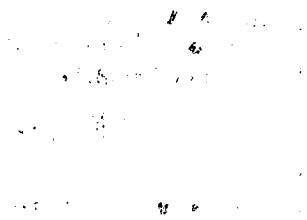
THE EFFECT OF LIPOSOMAL CHARGE ON THE DISTRIBUTION OF LIPOSOMES TO THE LIVER, BRAIN, LUNGS AND KIDNEYS IN A RAT MODEL



THE EFFECT OF LIPOSOMAL CHARGE ON THE DISTRIBUTION OF LIPOSOMES TO THE LIVER, BRAIN, LUNGS AND KIDNEYS IN A RAT MODEL

AJU MARY ABRAHAM

(B. Pharm.)

A dissertation submitted in accordance with the requirements for the degree:

MASTER OF MEDICAL SCIENCE (M.Med.Sc.)
IN PHARMACOLOGY

Faculty of Health Sciences
Department of Pharmacology

University of the Free State

Supervisor: Prof. A. Walubo

NOVEMBER, 2003.

DECLARATION OF INDEPENDENT WORK

I, AJU MARY ABRAHAM, declare that the dissertation hereby submitted by me for the MASTER OF MEDICAL SCIENCE: PHARMACOLOGY degree at the University of the Free State is my own independent work and has not been submitted by me at another university or faculty. I furthermore cede copyright of the dissertation in favour of the University of the Free State.

Aju M Abraham
.....
SIGNATURE OF STUDENT

26-03-2004
.....
DATE

CERTIFICATE OF APPROVAL

I, PROF. A. WALUBO, the supervisor of this dissertation entitled THE EFFECT OF LIPOSOMAL CHARGE ON THE DISTRIBUTION OF LIPOSOMES TO THE LIVER, BRAIN, LUNGS AND KIDNEYS IN A RAT MODEL hereby certify that the work in this research project was done by AJU MARY ABRAHAM at the Department of Pharmacology, University of the Free State.

I hereby approve submission of this dissertation and also affirm that it has not been submitted as a whole or partially to the examiners previously.

.....

SIGNATURE OF SUPERVISOR

26/7/2004

DATE

ACKNOWLEDGEMENTS

With a deep sense of gratitude, I wish to express my sincere thanks to my supervisor, Prof. A. Walubo for his expert guidance and motivation throughout the study and during the writing of this dissertation. I must appreciate his ever-ready helping, conversational and witty attitude as well as the example of hard work which has been of great encouragement to me.

My gratefulness to the University of the Free State for providing the facilities for this study and, particularly, the Faculty of Health Sciences for the financial support.

I specially thank all the staff members of the pharmacology department for help directly or indirectly enabling the completion of my thesis, especially Mrs. Christa Coetsee for her guidance and support during the initial phase of the study. My sincere thanks are due to Dr. Du Plessis and all the staff of the toxicology lab for their co-operation and assistance in many aspects, without which this work would not have been possible. The co-operation I received from other staff members of the Department of Plant sciences (Botany) and Medical microbiology as well as the assistance from Dr. Potgieter and staff of the animal house are gratefully appreciated. Special thanks are due to Mrs. S. Cooper, Anatomical pathology department, for help with the electron microscopy.

I am thankful to my colleague, Miss Shera Barr for her friendliness and timely support during the study.

I also want to thank my parents for the moral support, constant caring and their patience which has been tested to the utmost by the long working hours, as well as my sister and brother for their encouragement and valuable hints for the writing of the dissertation. In addition, I am thankful to friends for their interest and encouragement.

Finally, I thank the Lord Almighty for all the gifts, graces and the many answered prayers.

ABSTRACT

Liposomes are well known drug delivery systems and a lot of research has been done in this field. However, the notion that liposomes would enable selective delivery of drugs to tissues or organs still remains to be fulfilled. Although ligand-targeting of liposomes is receiving great attention today, manipulation of liposomal surface charge is the simplest means of achieving selective delivery of liposome encapsulated drugs. Unfortunately, the understanding of the influence of surface charge on the distribution of liposomes *in vivo* is still unclear. Therefore, this study was undertaken to evaluate the effect of negative, positive and neutral charge on the distribution of liposomes to the brain, lung, kidney and liver in a rat model.

Through a systematic approach, gentamicin was selected out of three drugs as the most appropriate liposomal marker based on its properties. Thereafter, a simple method for preparation of charged liposomes by rotary evaporation and hydration was adopted. Surface charge was induced by varying the lipid composition whereby neutral liposomes were prepared using phosphatidyl choline and cholesterol (9.7:6.9, molar ratio), negative and positive liposomes were prepared by addition of dicetyl phosphate (5:1:0.5, molar ratio) and stearylamine (5:1:0.5, molar ratio) to the neutral liposomes, respectively. The distribution of the encapsulated gentamicin to the specified organs in liposome treated groups was compared to a control group treated with free gentamicin at the following intervals: 1, 2, 4, 6 and 8 hours post injection. Gentamicin (60 mg/kg), the free and liposome entrapped, was administered intraperitoneally and five rats of each group were utilised at each time interval. Under ether anaesthesia, a blood sample was drawn and the relevant organs were harvested. The sodium hydroxide digestion method was used to extract gentamicin from the organs. Gentamicin in plasma and organ extracts was measured by fluorescence polarisation immunoassay.

Liposomal characterisation revealed multilammelar liposomes with a mean internal diameter of 3.17 ± 1.9 μm, and encapsulation efficiency was greater than 15 %. In the animal studies, liposomes delayed elimination of the encapsulated drug. The half life was 2.02 ± 0.5, 1.76 ± 0.1 and 2.04 ± 0.3 hours for the negative, positive and neutral liposome treated groups, respectively, versus 1.53 ± 0.02 hours for the control group. Peak plasma gentamicin

concentrations were higher with positive liposomes than negative and neutral liposomes at 1 hour, while the negative liposomes depicted a sustained release pattern between 4 and 8 hours.

Distribution of liposomes to the brain and liver was dependent on liposomal surface charge. Liposomes improved gentamicin concentrations in the brain with positive liposomes highest in this regard. A biphasic pattern of distribution to the brain, with lowest gentamicin concentration at 4 hours was observed in the three liposome groups. However, this was more marked in the negative liposome group. Generally, hepatic gentamicin concentrations were higher with liposomes than the control. Although, the average hepatic gentamicin concentrations were highest for positive liposomes, the negative liposomes were preferred for the liver because the concentrations were more consistent and increased with time. Uptake of gentamicin by the lungs was not enhanced by liposomes and was independent of surface charge of the liposomes. Renal concentrations of gentamicin were lower (3 to 5 folds) with liposomes, and uptake was not charge dependent.

In conclusion, a simple method for preparation of liposomes was adopted. The distribution studies suggested that positively charged liposomes had highest affinity for the brain and the negative liposomes for the liver. Also, liposomes irrespective of charge exhibited reduced renal concentration of gentamicin.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

GENERAL INTRODUCTION

Selective delivery of drugs to tissues or organs is a desirable therapeutic strategy in instances where a particular organ is exclusively affected by disease. Multi-drug resistant pulmonary tuberculosis, lung cancer and liver abscess, to mention but a few, are some of the clinical conditions where selective drug delivery would be preferred. In general, the use of formulations that permit selective drug delivery would not only enable clinicians to confidently provide better therapy to patients but also make it possible to revive some previously discarded drugs whose pharmacokinetic or toxicity profiles made them unsuitable for use. Even in the case of drugs in clinical use, there is growing concern of unintended exposure of healthy tissues to drugs, which would increase risk of toxicity. Furthermore, it has been postulated that due to the pharmacokinetic processes of absorption, distribution and elimination, effective drug concentrations in the target cell may not be achieved thereby reducing the pharmacological response. Therefore there is a need for selective delivery of drugs to tissues to attain effective drug concentrations in the diseased tissue and, at the same time, prevent unnecessary exposure of the drug to healthy tissues as well as reduce the required dose.

Although many drug delivery systems designed for selective tissue drug delivery have been extensively investigated in different laboratories, liposomes remain one of the most promising approaches to drug tissue targeting. Liposomes are microscopic vesicles consisting of a single or concentric lipid bilayer surrounding an internal aqueous compartment (Ostro, 1987). They are favoured as drug carriers because they are virtually non-toxic and can be loaded with a variety of medications. Liposomes containing different medications have been shown in animal and clinical studies to be effective and less toxic than free drugs.

Of importance here is that some surface properties of liposomes have been shown to play an important role on their disposition in the body particularly the surface charge. Originally, charged lipids were used in liposomes because they increased the aqueous space within the

liposome, hence the amount of entrapped solutes (Sessa and Weissman, 1970); they delayed diffusion of entrapped ions of homologous charge (Kalpan, 1972) and also reduced aggregation and increased stability of the liposomes. The effect of surface charge on liposomal disposition was not realised until the mid-seventies. Subsequent studies showed that the surface charge of liposomes influenced their clearance from circulation *in vivo* and this led to further research into the manipulation of liposomal surface charge for possible use in drug tissue targeting.

Unfortunately, the circulation clearance of liposomes with different surface charge observed by different investigators appeared to be in conflict. Juliano and Stamp (1975) reported more rapid clearance of negatively charged liposomes than positive or neutral liposomes and no appreciable differences between neutral or positively charged liposomes. Another study by Abraham et al. (1984) showed fastest clearance by neutral liposomes in the order of: neutral > negative > positive. On the other hand, there were disagreements on the effect of liposomal surface charge on their distribution to different tissues or organs. For instance, Kim et al. (1994) reported that negatively charged liposomes led to greater localisation of drug in liver, spleen, lung and lymph nodes at 2 hours after intravenous administration compared to neutral and positively charged liposomes, while Nabar and Nadkarni, (1998) showed that large positive liposomes were taken up to a greater extent in the liver compared to similar sized neutral and negative liposomes. The latter also observed that neutral liposomes exhibited greater uptake by the lungs than the charged liposomes and that uptake by the kidney and spleen was independent of charge. Although some of their observations appear to be in agreement with Colley and Ryman's (1975) who reported that positive liposomes exhibited greater uptake than negative ones to the liver and spleen, they differ in that the latter group did not observe any increase in uptake of charged liposomes by the lung, heart, brain or muscle.

Interestingly, all the above cited reports concurred that the increased clearance of liposomes is due to their removal from circulation by the reticuloendothelial system, hence their accumulation in the liver and spleen. In the liver, the mitochondrial-lysosomal fraction was shown to have highest involvement in clearing the liposomes (Gregoriadis and Ryman, 1972).

Briefly, the effect of liposomal surface charge on their disposition has not yet been completely resolved. There is a need for further investigations on the effect of surface charge of liposomes on their distribution into different tissues especially the brain.

Unfortunately undertaking such a study is compounded by the lack of a universally acceptable standard method for preparation of liposomes. In a review of the literature, over thirty methods for preparation of charged and neutral liposomes were found (see chapter 2, section 2.7). This could have contributed to the conflicting results cited above. This means that one would have to adopt or set up a new method for preparation of liposomes, and that a report on liposomal characteristics would not be complete without information on the method of preparation.

Therefore, the objective of this study was to set up a method for preparation of liposomes with different surface charge, viz.; negative, positive and neutral charge, and use them to study their distribution to the liver, brain, lungs and kidneys in a rat model. It was also envisaged that such a method could be useful in our laboratory for further studies on liposomes.

The study is described chronologically starting with a literature review on the preparation, properties and use of liposomes in chapter 2. This is followed by a description of experimental methods on the selection of an appropriate marker for liposomes in chapter 3, and on the adoption of a method for preparation of charged liposomes in chapter 4. Thereafter, in chapter 5, is described a study on the distribution of charged liposomes to the brain, lungs, kidneys and liver in rat. Finally, conclusions of the study are presented in chapter 6.

Chapter 2

LITERATURE REVIEW

2.1 HISTORY AND DISCOVERY OF LIPOSOMES

Although the carrier potential of phospholipid suspensions in medicine was predicted as far back as 1935, liposomes were first produced in 1961 by Dr. Alec D. Bengham of the Agricultural Research Council's Institute of Animal physiology in Cambridge, England. Bengham inadvertently produced liposomes while evaluating the effect of phospholipids on blood clotting. Quoting his own words; " I became fascinated with the way in which smears of egg lecithin reacted with water to form mobile fronds of delicate and quite intricate structure. In 1962, the institute of Animal physiology, with which I was affiliated, acquired an electron microscope. When we examined those dispersions, we discovered a multitude of unmistakable vesicles. The phospholipids were spontaneously assembling to form closed membrane systems. I called these tiny fat bubbles, which consisted of water surrounded by bilayered phospholipids membranes, smectic mesophases." (Bengham, 1992)

However, the word 'liposome' was coined by Weissmann (Sessa and Weissmann, 1968) 'rhyming with lysosome' according to Bengham's expression and is derived from the Greek word: 'lipo' referring to their fatty constitution and 'soma' referring to their structure.

In the mid-1960's, liposomes were used in research as simplified cells for study of biological processes of cell membranes. But by late 1960's, many investigators began to think liposomes might well prove to be efficient drug-delivery systems (Ostro, 1987). Because liposomes are made of the same phospholipids present in cell membranes, it seemed reasonable to assume that the spheres would be non-toxic and would escape recognition and removal by the body's immune system. In the early seventies, the potential of liposomes as drug carriers for safer chemotherapy was demonstrated for the first time (Patel and Russell, 1988). Since then liposomes have been studied extensively.

Unfortunately, early research in liposomal drug preparations was beset with problems due to insufficient understanding of liposome disposition *in vivo*, and inaccurate extrapolation of *in*

vitro liposome-cell interactions or liposome data. However, this was partly overcome by advances in the late 1980's and early 1990's that included detailed understanding of physiological mechanisms of *in vivo* liposome disposition, lipid-drug and lipid-protein interactions (Lian and Rodney, 2001). Consequently, the use of liposomes as drug carriers in pharmaceutical applications became a reality in the mid-1990's and, currently, several liposomal products are already in clinical use while some are still undergoing clinical trials (Table 5 and 6).

2.2 STRUCTURE OF LIPOSOMES

2.2.1 PHYSICAL STRUCTURE

Liposomes are microscopic vesicles with diameters between 20 nm and 20 μm that consist of a single or concentric lipid bilayer surrounding an aqueous core (Taylor and Newton, 1994), Fig 1 & 2. They can also be thought of as balloons because the outer shell is permeable and contains a hollow centre filled with air. To the naked eye, a suspension of liposomes is turbid white and has a milky consistency, but this may vary with the lipids used in their formulation.

2.2.2 CHEMICAL STRUCTURE

Phospholipids

The outer shells of liposomes are made of many individual molecules of phospholipids. Phospholipids are amphiphilic, i.e., they have a hydrophobic (water-insoluble) non-polar tail and a hydrophilic (water-soluble) or 'polar' head.

A phospholipid molecule is structurally divided into subunits (Fig. 3); a glycerol subunit linked to two fatty acid residues through two of its binding sites, and a bridging phosphate group at the third site. The other end of the phosphate bridge links to another organic subunit, most commonly a nitrogen-containing alcohol. Other organic subunits (head group) that may link at this position include an amino acid, serine, and a sugar, inositol. The two fatty acid chains, each containing from 10 to 24 carbon atoms make up the hydrophobic tail of most naturally occurring phospholipid molecules. Phosphoric acid bound to any of several water-soluble molecules composes the hydrophilic head. Phospholipids are named according to the head group and the fatty acid chains, for example, phospholipids with choline head

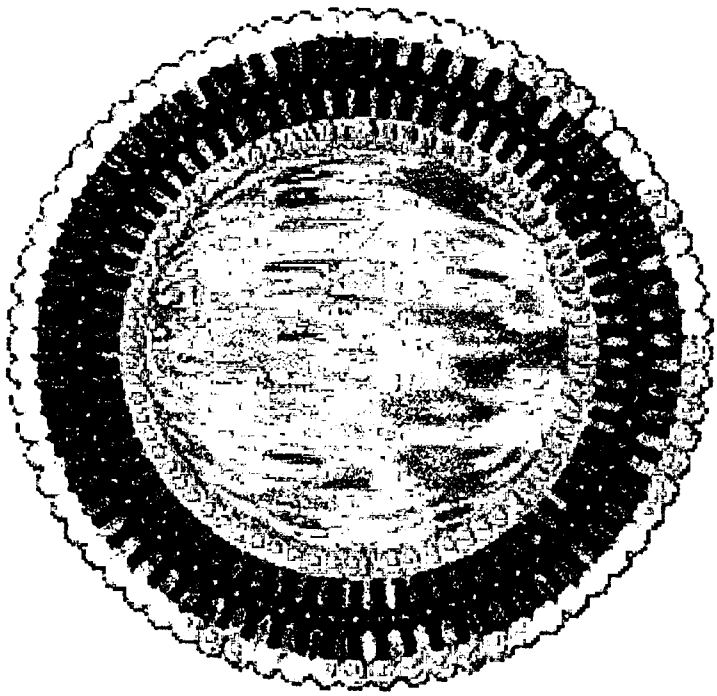


Fig 1 Cross-sectional view of a liposome. (From Collaborative Laboratories; wysiwyg://35/ http://www.collabo.com/liposome.htm)

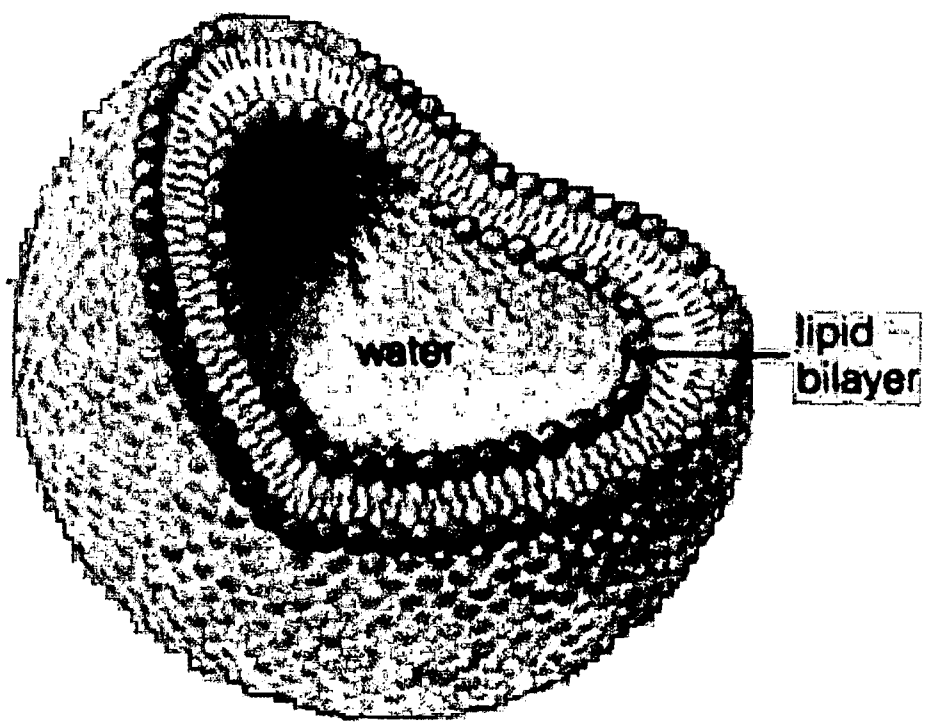


Fig. 2 Three-dimensional view of a liposome. (From; http://ntri.tamuk.edu/cell/lipid.html)

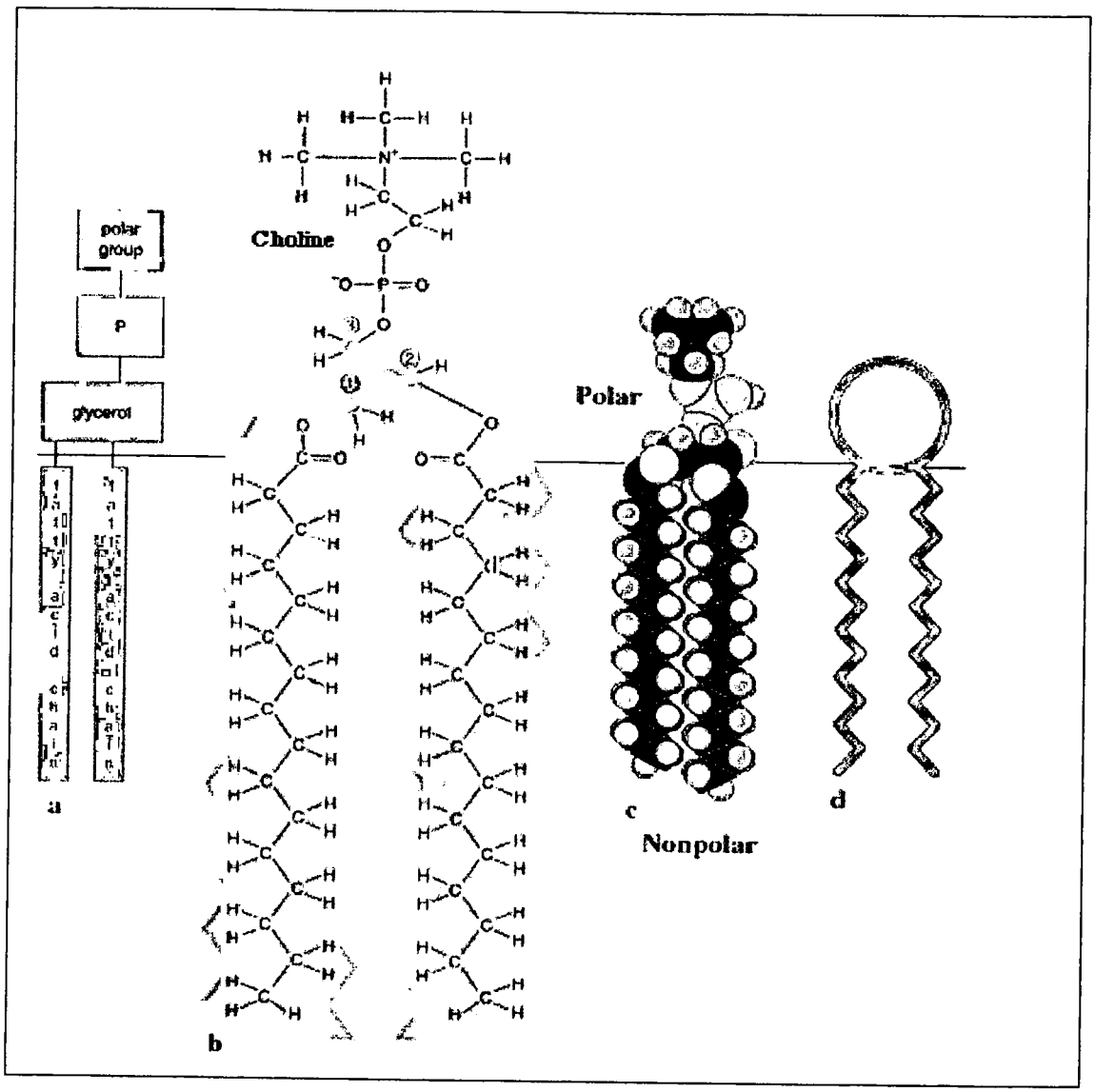


Fig. 3 An illustration of phospholipid molecules. (a) Structure; arrangement of the four subunits in a phospholipid molecule (b) The molecular structure of a phospholipid, phosphatidyl choline (c) Space-filling model of phosphatidyl choline (d) A diagram widely used to depict a phospholipid molecule; the circle represents the polar end of the molecule and the zigzag lines the nonpolar carbon chains of the fatty acid residues. (From; http://ntri.tamuk.edu/cell/lipid.html)

group are called phosphatidylcholine and those with an ethanolamine group are called phosphatidylethanolamine (Table1).

Liposome formation

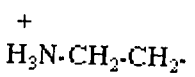
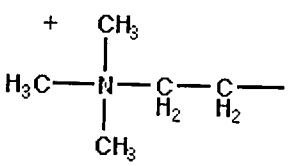
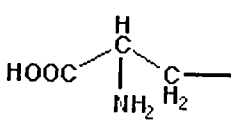
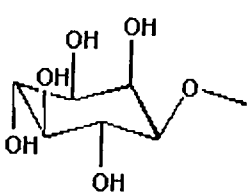
When an adequate amount of phospholipid is mixed with water, the hydrophobic tails spontaneously herd together to exclude water, whereas the hydrophilic heads bind to water. The result is a bilayer in which the fatty acid tail points into the membrane's interior and the polar head groups point outward. The polar head groups at one surface of the membrane point towards the liposome's interior and those at the other surface point towards the external environment. (Fig. 4)

As a liposome forms, any water-soluble molecules or drugs in the solvent are incorporated into the aqueous spaces in the interior of the spheres, while lipid-soluble molecules or drugs are incorporated into the lipid bilayer.

After the liposome is formed, the bilayer plays an important role of separating and protecting the entrapped fluid with its contents (drug molecules) from the outer environment.

Depending on the method or processes involved in preparing liposomes, the number of bilayers produced can vary from just one to as many as 20 lamellae. And this is a major source of variations in liposomal size and form as explained in the next section.

Table 1 Examples of some head groups that could be attached to the phosphate group and the corresponding phospholipids.

X-Structure	*Name*	*Type of phospholipid*
H_2O	Water	Phosphatidic Acid
	Ethanolamine	Phosphatidylethanolamine
	Choline	Phosphatidylcholine
	Serine	Phosphatidylserine
	Inositol	Phosphatidylinositol

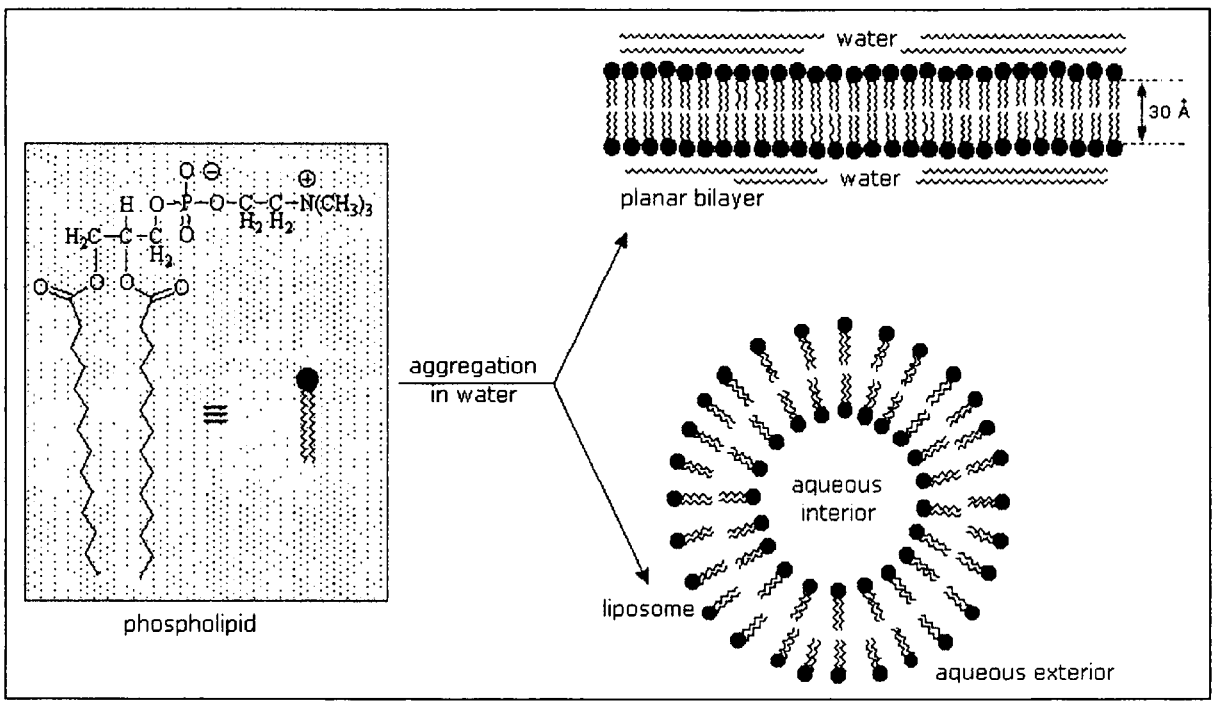


Fig. 4 An illustration of liposome formation. On the left is the phospholipid (phosphatidyl choline) with the head group (coloured blue) and the fatty acid tail (zigzag lines). The phospholipid molecules, in presence of aqueous medium, aggregate with the hydrophilic heads towards the aqueous region to form a bilayer and subsequently a liposome. (From: http://www.cem.msu.edu/~reusch/OrgPage/Virtual Text/lipids.htm)

2.3 SIZE AND FORMS OF LIPOSOMES

Liposomes vary greatly in size due to the number of bilayers and internal volume. As mentioned earlier, they can have a single or multiple bilayers. Based on this, two standard forms of liposomes are commonly described; (Fig. 5; Ostro, 1987; Taylor and Newton, 1994):

- *Multilamellar vesicles (MLV's)* - also known as "onion-skinned" due to the presence of several lipid bilayers separated by fluid. They vary from 0.5 to 20 μm in diameter.
- *Unilamellar vesicles* - consist of a single lipid bilayer surrounding an entirely fluid core. Unilamellar liposomes can be:
  - Small unilamellar vesicles (SUV's) - are 0.02 to 0.1 μm in diameter
  - Large unilamellar vesicles (LUV's) - are 0.1 to 1 μm in diameter

Liposomal size is an important factor in the vesicle's distribution and clearance in the body after systemic administration. Small unilamellar vesicles were cleared less rapidly than large multilamellar ones and are retained in circulation longer (Nabar and Nadkarni, 1998; Juliano and Stamp, 1975). It was also demonstrated that size affected the uptake of liposomes by the spleen (Abra and Hunt, 1981), as well as the targeting efficiency and retention of liposomal antitumor drugs (Nagayasu et al, 1999).

Liposomal size can be determined by different methods, and some of these were reviewed by Woodle and Papahadjopoulos, 1989. The most commonly used methods include light microscopy and coulter counter for large liposomes greater than 1 μm, and gel permeation chromatography (GPC), dynamic light scattering (DLS) or electron microscopy for small liposomes (smaller than 0.2 μm). Microscopy techniques allow not only the determination of size but also shape and structure (lamellarity) of the liposome. However, they could be difficult and tedious when used for quantification of images. Dynamic light scattering [also referred to as photocorrelation spectrometry (PCS) and quasielastic light scattering (QELS)] has been used extensively. Since it is based on the turbidity of the liposomal suspension it requires use of homogenous samples to reliably measure the size of liposomes. Indeed, it has been observed that the reliability and accuracy of the dynamic light scattering technique deteriorates rapidly with increasing heterogeneity. Therefore, it is limited to sizes smaller than 3 μm but its ability to resolve 1 μm particles in the presence of smaller ones is still questionable. Even then it is useful for SUV's and small LUV's, giving an average diameter

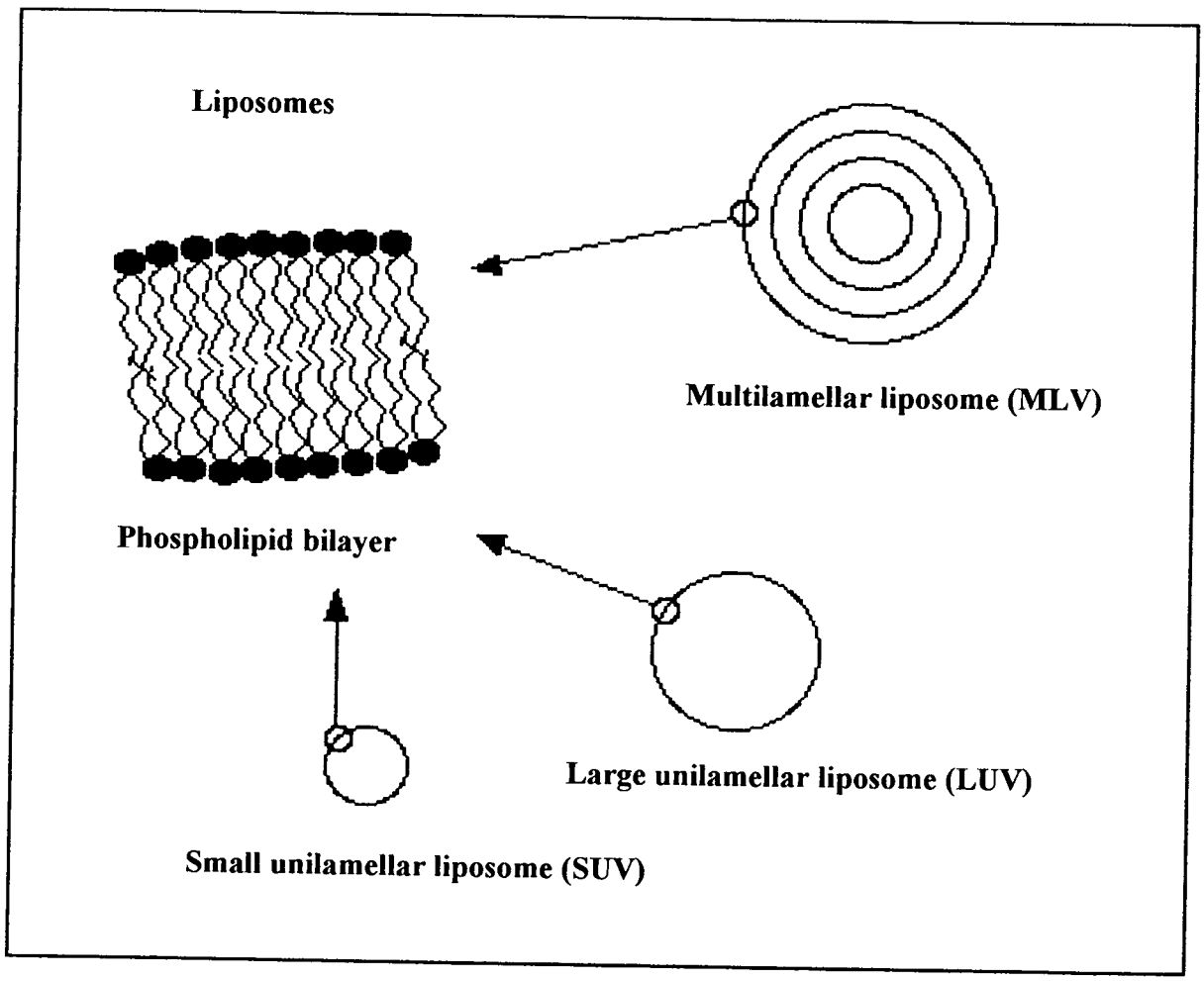


Fig 5 A diagrammatic illustration of the different forms of liposomes according to laminarity and size. A multilamellar liposomes (MLV) with several bilayers, a small unilamellar liposomes (SUV) and a large unilammelar liposome (LUV). (From http://www.its.caltech.edu/-sciwrite/2000-01/rodriguez.htm)

and polydispersity.

2.4 PROPERTIES OF LIPOSOMES

2.4.1 SURFACE PROPERTIES

In general, lipid composition is one of the major determinants of some of the surface properties of liposomes, particularly the surface charge, fluidity and stability.

Surface Charge

As described earlier, the type of phospholipid in the bilayers is a major determinant of liposomal surface charge. It is known that phospholipids exhibit a net charge whereby some are negative, neutral or positive (Table 2). Therefore, by using different combinations of phospholipids one can produce liposomes with a desired surface charge (described later).

Measuring liposomal surface charge

Liposomal surface charge (surface electrostatic potential) may be determined by the methylene, titrimetric and gel permeation methods. The methylene blue method is the most commonly used. It is based on the partitioning of methylene blue (MB), a positively charged dye, between the lipid bilayer. When the dye comes in contact with the lipid membrane its partitioning is dependent on the surface charge. Methylene blue partitions in membranes with a negative than with positive surface electrostatic potential. The extent of partitioning is a function of the strength of the surface potential. Due to this partitioning in the membrane, the absorbance declines as a consequence of the decrease in the concentration of methylene blue in the bulk solution. The membrane surface electrostatic potential (ψ) can be calculated using the equation below (Nakagaki et al., 1981);

$$\psi = 2.30(kT/e)(\log K_o - \log K)$$

where $2.30(kT/e)$ = 59.2 mV at 25 °C and K and K_o are defined by the partitioning of methylene blue into the membrane as a function of the concentration of acidic phospholipid (Ramasammy and Kaloyanides, 1988).

Table 2 Examples of phospholipids with their net charge.

Lipids instilling positive charge	Lipids instilling negative charge	Lipids instilling neutral/no charge
• Stearylamine • Monofatty acid ester of glucosamine • Dimyristol phosphatidylcholine (DMPC)	• Dicetyl phosphate • Cholesterol sulphate • Cardiolipin • Phosphatidic acid • Phosphatidyl serine • Phosphatidyl glycerol • Dioleoyl phosphoglcerol (DOPG) • Dipalmitoyl phosphatidylglycerol (DPPG) • Dimyristol phosphatidyl glycerol (DMPG)	• Phosphatidylcholine • Dipalmitoyl phosphatidylcholine (DPPC) • Cholesterol • Dioleoyl glycerophosphocholine

The surface charge was shown to reduce the tendency of liposomes to aggregate in aqueous suspensions and also to influence their kinetics, i.e., extent of biodistribution as well as interaction with and uptake of liposomes by target cells (Lian and Rodney, 2001). However, instilling charge to the liposome does not affect the physical properties; on electron microscopy the charged liposomes are indistinguishable from the neutral liposomes (Korn, 1970). But it has been demonstrated that the diffusion of ions across the lamellae of these phospholipid vesicles is dependent on the sign and magnitude of the charge prevailing at the membrane surface. It was reported that positively charged liposomes are totally impermeable to cations while negatively charged liposomes are more permeable to anions than positive liposomes (Kaplan, 1972). Also, it is known that charged liposomes interact electrostatically with opposite charged surfaces. The influence of charge on *in vivo* distribution is discussed in detail in section 2.11.

2.4.2 FLUIDITY

Membrane fluidity refers to the existence of thermal phase transitions in phospholipid aggregates (Fielding, 1991). Thermal phase transition refers to the ability of the phospholipid to change from the gel phase to the fluid phase due to change in the temperature. Below the phase transition temperature (Tc) the membranes exhibit a well-ordered or gel phase, but above the Tc, they move to a more disordered fluid-like liquid crystalline state. The phase transition temperature is directly affected by several factors including hydrocarbon length, unsaturation, charge and headgroup species of the lipid (Avanti polar lipids, Inc). As the hydrocarbon length is increased, van der Waals interactions become stronger requiring more energy to disrupt the ordered packing, thus the phase transition temperature increases. Likewise, introducing a double bond into the acyl group puts a kink in the chain, which requires much lower temperatures to induce an ordered packing arrangement.

In the gel state, liposomal membranes are more stable, less permeable to solutes and less likely to interact with destabilising macromolecules than in the lipid crystalline state. Maximum bilayer permeability occurs at the phase transition temperature (Tc) (Fielding, 1991). This is an important factor to be considered during the manufacture and storage of liposomal formulations. However, some investigators have taken advantage of this property, by engineering the liposomal formulation's transition temperature (Tc) to promote release of the encapsulated drug at inflamed or locally heated tissue sites. Unfortunately, it has been

reported that drugs or proteins bound to lipid membranes can affect the phase transition behaviour and this may make this technique less reliable.

2.5 STABILITY OF LIPOSOMES

Liposome stability may be defined as the ability of the liposomal membrane to retain its structural integrity and to remain associated with the incorporated drug. The short shelf life of liposomal delivery systems is one of its limiting drawbacks. This short shelf life of liposomal formulations is mainly due to physical and chemical instability. Physical instability includes drug leakage through the lipid bilayer and leakage associated with liposome aggregation or fusion. This could be alleviated by different ways, for instance, the inclusion of cholesterol in liposome formulations reduces permeability and increases the stability of the phospholipid bilayer by tighter packing of the bilayer. Chemical instability may occur due to hydrolysis of the ester bond or oxidation of unsaturated acyl groups. This however may be controlled by adjusting the pH and lowering temperature, excluding oxygen in the injection vials of formulations, addition of an antioxidant or by selecting saturated acyl-chain in the phospholipid. Also, storing or resuspending the vesicles in the original medium of preparation can prevent loss of the entrapped material, by passive leakage.

The stability of liposomes has been studied by measuring the release of drug or encapsulated material at different time intervals from stored liposomes. Higher stability was obtained when liposomes were stored at 4 °C than at room temperature or – 20 °C when studied for over 2 months (Hsieh et al., 2002; Przeworska et al., 2001). Results of these experiments emphasised that stability is dependent on lipid composition and storage conditions (e.g., temperature). When stored sterile, under nitrogen, liposomes can maintain their initial physical properties for months (Yatvin and Lelkes, 1982).

Scientists have come up with alternate means to deal with the instability of liposomal products when kept as an aqueous dispersion "on the shelf". This is by freeze drying them to a powder (Ostro, 1987; Fielding, 1991), such that they can be reconstituted prior to administration. This requires selection of proper lyoprotectants, ensuring that residual water content is sufficiently low and that temperatures are low enough (Crommelin and Storm, 2003). Also, "empty" (without the drug) liposomes can be prepared and loaded with drug immediately prior to use in order to avoid drug leakage.

In presence of biological fluids, however, liposomes, especially the cholesterol-poor liposomes rapidly lose their integrity, mainly due to the action of high-density serum lipoproteins. The interaction of serum proteins with the lipid membrane is dependent on the membrane fluidity and is maximal in the transition region of the lipids. Multilamellar vesicle stability in serum is somewhat different from that of unilamellar ones, in that, due to the larger number of bilayer shells, the structural integrity of the inner lamellae is better protected. However, absorption of other plasma proteins may lead to changes in the solute permeability rather than a structural disintegration of the liposomes by the high-density liposomes (Yatvin and Lelkes, 1982).

2.6 FORMULATION OF LIPOSOMES

As discussed earlier, formulation of liposomes involves combining different lipids which is also a determinant of the net surface charge; positive, negative or neutral (Table 3 a, b & c). As indicated in the table, a wide range of lipids have been used to produce liposomes of similar or different charge and non-charged liposomes. Precisely, there is no standard phospholipid combination by which liposomes are made.

2.7 PREPARATION OF LIPOSOMES

As liposomes have been around for longer than 30 years and are rapidly becoming accepted as pharmaceutical agents, it wouldn't be surprising that numerous preparation techniques are available. This has made the selection of a suitable method of preparation from these vast varieties an intricate procedure. Below is an overview of the procedures for preparation of liposomes.

2.7.1 SELECTION OF DRUG, LIPIDS AND SOLVENTS

1. **Selection of material (drug) for encapsulation and solvent**

   After the drug to be encapsulated is selected, a suitable solvent is identified based on the solubility of the drug. For instance, hydrophilic drugs are dissolved in aqueous medium (water/buffer). The osmolarity, pH and ionic strength of the aqueous media have to be adjusted to correspond to the medium in which the liposomes will be stored. Lipophilic drugs are dissolved in the organic solvent along with the lipids, as explained below.

Table 3 a Examples of phospholipids (in molar ratio) used in formulating neutral liposomes. *(Molar ratio: the comma separates different ratios of the same lipid combination used by different investigators).*

Phospholipid Combination	Molar ratio
• Phosphatidylcholine : Cholesterol	◦ 8:2, 2:1, 7:3
• Phosphatidylcholine : Cholesterol : α-tocopherol	◦ 8:4:0.1
• Dipalmitoyl phosphatidylcholine : ^{3}H-Triamcinolone Acetonide-21-palmitate (TRMAp)	◦ 0.87:0.13

Table 3 b Examples of phospholipids (in molar ratio) used in formulating positive liposomes. *(Molar ratio: the comma separates different ratios of the same lipid combination used by different investigators).*

Phospholipid Combination	Molar ratio
• Phosphatidylcholine : Cholesterol : Stearylamine	◦ 5:1:0.5, 8:5:1, 7:2:0.5
• Phosphatidylcholine : Cholesterol : α-tocopherol : Stearylamine	◦ 8:4:0.1:1
• Phosphatidylcholine : Stearylamine	◦ 4:1
• Dipalmitoyl phosphatidylcholine : TRMAp : Stearylamine	◦ 0.77: 0.13:0.1
• Dipalmitoyl phosphatidylcholine : Stearylamine	◦ -
• Dipalmitoyl phosphatidylcholine : Cholesterol : Stearylamine	◦ 14:7:4, 9:10:1

Table 3 c Examples of phospholipids (in molar ratio) used in formulating negative liposomes. *(Molar ratio: the comma separates different ratios of the same lipid combination used by different investigators).*

Phospholipid Combination	Molar ratio
• Phosphatidylcholine : Cholesterol : Phosphatidyl serine	◦ 7:2:1, 1:1:1, 8:6:2.
• Phosphatidylcholine : Cholesterol : Cardiolipin	◦ 7:2:1
◦ Phosphatidylcholine : Cholesterol : Dicetyl phosphate	◦ 5:1:0.5, 7:2:0.5
• Phosphatidylcholine : Cholesterol : α-tocopherol : Dicetyl phosphate	◦ 8:4:0.1:1
• Phosphatidylglycerol : Phosphatidylcholine : Cholesterol	◦ 1:4:5
• Phosphatidylglycerol : Phosphatidylcholine	◦ 1:4
• Dipalmitoyl phosphatidylcholine : TRMAp : Dicetyl phosphate	◦ 0.77: 0.13:0.1
• Dipalmitoyl phosphatidylcholine : Phosphatidylinositol	• -
• Egg phosphatidylcholine : Cholesterol : Dicetyl phosphate : distearoyl phosphatidylethanolamine -polyethylene glycol 2000	◦ 2:1.5:0.2:0.2
• Phosphatidylcholine : Dipalmytoyl phosphatidic acid : Cholesterol : α-tocopherol	◦ 4:1:5:0.1
• Phosphatidylcholine : Phosphatidyl serine : Cholesterol : α-tocopherol	◦ 4:1:5:0.1
• Phosphatidylcholine : Phosphatidyl glycerol : Cholesterol : α-tocopherol	• 4:1:5:0.1
• Egg lecithin : Cholesterol : Phosphatidic acid	◦ 7:2:1
• Phosphatidyl serine : Dipalmitoyl phosphatidylcholine: Distearylphosphatidylcholine	◦ 1:4.5:4.5

2. **Selection of lipid mixture and solvent (organic)**

The lipids to constitute the liposomes are dissolved in a suitable organic solvent to ensure a homogenous mixing of lipids. The organic solvent can either be miscible or immiscible with the aqueous phase, depending on the method to be used. For reverse phase evaporation method, immiscible solvents are used while miscible solvents are used for solvent injection method (see later). But, as indicated earlier, for encapsulation of lipophillic drugs, the drug is added directly to the organic solvent containing the lipid mixture.

2.7.2 FORMATION OF LIPOSOMES

1. **Hydration method**

In this method, the organic solvent is removed either by rotary evaporation or sublimation (freeze-drying) or spray drying to form a dry lipid film. Actually, the lipid film is a stack of lipid layers, the consistency of which is determined by both the solvent removal process and the surface of the container. The latter can ultimately affect the type of liposomes formed. For instance, multilamellar liposomes are usually a product of lipid films composed of many layers.

The aqueous solution of the drug is then added to the lipid film. This hydrates the stacks of lipid layers, which then become fluid and swell (Fig 6, steps A and B). The mixture is then sonicated. The hydrated lipid sheets detach during sonication (agitation) and self-close to form multilamellar vesicles (MLV's) (Fig 6, step C). The extent of sonication determines the type of liposomes formed. Sonication for short periods is associated with production of multilamellar vesicles (MLV's), while medium sonication is associated with large unilammellar liposomes (LUV's) and prolonged sonication with small unilamellar liposomes (SUV's) (Fig 6). The resultant aqueous suspension is processed by appropriate methods to separate the liposomes (section 2.7.4).

2. **Reverse phase evaporation technique**

When lipids are dissolved in an organic solvent immiscible with water (aqueous phase), e.g. chloroform, they form an emulsion in the aqueous phase. Removal of the organic solvent under the proper conditions leads to formation of multilamellar vesicles (MLV's) or large unilamellar vesicles (LUV's) and this is referred to as reverse phase evaporation.

