application of liposomes as potential cutaneous drug delivery systems: in vitro and in vivo investigation with radioactivity labelled vesicles. *Drug Target* 4:95–101.

Frohlich M., Volker B., Peschka-Suss R. “ Parameters influencing the determination of liposome lamellarity by ^{31}P-NMR” Chemistry and Physics of Lipids 109 (2001) 103 112

Fromming, K.H., Szejtli, J.: Cyclodextrins in Pharmacy, Kluwer Academic Publishers, Dordrecht (1994).

Furlanetto, S., Cirri, M., Maestrelli, F., Corti, G., Mura, P. (2006). Study of formulation variables influencing the drug release rate from matrix tablets by experimental design. *Eur J Pharm Biopharm* 62:77–84.

Gabizon, A., and Papahadjopoulos, D. (1988). Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc. Natl. Acad. Sci. USA 85, 6949–6953.

Gabrielsson, J., Lindberg, N.O., Lundstedt, T., 2002. Multivariate methods in pharmaceutical applications. J Chemometrics 16:141–160.

Garcia M.S.D., Lopez J.M., Bouza R., Abad M.J., Soto E.G. and Rodriguez M.V.G., Anal. Chim. Acta 521 (2004), p. 179.

Garcia-Villar N., Saurina J. and Hernandez-Cassou S., Anal. Chim. Acta 575 (2006), p. 97.

Garrido-Lopez A., Esquiu V.and Tena M.T., J. Chromatogr. A 1124 (2006), p. 51.

Garrido-Lopez A.and Tena M.T., J. Chromatogr. A 1099 (2005), p. 75.

Gelmon, K. A., Tolcher, A., Diab, A. R., Bally, M. B., Embree, L., Hudon, N., Dedhar, C., Ayers, D., Eisen, A., Melosky, B., Burge, C., Logan, P., and Mayer, L. D. (1999). Phase I of liposomal vincristine. J. Clin. Oncol. 17, 697–705.

Gferer M.and Lankmayr E., *Anal. Chim. Acta* 533 (2005), p. 203.

Ghelardini, C. Galeotti, N., Di Cesare Mannelli, L., Mazzanti, G., Bartolini, A., 2001. Local anaesthetic activity of □- caryophyllene. Il Farmaco, 56, 387-389.

Gideon K. MD "Topical skin anesthesia" Clinics in dermatology 7 (1989) 136-141

Gill, P. S., Wernz, J., Scadden, D. T., Cohen, P., Mukwaya, G. M., Von Roenn, J. H., Jacobs, M., Kempin, S., Silverberg, I., Gonzales, G., Rarick, M. U., Myers, A. M., Shepherd, F., Sawka, C., Pike, M. C., and Ross, M. E. (1996). Randomized phase III trial of liposomal daunorubicin versus doxorubicin, bleomycin, and vincristine in AIDS-related Kaposi's sarcoma. J. Clin. Oncol. 14, 2353–2364.

Glavas-Dodov M., Goracinova K., Mladenovska K., Fredro-Kumbaradzi E. "Release profile of Lidocaine HCl from topical liposomal gel formulation" Inter J Pharm 242 (2002) 381-384

Godin B, Touitou E. 2003 Ethosomes: new prospects in transdermal delivery. Crit Rev Ther Drug Carrier Syst.;20(1):63-102

Godin, B., Touitou E., 2005 Erytromycin ethosomal systems: physicochemical characterization and enhanced antibacterial activity. Curr. Drug Deliv., 2 (3) 269-275
Gokhale, P. C., Radhakrishnan, B., Husain, S. R., Abernethy, D. R., Sacher, R., Dritschilo, A., and Rahman, A. (1996). An improved method of encapsulation of doxorubicin in liposomes: Pharmacological, toxicological, and therapeutic evaluation. Br. J. Cancer 74, 43–48.

Goncalves C., Carvalho J.J., Azenha M.A. and Alpendurada M.F., J. Chromatogr. A 1110 (2006), p. 6.

Goodman L.S., Wintrobe M.M., Dameshek W., Goodman M.J. Gilman A., McLennan M.T. (1984). "Landmark article Sept. 21, 1946: Nitrogen mustard therapy. Use of methyl-bis(beta-chloroethyl)amine hydrochloride and tris(beta-chloroethyl)amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. By Louis S. Goodman, Maxwell M. Wintrobe, William Dameshek, Morton J. Goodman, Alfred Gilman and Margaret T. McLennan". JAMA 251 (17): 2255–61.

Goodman L.S., Wintrobe M.M., Dameshek W., Goodman M.J., Gilman A., McLennan M.T. (1946). "Nitrogen mustard therapy". JAMA 132: 26–32.

Gordon, A. N., Granai, C. O., Rose, P. G., Hainsworth, J., Lopez, A., Weissman, C., Rosales, R., and Sharpington, T. (2000). Phase II study of liposomal doxorubicin in platinum- and paclitaxel-refractory epithelial ovarian cancer. J. Clin. Oncol. 18, 3093–3100.

Goupy J., Plans d'Expériences pour Surfaces de Résponse, Dunod, Paris (1999).

Grabielle-Madelmont C., Lesieur S., Ollivon M. Characterization of loaded liposomes by size exclusion chromatography. Journal of Biochemical and Biophysical Methods. 2003; 56: 189-217.

Graham G.G., Scott K.F., Day R.O. Tolerability of paracetamol. Drug Saf 2005;28(3):227-40.

Gralla R., de Wit R., Herrstedt J., Carides A., Ianus J., Guoguang-Ma J., Evans J., Horgan K. (2005). "Antiemetic efficacy of the neurokinin-1 antagonist, aprepitant, plus a 5HT3 antagonist and a corticosteroid in patients receiving anthracyclines or cyclophosphamide in addition to high-dose cisplatin: analysis of combined data from two Phase III randomized clinical trials". Cancer 104 (4): 864–8.

Grant G.J., Bansinath M. Liposomal Delivery Systems for Local Anesthetics. Regional Anesthesia and Pain Medicine. *2001; 26: 61–63.*

Grant, G.J., Bansinath, M. (2001). Liposomal delivery systems for local anaesthetics. *Reg Anesth Pain Med* 26:61–63.

Green G.A. Understanding NSAIDS: from aspirin to COX-2. Clin Cornerstone 2001;3(5):50-59.

Gregoriadis G., AT Florence. In: G Gregoriadis editor. Liposome Technology, vol. I, 2nd ed. Boca Raton Press, (1993) 37-48

Grunaug, M., Bogner, J. R., Loch, O., and Goebel, F. D. (1998). Liposomal doxorubicin in pulmonary Kaposi's sarcoma: Improved survival as compared to patients without liposomal doxorubicin. Eur. J. Med. Res. 3, 13–19.

Grunberger, G.; Artiss, J.D.; Jen, K.L.C. (2007), "The benefits of early intervention in obese diabetic patients with FBCx TM-a new dietary fibre", Diabetes Metab Res Rev 23: 56–62,

Guidance for Industry: Liposome Drug Products - U.S. Department of Health and Human Services - Food and Drug Administration, August 2002

Guo J. *et al*, "Chitosan coated liposomes: characterization and interaction with leuprolide", Int J Pharm 260 (2003) 167-173

Gutteridge, J. M. (1984). Lipid peroxidation and possible hydroxyl radical formation stimulated by the self-reduction of a doxorubicin-iron (III) complex. Biochem. Pharmacol. 33, 1725–1728.

Gutteridge, J. M., and Quinlan, G. J. (1985). Free radical damage to deoxyribose by anthracycline, aureolic acid and aminoquinone antitumour antibiotics. An essential requirement for iron, semiquinones and hydrogen peroxide. Biochem. Pharmacol. 34, 4099–4103.

Hadjmohammadi M.R.and Safa F., *J. Sep. Sci.* 27 (2004), p. 997.

