

**SKIN DELIVERY OF SELECTED HYDROPHILIC
DRUGS
USED IN THE TREATMENT OF SKIN DISEASES
ASSOCIATED WITH HIV/AIDS BY USING ELASTIC
LIPOSOMES**

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**“ THE LORD LIVETH; AND BLESSED
BE MY ROCK; AND LET THE GOD OF
MY SALVATION BE EXALTED. “**

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ABSTRACT

Title: Skin delivery of selected hydrophilic drugs used in the treatment of skin diseases associated with HIV/AIDS by using elastic liposomes

Due to the immunocompromised status of AIDS patients, secondary infections and malignancies are common. Conditions secondary to AIDS for which patients require treatment include Kaposi's sarcoma (treated with methotrexate), varicella-zoster (treated with antivirals such as acyclovir) and herpes simplex (also treated with antivirals like acyclovir or idoxuridine). However the clinical efficacy of these drugs is limited by poor skin permeability.

Few reports, however, have dealt with the delivery of low molecular weight hydrophilic drugs from these vesicles (El Maghraby *et al.*, 2000). The aim of our study was to investigate *in vitro* permeation of methotrexate, acyclovir and idoxuridine across human epidermal membrane from elastic liposomes. The intent was to establish whether formulation of these hydrophilic drugs into elastic liposomes would enhance their skin permeation parameters.

We developed and validated high-performance liquid chromatographic techniques for quantitative analysis of methotrexate, idoxuridine and acyclovir. Elastic liposomes were prepared from various phospholipids- phosphatidylcholine 78.6%; phosphatidylcholine 50%; hydrogenated phosphatidylcholine 90%; phosphatidylcholine 95% and surfactants - sodium cholate, sodium deoxycholate, Span 20, 40, 60, 80. These vesicles were characterised by transmission electron microscopy. The solubilities of methotrexate, acyclovir and idoxuridine were determined. Phospholipon G (95% phosphatidylcholine) was chosen for the preparation of the liposomes with different surfactants. Permeation of methotrexate, acyclovir and idoxuridine from these vesicles across human epidermal membrane was investigated.

Flux values for methotrexate, acyclovir and idoxuridine values (J) obtained by curve-fitting of data using Easyplot® were compared to those obtained by linear regression. We used Student's t-test to determine statistically significant differences in the flux values of the formulations. A computer program http://www.physics.csbsju.edu/stats/t-test_bulk_form.html was used for this purpose. Our results indicate that there are no statistically significant differences between flux values from elastic liposomes and saturated aqueous solutions.

Key words: Skin delivery; elastic liposomes; solubility; permeation; hydrophilicity.

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UITTREKSEL

Dermale aflewering van geselekteerde hidrofiele geneesmiddels wat in die behandeling van veltoestande geassosieer met HIV/HIV/VIGS gebruik word deur middel van elastiese liposome

Sekondêre infeksies en maligniteite is algemene verskynsels in HIV/VIGS-pasiënte as gevolg van hul status van onderdrukte/verswakte immuniteit. Karposi se sarkoom (behandel met metotreksaat), varicella-zoster (behandel met antivirale middels soos asiklovir) en herpes simplex (ook behandel met antivirale middels soos asiklovir of idoxuridien) is voorbeelde van sommige sekondêre infeksies waarvoor HIV/VIGS-pasiënte behandeling benodig. Die swak permeabiliteit van bogenoemde geneesmiddels belemmer egter hul kliniese effektiwiteit en bruikbaarheid.

Sommige NAVORSERS het die aflewering van hidrofiele geneesmiddels met lae molekulêre gewig uit elastiese liposome ondersoek (El Maghraby *et al.*, 2001).

Die doel van ons studie was om die *in vitro* permeasie van metotreksaat, asiklovir en idoxuridien vanuit elastiese liposome oor menslike epidermis te ondersoek. Die oogmerk was om vas te stel of formulering van hierdie hidrofiele geneesmiddels in hierdie vesikels hul permeasie parameters oor die vel sou verbeter.

Kwantitatiewe hoë-druk vloeistofkromatografie analise metodes vir metotreksaat, idoxuridien en asiklovir is ontwikkel en gevalideer. Elastiese liposome is berei vanaf verskeie fosfolipiede- Phospholipon 80, Phospholipon 90G, Phospholipon 90H, Phosal (Nattermann Phospholipids) en surfaktante –natriumkolaat, natriumdeoksiekolaat, Span 20, 40, 60, 80. Transmissie elektron mikroskopie is gebruik vir die karakterisering van hierdie vesikels en wateroplosbaarheid van metotreksaat, asiklovir en idoxuridien is ook

bepaal. Phospholipon G (95% fosfatedielkolien) is gekies vir die bereiding van die liposome met verskillende surfaktante. Die permeasie van metotreksaat, asiklovir en idoxuridien oor membrane van menslike epidermis vanuit hierdie vesikels is ondersoek.

Metotreksaat, asiklovir en idoxuridien se voorspelde en eksperimenteel bepaalde fluks-waardes is met mekaar vergelyk. Die transdermale fluks-waardes (J) verkry deur kromme-passing van die data op Easyplot vir Windows was vergelykbaar met die eksperimenteel bepaalde waardes. Statisties betekenisvolle verskille in die fluks-waardes van die verskillende formulerings is deur 'n student t-toets bepaal. Die statistiese berekeninge is met behulp van rekenaar sagteware verwerk (http://www.physics.csbsju.edu/stats/t-test_bulk_form.html). Die resultate het bewys dat geen statisties betekenisvolle verskille tussen die J-waardes van elastiese liposome en versadige waterige oplossings bestaan nie.

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INTRODUCTION AND STATEMENT OF THE PROBLEM

1.1 Introduction and statement of the problem

Sub-Saharan Africa is considered home to more than 60% of human immunodeficiency virus (HIV) infected cases with an estimated adult prevalence of 8%. Although no country in Africa is spared the infection, the bulk is seen in Eastern and Southern Africa (Thomas, 2001). In South Africa, the infection rate is now 20% of the adult population (Goya & Gow, 2002). Bradshaw *et al.* report that in South Africa, without intervention, by the 2010, deaths resulting from AIDS will account for double all other causes of death in the country (Bradshaw *et al.*, 2002). Because of the immunocompromised status of AIDS patients, secondary infections and malignancies are common (Thomas, 2001). The treatment of skin diseases associated with AIDS therefore remains a critical pharmacotherapeutic challenge (Spano *et al.*, 2002; Gershon, 2001; Aoki, 2001; Matsuo *et al.*, 2001).

Conditions secondary to AIDS for which patients require treatment include Kaposi's sarcoma (treated with methotrexate), varicella-zoster (treated with antivirals such as acyclovir) and herpes simplex (also treated with antivirals like acyclovir or idoxuridine). However the clinical efficacy of these drugs is limited by poor permeability.

The clinical significance of low skin permeability can be explained with acyclovir as an example. When acyclovir is used orally in the dose of 200 mg five times daily, the serum concentration of about 1 µg/ml is achieved (Safrin, 2001). When applied topically, systemic concentrations are undetectable. This means that a substantial flux enhancement is required for an effective transdermal formulation of the drug. The same trend is observed for methotrexate and idoxuridine

Transport of drugs across the stratum corneum, the rate-controlling membrane of the skin, is slow and the mechanism appears complex. Diffusion is controlled by

fundamental physicochemical concepts, the predominant of which are partition (K), diffusion (D) and solubility (C) (Hadgraft, 2001). Several methods for circumventing the stratum corneum barrier have been reported. Steady state flux is described by the following equation (Barry 2001):

$$\frac{\partial m}{\partial t} = \frac{DCK}{h}$$

where $\frac{\partial m}{\partial t}$ is the steady state flux,

C -- concentration of the Drug in the donor solution,

D-- diffusion coefficient,

K-- partition coefficient and

h-- membrane thickness.

From this equation, the ideal properties of a molecule penetrating the stratum corneum can be deduced, namely:

- Low molecular mass of less than 500 Dalton;
- The log P should be in the range of 1-3 and
- Low melting point correlating with good solubility (Barry, 2002). The log octanol/water partition coefficients of idoxuridine, methotrexate and acyclovir (Chatterjee *et al.*, 1997; Kristl & Tukker, 1998; Bonina *et al.*, 2002) are given below in Table 1-1:

TABLE 1-1: Log octanol/water partition coefficient for selected drugs.

Drug	Log P
Idoxuridine	-0.95
Methotrexate	-1.2
Acyclovir	-1.5

These values are low and indicate poor skin permeation characteristics. Since these drugs are highly hydrophilic, one approach may be to use Elastic Liposomes for their transdermal delivery. Small unilamellar liposomes are rarely smaller than 50nm unless they are ultrasonically stressed and/or are supplemented with surfactants (Lesier *et al.*, 1991). In contrast, pores in the skin are normally 0.3nm across and can be opened without major skin damage to 20-30 nm at most (Aguillela *et al.*, 1994; Cevc *et al.*, 1996). It is therefore difficult for the conventional liposome to cross the skin barrier intact and participate in transdermal transport.

Cevc and co-workers have postulated that elastic liposomes can cross the skin due to their deformability. Aggregate deformability and the existence of an independent transbarrier water gradient are considered important for the successful passage of these liposomes through the stratum corneum. Transdermal hydration gradient enforces the widening of the weakest intercellular junctions in the barrier and creates 20-30 nm wide transcutaneous channels. These channels allow sufficiently deformed liposomes to cross the skin (Cevc *et al.*, 1992; Cevc *et al.*, 1998).

Elastic liposomes consist of natural amphipathic compounds suspended in a water-based solution, sometimes containing biocompatible surfactants. Similar to conventional liposomes, these vesicles have a lipid bilayer that surrounds an aqueous core. However in contrast to liposomes, they contain edge-activators that soften the membranes and make them more flexible. Cevc and coworkers have postulated that elastic liposomes can cross microporous barriers very efficiently, even when the pores are much smaller than the average vesicle size (Cevc *et al.*, 1998; Cevc *et al.*, 2001).

El Maghraby and co-workers also investigated the delivery of a hydrophilic molecule, 5-fluorouracil, from ultradeformable liposomes prepared from sodium cholate and phosphatidylcholine (El Maghraby *et al.*, 2001). The authors reported that there was no statistically significant difference in 5- fluorouracil permeation from the liposomes and an aqueous solution (El Maghraby *et al.*, 2000; El Maghraby *et al.*, 2001). Thus, there is still considerable scepticism regarding the benefits of skin delivery of drugs from liposomes (Redelmeier& Kitson, 1999).

It can be hypothesized that that formulation of hydrophilic compounds into elastic liposomes does not result in dramatic skin permeation enhancement. This project

attempts to delineate those factors responsible for the poor permeability and investigates the relationship between the physico-chemical properties of these compounds and their flux values. Because flux is a composite term that can be influenced by solubility, partition and diffusion processes, these three parameters have been studied in greater detail.

At room temperature, the phosphatidylcholine (PC) liposomal bilayer is in liquid crystalline state. This is because PC contains linoleic acid, a doubly unsaturated fatty acid. In the stratum corneum, the intercellular lipid matrix is in both solid and gel phases. The proportion of lipids in fluid liquid crystal phase is low. When PC is applied, it fuses with the intercellular lipids of the stratum and increases its fluidity. Molecules tend to traverse this fluid barrier depending on their partition and diffusion coefficients. In the case of hydrophilic molecules, however, the increase in SC fluidity does not substantially compensate for the low partition and diffusion coefficients.