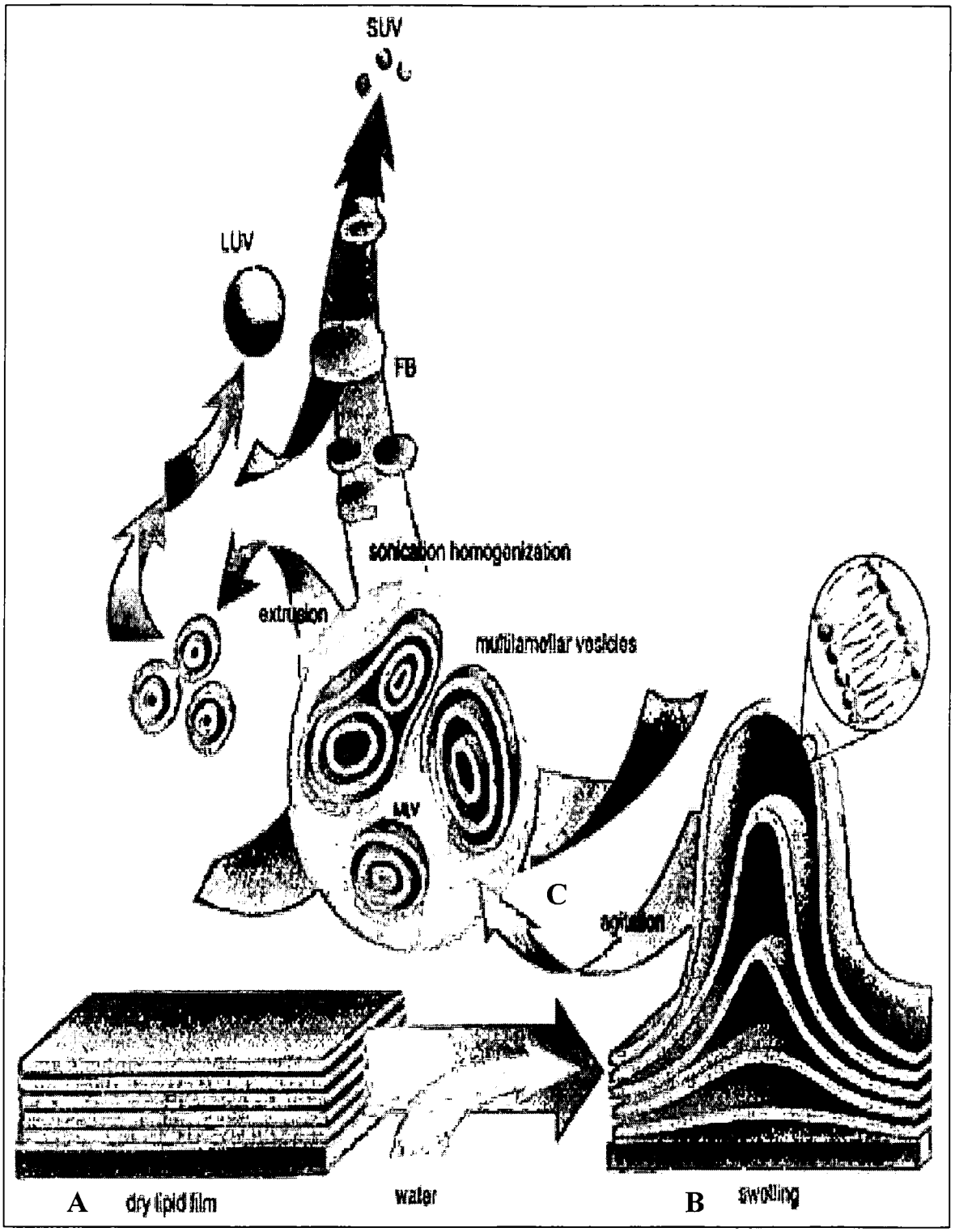


Fig 6 A representation of liposome formation by hydration of a dry lipid film (From http://www.avantilipids.com/PreparationOfLiposomes.html)

In this method, the aqueous solution of the drug to be encapsulated is added directly to the organic solution of lipids. The mixture is then sonicated resulting in a homogenous opalescent suspension. The organic solvent is then removed by rotary evaporation during which a viscous gel forms and gradually changes to an aqueous suspension of liposomes (Szoka and Papahadjopoulos, 1978). The resultant aqueous suspension is processed by appropriate methods to separate the liposomes (section 2.7.4).

In some cases, the lipids are solubilised in the organic solvent in two steps. The first involves dissolving the lipids in a mixture of immiscible (e.g. chloroform) and miscible (e.g. methanol) organic solvents. This, supposedly, is to improve solubilisation of the lipids. The organic solvent is then removed by rotary evaporation to form a lipid film. In the second step, the lipid film is dissolved in an organic solvent, usually diethylether, to which the aqueous phase is added and the procedure is continued as described in the previous paragraph.

Although reverse phase evaporation has been shown to produce stable vesicles with high entrapment efficiency, it is limited to entrapment of thermally stable material. Also, excess organic solvent may remain in the formulation, which could be deleterious to biologically active molecules (Yatvin and Lelkes, 1982).

3. Solvent injection method

This method involves first dissolving the lipids in an organic solvent that is miscible with water (aqueous phase), e.g. ethanol. The solution of lipids in ethanol is 'injected' into the aqueous phase using an infusion pump and syringe apparatus (Deamer, 1978). Liposomes of different sizes can be formed depending on several parameters viz.; lipid concentration, rate of injection and temperature of the aqueous phase. Small unilamellar vesicles can be produced by using low lipid concentrations, fast rate of injection and keeping the aqueous phase above the phase transition temperature of the lipids. Limitations of this method include the need for subsequent processing to remove the solvent, the inevitability of residual solvent, the low solubility of some lipid components in aqueous miscible organic solvents and its unsuitability for encapsulation of heat labile substances.

2.7.3 METHODS FOR SIZING OF LIPOSOMES

1. Mechanical fragmentation

a) Sonication/Ultrasonication - As mentioned under the two liposome preparation methods (hydration and reverse phase evaporation), sonication of the mixture of organic and aqueous phases is important for formation and sizing of liposomes. The sonicator may be of a tip or a bath-type. However, the tip sonicator can cause contamination and therefore the bath-type is more preferable. Sonication at high pulses or prolonged sonication can result in elevated temperature and in the presence of air which may cause chemical degradation and or oxidative degradation of unsaturated lipids. This type of lipid damage during sonication can be reduced by using lower power bath sonicators.

b) French press - Extrusion of MLV dispersions through a french press is a gentle method for producing SUV's of uniform size and stability. By varying the applied pressure during extrusion, the size of the vesicle as well as the number of lipid bilayers in each vesicle can be effectively controlled. But the characteristics of french press liposomes are not universally accepted and is also limited access to the equipment makes this method a not very widely used method.

c) Homogenisation - Microfluidizers and traditional homogenizers are usually used for sizing of liposomes by homogenisation. The size of the resulting liposomes depends on the conditions and frequency of homogenisation.

2. Physiochemically induced fragmentation

By temporarily altering the chemical nature of the aqueous phase, the physical properties of the head group are changed dramatically, thereby altering the organisation of the lipid dispersions. MLV's containing acidic lipids can be converted to LUV's by adjusting the pH leading to hydration changes i.e. by adding sufficient base (alkalisation) to alter the protonation of the lipid head followed by a return to neutral pH.

3. Fusion mediated size increase of SUV's

Due to their instability, SUV's can spontaneously increase in size during storage or by freezing and thawing or by addition of ions. Mostly divalent cations can result in conversion of SUV's to LUV's.

4. **Dehydration-Rehydration induced size increases**

Dehydration of the lipid head groups can be achieved by evaporation, lyophilization or even freezing. Reconstitution (rehydration of the dry lipid in the case of evaporation, and lyophilzation and thawing in case of freezing) results in substantial changes in the particle structure.

2.7.4 SEPARATION OF LIPOSOMES (Removal of unencapsulated material)

Liposomes are separated from the unencapsulated material either by dialysis, ultracentrifugation, gel-permeation chromatography or by ion-exchange resins. Selection of the appropriate method is determined by availability of facilities and expertise. Even then, fine tuning of the selected technique is necessary of production of liposomes of particular size. For example, smaller liposomes require ultracentrifugation at higher speed than larger liposomes.

2.7.5 METHODS FOR ENHANCING ENCAPSULATION

pH-gradient method

The pH-gradient technique is a method where liposomes containing a media of known pH are prepared and then subsequently loaded with the drug by pH-gradient. Therefore, it requires the drug to be a weak acid or base. For example, if the drug to be encapsulated is a weak base, the interior of the liposome should be acidic and the surrounding media in which the drug is dissolved, basic. In this way, the drug in the unionised form ($R\text{-}NH_2$), penetrates the liposome bilayer to the interior where it dissociates. The charged form ($R\text{-}NH_3^+$) of the drug is then trapped in the liposome. The unionised form can penetrate the liposome bilayer but not the charged form (ionic form). This movement of the drug molecules continues until equilibrium is reached or until the pH-gradient dissipates (Fig 7) (Allen, 1998). This method has been reported to give high encapsulation efficiencies (Haran G. et al., 1993).

2.8 ROUTE OF ADMINISTRATION OF LIPOSOMES

Because of their biocompatibility, liposomes have been administered by virtually every route of administration (Feilding, 1991).

- **Oral administration:** Orally administered liposome encapsulated insulin has been proven to be effective in diabetic rats (Ryman et al., 1978). However, concerns still remain regarding the efficacy of this route, as orally administered liposomes are easily

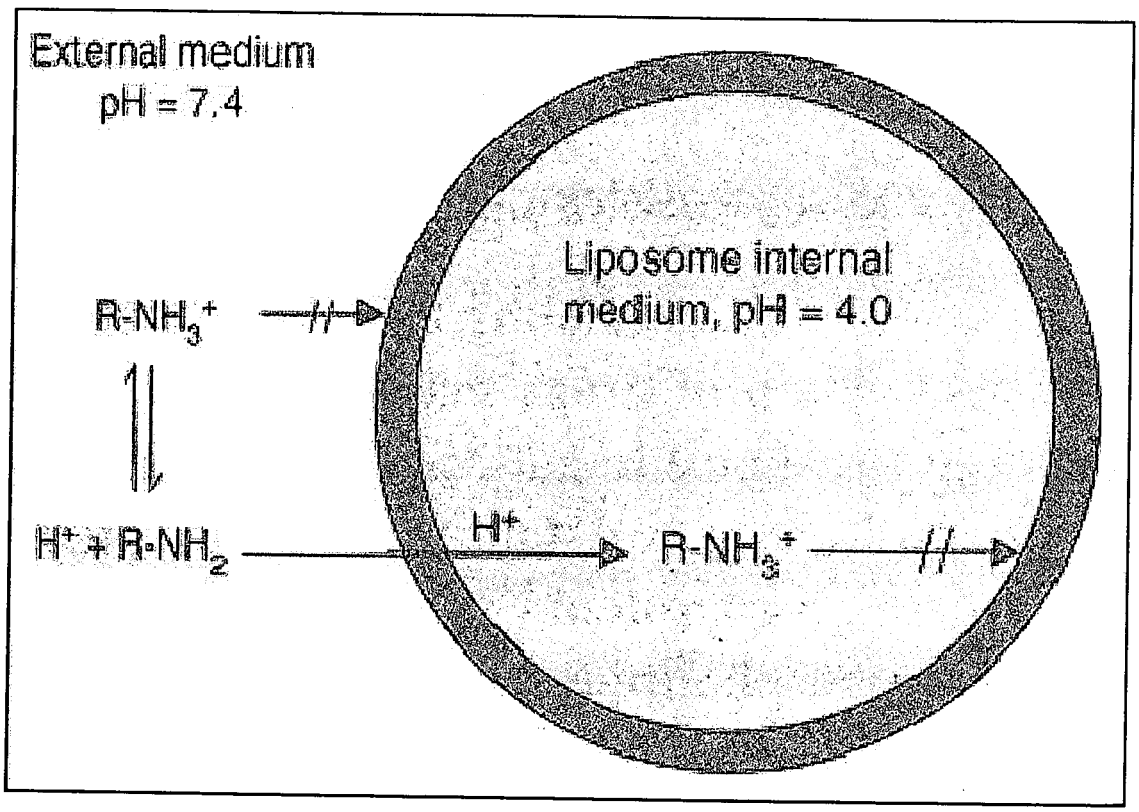


Fig 7 A representation of pH gradient method of loading drugs into liposomes. A weak acid (not depicted) or a weak base in the neutral form ($R\text{-}NH_2$), but not the charged form ($R\text{-}NH_3^+$), will penetrate the liposome bilayer and re-establish an equilibrium in favour of the charged form in the acid environment of the interior. The charged form of the drug is trapped in the liposome interior, and will be released as the pH dissipates (Allen T.M., 1998).

destabilised by several factors mainly pH, bile salts and pancreatic enzymes (Lian and Ho, 2001). Liposomes resistant to these conditions could be suitable candidates for oral use. Several membrane surface polymerisation chemistry methods have been developed to shield liposomes and their contents from gastrointestinal insults. However, incomplete polymerisation and toxicity of residual reagents and derivatives remain a concern.

- **Local and topical administration**: Local administration of liposomes is usually used when the target site can be accessed externally. Topically application of liposomal formulations increases absorption of the drug into the epidermis. Liposomal formulations have been used for administration of analgesics to joints in rheumatoid arthritis (Yatvin and Lelkes, 1982). Currently, liposomal formulations of topical antifungals, other medicinal agents and more prominently liposome-based cosmetics are being developed.

- **Subcutaneous injection (S.C.):** Allen et al. (1993) studied pharmacokinetics and distribution of liposome encapsulated polyethylene glycol administered subcutaneously and compared it to those observed after intravenous and intraperitoneal administration in mice. In general, they concluded that subcutaneously administered liposomes had longer half-lives and tended to move along the lymph node chains that drain the site of injection.

- **Intramuscular administration (I.M.):** Intramuscularly administered liposomes act as a depot for sustained slow release of the drug at the injection site. After intramuscular administration of liposomal formulations, high and prolonged concentrations of liposomal drug (enrofloxacin) in the plasma were observed by Cabanes et al. (1995) while Shinozawa et al. (1979) observed a similar trend with liposomal prednisolone but in the tissues.

- **Pulmonary administration:** Delivery of liposomes to the lung has been attempted by aerosol inhalation of either preformed or intact liposomes. Demaeyer et al. (1993) demonstrated greater retention of liposomal gentamicin compared to the free drug in the lungs when administered by intrabronchial pulmonary route. Pulmonary administration of liposome administration is useful in local treatment of lung tumours and or metastases (Yatvin and Lelkes, 1982).

- **Intraperitoneal administration:** Intraperitoneally (I.P) administered liposomes have a different mode of distribution. They reach the circulation via the lymphatic system, hence they take longer to appear in circulation than intravenously administered liposomes. However, it has been shown that they appear intact in circulation, which implied that intact liposomes are capable of crossing some membrane barriers (Yatvin and Lelkes, 1982). Intraperitoneal administration of liposomes has been shown to improve retention of the drug at the lymph nodes. Hirano and Hunt (1985) observed that absorption of liposomes from the peritoneal cavity was independent of the size of the liposomes but large liposomes were retained better at the lymph nodes than smaller liposomes.

- **Intravenous administration (I.V.):** This is the most extensively researched and most commonly used route for administration of liposomal formulations. Liposomes administered intravenously rapidly interact with the various constituents of blood, especially the plasma proteins. The plasma proteins coat the surface of the liposome, i.e. opsonization of the liposomes. The opsonized liposomes are then either cleared from circulation via the reticuloendothelial system or the process causes destabilisation of the liposomes thereby releasing the liposomal contents into the circulation. In most cases liposomes were administered via the tail vein in rats (Colley and Ryman, 1975; Juliano and Stamp, 1975; Steger and Desnick, 1977).

2.9 MECHANISM OF RELEASE OF LIPOSOMAL CONTENTS

Once in circulation, liposomes are taken up by almost any cell. The release of the entrapped material occurs by interaction of the liposome with the cell, which can occur by endocytotic or non-endocytotic mechanisms and this depends on the physical state, i.e. the fluidity and charge, of the lipids in the vesicle (Poste and Papahadjopoulos, 1975).

Interaction of liposomes with cells to release the encapsulated material may take place by adsorption, endocytosis, lipid exchange and fusion (Fig 8; Taylor and Newton, 1994; Fendler and Romero, 1977; Poste and Papahadjopoulos, 1975).

- **Adsorption:** This involves attaching of the liposome to the cell membrane and subsequent release of its contents into the surrounding. Some of the released contents

may then cross the cell membrane into the cell (Fig 8, label *a*).

- **Endocytosised:** The intact liposome is engulfed by the cell, particularly by phagocytic cells (Fig 8, label *b*), and the endocytosised liposomes are degraded intracellularly by lysosomal enzymes to release the contents.

- **Lipid exchange:** There is exchange between the liposomal membrane lipids and those of the cell membrane (Fig 8, label *c*) leading to in release of the liposomal contents, some of which may penetrate into the cell.

- **Fusion:** This involves adsorption of the liposome to the cell membrane followed by merging of the two membranes, leading to direct release of the liposome's contents into the cell's cytoplasm (Fig 8, label *d*).

2.10 PHARMACOKINETICS AND FATE OF LIPOSOMES *IN VIVO*

Once liposomes reach the circulation, either directly from I.V administration or gradually from depots (after I.M. or S.C. administration), they interact with plasma components. High-density lipoproteins (HDL) remove phospholipid molecules from the bilayer causing destabilisation of the liposome. Also, opsonins adsorb onto the surface of the vesicle to render them prey to the phagocytic cells of the reticuloendothelial system. The latter leads to accumulation of liposomes in organs with high macrophage concentration such as the liver, spleen and bone marrow. Their distribution also depends on the size of the vesicles; larger liposomes preferentially localise in the macrophages of the liver and spleen, while smaller vesicles pass through the fenestrae in the liver to reach the hepatic parenchymal cells by which they are endocytosed (Gregoriadis, 1991). However, the use of charged liposomes and, more recently, the development of new liposomal formulations having surface coatings such as polyethylene glycol (stealth liposomes) as well as the ligand target liposomes would enable distribution of the liposomes to less accessible tissues (Allen, 1998), discussed in following sections (2.11 and 2.12).

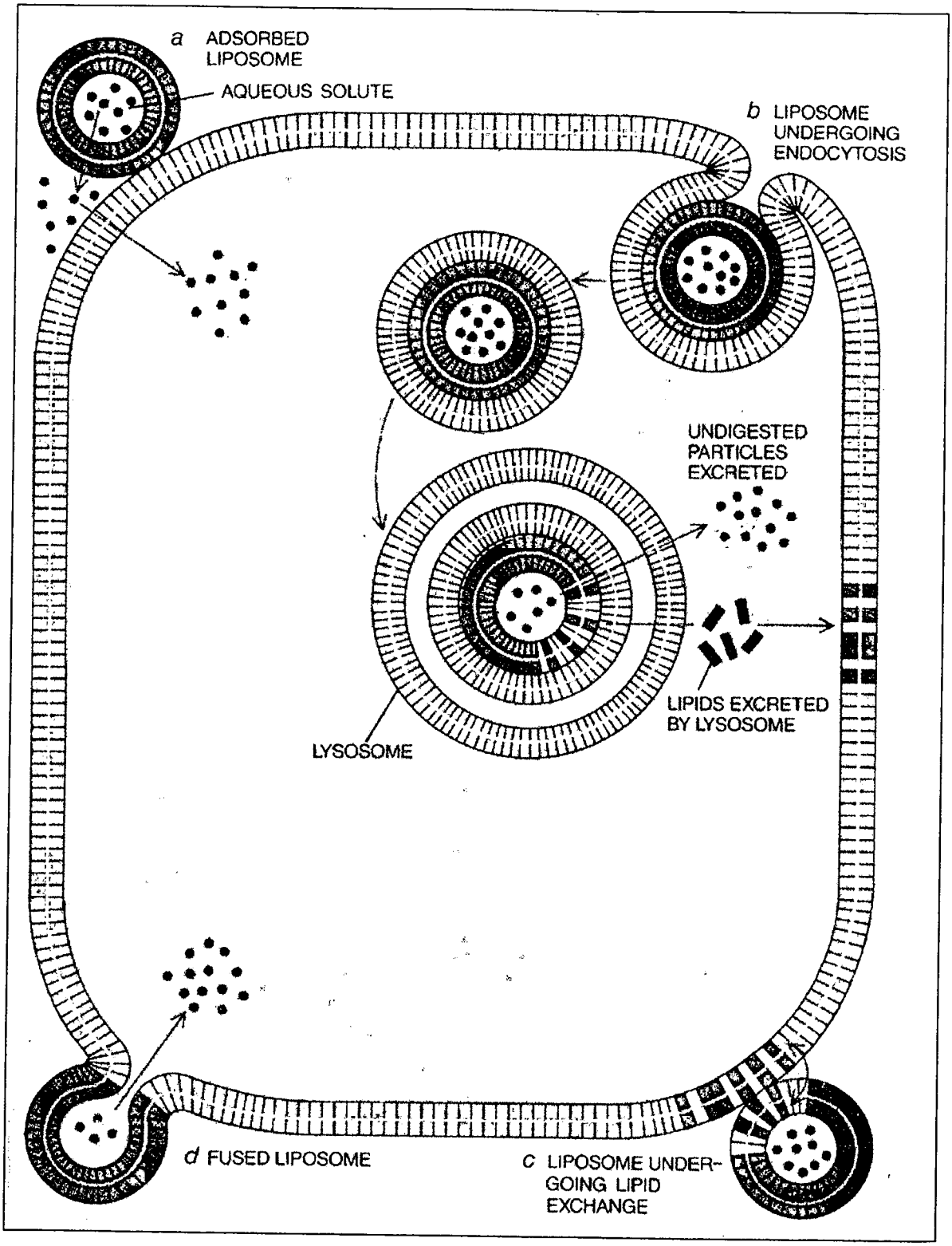


Fig 8 A representation of the types of interactions that can occur between a liposome and cell (Marc J. Ostro, 1987).

2.11 LIPOSOMAL SURFACE CHARGE AND DISPOSITION

Liposomal surface charge has been shown to influence the disposition of liposomes *in vivo* by mechanisms that are not yet known. A review of the literature (Table 4) revealed varying views by different authors on the influence of charge on the disposition of liposomes *in vivo*. Some of these are cited below.

2.11.1 Effect of charge on clearance of liposomes

Reports on the influence of liposomal surface charge on their clearance from circulation by different investigators are not in agreement. For example, Juliano and Stamp (1975), observed that negative liposomes were cleared fastest from circulation, and no difference in the clearance of positive and neutral liposomes. These authors postulated that this might be due to tendency of negative liposomes to coalesce in presence of proteins and calcium ions. Later, Kimelberg (1976) reported similar observations but they did not include neutral liposomes in their study. On the other hand, Abraham et al. (1984) reported the clearance of liposomes in the following order: neutral > negative > positive. Kim et al. (1994) demonstrated no significant difference in clearance of neutral, negative and positive liposomes. In general, the above contradictions have made it difficult to use liposomal surface charge to predict liposomal clearance.

2.11.2 Effect of charge on organ distribution of liposomes

Similarly, reports on the effect of liposomal surface charge on their distribution to different organs by different investigators appear to conflict. The main organs that have been studied include the liver, spleen, kidney, brain and lungs.

Distribution to liver

Steger and Desnick (1977) observed longer retention of positive liposomes than negative liposomes in the liver, while Colley and Ryman (1975) reported higher hepatic uptake of positive liposomes than negative liposomes. Although both of the above workers did not study neutral liposomes, Nabar and Nadkarni (1998) observed higher hepatic uptake of positive liposomes than negative and neutral liposomes. The previous observations appeared to be confirmed in a report by Harnandez-Caselles et al. (1989) who observed greater efficacy of gentamicin encapsulated in positive liposomes in suppressing infection in the liver than with negative liposomes. However, Jonah et al. (1975) reported contradicting

Table 4 A summary of some studies on the influence of charge on clearance and distribution of liposomes in different animal models. *Abbreviations are listed below the table.*

Parameter studied	Encapsulated drug	Lipid composition and route of liposome administration	Observation	Reference
Clearance (5 hours) in **rats**	^{3}H-colchicine	PC: Chol (neutral) PC: Chol: PS (negative) PC: Chol: SA (positive) (Intravenous injection)	• Negative liposomes cleared fastest from circulation • No difference in clearance rate between positive and neutral	Juliano & Stamp, 1975
Clearance and **distribution** (at end of 4hrs) in **monkeys**	[^{3}H] Methotrexate (MTX)	PC: Chol: PS (negative) PC: Chol: SA (positive) (Intravenous injection)	• Clearance: sonicated negative liposomes cleared faster than from positive sonicated liposomes • Spleen: greater uptake of positive non-sonicated liposomes than negative non-sonicated liposomes • Distribution to spleen, duodenum, bone marrow and brain: better for sonicated positive liposomes than negative sonicated liposomes	Kimelberg, 1976
Clearance (7 hrs) in **rabbits**	^{3}H-Triamcindone Acetonide-21-palmitate	DPPC: DCP (negative) DPPC: SA (positive) (Intravenous injection)	• At 1 hr clearance was neutral > negative > positive	Abraham et al., 1984
Tissue distribution (2 hrs) in **rats**	[^{3}H] Methotrexate (MTX)	PC: Chol: α-TP (neutral), PC: Chol: α-TP: DCP (negative), PC: Chol: α-TP: SA (positive) (Intravenous injection)	• Release of [^{3}H] MTX: negative liposomes greater compared than positive and neutral • Liver, lungs & lymph nodes: ↑ed localisation of negative liposomes than neutral/positive • Spleen: uptake highest from neutral & negative liposomes • Kidneys: ↑ed uptake of negative liposomes at 2 hrs	Kim et al., 1994
Distribution (11 days) in **mice**	β-glucuronidase (enzyme)	PC: Chol: PA(negative) PC: Chol: SA (positive) (Intravenous injection)	• Kidneys: negative liposomes showed higher dose (15 -20%) accumulation than positive • Liver: positive , activity detectable for up to 11 days and negative for 8 days	Steger & Desnick, 1977

Tissue distribution (1 hr) in **rats**	[^{3}H] Methotrexate	PC: Chol: DCP (negative) PC: Chol: SA (positive) Neutral - (? Lipids) (Intravenous injection)	• Liver & spleen: greater uptake of positive than negative • Spleen: highest uptake of neutral	Colley and Ryman, 1975
Distribution (1 hr) in **rats**	^{99m}Tc-DTPA	PC: Chol (neutral) PC: Chol: DCP (negative) PC: Chol: SA (positive) (Intravenous injection)	• Liver: greater uptake of positive MLV's than neutral & negative • Lungs: greater uptake of neutral MLV's at 1 hr cf. charged liposomes • Kidneys & spleen: uptake not affected by charge	Nabar & Nadkarni, 1998
Distribution: treatment of B.melitensis (13 days) in **mice**	Gentamicin	PC: Chol (neutral) PC: Chol: DCP (negative) PC: Chol: SA (positive) (Intraperiteoneal inj.)	• Positive liposomes completely suppressed B. melitensis in liver & spleen • Negative liposomes - also effective in liver but less in spleen	Hernandez-Caselles et al., 1989
Tissue distribution (5 min to 24 hr) in **mice**	EDTA	PC: Chol: PS (negative) PC: Chol: SA (positive) (Neutral) - PC: Chol (Intravenous injection)	• Spleen & marrow: highest uptake of negative liposomes • Lungs & brain: high levels of positive liposomes • Liver: highest uptake of neutral. Uptake of negative and positive liposomes were same • Kidneys & brain: showed similar decay curves	Jonah et al, 1975
Distribution (2.5 hr for tissues & 250 min for plasma) in **rats**	[^{3}H]-Amyloglucosidase & ^{131}I- albumin	PC: Chol: DCP(negative) (Intravenous injection)	• Clearance: bulk of liposomal radioactivity was removed within minutes & at later times, slower rate of disappearance was seen • Liver& spleen: most of the radioactivity was recovered here • Kidneys & lungs: minimal amount of radioacitvity	Gregoriadis & Ryman, 1972

Abbreviations: PC - phosphatidylchoine, Chol - cholesterol, DCP - dicetylphosphate, SA - stearylamine, DPPC - dipalmitoylphosphatylcholine, α-TP - α-tocopherol, PA - phosphatidic acid, PS - phosphatidyl serine, MLV's - multilamellar liposomes.

results in which they observed greater accumulation of neutral liposomes than charged liposomes in the liver. On the other hand, Kim et al. (1994) differed again; they observed greater hepatic uptake of negative liposomes than positive or neutral ones, while results of Kimelberg (1976) showed no general difference in uptake of charged liposomes by the liver.

Distribution to spleen

Colley and Ryman (1975) reported greater uptake by the spleen of positive liposomes than negative or neutral liposomes, while Kimelberg (1976) observed greater uptake by the spleen of positive liposomes than negative liposomes. Again, Harnandez-Caselles et al. (1989) supported their findings when they observed greater suppression of infection in the spleen when gentamicin was encapsulated in positive liposomes than in negative liposomes. On the contrary, Jonah et al. (1975) observed greater uptake of negative liposomes than positive or neutral to the spleen, while Kim et al. (1994) noted highest uptake by neutral and negative than positive liposomes, and Nabar and Nadkarni (1998) reported that charge had no effect on uptake by the spleen.

Distribution to kidney

Kim et al. (1994) described increased renal uptake of negative liposomes than positive or neutral liposomes. Steger and Desnick (1977) likewise observed higher renal accumulation of negative liposomes than positive liposomes. However, Jonah et al. (1975) observed high initial uptake of both charged liposomes with rapid subsequent decline, and Kimelberg (1976) reported general decreased renal excretion with liposomes. Nabar and Nadkarni (1998) reported that uptake by the kidney was not affected by charge.

Distribution to brain and lungs

Jonah et al. (1975) showed positive liposomes had higher uptake to the lung and brain than the other liposomes. Kim et al. (1994) reported that negative liposomes showed increased localisation to the lung than neutral or positive, the brain was not studied. Nabar and Nadkarni (1998) results were opposing in that they observed greater uptake to the lungs of neutral liposomes than the charged liposomes. They also did not analyse the brain. Colley and Ryman (1975) showed no marked increase in uptake of charged liposomes by the lung or brain. In general there are few studies on the distribution of liposomes to the brain.

Comment

From the above observations, the effect of liposomal surface charge on their clearance and distribution to the liver, lungs, brain and kidneys *in vivo* is not yet solved. These conflicting observations could arise, to mention but a few, from variations in liposomal lipid composition, size, study period, route of administration, liposomal preparation method, equipment and probe drug. This multitude of variables has made it difficult to undertake liposomal preparation and studies. Also, the high cost, material wastage and wide choice of methods from which to select compromise reproducibility. In conclusion, more studies are needed on liposomal charge and distribution *in vivo*.

2.12 RECENT DEVELOPMENTS IN LIPOSOME TECHNOLOGY

Optimisation of liposomal delivery systems over the years has lead to the development of stealth liposomes, ligand targeted liposomes, immunoliposomes and cationic liposomes, as explained below.

- **'Stealth'/long circulating/sterically stabilised liposomes:** Phagocytic uptake of liposomes could be reduced by rendering them invisible to the reticuloendothelial system in the circulating blood by making its hydrodynamic surface look more like the surface of a 'friendly' circulating cell (Bengham, 1992). Such liposomes have now been developed by coating them with hydrophilic polymers such as polyethylene glycol (PEG). This provides an additional surface hydration layer by attracting water to the liposome surface, presenting a barrier to the adherence of protein opsonins (fig 9 and 10). This camouflage enables the liposomes to remain in circulation for longer and alters the biodistribution of their associated drugs away from the phagocytic system. Hence the name 'Stealth liposomes'. They are also referred to as 'Sterically stabilised liposomes' due to the stearic stabilisation resulting from the local surface concentration of highly hydrated PEG groups that create a stearic barrier against interactions with molecular and cellular components in the biological environment. Although sterically stabilised liposomes are still cleared to a considerable extent by the phagocytic system, this clearance is at a significantly reduced rate and extent than seen for 'classical' liposomes (Allen, 1998).

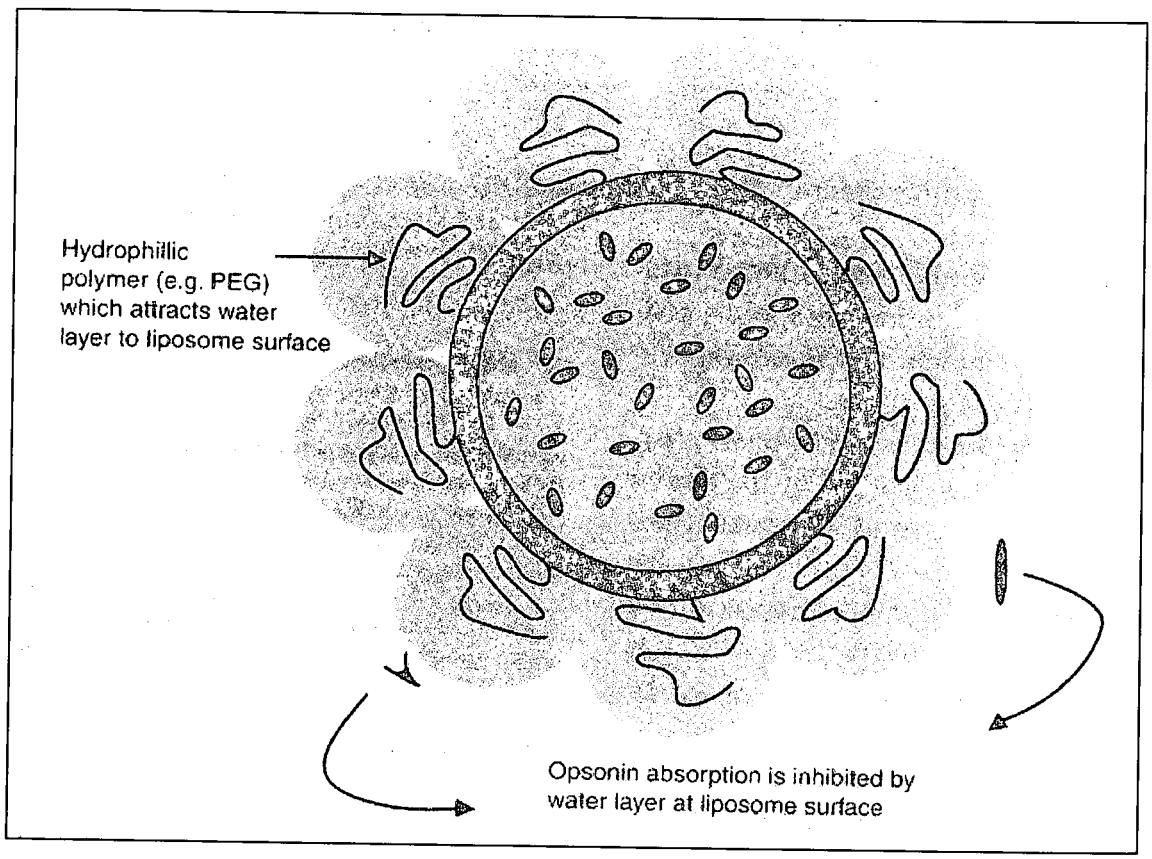


Fig 9 A representation of a stealth liposome. The surface coating of a hydrophillic polymer attracts water to the liposome surface, presenting a barrier to the adherence of protein opsonins, decreasing rate and extent of uptake by the phagocytic system. The hydrophillic barrier also retards disintegration of the liposomes through exchange and/or transfer of liposomal phospholipids to high density lipoproteins (Allen T.M., 1998).

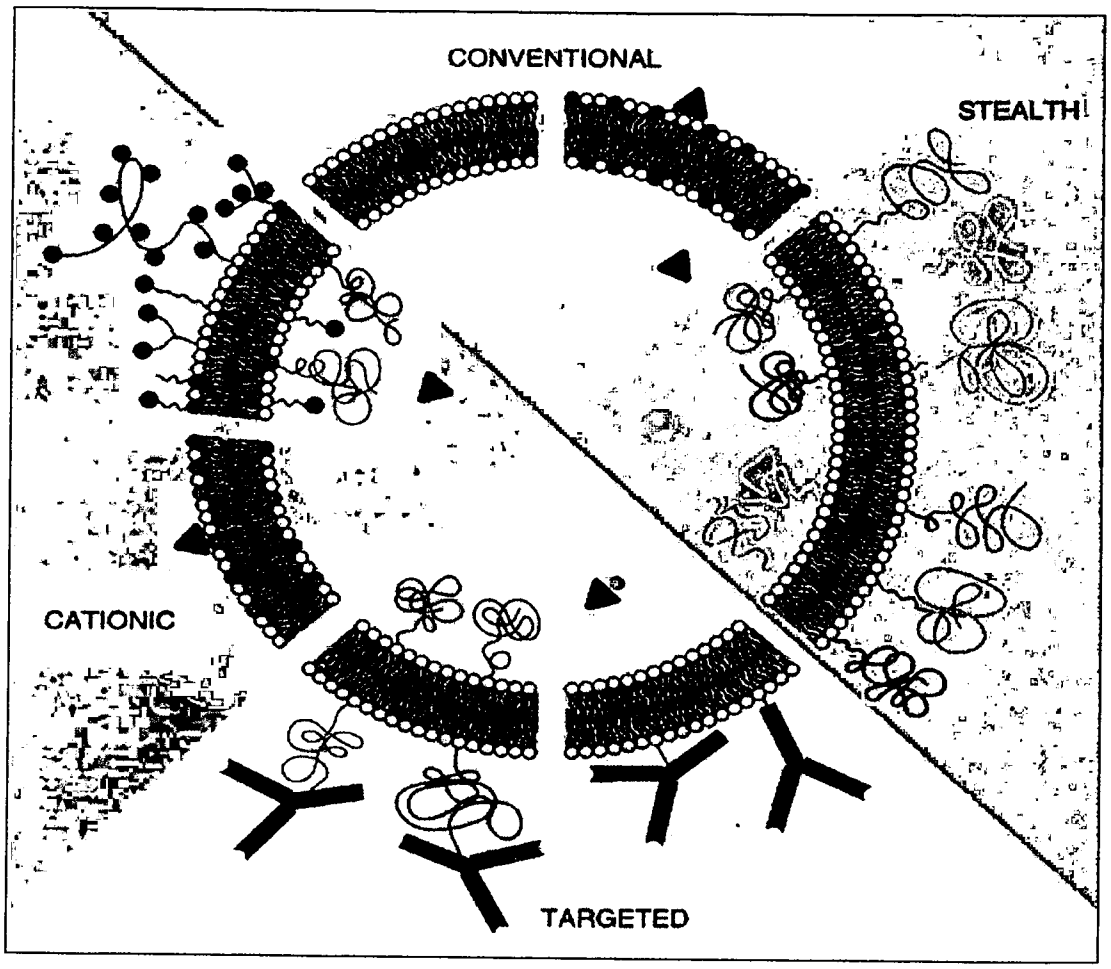


Fig 10 A representation of four major liposome types. Conventional liposomes are either neutral or negatively charged, stealth liposomes carry polymer coatings to obtain prolonged circulation times, immunoliposomes ('antibody targeted') may be either conventional or stealth. For cationic liposomes, several ways to impose positive charge are shown (mono-, di- or multivalent interactions) (Storm and Crommelin, 1998)

- **Ligand targeted liposomes and Immunoliposomes**: Enhanced target site can be attained by attaching specific ligands to the liposome surface (Fig 10). Chemically coupled ligands expressed on liposomal membranes are used for active targeting of liposomes. A variety of ligands or receptors such as antibodies or antibody fragments (immunoliposomes), growth factors, cytokinins, hormones and toxins have been anchored and expressed on the liposome surface to introduce drugs, proteins and nucleic acids into target cells (Lian and Ho, 2001). Active-targeting of liposomes using ligand-receptor interactions requires that;
  - The liposomes expressing specific targeting molecules must circulate in blood long enough to localise and penetrate into the target organ or tissue and eventually interact with and bind to the cells.
  - The ligand or receptor must provide sufficient specificity.
  - In addition the targeting molecule(s) expressed on the liposome surface must be sufficiently stable *in vivo* and exhibit minimum potential of being removed by serum proteins.

  Removal of immunoliposomes from the blood compartment, occurs through the liver and spleen as for any particle in the blood stream, therefore to guarantee accessibility of the target receptors local administration in body cavities and coating with PEG are being used (Storm and Crommelin, 1998).

  Although these approaches in anchoring the antibody, its derivatives and ligands on the liposome surface may provide target selectivity *in vitro* and *in vivo*, the cost and reproducibility of these derivatives in quality and quantity sufficient for pharmaceutical application are challenging problems, especially the practicality of pharmaceutical preparation of ligand targeted liposomes on a large scale.

- **Cationic liposomes:** (Fig 10) These are used in improving delivery of genetic material and are a newly developing area. The cationic lipid components of the liposome interact with and neutralise the negatively charged DNA, thereby condensing the DNA into a more compact structure. The resulting lipid-DNA complexes, rather than DNA encapsulated within liposomes, provide protection and promote cellular internalisation and expression of the condensed plasmid (Storm and Crommelin, 1998).

2.13 SOME APPLICATIONS OF LIPOSOMES

A number of applications for liposomes have been expressed in different reviews, the following are some of the important applications:

- **Vaccine immunoadjuvants** (Gregoriadis, 1990; Lian and Ho, 2001; Taylor and Newton, 1994; Lasic, 1998): Because macrophages are thought to be predominantly responsible for processing and presenting liposome-associated and encapsulated antigens, the liposome formulation provides an excellent way to enhance antigen delivery and presentation for both humoral and cellular immune stimulation of vaccines that is pertinent to human and veterinary immunisation.

  A large number of liposome-antigens have been studied for their immune adjuvant action. One of the recent is a liposome-based Hepatitis A vaccine (Epaxal®) which has been tested in humans. It consists of formalin-inactivated hepatitis A virus attached to phospholipid vesicles together with influenza virus hemaglutinin. Therefore the vaccine not only induces antibody to hepatitis A antigen but also influenza virus protein expressed on the liposome surface. The mechanism of liposome-mediated immune enhancement, however, is quite complex.

- **Gene therapy:** Gene therapy, the treatment of disease on the molecular level by switching genes on or off, is presently attainable by use of cationic liposomes, as discussed earlier. Successful expression of genes have been obtained *in vitro* and *in vivo*, however, absolute expression levels are still rather low, tissue specificity of expression upon *in vivo* administration rather uncontrollable and duration of expression short.

- **Anticancer therapy:** Liposomes have been researched widely in cancer treatment. It has been demonstrated that small, stable liposomes can passively target several different tumours, since their biological stability allows them to circulate for prolonged times and their small size to extravasate in tissues with enhanced vascular permeability, which is often the case in tumours (Gregoriadis et al., 1977; Nagayasu et al., 1999).

  Two anticancer liposomal formulations have been approved by the US Food and drug administration (FDA) and are commercially available in USA, Europe and Japan (Lian and Ho, 2001; Lasic, 1998). Doxil (Alza Corporation) is a formulation of doxorubicin precipitated in sterically stabilised liposomes (consisting of ^{2000}PEG-

distearoylphosphatidyl ethanolamine - hydrogenated-soya-bean phosphatidylcholine - cholesterol), loaded into preformed liposomes by ammonium-sulfate-gradient technique and is currently being used for AIDS-related Kaposi sarcoma and refractory ovarian cancer. While DaunoXome (Gilead Sciences) is daunorubicin encapsulated in small unilammelar liposomes consisting of distearoylphosphatidylcholine (DSPC)-cholesterol, loaded by a pH gradient, are mechanically strong (having strong and cohesive bilayers) and also used in Kaposi sarcoma related to AIDS.

- **Diagnostic imaging**: High concentrations of water-soluble iodinated contrast agents entrapped in liposomes directed to the liver have been designed, permitting more precise identification of small metastasised tumours by computed tomography (Bengham, 1992).

- **Liposome-based therapy for degenerated joint surfaces:** Liposomes formed by sonicating dipalmitoylphosphatidylcholine (the major lipid component of synovial fluid) and hyaluronic acid, significantly reduce friction lower than normally found in mammalian joints.

- **Topical creams:** Topical preparations are one of the fastest growing applications of liposomes. It offers selective distribution of the drug, prolonged retention of the drug on the skin and reduces entry of the drug to the blood stream causing systemic side effects.

2.14 COMMERCIAL LIPOSOMAL PRODUCTS

Liposomal products that have been approved for clinical use in the United States include liposomal amphotericin mainly for fungal infections, doxorubicin and duanorubicin for AIDS associated cancer treatment (Table 5). Also, liposomal products in clinical trials include annamycin, doxorubicin and tretinoin for cancer treatment, nystatin as antifungal and prostaglandin for acute respiratory distress syndrome (Table 6).

2.15 HURDLES IN LIPOSOME DELIVERY SYSTEM DEVELOPMENT

Liposomal delivery systems, like other delivery system are associated problems, some of which are yet to be solved. They include:

- **Raw materials:** use of raw materials of unpurified lipids and poor storage of lipids particularly if unsaturated bonds are present, leads to variations in the liposomal

Table 5 A list of liposome products for clinical use in the United States (Lian and Ho, 2001).

Product	Drug (lipid:drug ratio)	Lipid Formulation	Marketed By	Indication
Doxil™ Caelyx™	Doxorubicin (8:1)	PEG-DSPE: HSPC: Chol. (5:56:39)	Alza Corporation	Kaposi sarcoma in AIDS, Refractory ovarian cancer
DaunoXome™	Daunorubicin citrate (15:1)	DSPC: Chol. (2:1)	Gilead Sciences	Kaposi sarcoma in AIDS
Ambisome™	Amphotericin B (3:8:0.4)	HSPC: DSPG: Chol. (2:0.8:1)	Gilead Sciences	Serious fungal infections, Cryptococcal meningitis in patients HIV+
Amphotec™	Amphotericin B (1:1)	Cholesteryl sulfate	Alza Corporation	Serious fungal infections

Table 6 A list of liposome products in clinical trials in the United States (Lian and Ho, 2001).

Product	Drug	Formulation	Developed By	Status	Indication Sought
Annamycin	Annamycin	Liposomes	Aronex Pharmaceuticals	Phase I/II	Breast cancer
Antragen™	Tretinoin	Liposomes	Aronex Pharmaceuticals	Phase II/III Phase II Phase I	Kaposi's sarcoma in AIDS Recurrent acute promyelocytic leukaemia Cancer of blood
Nyotran™	Nystatin	Liposomes	Aronex Pharmaceuticals	Phase II/III Phase I	Candidemia Comparative study against Amphotericin B in suspected fungal infection
TLC-D99 Evacet™ Mycocet™	Doxorubicin	Liposomes	Elan Corporation	NDA filed	Metastatic breast cancer
Ventus™	Prostaglandin E1	Liposomes	Elan Corporation	Phase III	Acute respiratory distress syndrome

formulation. Improving purification schemes, the introduction of validated analytical techniques and better insight into lipid degradation mechanisms, should ensure quality.

- **Physiochemical properties:** The behaviour of liposomes both *in vitro* and *in vivo* strongly depends on their size, bilayer rigidity, charge and morphology. Hence complete physiochemical characterisation of pharmaceutical liposomes is required in early stages of research. Quality control assays should be used to obtain regulatory approval for liposomal products.

- **Poor encapsulation/Low 'pay load':** After liposomes are prepared, the unencapsulated material is separated from the liposomes. Low encapsulation efficiency, leads to large loss of the drug or material to be entrapped. Such loss should be avoided by using active or remote loading strategies to increase encapsulation efficiencies. Loading of drugs by pH gradient technique could be a possible option.

- **Shelf life:** The poor shelf life of liposomal formulations has always been of concern, but is now resolvable (discussed in section 2.6).

- **Scale up:** A wide variety of laboratory scale preparations of liposomes are known (section 2.8), but scaling up of these methods to an industrial scale is a challenge and comes with its own problems. However, a number of preparation approaches have been brought up to industrial scale. And has to be validated for different formulations.

2.16 RATIONALE

From the review, it can be contended that, despite the advancement in liposomal research there is still much more needed to be done, specifically;

- There are many methods for preparation of liposomes, whether neutral, positive or negative liposomes and there is no universally acceptable method for liposome preparation.
- Liposomal preparation methods are expensive and may be difficult to reproduce due to a lot of variables.
- There is a need for drug tissue targeting and liposomes are the most promising in this regard.
- Liposomal surface charge can influence the distribution of liposomes to different organs.
- There is no agreement on the effect of liposomal surface charge on their distribution to the different organs.

Therefore the objectives of this study are:

1. To develop or adopt a method for preparation of negative, positive and neutral liposomes in our laboratory. This will involve:
   - Selection and analysis of a marker compound
   - Liposomes preparation and characterisation

2. To study and compare the distribution of the prepared liposomes to the brain, kidney, liver and lungs in a rat model.

Expected Outcome

It is hoped that by the end of this study the specific liposomal surface charge required for drug targeting to the liver, brain, lungs and kidneys, will be determined. Furthermore, it is hoped that this knowledge will be useful in further development of liposomal drug formulations for selective delivery of drugs to each of these organs.

Chapter 3

SELECTION OF A MARKER DRUG FOR LIPOSOMES

3.1 INTRODUCTION

The marker or tracer drug serves as an indicator for the disposition of liposomes *in vivo*. For instance, the concentration of the marker in an organ would suggest the presence of liposome in that site. Specifically, a high concentration of the marker drug at the site would indicate greater affinity, while a low concentration would represent less affinity of the liposomes.