Haran, G., Cohen, R., Bar, L. K., and Barenholz, Y. (1993). Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim. Biophys. Acta 1151, 201–215.

Harang V. and Westerlund D., Chromatographia 50 (1999), p. 525.

Harang V., Jacobsson S.P. and Westerlund D., Electrophoresis 25 (2004), p. 1792.

Harrigan, P. R., Wong, K. F., Redelmeier, T. E., Wheeler, J. J., and Cullis, P. R. (1993). Accumulation of doxorubicin and other lipophilic amines into large unilamellar vesicles in response to transmembrane pH gradients. Biochim. Biophys. Acta 1149, 329–338.

Hasinoff, B. B. (1989a). The interaction of the cardioprotective agent ICRF-187 [þ)-1,2- bis(3,5-dioxopiperazinyl-1-yL)propane); its hydrolysis product (ICRF-198); and other chelating agents with the Fe(III) and Cu(II) complexes of adriamycin. Agents Actions 26, 378–385.

Hasinoff, B. B. (1989b). Self-reduction of the iron(III)-doxorubicin complex. Free Radic. Biol. Med. 7, 583–593.

Hasinoff, B. B., Davey, J. P., and O'Brien, P. J. (1989). The Adriamycin (doxorubicin)- induced inactivation of cytochrome c oxidase depends on the presence of iron or copper. Xenobiotica 19, 231–241.

Hatzi P., Mourtas S., Klepetsanis P.G., Antimisiaris S.G.. Integrity of liposomes in presence of cyclodextrins: effect of liposome type and lipid composition. International Journal of Pharmaceutics. 2007; 333: 167–176.

Hayakawa E., Naganuma M., Mukasa K., Shimozawa T., and Araiso T.“Change of Motion and Localization of Cholesterol Molecule during L-H_{II} Transition ‘’Biophys J, February 1998, p. 892-898, Vol. 74, No. 2

Hernandez-Borges J., Rodriguez-Delgado M.A., Garcia-Montelongo F.J. and Cifuentes A., J. Sep. Sci. 28 (2005), p. 948.

Higuchi T., Connors K.A. Phase-solubility techniques. Advanced Analitical Chemistry. *1965; 4: 212-217.*

Higuchi, T., Connors, K.A.: Phase-solubility techniques. Adv. Anal. Chem. Instrum. 4, 117-212 (1965).

Hillaert S., De Beer T.R.M., De Beer J.O.and Van den Bossche W., J. Chromatogr. A 984 (2003), p. 135.

Hillaert S., Vander Heyden Y. and Van den Bossche W., *J. Chromatogr. A* 978 (2002), p. 231.

Hillaert S.and Van den Bossche W., J. Chromatogr. A 979 (2002), p. 323.

Hirayama F., Uekama K. Cyclodextrin-based controlled drug release system. Advanced Drug Delivery Reviews. 1999; 36: 125-141.

Hirsch J. (September 2006). "An anniversary for cancer chemotherapy". JAMA 296 (12): 1518–20.

Hlabangana L., Hernandez-Cassou S. and Saurina J., J. Chromatogr. A 1130 (2006), p. 130.

Honzak L. *et al*, “In vivo EPR of topical delivery of a hydrophilic substance encapsulated in multilamellar liposomes applied to the skin of hairless and normal mice”, J Contr Rel 66 (2000) 221-228

Hope, M. J., and Wong, K. F. (1995). Liposomal formulation of ciprofloxacin. In ‘‘Liposomes in Biomedical Applications’’ (P. N. Shek, ed.), pp. 121–134. Harwood Academic Publishers, London.

Hope, M. J., Bally, M. B., Mayer, L. D., Janoff, A. S., and Cullis, P. R. (1986). Generation of multilamellar and unilamellar phospholipid vesicles. Chem. Phys. Lipids 40, 89–107.

Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985). Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochim. Biophys. Acta 812, 55–65.

Hows M.E.P., Perrett D.and Kay J., J. Chromatogr. A 768 (1997), p. 97.

Hu Y.and Massart D.L., J. Chromatogr. 485 (1989), p. 311.

Hu, Q., Shew, C. R., Bally, M. B., and Madden, T. D. (2001). Programmable fusogenic vesicles for intracellular delivery of antisense oligodeoxynucleotides: Enhanced cellular uptake and biological effects. Biochim. Biophys. Acta 1514, 1–13.

Huang, C. (1969). Studies on phosphatidylcholine vesicles. Formation and physical characteristics. Biochemistry 8, 344–352.

Imai, T., Irie,T., Otagiri, M., Uekama, K., Yamasaki, M.: Comparative study on inclusion complexations of antiinflammatory drug flurbiprofen with β-cyclodextrin and methylated β-cyclodextrins. J. Incl. Phenom Macrocycl. Chem. 2, 597-604 (1984).

Imura T.*et al*, "preparation and physicochemical properties of various soybean lecithin liposomes using supercritical reverse phase evaporation method" Coll Surf B: Biointerfaces 27 (2002) 133-140

Indu P. Kaur., Alka Garg, Anil K. Singla, Deepika Aggarwal "Vesicular systems in ocular drug delivery: an overview" International Journal of Pharmaceutics 269 (2004) 1–14

into large unilamellar vesicles, Biochimica et Biophysica Acta 1331(1997) 187–211
Iriarte G., Ferreiros N., Ibarrondo I., Alonso R.M., Maguregi M.I., Gonzalez L. and Jimenez R.M., J. Sep. Sci. 29 (2006), p. 2265.

Israel, V. P., Garcia, A. A., Roman, L., Muderspach, L., Burnett, A., Jeffers, S., and Muggia, F. M. (2000). Phase II study of liposomal doxorubicin in advanced gynecologic cancers. Gynecol. Oncol. 78, 143–147.

Jakobsson N., Degerman M. and Nilsson B., J. Chromatogr. A 1099 (2005), p. 157.
Joensuu H. (2008). "Systemic chemotherapy for cancer: from weapon to treatment". Lancet Oncol. 9 (3): 304.

Jorgensen K. *et al*, "Biophysical mechanism of phospholipase A_2 activation and their use in liposome-based drug delivery" FEBS Letters 531 (1) (2002) 23-27

Judson, I., Radford, J. A., Harris, M., Blay, J., van Hoesel, Q., le Cesne, A., van Oosterom, A. T., Clemons, M. J., Kamby, C., Hermans, C., Whittaker, J., Donato, d. P., Verweij, J., and Nielsen, S. (2001). Randomised phase II trial of pegylated liposomal doxorubicin (DOXIL(R)=CAELYX(R)) versus doxorubicin in the treatment of advanced or metastatic soft tissue sarcoma. A study by the EORTC Soft Tissue and Bone Sarcoma Group. Eur. J. Cancer 37, 870–877.

Junco S., Casimiro T., Ribeiro M., Nunes Da Ponte M., Cabral Marques H., J. Incl. Phenom. 44 (2002) 117-121.

Junco, S., Casimiro, T., Ribeiro, N., Nunes da Ponte, M., Cabral Marques H.: A comparative study of naproxen-beta cyclodextrin complexes prepared by conventional

methods and using supercritical carbon dioxide. J. Incl. Phenom Macrocycl. Chem. 44 117-121 (2002).

Kane G.C., Hoehn S.M., Behrenbeck T.R., Mulvagh S.L. Benzocaine-induced methemoglobinemia based on the Mayo Clinic experience from 28 478 transesophageal echocardiograms: incidence, outcomes, and predisposing factors. Arch Intern Med. 2007 Oct 8;167(18):1977-82.

Khan K.A., J. Pharm. Pharmacol. 27 (1975) 48-49.

Khan, K.A.: The concept of dissolution efficiency. J. Pharm. Pharmac. 27, 48-49 (1975).

Kirby C., Clarke J., Gregoriadis G. Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro. Biochemical Journal. 1980; 186: 591–598.