The utility of computational skin permeability prediction models will also be evaluated. This is important as accurate predicted estimates of solubility and partition coefficient can be used to predict skin permeability *ab initio* and reduce the use of *in vivo* experiments. This can considerably facilitate the development of new and effective transdermal drug delivery systems.

1.2 Research aims and objectives

The aim of this Research Project was to investigate the skin delivery of methotrexate, acyclovir and idoxuridine from elastic liposomes. The phospholipids and surfactants used in this study are shown in Table 1-1:

TABLE 1-2: Phospholipids and surfactants used in this study.

Phospholipids

Hydrogenated phosphatidylcholine 90% (Phospholipon 90 H)

Phosphatidylcholine 95% (Phospholipon G)

Phosphatidylcholine 78.6% (Phospholipon 80)

Phosphatidylcholine 50 % (Phosal PG)

Surfactants

Sodium cholate

Sodium deoxycholate

Sorbitan monolaurate (Span 20)

Sorbitan monopalmitate (Span 40)

Sorbitan monostearate (Span 60)

Sorbitan monooleate (Span 80)

The objectives of this study were to:

- validate high-performance liquid chromatographic techniques for quantitative analysis of methotrexate, idoxuridine and acyclovir;
- prepare and characterise elastic liposomes using various phospholipids- phosphatidylcholine 78.6%, phosphatidylcholine 50%, phosphatidylcholine 90%, phosphatidylcholine 95% and surfactants -sodium cholate, sodium deoxycholate, Span 20, 40, 60, 80;
- carry out in vitro permeation studies through human epidermal membrane using vertical Franz diffusion cells;
- evaluate computationally predicted values of solubility and partition coefficient
- deconvolute partition and diffusion parameters from flux values;
- postulate likely mechanism of skin permeation from elastic liposomes and propose future enhancement strategies.

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THE SKIN DELIVERY OF DRUGS FROM LIPOSOMES

2.1 Introduction

Interest in the skin delivery of drugs from liposomes has increased significantly over the last two decades. Mezei and co-workers were the first to report on enhanced accumulation in the epidermis of triamcinolone acetonide-loaded liposomes (Mezei & Gulasekharan, 1980; Mezei *et al.*, 1982). The localizing effect of liposomes has also been shown in a number of studies (Dayan *et al.*, 2002; Fresta & Puglisi, 1997). Depending on the type of liposomes, and or physicochemical properties of the drug as well as the additives, dermal and in some cases transdermal delivery has been demonstrated (El Maghraby *et al.*, 1999; 2000). A free drug mechanism, direct transfer between vesicles and skin or a combination of both mechanisms have also been suggested (Ganesan *et al.*, 1984). Diffusion studies with an aqueous solution of inulin, using in vivo liposomal treated skin, resulted in very small amounts of the drug delivered into the skin. It was suggested that drugs be encapsulated in liposomes or at least administered together with the lipids to achieve targeted delivery (Du Plessis *et al.*, 1994).

Many studies have been carried out on the use of elastic (ultraflexible) liposomes for skin delivery of drugs (Cevc *et al.*, 1992; 1998; 2002). They differ from conventional liposomes in composition because they contain so-called edge-activators, which impart elasticity to these vesicles. It has been reported that these vesicles, if applied non-occlusively, can penetrate intact skin and enhance flux (Cevc *et al.*, 1992; Cevc *et al.*, 2002). Other investigators have measured drug delivery from traditional and ultradeformable (elastic) liposomes using open and occluded conditions in vitro. Both liposomes types improved maximum flux and skin deposition compared to saturated aqueous drug solution. However, only 1-3% of drug was delivered (El Maghraby *et al.*, 1999; Barry, 2001). Although it has been generally accepted that the use of vesicles

with appropriate composition should result in increased drug transport across the skin, conflicting results have been reported (Van Kuijk-Meuwissen *et al.*, 1998; Cevc *et al.*, 2002) and many questions regarding the mechanisms of action remain unanswered (Bouwstra *et al.*, 2003). The feasibility of enhanced transdermal transport by means of liposomes has been recognised to be valid; therefore more studies are needed to elucidate underlying mechanisms and overcome the barrier posed by the stratum corneum.

2.2 The Skin As A Barrier To Drug Delivery

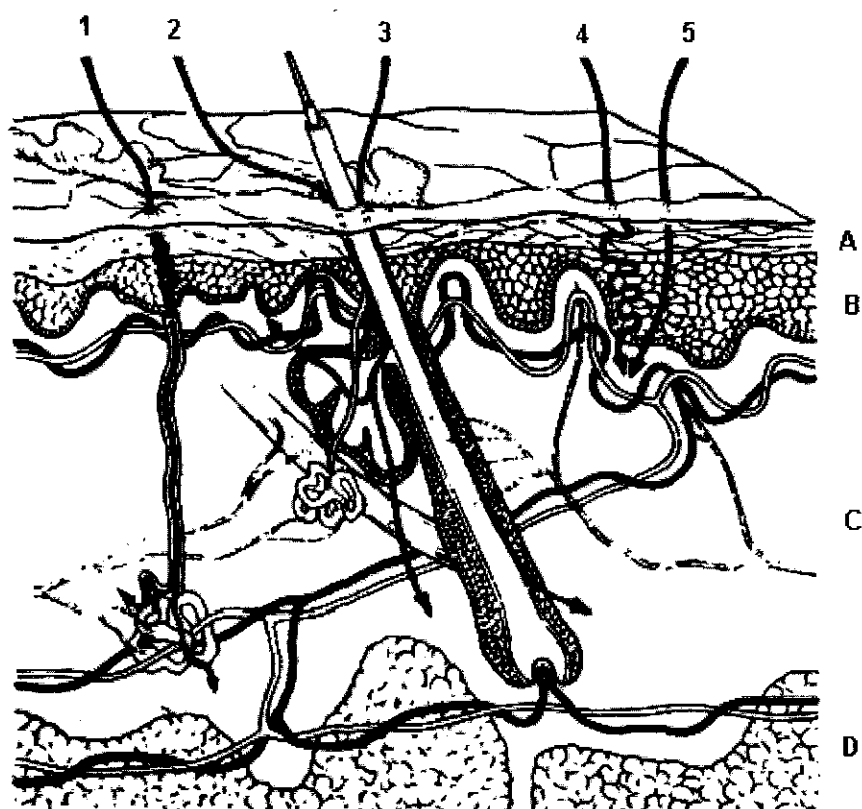


FIGURE 2-1. Schematic diagram of cross-section of the human skin Cross section of the skin. A - stratum corneum; B - viable epidermis; C - dermis; D - subcutaneous fat. 1 - transeccrine route; 2 - transsebaceous route; 3 - transfollicular route; 4 - intercellular; and 5 - transcellular route. (Junginger *et al.*, 1994).

Figure 2-1 shows the basic organisation of the skin. The superficial region, termed the stratum corneum or horny layer is between 10 and 20 μ m thick. Underlying this region is the viable epidermis (50-100 μ m), dermis (1-2mm) and hypodermis (1-2). The large

surface area as well as the volume of the compartments makes it the body's largest organ weighing more than 10% in total body mass. Although the skin overall comprises a very large compartment, the stratum corneum - the thinnest, outermost layer- forms the principal barrier to percutaneous absorption. The integrity of the stratum corneum is disrupted periodically by appendages such as hair follicles and sweat glands (Redelmeier & Kitson, 1999).

The dermis is directly adjacent to the epidermis and provides mechanical support. The viable epidermis is a stratified epithelium consisting of the basal, spinous and granular cell layers. Each layer is defined by position, shape morphology and state of differentiation of the keratinocytes. The epidermis is a dynamic, constantly self-renewing tissue, in which a loss of the cells from the surface of the stratum corneum (desquamation) is balanced by cell growth in lower epidermis. Upon leaving the basal layer, the keratinocytes start to differentiate and during migration through the stratum spinosum and stratum granulosum undergo a number of changes in both structure and composition. The keratinocytes synthesise and express numerous proteins and lipids during their last maturation. The last sequences of the keratinocytes differentiation result in their transformation into chemically and physically resistant cornified squames of the stratum corneum, called corneocytes. The corneocytes are flat anucleated squamous cells packed mainly with keratin filaments, surrounded by a cell envelope composed of cross-linked proteins and a covalently bound lipid envelope (Bouwstra *et al.*, 2003).

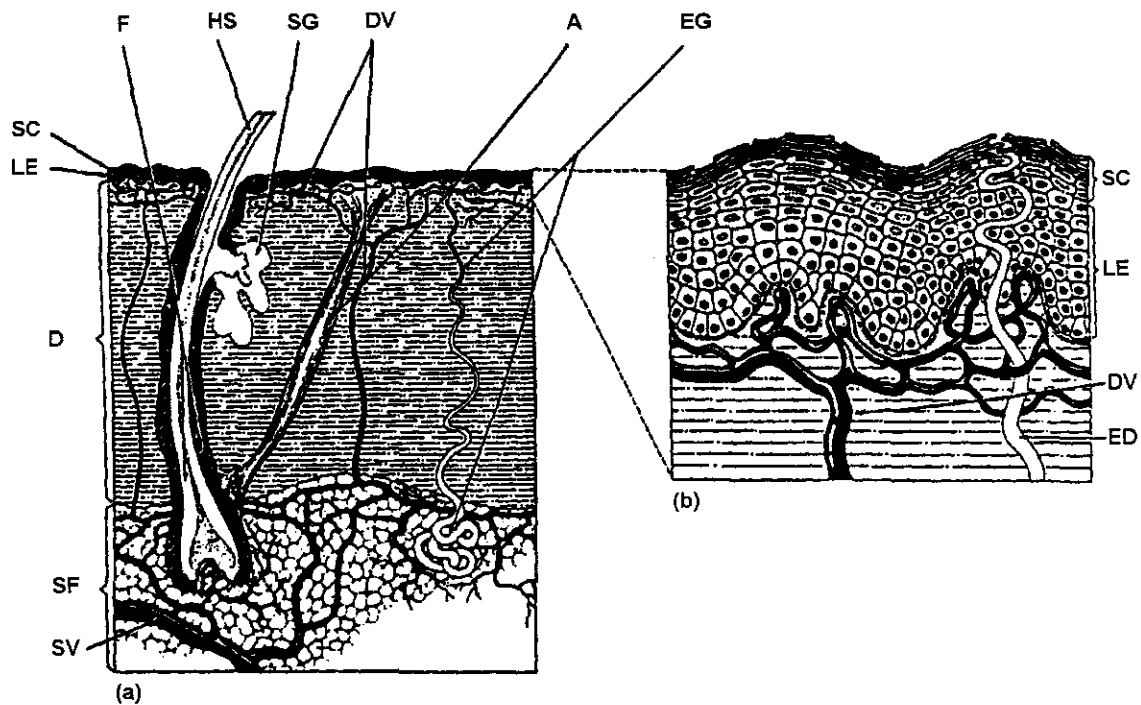


FIGURE 2-2: Sketch of the skin. SC = Stratum corneum (10 – 15 μm), LE = Living epidermis ($\approx 100 \mu\text{m}$), D = Dermis (2000 – 3000 μm), SF = Subcutaneous fatty tissue, SV = Subcutaneous vasculature, F = Follicle, HS = Hair shaft, SG = Sebaceous gland, DV = Dermal vasculature, A = Arrectores pilorum muscle, EG = Eccrine gland, ED = Eccrine gland duct (Flynn, 1996).