Various drugs and compounds encapsulated in liposomes have been used as markers for liposomal disposition *in vivo*. However, radiolabelled compounds or lipids have been the most commonly used for this purpose owing to their high sensitivity. Unfortunately, they have inherent disadvantage such as, radioactivity measured may not be the intact compound (could be a metabolite), are expensive, requires special skills and facilities for handling, and radioactivity is a potential risk to workers. Therefore, there is a need to select a marker drug that can be easily used in a simple laboratory setting for research on liposomes. An ideal marker should meet the following criteria:

- It should be easily and accurately measured in the different media, i.e., in liposomal or lipid suspension (non-biological medium) and in plasma and organ extracts (in biological media).
- It should be hydrophilic because lipophilic drugs alone may cross membranes even without liposomes thereby giving false results.
- It should have a relatively short half-life to enable studies over a short period.
- It should not be metabolised, to avoid loss of measurable drug in the organ.
- It should be devoid of side effects that may interfere with the experiment such as sedation or cardiovascular effects.
- It should be relatively cheap.
- It should be easily obtained i.e. not controlled compound.

- It should be easy to handle, preferably non-radioactive.

In this chapter, is presented a chronology of events that lead to the selection of an appropriate marker drug. It involved a practical evaluation of three drugs, diclofenac, piroxicam and gentamicin as markers for the liposomes based on the above criteria.

3.2 DICLOFENAC

Diclofenac, a non-steroidal anti-inflammatory, was chosen due to its ready availability, reasonable price and also because it did not have serious side effects such as sedation that could interfere with the animal study.

3.2.1 SPECTROPHOTOMETRIC ANALYSIS OF DICLOFENAC

Although several high performance liquid (HPLC) methods for assay of diclofenac have been published, most of them were found to be unsuitable for our purpose due to poor sensitivity, complexity, time consuming and tedious extraction steps (reviewed by Avgerinos et al., 1993). The method by Avgerinos et al. (1993) could not also be used here because, although less complex, it required injection of unprocessed sample in the high performance liquid chromatography, which would impact on the column performance. The spectrophotometric method reported by Agatonovic-Kustrin et al. (1997) was preferred because it appeared less complex and cheaper than the high performance liquid chromatography methods as well as the other two spectrophotometric methods (Sastry et al., 1989; Agrawal and Shivramchandran, 1991).

3.2.1.1 REAGENTS AND APPARATUS

Diclofenac sodium (Voltaren®) - 50mg tablets and diclofenac potassium (Cataflam®) - 50 mg tablets, Novartis were obtained from a local Pharmacy. Potassium chloride and sodium acetate were from Sigma Chemical Co. (Steinheim, Germany). Iron (III) chloride, acetic acid and sodium hydroxide from Merck (Darmstadt, Germany). Ammonium thiocyanate was obtained from Protea laboratory services (Pty.) Ltd (Johannesburg, S.A). A "LKB Biochrome Ultospec II" Spectrophotometer was used for UV absorption measurements, pH measurements were carried out on a 'inolab, pH level 1' pH meter and shaking on a GFL horizontal shaker.

3.2.1.2 PREPARATION OF STANDARD SOLUTIONS

Diclofenac stock solution containing 1 mg/ml of diclofenac was prepared by grinding a 50-mg tablet and dissolving it in 50 ml of water. Iron (III) chloride solution (81 mg/ml; 3×10^{-1} M) was prepared by dissolving 4.05 g of Iron (III) chloride in 40 ml of water and adding 1 ml of 1 M HCl, followed by dilution of the solution to 50 ml with water. Ammonium thiocyanate was prepared by dissolving 9.13 g of ammonium thiocyanate in 50 ml of water. Acetate buffer solution of pH 5.6 was prepared from acetic acid and sodium acetate, by mixing 4.8 ml of 0.2 M acetic acid and 45.2 ml of 0.2 M sodium acetate, followed by dilution of the solution to 100 ml with water (Lab FAQ's, Find a quick solution, Roche biochemicals).

3.2.1.3 ASSAY PROCEDURE

To 2 ml of diclofenac solution or blank (reference) was added 2 ml of iron (III) chloride, 2 ml of ammonium thiocyanate, 1 ml of 2 M potassium chloride and 3 ml of acetate buffer. Finally 5 ml of chloroform was added, the test tube was stoppered and the mixture was shaken gently for 10 min on a horizontal shaker. Thereafter, the chloroformic layer was separated using a separation funnel and its absorbance was measured at 481 nm against the reference. A concentration range of 0.2 to 1.5 mg/ml was used for calibration.

3.2.1.4 RESULTS AND DISCUSSION

Unfortunately, this procedure appeared to have failed from the start because the calibration obtained was grossly non-linear (fig 11). Since it was reported that diclofenac sodium (Voltaren®) was sparingly soluble in water, this was replaced by the more water soluble formulation, diclofenac potassium (Cataflam®). These changes did not alter the results.

However on further evaluation of this procedure, it was found to be unfavourable for our purpose because it required mixing large volumes of solutions and several liquid transfers which could lead to drug loss. Also, it involved use of chloroform which was not only inconvenient to handle but also required strict use of glassware apparatus, especially Quartz UV cuvettes. Furthermore, because chloroform can dissolve liposomal lipids, there was concern that this would interfere with the detection of the drug. In addition, it was realised that the procedure was developed for measuring diclofenac in pharmaceutical preparations, and therefore may not be suitable for biological media. Lastly, the choice of

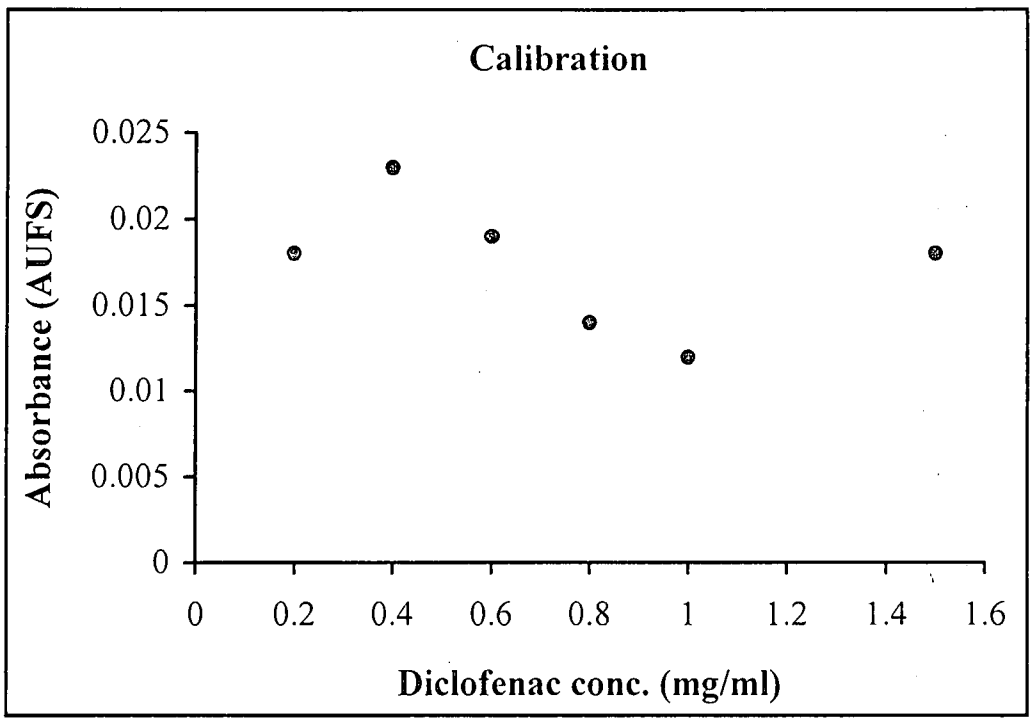


Fig 11 A plot of diclofenac concentration (0.2 – 1.5 mg/ml) versus absorbance (AUFS) measured. The unlinear line indicates a failed calibration.

diclofenac as a marker became questionable when it was realised that it is highly metabolised in the liver. Therefore, it was decided to abandon this method and no further steps to optimise the method were undertaken.

3.3 PIROXICAM

Piroxicam is a non-steroidal anti-inflammatory drug that exhibits low capacity metabolism, hence, it has a long half-life of approximately 50 hours in human. Its half life in rats was reported to vary with the gender. In males it averages to 13.3 hours while in females it was higher, 40.8 hours (Roskos and Boudinot, 1990).

3.3.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF PIROXICAM

Several high performance liquid chromatography analytical methods for piroxicam were reviewed and many were found to be unsuitable for this study either due complexity or lack of appropriate instruments e.g detector. (Donald and Twomey, 1979; Gillilan et al.,1989). The method by Dadashzadeh et al.(2002) was preferred because it is simple. Sample preparation is by protein precipitation only. However due to concerns of the likelihood of damaging the column with unprocessed samples, this method was compared with that reported by Klopas et al. (1998), in which the samples were purified by extraction and evaporation of an organic solvent.

3.3.1.1 REAGENTS AND APPARATUS

Piroxicam (Batch No. 547) and naproxen, (Batch No. 545, internal standard) were available at the laboratory, a left over from previous analytical studies. High performance liquid chromatography grade acetonitrile and methanol were obtained from Romil Ltd (England). Sodium acetate was from Sigma (Steinheim, Germany) while glacial acetic acid and triethylamine from Merck (Darmstadt, Germany). A "LKB Biochrome Ultospec II" Spectrophotometer was used to measure absorbance, a Mini Spin Eppendorf centrifuge to centrifuge samples and a 'inolab, pH level 1' pH meter for pH adjustments.

3.3.1.2 CHROMATOGRAPHIC CONDITIONS

The chromatographic system consisted of a Hewlett-Packard model 1100 high performance liquid chromatographic system equipped with an isocratic pump, an autosampler and a variable-UV detector. Chromatographic separations were done using a Synergi polar RP – 80A, 150 x 4.6 mm, 4 μ column and a mobile phase consisting of 0.1 M sodium acetate : acetonitrile : triethylamine (61:39:0.05 $^{v}/_{v}$), pH adjusted to 4 with glacial acetic acid and at a flow rate of 1ml/min. Total run time was 10 minutes and UV detection was done at 330 nm. The mobile phase was degassed before use and all chromatographic analysis was done at room temperature.

3.3.1.3 METHOD VALIDATION

Wave scan (Lambda max. determination): Absorption spectra of piroxicam was done to determine the most appropriate wavelength for detection. A spectrum scan from 200 to 400 nm was performed on a standard sample of piroxicam (1 mg/ml). The peak absorbance was at 387 nm but adequate sensitivity was obtained at 330 nm. The latter was adopted because it was also used by Dadashzadeh et al. (2002).

Sample preparation: Two extraction methods were compared.

Extraction 1 (S. Dahashzadeh et al., 2002) - By protein precipitation using acetonitrile and methanol: 200 μl of acetonitrile and 50 μl of methanol were added to 250 μl of sample (plasma), vortexed for 1 minute, centrifuged at 8 000 rpm for 10 minutes and the separated supernatant used for analysis.

Extraction 2 (Klopas et al., 1998) - By direct extraction with diethyl ether: 7 ml of ether was added to 2 ml of sample (plasma), vortexed for 10 minutes and centrifuged at 2,000 g for 10 minutes. The ether layer was then transferred to vials and evaporated at 40 °C under a stream of nitrogen gas to dryness. 200 μl of the mobile phase was added to the vials, vortexed for 1 minute and analysed.

Analysis of piroxicam in liver homogenate: Because the marker would have to be analysed in tissue homogenates, the method was tried on liver homogenate. Three grams of liver tissue was homogenised with distilled water (1:1) and centrifuged at 12 000 rpm for 30 minutes at 4 °C. The homogenate obtained was spiked with piroxicam (final concentration was 60 μg/ml). This was then vortexed, centrifuged (12 000 rpm, 30 minutes at 4 °C) and

extracted with acetonitrile (2 times volume), centrifuged (10 000 rpm, 30 minutes, 4 °C) and the separated supernatants were analysed.

3.3.1.4 RESULTS AND DISCUSSION

Chromatograms of standard methanolic solutions of piroxicam and naproxen (I.S.) are shown in fig 12 a and b. The peak were well resolved and symmetrical. Piroxicam resolved at 5.7 minutes and the naproxen at 8.7 minutes. Chromatograms of plasma extracts by procedure 1 and 2 are shown in fig. 13 & 14, respectively. Extraction by the first method (protein precipitation) gave better sensitivity. But overall, both methods did not give acceptable sensitivity. For a concentration of 10 μg/ml, extraction method 1 gave absorption units of 170 mAU while extraction method 2 gave only 24 mAU. Changing mobile phase to phosphate buffer (pH 7.0, 0.05 M) : methanol (60 : 40 $^{v}/_{v}$) mobile phase did not improve sensitivity.

Likewise, the results of the liver homogenate were not impressive with regard to sensitivity, fig 15.

In conclusion, the poor sensitivity of the method for detection of piroxicam in plasma and liver homogenate made it unsuitable for further use, as a result, another marker had to be sought.

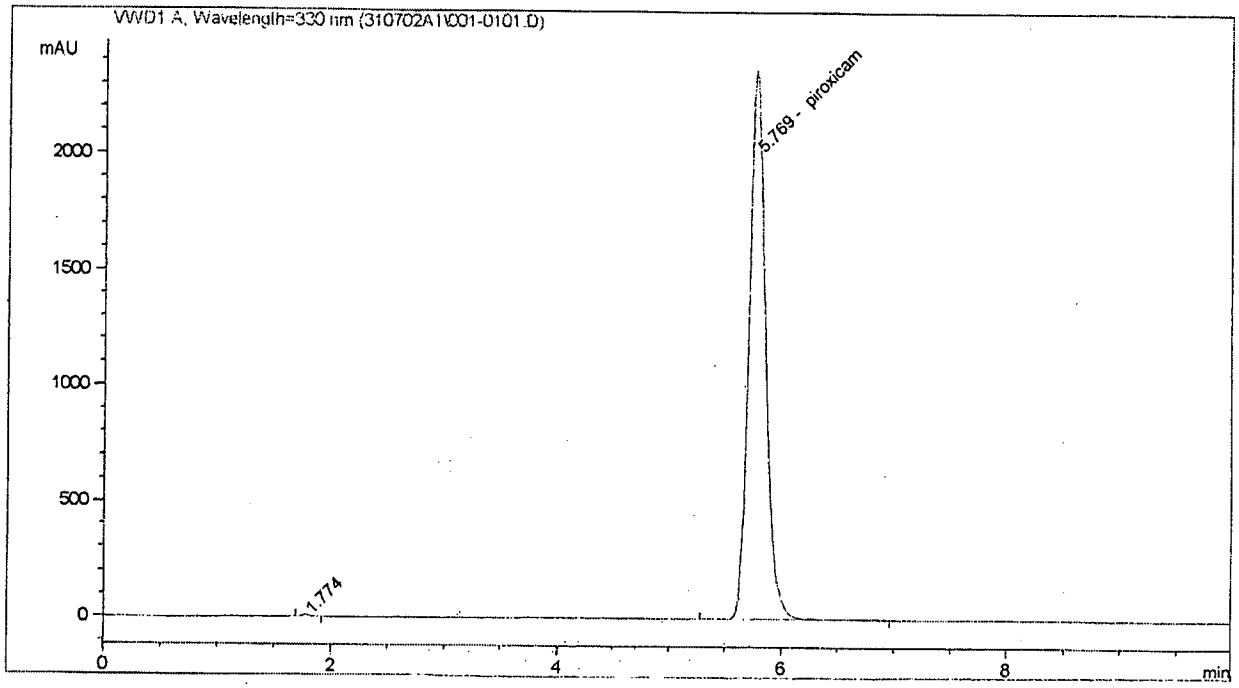


Fig 12a Chromatogram of piroxicam in methanol (1 mg/ml) with injection volume of 10 µl.

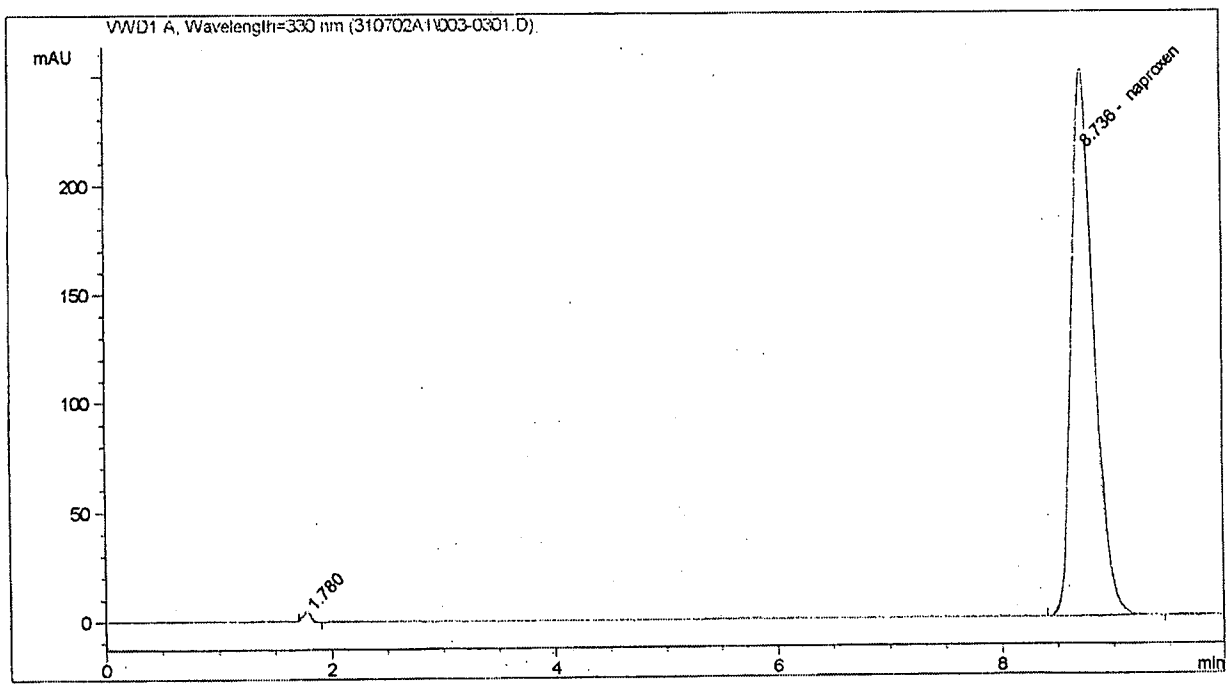


Fig 12b Chromatogram of naproxen in methanol (1 mg/ml) with injection volume of 10 µl.

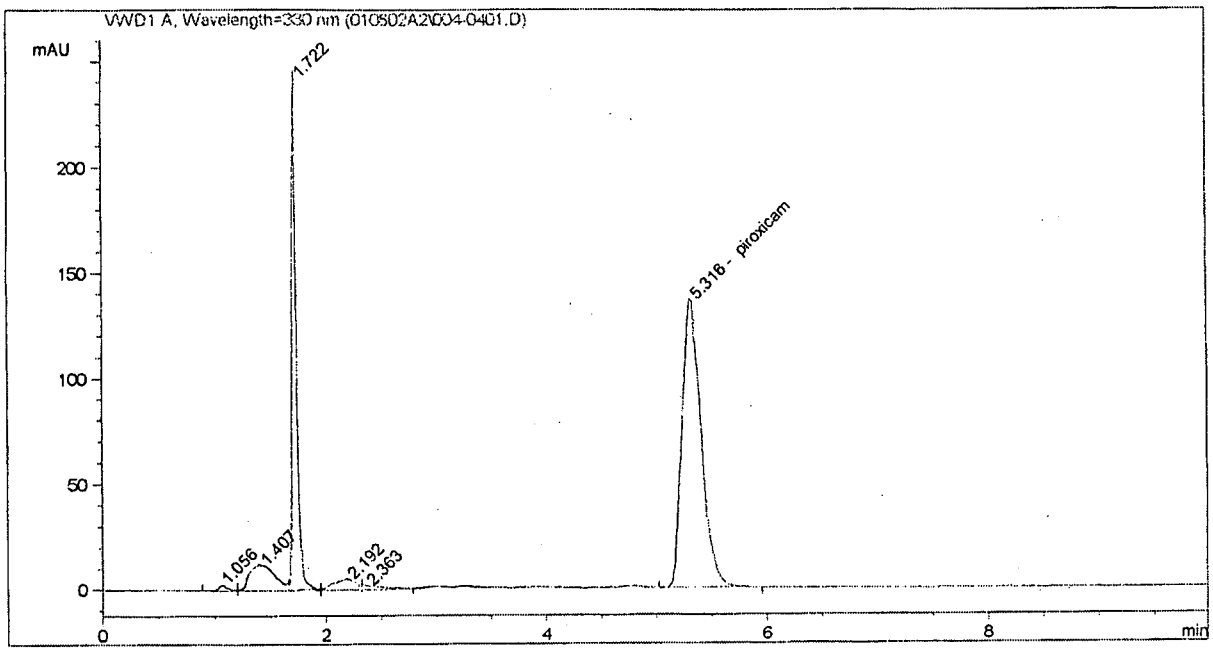


Fig 13 Chromatogram of spiked plasma, extracted by **extraction method 1**,with piroxicam concentration equivalent to 10 μg/ml and injection volume of 50 μl.

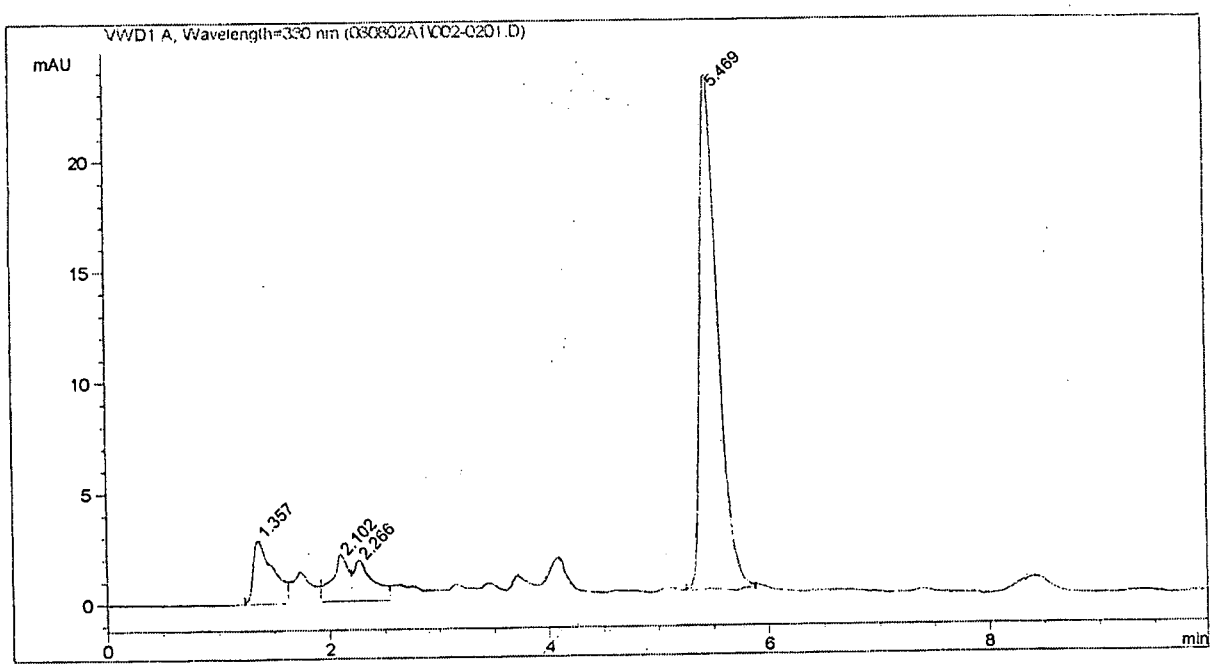


Fig 14 Chromatogram of spiked plasma, extracted by **extraction method 2**, with piroxicam concentration equivalent to 10 μg/ml and injection volume of 50 μl.

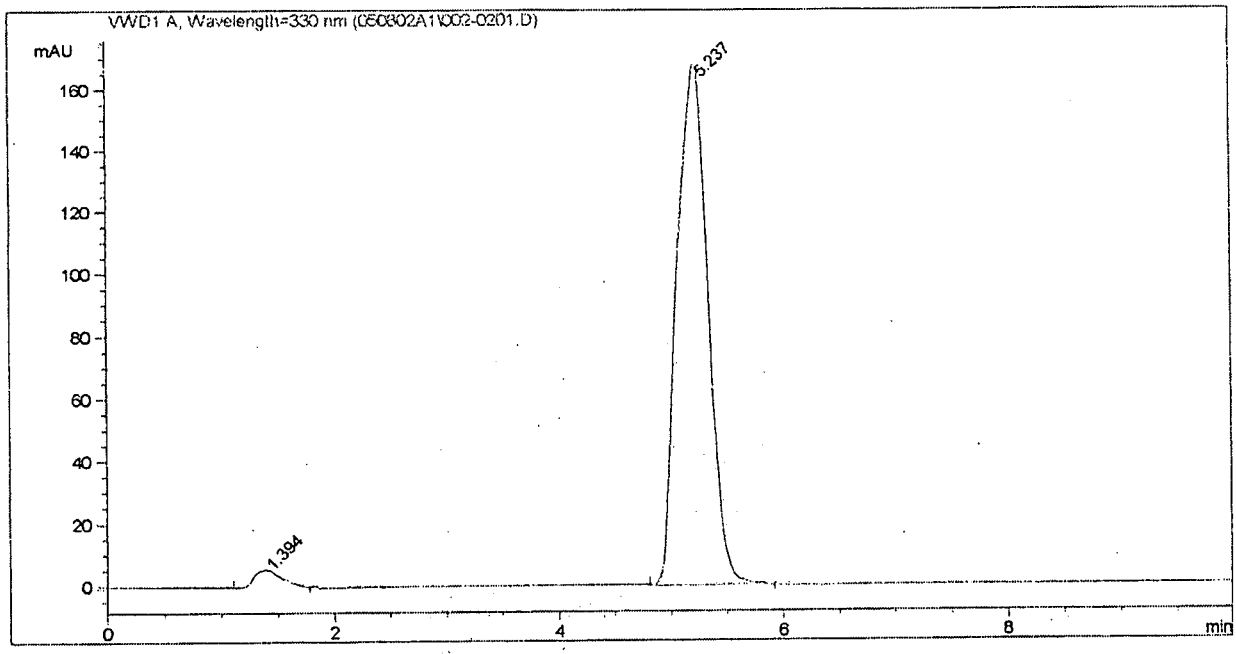


Fig 15 Chromatogram of spiked liver homogenate, with piroxicam concentration equivalent to 60 μg/ml and injection volume of 50 μl.

3.4 GENTAMICIN

Gentamicin, an aminoglycoside antibiotic was chosen for evaluation as a marker due to its favorable properties of being hydrophilic and highly charged, cationic with net charge of + 3.5 at pH 7.4 (Josepovitz et al., 1982). The latter reduces its ability to cross membranes thereby making the liposomal effect more vivid. Furthermore, because gentamicin is not appreciably metabolized, there will be no loss of measurable drug *in situ*. Also, its half life of 2 to 3 hours reduces the study period because changes in half life, e.g., prolongation of half life, can be observed within 8 hours (> 3 times half life) This is important when studying clearance and distribution, parameters that are closely related to half life. Lastly, gentamicin is devoid of interfering side effects, is non-radioactive, is not a controlled drug and is cheap. However, the major challenge was to identify an appropriate method for analysis for gentamicin both in biological and non-biological fluids.

3.4.1 FLUORESCENCE POLARISATION IMMUNOASSAY

Several methods for analysis of gentamicin by microbial assays (Sabath et al., 1971; Reamer et al., 1998), high performance liquid chromatography (Claes et al., 1984; Moreno et al.,1998) and immunoassays (Gurtler et al., 1995; Ara et al., 1995) have been published. Since the microbial assays are now obsolete and the high performance liquid chromatography methods are regarded as labor intensive, the immunoassays are now the most utilized because they are simple, rapid, sensitive, automated and hardly require any sample processing. The automation obviates errors caused by manual measurements. As such, the fluorescence polarization immunoassay (FPIA) was selected because, in addition to the above advantages, the analytical equipment was available in our laboratory.

The fluorescence polarization immunoassay (FPIA) is based on the principles of two technologies; competitive antibody binding and fluorescence polarization (Hong and Choi, 2002). Specifically there are three elements; the antibody, the tracer-antigen and the drug present in the sample. The antibody is specific for the drug and the tracer, hence, can bind to either, but not to both simultaneously. When polarized light is applied to the free tracer-antigen, it emits non-polarized fluorescence but when the tracer-antigen is bound to the antibody it emits polarized fluorescence. Measurement of the amount of polarized fluorescence emitted gives an estimate of the quantity of drug–antibody complexes in the sample, from which the exact drug concentration is derived.

In general, fluorescence polarization immunoassay involves, first, addition of a known amount of antibody to the sample to induce a drug-antibody reaction; whereby any drug present in the sample binds to the antibody. Secondly, the fluorescent antigen-tracer is added for detection of extent of drug–antibody binding. The fluorescent tracer-antigen binds to any unbound antibody. A high concentration of the drug in the sample would mean low concentration of unbound antibodies, and therefore a high amount of the free tracer-antigen, and vise versa. Finally the sample is illuminated with polarized light and the amount of polarized fluorescence emitted is measured.

Therefore, the quantity of drug in the sample is inversely proportional to the amount of polarized fluorescence emitted. When there is a high concentration of the drug in the sample, most of the antibody is bound to the drug leaving a high a concentration of the fluorescent antigen tracer free to rotate in the solution. Since the fluorescent antigen tracer is small in size and exhibits rapid Brownian motion, it emits non-polarized fluorescence. And when there is very little of the drug in the sample, most of the antibody is bound to the tracer-antigen. The antibody tracer-antigen complex is a large molecule and thus there is reduced the rotation of the tracer in the solution, causing it to emit polarized fluorescence. This principle is summarized in Fig 16.

As indicated earlier, this method had to be optimized for measurement of the drug in biological and in non- biological medium. The fluorescence polarization immunoassay method was developed to be used for measurement of drugs in biological fluids (plasma, urine etc). As such, the main concern was to validate the method to measure gentamicin in a non-biological medium i.e. in buffer, commonly used in liposome preparations.

3.4.1.1 REAGENTS AND APPARATUS

Gentamicin sulphate powder (Batch No. 455) was available in our laboratory, a left over from previous analytical studies and gentamicin sulphate ampoules (80 mg), Merck (Darmstadt, Germany), were obtained from a local pharmacy. Disodium hydrogen orthophosphate and sodium dihydrogen phosphate were obtained from BDH laboratory supplies (Poole, England) while sodium chloride was from Sigma Chemical Co. (Steinheim, Germany). Working plasma was obtained from healthy volunteers in the department. Gentamicin concentrations were measured on a fully automated Abbott AxSym® analyser.

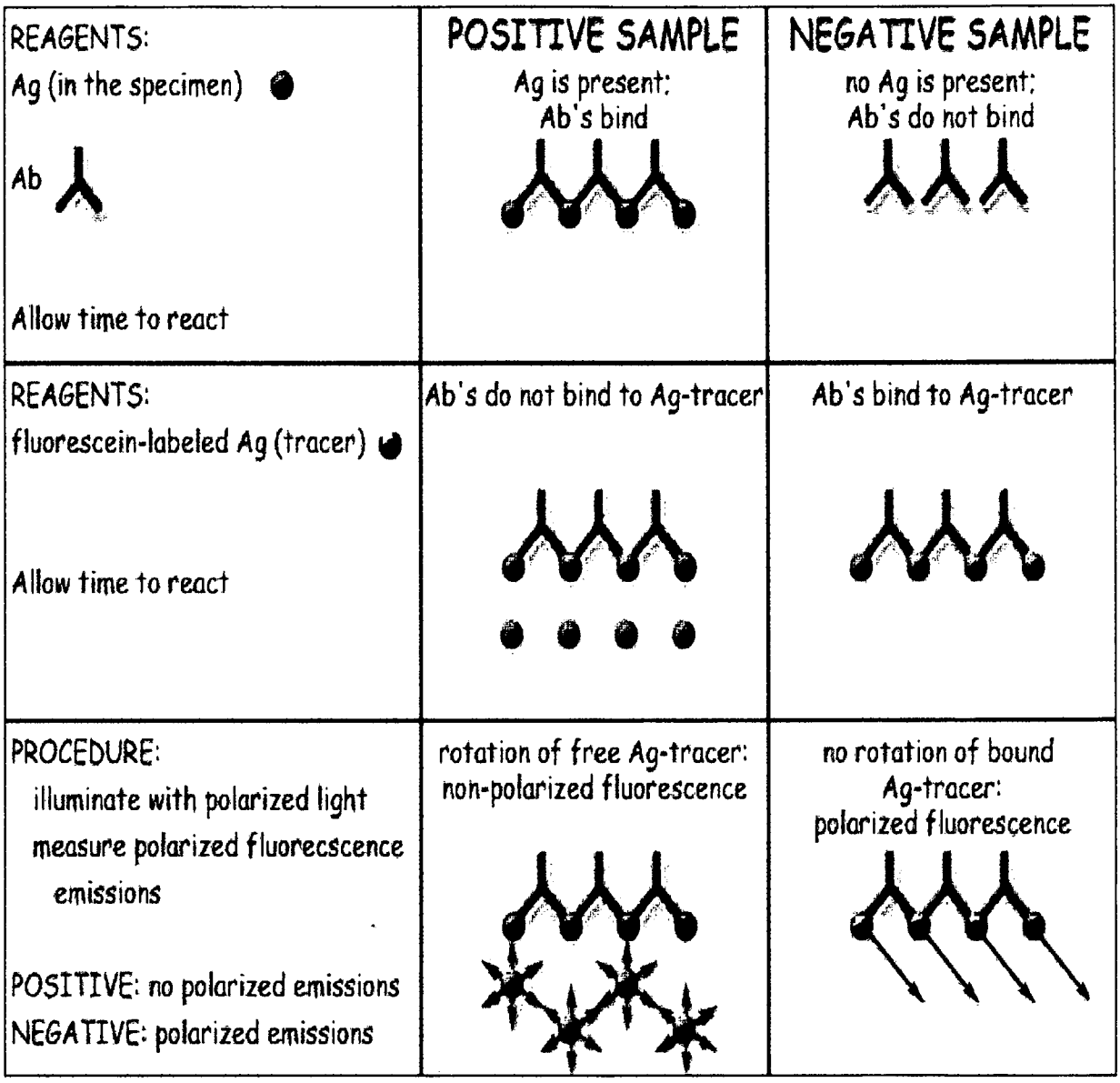


Fig 16 An illustration of the principle of fluorescence polarisation immunoassay (FPIA). [Ag = drug; in this case gentamicin; Ab = antibody; and Ag-tracer = tracer-antigen]. (From: http://vtpb-www.cvm.tamu.edu/vtpb/vet_micro/ serology/ fpia/default.html)

3.4.1.2 ANALYSIS OF GENTAMICIN

A stock solution of gentamicin (1 mg/ml) was prepared in PBS. This was used to make further dilutions that were run on the AxSym® system according to instructions. The AxSym® analyser undertakes auto-calibration, direct conversion and reporting of gentamicin concentration. Gentamicin concentrations were corrected for the salt (sulphate) as 0.6530 mg of gentamicin for 1 mg of gentamicin sulphate.

3.4.1.3 RESULTS AND DISCUSSION

Gentamicin concentrations read by the AxSym® system corresponded to the spiked concentrations when plain plasma samples were analysed. Since this is the standard practice by which patient samples for therapeutic drug monitoring are analysed in this laboratory, there was no need to validate this any further.

Unfortunately, gentamicin concentrations in the buffer did not correspond with the spiked concentrations, i.e., the measured concentrations were higher than the spiked (known) concentrations. For instance, a spiked concentration of 5 μg/ml gentamicin gave a reading of greater than 10 μg/ml. Even when a partial biological state was induced by diluting the samples with plasma in a 1:1 ratio, the measured concentrations still did not correspond with the spiked concentrations. Gentamicin injectable solution was also tested, as the purity of the powder sample was doubted, but this did not show any improvement. It was then concluded that the FPIA method could not be used on non-biological media. Hence, another method would have to be adopted for the measurement of gentamicin in non-biological media.

3.4.2 SPECTROPHOTOMETRIC ANALYSIS OF GENTAMICIN

In seeking for an alternative method, the principle was to select a method that incorporates techniques for enhancing gentamicin detection. Indeed, it was observed that the high performance liquid chromatography methods required derivatisation of gentamicin (Claes et al., 1984; Abdulsalam et al., 2002). However these methods were found to be complex, as such, the spectrophotometry method reported by Sampath and Robinson (1989) was preferred. This method involves derivatisation of gentamicin using o-phthaldialdehye. The principle is based on the reaction of o-phthaldialdehye with the amino group of gentamicin to yield a chromophoric product that can be detected at 332 nm.

3.4.2.1 REAGENTS AND APPARATUS

O-phthaldialdehyde (PHT) was obtained from Sigma Chemical Co. (Steinheim, Germany). Isopropanol and sodium hydroxide from Merck (Darmstadt, Germany). Absorbance measurements were made on a "LKB Biochrome Ultospec II" Spectrophotometer, samples were vortexed on a 'Vortex-2 Genie' vortexer and a 'Labcon' shaking water bath was used for temperature adjustment.

3.4.2.2 PREPARATION OF STANDARD SOLUTIONS

A 1 mg/ml solution of O-phthaldialdehyde (PHT) was prepared in deionised water and its pH was adjusted to 10.5 using 1 M NaOH. It was stored in a foil-covered glass bottle to protect it from light. Gentamicin stock solutions of 100 μg/ml and 40 mg/ml in saline were used to prepare appropriate concentrations for further use. All solutions were refrigerated when not in use and were used within 1 month of preparation.

3.4.2.3 ASSAY PROCEDURE

Below is outlined the assay procedure as published by Sampath and Robinson (1989). This procedure was optimised to suite our conditions as described next section 3.4.2.4.

1. To a 15 ml glass test tube, 1 ml of PHT and 1.5 ml of isopropanol were added and the test tube was stoppered.
2. An aliquot from the test sample (standard solution) was added such that final concentrations of gentamicin in the mixture ranged from 5 to 30 μg/ml after adjustment to 5 ml with deionised water. Final volume of the mixtures was 5 ml. A blank was also prepared.
3. Mixture was vortexed and allowed to stand at room temperature for 45 min.
4. Absorbance was measured at 332 nm, which corresponds to the maximum absorbance of PHT derivatised gentamicin .
5. A calibration curve of absorbance versus concentration was plotted for concentrations between 5 to 30 μg/ml.

Result: The calibration performed by using the procedure as described above (Fig 17), was linear. The regression equation was $y = 0.0097\ x + 0.1013$ with a regression coefficient of 0.9552. This method was used as standard in the optimisation of the conditions as described

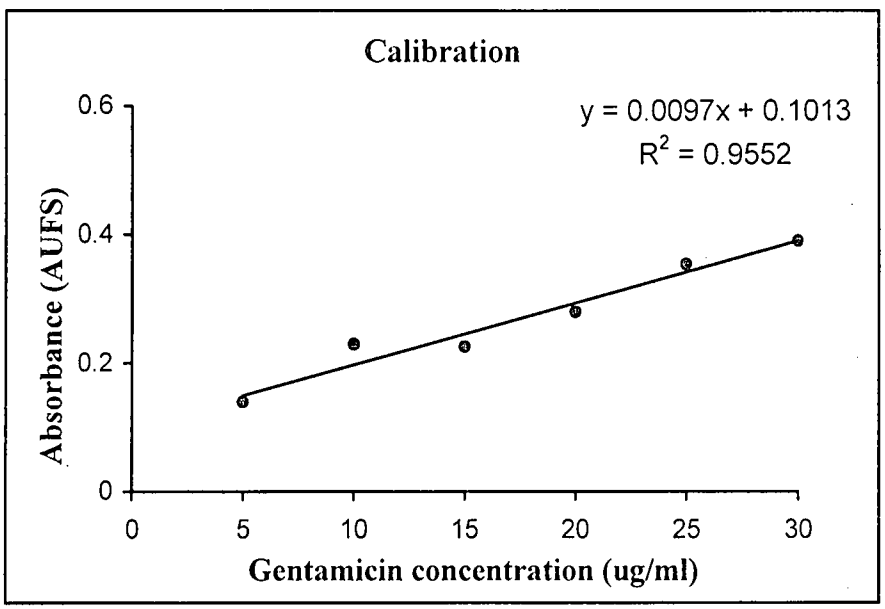


Fig 17 A plot of gentamicin concentration (5 - 30 μg/ml) versus absorbance (AUFS) measured.

below.

3.4.2.4 METHOD OPTIMISATION

1. **Reducing total reaction volume:** The total reaction volume was reduced from 5 ml to 3 ml and a calibration curve was repeated over the same range.

   Result:. Unfortunately, the resultant calibration curve was not acceptable due to poor linearly, as indicated by a poor regression coefficient (R^2 = 0.8973) (See appendix A 1). Hence it was decided to keep the final volume of 5 ml.

2. **Sensitivity at higher gentamicin concentrations:** As the concentration of gentamicin used in the liposome suspension is likely to be higher (> 30 μg/ml), it was decided to test the sensitivity of the assay at higher concentrations of gentamicin. This would reduce the dilutions required otherwise. Gentamicin concentrations of 10, 20, 40, 60, 80 and 100 μg/ml were used.

   Result: At concentrations of 10 to 100 μg/ml, it also showed compliance to Beer's law, regression coefficient was 0.9606 (Fig 18).

3. **Reducing reaction time:** In the procedure described by Sampath and Robinson (1989) a long reaction time of 45 minutes was used (step 5). Here the reaction time was reduced to 15 minute but at higher temperature of 40 °C to speed up the reaction. Concentrations used were 10, 20, 40, 60, 80 and 100 μg/ml.

   Result: : A linear graph was obtained with a regression coefficient was 0.9817 (Fig 19). Hence it was decided to incubate at 40 °C for 15 minutes.

4. **Fixing sample volume and testing sensitivity at higher gentamicin concentration (2 - 20 mg/ml):** The addition of sample aliquots followed by adjustment to 5 ml involves complex calculations to determine the volumes to be added to the reaction mixture and the concentration of drug in the sample. This can be avoided by using a fixed sample volume and, therefore, it was decided to use a sample volume of 100 μl. Concentrations of 2, 4, 8, 10, 15 and 20 mg/ml gentamicin were also tested.

   Result: The calibration curve was linear with regression coefficient of 0.9991 (Fig 20). Therefore, a fixed sample volume of 100 μl was accepted.

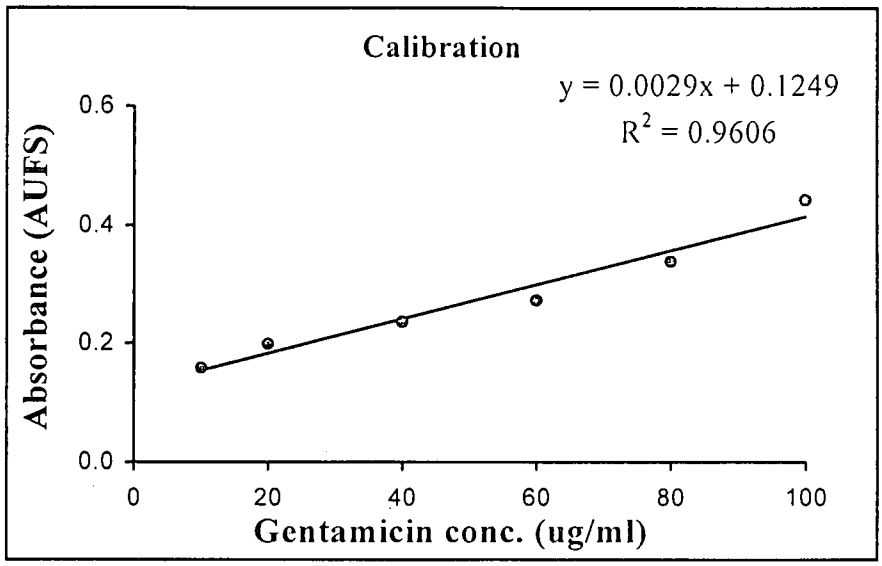


Fig 18 A plot of gentamicin concentration (10 - 100 μg/ml) versus absorbance (AUFS) measured.

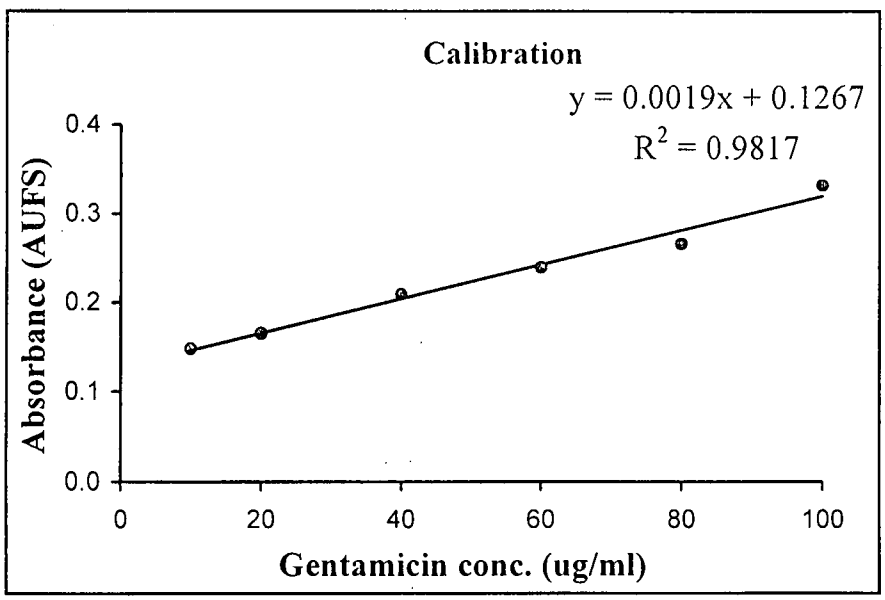


Fig 19 A plot of gentamicin concentration (10 - 100 μg/ml) versus absorbance (AUFS) measured, after incubation at 40 °C for 15 minutes.

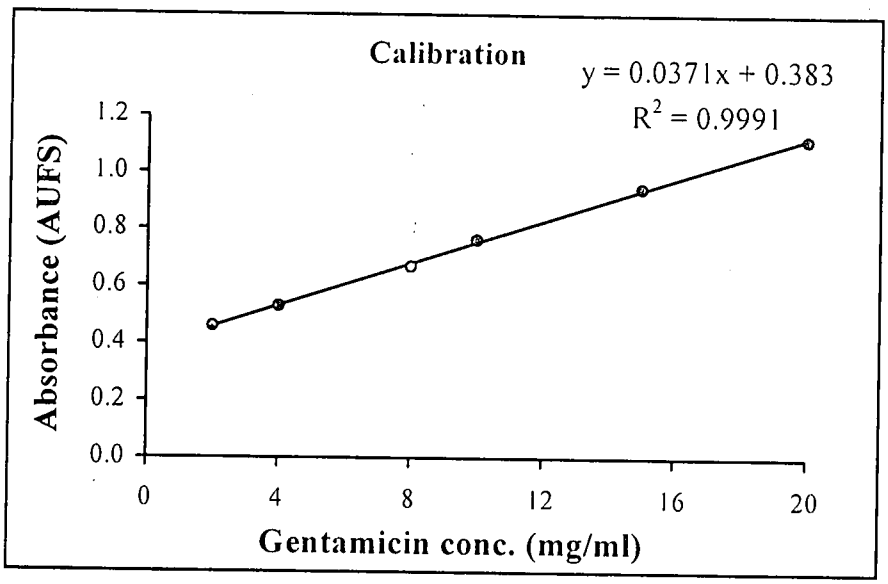


Fig 20 A plot of gentamicin concentration (2 - 20 mg/ml) versus absorbance (AUFS) measured and using a fixed sample volume (100 μl).

Summary of final procedure

Based on the results obtained above, the assay procedure was modified as summarised below:

1. Add O-phthaldialdehyde reagent- 1 ml and isopropanol - 1.5 ml to stoppered glass test tubes.
2. Add **100 µl** of the sample to be tested.
3. Adjust the final volume to 5 ml with deionised water.
4. Vortex the reaction mixtures.
5. Warm at **40 °C for 15 minutes** (including pre-incubation time). Shaking at 95 rpm.
6. Transfer reaction mixtures to cuvettes, measure absorbance at 332 nm.

NB: Please note that at this point, the assay is only applicable to non-biological medium.

3.4.2.5 METHOD VALIDATION

1. **Inter-day calibration:** A calibration was performed with concentrations of 2, 4, 8, 10, 15 and 20 mg/ml. This was done on 5 different days to determine the daily variability.

2. **Testing the effect of Triton X-100 on the assay:** Since triton X-100 would be used for lysis of liposomes in determining liposome encapsulation, it would be important to know whether triton X-100 would interfere with the assay. Therefore, concentrations of 10 %, 5 % and 1 % triton X-100 were tested. 1 ml of each of the above solutions was added to the respective reaction mixture and the final volume was adjusted to 5 ml in (step 3 of the procedure). A constant gentamicin concentration of 8 mg/ml was used for all samples.