Kirjavainen, M., Urtti, A., Jaaskelainen, I., Suhonen, T.M., Paronen, P., Valjakka Koskela, R., Kiesvaara, J., Monkkonen, J. (1996). Interaction of liposomes with human skin in vitro the Influence of lipid composition and structure. *Biochim Biophys Acta* 1304:179–189.

Kirjavainen, M., Urtti, A., Valjakka-Koskela, R., Kiesvaara, J., Monkkonen, J. (1999). Liposome-skin interactions and their effects on the skin permeation of drugs. *Eur J Pharm Sci* 7:279–286.

Kis B., Snipes, J.A., Busija D.W. Acetaminophen and the Cyclooxygenase-3 Puzzle: Sorting out Facts, Fictions, and Uncertainties. JPET 2005;315:1-7.

Komatsu H., Guy P.T. and Rowe E.S. Effect of unilamellar vesicle size on ethanol-induced interdigitation in dipalmitoylphosphatidylcholine Chemistry and Physics of Lipids Volume 65, Issue 1, April 1993, Pages 11-21

Kristoffersen L., Bugge A., Lundanes E. and Slordal L., *J. Chromatogr. B* 734 (1999), p. 229.

Kuo C.H. and Sun S.W., *Anal. Chim. Acta* 482 (2003), p. 47.

Lampidis, T.J. Castello C., del Giglio A.,. Pressman B.C,. Viallet P, Trevrrow K.W., Valet G.K., Tapiero H., Savaraj N., Relevance of the chemical charge of rhodamine dyes to multiple drug resistance, Biochem. Pharmacol. 38 (1989) 4267-4271.

Lasic, D. D., Ceh, B., Stuart, M. C., Guo, L., Frederik, P. M., and Barenholz, Y. (1995). Transmembrane gradient driven phase transitions within vesicles: Lessons for drug delivery. Biochim. Biophys. Acta 1239, 145–156.

Lasic, D. D., Frederik, P. M., Stuart, M. C., Barenholz, Y., and McIntosh, T. J. (1992). Gelation of liposome interior. A novel method for drug encapsulation. FEBS Lett. 312, 255–258.

Leclercq L., Bricout H., Tilloy S., Monflier E., "Biphasic aqueous organometallic catalysis promoted by cyclodextrins: Can surface tension measurements explain the efficiency of chemically modified cyclodextrins?", J. Colloid Interface Sci. 2007, 307, 481.

Leclercq L., Sauthier M., Castanet Y., Mortreux A., Bricout H., Monflier E., "Two-phase hydroformylation of higher olefins using randomly methylated α-cyclodextrin as mass transfer promoter: a smart solution for preserving the intrinsic properties of the rhodium / trisulfonated triphenylphoshine catalytic system", Adv. Synth. Catal. 2005, 347, 55.

Lee C.H., Lin W.C., Wang J. All-optical measurements of the bending rigidity of lipid-vesicle membranes across structural phase transitions. Physical Review. 2001; E64: 020901.

Levchenko T.S. *et al*, "Liposome clearance in mice: the effect of a separate and combined presence o surface charge and polymer coating", Int J Pharm 240 (2002) 95-102

Levy M.Y., Benita S., "Drug release from submicronized o/w emulsion: a new in vitro kinetic evaluation model" Int J Pharm 66 (1990) 29-37

Lewis, G.A., Mathieu, D., Phan-Tan-Luu, R. (1999). *Pharmaceutical experimental design*. New York: Marcel Dekker (ed).*Li et al* 2003 ."An in vitro permeation model to evaluate various benzocaine transoral formulations".

Li, X., Cabral-Lilly, D., Janoff, A. S., and Perkins, W. R. (2000). Complexation of internalized doxorubicin into fiber bundles affects its release rate from liposomes. J. Liposome Res. 10, 15–27.

Li, X., Hirsh, D. J., Cabral-Lilly, D., Zirkel, A., Gruner, S. M., Janoff, A. S., and Perkins, W. R. (1998). Doxorubicin physical state in solution and inside liposomes loaded via a pH gradient. Biochim. Biophys. Acta 1415, 23–40.

Lian T., Ho R.J.Y. Trends and Developments in Liposome Drug Delivery Systems. Journal of Pharmaceutical Science*. 2001; 90: n. 6*

Lichtenberg, D., and Barenholz, Y. (1988). Liposomes: Preparation, characterization, and preservation. Methods Biochem. Anal. 33, 337–462.

Lim, H. J., Masin, D., Madden, T. D., and Bally, M. B. (1997). Influence of drug release characteristics on the therapeutic activity of liposomal mitoxantrone. J. Pharmacol. Exp. Ther. 281, 566–573.

Lim, H. J., Masin, D., McIntosh, N. L., Madden, T. D., and Bally, M. B. (2000). Role of drug release and liposome-mediated drug delivery in governing the therapeutic activity of liposomal mitoxantrone used to treat human A431 and LS180 solid tumors. J. Pharmacol. Exp. Ther. 292, 337–345.

Lim, J.O., Kim, S.J., Pouliot, R., Baek, W.Y. (2005). Enhanced transdermal delivery of local anaesthetics by liposome formulation of local anaesthetic mixture. *Trans Tech Publ* 277:45–50.

Liu L., Zhu S., J. Pharm. Biomed. Anal. 40 (2006) 122-127.

Loftsson T., Duchêne D., Int. J. Pharm. 329 (2007) 1-11.

Loftsson T., Hreinsdóttir D., Másson M., Int. J. Pharm. 302 (2005) 18-28.

Loftsson T., Stefánsson E.. Effect of cyclodextrins on topical drug delivery to the eye. Drug Development and Industrial Pharmacy. 1997; 23: 473-481.

Loftsson, T., Duchêne, D.: Cyclodextrins and their therapeutic applications. Int. J. Pharm., 329, 1-11 (2007).

López-Pinto, J.M., Gonzalez-Rodriguez, M.L., Rabasco, A.M. (2005). Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. *Int J Pharm* 298:1–12.

Loukas Y.L. , Jayasekera P., Gregoriadis G. Novel liposome-based multicomponent systems for the protection of photolabile agents. International Journal of Pharmaceutics. 1995; 117: 85-94.

Loukas Y.L., Vraka V., Gregoriadis G.. Drugs, in cyclodextrins, in liposomes: a novel approach to the chemical stability of drugs sensitive to hydrolysis. International Journal of Pharmaceutics. 1998; 162: 137-142.

M'Hamdi R., Thiébaut D., Caude M., Robert E.and Grand C., *J. High Resolut. Chromatogr.* 21 (1997), p. 94.

Ma Q.,Rajewski R.A., Vander Velde D., Stella V.J.. Comparative effects of (SBE)7M-beta-CD and HP-beta-CD on the stability of two anti-neoplastic agents, melphalan and carmustine. Journal of Pharmaceutical Science. 2000; 89: 275-287.

Madden, T. D., Harrigan, P. R., Tai, L. C., Bally, M. B., Mayer, L. D., Redelmeier, T. E., Loughrey, H. C., Tilcock, C. P., Reinish, L. W., and Cullis, P. R. (1990). The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: A survey. Chem. Phys. Lipids 53, 37–46.

Maestrelli F., González-Rodríguez M.L., Rabasco A.M., Mura P. Effect of preparation technique on the properties of liposomes encapsulating ketoprofen–cyclodextrin complexes aimed for transdermal delivery International Journal of Pharmaceutics, Volume 312, Issues 1-2, 7 April 2006, Pages 53-60

Maestrelli F., Gonzalez-Rodrıguez M.L., Rabasco A.M., Mura P. Preparation and characterisation of liposomes encapsulating ketoprofen–cyclodextrin complexes for transdermal drug delivery. International Journal of Pharmaceutics. 2005; 298: 55–67.