Figure 2-2 shows a sketch of the skin. The stratum corneum consists of several layers of corneocytes with the intercellular spaces filled with lipid bilayers. About ten lipid bilayers are compacted between two adjacent corneocytes layers (Mitragotri, 2003). It is a multilamellar lipid milieu punctuated by protein-filled corneocytes that augment membrane integrity and significantly increase membrane tortuosity (Kalia & Guy, 2001). Light and scanning electron microscopy studies have established that there is considerable overlapping between adjacent corneocytes, which facilitates cohesion, and elasticity (Redelmeier & Kitson, 1999). The organisation of the stratum corneum has also been described by the brick and mortar model, in which extracellular lipids accounts for about 10% of the dry weight and 90% is intracellular protein (mainly keratin). The SC lacks phospholipids but is enriched in ceramides and neutral lipids

(cholesterol, fatty acids, cholesteryl esters) that are arranged in a bilayer format and form so-called lipid channels (Foldvari *et al.*, 2000).

The stratum corneum is the major permeability barrier to external materials and is regarded as the rate-limiting factor in the penetration of drugs through the skin ((Foldvari *et al.*, 2000; Hadgraft, 2001; Malan *et al.*, 2002; Tezel *et al.*, 2003). The lipophilic character of the stratum corneum, coupled with its intrinsic tortuosity, ensure that it almost always provides the principal barrier to entry of drug molecules; the only exceptions are highly lipophilic molecules that might encounter problems at the stratum corneum-epidermis interface where they must partition into a predominantly aqueous environment (Naik *et al.*, 2001).

Biophysical techniques have provided interesting insights into the nature of the barrier (Hadgraft, 2001; Bouwstra *et al.*, 2003). The major lipid classes in the stratum corneum are ceramides (CER), cholesterol (CHOL) and free fatty acids (FFA). The CER head groups are very small and contain several functional groups that can form lateral hydrogen bonds with adjacent ceramide molecules (Bouwstra *et al.*, 2003). The acyl chain length distribution in the CER is bimodal with the most abundant chain lengths being C24-C26. Only a small fraction of CER has an acyl chain length of C16-C18. The chain lengths of C24 and C26 are much longer than those in phospholipids in plasma membranes. In human stratum corneum, eight subclasses of ceramides (HCER) have been identified. These HCER, referred to as HCER 1-8, differ from each other by the head-group architecture (sphingosine, phytosphingosine or 6-hydroxysphingosine base) linked to a fatty acid or a α -hydroxy fatty acid of varying hydrocarbon chain length. In human stratum corneum, CER 1 and CER4 have a very exceptional molecular structure: a linoleic acid is linked to a fatty acid or an ω -hydroxy fatty acid with a chain length of approximately 30-32 carbon atoms. In this respect the HCER are different from ceramides isolated from pig stratum corneum (pigCER), in which only pigCER 1 has this exceptional molecular structure. The FFA fraction consists mainly of saturated acids. Another important lipid is cholesterol sulfate. Although this lipid is present in small amounts (2-5% w/w), it plays an important role in the desquamation process of the stratum corneum (Bouwstra *et al.*, 2003).

Three possible mechanisms have been proposed for the transport of drugs across the stratum corneum. The first can be described as the shunt route, which provides a parallel pathway through the sweat ducts and hair follicles. Under normal conditions the appendageal route is not thought to be very significant due to the low surface area occupied by the appendages (Hadgraft, 2001). The second and third pathways are the intercellular and intracellular routes. For the intracellular route, drugs pass directly through the cells of the stratum corneum, whereas in the intercellular route, they diffuse around the cells in a tortuous manner (Malan *et al.*, 2002).

2.3 Liposomes

Symmetric membranes prefer to be flat (spontaneous curvature $\equiv C_o = 0$) and energy is required to curve them. The bending elasticity per unit area for small distortions with both principal curvatures, $C_x = C_y = C$, being equal can be approximated by the equation (Lasic *et al.*, 2001):

$$E_b = (1/2) M_b (2C - C_o)^2 + gC^2$$

Equation 2-1

where M_b is the bending elastic modulus, g is the modulus of Gaussian curvature and C is the curvature ($= 1/\text{radius}, R$). Solving the above equation for a closed sphere with radius R , one gets $E_b = 4\pi (2M + g)$. Examining the above equation, it can be seen that liposomes can form spontaneously only in the case of extremely soft bilayers (very low values of $2M + g$, giving rise to $E_b \sim gT$) in which the excess energy is comparable to the thermal energy kT (k is the Boltzmann constant, T is the temperature) and such liposomes are entropically stable.

Classical phospholipid liposomes have rigid membranes ($E_b > 10\text{-}100\text{ kT}$) and can be formed in a dynamic kinetic process, such as sonication, homogenisation or high-pressure extrusion (Lasic *et al.*, 2001). In some instances, the self-assembly of lipids in water to minimise hydrophobic exposure results in the formation of closed continuous membranes which necessarily trap a portion of the aqueous solution in which they

form. It is this lipid bilayer with its captured volume that constitutes the entity we term liposome (Perkins, 1999).

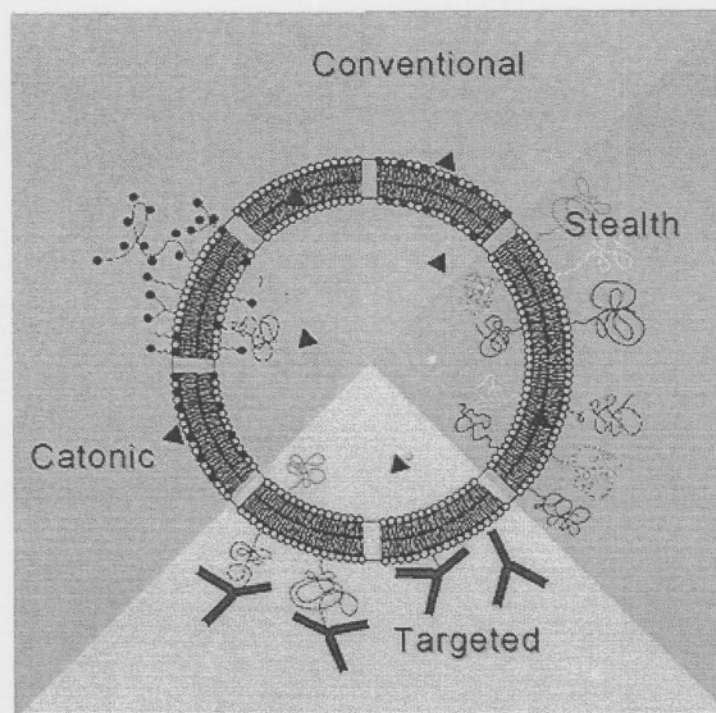


FIGURE 2-3. Schematic representation of four major liposome types. Conventional liposomes are either neutral or negatively charged. Sterically (stealth®) stabilised liposomes carry polymer coating to obtain prolonged circulation times. Immunoliposomes(‘antibody targeted’) may be either conventional or stealth. For cationic liposomes, several ways to impose a positive charge are shown (mono-, di- or multivalent) interactions (Cromellin *et al.*).

Liposomes are polymolecular aggregates formed in aqueous solution on the dispersion of certain bilayer-forming amphiphilic molecules. Under osmotically balanced conditions, the vesicles are spherical in shape and contain one or more concentric lamellae that are composed of amphiphiles. These shells are curved and self-enclosed molecular bilayers in which the hydrophobic part (polar head group) is in contact with the aqueous phase. The interior of the lipid vesicles is an aqueous core, the chemical composition of which corresponds in a first approximation to the chemical composition

of the aqueous solution in which the vesicles are prepared. Depending on the method of preparation, lipid vesicles can be multi-, oligo- or unilamellar, containing many, a few or one bilayer respectively (Walde & Ichikawa, 2001, Barry, 2001; Agarwal *et al.*, 2001; Imura *et al.*, 2002). Liposomes can also incorporate lipids grafted with polyethylene glycol (PEG). This is the so-called Stealth® strategy of creating a bound, highly solvated polymer layer at the membrane surface. The mechanism whereby such a PEG layer extends the liposome circulation time is due to the ability of the polymer layer to prevent the association and binding of opsonins, thereby inhibiting the body's molecular recognition processes from labelling the liposome as foreign for subsequent uptake and removal by cells of the reticuloendothelial system (Needham *et al.*, 1999). The structure of Stealth® liposome is illustrated in Figure 2-3.

Felgner *et al.* reported highly efficient in vitro gene transfection using cationic 2,3-dioleoyloxypropyl-1- trimethyl ammonium bromide (DOTMA) liposomes. After the introduction of DOTMA, Felgner et al. studied the effect of various chemical structure changes on the DOTMA molecule. Substitution of methyl in the charged group with hydroxyalkyl was shown to improve the activity of the lipid. The structure of a cationic liposome is also illustrated in Figure 2-3.

Liposomes can also be conjugated to monoclonal antibodies for targeting. Shaik *et al.* (2001) prepared monensin-loaded Stealth® liposomes which were conjugated to anti-My9 monoclonal antibody targeted against CD 33 antigen. In vitro cytotoxicity studies showed that antibody-conjugated monensin liposomes potentiated the cytotoxicity of anti-My9 immunotoxin by a factor of 2070 in comparison to 360-fold potentiation observed with unconjugated monensin against human HL-60 promyelocytic leukaemia cells.

Until recently, liposomes were used as models of biological membranes. Reconstitution of proteins into liposomes has allowed their function and structure to be studied with regard to the lipid environment and variation/manipulation of protein-protein interactions. For a better understanding of the lipid bilayer itself, protein-free liposomes have been examined quite extensively with much of the focus upon lipid-lipid interactions, mechanical behaviour, phase behaviour and membrane electrostatics (Perkins, 1999).

Liposomes have attracted a great deal of attention in the delivery of dermal drugs because of many advantages, like biodegradability, non-toxicity, amphiphilicity and modulation of drug release properties (Uchegbu & Vyas, 1998). Their structural characteristics like size, shape, lamellae nature and type of composition can be modified to meet drug delivery requirements (Agarwal *et al.*, 2001). It is possible to formulate different types of liposomes with widely differing properties. Many methods also exist for the preparation of drug-containing liposomes:

- ❖ Rotary evaporation
- ❖ Reverse phase evaporation technique
- ❖ Dehydration/rehydration
- ❖ Extrusion
- ❖ Sonication
- ❖ Detergent solubilization
- ❖ pH-induced vesiculation
- ❖ Ethanol injection
- ❖ Cross-flow technique
- ❖ Supercritical fluid Technology

2.3.1 Rotary evaporation

Bangham and co-workers first introduced this method. Liposomes were prepared using this technique and it was demonstrated that lamellae composed of swollen phospholipids could differentially impede the diffusion of ions (Bangham, 1965). The method involves dissolution of lipids and other components in an organic solvent; evaporation of the solvent in a rotary evaporator fitted with a cooling coil and a thermostatically-controlled water bath (New 1990; Du Plessis *et al.*, 1996; Trotta *et al.*, 2002; Verma *et al.*, 2003). Rapid evaporation of solvents is usually carried out by gentle warming (20-40°) under reduced pressure (400-700 mmHg). The temperature for drying down should be regulated so that it is above the phase transition temperature of the lipids. Rapid rotation of the solvent containing flask increases the surface area from evaporation. Solvent traces are removed by maintaining the lipid film under vacuum overnight. The film is then hydrated with an aqueous solution of the drug by shaking; if the drug is lipid soluble it is added to the organic solution. When large volumes of lipid

and aqueous solution are used, the hydration can be carried out by vigorous vibratory motion in a mechanical shaker. The process may last for several hours to ensure homogenous dispersion. Even before exposure to water, the lipids in the dried-down film are thought to be oriented in such a way as to separate hydrophilic and hydrophobic regions from each other, in a manner not unlike their conformation in the finished membrane preparation. Upon hydration, the lipids form multilamellar vesicles.