3. **Testing performance of the assay in biological media:** Plasma was spiked with gentamicin to obtain concentrations of 2, 5 and 10 µg/ml and then treated with isopropanol or 2.5 % zinc sulphate (3:1) to precipitate proteins. These samples were then vortexed (1 min) and centrifuged (12 100 rcf for 10 min). A blank was also prepared. This was done in duplicate. The spiked plasma samples were used in the assay using the same procedure as described earlier.

3.4.2.6 RESULTS AND DISCUSSION

Inter-day calibration:

The average equation was $y = 0.0368x + 0.3924$ ($y = mx + c$) with $r^2 = 0.9874$ (Fig. 21) and coefficient of variation after regression analysis are shown in Table 7. (Also see appendix A 2)

This assay was found to be applicable for measuring gentamicin in a non-biological medium. To avoid variations, it was decided to run a calibration curve for each experiment.

Testing the effect of Triton X-100 on the assay: Triton X-100 interfered with the gentamicin assay. The measured concentrations were lower than the spiked concentration. For a spiked concentration of 8 mg/ml, the measured values were 4.2 mg/ml for 10 %, 4.95 mg/ml for 5% and 7.08 mg/ml for 1 % triton X-100.

Testing sensitivity of the assay in biological medium: The spiked plasma samples (2, 5 and 10 µg/ml) showed opposite trend i.e. the absorbance decreased with increasing gentamicin concentration (Fig 22).

In conclusion, this gentamicin assay is not applicable to biological media and samples containing triton X-100.

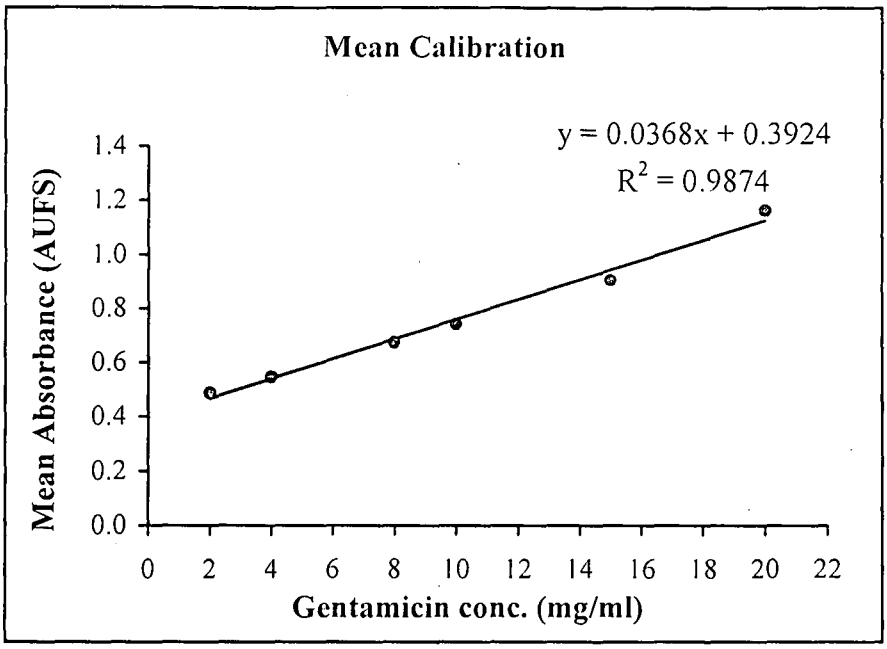


Fig 21 Mean plot of gentamicin concentration (2 - 20 mg/ml) versus absorbance (AUFS), each point represents mean of 5 readings.

Table 7 Regression data for mean calibration plots for gentamicin (n = 5).

Gentamicin concentration (mg/ml)	Mean Absorbance	Standard deviation	Coefficient of variation (%)
2	0.489	0.039	7.93
4	0.546	0.012	2.11
8	0.674	0.021	3.18
10	0.743	0.042	5.59
15	0.906	0.070	7.77
20	1.167	0.123	10.5
Mean		0.051	6.18
Average regression equation, regression coefficient		$Y = 0.0368\ x + 0.3924$, $r^2 = 0.9874$	

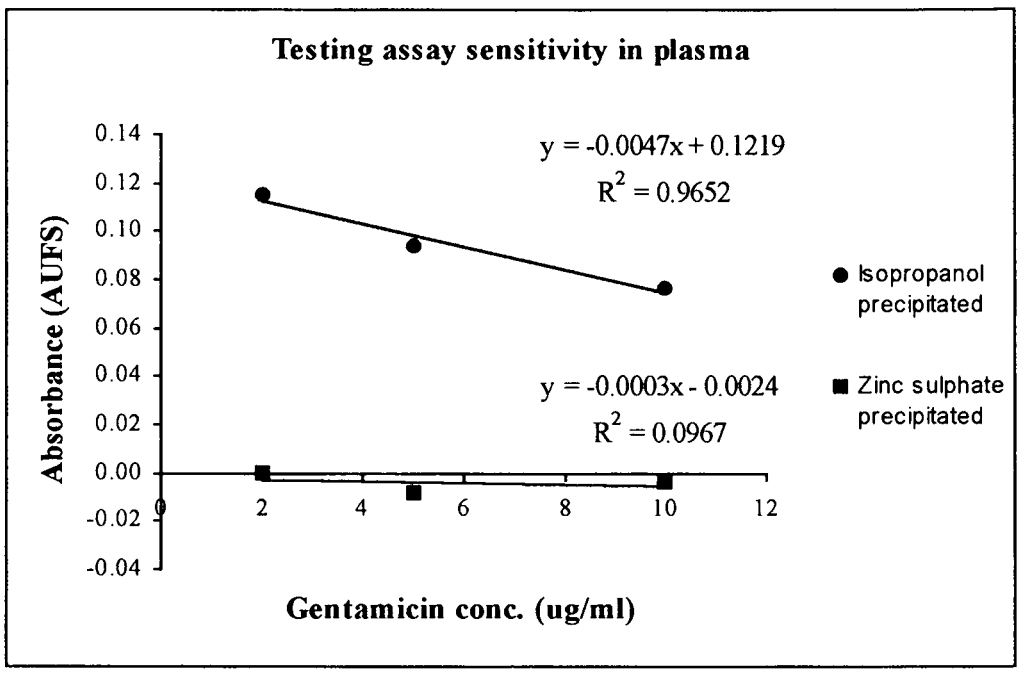


Fig 22 A plot of gentamicin concentration (2, 5, 10 μg/ml) versus absorbance measured of spiked plasma treated with isopropanol or 2.5 % zinc sulphate.

3.5 CONCLUSION

In conclusion, gentamicin was selected as the most appropriate marker drug for further use, and it was decided that gentamicin was to be analysed by two methods, the spectrophotometric analysis for measuring gentamicin in non-biological medium and fluorescence polarisation immunoassay for biological medium (in plasma and organ extracts).

Chapter 4

LIPOSOME PREPARATION AND CHARACTERIZATION

4.1 INTRODUCTION

This part describes methods by which liposomes were prepared and characterised. Due to availability of many methods for preparation of liposomes (chapter 2, section 2.8), selection of the most appropriate method is crucial, as it may determine the quality of liposomes produced. It requires consideration of several factors such as, to mention but a few, facilities available, simplicity, cheapness, run time, reproducibility and ultimate purpose of the liposomes.

Characterisation of liposomes is also as important, since the characteristics of the liposomes governs their behaviour *in vitro* and *in vivo*. Liposomes are commonly characterised by their size, morphology (laminarity), encapsulation efficiency and stability.

4.2 REAGENTS AND APPARATUS

Liposomal preparation: Lipids; L-α-phosphatidyl choline (lyophilised powder from fresh egg yolk), cholesterol, dicetyl phosphate, and stearylamine were obtained from Sigma Chemical Co. (Steinheim, Germany). Gentamicin sulphate ampoules (80 mg/2 ml), Merck (Darmstadt, Germany), were obtained from a local pharmacy. Physiological saline from Adock Ingram Critical Care Ltd (Johannesburg, South Africa). Lipids were weighed on a Scaltec, SBC 31 fine balance, rotary evaporation was done on a Rotavapo-RE-Optolabor with an Eyela A3S (Tokyo Rikakikai) aspirator, sonication on a Bandelin Sonorex RK100, bath type sonicator and samples were ultracentrifuged using a Beckman, L8-70M ultracentrifuge.

Characterisation: Phosphotungstic acid (PTA) and formvar, 3-mm 200-mesh carbon coated copper grids were obtained from Wirsam (Johannesburg, S.A.). A Philips Tecnai 10 Transmission Electron Microscope was used to examine the liposome suspension. Patent

blue violet and triton X-100 were obtained from Sigma Chemical Co. (Steinheim, Germany). Physiological saline from Adock Ingram critical care ltd (Johannesburg). Absorbency measurements were made on a "LKB Biochrome Ultospec II" Spectrophotometer.

4.3 METHODS

4.3.1 LIPOSOME PREPARATION

Two methods of liposome preparation were compared; reverse phase evaporation method and hydration method. These two are the most commonly used liposome preparation techniques. Reverse phase evaporation was explained earlier (chapter 2). In the current experiment, it was successfully used to prepare liposomes, but the encapsulation rates varied widely and it involved a longer and technically more complex procedure. This was unacceptable considering that many liposomal preparations would have to be done daily. Therefore, another method, specifically the hydration method was sought. The hydration method also offers the advantage, in that it can be used for thermally unstable drugs because it does not involve heating of the drug solution. Hence, the hydration procedure was selected for preparing liposomes as described below (Wasserman et al., 1986).

4.3.1.1 GENERAL PROCEDURE OF LIPOSOME PREPARATION BY HYDRATION

1. Lipids were weighed and transferred to a clean dry round bottom flask.
2. Chloroform (17 ml) was added to dissolve the lipids (Fig 23, step 1).
3. The organic solvent (chloroform) was removed under vacuum in a rotary evaporator at 37 °C until a thin opaque lipid film remained on the walls of the flask (Fig 23, step 2).
4. The aqueous phase containing the marker compound (gentamicin, 20 mg/ml) was then added to the lipid film (1 ml per 14.7 mg lipid) i.e. 8 ml of 20 mg/ml gentamicin (prepared by diluting 40 mg/ml gentamicin with physiological saline) (Fig 24, step 3) and vortexed for 1 minute.
5. The suspension was sonicated in a bath-type sonicator for 10 minutes (Fig 24, step 4 & 5).
6. The milky suspension obtained was then transferred to an ultracentrifugation tube, the round bottom flask was rinsed with 2 ml of physiological saline and this was added to the rest of the suspension.

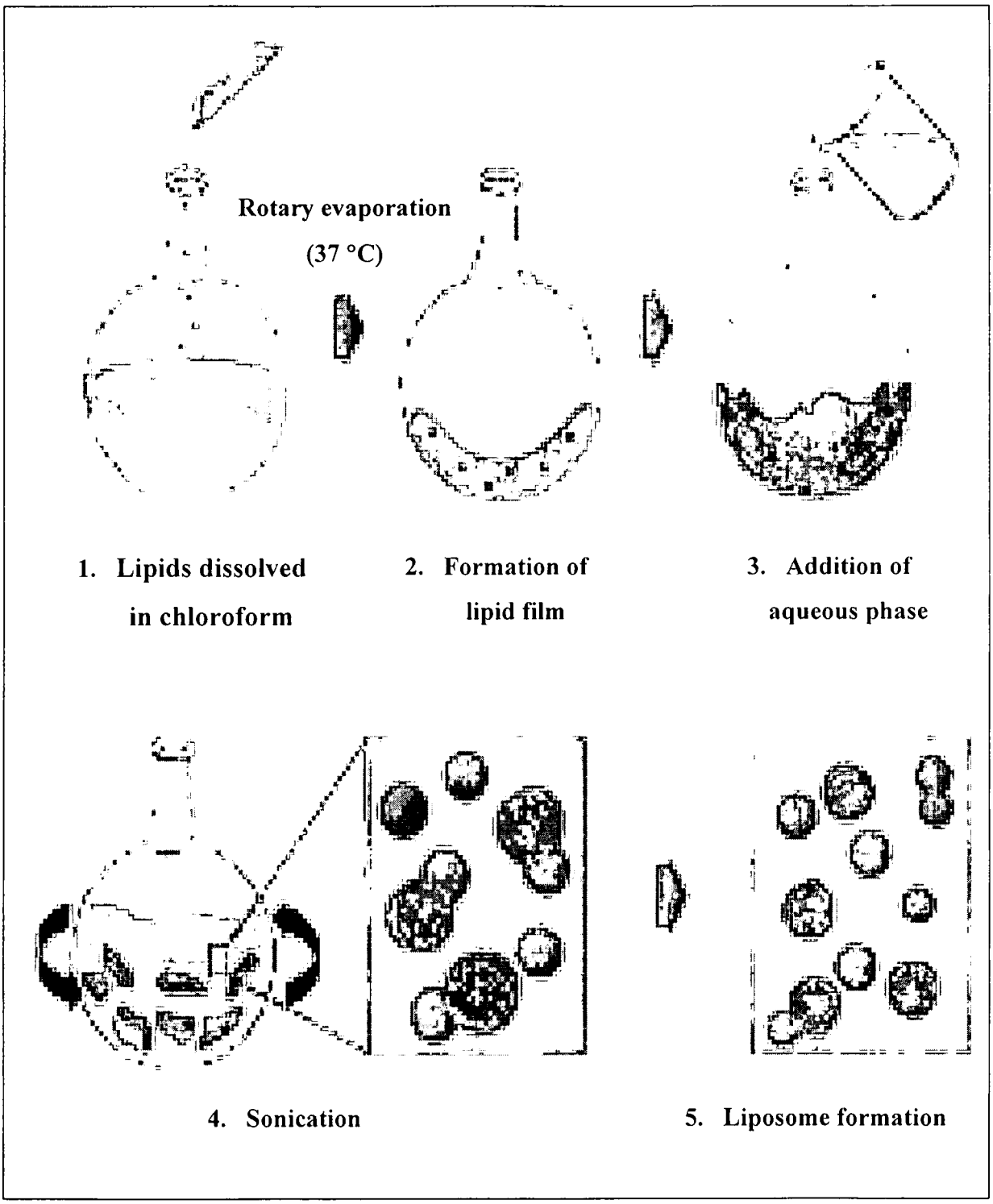


Fig 23 An illustration of stages involved in liposome preparation by hydration. (From http://www.avantilipids.com/PreparationOfLiposomes.html)

7. This was ultracentrifuged at 100 000 g (26 000 rpm) for 45 minutes at 4 °C. The supernatant was removed using a Pasteur pipette and its volume measured.
8. The pellet (liposomes) was resuspended in physiological saline (4 ml) until use.

4.3.1.2 LIPID COMPOSITION FOR THE DIFFERENT LIPOSOMES

The above procedure was used to prepare liposomes of different surface charge by varying the lipid proportions and quantity.

Negative liposomes were prepared using phosphatidyl choline, cholesterol and dicetyl phosphate in the molar ratio 5:1:0.5 respectively. 100 mg of phosphatidyl choline and appropriate amounts of cholesterol and dicetyl phosphate were used i.e.10.25 mg and 7.25 mg respectively.

Positive liposomes were prepared using phosphatidyl choline, cholesterol and stearylamine in the molar ratio of 5:1:0.5 respectively. 100 mg of phosphatidyl choline and appropriate amounts of cholesterol and stearylamine i.e. 10.2 mg and 3.5 mg respectively were used. Stearylamine concentration used is non-toxic, high stearylamine concentration i.e. > 0.5 μmol/ml (134.75 μg/ml) in plasma have been reported to be toxic to cells (Senior et al., 1991; Yoshihara and Nakae, 1986).

Neutral liposomes were initially prepared using phosphatidyl choline and cholesterol in the molar ratio of 8:2 (i.e, phosphatidyl choline, 100 mg and 12.7 mg of cholesterol). During the characterisation process, the molar ratio was changed to 9.7:6.9, such that 100 mg and 36.2 mg of phosphatidyl choline and cholesterol respectively were utilised.

4.3.2 LIPOSOME CHARACTERISATION

4.3.2.1 SIZE AND MORPHOLOGY

Electron microscopy was used to verify that liposomes had been formed, as well as to determine their size and morphology. The liposomes were examined by a negative staining technique using transmission electron microscopy. The whole process was done with the help of a specialist in electron microscopy.

Staining: A drop of liposome suspension, diluted to approximately 40 times with physiological saline, was mixed with one drop of 2 % phosphotungstic acid (PTA) reagent. Phosphotungstic acid reagent was prepared by dissolving 2 g of PTA in 100 ml distilled water, pH adjusted to 7. A drop of the final mixture was placed on a dental wax surface. The carbon side of the grid was placed on the drop, so that the grid floated on the drop for 30 seconds. The excess fluid on the grid was sucked off using a filter paper and the grid was dried. The grid was then examined on the electron microscope (method adapted from Wasserman et al., 1986).

Verification of liposome formation and morphology were by visualisation of the electron micrographs, while the size of liposomes were determined by manually measuring the internal diameters using a ruler. The internal diameter for each liposome was computed by taking the mean of four diameters at different cross sections.

4.3.2.2 ENCAPSULATION EFFICIENCY

A. ESTIMATION OF ENCAPSULATION EFFICIENCY USING GENTAMICIN

Encapsulation efficiency (EE) expresses the competence of liposomes to entrap the added drug. It was calculated using the following equation:

$$EE\% = \left(\frac{Z-X}{Z}\right) \times 100 \qquad \cdots\cdots \text{Eq 1}$$

Z is total amount of drug in the original aqueous phase that was added to the lipid film and X is the total amount drug in the supernatant after ultracentrifugation. Thus, (Z – X) is the total amount of drug associated with liposomes.

The concentration of the marker compound (gentamicin) was determined using the spectrophotometric assay of gentamicin (described in part I, section 3.1.4B). The total amount of gentamicin in the supernatant was obtained by multiplying the gentamicin concentration with the volume of the supernatant. Whenever there was turbidity in the supernatant, it was centrifuged at 12 000 g (13, 400 rpm) for 10 minutes.

B. ESTIMATION OF ENCAPSULATION EFFICIENCY USING METHYL VIOLET

The aim of using methyl violet was to utilise another agent to corroborate the results obtained using the marker compound. Methyl violet is a triarylmethane dye that can be measured directly by spectrophotometry. Methyl violet in aqueous phase was used in liposome preparation and encapsulation efficiency was calculated as described above.

Conditions for assay of methyl violet were standardised in preliminary experiments. The absorption spectra of methyl violet showed maximum absorbance at 584 nm and this wavelength was selected for subsequent reading. Calibration data exhibited linearity between concentration range of 0 to 5 μg/ml ($y = 0.0885\ x - 0.0006$, regression coefficient = 0.9808).

Negative liposomes were prepared as described earlier and a concentration of methyl violet, 3.5 μg/ml, was used in the aqueous phase. The concentration of methyl violet was determined in the supernatant and percentage encapsulation was computed as explained earlier.

4.3.2.3 STABILITY OF LIPOSOMES

It is generally known that liposomes show greater stability when stored at 4 °C than when kept at room temperature or at freezing temperatures, the latter causing greater damage to the liposomes. Therefore stability of the liposomes were tested only at 4 °C. Patent blue violet, a blue coloured dye, was used to determine stability. Of note, methyl violet was used for determination of encapsulation efficiency because by then patent blue violet was not available. Patent blue violet was preferred because it was more water soluble than methyl violet, 8.4 % versus 2.93 %, respectively (Sigma Chemical Co. catalogue). Liposomes containing patent blue violet were stored at 4 °C and were checked for leakage of patent blue violet at 2 weeks.

Conditions for assay of patent blue violet were standardised in preliminary experiments. The absorption spectra of patent blue violet showed maximum absorbance at 685 nm and this wavelength was selected for subsequent reading. Calibration data exhibited linearity between concentration range of 0 to 0.6 mg/ml ($y = 4.7871\ x + 0.0291$, regression coefficient = 0.9982).

Negative liposomes were prepared as described earlier and a concentration of patent blue violet, 0.6 mg/ml (1.06 mM), was used in the aqueous phase. The liposomes were resuspended in 8 ml of plain physiological saline and kept at 4 °C (fridge). After 2 weeks, 100 µl of the supernatant was separated and the dye concentration in it was determined.

4.4 RESULTS

Liposome characterisation: The electron micrographs of the liposomes are shown in Fig 24 to 28. These were multilamellar (i.e., had several lamella) in morphology, up to 18 lamella could be counted. This confirmed a successful preparation of liposomes by the hydration method. The liposomes varied in size from 0.41 to 10 µm (mean diameter) with a mean of 3.17 ± 1.9 µm (n = 75). Ranking of the diameters of the liposomes that were measured revealed that most of the liposomes (78 %) were within 1.38 to 6 µm range, Fig 29 (Appendix B 1).

Encapsulation efficiency: To avoid wastage of the liposomes and expense, encapsulation efficiency was determined on batches of liposomes prepared for the animal experiment. However for demonstration purposes two preparation are presented here. Encapsulation efficiency for two preparations of negative liposomes was 22.01 % for preparation 1 and 21.47 % for preparation 2. This in agreement with the 22.8 % observed with methyl violet.

Liposome stability: On investigation of the supernatant after 2 weeks, it was observed that release of patent blue violet from the liposomes was negligible. Negative readings of -0.2602 and -0.2908 were obtained, notably because concentrations were far below detectable range. Fig 30 shows the two tubes with the supernatant and the patent blue violet entrapped liposomal pellet at 2 weeks. It is obvious from the photograph that the dye colour (concentration) is insignificant in the supernatant compared to the liposomes at the base.

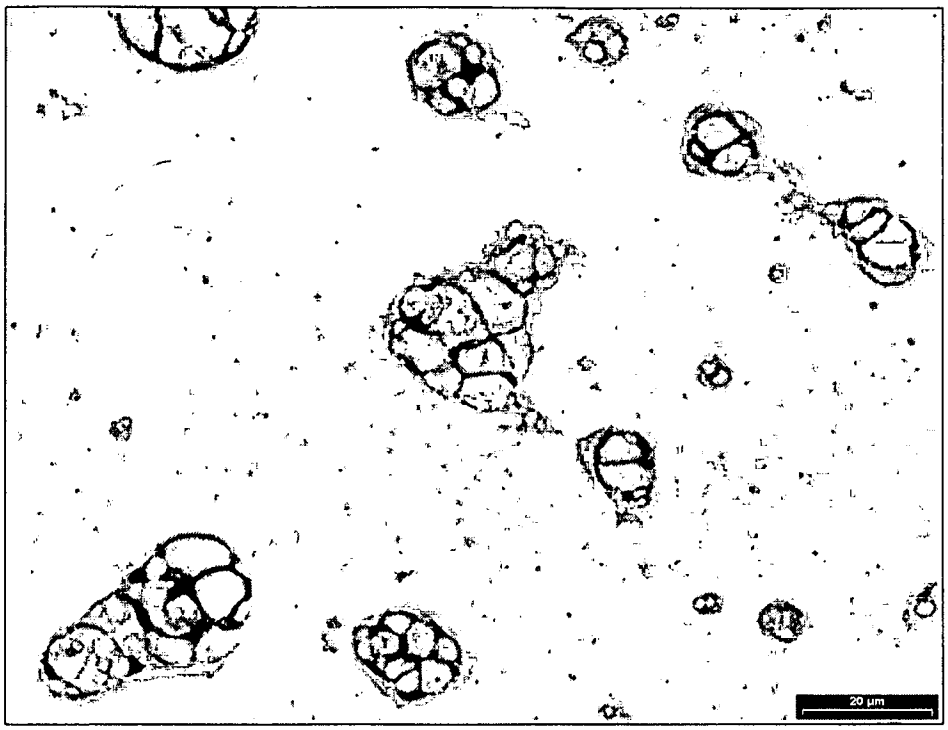


Fig 24 An electron micrograph of a liposome sample, analysed by negative staining technique. Clusters of liposomes are visible, the bar represents 20 µm.

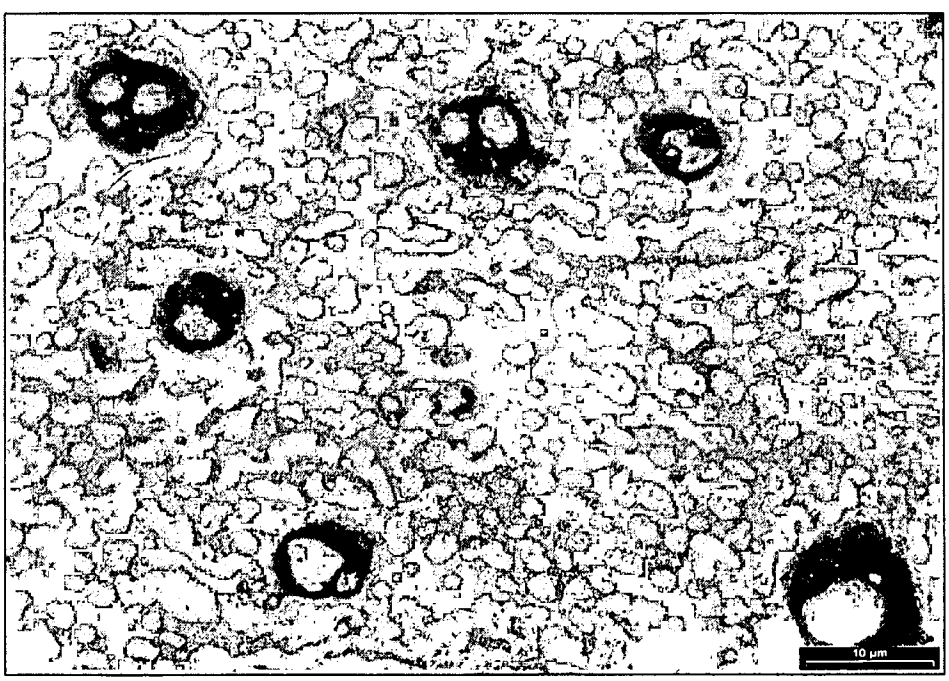


Fig 25 An electron micrograph of a liposome sample, few scattered liposomes are visible, the bar represents 10 µm.

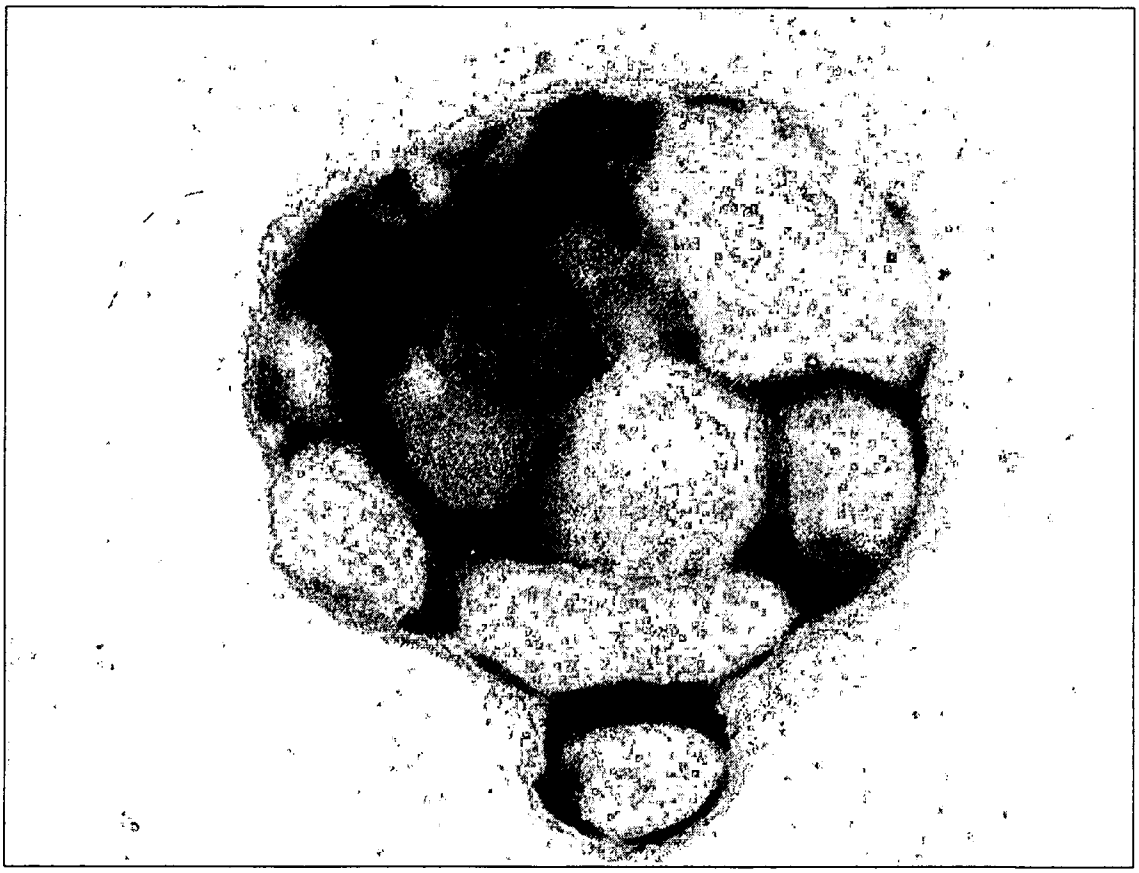


Fig 26 An electron micrograph of a liposome sample, a group of liposomes can be seen, magnification of x3000 has been used.

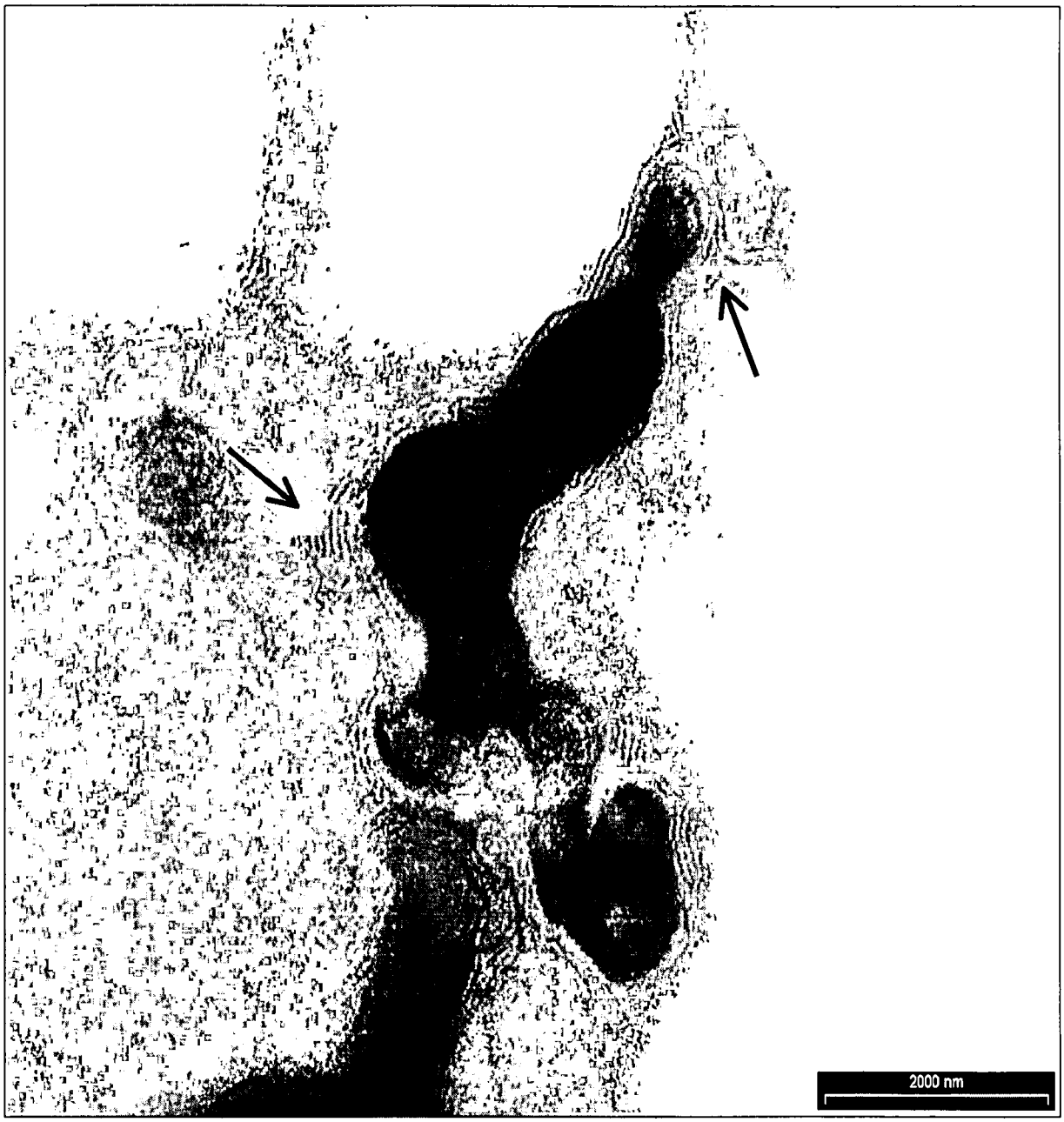


Fig 27 An electron micrograph of a liposome sample, a group of liposomes can be seen and surrounding lamella are also visible (indicated by the arrows), the bar represents 2000 nm.

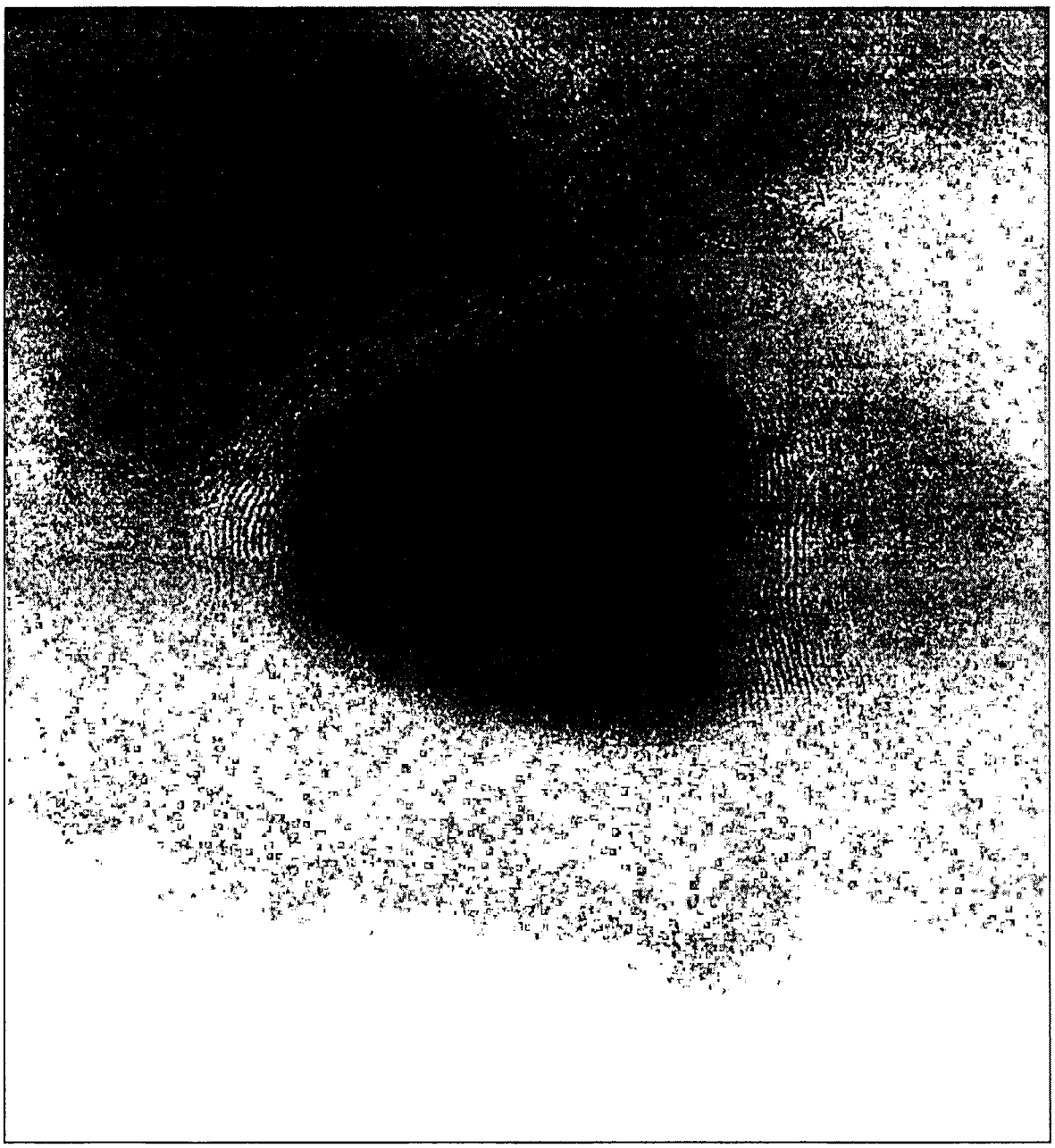


Fig 28 An electron micrograph of a liposome sample, lamella surrounding the liposomes are clearly visible, magnification of 93000 has been used.

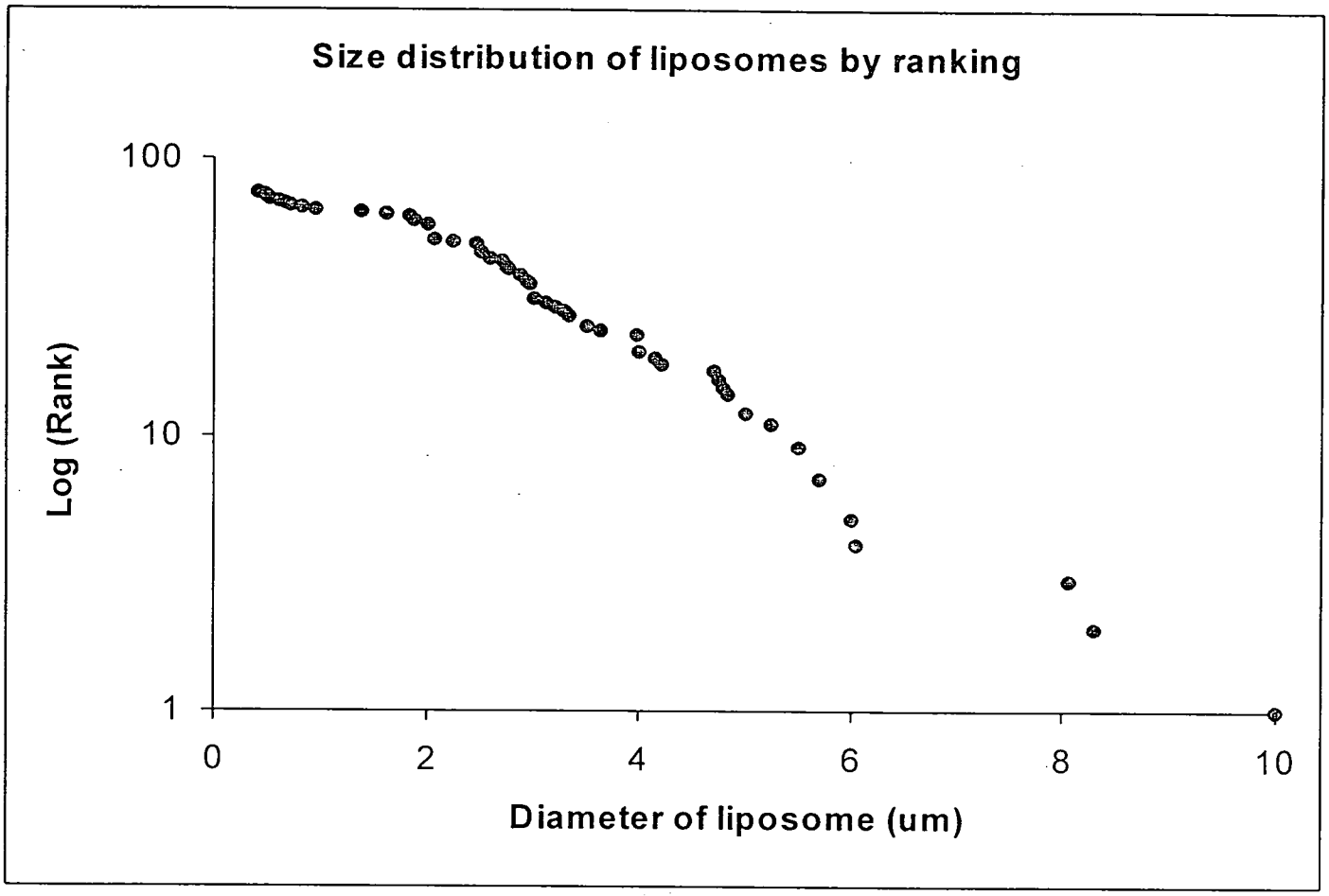


Fig 29 A plot of diameter of liposomes (μm) versus log rank. Most of the liposomes were within 6 μm.

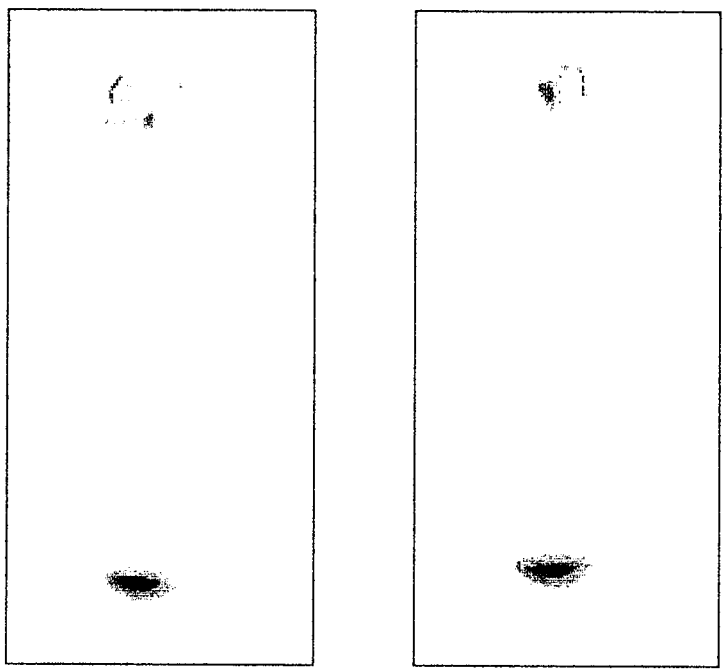


Fig 30 Tubes containing patent blue violet entrapped liposomes stored at 4 °C for 2 weeks.

4.5 DISCUSSION

A simple method for preparation of liposome by hydration was successfully adopted. It produced mutlilammellar liposomes with good encapsulation efficiency that were stable for at least 2 weeks. The method required a short preparation time, approximately 2 hrs including ultracentrifugation of 45 minutes and would be suitable for thermally unstable drugs because it did not involve heating of the aqueous phase. Furthermore, a higher encapsulation efficiency was obtained than observed by other workers (Steger and Desnick, 1977; Jonah et al., 1975; Nabar and Nadkarni, 1998; Wasserman et al., 1986). This could have been probably due to high amount of lipids used.

Electron microscopy revealed that liposomes were of multilamellar nature and although they were heterogeneous, most liposomes were medium in size (1.38 to 6 μm) (Fig 29). The presence of random large liposomes may have be due to fusion of some liposomes, which cannot be avoided Aggregation of liposomes was observed in few of the micographs indicating grouping of the liposomes to a small extent.

Since the liposomes were stable as exhibited by the negligible loss of the drug over 2 weeks, the use of the liposomes within 24 hours meant that there would be practically no loss of drug.

Of note, it would have been desirable to confirm liposomal surface charge by established methods such as the methylene blue partitioning technique or the electrophoretic mobility method but this was not possible due to lack of expertise and facilities. Nevertheless, this has not been done in all the investigations of liposomes reviewed earlier.

In conclusion, this procedure was found acceptable and was used in the next phase of the study: a study of liposomal distribution to different tissues in rats.

Chapter 5

DISTRIBUTION OF LIPOSOMES TO THE RAT BRAIN, LUNGS, KIDNEYS AND LIVER

5.1 INTRODUCTION

In this chapter, studies on the distribution of liposomes to the rat brain, kidneys, liver and lungs are described. These organs were selected because they are among the vital organs in the body that are highly perfused and hence are likely to be exposed to high concentrations of the drug, particularly in the initial distribution phase. Whereas this would be necessary for the diseased organ, it predisposes healthy organs to toxicity. This calls for selective or target therapy whereby liposomes are used to target a diseased organ, thereby reducing exposure of the drug to healthy organs. This would improve the drug's ratio of effectiveness to toxicity.

Therefore, this study was undertaken with a hope that knowledge of the distribution of liposomes with specific surface charge could be used in management of organ specific disease.

5.2 REAGENTS AND APPARATUS

The following materials were used for dissection; ether was obtained from Merck (Darmstadt, Germany), needles (26G x ½", 20 G x ½") and syringes (2 ml and 10 ml) were obtained from Sigma Chemical Co.(Steinheim, Germany). An eppendorf 5810 R centrifuge was used to centrifuge blood samples.

The following were used for extraction of gentamicin from the isolated organs; sodium hydroxide and glacial acetic acid were obtained from Merck (Darmstadt, Germany), sodium dihydrogen phosphate and disodium hydogen phosphate were from BDH laboratory supplies (Poole, England). Sodium chloride was from Sigma Chemical Co. (Steinheim, Germany). A Scaltec normal balance was used to weigh the organs. A 'Labcon' shaking water bath, a 'Ultra-Turrax' (Janke & Kunkel, Kika-Werk) homogeniser and a Beckman, J2-21 centrifuge

were also used. A fully automated Abbott AxSym® analyser was used for gentamicin concentrations measurements.

5.3 METHODS

5.3.1 LIPOSOME PREPARATION

Liposome preparation and their encapsulation efficiency were determined as described in the previous chapter. Encapsulation efficiency (%) of negative, positive and neutral liposomes were determined for each preparation (Table 8, Appendix B 2 to B 5). The corresponding total gentamicin encapsulated for each preparation are presented in table 9. The prepared liposome suspension was stored at 4 °C until use, within 24 hours.

5.3.2 ANIMAL EXPERIMENT

Male Spraque Dawley rats weighing 300 to 350 g were obtained from the animal house, University of the Free State and the study was approved by the University Animal Ethics Committee.

The control consisted of 25 rats that were divided into 5 equal groups. The animals were treated with gentamicin (60 mg/kg) intraperitoneally, after which five animals (one group) were sacrificed at intervals of 1, 2, 4, 6 and 8 hours after dosing (Fig 31).This was repeated but using encapsulated gentamicin in negative, positive and neutral liposomes. All together, 100 rats were used. The dose of the liposomal gentamicin was estimated based on the total amount of gentamicin encapsulated. The average gentamicin encapsulated was approximately 40 mg, so it was possible to use two animals per preparation at a dose of 20 mg of gentamicin. Two liposomal preparations were made on every experimental day such that four animals were tested on every occasion. However, in some instances particularly with neutral liposomes where low encapsulation was obtained, the dose was adjusted so that the same dose per kilogram was given, but to fewer animals. The actual doses of gentamicin administered are given in table 10 (also see appendix B 6).

Table 8 Average encapsulation efficiency (mean ± S.D) of negative, positive and neutral liposomes.

Liposomes	Negative (n = 13)	Positive (n = 13)	Neutral (n = 17)
Encapsulation efficiency (%)	24.1 ± 4.4	23.2 ± 6.2	15.7 ± 4.5

Table 9 Total gentamicin encapsulated (mean ± S.D) in negative, positive and neutral liposomes.