Maestrelli, F., González-Rodríguez, M.L., Rabasco, A.M., Mura, P. (2005). Preparation and characterisation of liposomes encapsulating ketoprofen-cyclodextrin complexes for transdermal drug delivery. *Int J Pharm* 298:55–67.

Mangelinas D., Maftouh M., Massart D.L. and Heyden Y.V., J. Pharm. Biomed. Anal. 35 (2004), p. 789.

Manosroi, A., Kongkaneramit, L.L., Manosroi, J., 2004. Characterization of amphotericin B liposome formulation. Drug Dev. Ind. Pharm. 30, 535-543.

Marathe P.H., Wen Y., Norton J., Greene D.S., Barbhaiya T.F., Wilding I.R., Br. J. Clin. Pharmacol. 50 (2000) 325-332.

Marchetti P., Navalesi R., Clin. Pharmacokinet. 16 (1989) 100-128.

Margalit, R. (1995). Liposome-mediated drug targeting in topical and regional therapies. *Crit Rev Ther Drug Carrier Syst* 12:233–261.

Marques Fernandes C., Ramos P., Celta Falcão A., Veiga F.J.B., J. Control Rel. 88 (2003) 127-134

Martínez-Uruñuela A., González-Sáiz J.M.and Pizarro C., J. Chromatogr. A 1056 (2004), p. 49.

Massart D.L., Vandeginste B.G.M., Buydens L.M.C., de Jong S., Lewi P.J. and Smeyers-Verbeke J., Handbook of Chemometrics and Qualimetrics. Part A, Elsevier, Amsterdam (1977).

Mathieu, D., Nony, J., Phan-Tan-Luu, R. (2000). NEMRODW software, LPRAI sarl, Marseille, F-13331, France.

Matsubara K., Irie T., Uekama K. Controlled release of the LHRH agonist buserelin acetate from injectable suspensions containing triacetylated cyclodextrins in an oil vehicle. Journal of Controlled Release. 1994; 31: 173-180.

Matsuda H., Arima H.. Cyclodextrins in transdermal and rectal delivery. Advanced Drug Delivery Reviews. 1999; 36: 81-99.

Maurer, N., Mori, A., Palmer, L., Monck, M. A., Mok, K. W., Mui, B., Akhong, Q. F., and Cullis, P. R. (1999). Lipid-based systems for the intracellular delivery of genetic drugs. Mol. Membr. Biol. 16, 129–140.

Maurer, N., Wong, K. F., Hope, M. J., and Cullis, P. R. (1998). Anomalous solubility behaviour of the antibiotic ciprofloxacin encapsulated in liposomes: A 1H-NMR study. Biochim. Biophys. Acta 1374, 9–20.

Maurer, N., Wong, K. F., Stark, H., Louie, L., McIntosh, D., Wong, T., Scherrer, P., Semple, S. C., and Cullis, P. R. (2001). Spontaneous entrapment of polynucleotides

upon electrostatic interaction with ethanol-destabilized cationic liposomes. Biophys. J. 80, 2310–2326.

Maurer-Spurej, E., Wong, K. F., Maurer, N., Fenske, D. B., and Cullis, P. R. (1999). Factors influencing uptake and retention of amino-containing drugs in large unilamellar vesicles exhibiting transmembrane pH gradients. Biochim. Biophys. Acta 1416, 1–10.

Mayer L.D. *et al*, "Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles", Biochim. Biophys. Acta, 817 (1985) 193-196

Mayer L.D., Hope M.J., Cullis P.R., Janoff A.S. Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles.Biochim Biophys Acta. 1985 Jul 11;817(1):193-6.

Mayer, L. D., Bally, M. B., and Cullis, P. R. (1986a). Uptake of Adriamycin into large unilamellar vesicles in response to a pH gradient. Biochim. Biophys. Acta 857, 123–126.

Mayer, L. D., Bally, M. B., and Cullis, P. R. (1990a). Strategies for optimizing liposomal doxorubicin. J. Liposome Res. 1, 463–480.

Mayer, L. D., Bally, M. B., Cullis, P. R., Wilson, S. L., and Emerman, J. T. (1990b). Comparison of free and liposome encapsulated doxorubicin tumor drug uptake and antitumor efficacy in the SC115 murine mammary tumor. Cancer Lett. 53, 183–190.

Mayer, L. D., Bally, M. B., Loughrey, H., Masin, D., and Cullis, P. R. (1990c). Liposomal vincristine preparations which exhibit decreased drug toxicity and increased activity against murine L1210 and P388 tumors. Cancer Res. 50, 575–579.

Mayer, L. D., Hope, M. J., and Cullis, P. R. (1986b). Vesicles of variable sizes produced by a rapid extrusion procedure. Biochim. Biophys. Acta 858, 161–168.

Mayer, L. D., Nayar, R., Thies, R. L., Boman, N. L., Cullis, P. R., and Bally, M. B. (1993). Identification of vesicle properties that enhance the antitumour activity of liposomal vincristine against murine L1210 leukemia. Cancer Chemother. Pharmacol. 33, 17–24.

Mayer, L. D., Tai, L. C., Ko, D. S., Masin, D., Ginsberg, R. S., Cullis, P. R., and Bally, M. B. (1989). Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. Cancer Res. 49, 5922–5930.

Mc Cormack B., Gregoriadis G.. Entrapment of Cyclodextrin-Drug Complexes into Liposomes: Potential Advantages in Drug Delivery. Journal of Drug Targeting. 1994; 2, 449-454.

Mc Cormack B., Gregoriadis G.. Comparative studies of the fate of free and liposome-entrapped hydroxypropyl-β-cyclodextrin/drug complexes after intravenous injection into rats: implications in drug delivery. Biochimica et Biophysica Acta. 1996; 1291: 237-244.

Melani, C., Rivoltini, L., Parmiani, G., Calabretta, B., and Colombo, M. P. (1991). Inhibition of proliferation by c-myb antisense oligodeoxynucleotides in colon adenocarcinoma cell lines that expRes.s c-myb. Cancer Res. 51, 2897–2901.

Meng M., Liu Y., Wang Y., Wang J., Zhang H., Wang X., Zhang Z., Lu W., Zhang Q., Increase of the pharmacological and pharmacokinetic efficacy of negatively charged polypeptide recombinant hirudin in rats via parenteral route by association with cationic liposomes Journal of Controlled Release (2008).

Mezei, M., Gulasekharam, V. (1982). Liposomes, a selective drug delivery system for the topical route of administration: gel dosage form. *J Pharm Pharmacol* 34:473–474.

Millar, J. L., Millar, B. C., Powles, R. L., Steele, J. P., Clutterbuck, R. D., Mitchell, P. L., Cox, G., Forssen, E., and Catovsky, D. (1998). Liposomal vincristine for the treatment of human acute lymphoblastic leukaemia in severe combined immunodeficient (SCID) mice. Br. J. Haematol. 102, 718–721.

Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., and Reynolds, J. A. (1981). Phospholipid vesicle formation and transmembrane protein incorporation using octyl glucoside. Biochemistry 20, 833–840.

Mirza T. and Tan H.S.I., J. Pharm. Biomed. Anal. 25 (2001), p. 39.

Mohammed, A.R., Weston, N., Coombes, A.G.A., Fitzgerald, M., Perrie, Y. (2004). Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. *Int J Pharm* 285:23–34.

Mok, K. W., Lam, A. M., and Cullis, P. R. (1999). Stabilized plasmid-lipid particles: Factors influencing plasmid entrapment and transfection properties. Biochim. Biophys. Acta 1419, 137–150.

Monbaliu J., Van Beijsterveldt L., Meuldermans W., Szathmary S. and Heykants J. Disposition of hydroxy- ropyl B-cyclodextrin in experimental animals. D. Duchene (ed.) 5th International Symposium on Cyclodextrins. 1990. pp. 514-517.