The simplest and most widely used method of mechanical dispersion is commonly known as hand-shaking, since the lipids are suspended off the sides of a glass vessel into the aqueous medium by gentle manual agitation. In order to increase entrapment volume, it is advisable to start with a round-sided glass vessel of large volume, so that the lipids will be dried down onto as large a surface area as possible to form a very thin film. Thus even though the volumes of organic or aqueous starting solutions may be only 1ml each, it is recommended that a 50- or 100-ml vessel be used for drying down (New, 1990).

2.3.2 Reverse phase evaporation technique

A very popular technique developed for liposome preparation is the reverse phase evaporation technique (Du Plessis, 1992; Perkins, 1999). Lipid dissolved in ether is usually mixed with an aqueous solution, briefly sonicated to form water-in-oil type dispersion and subjected to rotary evaporation to remove solvent. The resulting reverse phase evaporation vesicles (MLV-REVs) have substantial captured volumes (8-20 $\mu\text{l}/\mu\text{mole}$) that can be manipulated by process variation to produce unilamellar structures (Perkins, 1999). These vesicles have a high aqueous space to lipid ratio and therefore are able to encapsulate a high percentage of the initial aqueous phase. The entrapment usually ranges from 20-60% depending upon lipid concentration and the ionic strength of the aqueous phase. As the organic solvent evaporates, a viscous gel-like intermediate forms and when solvent removal is complete, liposomes form spontaneously. A relatively uniform size distribution can be obtained with most phospholipid mixtures with the exception of preparations containing cholesterol, which tend to have more heterogenous size distribution (Du Plessis, 1992).

Gruner *et al.* (1985) have developed a method that purposely produced multilamellar ("plurilamellar") vesicles but avoided solute exclusion that occurs with conventional vesicles. The procedure is similar to reverse phase evaporation but differs in that solvent is removed via sparging with nitrogen gas. The resulting liposomes are termed stable plurilamellar vesicles (SPLVs). The internal structure of the vesicles differs from MLV-REVs in that they lack a large aqueous core, the majority of the entrapped aqueous medium being located in compartments in between adjacent lamellae (New, 1990).

Kim *et al.* used a different solvent removal approach to make unilamellar vesicles of remarkable captured volumes (nearly 100 $\mu\text{l}/\mu\text{mole}$ and 80 $\mu\text{l}/\mu\text{mole}$, for unilamellar and multilamellar vesicles respectively). These structures were formed via creation of a water-in-oil-in-water emulsion. That is, water was first added to a lipid containing chloroform ($\pm$ ether) solution to form a water-in-oil emulsion and then this chloroform solution was mixed with a second aqueous phase to form solvent spherules. The internal bilayer structure of the MVLs was quite unusual in that interior vesicles structures were not concentrically arranged but rather appeared as connected compartments, much like a honey comb. It is this arrangement that explains the high captured volume since it is basically a conglomerate of many large vesicles each with its own high captured volume (Perkins, 1999).

2.3.3 Dehydration/rehydration

Rehydration of lyophilised or dried lipid/solute dispersions produces liposomes capable of encapsulating large macro-molecular structures and capable achieving high trapping efficiencies. The method consists of dehydrating or freeze-drying an initial liposome dispersion containing the drug to be encapsulated. As the liposome dispersion is dehydrated, the vesicles fuse to form a multilamellar film, sandwiching the solutes between successive lipid layers. Rehydration of the dried lipid/solute film with the desired aqueous solution forms a heterogenous population of large unilamellar and multilamellar vesicles (Du Plessis, 1992). The method is another way of dispersing the solid lipid in a finely divided form before contact with the aqueous fluid, which will form the medium for the final suspension. Freeze-drying is used to freeze and lyophilise empty small unilamellar vesicles (SUVs). In contrast to other solvent evaporation

techniques, where lipid molecules are in a random mix, the SUV-dried lipid is already very highly organised into membrane structures (New, 1990).

After preparation of multilamellar vesicles, liposomes are sometimes processed further to modify their size and other characteristics. For many purposes, MLV are too large or too heterogeneous so many methods have been devised to reduce their size. These include extrusion (Singh *et al.*, 1999; Imura *et al.*, 2002; Shabbits *et al.*, 2002; Verma *et al.*, 2003) and ultrasonication (Guo *et al.*, 2000; Cevc *et al.*, 2002; Trotta *et al.*, 2002).

2.3.4 Extrusion

This method produces size reduction of liposomes by passing them through membrane filters of defined pore size. This can be achieved at a high pressure using extrusion devices (Singh *et al.*, 1999; Shabbits *et al.*, 2000). Shabbits and co-workers reported the production of homogenously sized liposomes following a 10-cycle extrusion through 100nm polycarbonate filters mounted on a Lipex extruder (Northern Lipids, Vancouver, Canada). The upper size limit of extruded liposomes depends on the pore size of the membrane filter used. Nucleation (Nucleopore®) track membranes are frequently used. This type of membrane consists of a thin continuous sheet of polymer (usually polycarbonate) in which straight-sided pore holes of exact diameter have been bored through by a combination of laser and chemical etching. Because the pores go straight through from one side to the other, they offer little resistance to the liposomes passing through. Inherent flexibility of phospholipid lamellae enables liposomes to change their conformation so that they can squeeze through the pores; in the process significant size reduction is achieved. If MLVs are extruded through membranes of pore size 0.1µm or smaller, then upon repeated extrusions, the liposome suspension becomes progressively more unilamellar in character, with the vesicles still maintaining a size distribution around membrane pore size but possessing a considerable internal aqueous volume (New, 1990).

2.3.5 Sonication

Hydrated lipids can also be reduced to the smallest possible size by using sonication (New, 1990; Cevc *et al.*, 2002; Guo *et al.*, 2000). There are two methods of sonication-

bath and probe. The probe is usually employed for suspensions, which require high energy in a small volume while the bath is more suitable for large volumes of dilute lipids. For the most efficient transfer of energy from the probe, it is advisable to hold the fluid in a round-bottomed tube with straight sides, having a diameter just slightly greater than that of the probe. The probe is usually immersed in fluid, approximately 4mm below the surface of the suspension. Because a lot of heat is generated in the process, the vessel containing the suspension is normally immersed in a cooling bath. A flat tip probe of diameter 19mm (3/4 inch) is convenient to use for samples volumes of between 5 and 10ml.

Because of the high input of energy in this method, there is considerable risk of degradation of lipids resulting from high temperatures and increased gas exchange associated with the operation of the probe. Lipids are usually sonicated above their transition temperature T_c (El Maghraby *et al.*, 1999). For phosphatidylcholine, the T_c is less than ambient temperature. Aerosols are also formed during sonication. It is therefore necessary to keep the sonication vessel sealed to avoid contamination of the environment with potentially hazardous chemicals or isotopes. For large samples, bath sonication is usually the technique of choice. The method is milder than probe sonication and there is less risk of degrading the liquid. Sample volume is larger, the field of ultrasonic irradiation more homogenous and reproducibility greater. However, because the energy is dispersed over a much larger area, it may not be possible to reach the minimum size limit for sonicated vesicles (New 1990).

2.3.6 Detergent solubilization

Mixing an aqueous dispersion of a bilayer-forming lipid (e.g. egg phosphatidylcholine) with an aqueous (micellar) solution of a micelle-forming detergent (e.g. sodium cholate) under appropriate conditions (e.g. excess of detergent over lipid) results in the formation of mixed detergent-lipid micelles, which are in equilibrium with nonmicellized (monomeric) detergent molecules. On a controlled and continuous removal of the detergent by dialysis or gel filtration, the mixed detergent-lipid micelles transform into mixed-detergent vesicles and finally into almost detergent-free lipid vesicles. The resulting vesicles are abbreviated as DDV, which stands for 'detergent dialysed vesicles' (Walde & Ichikawa, 2001).

Removal of detergent molecules from aqueous dispersions of phospholipid/detergent mixed micelles represents a radically different approach to producing liposomes. As the detergent is removed, the micelles become progressively richer in phospholipid, and finally coalesce to form closed, single-layer vesicles. Shortcomings of the approach include leakage and dilution of the drug during liposome formation, high cost and the difficulty of removing the last traces of the detergent once liposomes have formed (Betageri *et al.*, 1993).

In contrast to phospholipids, detergents are highly soluble in both aqueous and organic media, and there is equilibrium between the detergent molecules in the water phase, and in the lipid environment of the micelle. The critical micelle concentration can give an indication of the position of this equilibrium, and from that conclusions can be drawn about the ease of detergent removal from micelles (New, 1990). Detergent dialysis is commonly used as a method for liposome preparation. Detergents commonly used for this purpose exhibit a relatively high critical micelle concentration. This property facilitates their removal. Representative detergents used for this purpose include the bile salts and octylglucoside. During dialysis, liposomes about 100nm in diameter form within a few hours (Betageri *et al.*, 1993).

Liposomes can also be formed by the removal of surfactants using column chromatography. The method entails the mixing of phospholipid in the form of either small sonicated vesicles or a dry lipid film, with deoxycholate at a molar ratio of 1:2, respectively. Subsequent removal of the detergent is accomplished by the passage of the dispersion over a Sephadex G-25 column resulting in the formation of uniform 100-nm vesicles (Betageri *et al.*, 1993).

2.3.7 pH-induced vesiculation

Multilamellar vesicles can be induced to reassemble into unilamellar vesicles without the need for sonication or high pressure, simply by changing the pH (New 1990; Betageri *et al.*, 1993; Li *et al.*, 1998). These liposomes are formed when phospholipid mixtures are dispersed either directly in sodium hydroxide at pH ~ 10 or in water the pH of which is then rapidly (~ 1 sec) increased. Exposure of the phospholipids to high

pH is short (< 2min) and during this time no degradation is detectable. The small liposomes can be separated from the large ones by centrifugation, gel chromatography or filtration.

Transmembrane pH gradients can also be created by forming liposomes in a well-buffered solution of low pH (e.g. 300mM citrate at pH 4) and then adding a more basic solution to raise the external solution pH. Li *et al.* (1998) used this technique to increase encapsulation efficiency of doxorubicin. pH-induced vesiculation is an electrostatic phenomenon. The transient change in pH brings about an increase in the surface charge density of the lipid bilayer; once this exceeds a threshold value of around $1\text{--}2\ \mu\text{C cm}^{-2}$, spontaneous vesiculation will occur. Liposome size is dependent on acidic phospholipid used, the molar ratio of acidic phospholipid to phosphatidylcholine and the extent of pH change. However the technique is limited to charged phospholipids and their mixtures with neutral phospholipids (Betageri *et al.*, 1993).

2.3.8 Ethanol injection

This is a solvent dispersion technique, which was first reported by Batzri and Korn (Batzri & Korn, 1973). An ethanol solution of lipids is injected rapidly into an excess of saline or other aqueous medium through a fine needle (New, 1990; Betz *et al.*, 2001). The force of the injection is usually sufficient to achieve complete mixing, so that the ethanol is diluted almost instantaneously in water, and phospholipid molecules are dispersed evenly throughout the medium. This procedure can yield a high proportion of small unilamellar vesicles with a mean diameter of about 25nm (250 angstroms) although lipid aggregates and larger vesicles may form if the mixing is not thorough. The method has the advantage of extreme simplicity and a very low risk of lipid degradation. Its major shortcoming is the limitation of solubility of lipids in ethanol (40 mM for phosphatidylcholine), and on the volume of ethanol that can be introduced into the medium (7.5 % v/v maximum). Encapsulation efficiency is thus extremely low if the entrapped drug is dissolved in the aqueous phase (New, 1990).