Liposomes	Negative (n = 13)	Positive (n = 13)	Neutral (n = 17)
Total gentamicin encapsulation (mg)	38.6 ± 7.0	37.5 ± 9.3	26.5 ± 7.5

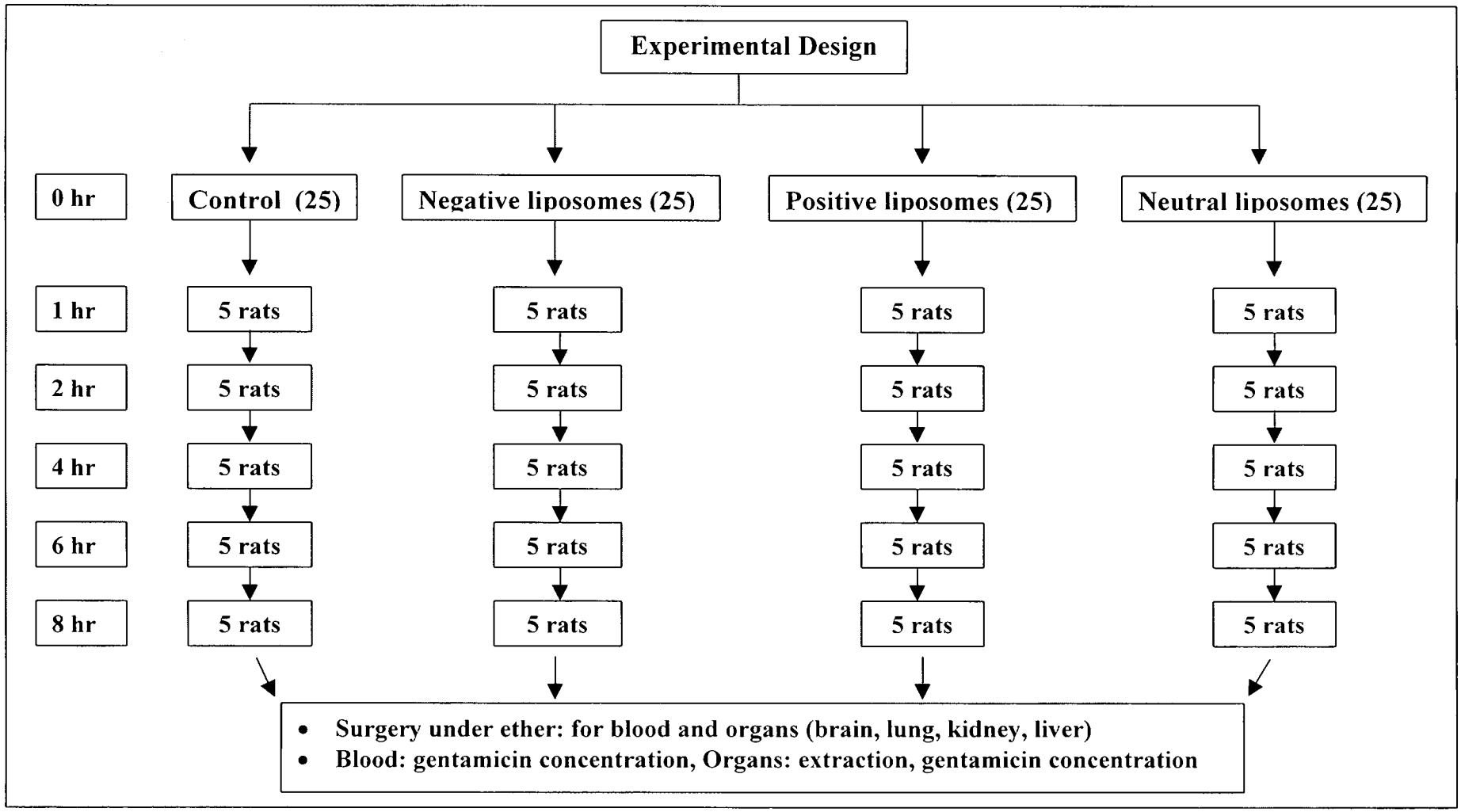


Fig 31 An illustration of the experimental design for the animal experiment.

Table 10 Dose of gentamicin administered (mean ± S.D) in control, negative liposome, positive liposome and neutral liposome treated rats.

Group	Control (n = 25)	Negative liposomes (n = 25)	Positive liposomes (n = 25)	Neutral liposomes (n = 25)
Gentamicin administered (mg)	20	19.7 ± 3.26	19.9 ± 2.93	19.4 ± 4.05

5.3.2.1 SURGICAL PROCEDURE

At the time of sacrifice, rats were anaesthetised with ether, limbs were held down by tapes and the skin over the abdomen was incised and slit followed by the abdominal walls which were held aside (Fig 32). The abdominal vein (posterior vena cava) was exposed by gently clearing aside the covering organs (alimentary canal), fat and soft tissue. Blood was then drawn from the abdominal vein (posterior vena cava) (Fig 33) and transferred to heparinised tubes. These were kept on ice until they were centrifuged (3220 g for 10 min) and plasma separated. Subsequently, the rat was bled by cutting the abdominal artery to remove most of the blood from the organs. The liver, kidneys and lungs were gently detached (Fig 34), washed, dried and placed in individual labelled bags. The rat was then turned over, dorsal side up. The skin covering the skull area was incised and cleared by gently detaching it using a scalpel. The skull was removed in pieces by piercing the interperiatal bone and chipping the bone, using a scalpel. The brain was carefully disassociated and transferred to a labelled bag. The organ bags were stored in liquid nitrogen and extracted the following day.

5.3.2.2 PHARMACOKINETIC ANALYSIS OF PLASMA SAMPLES

Plasma samples were analysed for gentamicin on the same day by fluorescence polarisation immunoassay. Gentamicin plasma concentrations, half life and clearance of the control group were compared with the liposome treated groups. The half life ($T_{1/2}$) of gentamicin between 1 and 4 hours was calculated manually using the formula below;

$$T_{1/2} = \left(\frac{\Delta T}{C_1 - C_2}\right) \times \left(\frac{C_1}{2}\right) \qquad \cdots\cdots \text{Eq 2}$$

where C_1 was the initial concentration (at 1 hour), C_2 was the final concentration (at 4 hours) and ΔT was time taken for the concentration to fall from C_1 to C_2.

Clearance of the control group (Cl_c) between 1 and 4 hours was calculated using the formula (Gibaldi and Perrier, 1982):

$$Cl_c = \frac{D}{AUC_{1-4}} \qquad \cdots\cdots \text{Eq 3}$$

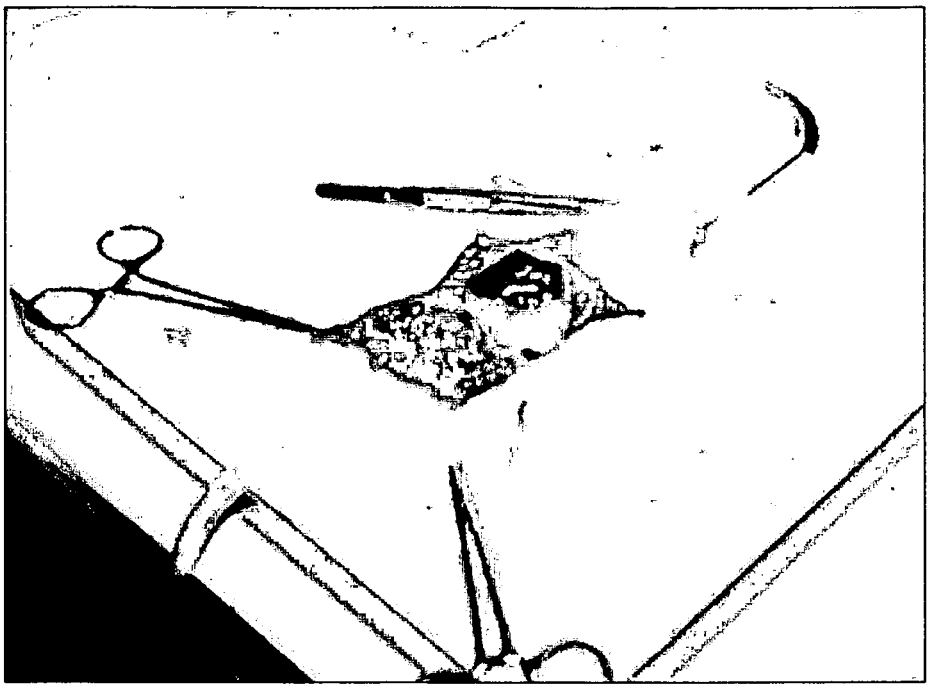


Fig 32 A photograph taken during the surgical procedure on the rat. The rat has been anaesthetised with ether, limbs held down by tapes, and the skin and abdominal walls have been slit and held aside.

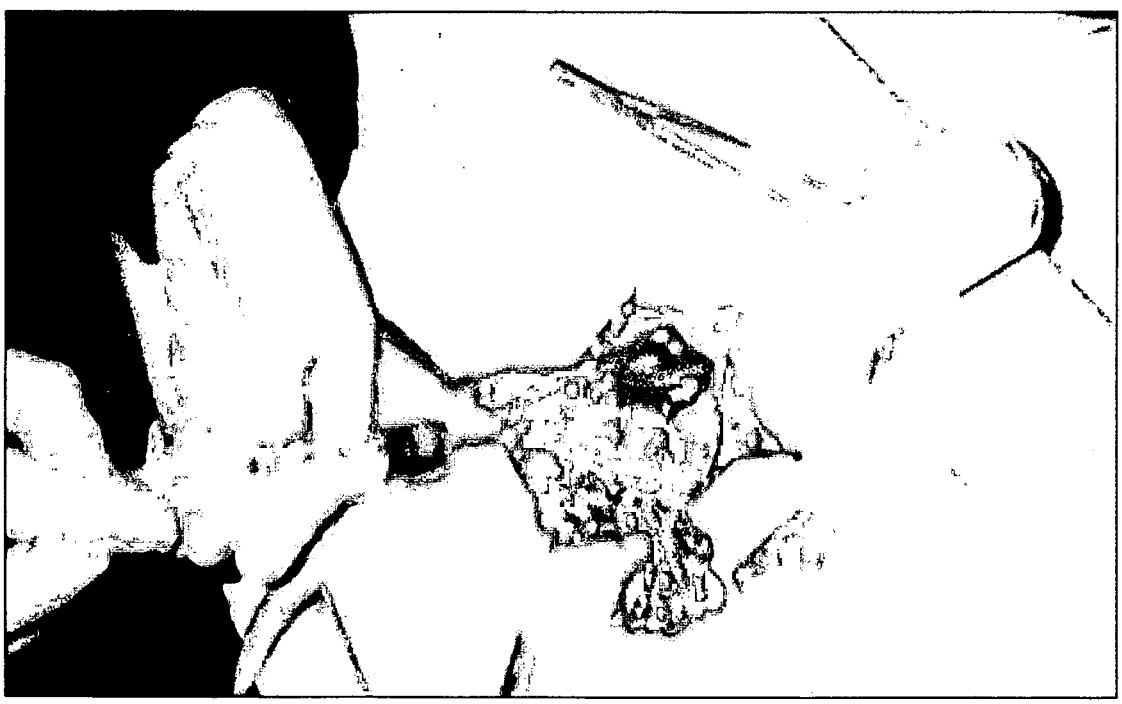


Fig 33 A photograph showing blood being drawn from the abdominal vein.

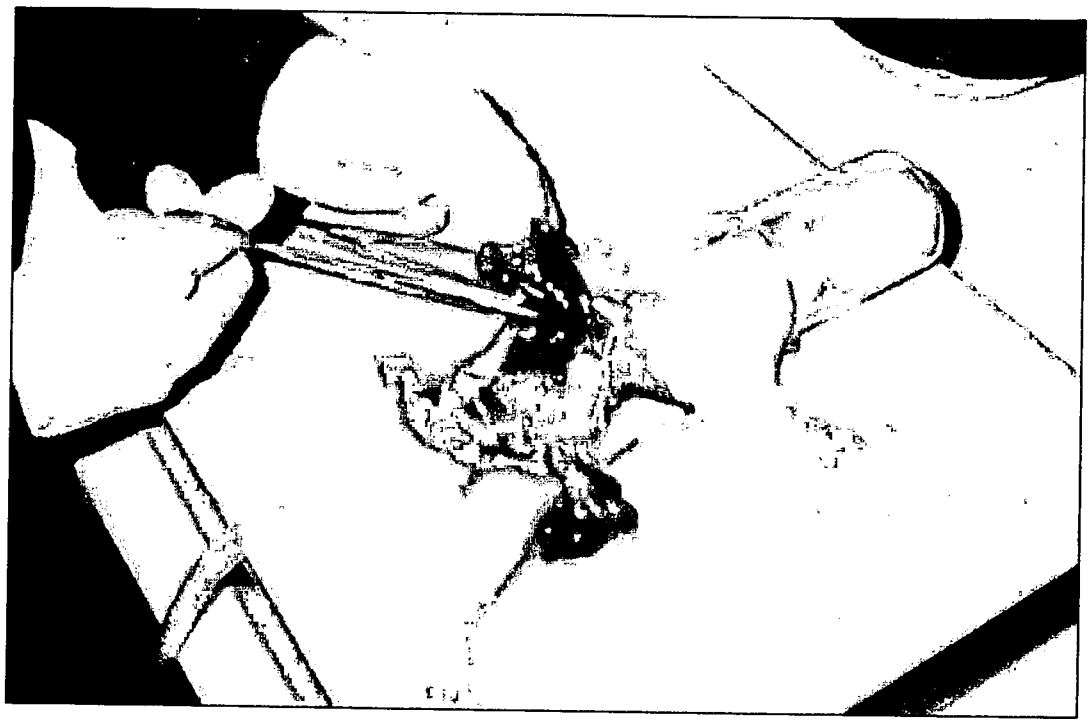


Fig 34 A photograph showing the liver being detached from the abdomen.

Where D was the dose of gentamicin administered, AUC_{1-4} was the area under the curve between 1 and 4 hours, which was calculated using the formula below:

$$AUC_{1-4} = AUC_1 - AUC_4 = \left(\frac{C_1}{k_e} - \frac{C_4}{k_e}\right) \quad \cdots\cdots \text{Eq 4}$$

Where AUC_1 was the area under the curve from 1 hour to infinity, AUC_4 was the area under the curve from 4 hours to infinity, C_1 was the plasma concentration at 1 hour, C_4 was the plasma concentration at 4 hours and k_e was elimination rate constant. Derivation of equation 4 (AUC = C/k) is shown in the Appendix C 4.

k_e was calculated using the formula below (Gibaldi and Perrier, 1982):

$$k_e = \frac{0.693}{T_{1/2}} \quad \cdots\cdots \text{Eq 5}$$

Where $T_{1/2}$ is the half life, calculated as mentioned earlier (Eq 2).

Clearance of gentamicin in the liposome treated groups (Cl_L) was calculated differently, because the concentration of gentamicin measured in plasma may not account for the total dose administered. Clearance (Cl_L) was calculated using the following formula (Gibaldi and Perrier, 1982):

$$Cl_L = V_d \times k_e \quad \cdots\cdots \text{Eq 6}$$

Where k_e was elimination rate constant for each group calculated as mentioned earlier (Eq 5) and V_d was the volume of distribution derived from the control group using the equation below.

$$V_{d(c)} = \frac{Cl_c}{k_{e(c)}} \quad \cdots\cdots \text{Eq 7}$$

$k_{e(c)}$ was elimination rate constant obtained for the control group.

5.3.2.3 EXTRACTION OF GENTAMICIN FROM THE ORGANS

5.3.2.3 A SELECTION OF A METHOD FOR EXTRACTION

The most common method used for extraction of drugs from organs is by homogenisation, but other extraction techniques have also been used. Brown et al. (1988) compared four extraction methods of gentamicin from renal tissue, viz. simple homogenisation, trichloroacetic acid precipitation, sodium hydroxide homogenisation-digestion and sodium hydroxide digestion methods. The methods are described below.

- **Simple homogenisation**: Phosphate buffered saline (PBS) was added to the sample tissue (2 ml/g tissue) and incubated at 25 °C for 1 hour. The mixture was then homogenised. The homogeniser was rinsed and the rinses were pooled with the homogenate. This was then centrifuged (1,000 x g, 10 min) and the supernatant was used for measurement of gentamicin.
- **Trichloroacetic acid precipitation**: Phosphate buffered saline (PBS) was added to the sample tissue (1 ml/g tissue) and incubated at 25 °C for 1 hour. To this 50 % trichloroacetic acid (TCA) was added, 3.3 ml/g tissue. The mixture was then homogenised. The homogeniser was rinsed and the rinses were pooled with the homogenate and then centrifuged (1,000 x g, 10 min). The supernatant was transferred to another tube and the pH adjusted to approximately 7.5 with either 10 N or 1 N sodium hydroxide, and then used for measurement of gentamicin.
- **Sodium hydroxide (NaOH) homogenisation-digestion**: Phosphate buffered saline (PBS) was added to the sample tissue (1 ml/g tissue) and incubated at 25 °C for 1 hour. The mixture was then homogenised, the homogeniser was rinsed with 2 volumes of PBS, which was pooled with the original homogenate. To this an equal volume of 2 N NaOH was added and the mixture was incubated at 70 °C for 20 min. The digested homogenate was cooled to room temperature, pH adjusted to 7.5 ± 0.2 (with either 10 % acetic acid or 1 % acetic acid and 1 N NaOH) and used for measurement of gentamicin.
- **Sodium hydroxide (NaOH) digestion**: Phosphate buffered saline (PBS) was added to the sample tissue (4 ml/g tissue) and incubated at 25 °C for 1 hour. An equal volume of 2 N NaOH was then added and the mixture was incubated at 70 °C for 20 min. The digested homogenate was cooled to room temperature, pH adjusted to 7.5 ± 0.2 (with either 10 % acetic acid or 1 % acetic acid and 1 N NaOH) and used for measurement of gentamicin.

In all methods, gentamicin was measured by fluorescence polarization immunoassay (FPIA).

Below, the simple homogenisation, NaOH digestion and NaOH homogenisation-digestion methods were compared, after which a more appropriate procedure was adopted. For the purpose of this study, trichloroacetic acid precipitation method was excluded due to the reagent's corrosive nature and price, considering the large volumes that would be required.

Preparation of standard solutions

Phosphate buffered saline, pH 7.4 ± 0.1, was prepared by dissolving sodium chloride 8.18 g (140 mM), sodium dihydrogen phosphate 0.27 g (1.72 mM) and disodium hydrogen phosphate 1.28 g (9.05 mM) in 1 litre of deionised water. 2 N NaOH was prepared by dissolving 80 g of NaOH in 1 litre of deionised water. And one litre of 10 % acetic acid, was prepared by mixing 100 ml of glacial acetic acid with 900 ml of deionised water.

Comparison of simple homogenisation and NaOH digestion methods

Gentamicin 8 mg/kg was administered intravenously to a rat, the relevant organs were harvested 40 minutes post injection. The organs were cut into approximately equal parts, weights of which were recorded. One half of each organ was extracted by simple homogenisation and the other half by NaOH digestion method. The procedures were used as described above, except that, for the NaOH digestion the kidney and the liver samples were cut to smaller fractions since they were heavier. Gentamicin in the extracted samples was measured by fluorescence polarisation immunoassay on an AxSym® analyser (Abbott laboratories).

Results: Compared to the NaOH digestion method, gentamicin concentrations determined by simple homogenisation method were 3.6 times less in the brain, 2. times less in the kidney and lungs, and 3 times less in the liver (Table 11). In conclusion, the NaOH digestion method was superior to simple homogenisation method for all organs. However, it was observed that the lungs remained intact during the NaOH digestion while the other organs had completely disintegrated. which raised concern regarding extent of recovery of gentamicin. On the other hand, although the liver was cut to smaller fragments before the digestion process, it was essential to determine if further size reduction by homogenisation would improve recovery of gentamicin. Therefore, it was decided to compare recovery of gentamicin by the NaOH digestion and the NaOH digestion – homogenisation methods.

Table 11 Comparison of gentamicin concentration after extraction by simple homogenisation and NaOH digestion methods.

Organ	Gentamicin concentration / weight of organ (μg/g)	
	Simple homogenisation	**NaOH digestion**
Brain	0.58	2.08
Kidneys	1.08	2.40
Liver	36.92	112.16
Lungs	1.66	3.52

Comparison of NaOH digestion and NaOH digestion-homogenisation methods

For this experiment the rat was dosed with gentamicin, 8 mg/kg intraperitoneally and the organs were harvested 2 hours post injection. The organs were similarly cut into approximately equal parts, weights of which were recorded. One half of each organ was extracted by NaOH digestion and the other half by NaOH digestion-homogenisation method. Gentamicin in the extracted samples was also measured by fluorescence polarisation immunoassay on an AxSym® analyser (Abbott laboratories).

Results: The results showed that the lungs had higher gentamicin concentrations when it was extracted by NaOH digestion-homogenisation than NaOH digestion method (Table 12). For the liver, the NaOH digestion-homogenisation method yielded lower gentamicin concentrations.

5.3.2.3 B ADOPTED EXTRACTION PROCEDURE

Based on the above results, the NaOH digestion method was used for extraction of gentamicin from the brain, liver and kidney, while the NaOH digestion-homogenisation method was used for the lungs. This is described below.

The organs were weighed in beakers and the weights were recorded. The kidney and the liver were then cut into smaller pieces. Scissors were washed after each cut to avoid contamination. Phosphate buffered saline (PBS) was added to the organs in a ratio of 4 ml per gram of tissue, except for the lungs where it was added in the ratio of 1 ml per gram of tissue. Since it was observed that the organs were usually within specific weight ranges, fixed volumes of PBS were allotted for each organ (table 13). If the weight of the organ varied from this range, the volume was calculated using the 4 ml/g ratio. Of note, the ratio of PBS added to the lung was different because it was extracted by NaOH digestion-homogenisation method.

The beakers were then covered with laboratory sealing film and incubated at 25 °C for 1 hour in a shaking water bath, after which equal volumes of 2 N NaOH were added to each organ. The lungs were first homogenised before the addition of 2 N NaOH. These were transferred to plastic tubes and homogenised at medium speed until complete disintegration of the organ was achieved. The homogeniser was rinsed with 2 ml of PBS, and rinse was pooled with the homogenate.

Table 12 Comparison of recovery of gentamicin after extraction by NaOH digestion and NaOH digestion-homogenisation methods.

Organ	Gentamicin concentration / weight of organ (ug/g)	
	NaOH digestion	NaOH digestion-homogenisation
Lungs	0.23	3.25
Liver	2.83	0.75

Table 13 Weight range of organs: brain, kidneys, liver and lungs observed in the animals and the volume of phosphate buffered saline (PBS) that was allotted for each organ in that range.

Organ	Weight range (g)	Volume of PBS added (ml)
Brain	1 - 2	6
Kidneys	1.5 – 2.5	7
Liver	8 -10	35
Lungs	1 - 2	2

Next, the contents were incubated at 70 °C for 20 min. After incubation, the digested extracts were cooled to room temperature. Samples were handled in a fume hood using masks and gloves at all times. The pH of the organ digests were adjusted to 7.4 ± 0.2 by adding equal volumes of 10 % acetic acid (standardised previously), after which, the samples were centrifuged (10,000 g) for 15 min. Supernatants were separated and analysed for gentamicin by fluorescence polarisation immunoassay (FPIA). Gentamicin concentrations in the organs were calculated as amount of gentamicin per weight of the organ, using the formula below.

$$\text{Gentamicin/Organ weight } (\mu g/g) = \left(\frac{Cs\ (\mu g/ml) \times Vs\ (ml)}{Wo\ (g)} \right) \quad \cdots\cdots \text{Eq 8}$$

Where, Cs is the concentration of gentamicin in the supernatant, Vs is the volume of the supernatant and Wo is the weight of the organ. Gentamicin in the different organs of the control group were compared with the liposome treated groups.

5.4 DATA ANALYSIS

Results were expressed as mean ± standard deviation (S.D.). Comparison of groups were done by the Mann-Whitney test (non-parametric test), with P = 0.05 as limit of significance using the Graph pad-InStat program.

5.5 RESULTS

5.5.1 ENCAPSULATION EFFICIENCY OF LIPOSOMES

Encapsulation efficiencies achieved appeared to be satisfactory (> 15 %). In general, encapsulation efficiency was in order of: negative > positive > neutral (Table 8), but there was no significant difference (P > 0.9999) between negative and positive liposomes. On the other hand, when encapsulation in negative and positive liposomes was compared to neutral liposomes, negative liposomes exhibited significantly higher encapsulation (P < 0.0001) than positive liposomes.

5.5.2 GENTAMICIN PROFILES

Results are reported below in separate sections under plasma and organs viz. brain, kidney, liver and lung.

5.5.2.1 PLASMA GENTAMICIN LEVELS

In the control group, high plasma concentrations of gentamicin were observed initially, 112.3 ± 34.1 μg/ml at 1 hour and this declined rapidly (Fig 35 a & b). The elimination of gentamicin was biphasic with an initial rapid elimination phase from 1 to 4 hours with half-life (t ½) of 1.53 ± 0.02 hours, followed by a slower elimination phase (t ½ = 2.29 ± 0.23 hours) between 4 and 8 hours (Also see semi-log plot, appendix C 3a). Gentamicin plasma concentration was 2.1 ± 0.7 μg/ml at 4 hours and 0.2 ± 0.2 μg/ml at 8 hours (Table 14).

On the other hand, the liposome groups exhibited significantly lower plasma gentamicin concentrations than the control during the initial period. Specifically, for negative and positive liposome treated groups, gentamicin concentrations were lower at 1 to 2 hours ($P = 0.008$), while the neutral liposomes was lower between 1 and 4 hours ($P = 0.008$) (Fig 35 to 38, Table 14).

In the later hours, there was no significant difference in gentamicin concentrations between the control and liposome treated groups, specifically, from 4 to 6 hours in the negative liposome treated group, 4 to 8 hours in the positive liposome treated group and 6 to 8 hours in the neutral liposome treated group. Interestingly, the negative liposome group exhibited a sustained gentamicin concentration between 4 and 8 hours, of 1.33 ± 1.0 to 1.31 ± 0.3 μg/ml, respectively (Fig 36 a & b). Also, gentamicin plasma concentration in the negative liposome treated group was significantly higher ($P < 0.05$) than the control and other liposome treated groups during this period. These observations indicate that liposomal formulations lead to lower concentrations of gentamicin in plasma and negative liposomes lead to greater retention of gentamicin in plasma than other liposomes.

Among the liposome treated groups, plasma gentamicin concentration between 1 to 4 hours was highest in the positive liposome treated group (Fig 35 to 38, Table 14). Gentamicin plasma concentrations were in the order: positive > negative > neutral. However, there was no significant difference between the three liposome groups during this time. Between 6 and 8 hours, the negative liposome treated group exhibited higher gentamicin plasma concentrations but this was only significant ($P < 0.05$) from those of positive and neutral liposome treated groups at 8 hours. These observations suggest that positive liposomes released gentamicin in plasma faster than negative and neutral liposomes.

The half life of gentamicin in the liposome treated groups between 1 and 4 hours was longer than in the control (Table 15, Appendix C 5a to C 5d). A similar trend was also observed in the other elimination parameters, i.e., clearance and the elimination rate constant were lower (Table 15, Appendix C 5a to C 5d). However, there was no difference between the elimination parameters (half life, clearance and elimination rate constant) among the liposomal treated groups. Of note, variations in area under curves for the different groups did not relate to the elimination parameters. Pharmacokinetic analysis was not done after 4 hours because individual plots of the liposome treated groups did not exhibit uniform elimination characteristics; in most cases liposome treated groups exhibited a rise in concentration at 8 hours (? second peak) thereby giving negative values for the half life.

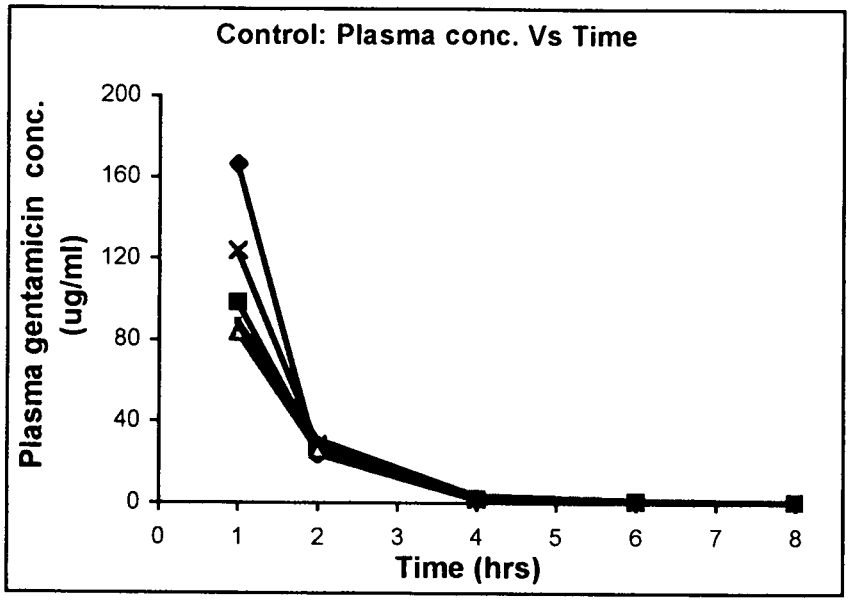


Fig 35a A plot of plasma gentamicin concentration versus time for the control group (treated with free gentamicin). Each point on the graph represents an individual animal (Appendix C 2a, plasma).

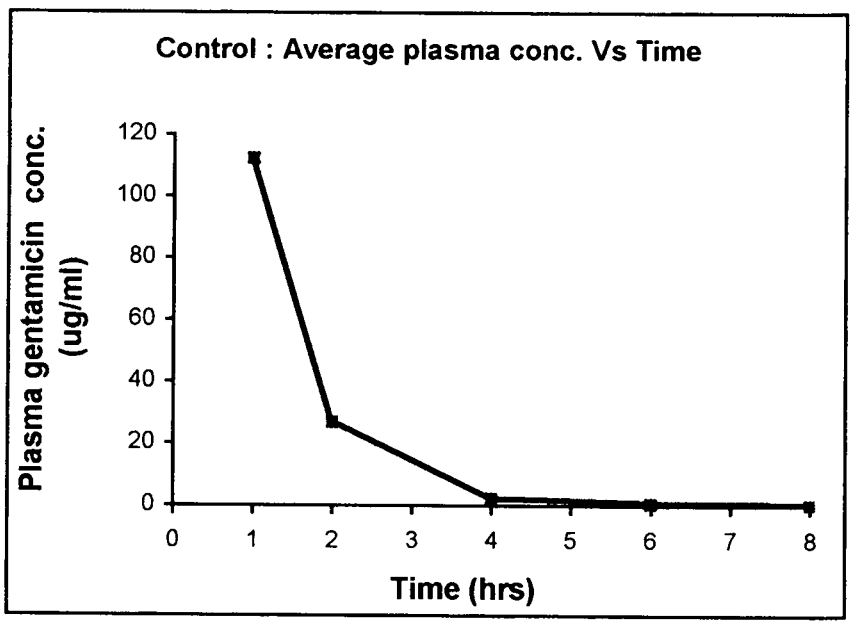


Fig 35b A plot of average plasma gentamicin concentration versus time for the control group (treated with free gentamicin). Each point on the graph represents an average concentration for five animals.

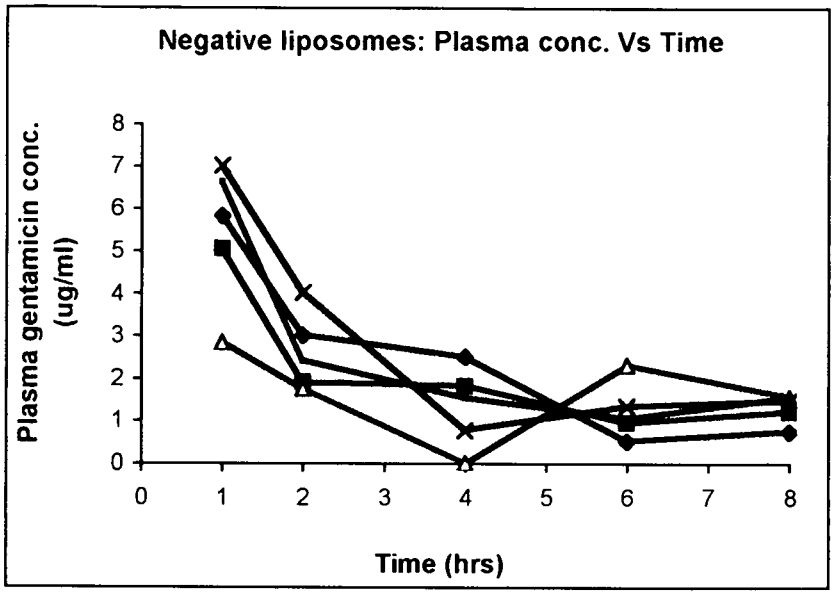


Fig 36a A plot of plasma gentamicin concentration versus time for the negative liposome group. Each point on the graph represents an individual animal (Appendix C 2b, plasma).

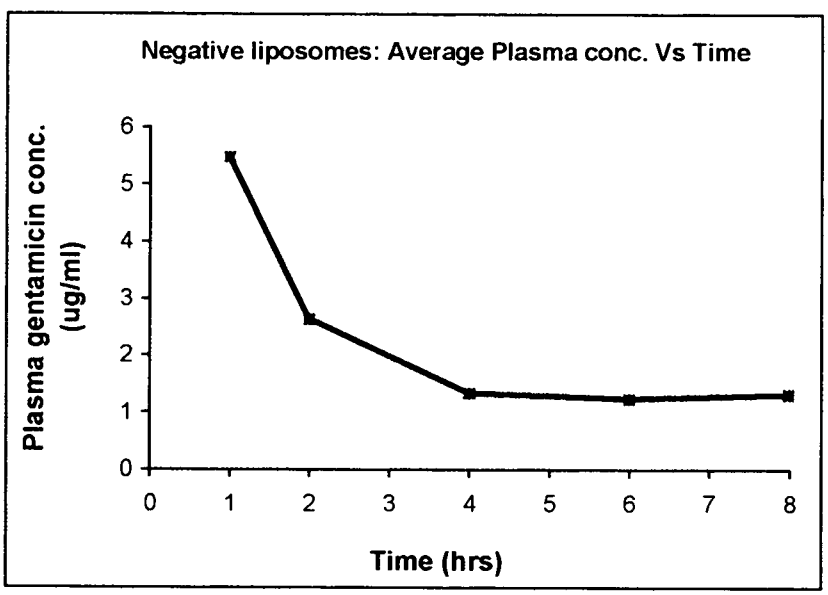


Fig 36b A plot of average plasma gentamicin concentration versus time for the negative liposome group. Each point on the graph represents an average concentration for five animals.

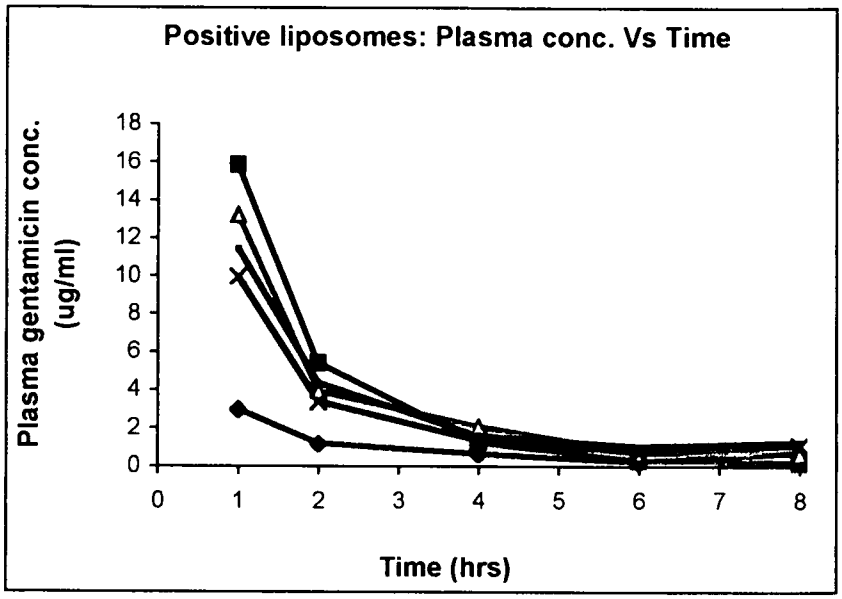


Fig 37a A plot of plasma gentamicin concentration versus time for the positive liposome group. Each point on the graph represents an individual animal (Appendix C 2c, plasma).

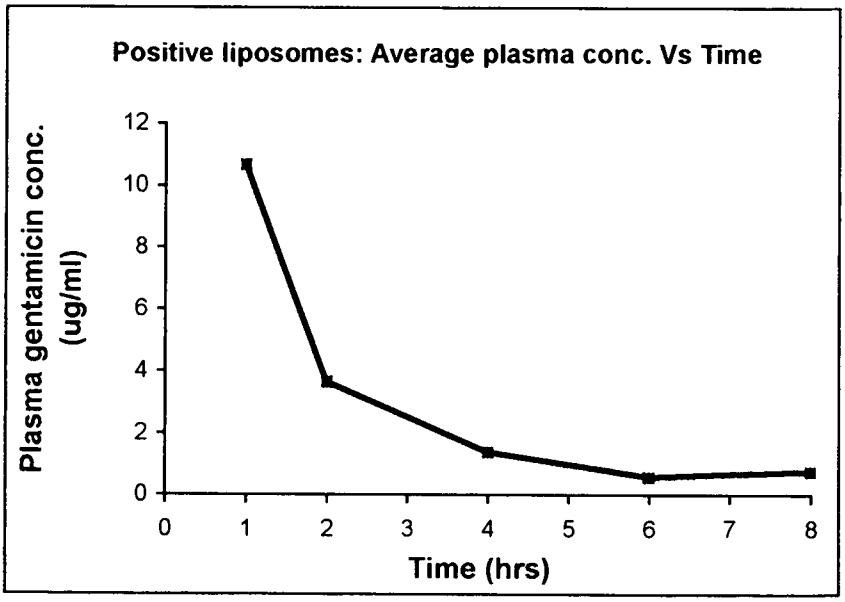


Fig 37b A plot of average plasma gentamicin concentration versus time for the positive liposome group Each point on the graph represents an average concentration for five animals.

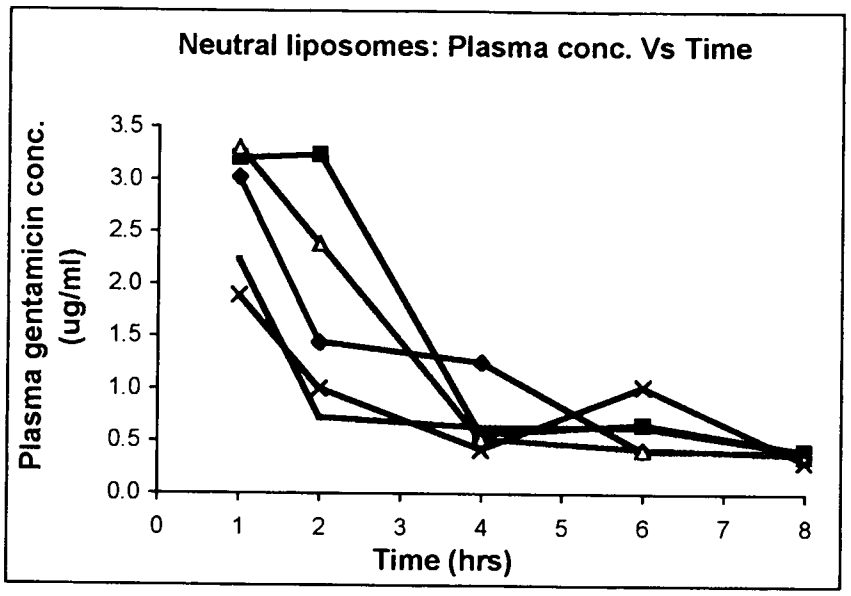


Fig 38a A plot of plasma gentamicin concentration versus time for the neutral liposome group. Each point on the graph represents an individual animal (Appendix C 2d, plasma).

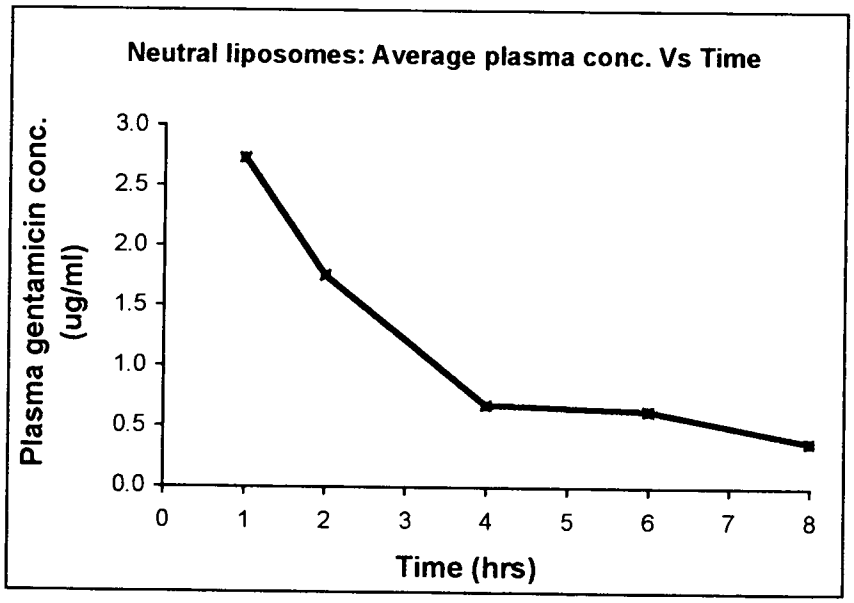


Fig 38b A plot of average plasma gentamicin concentration versus time for the neutral liposome group. Each point on the graph represents an average concentration for five animals.

Table 14 Plasma gentamicin concentration (mean ± S.D) obtained for the control, negative liposome, positive liposome and neutral liposome treated groups at different time intervals (1 to 8 hours). P values obtained by comparison with the control group are indicated in parenthesis.

Time (hours)	Plasma gentamicin concentration (μg/ml)			
	Control	Negative liposomes	Positive liposomes	Neutral liposomes
1	112.3 ± 34.1	5.47 ± 1.7 (0.008)	10.67 ± 4.9 (0.008)	2.73 ± 0.6 (0.008)
2	26.9 ± 2.8	2.63 ± 0.9 (0.008)	3.63 ± 1.6 (0.008)	1.75 ± 1.0 (0.008)
4	2.1 ± 0.7	1.33 ± 1.0 (0.310)	1.37 ± 0.5 (0.095)	0.68 ± 0.3 (0.008)
6	0.6 ± 0.2	1.24 ± 0.7 (0.056)	0.57 ± 0.3 (0.841)	0.63 ± 0.2 (0.917)
8	0.2 ± 0.2	1.31 ± 0.3 (0.008)	0.75 ± 0.4 (0.095)	0.38 ± 0.0 (0.151)

Table 15 Pharmacokinetic parameters (mean ± S.D) of the control, negative liposome, positive liposome and neutral liposome treated rats between 1 and 4 hours.

Parameter	Control	Liposomes		
		Negative	Positive	Neutral
Half life - T½ (hr)	1.53 ± 0.02	2.02 ± 0.5	1.76 ± 0.1	2.04 ± 0.3
Clearance – Cl (ml/min)	1.46 ± 0.4	1.16 ± 0.3	1.28 ± 0.1	1.12 ± 0.2
Elimination rate constant - K_e (h^{-1})	0.45 ± 0.01	0.36 ± 0.1	0.4 ± 0.02	0.35 ± 0.05
Area under the curve from 1 to 4 hours - AUC_{1-4} (μg.hr/ml)	242.89 ± 73.4	11.85 ± 3.6	23.09 ± 10.5	5.91 ± 1.4

5.5.2.2 ORGAN DISTRIBUTION

A. Brain gentamicin levels

In the control group, gentamicin was detectable only at 1 and 2 hours (Fig 39 a & b, Table 16), while it was detectable at almost all time points in the liposome treated groups. The distribution to the brain exhibited a biphasic pattern in the individual plots of the liposome groups, which was more marked in the negative liposomes.

Compared to the control, the positive liposomes exhibited highest gentamicin levels in brain (Fig 40 a & b), followed by neutral liposomes (Fig 41 a & b). The negative liposomes, interestingly, demonstrated a biphasic pattern of distribution. There was a rapid decline in gentamicin concentration such that by 4 hours the concentration was zero, followed by a rapid increase (Fig 42 a & b).

In general, the average concentrations of gentamicin in the brain at different times for the liposome treated groups was in the order: positive > neutral > negative (Table 16). These results signify that positive liposomes had greater affinity to the brain than negative or neutral liposomes.

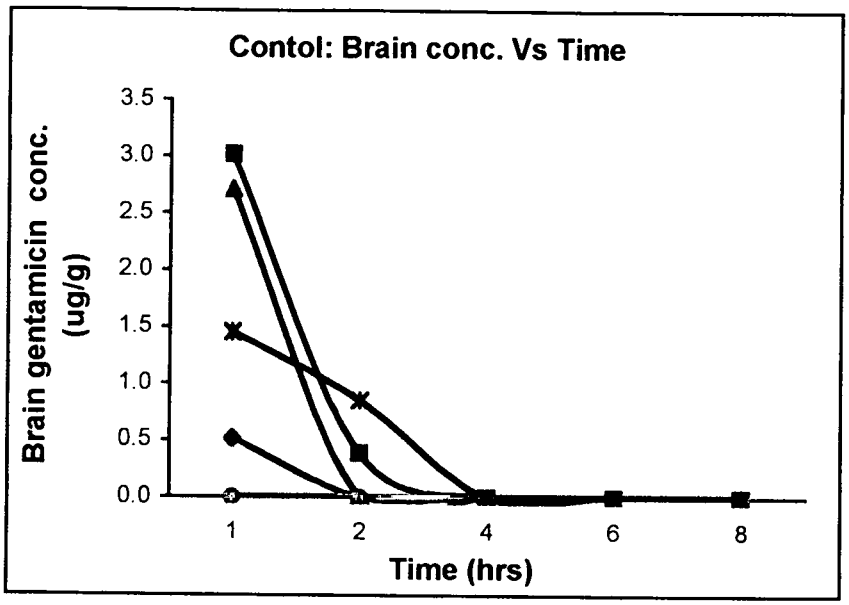


Fig 39a A plot of brain gentamicin concentration versus time for the control group (treated with free gentamicin). Each point on the graph represents an individual animal (Appendix C 2a, brain).

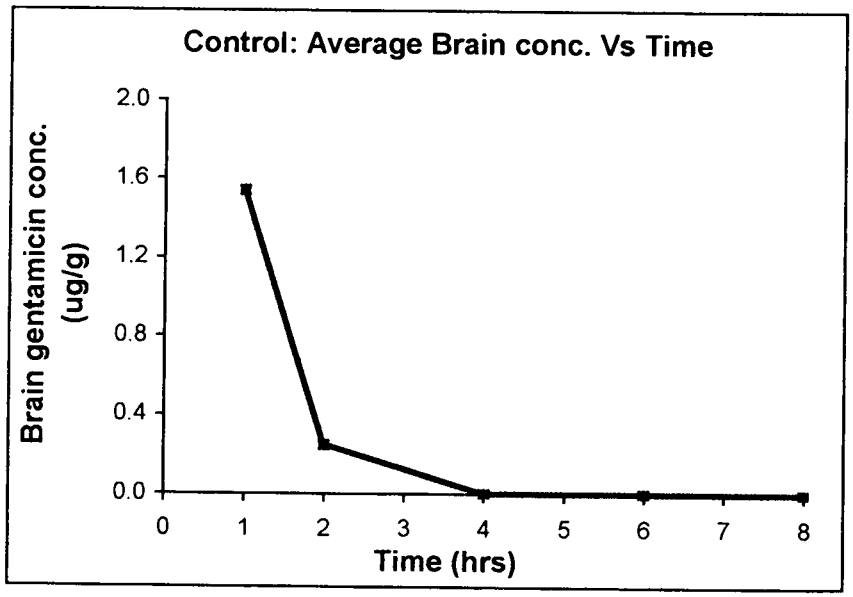


Fig 39b A plot of average brain gentamicin concentration versus time for the control group (treated with free gentamicin). Each point on the graph represents an average concentration for five animals.

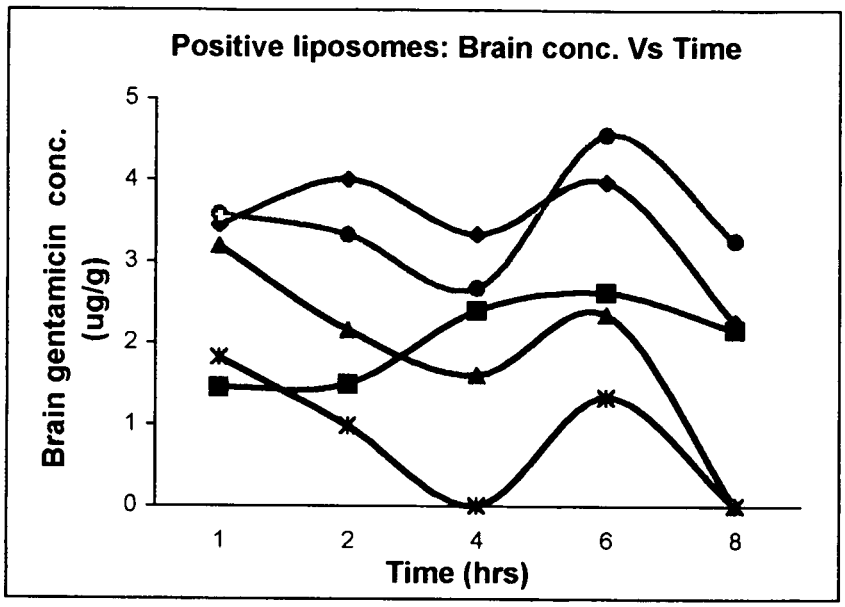


Fig 40a A plot of brain gentamicin concentration versus time for the positive liposome treated group. Each point on the graph represents an individual animal (Appendix C 2c, brain).