Montgomery D.C., Design and Analysis of Experiments (fourth ed.), Wiley, New York (1997).

Moore D.E.. Drug-induced cutaneous photosensitivity. Drug Safety 2002;25:345-72.

Moore T.J.; Walsh C.S.; Cohen M.R. Reported Adverse Event Cases of Methemoglobinemia Associated With Benzocaine Products Arch Intern Med. 2004;164:1192-1196.

Morrison, R. S. (1991). Suppression of basic fibroblast growth factor expression by antisense oligodeoxynucleotides inhibits the growth of transformed human astrocytes. J. Biol. Chem. 266, 728–734.

Muggia, F. M. (2001). Liposomal encapsulated anthracyclines: New therapeutic horizons. Curr. Oncol. Rep. 3, 156–162.

Muindi, J. R., Sinha, B. K., GiAnni, L., and Myers, C. E. (1984). Hydroxyl radical production and DNA damage induced by anthracycline-iron complex. FEBS Lett. 172, 226–230.

Muir B., Carrick W.A. and Cooper D.B., Analyst 127 (2002), p. 1198.

Müller M., Mackeben S., MüllerGoymann C.C."Physicochemical characterisation of liposomes with encapsulated local anaesthetics" International Journal of Pharmaceutics 274 (2004) 139–148

Munot V.B. – Solubility studies of prilocaine and lignocaine with HPβCD. Curtin University of Technology (Australia) – Archive of thesis presented for Degree of Master of Pharmacy - 2007.

Mura P., Adragna E., Rabasco A.M., Moyano J.R., Pérez-Martìnez J.I., Arias M.J., Ginés J.M., Drug Dev. Ind. Pharm. 25 (1999) 279-287.

Mura P., Capasso G., Maestrelli F., Furlanetto, S. 2008 "Optimization of formulation variables of benzocaine liposomes using experimental design" Journal of Liposome Research, 18, 1-13.

Mura P., Faucci M.T., Parrini P.L., Furlanetto S., Pinzauti S., Int. J. Pharm. 179 (1999) 117-128.

Mura P., Furlanetto S., Cirri M., Maestrelli F., Corti G., Pinzauti S., J. Pharm. Biomed. Anal. 37 (2005) 987-994.

Mura P., Maestrelli F., González-Rodríguez M. L., Michelacci I., Ghelardini C., Rabasco A. M. 2007."Development, characterization and in vivo evaluation of benzocaine-loaded liposomes". Eur.J.Pharm.Sci. 67, 1, pag. 86-95.

Mura P., Maestrelli F., Neri C., Capasso G., Gonzales M.L., Rabasco A.M. - Poster presentato al PBP 6th World Meeting – Benzocaine-cyclodextrin complexes encapsulated in liposomes: pre-formulation and characterization studies. ISO Standard 9276-5. Representation of results of particle size analysis. 2005.

Mura, P., Adragna, E., Rabasco, A.M., Moyano, J.R., Pérez-Martìnez, J.I., Arias, M.J., Ginés, J.M.: Effects of the Host Cavity Size and the Preparation Method on the Physicochemical Properties of Ibuproxam-Cyclodextrin Systems Drug Dev. Ind. Pharm. 25, 279-287 (1999).

Mura, P., Bettinetti, G.P., Faucci, M.T., Sorrenti, M., Negri, A.: Interaction of naproxen with crystalline and amorphous methylated ß-cyclodextrin in the liquid and in the solid state. Supramolecular Chemistry, 11, 379-391 (2001).

Mura, P., Bettinetti, G.P., Manderioli, A., Faucci, M.T., Bramanti, G., Sorrenti, M.: Interactions of ketoprofen and ibuprofen with ß-cyclodextrins in solution and in the solid state. Int. J. Pharm., 166, 189-203 (1998).

Mura, P., Bettinetti, G.P., Melani, F., Manderioli, A.: Interaction between naproxen and chemically-modified □-cyclodextrins in the liquid and solid state. Eur. J. Pharm. Sci. 3 347-355 (1995).

Mura, P., Celesti, L., Murratzu, C., Corsi, S., Furlanetto, S., Corti, P. (1993). In vitro studies of simulated percutaneous absorption: influence of artificial membrane impregnation agent. *Acta Techn Leg Med* 4:121–136.

Mura, P., Cirri, M., Faucci, M.T., Ginès-Dorado, J.M., Bettinetti, G.P.: Investigation of the effects of grinding and co-grinding on physicochemical properties of glisentide. J. Pharm. Biomed. Anal. 30 227-237 (2002).

Mura, P., Faucci, M.T., Manderioli, A., Bramanti, G.: Influence of the preparation method on the physicochemical properties of binary systems of econazole with cyclodextrins. Int. J.Pharm. 193, 85-95 (1999).

Mura, P., Faucci, M.T., Parrini, P.L., Furlanetto, S., Pinzauti, S.: Influence of the preparation method on the physicochemical properties of ketoprofen-cyclodextrin binary systems. Int. J. Pharm. 179, 117-128 (1999).

Mura, P., Faucci, M.T., Parrini, P.L.: Effects of grinding with microcrystalline cellulose and cyclodextrins on the ketoprofen physico-chemical properties. Drug Dev. Ind. Pharm. 27 119-128 (2001).

Mura, P., Maestrelli, F., Michelacci, I., Ghelardini, C., Gonzalès, M.L., Rabasco, A. (2007). Development, characterization and *in vivo* evaluation of benzocaine-loaded liposomes. *Eur J Pharm Biopharm* 67:86–95.

Mura, P., Zerrouck, N., Faucci, M.T., Maestrelli, F., Chemtob, C.: Comparative study of ibuproxam complexation with amorphous ß-cyclodextrin derivatives in solution and in the solid state. Eur. J. Pharm. Biopharm. 54 181-191 (2002).

Myers R.H. and Montgomery D.C., Response Surface Methodology: Process and Product Optimization Using Designed Experiments (second ed.), Wiley, New York (2002).

Nagumo A. *et al*, "Electron spin resonance studies of phosphatidylcholine interacted with cholesterol and with hopanoid in liposomal membrane" Chem Pharm Bull, 39 (1991) 3071-3074

Nakanishi K., Masukawa T., Nadai T. , Yoshii K., Okada S., Miyajima K., Biol. Pharm. Bull. 20 (1997) 66-70.

Nallamothu R., Wood G.C., Pattillo C.B., Scott R.C., Kiani M.F., Moore B.M., Thoma L.A.. A Tumor Vasculature Targeted Liposome Delivery System for Combretastatin. AAPS PharmSciTech. 2006; 7: n. 32

Navalon A., Prieto A., Araujo L. and Vílchez J.L., J. Chromatogr. A 946 (2002), p. 239.

Nelder J.A.and Mead R., Comput. J. 7 (1965), p. 308.

Neto B.B., Scarminio I.S. and Bruns R.E., Quim. Nova 29 (2006), p. 1401.

Nicolazzi C., Venare V., Le Faou A., Finance C. In vitro antiviral activity of the gancyclovir complexed with beta cyclodextrin on human cytomegalovirus strains. Antiviral Research. 2002; 54: 121-127.

Northfelt, D. W., Dezube, B. J., Thommes, J. A., Miller, B. J., Fischl, M. A., Friedman-Kien, A., Kaplan, L. D., Du, M. C., Mamelok, R. D., and Henry, D. H. (1998). Pegylatedliposomal doxorubicin versus doxorubicin, bleomycin, and vincristine in the treatment of AIDS-related Kaposi's sarcoma: Results of a randomized phase III clinical trial. J. Clin. Oncol. 16, 2445–2451.

Novotna K., Havlis J. and Havel J., J. Chromatogr. A 1096 (2005), p. 50.

Nowak M. and Seubert A., *J. Chromatogr. A* 855 (1999), p. 91.