2.3.9 Cross-flow technique

A new technique (cross-flow) based on the principles of ethanol injection has been developed (Wagner *et al.*, 2002; Vorauer-Uhl *et al.*, 2002). The principal item here is the cross-flow injection module which has the benefit of well-defined and characterized injections streams and permits liposome manufacture regardless of production scale, as scale is determined only by free disposable vessel volumes. Other parts of the production system are vessels for the polar phase, an ethanol/lipid solution vessel and a nitrogen pressure device. The crossflow injection module used for liposome manufacture is made of two stainless steel tubes welded together to form a cross. At the connecting point, the module has an injection (250 μm drill hole) drilled by spark erosion (Wagner *et al.*, 2002).

All reagents such as the buffer, drug and lipid-ethanol solutions are usually transferred into containers by filtration through 0.22 μm filters. Nitrogen, which is used for the injection process, is also filtered through a 0.22 μm filter (Vorauer-Uhl *et al.*, 2002). Lipid vesicles are formed in the cross-flow injection module at 55 °C by injection of lipids solubilised in ethanol into the drug-containing buffer. Immediately after the lipids are distributed into the aqueous drug solution, planar bilayer fragments are formed. These fragments reassemble to form liposomes. Non-entrapped drug is usually separated by ultra/diafiltration equipment.

2.3.10 Supercritical fluid Technology

Rotary evaporation and solvent injection techniques require large amounts of organic solvents that are harmful to the environment and human body. Very few methods have been developed that yield liposomes having a high trapping efficiency for water-soluble substances without using any organic solvent (Imura *et al.*, 2002). As a possible alternative to reduce health, environmental and safety risks, supercritical or near critical fluids have been introduced for liposome preparation (Frederiksen *et al.*, 1997; Imura *et al.*, 2002).

Supercritical fluids are non-condensable and highly dense at temperatures and pressures beyond their critical point. They are highly functional solvents whose properties can be

altered remarkably by varying temperature and pressure. Supercritical carbon dioxide (scCO₂) in particular has attracted attention as an environmentally-friendly alternative solvent that can replace organic solvents because it has a low critical temperature ($T_c = 31\text{ }^\circ\text{C}$) and pressure ($P_c = 73.8\text{ bar}$), and because it is non-toxic and cheap (Imura *et al.*, 2002).

Frederiksen has described a liposome preparation technique using supercritical carbon dioxide (Frederiksen *et al.*, 1997). The apparatus used consisted of two main parts: the high-pressure part, in which the lipid components were dissolved under pressure in supercritical carbon dioxide, and a low-pressure part, in which the homogenous supercritical solution was expanded and simultaneously mixed with the aqueous phase to yield liposomes. Supercritical carbon dioxide can be used either as a solvent to dissolve liposomal components, or an antisolvent to promote rapid and uniform precipitation of phospholipids from saturated ethanolic solutions.

2.4 Characterization Of Liposomes

The behaviour of liposomes in both physical and biological systems is determined to a large extent by factors such as physical size, chemical composition, membrane permeability, quantity of entrapped solutes, as well as the quality and purity of the starting material (New 1990). Particle size, lamellarity, trapping efficiency, zeta potential and chemical composition are important properties.

2.4.1 Particle size

2.4.1.1 Transmission Electron Microscopy

Particle size can be determined by transmission electron microscopy (Ganesan *et al.*, 1984; Du Plessis, 1992; Imura *et al.*, 2002). Samples are usually prepared at room temperature by conventional negative staining methods. It is then placed on a copper grid mesh and observed with a transmission electron microscope. Negative stain electron microscopy is used to study vesicle size and size distribution when a significant fraction of the liposomes have diameters below the resolution of a light microscope (Du Plessis, 1992). Materials used as negative stains should fulfil the following criteria:

- Non-reactivity with the specimen and its surrounding buffer
- High solubility
- High electron density, and
- Stability under the electron beam

Two heavy metal salts, which meet these criteria and have been used extensively with liposomes, are phosphotungstic acid and ammonium molybdate ((New 1990). Both reagents are used at concentrations between 0.5% and 2%.

The transmission electron microscope allows the investigation of the internal microstructure of samples, provided that they are thin enough to transmit electrons. The source of illumination in a TEM is contained in the electron gun, situated at the top of the column. Electrons travel at high speed down the column, and are focussed onto the specimen using a combination of magnetic lenses. The microscope can be operated in a number of modes, both image and diffraction, and can be fitted with a variety of detectors, making it a versatile and powerful analytical tool for microstructural investigation at high spatial resolution (Tiedt & Pretorius).

2.4.1.2 Photon correlation spectroscopy

Photon correlation spectroscopy (PCS) is the analysis of time dependence of intensity fluctuations in scattered laser light due to the Brownian motion of particles in solution or suspension. Since small particles diffuse more rapidly than large particles, the rate of fluctuation of scattered light intensity varies accordingly. Thus, the translational diffusion coefficient (D) can be measured and used to determine mean hydrodynamic radius (R_h) of the particles using the Stokes-Einstein equation (New 1990). The technique, which is also called dynamic laser scattering, can be used to measure particles in the range of about 3nm to 3 μ m. It is widely used for liposome characterization (Cevc *et al.*, 2002; Trotta *et al.*, 2002). A laser light source (helium-neon or argon) is focused on the contents of a highly polished, fine quality glass cuvette. The cuvette is housed within a thermostatically-controlled goniometer cell. The sample can be suspended in a range of dispersion media with known viscosity and refractive indices. To eliminate potential light flare, it is normal to immerse the sample

cell in a liquid, which matches the refractive index of the dispersion fluid. Scattered laser light from the sample is detected by a photomultiplier assembly usually situated at an angle of 90 ° relative to the laser beam.

2.4.1.3 Multiangle laser light scattering

Multiangle laser light scattering (MALLS) has also been used to characterise vesicle size (Van Zanten *et al.*, 2002). The authors used a DAWN DSP® Laser photometer (Wyatt Technology Inc., USA) to monitor lipoplex geometric sizes and molar masses over time. The Photometer determines excess Rayleigh ratios (scattered light intensity) at 18 scattering angles simultaneously. The size is determined from angular variation of the excess Rayleigh ratio extrapolated to zero scattering angle.

2.4.2 Lamellarity

Several techniques have been developed to measure lamellarity of liposomes. They rely upon distinguishing the lipid present in the outer monolayer from the total lipid (Perkins, 1999). The techniques fall into two categories in which either no marker lipid is added (e.g. ^1H or ^{31}P nuclear magnetic resonance) or a marker is included (e.g. nitroxide labelled lipid, or phosphatidylethanolamine). For NMR, additions of broadening or shift reagents then distinguish that fraction of lipid residing in the outer monolayer. Frequently, a nonpermeable broadening agent such as Mn^{2+} is added. Manganese ions interact with the outer leaflet of the outermost bilayer. Thus, a 50% reduction in NMR signal means that the liposome preparation is unilamellar, and 25% reduction in the intensity of the original NMR signal means there are two bilayers in the liposome (Betageri *et al.*, 1993).

However, Fröhlich *et al.* (2001) investigated the influence of various parameters on lamellarity measurement, such as different buffers with changing ion concentrations, varying pH and different shift reagents at increasing concentrations. Results were discussed by using cryo-electron microscopy as a reference method. The data of the study showed that ^{31}P -NMR might not result in the correct determination of liposome lamellarity depending on the experimental settings and the shape of the liposomes

(Fröhlich *et al.*, 2001). Lamellarity has also been shown to be an important indicator of liposome stability (Du Plessis *et al.*, 1996).

2.4.3 Trapping efficiency

Trapping efficiency is the percentage of a water-soluble drug in the aqueous phase of liposomes and is usually expressed as percent entrapment per mg or μ mole of lipid (Du Plessis, 1992; Nagarsenker *et al.*, 2003). Several techniques have been described in the literature for measuring trapping efficiency (Imura *et al.*, 2002; Nagarsenker *et al.*, 2003; Gabriels *et al.*, 2003). Imura *et al.* (2002) carried out glucose trapping experiments by dialyzing a liposome suspension against water with a cellophane tube to remove the non-encapsulated drug. Liposomes inside the tube were then destroyed by addition of ethanol. The amount of glucose in the tube was then determined spectrophotometrically.

Nagarsenker *et al.* (2003) measured trapping efficiency by diluting a liposomal dispersion with buffer and centrifuging at 15,000 rpm for 30 min. The supernatant was then analysed for drug content. Knowledge of total drug in the preparation allowed the amount of the drug associated with the liposomes to be determined. Another procedure for measuring trapping efficiency has also been reported (Gabriels *et al.*, 2003). To determine total drug content, an exactly weighed amount of liposomal suspension (500 mg) was vigorously mixed with 250.0 μ l methanol; 150 ml of acetonitrile was added to precipitate the lipids. The solution was then centrifuged during 15 min at 3000 rpm (1512 $\times$ g). The supernatant was injected in the HPLC system after appropriate dilution. For the determination of the free drug, approximately 1 ml suspension was centrifuged at 4000 rpm (2500 $\times$ g) for 20 min and an aliquot of the supernatant was analysed with HPLC. Trapping efficiency is normally calculated according to the equation: Trapping efficiency (%) = encapsulated drug in liposome/drug in liposomal solution $\times$ 100.

2.4.4 Zeta potential

Zeta potential of liposomes can be measured by a laser Doppler method (Du Plessis *et al.*, 1996; Guo *et al.*, 2000; Imura *et al.*, 2002). The method gives the electrophoretic mobility and zeta potential of particles moving in an electric field based on the Doppler

shift, a frequency shift of laser light scattered by the particles (Imura *et al.*, 2002). The liposome is usually diluted and placed in an electrophoresis chamber consisting of two electrode compartments and a connecting chamber. Voltage is then applied between the two electrodes, one located in each compartment (Du Plessis *et al.*, 1996). Zeta potential values affect liposome interaction with other surfaces, hydration and stability (Du Plessis, 1992).

2.4.5 Chemical composition

Accurate measurements of phospholipid concentrations are difficult to make directly, since dried lipids can often contain considerable quantities of residual solvents. Consequently the most widely used method for phospholipid determination is an indirect one, in which the phosphate content of the sample is first measured (New 1990).

2.4.5.1 Bartlett Assay

This method is widely used for the analysis of phosphorus. Phospholipid phosphorus in the sample is first acid-hydrolysed to inorganic phosphate. This is converted to phospho-molybdic acid by addition of ammonium molybdate, and the phospho-molybdic acid is quantitatively reduced to a blue-coloured compound by amino-naphthyl sulphonic acid. The intensity of the blue colour is measured spectrophotometrically. The majority of phospholipids used for preparing liposomes contain one mole of phosphorus per mole of phospholipid; hence the concentration of phospholipid can be derived directly from a measurement of the phosphorus content of the sample. An exception is cardiolipin, which contains two moles of phosphorus per mole of phospholipid (New 1990).