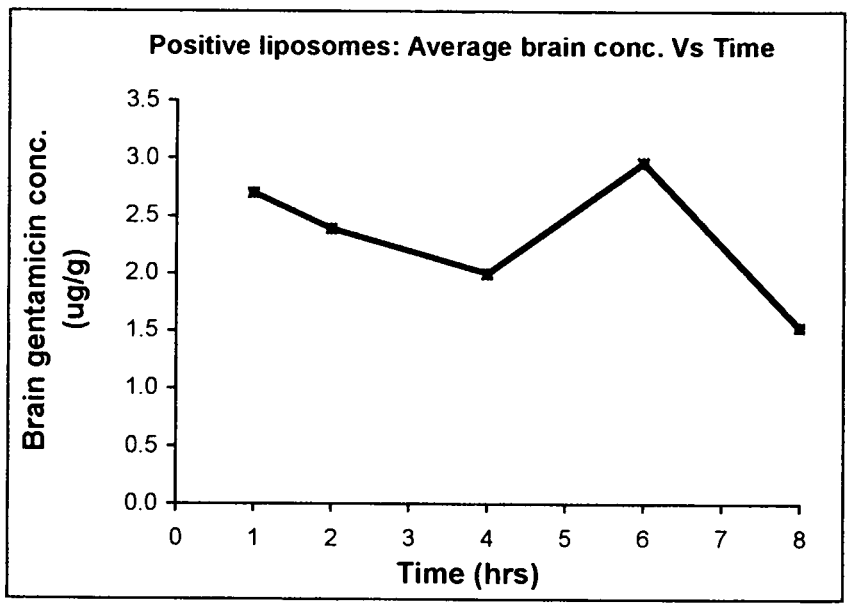


Fig 40b A plot of average brain gentamicin concentration versus time for the positive liposome treated group. Each point on the graph represents an average concentration for five animals.

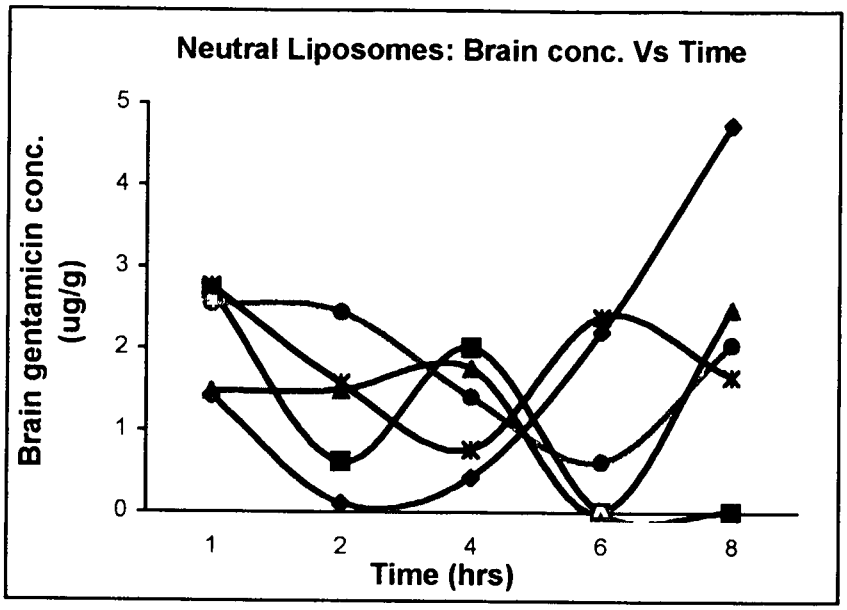


Fig 41a A plot of brain gentamicin concentration versus time for the neutral liposome treated group. Each point on the graph represents an individual animal (Appendix C 2d, brain).

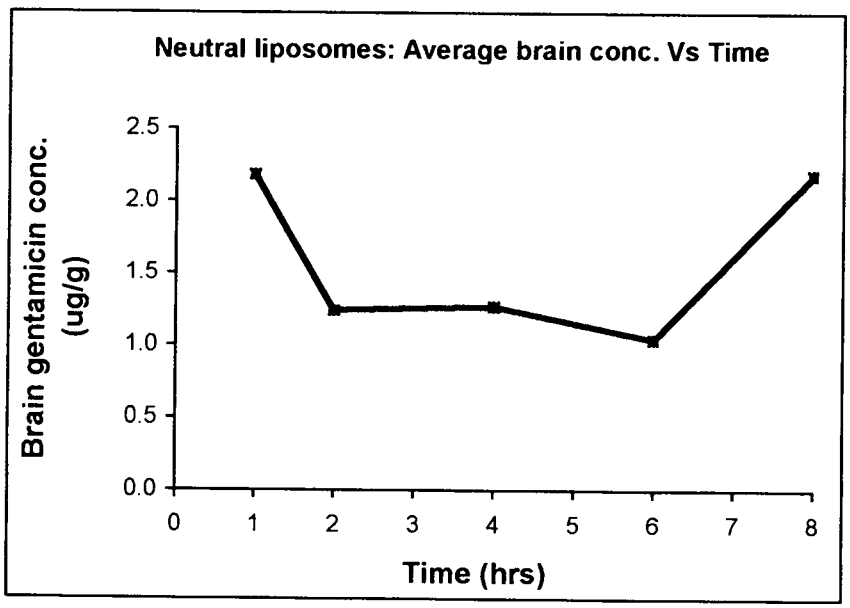


Fig 41b A plot of average brain gentamicin concentration versus time for the neutral liposome treated group. Each point on the graph represents an average concentration for five animals.

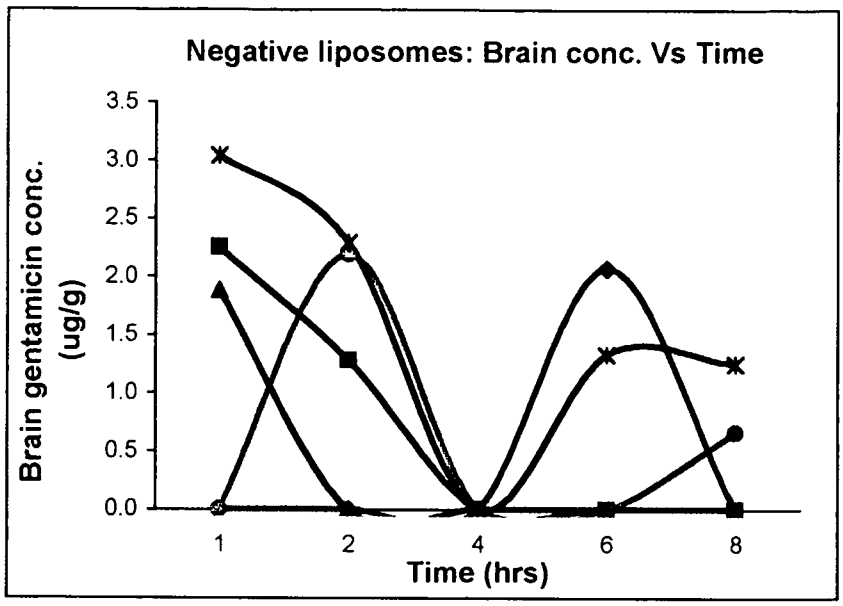


Fig 42a A plot of brain gentamicin concentration versus time for the negative liposome treated group. Each point on the graph represents an individual animal (Appendix C 2b, brain).

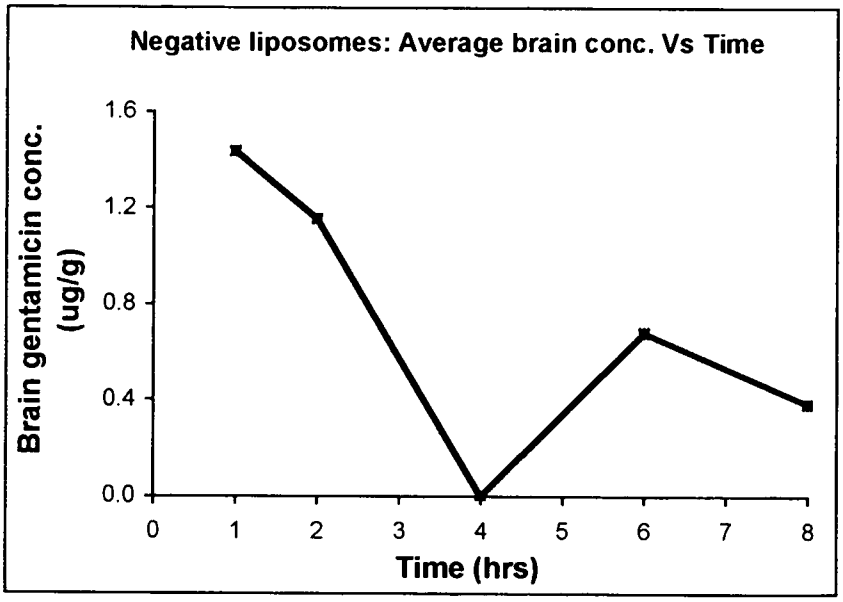


Fig 42b A plot of average brain gentamicin concentration versus time for the negative liposome treated group. Each point on the graph represents an average concentration for five animals.

Table 16 Brain gentamicin concentrations (mean ± S.D) obtained for the control, negative liposome, positive liposome and neutral liposome treated groups at different time intervals (1 to 8 hours). P values obtained by comparison with the control group are indicated in parenthesis.

Time (hours)	Brain gentamicin concentration (μg/g)			
	Control	Negative liposomes	Positive liposomes	Neutral liposomes
1	1.54 ± 1.3	1.43 ± 1.4 (> 0.9999)	2.7 ± 0.99 (0.117)	2.18 ± 0.7 (0.601)
2	0.25 ± 0.4	1.15 ± 1.1 (0.290)	2.39 ± 1.26 (0.008)	1.24 ± 0.9 (0.056)
4	0.0 ± 0.0	0.0 ± 0.0	2.00 ± 1.3	1.27 ± 0.7
6	0.0 ± 0.0	0.68 ± 0.97	2.96 ± 1.3	1.04 ± 1.2
8	0.0 ± 0.0	0.38 ± 0.6	1.53 ± 1.5	2.18 ± 1.7

B. Lung gentamicin levels

Initially, the concentration of gentamicin in the lungs was higher in the control group than the liposome treated groups, particularly at 1 and 2 hours (Fig 43 a & b, Table 17). However the concentrations fell rapidly such that by 4 hours it was not different from that of negative and positive liposome treated groups (Fig 44 and 45, Table 17). Concentrations in the lungs for the neutral liposomes were significantly lower than the control group from 1 to 6 hours (Fig 46, Table 17). However, by 8 hours, concentrations in all the liposome treated groups were not different ($P > 0.05$) from the control group. Of note, there was wide variations in concentrations for the negative and neutral liposome treated groups as evidenced by the large standard deviations (Table 17).

Although the positive liposome treated group had a higher gentamicin concentration than negative and neutral liposome treated groups, there was no significant difference. Therefore it may be deduced that the surface charge of the liposomes did not influence the distribution of liposomes to the lungs.

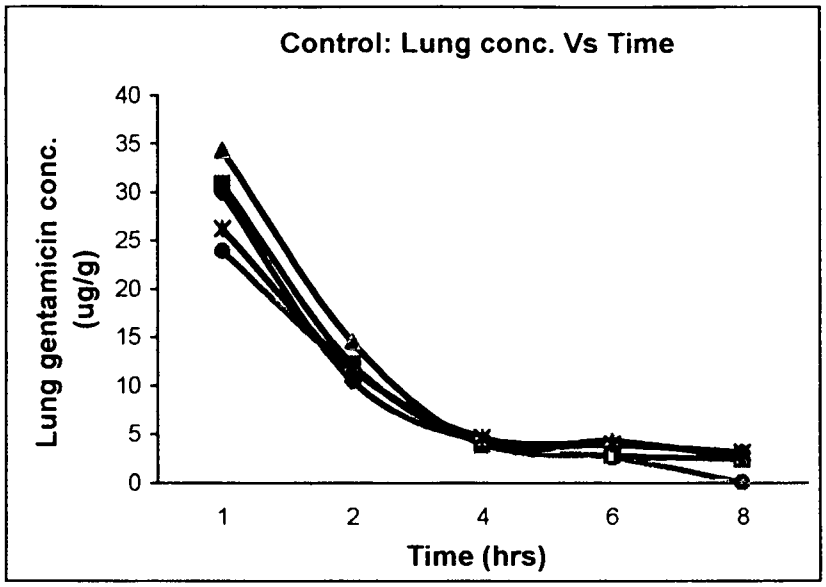


Fig 43a A plot of lung gentamicin concentration versus time for the control group (treated with free gentamicin). Each point on the graph represents an individual animal (Appendix C 2a, lungs).

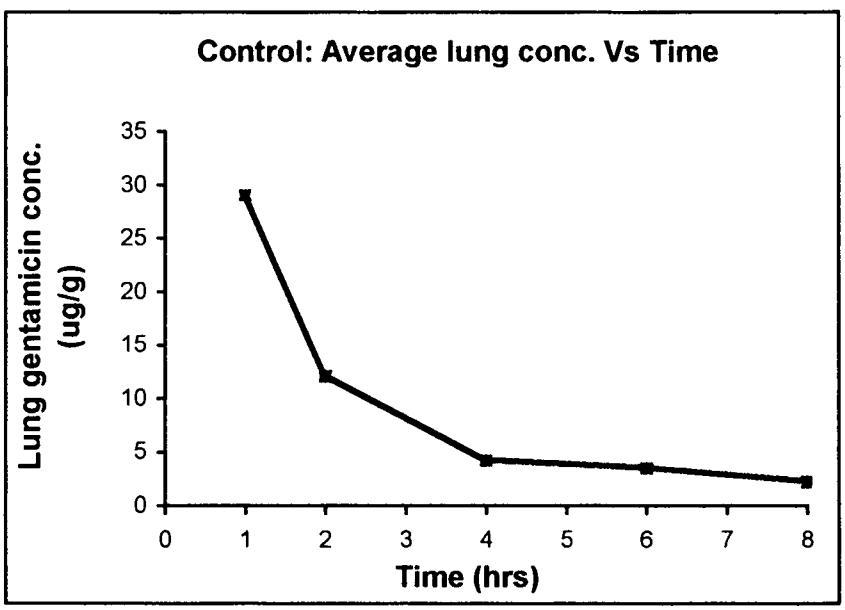


Fig 43b A plot of average lung gentamicin concentration versus time for the control group (treated with free gentamicin). Each point on the graph represents an average concentration for five animals.

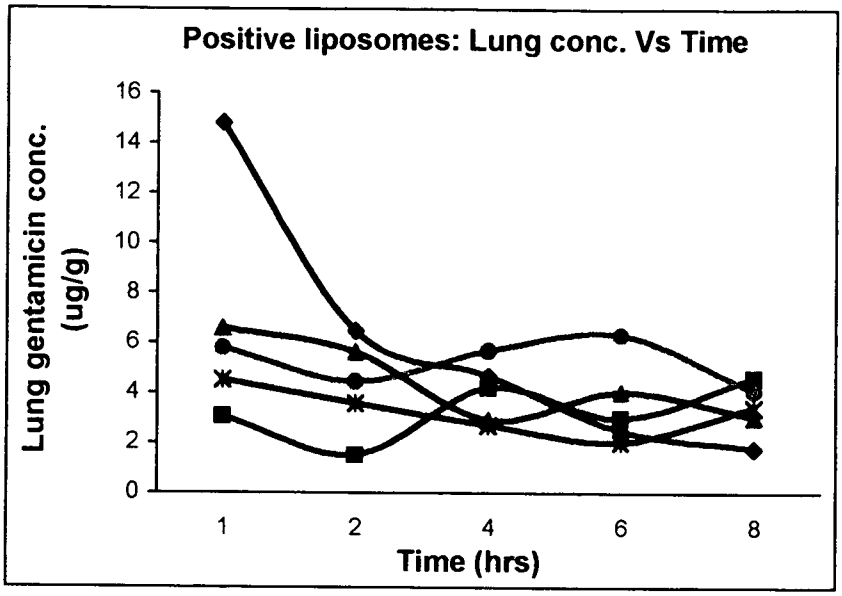


Fig 44a A plot of lung gentamicin concentration versus time for the positive liposome treated group. Each point on the graph represents an individual animal (Appendix C 2c, lungs).

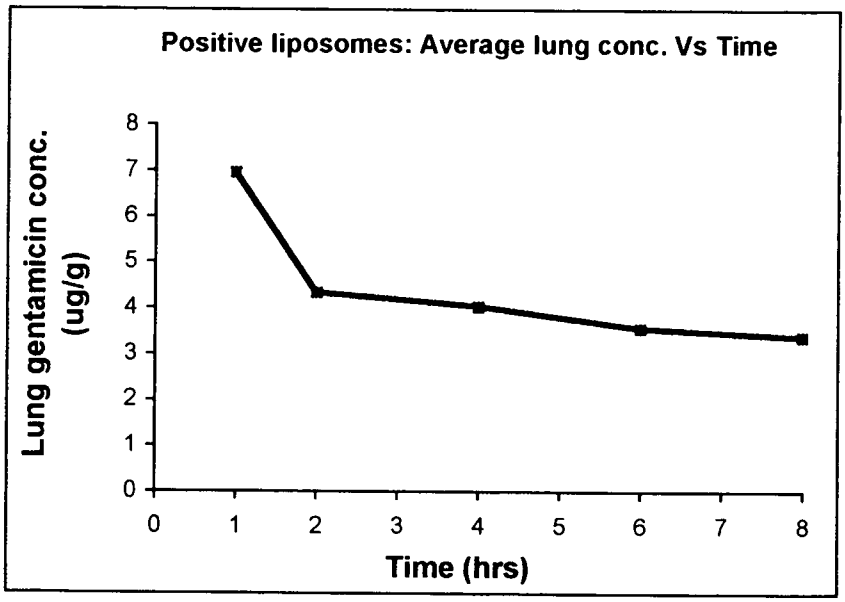


Fig 44b A plot of average lung gentamicin concentration versus time for the positive liposome treated group. Each point on the graph represents an average concentration for five animals.

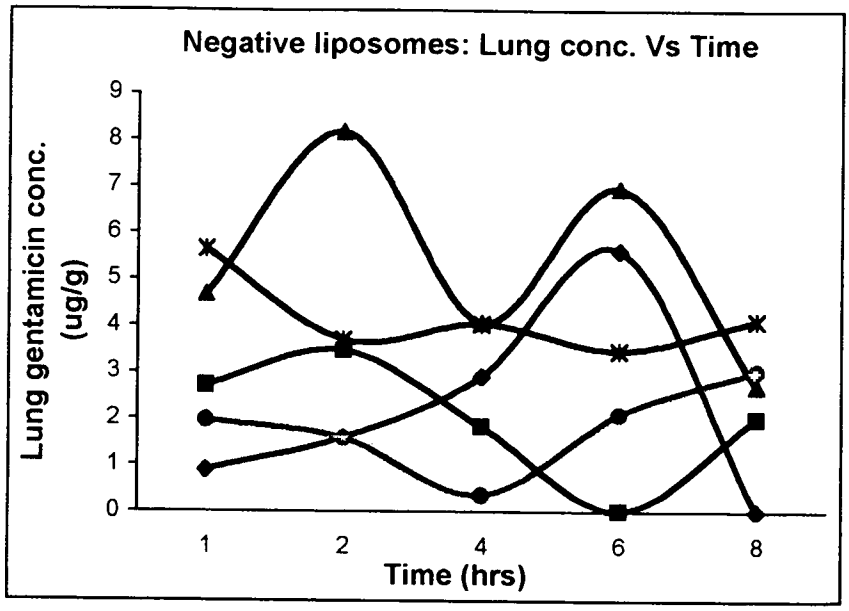


Fig 45a A plot of lung gentamicin concentration versus time for the negative liposome treated group. Each point on the graph represents an individual animal (Appendix C 2b, lungs).

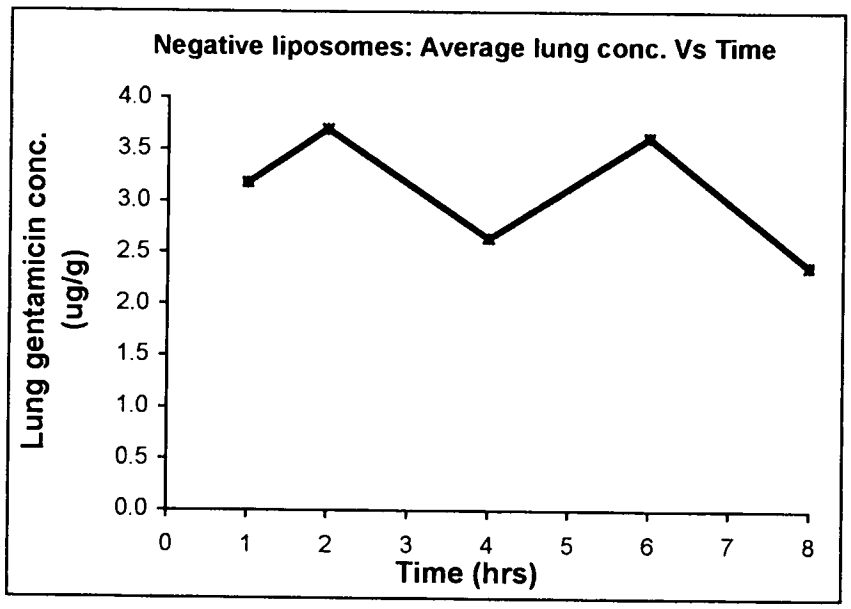


Fig 45b A plot of average lung gentamicin concentration versus time for the negative liposome treated group. Each point on the graph represents an average concentration for five animals.

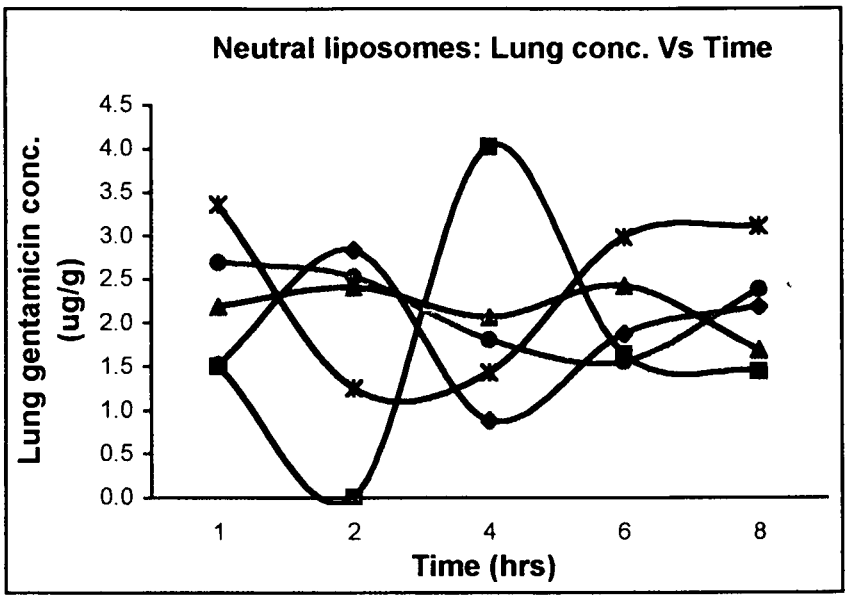


Fig 46a A plot of lung gentamicin concentration versus time for the neutral liposome treated group. Each point on the graph represents an individual animal (Appendix C 2d, lungs).

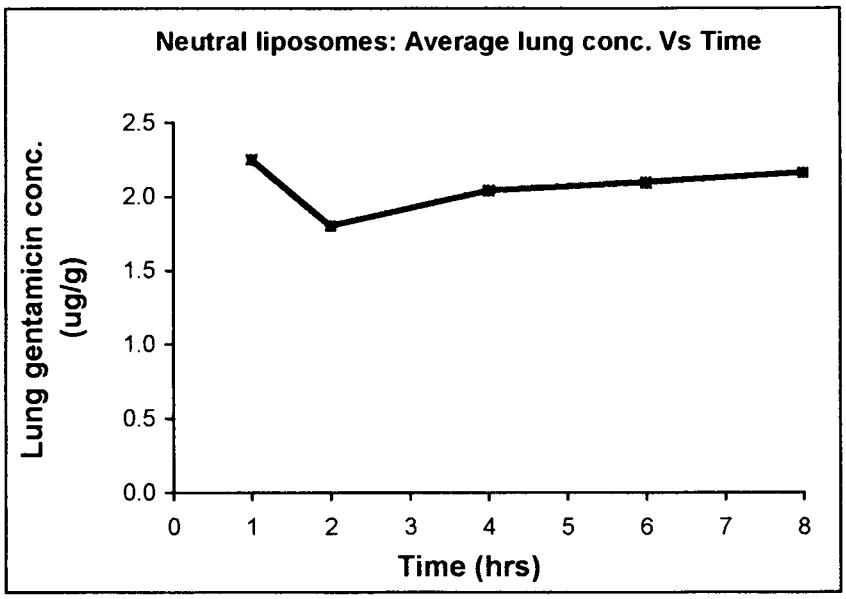


Fig 46b A plot of average lung gentamicin concentration versus time for the neutral liposome treated group. Each point on the graph represents an average concentration for five animals.

Table 17 Lung gentamicin concentration (mean ± S.D) obtained for the control, negative liposome, positive liposome and neutral liposome treated groups at different time intervals (1 to 8 hours). P values obtained by comparison with the control group are indicated in parenthesis.

Time (hours)	Lung gentamicin concentration (μg/g)			
	Control	Negative liposomes	Positive liposomes	Neutral liposomes
1	29.05 ± 4.1	3.17 ± 1.95 (0.008)	6.95 ± 4.6 (0.008)	2.25 ± 0.8 (0.008)
2	12.08 ± 1.5	3.69 ± 2.7 (0.008)	4.32 ± 1.9 (0.008)	1.8 ± 1.2 (0.008)
4	4.19 ± 0.3	2.63 ± 1.6 (0.095)	4.03 ± 1.2 (> 0.9999)	2.04 ± 1.2 (0.032)
6	3.51 ± 0.8	3.62 ± 2.8 (> 0.9999)	3.55 ± 1.7 (0.691)	2.09 ± 0.6 (0.032)
8	2.23 ± 1.3	2.37 ± 1.5 (0.917)	3.38 ± 1.1 (0.222)	2.16 ± 0.7 (0.347)

C. Kidney gentamicin levels

The control group exhibited higher levels (P = 0.008) of gentamicin in the kidneys than the liposome treated groups, ranging from 383.97 ± 69.1 μg/ml at 1 hour to 315.44 ± 59.7 μg/ml at 8 hours (Fig 47 a & b, Table 18). These concentrations were 4.4, 3.1 and 5 folds higher than those of the negative, positive and neutral liposome treated groups, respectively (Fig 48 to 50, Table 18).

Although a trend of increasing renal gentamicin concentration with time was observed with the negative and neutral liposome treated groups, these concentrations were lower compared to those of the positive liposome treated group. In general, renal gentamicin concentration in the liposome treated groups were in the order of: positive > negative > neutral. However, there was no significant difference between positive liposome treated group and negative liposome treated group except at 4 hours, and at 1 and 4 hours with the neutral liposome treated group. The significance of these differences could not be ascertained.

These observation indicate that the surface charge of liposomes did not influence their distribution to the kidneys. Also, liposomal formulations reduced the concentration of gentamicin in the kidneys.

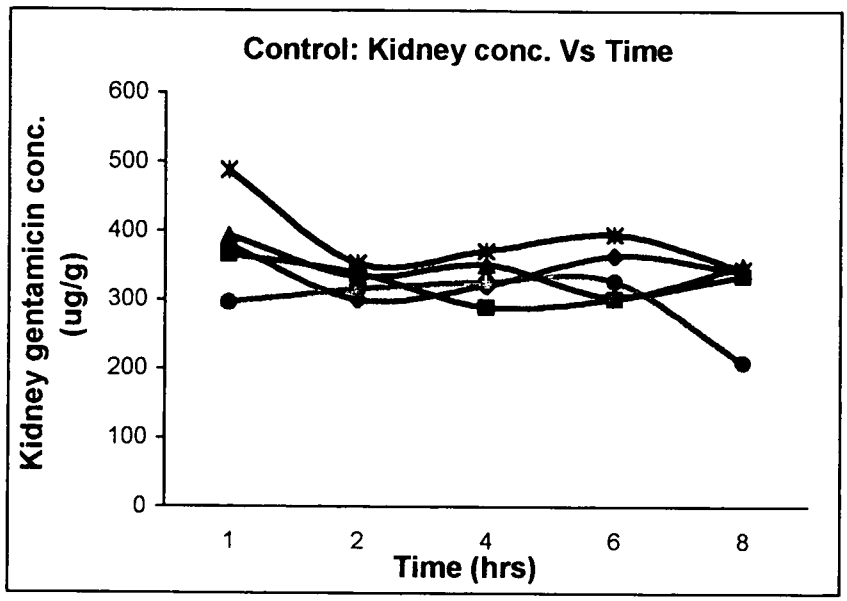


Fig 47a A plot of kidney gentamicin concentration versus time for the control group (treated with free gentamicin). Each point on the graph represents an individual animal (Appendix C 2a, kidneys).

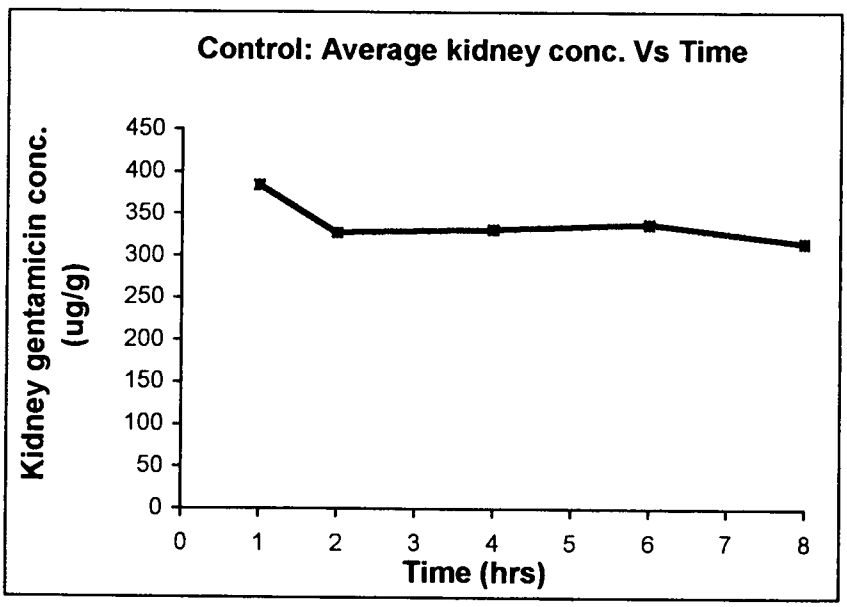


Fig 47b A plot of average kidney gentamicin concentration versus time for the control group (treated with free gentamicin). Each point on the graph represents an average concentration for five animals.

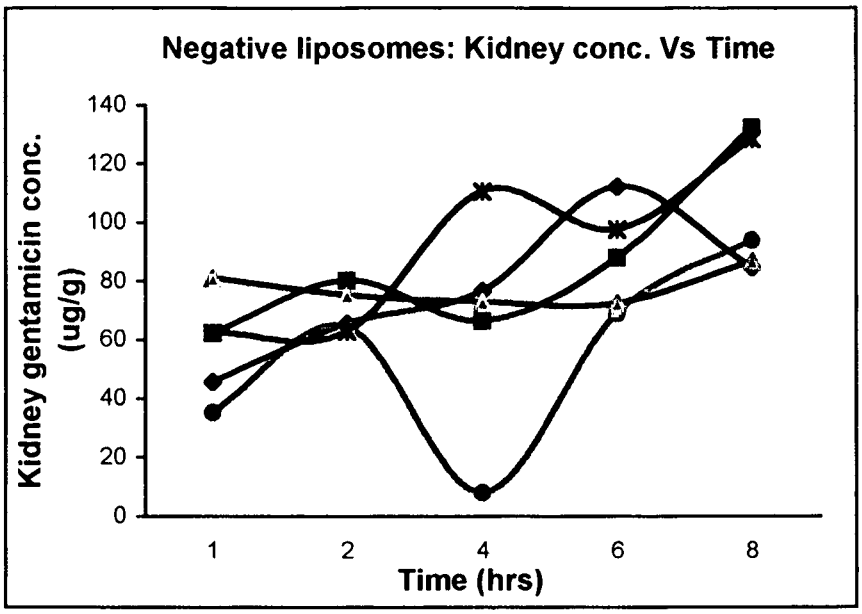


Fig 48a A plot of kidney gentamicin concentration versus time for the negative liposome treated group. Each point on the graph represents an individual animal (Appendix C 2b, kidneys).

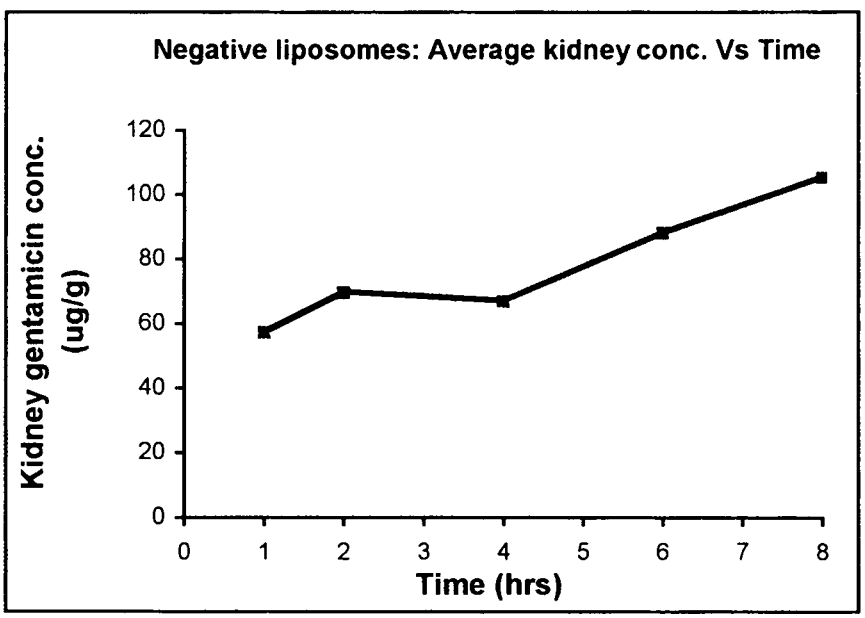


Fig 48b A plot of average kidney gentamicin concentration versus time for the negative liposome treated group. Each point on the graph represents an average concentration for five animals.

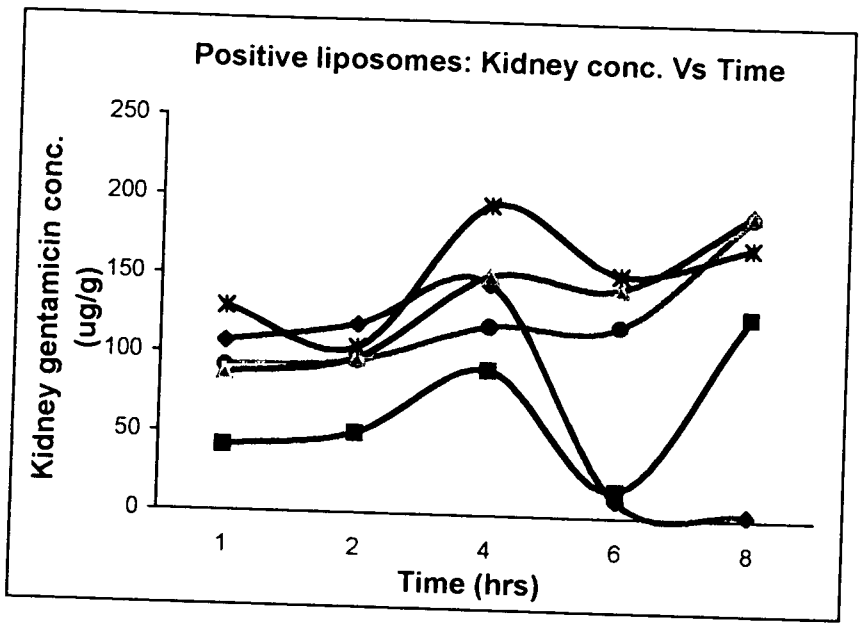


Fig 49a A plot of kidney gentamicin concentration versus time for the positive liposome treated group. Each point on the graph represents an individual animal (Appendix C 2c, kidneys).

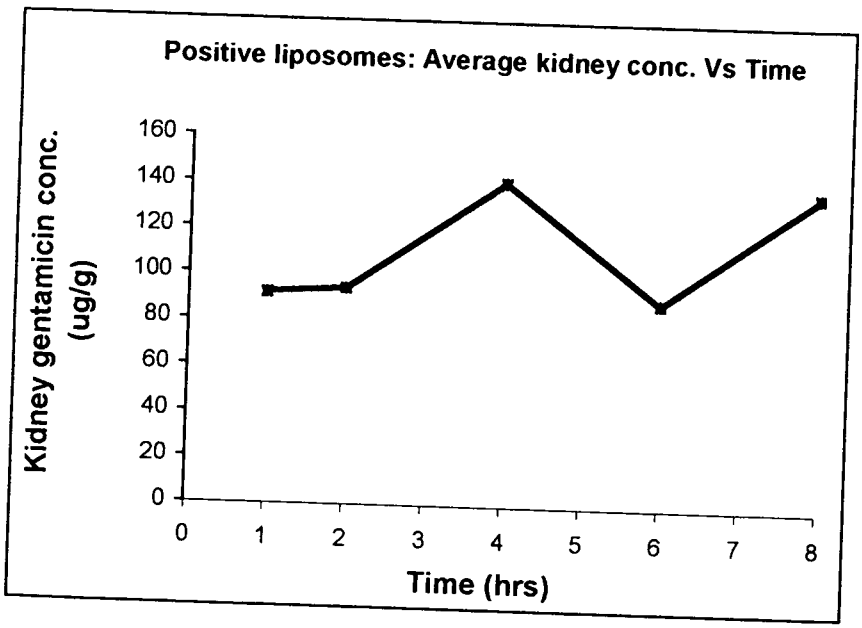


Fig 49b A plot of average kidney gentamicin concentration versus time for the positive liposome treated group. Each point on the graph represents an average concentration for five animals.

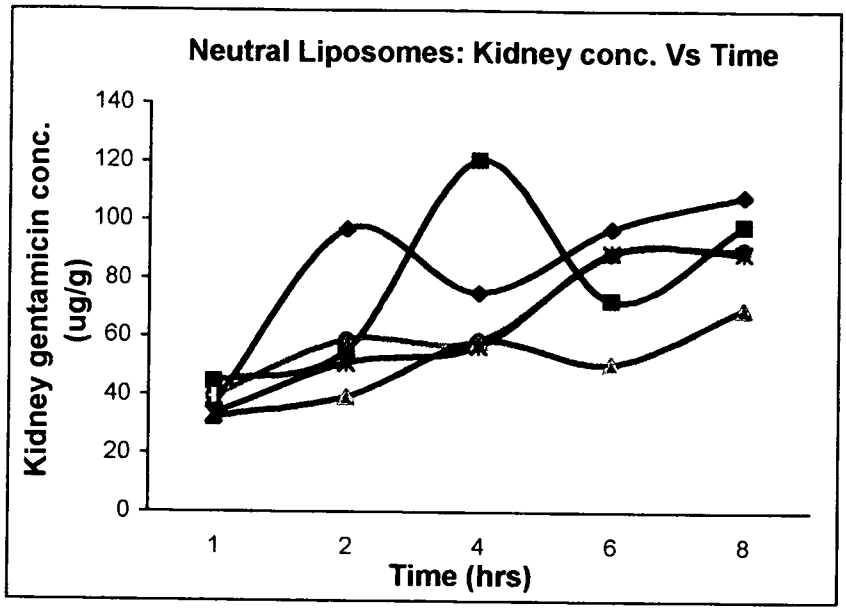


Fig 50a A plot of kidney gentamicin concentration versus time for the neutral liposome treated group. Each point on the graph represents an individual animal (Appendix C 2d, kidneys).

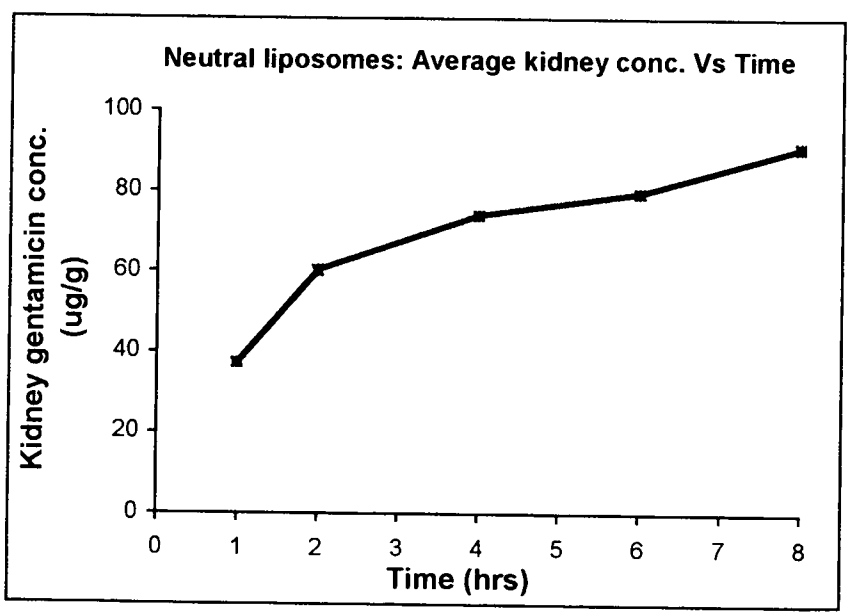


Fig 50b A plot of average kidney gentamicin concentration versus time for the neutral liposome treated group. Each point on the graph represents an average concentration for five animals.

Table 18 Kidney gentamicin concentration (mean ± S.D) obtained for the control, negative liposome, positive liposome and neutral liposome treated groups at different time intervals (1 to 8 hours).

Time (hours)	Kidney gentamicin concentration (μg/g)			
	Control	Negative liposomes	Positive liposomes	Neutral liposomes
1	383.97 ± 69.1	57.26 ± 17.7	91.5 ± 32. 5	37.2 ± 4.9
2	327.52 ± 20.7	69.59 ± 7.7	93.75 ± 25.8	60.15 ± 21.7
4	331.14 ± 31.2	66.86 ± 37.2	140.88 ± 38.97	73.82 ± 27.1
6	337.91 ± 40.4	87.89 ± 17.9	89.11 ± 69.8	79.39 ± 18.4
8	315.44 ± 59.7	105.21 ± 23.3	137.12 ± 79.6	90.87 ± 14.2

P value for negative, positive and neutral liposomes in comparison to the control group was 0.0079, during all time intervals (1 to 8 hrs).

D. Liver gentamicin levels

Whereas hepatic gentamicin concentration in the control group decreased with time, from 9.87 ± 2.4 μg/ml at 1 hour to 2.73 ± 1.7 μg/ml at 8 hours, it increased with time in the liposome treated groups (Fig 51 to 55, Table 19). Of note, the initial gentamicin concentrations in the liver at 1 and 2 hours for the control were higher ($P < 0.02$) than for all the liposome treated groups. Thereafter, variations in gentamicin concentrations from the control were different for the different liposome treated groups. For instance, the negative and neutral liposome treated groups exhibited significant difference from the control at 6 and 8 hour, while the positive liposome treated groups did not. The positive liposome treated group exhibited the highest average hepatic gentamicin concentration at 4 to 8 hours (Fig 52 to 54, Table 19). However, due to wide variations, there was no significant difference from the control and other liposome treated groups by 6 and 8 hours.

Whereas between 1 to 4 hours hepatic concentrations of gentamicin were higher in the positive treated group than the negative treated group, concentrations in the former group were very variable, particularly at 4 hours. No significant difference was observed between the two groups at 6 and 8 hours. On the other hand, there was no significant difference in hepatic gentamicin concentration between the negative and neutral liposome treated groups except at 8 hours, signifying that higher concentrations were achieved with negative liposomes. Also, there was no significant difference between concentrations in the positive and neutral liposome treated groups, except at 2 and 4 hours.

These observations show that there were variations in the pattern of liposome distribution to the liver in the initial hours after administration. Therefore the order of affinity of the liposomes to the liver between 1 and 4 hours was; positive > negative = neutral, while at 8 hours it was; negative > neutral (Fig 55). The positive liposomes were excluded in this analysis because of wide variations that made the interpretations difficult. The increase in gentamicin concentrations in the liver was less variable for the negative liposomes than positive. In general, there was no difference in affinity for the liver between positive and negative liposomes by 8 hours.

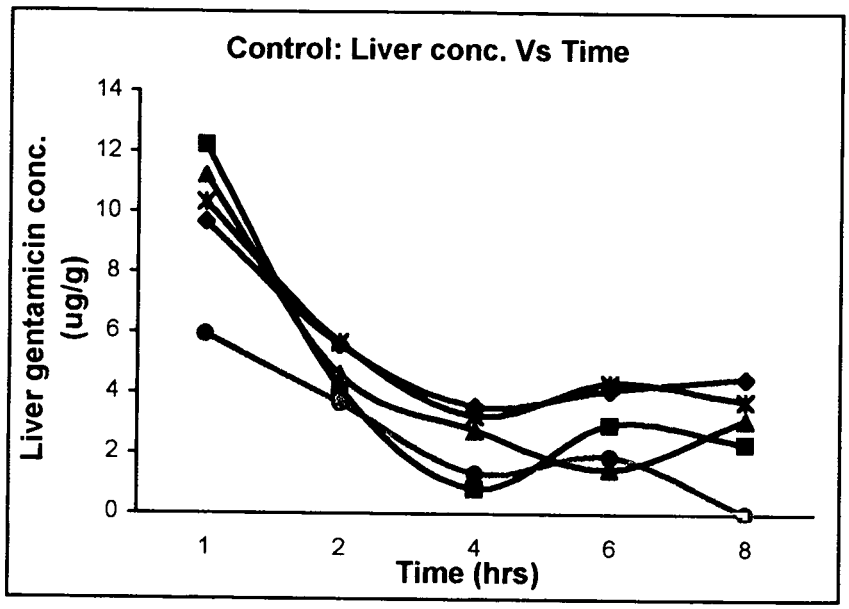


Fig 51a A plot of liver gentamicin concentration versus time for the control group (treated with free gentamicin). Each point on the graph represents an individual animal (Appendix C 2a, liver).

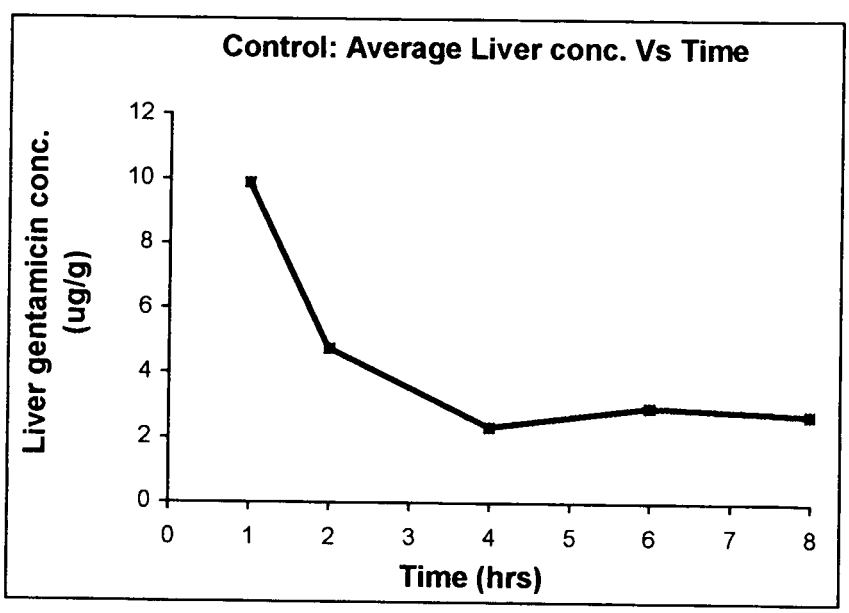


Fig 51b A plot of average liver gentamicin concentration versus time for the control group (treated with free gentamicin). Each point on the graph represents an average concentration for five animals.