Olson F., Mathew E., Maslow D.,Rustums Y., Szoka F. Characterization, Toxicity and Therapeutic Efficacy of Adriamycin Encapsulated in Liposomes Eur J Cancer Clin Oncol. Vol. 16. No. 2, pp. 167-176. 1982.

Olson, F., Hunt, C. A., Szoka, F. C., Vail, W. J., and Papahadjopoulos, D. (1979). Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim. Biophys. Acta 557, 9–23.

Ortiz-Boyer, T., Luque de Castro, V. "Development and validation of chromatographic methods (HPLC and GC) for the determination of the active components (benzocaine, tyrothricin and menthol) of a pharmaceutical preparation" J Pharm and Biom Analisys 13 (1995) 1297-1303

Ostensen M.E., Skomsvoll J.F. Anti-inflammatory pharmacotherapy during pregnancy. Expert Opin Pharmacother 2004;5(3):571-80.

Otero Espinar, F.J., Anguia Igea A., S., Blanco Mendez, J., Vila Jato, J.L.: Reduction in the ulcerogenicity of naproxen by complexation with ß-cyclodextrin. Int. J. Pharm. 70, 35-41 (1991).

Otero-Espinar, F.J., Anguiano-Igea, S., Garcia-Gonzalez, N., Vila-Jato, J.L., Blanco-Mendez, J.: Oral bioavailability of naproxen-ß-cyclodextrin inclusion compound. Int.J.Pharm. 75, 37-44 (1991).

Outinen K., Haario H., Vuorela P., Nyman M., Ukkonen E. and Vuorela H., Eur. J. Pharm. Sci. 6 (1998), p. 197.

Padilla A., Palma M. and Barroso G.C., *J. Chromatogr. A* 1091 (2005), p. 83.

Paolino, D. Lucania G., Mardente, D. Alhaique, F. Fresta M. Ethosomes for skin delivery of ammonium glycyrrhizinate: In vitro percutaneous permeation through human skin and in vivo anti-inflammatory activity on human volunteers Journal of Controlled Release 106 (2005) 99–110

Papahadjopoulos D., “Sterically stabilized liposomasis improvements in pharmacokinetics and antitumor therapeutic efficacy”, PROC Natl Acad Sci USA, Vol 88 (1991) 11460-11464

Pellati F., Benvenuti S., Yoshizaki F., Bertelli D. and Rossi M.C., J. Chromatogr. A 1087 (2005), p. 265.

Perrie Y. *et al*, “Liposome-mediated DNA vaccination: the effect of vesicle composition” Vaccine, 19 (23-24) (2001) 3301-3310

Pickholz M., Fernandes Fraceto L., De Paula E.. Interactions of neutral and protonated prilocaine with phospholipid bilayers. Abstracts / Chemistry and Phisics of lipids. 2007; 149S: S23-S49.

Pinheiro P.B.M. and Esteves da Silva J.C.G., Anal. Bioanal. Chem. 382 (2005), p. 341. Pinto L.M.A., Fernandes Fraceto L., Santana M.H.A., Pertinhez T.A., Oyama Junior S., de Paula E., J. Pharm. Biomed. Anal. 39 (2005) 956-963.

Pizarro C., Gonzalez-Saiz J.M. and Perez-del-Notario N., J. Chromatogr. A 1132 (2006), p. 8.

Polo M., Llompart M., Garcia-Jares C., Gomez-Noya G., Bollain M.H. and Cela R., J. Chromatogr. A 1124 (2006), p. 11.

Pratt, G., Wiles, M. E., Rawstron, A. C., Davies, F. E., Fenton, J. A., Proffitt, J. A., Child, J. A., Smith, G. M., and Morgan, G. J. (1998). Liposomal daunorubicin: In vitro and in vivo efficacy in multiple myeloma. Hematol. Oncol. 16, 47–55.

Preu M., Guyot D.and Petz M., *J. Chromatogr. A* 818 (1998), p. 95.

Puglisi “I liposomi” 1° corso della scuola dottorale per la formazione avanzata in discipline tecnologico-farmaceutiche (2001) 7-48

Qiu J.S. and Jin X.H., *J. Chromatogr. A* 950 (2002), p. 81.

Qiu R., MacDonald R.C.A metastable state of high surface activity produced by sonication of phospholipids Biochimica et Biophysica Acta (BBA) - Biomembranes, Volume 1191, Issue 2, 11 May 1994, Pages 343-353

Ramsay E., Alnajim J., Anantha M., Zastre J., Yan H., Webb M., Waterhouse D., Bally M., A novel liposomal irinotecan formulation with significant anti-tumor activity: Use of the divalent cation ionophore A23187 and copper-containing liposomes to improve drug retention. European Journal of Pharmaceutics and Biopharmaceutics 68 (2008) 607-617

Ranjit Singh, S.P. Vyas “Topical liposomal systemfor localized and controlled drug delivery” Journal of dermatological Science 13 (1996) 107-111

Rendell M. (2004). "Advances in diabetes for the millennium: drug therapy of type 2 diabetes". MedGenMed 6 (3 Suppl): 9.

Rodil R., Carro A.M., Lorenzo R.A., Abuin M. and Cela R., *J. Chromatogr. A* 963 (2002), p. 313.

Rodrigues M.V.N., Reyes F.G.R., Rehder V.L.G. and Rath S., *Chromatographia* 61 (2005), p. 291.

Rodriguez I., Quintana J.B., Carpinteiro J., Carro A.M., Lorenzo R.A. and Cela R., *J. Chromatogr. A* 985 (2003), p. 265.

Romero R., Bagur M.G., Sanchez-Vinas M. and Gazquez D., Chromatographia 51 (2000), p. 404.

Rossi S, editor. Australian Medicines Handbook 2006. Adelaide: Australian Medicines Handbook; 2006.

Rothstein, R.: Safety profiles of leading nonsteroidal anti-inflammatory drugs. Am. J. Med. 105, 39-43 (1998).

Rottenberg, H. (1979). The measurement of membrane potential and deltapH in cells, organelles, and vesicles. Methods Enzymol. 55, 547–569.

Routh M.W., Swartz P.A.and Denton M.B., Anal. Chem. 49 (1977), p. 1422.

Ruzica G.R., Barisic, Pavelic, Grubisis."Hight efficiency entrapment of superoxide dismutase into mucoadhesive chitosan-coated liposomes" Europ J Pharm Sci 15 (2002) 441-448

Saia Cereda C.M., De AraujJo D.R., Brunetto G.B., De Paula E. Liposomal prilocaine: preparation, characterization and in vivo evaluation. Journal of Pharmaceutical Science. 2004; 7: 235-240.

Saravolac, E. G., Ludkovski, O., Skirrow, R., Ossanlou, M., Zhang, Y. P., Giesbrecht, C., Thompson, J., Thomas, S., Stark, H., Cullis, P. R., and Scherrer, P. (2000). Encapsulation of plasmid DNA in stabilized plasmid-lipid particles composed of different cationic lipid concentration for optimal transfection activity. J. Drug Target. 7, 423–437.

Schätzlein A., Cevc G., Paul A., Blume G. Ultraflexible vesicles, transferosomes, as transdermal drug carriers for peptides and proteins. Journal of Controlled Release. 1997; 46: 179–180.

Scheen A.J., Clin. Pharmacokinet. 30 (1996) 359-371.

Semple, S. C., Klimuk, S. K., Harasym, T. O., and Hope, M. J. (2000). Lipid-based formulations of antisense oligonucleotides for systemic delivery applications. Methods Enzymol. 313, 322–341.

Semple, S. C., Klimuk, S. K., Harasym, T. O., Dos, S. N., Ansell, S. M., Wong, K. F., Maurer, N., Stark, H., Cullis, P. R., Hope, M. J., and Scherrer, P. (2001). Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable

aminolipids: Formation of novel small multilamellar vesicle structures. Biochim. Biophys. Acta 1510, 152–166.