Rao & Murthy recently described a technique for phosphatidylcholine (PC) analysis. The PC was transferred to a calibrated centrifuge tube and chloroform added. Ammonium ferrothiocyanate was also added and the mixture vortexed and centrifuged at 2750 rev min⁻¹. The chloroform layer was separated and absorbance measured at 485nm (Rao & Murthy, 2000).

2.5 Liposomes as skin delivery systems

Interest in the use of liposomes for dermal drug delivery has been spurred by the publication of reports showing enhanced drug delivery from elastic liposomes (Cevc & Blume, 1992; Guo *et al.*, 2000; Cevc *et al.*, 1998; El Maghraby *et al.*, 1999, 2002; Cevc *et al.*, 2002; Verma *et al.*, 2003). However, there is still controversy regarding the benefits of skin delivery of drugs from liposomes (Van Kuijk-Meuwissen *et al.*, 1998; Redelmeier & Kitson, 1999). Cevc & Blume (1992) prepared elastic vesicles from phospholipids and edge activators- surfactant molecules such as sodium cholate. They postulated that the transepidermal water gradient was sufficiently strong to push at least 0.5mg/hr/cm of lipids through the stratum corneum (Cevc & Blume, 1992; Cevc *et al.*, 2002). They concluded that such vesicles, when applied to the skin non-occlusively, could enhance drug permeation.

The maximum flux rate for spontaneous skin penetration by the elastic vesicles is of the order of 0.1 mg/cm²/h (Schatzlein & Cevc, 1995). This value is substantially higher than that which is typically driven by the transdermal concentration gradients. Considering the effective mass of a typical lipid vesicle with a radius of 100nm, which is 5×10^7 Dalton and the maximum achievable lipid concentration on the skin surface (≈ 0.3 mol/L), the expected concentration gradient driven flux is approximately 1 nanogram of lipid /hr/cm². The transdermal lipid concentration gradients, consequently, cannot provide a sufficiently strong force for the practically significant and spontaneous material transport across the stratum corneum. Transdermal osmotic gradient creates a more substantial transdermal flux for colloidal suspensions. The gradient arises due to the water-activity difference between the viable epidermis ($\sim 75\%$ water content) and the stratum corneum ($\sim 15\%$ water content). This gradient is very stable because ambient air is a perfect sink for water molecules, even when the transepidermal water loss is unphysiologically high. Owing to the energetically favourable interactions between the hydrophilic parts of the amphiphilic membrane components and the surrounding water, most lipid bilayers try to avoid dehydration. Nonocclusively applied suspensions of the phospholipid vesicles on the skin surface thus have to face the problem of partial dehydration in an extremely arid surrounding. Finally, this surrounding would deprive the lipids of most of the water necessary for maximum

hydration (Schatzlein & Cevc, 1995). The only escape is to move along the water activity gradient, that is, into the skin.

However the size of phospholipid vesicles is very large when compared to the spacing between the intercellular lipid lamellae in the stratum corneum. Epicutaneously applied vesicles must therefore deform strongly if they are to pass through this barrier. By adding at least one component to a membrane with a high affinity for the strongly curved surfaces, the elastic energy associated with such a deformation may be lowered by several orders of magnitude (Cevc & Schatzlein, 1995).

Other investigators have subsequently published data demonstrating enhanced skin delivery of insulin (Guo *et al.*, 2000) and oestradiol (El Maghraby *et al.*, 2002) from elastic liposomes. The enhancing effect of flexible lecithin vesicles on transdermal delivery of insulin has also been investigated. The vesicles were prepared by reverse-phase evaporation technique and treated further by sonication. There was 21.42 % drop in blood glucose when the vesicles were applied non-occlusively onto abdominal mice skin (Guo *et al.*, 2000). El Maghraby *et al.* compared in vitro skin delivery of oestradiol from conventional and deformable (elastic) liposomes containing 1mg/ml of the drug. Flux values of oestradiol from elastic vesicles containing sodium cholate, Span 80 and Tween 80 were 171, 161 and 128 ng cm⁻² h⁻¹ respectively. Flux values from conventional phosphatidylcholine and dipalmitoylphosphatidylcholine vesicles were 98.8 and 109 ng cm⁻² h⁻¹ respectively (El Maghraby *et al.*, 1999). They however noted that the difference between the two types of vesicles did not reflect a highly effective action as previously reported by Cevc and co-workers. Also, only low percentages of the applied dose (1.3-2%) permeated through and deposited into the skin. It was concluded that liposome adhesion, fusion or even penetration into the stratum corneum were possible with the vesicles.

Verma and co-workers (2003) investigated skin penetration from liposomes of two fluorescently labelled substances. Liposomes with a size of 120 nm diameter showed statistically enhanced penetration of carboxyfluorescein (CF) into the skin as compared to larger ones. The results indicated that the CF penetration was inversely related to the size of the liposomes, which was confirmed by data from confocal laser scanning microscopy studies (Verma *et al.*, 2003). Honeywell-Nguyen *et al.* also reported on

enhanced skin permeation of pergolide from elastic vesicles prepared from a bilayer-forming surfactant (sucrose laurate ester) and the micelle-forming surfactant (octaoxyethylene laurate ester). Non-occlusive co-treatment with the vesicles improved skin delivery of pergolide compared to the no-occlusive buffer control by more than two-fold. Occlusive application of vesicles diminished their action resulting in a lower flux. However the highest pergolide skin permeation was from an occluded saturated solution with a steady-state flux of 137.9 ng/cm²/h (Honeywell-Nguyen *et al.*, 2003). The authors proposed four essential steps in the transport of drugs from elastic vesicle formulations. Step 1 involves association of drugs with vesicle bilayers. Step 2-partitioning of vesicles into the stratum corneum. This aids the partitioning of drugs into the skin, since vesicles will 'carry' vesicle-bound drug into the stratum corneum. Vesicles themselves remain in the stratum corneum and do not penetrate into deeper layers of the skin. Vesicle-bound drug molecules are released from the vesicles (step 3) after which free drug molecules can diffuse through the stratum corneum and partition into the viable skin tissue (step 4). Several factors can have influence on each of these steps (Honeywell-Nguyen *et al.*, 2003).

Kitagawa *et al.* (2001) studied the effects of a double-chained cationic surfactant dimethyldidecyl ammonium on the permeation of benzoic acid through excised guinea pig dorsal skin and showed that the flux of benzoic acid was increased three-fold. In spite of these remarkable results, there is still scepticism over the benefits of liposomal skin delivery of drugs. Van Kuijk-Meuwissen *et al.* (1998) studied the penetration profiles of fluorescent labels from liquid-state liposomes and concluded that intercalation of a surfactant in the liposomal bilayer did not result in a dramatic increase in penetration of a fluorescent label into human skin.

The transport of the entrapped spin labelled hydrophilic compounds into the skin from non-hydrogenated (NSL) soyabean lecithin, hydrogenated (HL) soyabean lecithin, and dipalmitoylphosphatidylcholine liposomes (DPPC) was also measured by electron paramagnetic resonance imaging methods (Sentjurs *et al.*, 1999). No significant transport into the deeper skin layers (more than 100 µm deep) was observed for NSL liposome, irrespective of vesicle size. For all other vesicular systems some transport into the deeper skin layers was observed, which did not depend on vesicle size significantly until the vesicle diameter of approximately 200 nm was reached.

However, for small vesicles (with diameter less than 200 nm) the transport was significantly decreased. The authors hypothesised that small vesicles are not stable, disintegrate immediately in contact with other surfaces and as a consequence, they lose an important influence on topical delivery of entrapped hydrophilic drugs.

There is also continued scepticism regarding the degree to which liposomes can penetrate the stratum corneum and influence drug pharmacokinetics. Arguments are centred around conflicting claims regarding the impact of liposomes on percutaneous absorption, and whether liposomes limit the clearance of compounds from viable skin tissues. There is also suspicion regarding how liposomes with diameters in excess of 50nm can possibly penetrate the stratum corneum in sufficient amounts needed to enhance drug delivery (Redelmeier & Kitson, 1999). Despite these conflicting results from different investigators, there is no doubt regarding the potential utility of liposomes for dermal drug delivery. More studies are needed to quantify the effect of liposome size, composition and other characteristics on drug permeation across the stratum corneum.

Summary

The use of liposomes for the skin delivery of drugs has increased tremendously over the last two decades. The therapeutic potential of these vesicles is enormous because of the versatility of liposomes. Liposome composition, size and charge can be manipulated to obtain vesicles for dermal or transdermal drug delivery. In spite of the tremendous progress in the field, the use of these vesicles has attracted considerable controversy in the literature. The underlying mechanisms governing skin permeation from these vesicles are yet to be fully elucidated. It is evident that the physico-chemical properties of drugs formulated into these vesicles play a significant role in their skin delivery. More studies are needed for a better understanding of these complex processes.

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PHYSICOCHEMICAL PROPERTIES OF DRUGS INCLUDED IN THIS STUDY

3.1 Introduction

Before a drug can be considered seriously as a candidate for transdermal delivery, it is necessary to have a thorough understanding of a drug's physico-chemical properties, particularly its absolute and relative solubilities and related partitioning tendencies (Flynn & Yalkowsky, 1972; Sloan *et al.*, 1986). The amphiphilic nature of the skin dictates that its permeability will be highly dependent on the lipophilicity of a penetrant. Also, the greater a drug's innate tendency to dissolve, the more likely it is that the drug can be delivered at an adequate rate across the skin (Spruance *et al.*, 1984). Given the unique barrier function of the skin, the key requirements for transdermal application of a therapeutic agent are a good solubility in both lipoidal and aqueous systems, a low molecular weight and volume, a high diffusivity in the stratum corneum and little or no accumulation in the stratum corneum (Wiechars, 1989). It is necessary to understand the physico-chemical determinants, which affect the skin delivery of methotrexate, acyclovir and idoxuridine. The most important physicochemical properties, which affect skin delivery of drugs, are:

- Drug solubility
- Diffusion coefficient
- Partition coefficient
- Hydrogen bonding
- Melting point
- Ionisation
- Molecular weight and size (Hadgraft, 2001; Du Plessis *et al.*, 2001; Du Plessis *et al.*, 2002; Malan *et al.*, 2002)

3.2 Solubility

The aqueous solubility of a drug molecule is partly dependent on other physicochemical properties like partition coefficient and other molecular surface features. Solubility is a dominant factor in skin penetration: compounds that are soluble in both lipid and water penetrate better than substances manifesting either high water or lipid solubility (Liron & Cohen, 1984; Malan *et al.*, 2002). The thermodynamic activity (TA) of a drug also plays an important role in skin delivery. TA is usually derived from the ratio of its concentration in the formulation to that of its saturation solubility in that formulation (Thomas *et al.*, 2003). Since a saturated solution has maximum TA it is usually preferred for skin delivery studies.

The solubility parameter of an organic solute in the stratum corneum is also considered important for skin delivery. It can be estimated by using the Hildebrand equation (Equation 3-1) (Hildebrand *et al.*, 1970):

$$\ln X_2 = \frac{-\Delta H_f}{RT} \left(\frac{T_f - T}{T_f} \right) + \frac{\Delta C_p}{R} \left[\frac{T_f - T}{T} - \ln \frac{T_f}{T} \right] - \frac{V_2 \phi_1^2}{RT} (\delta_1 - \delta_2)^2 \quad \text{Equation 3-1}$$

Where

- X_2 is the solute's mole fraction solubility in hexane
- ΔH_f is the heat of fusion of a solid
- R is the gas constant
- T_f is the melting point of the solid in degrees Kelvin
- T is any experimental temperature less than T_f
- ΔC_p is the difference in heat capacity between the solid form and the hypothetical supercooled liquid form of the compound, both at the same temperature
- V_2 is the molar volume of the liquid solute
- ϕ_1 is the volume fraction of the solvent

- δ_1 is the solubility parameter or square-root of the cohesive energy density of the solvent (hexane)
- δ_2 is the solubility parameter or square-root of the cohesive energy density of the solute.