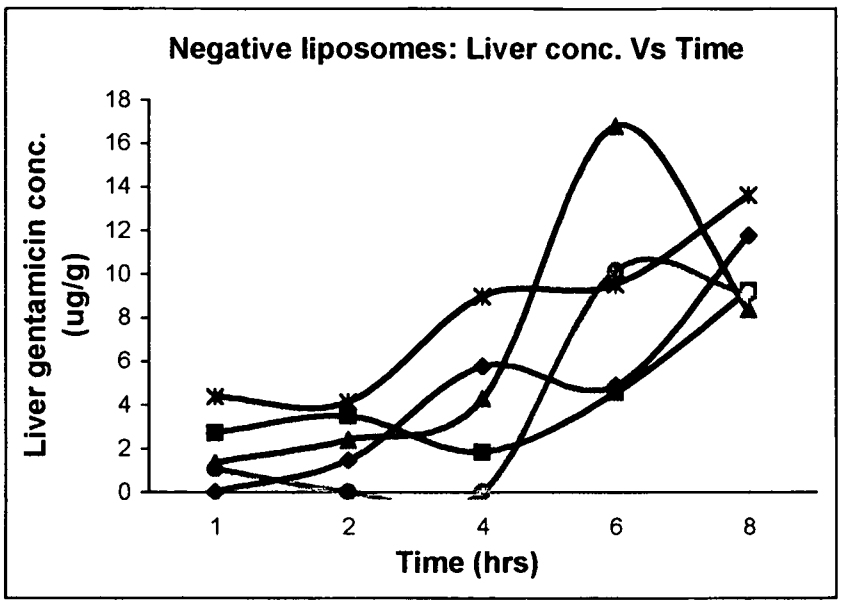


Fig 52a A plot of liver gentamicin concentration versus time for the negative liposome treated group. Each point on the graph represents an individual animal (Appendix C 2b, liver).

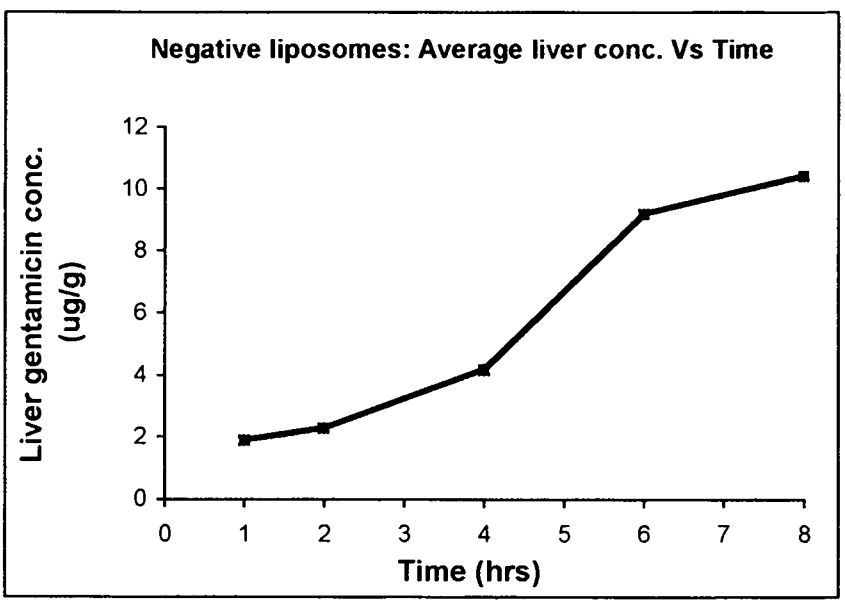


Fig 52b A plot of average liver gentamicin concentration versus time for the negative liposome treated group. Each point on the graph represents an average concentration for five animals.

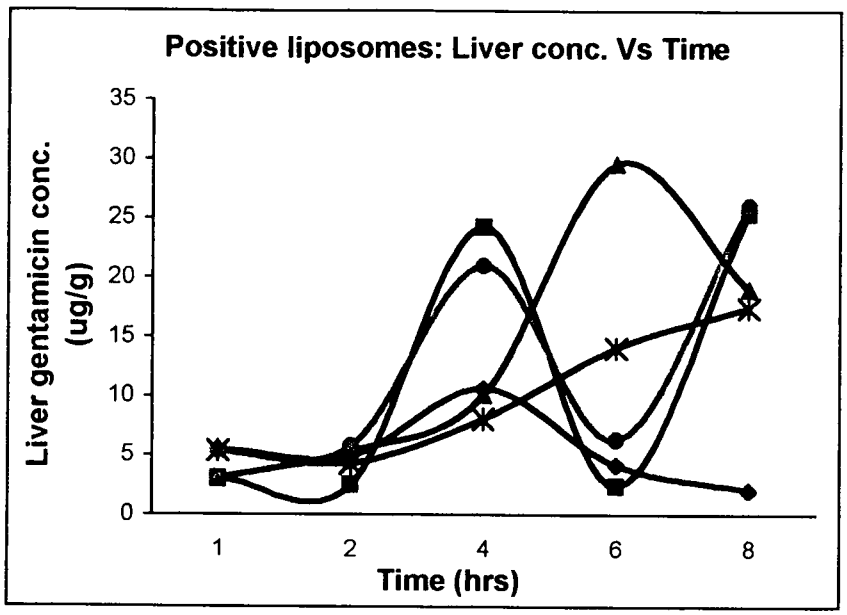


Fig 53a A plot of liver gentamicin concentration versus time for the positive liposome treated group. Each point on the graph represents an individual animal (Appendix C 2c, liver).

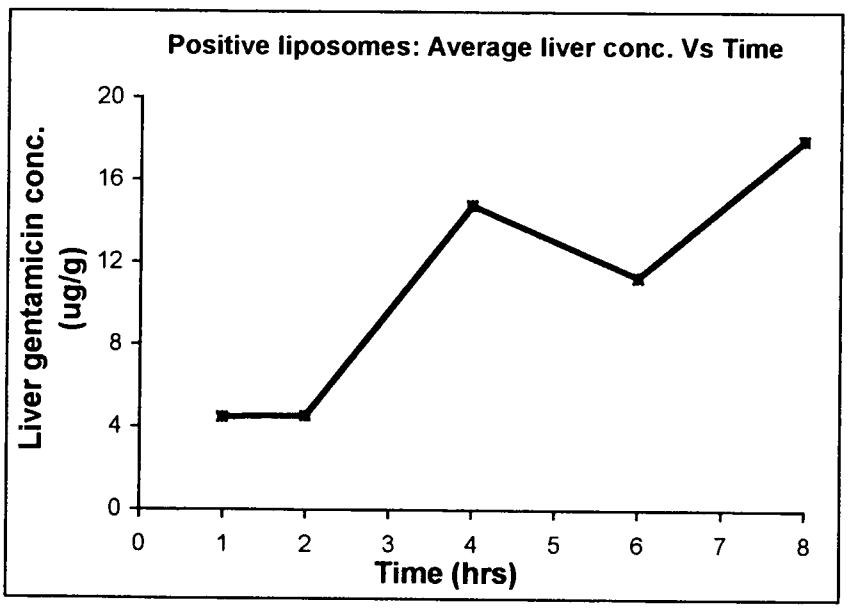


Fig 53b A plot of average liver gentamicin concentration versus time for the positive liposome treated group. Each point on the graph represents an average concentration for five animals.

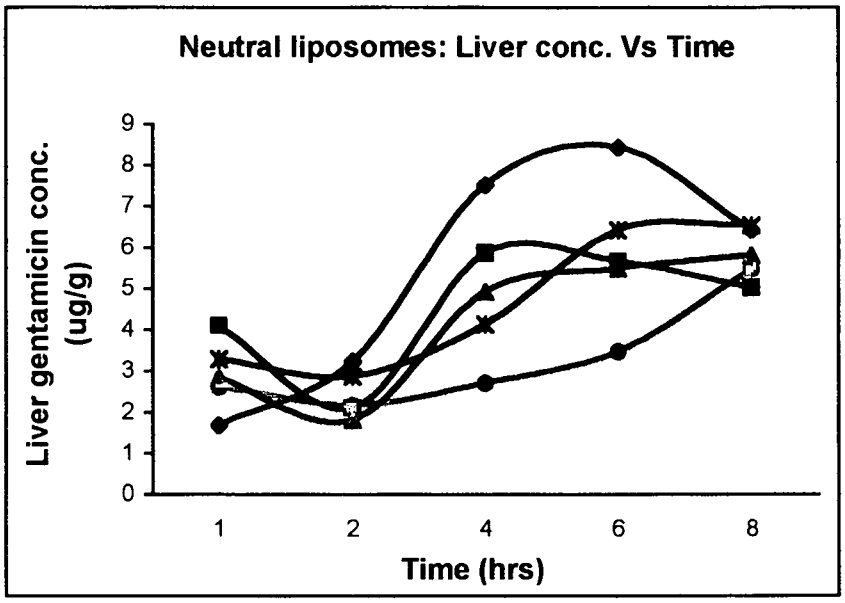


Fig 54a A plot of liver gentamicin concentration versus time for the neutral liposome treated group. Each point on the graph represents an individual animal (Appendix C 2d, liver).

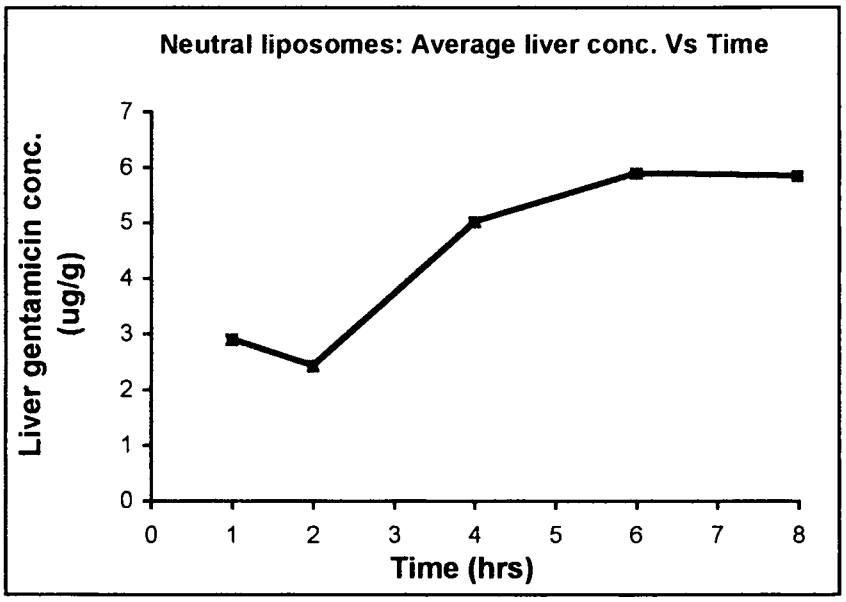


Fig 54b A plot of average liver gentamicin concentration versus time for the neutral liposome treated group. Each point on the graph represents an average concentration for five animals.

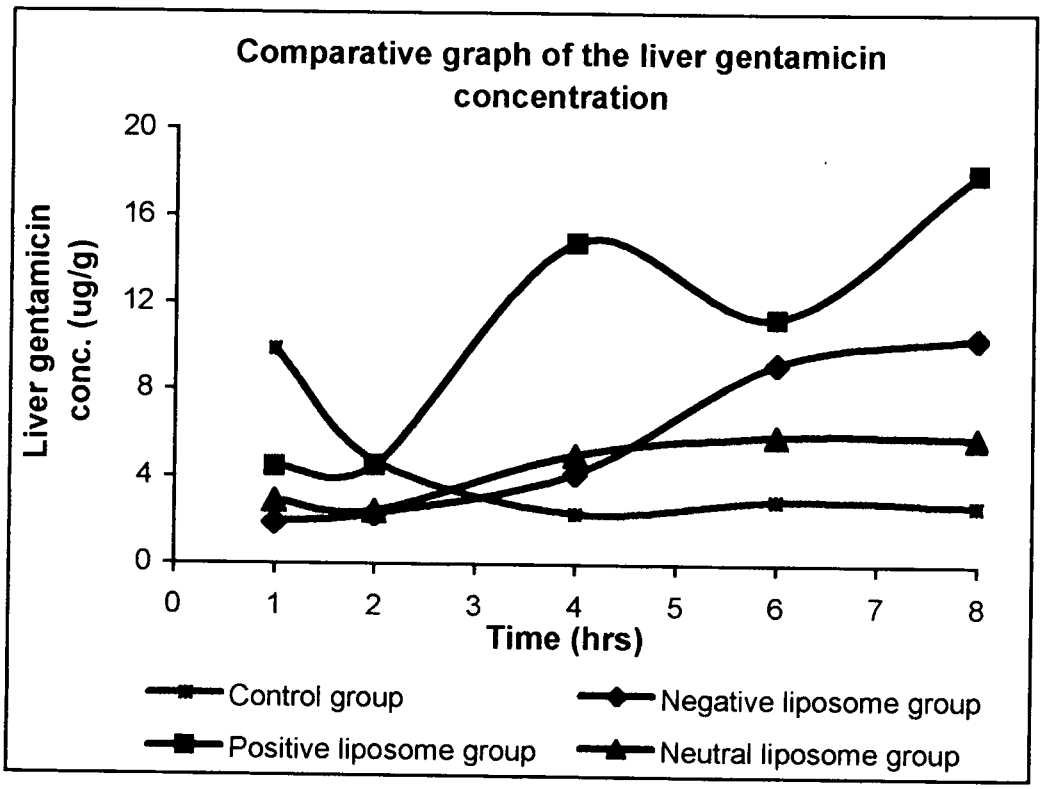


Fig 55 A comparative plot of average liver gentamicin concentration versus time for the control and liposome treated groups.

Table 19 Liver gentamicin concentration (mean ± S.D) obtained for the control, negative liposome, positive liposome and neutral liposome treated groups at different time intervals (1 to 8 hours). P values obtained by comparison to the control group are indicated in parenthesis.

Time (hours)	Liver gentamicin concentration (μg/g)			
	Control	Negative liposomes	Positive liposomes	Neutral liposomes
1	9.87 ± 2.4	1.89 ± 1.7 (0.008)	4.47 ± 1.3 (0.008)	2.90 ± 0.9 (0.008)
2	4.73 ± 0.9	2.28 ± 1.6 (0.016)	4.53 ± 1.3 (0.008)	2.42 ± 0.6 (0.008)
4	2.32 ± 1.2	4.17 ± 3.5 (0.421)	14.78 ± 7.3 (0.008)	5.02 ± 1.8 (0.056)
6	2.93 ± 1.3	9.18 ± 4.9 (0.008)	11.28 ± 11.1 (0.095)	5.89 ± 1.8 (0.032)
8	2.73 ± 1.7	10.41 ± 2.2 (0.008)	17.98 ± 9.7 (0.095)	5.84 ± 0.6 (0.008)

5.6 DISCUSSION

In this study it has been shown that the surface charge of liposomes can influence their distribution to different organs in rats as well as their circulation time in plasma and encapsulation efficiency.

The liposome encapsulation efficiency attained during the animal experiment was in agreement to that obtained in the standardisation phase. Specifically, encapsulation efficiency for the negative liposomes was in the range predicted with methyl violet (22.8 %) indicating that the method was robust. The positive liposomes also gave similar encapsulation efficiencies to the negative liposomes. Encapsulation efficiency was lowest for neutral liposomes, indicating that surface charge may enhance encapsulation. This is in agreement with earlier observations suggesting that charged lipids may increase the volume of entrapped material in the liposome (Sessa and Weissman, 1970; Morgan and Williams, 1980). It was also postulated that, probably, the existence of similar charges on the lipid bilayers leads to repulsion between them, thereby creating a larger distance or gap between the bilayers where an additional amount of the material can be enclosed (Sessa and Weissman, 1970).

According to Kaplan's theory (Kaplan, 1972), the negative liposomes should have exhibited a higher encapsulation efficiency than positive liposomes. Kaplan claimed that positively charged liposomes are impermeable to cations (explained in chapter 2) of which gentamicin is one. It is assumed that since gentamicin has a positive charge, the repulsion between the like charges of gentamicin and the lipid in the positive liposome (stearylamine) may be responsible for the decreased encapsulation. However, it seems that this factor did not play a major role here, as no significant difference in encapsulation efficiency was observed between negative and positive liposomes. Therefore, it is suffice to say that charged liposomes were better candidates for encapsulation of gentamicin.

Of note, the method used for deriving encapsulation efficiency estimated both the entrapped and phospholipid associated drug (Fig 56). It is possible that this was responsible for the high encapsulation efficiency because other studies using similar methods have reported even higher values. Hsieh et al. (2002) and Turrens et al. (1984) reported encapsulation efficiencies of 44 - 51 % and 30 %, respectively. The higher values than those obtained

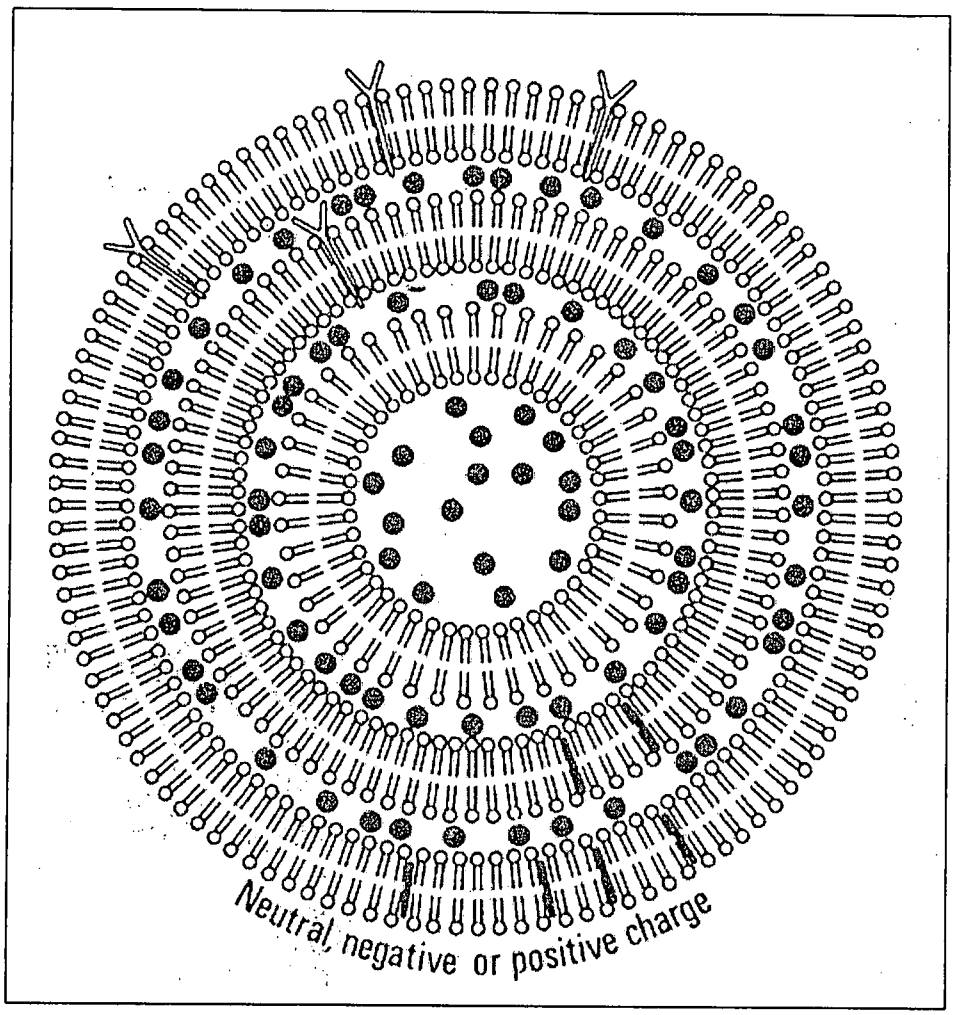


Fig 56 A diagram displaying the distribution of drug molecules (•) within the liposome structure. (Gregoriadias, 1976)

in this study could be due to differences in other factors such as lipid composition and preparation methods. On the other hand, workers who determined encapsulation by lysis of liposomes with triton X-100 obtained lower encapsulation efficiencies. For instance, Morgan and Willams (1980) obtained encapsulation efficiencies of 3.7 %, 4 % and 1.8 % for positive, negative and neutral liposomes, respectively. This could be because only the entrapped drug but not the phospholipid associated drug was estimated. This though could be dangerous, since in reality the total amount of drug administered would be higher than reported.

The high dose of gentamicin (60 mg/kg) used in this study was necessary to enable a longer sampling period (> 6 hours). Higher doses of gentamicin have been used before and 60 mg/kg is considered non-toxic because gentamicin induced nephrotoxicty in rats occurred only at doses of 80 mg/kg administered for 5 days (Julien et al., 2000). Moreover in the present study, rats were sacrificed before toxicity would occur.

The NaOH-digestion method was superior in the extraction of gentamicin from the organs except for the lungs where the NaOH-digestion-homogenisation method was better. This was in agreement with Brown et al. (1988) who observed highest recovery of gentamicin from renal cortical samples with the NaOH-digestion method. Further, they recommended that homogenisation was unnecessary with NaOH-digestion for tissue samples less than 0.45 g. They also suggested that the NaOH-digestion method could be used on other tissues for extraction of gentamicin, and that fibrous tissues may require homogenisation. This may explain the observation here that the NaOH-digestion-homogenisation method gave better results for the lung. The superiority of the NaOH-digestion method in extracting gentamicin could be explained by the theory that the alkaline digestion additionally releases gentamicin, a polycationic aminoglycoside, from its electrostatic binding with anionic phospholipids, (Gilbert and Kohlhepp, 1986; Brown et al.,1988).

Plasma gentamicin levels

In general, the elimination characteristics of gentamicin exhibited a biphasic pattern (Appendix 3a to 3d), with the first phase between 1 and 4 hours, and the second phase after that. However, the half life of gentamicin in the first phase was longer than that reported by Swenson et al. (1990), who observed a half life of 0.6 hours in rats treated with a single dose of 20 mg/kg gentamicin. The difference could also arise from differences in age of the rats

and route of administration. The rats used by Swenson et al. (1990) were younger (6 – 8 weeks, 150 to 250 g) and gentamicin was administered intravenously, while for the present study rats were older (10 –12 weeks, 300 to 350 g) and the drug was given intraperitoneally. Of note, they did not mention anything on the biphasic elimination pattern of gentamicin, yet a two-compartment model was used for kinetic calculations. This implied that gentamicin exhibited a biphasic elimination characteristic in their study.

Contrary to observations by other workers (Kim et al., 1994; Swenson et al.,1990; Kimelberg, 1976; Colley and Ryman, 1975), the plasma concentrations of gentamicin in the liposome treated groups were lower than in the control for this study. This could be due to differences in the processing of plasma samples. The afore mentioned workers measured the total amount of drug in the plasma, i.e., the free and liposome associated drug. Kim et al. (1994); Kimelberg (1976) and Colley and Ryman (1975), used radiolabelled drugs, while Swenson et al. (1990) disrupted the liposomes before drug assay, thereby measuring both the free and entrapped drug in plasma. In the current study, plasma samples were not subjected to any process that would disrupt the liposomes. Therefore, only the drug released from the liposomes may have been measured thereby substantiating the lower concentrations obtained. This notion was supported by studies in which it was observed that most of the drug in plasma was intact in the liposomes (Kim et al., 1994, Abraham et al., 1984).

Furthermore, the trends for clearance and half life agreed with other studies that liposomes delay elimination of encapsulated drugs (Swenson et al., 1990; Kim et al., 1994; Morgan and Williams, 1980). Since, in this study, only liposome-free gentamicin in plasma was measured, the area under curves (AUC) did not relate to the clearance and half life parameters because of variations in the release of gentamicin in plasma by different liposomes. For instance, area under curve was highest for positive liposomes and these were associated with faster release of gentamicin in plasma.

The higher concentrations of gentamicin in plasma for the positive liposome treated group could probably be due to the stronger interaction of positive liposomes with serum components which results in destabilisation of the liposome and release of the drug (Foradada et al., 2000). Therefore, it may be suggested that positive liposomes would be a better choice for treatment of diseases or infections that may require high concentration of the drug in the blood such as septicaemia.

Brain gentamicin levels

The limited entry of gentamicin into the brain for the control group confirms the poor permeability of the blood brain barrier to gentamicin. This was also observed by Neuwelt et al. (1984), who reported brain gentamicin of approximately 1 % to 10 % of the serum concentration in a rat model. Conversely, the high concentrations of brain gentamicin in the liposome treated groups indicate that, contrary to some reports (Sakamoto and Ido, 1993), liposomes contributed to the entry of the drug into the brain. This could be by either the liposomes crossing the blood brain barrier or undergoing fusion with the blood brain barrier thereby releasing the drug into the brain, the exact process is not clear.

The reason why positively charged liposomes exhibited highest affinity to the brain than the other liposomes is also not clear. However, Jonah et al. (1975) observed similar results and postulated that this could be due to the positive charge and their lipophilicity, in view of the earlier observations that positively charged dyes had greater ability to cross the blood brain barrier (Bakay, 1956). In this case, it is possible that greater interaction of liposomal lipid with the blood brain barrier was enhanced by stearylamine, a positively charged lipid. Of note, Jonah et al. (1975) also used stearylamine containing positive liposomes. The importance of 'positive charge' in this process was supported by recent findings that positively charged peptides or peptides into which positive charges have been induced are transported into the brain via specific processes (Tamai and Tsuji, 1996). Nevertheless, there is a need for more studies on the disposition of liposomes to the brain.

The biphasic pattern of distribution of gentamicin to the brain observed in the liposome treated groups, particularly by the negative liposomes, suggests probably a saturable uptake mechanism of the liposomes. It was suggestive of a gated mechanism whereby the first bout of liposome uptake lead to saturation at 1 to 2 hours (gates closed), followed by a period of recovery (2 to 4 hours) during which the internalised drug was eliminated from the brain as indicated by concentration decline from 2 to 4 hours. Thereafter the second liposome uptake occurred and saturation was observed at 6 hours. However, the validity of this hypothesis remains to be proven. Nevertheless, from these results, it can be concluded that positive liposomes would be better candidates for delivery of drugs into the brain.

Lung gentamicin levels

The observations that none of the liposomes showed significant affinity to the lungs agreed with the findings by Colley and Ryman (1975). However, it differed from observations by others who reported highest affinity to the lungs by neutral liposomes (Nabar and Nadkarni, 1998), by negative liposomes (Kim et al.,1994) and positive liposomes (Jonah et al., 1975). The disparity in some of the observations could have risen from the short study periods of 1 hour by Nabar and Nadkarni (1998), and 2 hours by Kim et al. (1994). As a result, their data would relate more to the fast distribution in the initial phase but not to the ultimate distribution of the liposomes. The difference also could have arisen from use of markers with different solubility properties; the gentamicin used in this study is more hydrophilic than methotrexate used by Kim et al.(1994), technetium used by Nabar and Nadkarni (1998) and probably EDTA used by Jonah et al. (1975). Lipophilic drugs are more likely to cross cell membranes when released in proximity to the tissue than hydrophilic drugs.

Since there was no difference in the concentration of gentamicin in the lungs of the control and the liposome treated groups, this implies that liposomes surface charge has no influence on the distribution of liposomes to the lungs and liposomes did not offer any advantage over the free drug.

Kidney gentamicin levels

It is well known that gentamicin excretion occurs mainly via the kidneys, and most probably this could account for the high renal gentamicin concentrations observed in the control group. Also, the observations in this study, that distribution to the kidneys was not influenced by the charge of the liposome agreed with those of Nabar and Nadkarni (1998). Furthermore, the lower renal concentrations of gentamicin in the liposome treated groups than the control concurred with observations by Kimelberg (1976). This combination of limited distribution of liposomal gentamicin to the kidneys and reduced concentrations of the drug in the kidneys has been attributed for the renal protective effects of liposomal formulations. But this has been challenged by findings of Kim et al. (1994) and Steger and Desnick (1977) who observed greater affinity of negative liposomes to the kidneys. Most probably this could account for the increase in renal gentamicin concentration at 8 hours observed in this study.

Liver gentamicin levels

In this study, uptake of positive liposomes by the liver in the initial period was higher than other liposome groups probably because of their higher clearance from plasma. But the wide variations in hepatic gentamicin of the positive liposome treated group after 4 hours could not be explained. These observations in the initial period appear to agree with those by Nabar and Nadkarni (1998) and Colley and Ryman (1975) where a short study period of 1 hour was used. Nevertheless, this implies that the hepatic uptake of positive liposomes in the initial period is different from the subsequent period.

Although positive liposomes appeared to achieve higher concentrations of gentamicin in the liver than the negative liposomes, their dubious kinetics in the later period (after 4 hours) made them unfavourable for drug delivery to the liver. The consistency observed in hepatic gentamicin concentrations with the negative liposomes reflected a predictable drug delivery system to the liver. As such, even though there was no difference in affinity of positive and negative liposomes to the liver, the afore mentioned observations imply that negative liposomes would be the best choice for hepatic drug delivery.

The results also indicate that charged liposomes were more effective drug carriers than the neutral liposomes. This implies that liposomal surface charge is a major determinant of hepatic uptake of liposomes and forms a basis on which mechanisms of liposomal tissue uptake involving surface charge may be described. One such mechanism was described by Jonah et al. (1975) in which he described that because of the net negative charge on cell surfaces, positive liposomes would be taken up more than negative or neutral liposomes. But the lack of significant difference between the hepatic concentration of gentamicin in the positive and negative groups challenges the above theory. This suggests the existence of more than one mechanism by which liposomes can interact with the hepatic cells. It was suggested that the presence of calcium ions promotes fusion of negatively charged liposomes with cells (Papahadjopoulos et al., 1974). Generally it is accepted that the increased uptake of liposomes to the liver is due to uptake by the reticuloendothelial system, probably one of the mechanism by which neutral liposomes were taken up.

The accumulation of gentamicin in the liver for the liposome groups would be due to retention of the drug in liposomes because free gentamicin is not stored or bound in the liver as exemplified by the control group. This would also imply that liposomes were taken up

intact and suggests that gentamicin was a good marker for liposomal hepatic uptake. This was also observed by Streger and Desnick (1977) on the hepatic uptake of liposome encapsulated enzyme. They also explained that the prolonged retention of positive liposomes in the liver may be due to the inhibitory effect of stearylamine on the phospholipase enzyme responsible for liposomal lipids degradation thereby retarding release of the entrapped drug. However the reason why negative liposomes were also retained in the liver is not clear. In conclusion, negative liposomes would be the best choice for the delivery of drugs to the liver.

Shortcomings of the study

The use of different animals per time interval could have been a major source of the variations in plasma gentamicin concentrations compared to when sampling is done in the same animal over the entire study period. But this would not be possible because the animals had to be sacrificed to remove the organs in order to measure the organ concentrations of gentamicin. Also, whereas renal function is the major determinant of gentamicin elimination, renal function tests were not monitored here. As such, the effect of this treatment on the kidney could not be ascertained. But this may not be of major concern as it has not been routinely done in similar experiments with gentamicin.

Chapter 6

GENERAL CONCLUSIONS AND FUTURE RESEARCH POTENTIAL

6.1 CONCLUSIONS

1. An appropriate non-radioactive marker (gentamicin) for studying the disposition of liposomes *in vivo* was successfully selected based on its properties and the practicability to set up assays for its measurement.

2. A simple and short method for preparation of stable, multilamellar, medium sized liposomes of different surface charges with appreciably good encapsulation efficiency was successfully adopted.

3. An appropriate experimental design for the study of the distribution of liposomes in rats was successfully developed and utilised

4. The NaOH-digestion method for extraction of gentamicin from organs was adopted.

5. The brain exhibited a preferential uptake up of positively charged liposomes than the negative and neutral liposomes, hence, the positively charged liposomes would be the preferred choice for delivery of drugs to the brain.

6. Surface charge of the liposomes did not influence their affinity to lungs because no difference in gentamicin lungs concentrations were observed between the negative, positive and neutral liposome treated rats. Also, gentamicin lung concentrations were not different from the control group, which implied that liposomes did not improve delivery of the drug to the lung than the free drug.

7. Surface charge of the liposomes did not influence their affinity to the kidneys since there was no difference in renal gentamicin concentrations between the liposome treated rats.

Also, renal gentamicin concentration was lower in liposomes treated rats than the control group, and this has been attributed for the renal protective effects of liposomal formulations.

8. The liver exhibited higher uptake of positive and negative liposomes than the neutral liposomes. However, because of low variability in the hepatic marker drug concentrations for the negative liposomes, they would be preferred to positive liposomes for hepatic delivery of drugs. The higher hepatic gentamicin concentrations of the liposome treated rats than the control indicated that liposomes improved delivery of the marker drug to the liver.

9. To summarise, the surface charge of liposomes can influence their distribution to the brain and the liver but not to the lungs and kidneys.

6.2 FUTURE RESEARCH POTENTIAL

The following were identified as possible future research projects arising from this study:

1. Although several mechanisms of liposomal uptake by tissues have been described, none of these mechanisms has been correlated to a specific liposomal uptake. In all studies, this has been the major cause of difficulty in explaining the observed results. Furthermore, the contribution of each mechanism of liposome uptake has not been quantified. Understanding the afore mentioned processes is a prerequisite to rational and accurate dosing or use of liposomal formulations. Therefore there is a need for further studies on the mechanism of tissue uptake of liposomes.

2. Although the concept of tissue drug targeting with liposome is attractive for treatment of organ selective disease or disorders, there is a need for further studies on optimisation of dose to avoid drug toxicity.

3. More studies are needed on mechanisms of liposomal uptake by the brain, and the role of the blood brain barrier in this process. Specifically, for the present study, the concept of saturable uptake of liposomes to the brain elucidated needs further investigations.

4. Since results of animal studies do not necessarily apply to humans, there is a need to undertake clinical studies in humans before definite conclusions can be made.

REFERENCES

Abra, R.M. and Hunt, C.A. (1981) Liposome disposition *in vivo*. III. Dose and vesicle size effects. *Biochimica et Biophysica Acta* 666, 493-503.

Abraham, I., Goundalkar, A. and Mezei, M. (1984) Effect of liposomal surface charge on the pharmacokinetics of an encapsulated model compound. *Biopharmaceutics and Drug Disposition* 5, 387-398.

Abdulsalam, I.A.A., Clark, B.J. and Chrystyn, H. (2002) Determination of gentamicin in urine sample after inhalation by reversed-phase high-performance liquid chromatography using pre-column derivatisation with *o*-phthalaldehyde. *Journal of Chromatography B* 769, 89-95.

Agatonovic-Kustrin, S., Zivanovic, Lj., Zecevic, M. and Radulovic, D. (1997) Spectrophotometric study of diclofenac-Fe (III) complex. *Journal of Pharmaceutical and Biomedical Analysis* 16, 147-153.

Agrawal, Y.K. and Shivramchandran, K. (1991) Spectrophotometric determination of diclofenac sodium in tablets. *Journal of Pharmaceutical and Biomedical analysis* 9 (2), 97-100.

Allen, T.M., Hansen, C.B., Guo, L.S. (1993) Subcutaneous administration of liposomes: a comparison with the intravenous and intraperitoneal routes of injection. *Biochimica et Biophysica Acta* 1150 (1), 9-16.

Allen, T.M. (1998) Liposomal drug formulations. Rationale for development and what we can expect for the future. *Drugs* 56 (5), 747-756.

Ara, J., Gans, Z., Sweeney, R. and Wolf, B. (1995) Dot-ELISA for the rapid detection of gentamicin in milk. *Journal of Clinical and Laboratory Analysis* 9, 320-324.

Avanti polar lipids, Inc. *http: //www.avantilipids.com/PreparationOfLiposomes.html*

Avgerinos, A., Karidas, Th. and Malamataris, S. (1993) Extractionless high-performance liquid chromatographic method for the determination of diclofenac in human plasma and urine. *Journal of Chromatography* 619, 324-329.

Bakay, L. (1956) The blood brain barrier, C. Thomas, Springfield, III.

Bengham, A.D. (1992) Liposomes: realizing their promise. *Hospital Practice* December 15, 51-62.

Brown, S.A., Sugimoto, K., Smith, G.G. and Garry, F.B. (1988) Improved sodium hydroxide digestion method without homogenisation for extraction of gentamicin from renal tissue. *Antimicrobial Agents and Chemotherapy* 32 (4), 595-597.

Cabanes, A., Reig, F., Anton, J.M. and Arboix, M. (1995) Sustained release of liposome-encapsulated enrofloxacin after intramuscular administration in rabbits. *American Journal of Veterinary Research* 56 (11), 1498-1501.

Claes, P.J., Busson, R. and Vanderhaeghe,H. (1984) Determination of the component ratio of commercial gentamicins by high-performance liquid chromatography using pre-column derivatization. *Journal of Chromatography* 298, 445-457.

Colley, C.M. and Ryman, B.E. (1975) Liposomes as carriers *in vivo* for methotrexate. *Biochemical Society Transactions. 553 rd Meeting, London*, 157-159.

Crommelin, D.J.A and Storm, G. (2003) Liposomes: From the bench to the bed. *Journal of Liposome Research* 13 (2), 33-36.

Dadashzadeh, S., Vali, A.M. and Rezagholi, N. (2002) LC determination of piroxicam in human plasma. *Journal of Pharmaceutical and Biomedical Analysis* 28, 1201-1204.

Deamer, D.W. (1978) Preparation and properties of ether-injection liposomes. *Annals of the New York Academy of Sciences* 308, 250-257.

Demaeyer, P., Akodad, E.M., Gravet, E., Schietecat, P., Van-Vooren, J.P., Drowart, A., Yernault, J.C. and Legros, F.J. (1993) Disposition of liposomal gentamicin following intrabronchial administration in rabbits. *Journal of Microencapsualtion* 10 (1), 77-88.

Donald, C.H. and Twomey, T.M. (1979) Piroxicam pharmacokinetics in man: aspirin and antacid interaction studies. *Journal of Clinical Pharmacology* 19 (5-6), 270-281.

Fendler, J.H. and Romero, A. (1977) Liposomes as drug carriers. *Life Sciences* 20, 1109-1120.

Fielding, R.M. (1991) Liposomal drug delivery. Advantages and limitations from a clinical pharmacokinetic and therapeutic perspective. *Clinical Pharmacokinetics* 21, (3) 155-164.

Foradada, M., Pujol, M.D., Bermudez, J. and Estelrich, J. (2000) Chemical degradation of liposomes by serum components detected by NMR. *Chemistry and Physics of Lipids* 104, 133-148.

Gibaldi, M. and Perrier, D. (1982) Pharmaceutics. In: Drugs and Pharmaceutical Sciences vol. 15, 2nd edn., New York, Marcel Dekker, U.S.A.

Gilbert, D.N. and Kohlhepp, S.J. (1986) New sodium hydroxide digestion method for measurement of renal tobramycin concentrations. *Antimicrobial Agents and Chemotherapy* 30, 361-365.

Gillilan R.B., Mason, W.D. and Fu, C.H.J. (1989) Rapid analysis of piroxicam in dog, rat and human plasma by high-performance liquid chromatography. *Journal of Chromatography* 487 (1), 232-235.

Gregoriadis, G. and Ryman, B.E. (1972) Fate of protein-containing liposomes injected into rats. An approach to the treatment of storage diseases. *European Journal of Biochemistry* 24, 485-491.

Gregoriadis, G. (1976) The carrier potential of liposomes in biology and medicine. *The New England Journal of Medicine* 295 (13), 704-710.

Gregoriadis, G., Neerunjun, D. E. and Hunt, R. (1977) Fate of liposome-associated agent injected into normal and tumour-bearing rodents. Attempts to improve localisation in tumour tissues. *Life Sciences* 21, 357-370.

Gregoriadis, G. (1990) Immunological adjuvants: a role of liposomes. *Immunology Today* 11 (3), 89-97.

Gregoriadis, G. (1991) Overview of liposomes. *Journal of Antimicrobial Chemotherapy* 28, 39-48

Gurtler, F., Kaltsatos, V., Biosrame, B., Deleforge, J., Gex-Fabry, M., Balant, L.P. and Gurny R. (1995) Ocular availability of gentamicin in small animals after topical administration of a conventional eye drop solution and a novel long acting bioadhesive ophthalmic drug insert. *Pharmaceutical Research* 12 (11), 1791-1795.

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. *Biochimica et Biophysica Acta* 1151, 201-215.

Hernandez-Caselles, T., Vera, A., Crespo, F., Villalain, J. and Gomez-Fernandez, J.C. (1989) Treatment of *Brucella melitensis* infection in mice by use of liposome-encapsulated gentamicin. *American Journal of Veterinary Research* 50 (9), 1486-1488.

Hirano, K. and Hunt, C.A. (1985) Lymphatic transport of liposome-encapsulated agents: Effects of liposome size following intraperitoneal administration. *Journal of Pharmaceutical Sciences* 74 (9), 915-921.

Hong, J.Y. and Choi, M.J. (2002) Development of one-step fluorescence polarization immunoassay for progesterone. *Biological and Pharmaceutical Bulletin* 25 (10), 1258-1262.

Hsieh, Y.F., Chen, T.L, Wang, Y.T., Chang, J.H. and Chang, H.M. 2002. Properties of liposomes prepared with various lipids. *Journal of Food Science* 67 (8), 2808-2813.

Jonah, M.M., Cerny, E.A. and Rahman, Y.E. (1975) Tissue distribution of EDTA encapsulated within liposomes of varying surface properties. *Biochimica et Biophysica Acta* 401, 336-348.

Josepovitz, C., Pastoriza-Munoz, E., Timmerman, D., Scott, M., Feldman, S. and Kaloyanides, G.J. (1982) Inhibition of gentamicin uptake in rat renal cortex *in vivo* by aminoglycosides and organic polycations. *Journal of Pharmacology and Experimental Therapeutics* 223 (2), 314-321.

Juliano, R.L. and Stamp, D. (1975) The effect of particle size and charge on the clearance rates of liposomes and liposome encapsulated drugs. *Biochemical and Biophysical Research Communications* 63 (3), 651-658.

Julien, N., Karzazi, M., Labrecque, G., Beauchamp, D. and Thibault, L. (2000) Temporal modulation of nephrotoxicty, feeding and drinking in gentamicin-treated rats. *Physiology and Behavior* 68, 533-541.

Kaplan, J.H. (1972) Anion diffusion across artificial lipid membranes: The effect of lysozyme on anion diffusion from phospholipid liposomes. *Biochimica et Biophysica Acta* 290, 339-347.

Kim, C.-K., Lee, M.-K., Han, J.-H. and Lee B.-J. (1994) Pharmacokinetics and tissue distribution of methotrexate after intravenous injection of differently charged liposome-entrapped methotrexate to rats. *International Journal of Pharmaceutics* 108, 21-29.

Kimelberg, H.K. (1976) Differential distribution of liposome-entrapped [3H]methotrexate and labelled lipids after intravenous injection in a primate. *Biochimica et Biophysica Acta* 448, 531-550.

Klopas, A., Panderi, I. And Parissi-Poulou, M. (1998) Determination of piroxicam and its major metabolite 5-hyroxypiroxicam in human plasma by zero-crossing first-derivative

spectrophotometry. *Journal of Pharmaceutical and Biomedical Analysis* 17 (3), 515-524.

Korn, E.D. (1970) The effect of charge on the physical and biological properties of liposomes. A report of work in progress. *Proceedings of the Physiological Society* 112*P*-113*P*.

Lasic, D.D. (1998) Novel applications of liposomes. *Trends in Biotechnology* 16, 307-321.

Lian, T and Rodney, J.Y. Ho. (2001) Trends and developments in liposome drug delivery systems. *Journal of Pharmaceutical Sciences* 90 (6), 667-680.

Moreno, M.A., Frutos, P. and Ballesteros, M.P. (1998) Extraction and liquid chromatographic determination of amphotericin B in oil-water lecithin-based microemulsions. *Chromatograpia* 48 (11-12), 803-806.

Morgan, J.R. and Williams, K.E. (1980) Preparation and properties of liposome-associated gentamicin. *Antimicrobial Agents and Chemotherapy* 17 (4), 544-548.

Nabar, S.J. and Nadkarni, G.D. (1998) Effect of size and charge on biodistribution of encapsulated ^{99m}Tc-DTPA in rats. *Indian Journal of Pharmacology* 30, 199-202.

Nagayasu, A., Uchiyama, K. and Kiwada, H. (1999) The size of liposomes: a factor which affects their targeting efficiency to tumors and therapeutic activity of liposomal antitumor drugs. *Advanced Drug Delivery Reviews* 40 (1-2), 75-87.

Nakagaki, M., Katoh, I. and Handa, T. (1981) Surface potential of lipid membrane estimated from the partitioning of methylene blue into liposomes. *Biochemistry* 20, 2208-2212.

Neuwelt, E.A, Baker, B.E., Pagel, M.A. and Blank, N.K. (1984) Cerebrovascular permeability and delivery of gentamicin to normal brain and experimental brain abscess in rats. *Journal of Neurosurgery* 61 (3), 430-439.

Ostro, M.J. (1987) Liposomes. *Scientific American* 91-99.

Papahadjoulos, D., Poste, G., Schaeffer, B.E. and Vail, W.J. (1974) Membrane fusion and molecular segregation in phospholipid vesicles. *Biochimica et Biophysica Acta* 352, 10-28.

Patel, H.M and Russell, N.J. (1988) Liposomes: From membrane model to therapeutic applications. *Biochemical Society Transactions, 625th Meeting, London* 16, 909-922.

Poste, G. and Papahadjopoulos, D. (1975) Lipid vesicles as carriers for introducing materials into culture cells: Influence of vesicle lipid composition on mechanism (s) of vesicle incorporation into cells. *Proceeding of National Academy of Sciences* 73 (5), 1603-1607.

Przeworska, E., Gubernator, J. and Kozubek A. (2001) Formation of liposomes by resorcinolic lipids, single-chain phenolic amphiphiles from *Anacardium occidentale* L. *Biochimica et Biophysica Acta* 1513, 75-81.

Ramasammy, L.S. and Kaloyanides, G.J. (1988) The effect of gentamicin on the biophysical properties of phosphatidic acid liposomes is influenced by the O-C=O group of the lipid. *Biochemistry* 27, 8249-8254.

Reamer, R.H., Dey, B.P., White, C.A. and Mageau, R.P. (1998) Comparison of monolayer and bilayer plates used in antibiotic assay. *Journal of AOAC. International.* 81 (2), 398-402.

Roche molecular biochemicals, lab FAQ's, Germany.

Roskos, L.K. and Boudinot, F.D. (1990) Effects of dose and sex on the pharmacokinetics of piroxicam in the rat. *Biopharmaceutics and Drug Disposition* 11 (3), 215-225.

Ryman, B.E., Jewkes, R.F., Jeyasingh, K., Osborne, M.P., Patel, H.M., Richardson, V.J., Tattersall, M.H.N., Tyrrell, D.A. (1978) Lipid vesicles as carriers for introducing drugs and other biologically active materials into cells. II. *In vivo* systems. Potential applications of liposomes to therapy. *Annals of the New York Academy of Sciences* 308, 281-307.

Sabath, L.D., Casey J.I., Ruch, P.A., Stumpf, L.L. and Finland, M. (1971) Rapid microassay of gentamicin, kanamycin, neomycin, streptomcin and vancomycin in serum or plasma. *Journal of Laboratory and Clinical Medicine* 78 (3), 457-463.

Sakamoto, A. and Ido, T. (1993) Liposome targeting to the rat brain: effect of osmotic opening of the blood brain barrier. *Brain Research* 629 (1), 171-175.

Sampath, S.S. and Robinson, D.H. (1990) Comparison of new and existing spectrophotometric methods for the analysis of tobramycin and other aminoglycosides. *Journal of Pharmaceutical Sciences* 79 (5), 428-431.

Sastry, C.S.P., Tipirneni, A.S.R.P. and Suryanarayana, M.V. (1989) Extractive spectrophotometric determination of some anti-inflammatory agents with methylene violet. *Analyst* 114, 513-515.

Senior, J.H., Trimble, K.R. and Maskiewicz, R. (1991) Interaction of positively-charged liposomes with blood: implications for their applications *in vivo*. *Biochimica et Biophysica Acta* 1070, 173-179.

Sessa, G. and Weissman, G. (1968) Phospholipid spherules (liposomes) as a model for biological membranes. *Journal of Lipid Research* 9, 310-318.

Sessa, G. and Weissman G. (1970) Incorporation of lysozyme into liposomes. *Journal of Biological Chemistry* 245 (13), 3295-3301.

Shinozawa, S., Araki, Y. and Oda, T. (1979) Distribution of [3H] prednisolone entrapped in lipid layer of liposomes after intramuscular administration in rats. *Research Communications in Chemical Pathology and Pharmacology* 24 (2), 223-232.

Steger L.D. and Desnick R.J. (1977) Enzyme therapy VI: Comparative *in vivo* fates and effects on lysosomal integrity of enzyme entrapped in negatively and positively charged liposomes. *Biochimica et Biophysica Acta* 464, 530-546.

Storm, G. and Crommelin, D.J.A. (1998) Liposomes: qua vadis? *Pharmaceutical Science and Technology Today* 1 (1), 19-31.

Swenson, C.E., Stewart, K.A., Hammett, J.L., Fitzsimmons, W.E. and Ginsberg, R.S. (1990) Pharmacokinetics and *in vivo* activity of liposome-encapsulated gentamicin. *Antimicrobial Agents and Chemotherapy* 34 (2), 235-240.

Szoka, F.Jr. and Papahadjopoulos, D. (1978) Procedure for preparation of liposomes with large internal aqueous space and high-capture by reverse phase evaporation. *Proceedings of the National Academy of Science* 75 (9), 4194-4198.