Sentjurc M. *al*, "Role of cholesterol in the transport of liposome encapsulated substances into skin" Prog Drug Del Syst 5 (1996) 157-160

Sessa, G., and Weissmann., G. (1968). Phospholipid spherules (liposomes) as a model for biological membranes. J. Lipid Res. 9, 310–318.

Seth, A.K., Misra, A. (2002). Matematical modelling of preparation of acyclovir liposomes: reverse phase evaporation method. *J Pharm Pharmaceut Sci* 5:285–291.

Shields, A. F., Lange, L. M., and Zalupski, M. M. (2001). Phase II study of liposomal doxorubicin in patients with advanced colorectal cancer. Am. J. Clin. Oncol. 24, 96–98.

Sieffert N., Wipff G. Chem. Eur. J. 2007, 13, 1978–1990.

Sinha V.R., Nands A., KumriaR. Cyclodextrins as sustained release carriers. Pharmaceutical Technologies. 2002: 36-46.

Siouffi A.M. and Phan-Tan-Luu R., *J. Chromatogr. A* 892 (2000), p. 75.

Skalko N., Brandl M., Ladan M.B., Grid J.F., Genjak J. Liposomes with nifedipine and nifedipine-cyclodextrin complex: calorimetrical and plasma stability comparison. European Journal of Pharmaceutical Science. 1996; 4: 359-366.

Spendley W., Hext G.R. and Himsworth F.R., Technometrics 4 (1962), p. 441.

Stafiej A., Pyrzynska K., Ranz A. and Lankmayr E., *J. Biochem. Biophys. Methods* 69 (2006), p. 15.

Stalikas C.D. and Pilidis G.A., J. Chromatogr. A 872 (2000), p. 215.

Stanier C.A., O Connell M.J., Anderson H.L. and Clegg W.(2001). "Synthesis of fluorescent stilbene and tolan rotaxanes by Suzuki coupling". Chem. Commun. (5): 493–494.

Stein, C. A., and Cohen, J. S. (1988). Oligodeoxynucleotides as inhibitors of gene expression: A review. Cancer Res. 48, 2659–2668.

Stern A.(2002). Pharmacology: PreTest self-assessment and review. New York: McGraw-Hill, Medical Pub. Division.

Storm G., Oussoren C., Peeters P., Barenholz Y.. Liposome technology. CRC Press; p. 345.

Storm, Oussoren, Peeters, Barenholz (1993) In " Liposome technology" Vol. III 345

Stuart, D.D., and Allen, T. M. (2000). A new liposomal formulation for antisense oligodeoxynucleotides with small size, high incorporation efficiency and good stability. Biochim. Biophys. Acta 1463, 219–229.

Stuart, D. D., Kao, G. Y., and Allen, T. M. (2000). A novel, long-circulating, and functional liposomal formulation of antisense oligodeoxynucleotides targeted against MDR1. Cancer Gene Ther. 7, 466–475.

Sun, X., Zhang, Z. (2004). Optimizing the novel formulation of liposome-polycation DNA complexes (LPD) by central composite design. *Arch Pharm Res* 27:797–805.

Szczylik, C., Skorski, T., Nicolaides, N. C., Manzella, L., Malaguarnera, L., Venturelli, D., Gewirtz, A. M., and Calabretta, B. (1991). Selective inhibition of leukemia cell proliferation by BCR-ABL antisense oligodeoxynucleotides. Science 253, 562–565.

Szejtli J. (1988). "Cyclodextrin Technology" vol 1. Springer, New York"

Szente L., Szejtli J. Highly soluble cyclodextrin derivatives: chemistry, properties, and trends in development. Advanced Drug Delivery Reviews. 1999; 36: 17-38

Szoka F. *et al*, "Procedures for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation" Proc Natl Acad Sci ESA, 75 (1978) 4194-198

Szoka F.C. Antisense Drug Technology Journal of Controlled Release, Volume 89, Issue 2, 29 April 2003, Pages 355-356

Szoka F.C. Review of Liposomes. Journal of Controlled Release, Volume 96, Issue 2, 28 April 2004, Page 353

Szoka F.C., Olson F. , Heath T., Vail W., Mayhew E., Papahadjopoulos D., Preparation of unilamellar liposomes of intermediate size (0.1-0.2 pm) by a combination of reserve phase evaporation and extrusion through polycarbonate membranes, Biochimica et Biophysica Acta, 601 (1980) 559—571.

Szoka F.*et al*, "Comparative studies and methods of preparation of lipid vesicles (liposomes)", Ann Rev Bioeng, 9 (1980) 467-508

Szoka, F. C., and Papahadjopoulos, D. (1978). Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. USA 75, 4194–4198.

Szoka, F.C., 1978 Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse phase evaporation. Proc. Natl. Acad. Sci. U.S.A. 75, 4194-4198

Tam, P., Monck, M., Lee, D., Ludkovski, O., Leng, E. C., Clow, K., Stark, H., Scherrer, P., Graham, R. W., and Cullis, P. R. (2000). Stabilized plasmid-lipid particles for systemic gene therapy. Gene Ther. 7, 1867–1874.

Tannock I.F., Ahles T.A., Ganz P.A., Van Dam FS.. (June 2004). "Cognitive impairment associated with chemotherapy for cancer: report of a workshop". J. Clin. Oncol. 22 (11): 2233–9.

Tardi P.G., Gallagher R.C., Johnstone S., Harasym N., Webb M., Bally M.B., Mayer L.D., Coencapsulation of irinotecan and floxuridine into low cholesterol-containing liposomes that coordinate drug release in vivo. Biochimica et Biophysica acta 1768 (2007) 678-687.

Tardi, P., Choice, E., Masin, D., Redelmeier, T., Bally, M., and Madden, T. D. (2000). Liposomal encapsulation of topotecan enhances anticancer efficacy in murine and human xenograft models. Cancer Res. 60, 3389–3393.

Thomas M.C. Diuretics, ACE inhibitors and NSAIDs – the triple whammy. Med J Aust 2000;172:184-185.

Tokudome, Y., Oku, N., Doi, K., Namba, Y., and Okada, S. (1996). Antitumor activity of vincristine encapsulated in glucuronide-modified long-circulating liposomes in mice bearing Meth A sarcoma. Biochim. Biophys. Acta 1279, 70–74.

Torchilin V.P. and Weissing V. “Liposomes- Sonicated Vesicles” Second Edition (2002) 7-8

Torchilin V.P. and Weissing V. “Liposomes-Chemical analysis of degradation of liposomal phospholipids” Second Edition (2002) 45-50

Torchilin V.P. and Weissing V. “Liposomes-HPLC analysis of cholesterol and α-tocopherol” Second Edition (2002) 41-42

Torchilin V.P. and Weissing V. “Liposomes-Physical characterization of liposomes” Second Edition (2002) 50-54

Torchilin V.P., V. Weissig. Liposomes 2nd edition. Oxford University Press, 2003.

Touitou, E. Dayan, N. L. Bergelson, B. Godin, M. Eliaz Ethosomes — novel vesicular carriers for enhanced delivery: characterization and skin penetration properties. Journal of Controlled Release 65 (2000) 403–418

Touitou, E., Alkabes, M., Dayan, N., Elbaz, M. (1997). Ethosomes: novel vesicular carriers for enhanced skin delivery. *Pharm Res* 14:305–306.

Tramer M.R., Carroll D., Campbell F.A., Reynolds D.J., Moore R.A., McQuay H.J. Cannabinoids for control of chemotherapy induced nausea and vomiting: quantitative systematic review. BMJ 2001;323:16-21.

Trotta M., Peira E., Parlotti M.E., Gallarate M.. Deformable liposomes for dermal administration of methotrexate. International Journal of Pharmaceutics. 2004; 270: 119-125.