The solubility parameter of the skin has been estimated at about 10 and drugs, which have equivalent values, are usually expected to dissolve readily in the stratum corneum and exhibit higher fluxes (Liron&Cohen, 1984). There is also a relationship between aqueous solubility and n-octanol water partition (P). One of the most well-known equations for predicting the solubility of liquid and solid organic solutes was derived by Yalkowsky and Valvani:

$$\log S_w \approx -1.00 \log P - 1.11 \frac{\Delta S_f (mp - 25)}{1364} + 0.54 \quad \text{Equation 3-2}$$

For rigid molecules, where ΔS_f was taken as 56.7 J/molK, Kristl obtained

$$\log S_w \approx -1.05 \log P - 0.012 mp + 0.87 \quad \text{Equation 3-3}$$

where S_w is aqueous solubility in mol/L, mp is the solute melting point temperature in °C, and ΔS_f is the entropy of fusion. The parameter P is given as the concentration ratio in octanol and aqueous phases (C_o/C_w).

Essentially, the stratum corneum (SC) barrier is lipophilic. For this reason, lipophilic molecules are better accepted by the SC. A molecule must first be liberated from the formulation and partition into the uppermost SC layer before diffusing through the entire thickness, and must then repartition into the more aqueous viable epidermis beneath. Ideally, a drug must possess both lipoidal and aqueous solubilities: if it is too hydrophilic, the molecule will be unable to transfer into the SC; if it is too lipophilic, the drug will tend to remain in the SC layers. This limitation imposes the criterion of adequate aqueous solubility (> 1mg/ml) for a successful transdermal drug candidate (Naik *et al.*, 2000).

Experimentally determined aqueous solubilities for acyclovir and idoxuridine, reported in the literature were 2.5mg/ml and 2mg/ml respectively (Shao *et al.*, 1994; Gosh & Mitra, 1991). These solubility values meet one of the requirements for successful dermal delivery. However, it must be emphasized that aqueous solubility alone does not determine successful passive transdermal drug delivery. Partition coefficient values and degree of ionisation are also important. Reported solubility values for methotrexate are highly variable due to the different pH of the solutions used by different investigators. Methotrexate solubility in a pH 4.0 buffer was 0.32mg/ml (Chatterjee *et al.*, 1997) while Wallace & Barnett (1978) reported a solubility value of 0.75mg/ml at the pH of 6.5. However, at pH 7.4, Alvarez-Figuerova *et al.* (2001) reported a solubility value of 4mg/ml. This value also meets one of the criteria for successful dermal delivery.

Solubility values can also be predicted for methotrexate, idoxuridine and acyclovir using a number of algorithms available in software packages. Three different software packages were chosen to estimate the solubility values for methotrexate, idoxuridine and acyclovir: EPIWIN™ (United States Environmental Protection Agency) ACD/CHEMSKETCH™ (Advanced Chemistry development, Canada) and IALOGP™ (Interactive Analysis, USA). Table 3-1 shows solubility values calculated with different software packages.

TABLE 3-1: Solubility values calculated from different software packages

Permeant	AD™ (mg/ml)	EPIWIN™ (mg/ml)	IALOGP™ (mg/ml)	Experimental values reported in the literature (mg/ml)
Methotrexate	31.00	0.063	0.002	4.00
Idoxuridine	2.17	0.014	19.05	2.00
Acyclovir	i.s.	11.38	1.32	2.50

(i.s. = infinitely soluble)

There is a good agreement between predicted solubility value for idoxuridine (ACD™) and the experimental value reported in the literature (Gosh&Mitra, 1991). There is also a good agreement between predicted (IALOGP™) and literature solubility values for acyclovir (Shao *et al.*, 1994). However, rather large discrepancies were observed between literature and EPIWIN™ solubility values for methotrexate and idoxuridine. This can be explained by the fact that the partition coefficient values from KOWWIN™ (also incorporated into EPIWIN™), which were used for solubility prediction, differ from reported experimental values. Poor predictive ability could also result from the inability of some of the programs to incorporate all the effects of molecular surface area and connectivity into the calculation procedure.

It is a common misconception that an increase in the applied concentration of a drug always results in an increase of drug flux. If a drug is presented as a suspension, the flux will be invariant with applied concentration. The driving force for diffusion through the skin is the chemical potential gradient (Hadgraft, 1999). A saturated formulation of the drug (with thermodynamic activity of unity) will in fact provide the maximum flux, irrespective of the selected vehicle and drug solubility therein.

Cooper *et al.* (1985) studied the effect of various vehicles on the delivery of acyclovir. Propylene glycol was evaluated as a vehicle. Without the addition of penetration enhancers (oleic acid and oleyl alcohol), penetration of acyclovir was very low.

However, Okamoto *et al.* (1990) also studied the effects of vehicles and penetration enhancers on the skin delivery of acyclovir. Four solvents- propylene glycol (PG), ethanol (ET), isopropanol (IPA) and isopropyl myristate (IPM)- were evaluated. When the results of the experiments were compared, acyclovir penetration from the vehicle with the higher lipophilicity was faster (IPM>IPA>ET>PG). Also the amount of acyclovir remaining in the hydrophobic vehicle was higher than that in the hydrophilic vehicle. Thus, the vehicles themselves affected the permeability of the skin to some extent. The permeation rate of the solvent can also be an important factor influencing skin permeation of drugs. The results of increased solvent penetration into the skin may include increased drug solubility in the skin and increased barrier disruption if the solvent itself is a penetration enhancer (Aungst *et al.*, 1990).

Chatterjee *et al.* (1997) also correlated the effect of different vehicles -propylene glycol (PG), ethanol (ET), Polyethylene glycol 400(PEG 400) and isopropanol (IPA)- with methotrexate flux. The order for methotrexate flux from the different vehicles was IPA>ET>PG>PEG 400. Permeability coefficient also followed a similar trend. It has been suggested that the increased flux may be as a result of an ion-pairing mechanism. Ion pairing could substantially increase the penetration of ionic drugs through synthetic hydrophobic membranes using nonaqueous vehicles with a low dielectric constant (Aungst *et al.*, 1990).

3.3 Diffusion coefficient

Diffusion coefficient is the number of moles of a drug that diffuse across a membrane or within the various membrane strata of a given unit area per unit time (Idson, 1975). The diffusion coefficient of a drug in the skin is dependent on properties of the drug and the medium through which it diffuses (Barry, 1988). The epidermal transport for most solutes is limited by passive diffusion across stratum corneum (Roberts *et al.*, 1995).

Drug diffusion is an integral part of the membrane permeation process. Permeant molecules partition from the applied vehicle solution across the vehicle-membrane interface and concentrate in the membrane. A concentration gradient is thus established. This serves as the impetus for the net movement (diffusion) of the molecules between proximal and distal surfaces of the membrane. There are essentially two diffusion environments for the drug molecules in solution: the solvent system of the donor vehicle and the solvent system of the resistant barrier membrane. The ease with which a specific molecule type diffuses through a solvent system is described by the magnitude of its diffusion coefficient, either in the donor vehicle or in the membrane. This depends on the physicochemical properties of (and the extent of interaction between) the drug and the respective diffusion medium (Smith & Surber, 2000).

The mechanism by which drugs passively permeate the stratum corneum (SC) remains a subject of considerable debate (Bunge *et al.*, 1999). The heterogenous nature of the SC suggests that mass transfer can occur (at least) via transcellular and intercellular pathways. Furthermore, the intercellular regions are full of lipids structured in

multilamellar arrays, through which more than one route of molecular diffusion may be envisaged.

Lately, there has been a preponderance of opinion leaning towards the dominance of intercellular diffusion, based on supporting evidence from diverse sources. Albery and Hadgraft applied a detailed mathematical analysis to the results of in vivo skin permeation experiments to deduce that the intercellular path was likely to dominate for small electrolytes (Albery & Hadgraft, 1979a,b). The most compelling argument originated from combined permeability and SC biophysical measurements which led, ultimately, to the deduction that the molecular diffusion pathlength (h) of water across the SC is manifold greater than the membrane's simple thickness (Potts & Francouer, 1991). Pellet *et al.* (1997) re-examined the issue of diffusion pathlength, and determined a value of h much closer to that of h_{sc} , opening the question once more of the importance of transcellular diffusion. However, Bunge *et al.* have observed that it is incorrect to conclude from Pellet's studies that molecular transport occurs via a specific route. The authors noted that it is not possible to determine unequivocally the length of the actual SC diffusion pathway (Bunge *et al.*, 1999).

There has been little evidence to suggest that active processes are involved in skin permeation. The underlying transport process is controlled by simple passive diffusion (Hadgraft, 2001). Fick's law of diffusion can be used to describe steady state diffusion and can be simplified to:

$$J = \frac{DK\Delta c}{h} \quad \text{Equation 3-4}$$

Where J is the flux per unit area, D is the diffusion coefficient in the skin, K is the skin-vehicle partition coefficient, Δc is the concentration gradient across the skin and h is the diffusional pathlength. Under normal circumstances, the applied concentration (c_{app}) is larger than the concentration under the skin and Equation 3-4 can be simplified to

$$J = k_p \cdot c_{app} \quad \text{Equation 3-5}$$

where k_p is the permeability coefficient ($=KD/h$) and is a heterogeneous rate constant having the units cm h^{-1} . It is often difficult to separate D and K and their calculated magnitude depends on h . And h cannot be accurately estimated, as it is the tortuosity of intercellular channels, which is imprecise (Hadgraft, 2001).

The following equation can be used to determine diffusion coefficient (Barbar *et al.*, 1990):

$$D = \frac{k_p}{K} \quad \text{Equation 3-6}$$

Where:

- D is the diffusion coefficient (cm^2)
- k_p is the permeability coefficient
- K is the partition coefficient

Due to the dense nature of the stratum corneum, values of diffusion coefficients in this tissue are 1000 times smaller than anywhere else in the skin. This factor contributes to high resistance and low penetrability (Flynn, 1990).

Diffusion within the confines of the stratum corneum has been modelled with the aid of three simplified modelling processes (Rieger, 1993):

- (1) The drug must pass through the vehicle to the surface of the stratum corneum. The step controlling this process is diffusion.
- (2) The second step, passage into the stratum corneum, is controlled by the distribution coefficient K .
- (3) In the third step, the permeant diffuses through the stratum corneum. This is generally the rate-determining step.

The speed with which materials diffuse depends first and foremost on the state of matter of the diffusing medium. In gases and air, typical diffusion coefficients have high values (in the order of $0.05\text{-}1.00 \text{ cm}^2/\text{sec}$) due to the free volume or void space available to the molecules in comparison to their size. In this case, the mean free path between molecular collisions is high. In liquids, the void space is smaller, meaning decreased free path and reduced diffusivities. Thus for an aqueous lotion on the skin, diffusion coefficients within the vehicle would be in the order of 10^{-5} to $10^{-6} \text{ cm}^2/\text{sec}$.