Tamai, I. and Tsuji, A. (1996) Drug delivery through the blood brain barrier. *Advanced Drug Delivery Reviews* 19 (3), 401-424.

Taylor, K.M.G. and Newton, J.M. (1994) Liposomes as a vehicle for drug delivery. *British Journal of Hospital Medicine* 51 (1/2), 55-60.

Turrens, J.F, Crapo, J.D. and Freeman, B.A. (1984) Protection against oxygen toxicity by intravenous injection of liposome-entrapped catalase and superoxide dismutase. *Journal of Clinical Investigation* 73, 87-95.

Wasserman, M., Beltran, R.M., Quintana, F.O., Mendoza, P.M., Orozco, L.C. and Rodriguez, G. (1986) A simple technique for entrapping rifampicin and isoniazid into liposomes. *Tubercle* 67, 83-90.

Woodle, M.C. and Papahadjoulos, D. (1989) Liposome preparation and size characterization. *Methods in Enzymology* 171, 193-217.

Yatvin, M.B. and Lelkes P.I. (1982) Clinical prospects of liposomes. *Medical Physics* 9 (2), 149-175.

Yoshihara, E. and Nakae, T. (1986) Cytolytic activity of liposomes containing stearylamine. *Biochimica et Biophysica Acta* 854 (1), 93-101.

SUMMARY

THE EFFECT OF LIPOSOMAL CHARGE ON THE DISTRIBUTION OF LIPOSOMES TO THE LIVER, BRAIN, LUNGS AND KIDNEYS IN A RAT MODEL

Gentamicin was selected out of three drugs as the most appropriate liposomal marker based on its properties There after, a simple method for preparation of charged liposomes by rotary evaporation and hydration was adopted. Surface charge was induced by varying the lipid composition whereby neutral liposomes were prepared using phosphatidyl choline and cholesterol (9.7:6.9, molar ratio), negative and positive liposomes were prepared by addition of dicetyl phosphate (5:1:0.5, molar ratio) and stearylamine (5:1:0.5, molar ratio) to the neutral liposomes, respectively. The distribution of the encapsulated gentamicin to the specified organs in liposome treated groups was compared to a control group treated with free gentamicin at the following intervals: 1, 2, 4, 6 and 8 hours post injection. Gentamicin (60 mg/kg) free and liposome entrapped was administered intraperitoneally and five rats of each group were utilised at each time interval. Under ether anaesthesia, a blood sample was drawn and the relevant organs were harvested. The sodium hydroxide digestion method was used to extract gentamicin from the organs, and gentamicin in plasma and organ extracts was measured by fluorescence polarisation immunoassay.

Liposomal characterisation revealed multilammelar liposomes with a mean internal diameter of 3.17 ± 1.9 μm, and encapsulation efficiency greater than 15 %. In the animal studies, liposomes delayed elimination of the encapsulated drug. The half life was 2.02 ± 0.5, 1.76 ± 0.1 and 2.04 ± 0.3 hours for the negative, positive and neutral liposome treated groups, respectively, versus 1.53 ± 0.02 hours for the control group. Peak plasma gentamicin concentrations were higher with positive liposomes than negative and neutral liposomes at 1 hour, while the negative liposomes depicted a sustained release pattern between 4 and 8 hours.

Distribution of liposomes to the brain and liver was dependent on liposomal surface charge. Liposomes improved gentamicin concentrations in the brain with positive liposomes highest in this regard. A biphasic pattern of distribution to the brain, with lowest gentamicin concentration at 4 hours was observed in the three liposome groups, and this was more

marked in the negative liposome group. Generally, hepatic gentamicin concentrations were higher with liposomes than the control. Although, the average hepatic gentamicin concentrations were highest for positive liposomes, the negative liposomes were preferred for the liver because the concentrations were more consistent and increased with time. Uptake of gentamicin by the lungs was not enhanced by liposomes and was independent of surface charge of the liposomes. Renal concentrations of gentamicin were lower (3 to 5 folds) with liposomes, and uptake was not charge dependent.

In conclusion, a simple method for preparation of liposomes was adopted. The distribution studies suggested that positively charged liposomes had highest affinity for the brain and the negative liposomes for the liver. Also, liposomes irrespective of charge exhibited reduced renal concentration of gentamicin.

Key Words: Liposomes, surface-charge, positive-liposomes, negative-liposomes, neutral-liposomes, brain, kidneys, liver, lungs, organ-targeting.

OPSOMMING

DIE EFFEK VAN LIPOSOME MET LADING OP DIE VERSPREIDING VAN LIPOSOME NA DIE LEWER, BREIN, LONGE EN NIERE IN 'N DIERMODEL

Gentamisien is uit drie middels gekies as die beste liposoommerker. 'n Eenvoudige metode is aangepas vir die voorbereiding van liposome met lading deur middel van rotasieverdamping en hidrering. Oppervlaklading is moontlik gemaak deur die lipiedsamestelling van die liposome te varieer. Neutrale liposome is voorberei deur fosfatidielcholien en cholesterol (9.7:6.9, mol verhouding) te gebruik. Negatiewe en positiewe liposome is voorberei deur byvoeging van disetielfosfaat (5:1:0.5, mol verhouding) en stearielamien (5:1:0,5, mol verhouding) by neutrale liposome onderskeidelik. Die verspreiding van die gekapsileerde gentamisien na die spesifieke teikenorgane in die liposoombehandelde groepe is vergelyk met 'n kontrole groep wat behandel is met vrye gentamisien met tydsintervalle van 1, 2, 4, 6 en 8 ure na toediening. Vry gentamisien (60 mg/kg) en gentamisien bevattende liposome is intraperitoriaal toegedien en vyf rotte van elke groep is gebruik tydens elke tydsinterval. Bloedmonsters is tydens eterverdowing geneem en die spesifieke organe is verwyder. 'n Natrium Hidroksied verterings metode is gebruik om die gentamisien uit die organe te ekstraheer en die gentamisien in die plasma en organe is bepaal deur fluoreserende polarisasie immunobepaling.

Liposoomkarakterisering toon 'n veelvoudige laag liposoom met 'n gemiddelde radius van 3.17 ± 1.9 µm met 'n inkapselering effektiwiteit van meer as 15 %. Tydens die dierestudies het die liposome die eliminasie van die gekapsileerde middel vertraag. Die half-leeftyd was 2.02 ± 0.5, 1.76 ± 0.1 en 2.04 ± 0.3 ure vir die negatiewe, positiewe en neutrale liposoombehandelde groepe onderskeidelik teenoor 1.53 ± 0.02 ure vir die kontrole groep. Die plasma gentamisien konsentrasies was hoër vir die positiewe liposome as vir die negatiewe en neutrale liposome by 1 uur, terwyl die negatiewe liposome 'n konstante vrystellingspatroon tussen 4 en 8 ure getoon het.

Die verspreiding van die liposome na die brein en die lewer was afhanklik van die oppervlaklading van die liposome. Liposome met 'n positiewe lading verbeter die gentamisienkonsentrasie in die brein. 'n Dubbelfasige verspreidingspatroon na die brein in

die drie liposoomgroepe is waargeneem met die laagste gentamisien konsentrasie by 4 ure. Die patroon was meer sigbaar in die negatiewe liposoomgroep. In die algemeen is die hepatiese gentamisien konsentrasie hoër in die teenwoordigheid van liposome as in die kontrole groep. Die gemiddelde hepatiese gentamisien konsentrasie was die hoogste vir die positief gelaaide liposome. Dit blyk egter dat die lewer die negatiewe liposome verkies, omdat die verhoogde gentamisien konsentrasie in die lewer meer konstant is gedurende die tydsinterval. Die opname van gentamisien deur die longe was nie verhoog deur die liposome nie en was onafhanklik van die oppervlaklading van die liposome. Gentamisienkonsentasie in die niere was drie tot vyf maal laer in die teenwoordigheid van liposome en is dus nie ladingafhanklik nie.

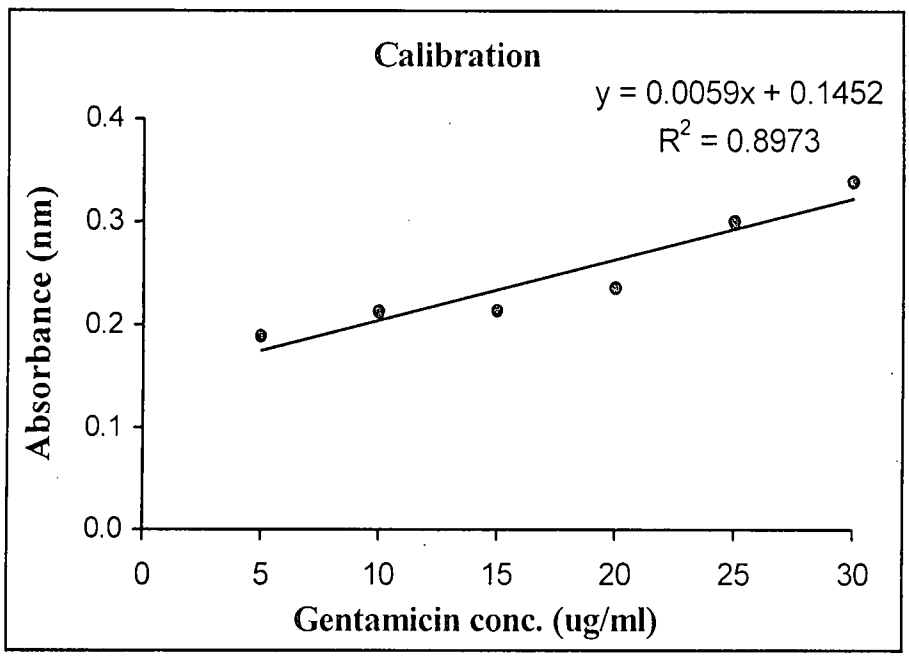
In gevolgtrekking is 'n eenvoudige metode vir die voorbereiding van liposome aangepas. Die verspreidingstudies toon dat liposome met positiewe lading die hoogste affiniteit het vir die brein en die negatiewe liposome vir die lewer. 'n Verlaagde gentamisienkonsentrasie is in die niere waargeneem ongeag die oppervlaklading van die liposome.

Sleutelwoorde: Liposome, oppervlaklading, positiewe liposome, negatiewe liposome, neutrale liposome, teikenorgaan, brein, niere, lewer, longe.

APPENDICES

APPENDIX A

Appendix A 1: Development of spectrophotometric assay of gentamicin, a plot of gentamicin concentrations (5 – 30 μg/ml) versus absorbance (nm), when total reaction volume was adjusted to 3 ml.



Appendix A 2: Interday calibration data for the spectrophotometric assay of gentamicin.

S/No	Gent. Conc. (mg/ml)	Absorbance on day					Mean Absorbance	S.D	% C.V
		1	2	3	4	5			
1	2	0.459	0.463	0.461	0.542	0.518	0.489	0.039	7.93
2	4	0.529	0.554	0.541	0.549	0.558	0.546	0.012	2.11
3	8	0.669	0.698	0.686	0.677	0.641	0.674	0.021	3.18
4	10	0.763	0.775	0.768	0.734	0.674	0.743	0.042	5.59
5	15	0.949	0.907	0.965	0.924	0.787	0.906	0.070	7.77
6	20	1.119	1.04	1.12	1.364	1.191	1.167	0.123	10.50
				Average				**0.051**	**6.18**

APPENDIX B

Appendix B 1: Liposome size (internal diameter in μm) from the electron micrographs, and ranking and percentile data.

Photo No.	S/No.	Mean diameter (um)
1	1	4.70
10	2	0.94
	3	0.81
	4	0.50
	5	0.41
	6	0.61
	7	0.67
	8	0.52
	9	0.52
	10	0.72
	11	0.47
5	12	5.70
	13	8.30
	14	6.05
	15	5.70
	16	8.05
	17	4.80
	18	2.95
	19	3.20
	20	10.0
	21	2.70
14	22	2.88
	23	2.50
	24	2.75
	25	2.00
	26	1.63
	27	2.25
	28	2.50
	29	6.00
	30	3.50
	31	4.75
	32	5.25
	33	5.50
	34	1.88
	35	2.75
	36	3.00
	37	2.50
	38	1.88
	39	3.00
	40	2.88
9	41	0.95

Mean diameter (um)	Rank	Percent
10.0	1	100.00%
8.30	2	98.60%
8.05	3	97.20%
6.05	4	95.90%
6.00	5	93.20%
6.00	5	93.20%
5.70	7	90.50%
5.70	7	90.50%
5.50	9	87.80%
5.50	9	87.80%
5.25	11	86.40%
5.00	12	83.70%
5.00	12	83.70%
4.83	14	82.40%
4.80	15	81.00%
4.75	16	79.70%
4.70	17	78.30%
4.21	18	77.00%
4.14	19	75.60%
4.00	20	71.60%
4.00	20	71.60%
4.00	20	71.60%
3.97	23	70.20%
3.62	24	68.90%
3.50	25	66.20%
3.50	25	66.20%
3.33	27	64.80%
3.28	28	63.50%
3.20	29	62.10%
3.10	30	60.80%
3.00	31	55.40%
3.00	31	55.40%
3.00	31	55.40%
3.00	31	55.40%
2.95	35	54.00%
2.93	36	51.30%
2.93	36	51.30%
2.88	38	48.60%
2.88	38	48.60%
2.76	40	47.20%
2.75	41	44.50%

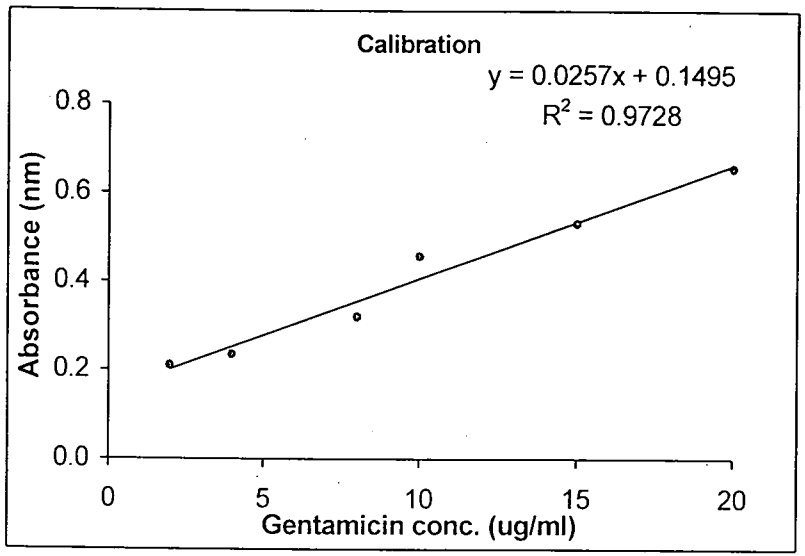
(Table continued)

Photo No.	S/No.	Mean diameter (um)
	42	2.07
	43	2.07
	44	2.07
	45	3.97
	46	2.93
	47	2.93
	48	2.59
	49	4.14
	50	3.28
	51	2.59
	52	4.83
	53	3.62
	54	2.07
	55	2.07
	56	3.10
	57	2.76
	58	2.07
	59	1.38
	60	2.07
7	61	4.00
	62	3.00
	63	4.00
13	64	5.50
	65	4.21
	66	3.33
	67	1.84
	68	2.46
4	69	4.00
	70	5.00
	71	5.00
	72	3.00
	73	6.00
12	74	2.00
	75	3.50
Mean		**3.17**
S.D.		**1.90**
Maximum		**10**
Minimum		**0.41**

Mean diameter (um)	Rank	Percent
2.75	41	44.50%
2.70	43	43.20%
2.59	44	40.50%
2.59	44	40.50%
2.50	46	36.40%
2.50	46	36.40%
2.50	46	36.40%
2.46	49	35.10%
2.25	50	33.70%
2.07	51	24.30%
2.07	51	24.30%
2.07	51	24.30%
2.07	51	24.30%
2.07	51	24.30%
2.07	51	24.30%
2.07	51	24.30%
2.00	58	21.60%
2.00	58	21.60%
1.88	60	18.90%
1.88	60	18.90%
1.84	62	17.50%
1.63	63	16.20%
1.38	64	14.80%
0.95	65	13.50%
0.94	66	12.10%
0.81	67	10.80%
0.72	68	9.40%
0.67	69	8.10%
0.61	70	6.70%
0.52	71	4.00%
0.52	71	4.00%
0.50	73	2.70%
0.47	74	1.30%
0.41	75	.00%

Appendix B 2: Sample template for liposome encapsulation efficiency determination.

Gent. Conc. mg/ml	Absorbance (nm)	Net Absorbance (nm)
0	0.201	0.000
2	0.412	0.211
4	0.437	0.236
8	0.522	0.321
10	0.659	0.458
15	0.733	0.532
20	0.858	0.657
Sp - 1_1	0.670	0.469
Sp - 1_2	0.683	0.482
Sp - 2_1	0.709	0.508
Sp - 2_2	0.719	0.518



Sample	Net Absorbance (nm)	Gent. conc. from graph** (mg/ml)	Volume of supernantant (ml)	Total Conc. (Conc.*Vol.) (mg)	Encapsulated gent. (mg) (160-Total conc.)	Encapulation efficiency (%) (Conc./160) *100	Average encapsulated gent. (mg)	Average encapsulation efficiency (%)
Sp - 1_1	0.469	12.43	9.4	116.86	43.14	26.96	40.76	25.48
Sp - 1_2	0.482	12.94	9.4	121.61	38.39	23.99		
Sp - 2_1	0.508	13.95	9.2	128.33	31.67	19.79	29.88	18.67
Sp - 2_2	0.518	14.34	9.2	131.91	28.09	17.55		

** x = (Net absorbance - 0.1495) / 0.0257

Sp - 1_1 and Sp 1_2 are reaction mixutres obtained using aliquots of supernatant of liposome preparation 1

Sp - 2_1 and Sp 2_2 are reaction mixutres obtained using aliquots of supernatant of liposome preparation 2

Appendix B 3: Encapsulation efficiency and total gentamicin encapsulated for negative liposomes.

S/No.	Date of preparation	Encapsulation efficiency (%)	Total gentamicin encapsulated (mg)
1	21/01/2003	25.80	41.28
2	17/02/2003	22.01	35.21
3	23/04/2003	25.48	40.77
4	23/04/2003	18.67	29.88
5	21/02/2003	27.86	44.59
6	21/02/2003	21.47	34.35
7	25/02/2003	25.45	40.77
8	25/02/2003	17.05	27.27
9	29/04/2003	26.28	42.05
10	29/04/2003	30.94	49.50
11	05/06/2003	31.00	49.60
12	05/06/2003	20.32	32.52
13	10/06/2003	21.58	34.53
	Mean	24.15	38.64
	S.D	4.39	7.02
	% C.V.	18.17	18.17

Appendix B 4: Encapsulation efficiency and total gentamicin encapsulated for positive liposomes.

S/No.	Date of preparation	Encapsulation efficiency (%)	Total gentamicin encapsulated (mg)
1	10/06/2003	27.32	43.71
2	13/06/2003	30.11	48.17
3	13/06/2003	25.76	41.21
4	17/06/2003	9.37	14.99
5	17/06/2003	21.78	34.85
6	23/06/2003	21.45	34.31
7	23/06/2003	28.43	45.49
8	26/06/2003	27.94	44.70
9	26/06/2003	19.76	31.62
10	30/06/2003	22.19	39.63
11	30/06/2003	14.10	22.64
12	03/07/2003	23.43	41.20
13	07/07/2003	29.79	44.69
	Mean	23.19	37.48
	S.D	6.17	9.33
	% C.V.	26.60	24.89

Appendix B 5: Encapsulation efficiency and total gentamicin encapsulated for neutral liposomes.

S/No.	Date of preparation	Encapsulation efficiency (%)	Total gentamicin encapsulated (mg)
1	07/07/2003	13.64	21.82
2	14/07/2003	17.91	28.66
3	14/07/2003	13.55	21.68
4	16/07/2003	8.98	14.37
5	16/07/2003	13.01	20.81
6	17/07/2003	21.96	38.52
7	21/07/2003	12.71	20.94
8	21/07/2003	15.90	25.87
9	22/07/2003	19.98	38.56
10	22/07/2003	20.42	34.43
11	25/07/2003	23.08	36.92
12	25/07/2003	20.63	33.00
13	28/07/2003	8.23	22.57
14	29/07/2003	17.48	27.97
15	29/07/2003	14.31	24.53
16	01/08/2003	14.30	22.88
17	01/08/2003	10.07	16.12
	Mean	15.66	26.45
	S.D	4.53	7.53
	% C.V.	28.93	28.46

Appendix B 6: Gentamicin dose data for negative, positive and neutral liposomes treated rats.

S/No.	Gentamicin administered (mg)		
	Negative	Positive	Neutral
1	20.64	21.85	10.91
2	17.61	24.09	28.66
3	20.38	20.61	21.68
4	20.17	19.94	14.37
5	22.29	19.94	20.81
6	17.18	19.95	19.26
7	20.38	19.95	20.94
8	13.64	19.08	25.87
9	21.03	19.08	18.25
10	24.75	19.82	18.25
11	24.8	11.32	17.48
12	16.26	20.60	17.48
13	17.27	22.35	22.57
14	N/A	N/A	17.50
15	N/A	N/A	17.50
16	N/A	N/A	19.50
17	N/A	N/A	19.50
Mean	19.7	19.9	19.4
S.D	3.26	2.93	4.05
% C.V.	16.52	14.75	20.81

APPENDIX C

Appendix C 1: Sample template for organ concentration determination for a specific rat (Control, group 2, 1 hr)

14/05/2003

Distribution of Gentamicin after Intraperitoneal injection - 1 hr

Weight of rat = 319 g
Dose - 2 ml of 10 mg/ml (20mg)

Conc.of gentamicin in plasma = 98.5 ug/ml

S/No.	Organ	Weight (g)	Volume of PBS-NaOH-CH3COOH (ml)	Conc. read by FPIA (ug/ml)	Total gent. conc (Conc.*Volume) (ug)	Conc. / Organ weight (ug/g)
1	Brain	1.75	18	0.05	0.90	0.51
2	Kidney	2.07	21	37.38	784.98	379.22
3	Liver	11.46	135	0.82	110.70	9.66
4	Lung	1.42	12	3.54	42.48	29.92

Appendix C 2a: Plasma and organ gentamicin concentrations (μg/ml) in rats of the **control** group treated with free gentamicin.

Time (hrs)	Plasma Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	166.5	98.5	83.4	124	89.1	112.3	34.05
2	23.57	25.76	25.76	28.63	30.76	26.90	2.81
4	1.45	1.89	2.34	1.71	3.30	2.14	0.73
6	0.53	0.70	0.46	0.41	0.80	0.58	0.16
8	0.27	0.42	0.00	0.19	0.23	0.22	0.15

Time (hrs)	Brain Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	3.02	0.51	0.00	2.71	1.45	1.54	1.32
2	0.38	0.00	0.00	0.00	0.85	0.25	0.38
4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Time (hrs)	Lungs Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	30.87	29.92	23.91	34.31	26.23	29.05	4.07
2	12.16	10.48	11.64	14.49	11.65	12.08	1.48
4	3.97	4.24	4.18	3.94	4.64	4.19	0.28
6	2.80	3.85	2.59	4.26	4.03	3.51	0.76
8	2.41	3.12	0.00	2.51	3.11	2.23	1.29

Time (hrs)	Kidneys Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	365.09	379.22	295.36	392.39	487.80	383.97	69.07
2	337.89	299.44	314.53	333.27	352.48	327.52	20.74
4	288.80	320.25	325.45	350.33	370.87	331.14	31.19
6	300.64	363.42	326.40	304.41	394.68	337.91	40.37
8	334.16	340.32	209.20	349.35	344.18	315.44	59.65

Time (hrs)	Liver Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	12.22	9.66	5.93	11.21	10.32	9.87	2.40
2	4.17	5.57	3.66	4.59	5.66	4.73	0.87
4	0.80	3.55	1.31	2.74	3.22	2.32	1.21
6	2.94	4.08	1.87	1.45	4.32	2.93	1.28
8	2.31	4.48	0.00	3.11	3.74	2.73	1.72

Appendix C 2b: Plasma and organ gentamicin concentrations (μg/ml) in rats treated with **negative liposomes**.

Time (hrs)	Plasma Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	5.83	5.05	2.85	7.01	6.63	5.47	1.65
2	3.02	1.91	1.76	4.03	2.41	2.63	0.93
4	2.51	1.82	0.00	0.78	1.54	1.33	0.97
6	0.52	0.95	2.31	1.34	1.06	1.24	0.67
8	0.75	1.22	1.57	1.48	1.55	1.31	0.34

Time (hrs)	Brain Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	2.25	0.00	0.00	1.88	3.04	1.43	1.37
2	1.28	0.00	2.19	0.00	2.29	1.15	1.12
4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	0.00	2.07	0.00	0.00	1.33	0.68	0.97
8	0.00	0.00	0.66	0.00	1.25	0.38	0.56

Time (hrs)	Lungs Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	2.71	0.89	1.96	4.67	5.64	3.17	1.95
2	3.46	1.58	1.57	8.17	3.69	3.69	2.70
4	1.83	2.90	0.35	4.06	4.03	2.63	1.57
6	0.00	5.60	2.08	6.95	3.45	3.62	2.76
8	2.00	0.00	3.02	2.71	4.10	2.37	1.52

Time (hrs)	Kidneys Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	62.24	45.47	35.1	81.06	62.41	57.26	17.66
2	80.19	65.45	64.31	75.21	62.81	69.59	7.66
4	66.35	76.62	7.84	72.96	110.52	66.86	37.16
6	88.05	112.16	69.11	72.45	97.66	87.89	17.86
8	132.35	84.75	93.89	86.66	128.42	105.21	23.27

Time (hrs)	Liver Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	2.71	0.00	1.04	1.33	4.38	1.89	1.69
2	3.46	1.46	0.00	2.37	4.13	2.28	1.64
4	1.83	5.76	0.00	4.27	8.98	4.17	3.48
6	4.56	4.89	10.14	16.80	9.52	9.18	4.97
8	9.24	11.77	9.07	8.36	13.61	10.41	2.21

Appendix C 2c: Plasma and organ gentamicin concentrations (μg/ml) in rats treated with **positive liposomes.**

Time (hrs)	Plasma Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	2.95	15.83	13.2	9.95	11.4	10.67	4.84
2	1.16	5.38	3.92	3.35	4.34	3.63	1.6
4	0.63	1.24	2.04	1.35	1.59	1.37	0.5
6	0.18	0.27	0.69	0.68	1.01	0.57	0.3
8	0.70	0.16	0.63	1.05	1.22	0.75	0.4

Time (hrs)	Brain Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	1.45	3.45	3.58	3.19	1.82	2.70	0.99
2	1.49	4.00	3.32	2.15	0.98	2.39	1.26
4	2.39	3.33	2.67	1.60	0.00	2.00	1.28
6	2.61	3.97	4.55	2.34	1.33	2.96	1.30
8	2.15	2.25	3.25	0.00	0.00	1.53	1.46

Time (hrs)	Lungs Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	3.06	14.8	5.79	6.58	4.51	6.95	4.59
2	1.50	6.46	4.44	5.62	3.57	4.32	1.92
4	4.19	4.66	5.67	2.91	2.70	4.03	1.24
6	2.97	2.47	6.29	4.02	2.01	3.55	1.70
8	4.60	1.77	4.08	3.02	3.42	3.38	1.08

Time (hrs)	Kidneys Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	41.39	107.36	92.0	87.39	129.36	91.50	32.45
2	50.53	119.2	96.27	98.13	104.60	93.75	25.78
4	91.68	146.1	119.28	151.20	196.14	140.88	38.97
6	16.95	10.8	120.26	144.22	153.32	89.11	69.77
8	127.51	2.81	190.98	192.93	171.38	137.12	79.56

Time (hrs)	Liver Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	3.07	5.49	5.34	3.05	5.38	4.47	1.28
2	2.48	4.88	5.73	5.27	4.30	4.53	1.26
4	24.24	10.59	20.99	10.06	8.03	14.78	7.31
6	2.41	4.16	6.29	29.53	14.02	11.28	11.12
8	25.38	2.02	26.05	19.00	17.43	17.98	9.69

Appendix C 2d: Plasma and organ gentamicin concentrations (μg/ml) in rats treated with **neutral liposomes.**

Time (hrs)	Plasma Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	3.02	3.2	3.3	1.89	2.23	2.73	0.63
2	1.44	3.23	2.38	1.00	0.72	1.75	1.0
4	1.25	0.56	0.52	0.42	0.63	0.68	0.3
6	0.41	0.66	0.42	1.02	0.63	0.63	0.2
8	0.39	0.41	0.37	0.31	0.4	0.38	0.0

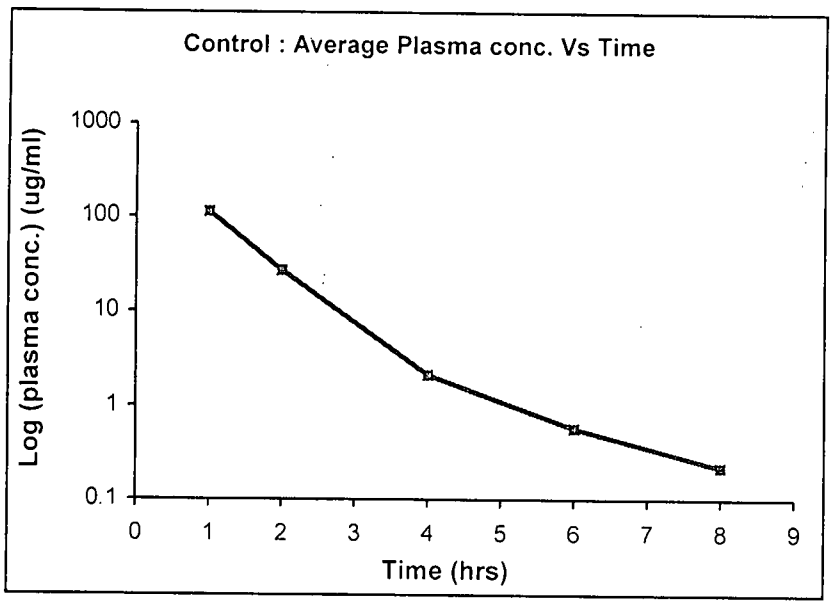
Time (hrs)	Brain Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	2.71	1.42	2.54	1.47	2.76	2.18	0.68
2	0.61	0.1	2.44	1.48	1.57	1.24	0.91
4	2.01	0.42	1.40	1.75	0.76	1.27	0.67
6	0.00	2.21	0.61	0.00	2.39	1.04	1.18
8	0.00	4.74	2.05	2.48	1.65	2.18	1.71

Time (hrs)	Lungs Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	1.50	1.52	2.69	2.18	3.36	2.25	0.79
2	0.00	2.83	2.52	2.40	1.25	1.80	1.17
4	4.02	0.88	1.80	2.06	1.43	2.04	1.19
6	1.64	1.87	1.55	2.42	2.98	2.09	0.60
8	1.44	2.18	2.38	1.69	3.11	2.16	0.65

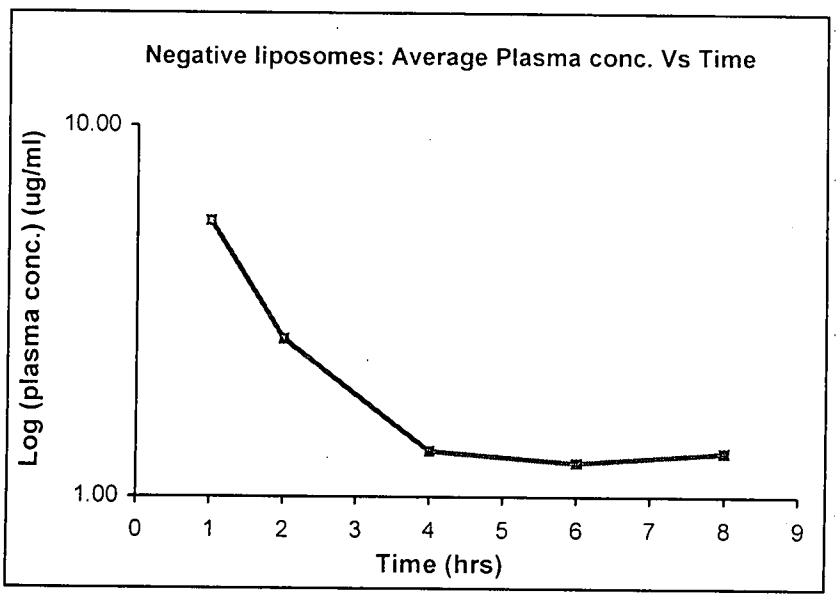
Time (hrs)	Kidneys Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	44.47	36.49	39.5	32.32	33.27	37.20	4.94
2	55.28	96.66	58.82	39.25	50.76	60.15	21.70
4	120.47	75.08	58.58	58.26	56.69	73.82	27.14
6	72.38	96.92	88.24	50.58	88.85	79.39	18.40
8	97.93	108.12	89.93	69.52	88.85	90.87	14.21

Time (hrs)	Liver Conc. (ug/ml)						
	1	2	3	4	5	Mean	S.D.
1	4.1	1.67	2.6	2.83	3.28	2.90	0.89
2	2.09	3.22	2.15	1.80	2.85	2.42	0.59
4	5.85	7.51	2.69	4.91	4.12	5.02	1.81
6	5.66	8.43	3.46	5.48	6.41	5.89	1.79
8	5.01	6.42	5.45	5.80	6.53	5.84	0.64

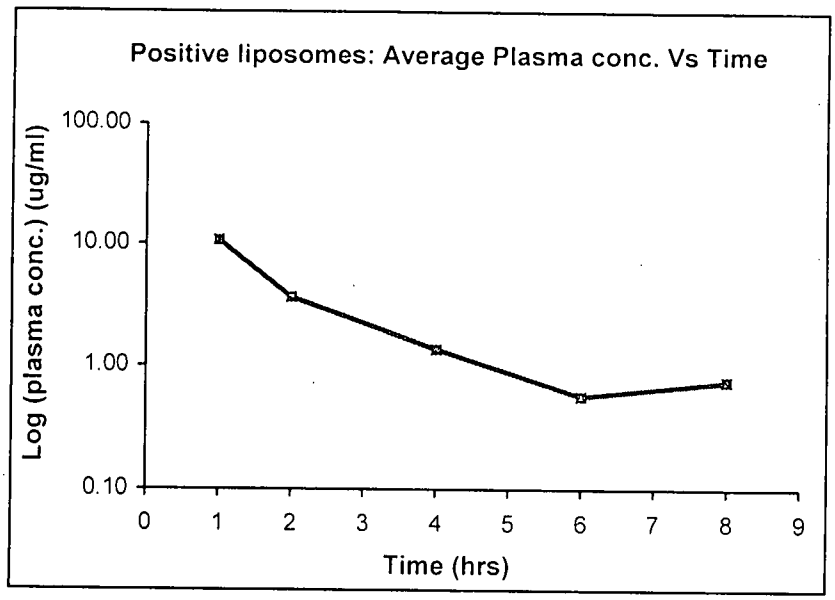
Appendix C 3a: Log graph of gentamicin plasma concentration for the control.



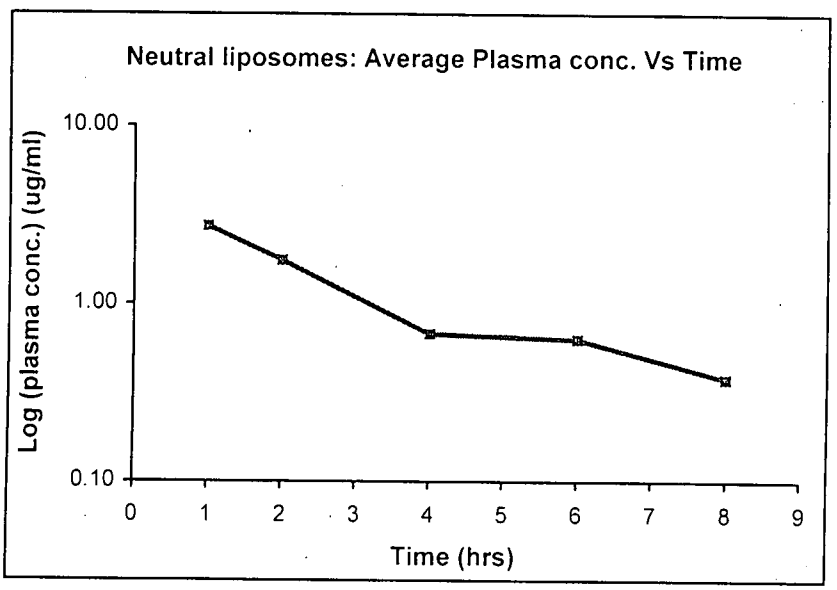
Appendix C 3b: Log graphs of gentamicin plasma concentrations for the negative liposome treated group.



Appendix C 3c: Log graphs of gentamicin plasma concentrations for the positive liposome treated group.



Appendix C 3d: Log graphs of gentamicin plasma concentrations for the neutral liposome treated group.



Appendix C 4: Derivation of equation for area under the curve.

From the known equation:

$$Cl = \frac{D}{AUC} \qquad \cdots\cdots \text{Eq } 3$$

Where Cl is the clearance, D is the dose of gentamicin administered and AUC is the area under the curve

Aware that clearance is also

$$Cl = V_d \times k_e \qquad \cdots\cdots \text{Eq } 6$$

Where V_d is the volume of distribution and Ke is the elimination rate constant.

Also dose is

$$D = V_d \times C_t \qquad \cdots\cdots \text{Eq } 8$$

Where C_t is concentration of the drug at time t.

Substituting equation 6 and 8 in equation 3:

$$V_d \times k_e = \frac{V_d \times C_t}{AUC} \qquad \cdots\cdots \text{Eq } 9$$

Therefore the equation may be reduced to:

$$k_e = \frac{C_t}{AUC} \qquad \cdots\cdots \text{Eq } 10$$

Rearranging equation 10:

$$\underline{\underline{AUC = \frac{C_t}{k_e}}} \qquad \cdots\cdots \text{Eq } 11$$

Appendix C 5a: Pharmacokinetic parameters of gentamicin in plasma between 1 and 4 hours for the control group.

Group	Conc.(ug/ml)		T 1/2 (hr)	K el (hr -1)	AUC 1 (ug.hr/ml)	AUC 4 (ug.hr/ml)	AUC 1-4 (ug.hr/ml)	Dose (ug)	Cl = Dose/AUC (ml/hr)	Cl (ml/min)	Vd (ml)
	1 hr	4 hr									
1	166.5	1.45	1.51	0.46	362.8	3.2	359.6	20000	55.61	0.93	121.18
2	98.5	1.89	1.53	0.45	217.5	4.2	213.3	20000	93.77	1.56	207.02
3	83.4	2.34	1.54	0.45	185.3	5.2	180.1	20000	111.03	1.85	246.73
4	124	1.71	1.52	0.46	272.0	3.8	268.2	20000	74.56	1.24	163.55
5	89.1	3.3	1.56	0.44	200.6	7.4	193.1	20000	103.55	1.73	233.10
Mean	112.30	2.14	1.53	0.45	247.63	4.74	242.89		87.70	1.46	194.31
S.D	34.1	0.7	0.02	0.01	72.2	1.7	73.4		22.6	0.4	51.7

Appendix C 5b: Pharmacokinetic parameters of gentamicin in plasma between 1 and 4 hours for the negative liposome treated group.

Group	Conc.(ug/ml)		T 1/2 (hr)	K el (hr -1)	AUC 1 (ug.hr/ml)	AUC 4 (ug.hr/ml)	AUC 1-4 (ug.hr/ml)	Dose (ug)	Cl = Dose/AUC (ml/hr)	Cl (ml/min)
	1 hr	4 hr								
1	5.83	2.51	2.63	0.26	22.1	9.5	12.6	19700	51.20	0.85
2	5.05	1.82	2.35	0.29	17.1	6.2	11.0	19700	57.30	0.96
3	2.85	0	1.5	0.46	6.2	0.0	6.2	19700	89.77	1.50
4	7.01	0.78	1.69	0.41	17.1	1.9	15.2	19700	79.68	1.33
5	6.63	1.54	1.95	0.36	18.7	4.3	14.3	19700	69.05	1.15
Mean	5.47	1.33	2.02	0.36	16.23	4.39	11.85		69.40	1.16
S.D	1.6	1.0	0.5	0.1	6.0	3.7	3.6		15.8	0.3

Appendix C 5c: Pharmacokinetic parameters of gentamicin in plasma between 1 and 4 hours for the positive liposome treated group.

Group	Conc.(ug/ml)		T 1/2 (hr)	K el (hr -1)	AUC 1 (ug.hr/ml)	AUC 4 (ug.hr/ml)	AUC 1-4 (ug.hr/ml)	Dose (ug)	Cl = Dose/AUC (ml/hr)	Cl (ml/min)
	1 hr	4 hr								
1	2.95	0.63	1.91	0.36	8.1	1.7	6.4	19900	70.50	1.18
2	15.83	1.24	1.63	0.43	37.2	2.9	34.3	19900	82.61	1.38
3	13.2	2.04	1.77	0.39	33.7	5.2	28.5	19900	76.08	1.27
4	9.95	1.35	1.74	0.40	25.0	3.4	21.6	19900	77.39	1.29
5	11.4	1.59	1.74	0.40	28.6	4.0	24.6	19900	77.39	1.29
Mean	10.67	1.37	1.76	0.40	26.54	3.45	23.09		76.79	1.28
S.D	4.8	0.5	0.1	0.02	11.3	1.3	10.5		4.3	0.1

Appendix C 5d: Pharmacokinetic parameters of gentamicin in plasma between 1 and 4 hours for the neutral liposome treated group.

Group	Conc.(ug/ml)		T 1/2 (hr)	K el (hr -1)	AUC 1 (ug.hr/ml)	AUC 4 (ug.hr/ml)	AUC 1-4 (ug.hr/ml)	Dose (ug)	Cl = Dose/AUC (ml/hr)	Cl (ml/min)
	1 hr	4 hr								
1	3.02	1.25	2.56	0.27	11.2	4.6	6.5	19400	52.60	0.88
2	3.2	0.56	1.82	0.38	8.4	1.5	6.9	19400	73.99	1.23
3	3.3	0.52	1.78	0.39	8.5	1.3	7.1	19400	75.65	1.26
4	1.89	0.42	1.93	0.36	5.3	1.2	4.1	19400	69.77	1.16
5	2.23	0.63	2.09	0.33	6.7	1.9	4.8	19400	64.43	1.07
Mean	2.73	0.68	2.04	0.35	8.01	2.10	5.91		67.29	1.12
S.D	0.6	0.3	0.3	0.05	2.2	1.4	1.4		9.3	0.2

Appendix C 6a: P-values from comparison of gentamicin plasma concentration between liposome treated groups using the Mann-Whitney test (on graph pad instat program).

Time (hrs)	Two-tailed P value in plasma between		
	Negative lipo.Vs Positive lipo.	Negative lipo.Vs Neutral lipo.	Positive lipo. Vs Neutral lipo.
1	0.0952	0.0556	0.0556
2	0.3095	0.2222	0.0556
4	> 0.9999	0.2222	0.0469 *
6	0.0952	0.0952	> 0.9999
8	0.0469 *	0.0079 *	0.1508

* $P < 0.05$

Appendix C 6b: P-values from comparison of gentamicin brain concentration between liposome treated groups using the Mann-Whitney test.

Time (hrs)	Two-tailed P value in brain between		
	Negative lipo.Vs Positive lipo.	Negative lipo.Vs Neutral lipo.	Positive lipo. Vs Neutral lipo.
1	0.3095	0.222	0.5476
2	0.2222	0.3095	0.6905
4	0.3095	-	-
6	0.0556	0.0281 *	0.5245
8	0.8339	0.2889	0.0736

* $P < 0.05$

Appendix C 6c: P-values from comparison of gentamicin lung concentration between liposome treated groups using the Mann-Whitney.

Time (hrs)	Two-tailed P value in lung between		
	Negative lipo.Vs Positive lipo.	Negative lipo.Vs Neutral lipo.	Positive lipo. Vs Neutral lipo.
1	0.0952	0.5476	0.0159 *
2	0.6905	0.2222	0.0556
4	0.1508	0.4206	0.0317 *
6	> 0.9999	0.3095	0.0952
8	0.3465	0.6905	0.0952

* $P < 0.05$

Appendix C 6d: P-values from comparison of gentamicin kidney concentration between liposome treated groups using the Mann-Whitney.

Time (hrs)	Two-tailed P value in kidney between		
	Negative lipo.Vs Positive lipo.	Negative lipo.Vs Neutral lipo.	Positive lipo. Vs Neutral lipo.
1	0.0952	0.0556	0.0159 *
2	0.1508	0.1508	0.1508
4	0.0159 *	0.8413	0.0317 *
6	0.6905	0.6905	0.6905
8	0.3095	0.3095	0.1508

* $P < 0.05$

Appendix C 6e: P-values from comparison of gentamicin liver concentration between liposome treated groups using the Mann-Whitney.

Time (hrs)	Two-tailed P value in liver between		
	Negative lipo.Vs Positive lipo.	Negative lipo.Vs Neutral lipo.	Positive lipo. Vs Neutral lipo.
1	0.0317 *	0.3095	0.0952
2	0.0317 *	> 0.9999	0.0317 *
4	0.0159 *	0.6905	0.0079 *
6	0.8413	0.4206	0.8413
8	0.1508	0.0079 *	0.1508

* $P < 0.05$

APPENDIX D

Abstract of presentation given at the Faculty forum (Faculty of Health Sciences, University of Free State), August 2003. Only a portion of the study, that been completed at the time, was presented.

THE EFFECT OF 'NEGATIVE CHARGE' ON THE DISTRIBUTION OF LIPOSOMES TO THE LIVER, BRAIN, LUNGS AND KIDNEY IN A RAT MODEL

A.M. Abraham and A. Walubo

Department of Pharmacology, University of the Free State, South Africa.

Introduction and aim: Selective delivery of drugs to tissues or organs is a desirable therapeutic strategy in instances where a particular organ is selectively affected by disease e.g. cancer of liver or lungs, tuberculosis of the lungs and liver abscess. It would enable the clinician to achieve effective drug concentrations in the diseased tissue or organ without unnecessary exposure of the drug to healthy tissues. To attain this, different drug delivery systems have been formulated and of these liposomes are regarded as one of the most promising. Liposomes are microscopic vesicles consisting of one or more phospholipid bilayers surrounding an internal aqueous compartment. They are nontoxic and can be loaded with a variety of medications, to protect it from dilution or degradation in the body, thus providing a means by which sustained delivery and selective delivery of drugs to tissues can be attained. It is now known that manipulation of the liposomal surface charge can modify their uptake by some organs and is one of the simplest means of attaining organ selectivity. This study was undertaken to evaluate the effect of negative charge on the distribution of liposomes to the brain, kidney, liver and lungs of rat.

Methodology: Using gentamicin as a marker, negatively charged multilamellar liposomes were prepared with phosphatidyl choline, cholesterol and dicetyl phosphate (5:1:0.5, molar ratio) by rotary evaporation and hydration. Two groups of 25 rats each were administered with gentamicin (19.7 ± 3.26 mg) intraperitoneally as follows; the liposome encapsulated gentamicin for the test group and the free or unencapsulated gentamicin for the control group. Five rats were killed at each of the following times; 1, 2, 4, 6 and 8 hours after administration of gentamicin. Under ether anesthesia, a blood sample was drawn from the

abdominal vein and the relevant organs were harvested. The organs were homogenized by sodium hydroxide digestion method. The concentrations of gentamicin in plasma and organ extracts were measured by fluorescence polarisation immunoassay and they were compared using non-parametric statistics.

Results: Gentamicin peak plasma concentration at one hour was higher in the control group (112.3 ± 34.05 μg/ml) than in the test group (5.47 ± 1.65 μg/ml). Thereafter, plasma concentrations declined linearly in the control group (half-life was 1.53 ± 0.02 hours), such that by 4 hours it was 2.1 ± 0.7 μg/ml. On the contrary, plasma concentrations in the test group were sustained over the study time with no pattern of elimination; gentamicin concentrations were 1.33 ± 1.0 μg/ml and 1.31 ± 0.3 μg/ml at 4 and 8 hours, respectively, indicating a sustained drug delivery. There were differences in the distribution of gentamicin to the different organs. Whereas the liver exhibited increased gentamicin concentration with time from 2.28 ± 1.64 μg/ml at 2 hours to 10.4 ± 2.21 μg/ml by at 8 hours, gentamicin concentrations in the brain and lungs were not different from the control ($P > 0.05$). On the other hand, the concentrations of gentamicin in the kidneys were, at all times, much higher (x 4) in the control than in the test group, e.g., 331.14 ± 31.19 μg/ml at 4 hours in the control group versus 66.86 ± 37.16 μg/ml in test group ($P < 0.05$).

Conclusion: Negatively charged multilamellar liposomes distribute mainly to the liver and least to the kidneys, and this indicates their potential for use in the selective delivery of drugs to the liver and minimization of renal exposure to nephrotoxic.