Trotta, M., Peira, E., Carlotti, M.E., Gallarate, M. (2004). Deformable liposomes for dermal administration of methotrexate. *Int J Pharm*.270:119–125.

Turner C., Whitehand L.C., Nguyen T. and McKenon T., J. Agric. Food Chem. 52 (2004), p. 26.

Uekama K., Hirayama F., Irie T., Chem. Rev. 98 (1998) 2045-2076.

Uekama, K., Hirayama, F., Tetsumi, I.: Cyclodextrin drug carrier systems. Chem. Rev. 98, 2045-2076 (1998).

Van den Bergh B., Wertz P.W., Junginger H.E., Bouwstra J.A.. Elasticity of vesicles assessed by electron spin resonance, electron microscopy and extrusion measurements. International Journal of Pharmaceutics. 2001; 217: 13-24.

Vanbel P.F., Tilquin B.L. and Schoenmakers P., *Chemom. Intell. Lab. Syst.* 35 (1996), p. 67.

Vander Heyden Y., Perrin C. and Massart D.L. In: K. Valkó, Editor, Handbook of Analytical Separations 1, Elsevier, Amsterdam (2000) Ch. 6.

Veiga, M.D., Merino, M., Cirri, M., Maestrelli, F., Mura, P.: Comparative study on triclosan interactions in solution and in the solid state with naturally and chemically modified cyclodextrins. J. Incl. Phenom. Macrocycl. Chem. 53, 77-83 (2005).

Venkatesan N., Vyas S.P."Polysaccharide coated liposomes for oral immunization development and characterization" Inter J Pharm 203 (2000) 169-177

Verma D.D. and Fahr A. "Confocal laser scanning microscopy study using lipophilic fluorescent probe DiI incorporated in liposomes for investigating the efficacy of a new device for substance deposition into deeper layers of the skin : Dermaroller". Philipps-Universität Marburg

Verma D.D. *et al*, "Particle size liposomes influences dermal delivery of substances into skin" Int J Pharm 258 (2003) 141-151

Verma D.D., Verma S., Blume G., Fahr A., Particle size of liposomes influences dermal delivery of substances into skin, Int. J. Pharm. 258 (2003) 141-151.

Verma, D.D., Verma, S., Blume, G., Fahr, A. (2003). Liposomes increase skin penetration of entrapped and non-entrapped hydrophilic substances into human skin: a skin penetration and confocal laser scanning microscopy study. *Eur J Pharm Biopharm* 55:271–277.

Vidon N., Chaussade S., Noel M., Franchisseur C., Huchet B., Bernier J.J., Diab. Res. Clin. Pract. 4 (1988) 223-229.

Villiers A., Sur la transformation de la fécule en dextrine par le ferment butyrique, Compt. Rend. Fr. Acad. Sci. 1891:435-8

Walde P., Ichikawa S. Enzymes inside lipid vesicles: preparation, reactivity and applications Biomolecular Engineering 18 (2001) 143–177

Walters F.H. and Qiu H.C., Anal. Lett. 25 (1992), p. 1131.

Wang, S., Lee, R. J., Cauchon, G., Gorenstein, D. G., and Low, P. S. (1995). Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol. Proc. Natl. Acad. Sci. USA 92, 3318–3322.

Watanabe M., Kawano K., Toma K., Hattori Y., Maitani Y., In vivo antitumor activity of camptothecin incorporated in liposomes formulated with an artificial lipid and human serum albumin, Journal of Controlled Release xx (2008) xxx–xxx

Waugh R.E. and Hochmuth R.M. Mechanical equilibrium of thick, hollow, liquid membrane cylinders. Biophysical Journal. 1987; 52: 391-400

Webb, M. S., Boman, N. L., Wiseman, D. J., Saxon, D., Sutton, K., Wong, K. F., Logan, P., and Hope, M. J. (1998b). Antibacterial efficacy against an in vivo Salmonella typhimurium infection model and pharmacokinetics of a liposomal ciprofloxacin formulation. Antimicrob. Agents Chemother. 42, 45–52.

Webb, M. S., Harasym, T. O., Masin, D., Bally, M. B., and Mayer, L. D. (1995). Sphingomyelin cholesterol liposomes significantly enhance the pharmacokinetic and therapeutic properties of vincristine in murine and human tumour models. Br. J. Cancer

Webb, M. S., Logan, P., Kanter, P. M., St-Onge, G., Gelmon, K., Harasym, T., Mayer, L. D., and Bally, M. B. (1998a). Preclinical pharmacology, toxicology and efficacy of sphingomyelin=cholesterol liposomal vincristine for therapeutic treatment of cancer. Cancer Chemother. Pharmacol. 42, 461–470.

Webb, M.S., Wheeler, J.J., Bally, M.B., Mayer, L.D. (1995). Cationic lipid stearylamine reduces permeability of cationic drugs verapamil and prochlorperazine to lipid bilayer: implications for drug delivery. *Biochim Biophys Acta* 1238:147–155.

Wheeler, J. J., Palmer, L., Ossanlou, M., MacLachlan, I., Graham, R. W., Zhang, Y. P., Hope, M. J., Scherrer, P., and Cullis, P. R. (1999). Stabilized plasmid-lipid particles: Construction and characterization. Gene Ther. 6, 271–281.

Wheeler, J. J., Veiro, J. A., and Cullis, P. R. (1994). Ionophore-mediated loading of Ca2þ into large unilamellar vesicles in response to transmembrane pH gradients. Mol. Membr. Biol. 11, 151–157.

Wickstrom, E. L., Bacon, T. A., Gonzalez, A., Freeman, D. L., Lyman, G. H., and Wickstrom, E. (1988). Human promyelocytic leukemia HL-60 cell proliferation and c-myc protein expression are inhibited by an antisense pentadecadeoxynucleotide targeted against c-myc mRNA. Proc. Natl. Acad. Sci. USA 85, 1028–1032.

Wilkes J.M., Clark L.E., Herrera J.L. Acetaminophen overdose in pregnancy. South Med J 2005;98(11):1118-22.

Woodle, M. C., Newman, M. S., and Cohen, J. A. (1994). Sterically stabilized liposomes: Physical and biological properties. J. Drug Target. 2, 397–403.

Yekkala R., Vandenwayenberg S., Hoogmartens J. and Adams E., *J. Chromatogr. A* 1134 (2006), p. 56.

Yi-Ping F., Yi-Hung T., Pao-Chu W., Yaw-Bin H. Comparison of 5-aminolevulinic acid-encapsulated liposome versus ethosome for skin delivery for photodynamic therapy International Journal of Pharmaceutics, Volume 356, Issues 1-2, 22 May 2008, Pages 144-152

Yu H., Sun P., Hou W. "Prolonged local anesthetic effect of bupivacaine liposomes in rats" International Journal of Pharmaceutics 176 (1998) 133–136

Yu-Min Y., Kuo-Chang W., Zheng-Lin H., and Chien-Hsiang C. On the Stability of Liposomes and Catansomes in Aqueous Alcohol Solutions Langmuir, 2008, 24, (5), pp 1695–1700.

Yusa V., Pastor A.and Guardia de la M., Anal. Chim. Acta 565 (2006), p. 103.

Zhang Y. O.(1993) J.Clin. Biochem. Nutr. 14-83

Zhang Y.P., Zhang Y.J., Gong W.J., Gopalan A.I. and Lee K.P., J. Chromatogr. A 1098 (2005), p. 183.

Zhang, Y. P., Sekirov, L., Saravolac, E. G., Wheeler, J. J., Tardi, P., Clow, K., Leng, E., Sun, R., Cullis, P. R., and Scherrer, P. (1999). Stabilized plasmid–lipid particles for regional gene therapy: Formulation and transfection properties. Gene Ther. 6, 1438–1447.

Zuidam N.J., Talsma H., Crommelin D.J.A. Handbook of nonmedical applications of liposomes. CRC Press; p.71.