Diffusivities progressively drop as the consistency of the material increases until, for a true crystalline solid with no free volume, molecules are totally inhibited (Barry, 1983). The amount of drug flowing through a unit cross section of a membrane in unit time, is known as the flux (J) (Hadgraft, 2001):

$$J = \frac{dM}{S \cdot dt} \quad \text{Equation 3-7}$$

Where:

- M is the amount of drug (mg)
- S is the area (cm²)
- t is the time
- J is the flux (mg/cm²/h).

The flux is proportional to concentration gradient and inversely proportional to membrane thickness (dC/dh).

$$J = -D \frac{dC}{dh} \quad \text{Equation 3-8}$$

Where:

- D is the diffusion coefficient of the drug (cm²/sec)
- h is membrane thickness (cm).

Lashmer and Manger investigated the skin delivery of acyclovir. The amount of the drug, which permeated in vitro across nude mouse skin by means of passive diffusion, was 1.66 µg/cm²/hr (Lashmer & Manger, 1994). The flux of methotrexate through hairless mouse skin as calculated by Wallace and Barnett at pH 6.5 was 0.108 µg/cm²/hr (Wallace & Barnett, 1978). Bonina *et al.* (2002) carried out in vitro experiments to evaluate the flux of idoxuridine and its derivatives through human skin. The cumulative amount of IDU that penetrated through excised human skin was 0.76 µg/cm²/hr.

3.4 Partition coefficient

An ideal drug candidate would have sufficient lipophilicity to partition into the SC, but also sufficient hydrophilicity to enable the second partitioning step into the viable epidermis and eventually the systemic circulation (Kalia & Guy, 2001). When the drug reaches the viable tissue it encounters a phase change. It has to transfer from the predominantly lipophilic intercellular channels of the stratum corneum into the living cells of the epidermis, which will be largely aqueous in nature and essentially buffered to pH 7.4 (Hadgraft & Wolff, 1993).

The lipophilicity of a molecule is related to its free energy of partitioning; partition coefficients are directly related to the free energy of transfer of a substance between two immiscible phases. The most common expression of lipophilicity is the logarithm of the n-octanol-water partition coefficient ($\log P_{\text{oct}}$). The n-octanol-water two-phase system is a popular model for assessing partitioning at lipid membranes because of the similarities of n-octanol (long hydrophobic chain and polar hydroxyl group) and membrane lipids (Malan *et al.*, 2002).

Relatively few measurements of drug partition coefficient in the stratum corneum (SC) have been performed. This is because direct measurements are more challenging due to the heterogeneous nature of the SC (Mitragotri, 2001). Accordingly, attempts have been made to extract SC lipids and form bilayers of these lipids on a solid support. Partition coefficients of solutes are then measured. Although this method has been greatly helpful in understanding partition coefficients of drugs, its applicability is limited due to the requirement of lipid extraction, which removes SC lipids from their natural environment. Mitragotri recently developed a non-intrusive method to determine partition coefficient in the SC lipid bilayers. This method is based on two independent measurements of transport properties of the SC: (i) steady-state permeability in the presence of a concentration gradient, and (ii) release of drugs from the SC when the concentration gradient is in the opposite direction. This information is then analysed to determine partition coefficient (Mitragotri, 2000).

Since there are difficulties in determining the actual skin/water partition coefficients of drugs, octanol/water partition coefficients are used for ranking the lipophilicities of compounds for skin permeation (Takahashi *et al.*, 1993). Partition coefficients are determined by dissolving drugs in an organic solvent, and assaying both phases for

drug content. The partition coefficient is taken as the organic solvent: water-drug concentration ratio (Chattarjee *et al.*, 1997).

A convenient procedure has been developed for the determination of partition coefficient using potentiometric titration. The method involves the potentiometric titration of the compound, first in water and then in a rapidly stirred mixture of water and octanol. An automatic titrator is used and the data collected and analysed by non-linear regression (Clarke & Cahoon, 1987; Geinoz *et al.*, 2002). The method is rapid and accurate for compounds with pK_a values between 4 and 10. Partition coefficients can be measured for monoprotic and diprotic acids and bases. The partition coefficients of neutral compounds and their ions can be determined by varying the ration of octanol to water. It has been shown that subtraction of the titration curve of solvent alone from that of the compound in the solvent offers advantages for pK_a determination by curve fitting for compounds of low aqueous solubility. Potentiometry is especially useful for analysing substances, which are poorly soluble in water. However the solubility must be at least 10^{-4} M. Solutions as dilute as 10^{-5} can still be analysed, but special attention must be given to electrode calibration and ambient carbon dioxide must be excluded or corrections for its presence incorporated (Avdeef *et al.*, 1993).

Mixed-solvent approaches can also be used for compounds that are virtually insoluble. Methanol is often the solvent of choice since its effect has been studied extensively. Mixed-solvent solutions of various methanol-water proportions are prepared, and the p_sK_a (apparent pK_a) is determined in each mixture. The aqueous pK_a is deduced by extrapolation of the p_sK_a values to zero methanol. Plots of p_sK_a versus weight percent organic solvent, $R = 0-60$ wt %, show either a “hockey-stick” shape or a “bow” shape, but rarely a straight line (Avdeef *et al.*, 1993).

Instead of measuring $\log P_{oct}$ by the standard but time-consuming shake-flask method, reversed-phase chromatography is increasingly being used as a powerful tool to predict $\log P_{oct}$ from readily accessible retention factor, $\log k$, given by

$$k = (t_R - t_0) / t_0$$

Equation 3-9

where t_0 and t_R express retention times of the un-retained solvent and analyte, respectively (Pignatello & Puglisi 2000; Yamagami *et al.*, 2002). Toon *et al.* (1984) proposed an alternative isocratic chromatographic parameter,

$$R_Q = \log \left(\frac{R_T - R_0}{R_T} \right) \quad \text{Equation 3-10}$$

Where $R_T = t_R$ and $R_0 = t_0$

R_Q was found to vary linearly with the mobile phase composition, in particular with the increase of organic modifier fraction in a water/solvent eluent mixture. By plotting the R_Q values obtained for a homologous series of solutes vs. the amount of the organic solvent in the mobile phase, extrapolations from experimental R_Q values to its values at any desired mobile phase composition can be achieved. R_Q has been well correlated with $\log P$ values (Pignatello & Puglisi, 2000).

Yamagami *et al.* also demonstrated a linear relationship between $\log k$ derived from an eluent containing 50% methanol and $\log P$ (Yamagami *et al.*, 2002). It has also been suggested by Rodrigues *et al.* that liposomes are a better model membrane to predict drug/membrane partition coefficients than an isotropic two-phase solvent system, since they mimic better the hydrophobic part and the outer polar and negatively charged surface of the phospholipids of natural membranes (Rodrigues *et al.*, 2001). The authors used derivative spectrophotometry to obtain partition coefficients of rifampicin in water/dimyristoyl-L- α -phosphatidylglycerol (DMPG). The molar partition coefficient of drugs between lipid bilayer vesicle suspensions and aqueous solution is defined as:

$$K_p = \frac{(C_m / C_t) / [lipid]}{(C_w / C_t) / [water]} \quad \text{Equation 3-11}$$

Where C_t is the drug molar concentration, the subscripts m and w stand for drugs in lipid and in aqueous media, $[lipid]$ and $[water]$ represent lipid and water molar concentrations, respectively. Partition coefficients can be determined from changes in drug absorbance caused by binding to vesicles. As absorbance is proportional to solute concentration, at a specific wavelength,

$$A = \epsilon_m C_m + \epsilon_w C_w, \quad \text{Equation 3-12}$$

where ϵ_m and ϵ_w are the drug extinction coefficients in lipid bilayer and water, respectively.

The difference between absorption in the presence and absence of liposomes (ΔA) and can be related to K_p by the following equation:

$$\Delta A = \frac{K_p \epsilon C_i [\text{lipid}]}{[\text{water}] + K_p [\text{lipid}]} \quad \text{Equation 3-13}$$

where $\epsilon = \epsilon_m - \epsilon_w$.

Derivative intensity, like absorbance, is proportional to solute concentration, as long as the background signal caused by the liposomes is entirely eliminated in the derivative spectrum. The values of partition coefficients, K_p , can be obtained by fitting Equation 3-13 to the experimental data (ΔA vs. $[\text{lipid}]$) for a given drug concentration using non-linear regression methods (Rodrigues *et al.*, 2001). Besides direct measurements, partition coefficients can be predicted by using one of several estimation techniques (Kristl *et al.*, 1999). However, the estimation of $\log P$ for complex structures may have a restricted importance because, so far, none of the available methods can include all the effects of molecular conformation, proximity and hydrogen bonds into the calculation procedure.

There are many different commercially available computer programs, which have simplified such computations. They are arranged into three major groups: programs based on fragmental methods, those based on atomic contributions and those based on molecular (conformation dependent) properties. Different reports are available in the literature where the authors compare experimentally determined $\log P$ values with those estimated by different programs such as CLOGP (Biobyte) and SYBYL (Tripos) and HYPERCHEM (Hyperchem). Kristl *et al.* compared $\log P$ values for of n-octanol/water for some guanine derivatives (acyclovir, deoxyclovir and their acetyl congeners) obtained by conventional shake-flask methods with the values calculated by some commercially available computer programs. It was established that the calculations did

not give reliable results (Kristl *et al.*, 1999). However, the same authors did indicate that good correlations have been obtained between lipophilicity indices obtained by HPLC and log P values computed by MOLGEN (CHERS, Slovak Republic) and SYBYL (Tripos).

Skin permeants must have reasonable solubilities in oil and water, but should favour the oil. A preferential oil soluble drug may have difficulty leaving the stratum corneum while an extremely polar drug will have trouble partitioning into the stratum corneum from its vehicle (Goosen, 1998). The lipid/water partition coefficient of a drug is the basic determinant of drug permeability through the stratum corneum. A drug with a log P_{oct} value of approximately 2 is considered to be a potential candidate for transdermal drug delivery. Compounds with a log P_{oct} value between 1 and 3, with relatively low melting points are likely to display optimum passive skin permeation ((Barry, 2002).

Kamlet *et al.* (1983) developed a linear solvation relationship (LSER) for the correlation of various physicochemical and biochemical phenomena. Their initial equation for log K_{oct} used four descriptors. For log K_{oct} they established the following equation:

$$\text{Log}K_{oct} = -0.02 - 0.74\pi - 0.15\alpha - 3.5\beta + 5.83 V_1 \quad \text{Equation 3-14}$$

Where:

π = solute dipolarity/polarizability,

α = solute hydrogen- bond acidity,

β = solute hydrogen- bond basicity and

V_1 = intrinsic volume in units of $(\text{cm}^3\text{mol}^{-1})/100$.

The two main factors governing log K_{oct} values are solute hydrogen-bond basicity, which favours water, and solute size, which favours octanol, with solute hydrogen-bond acidity playing almost no part (Abraham *et al.*, 1995). Experimental techniques have been widely used for the measurement of log K. Kristl and Vesnaver used the shake flask method to determine the logP of acyclovir. The logP determined by this technique was -1.57 (Kristl & Vesnaver, 1995; Kristl & Tukker, 1998). Chatterjee *et al.*(1997) determined the logP of methotrexate using the same method. It was found to be -1.2.

Log P for idoxuridine as determined by Ghosh and Mitra was -0.95 (Ghosh & Mitra, 1991).

3.5 Hydrogen bonding

The presence of H-bonding groups on the permeant dramatically retards permeation through the stratum corneum in vitro. It has been suggested that, whereas lipophilicity of a solute is the major determinant for solute partitioning into the stratum corneum from aqueous solutions, hydrogen bonding of the solute is the main determinant of solute diffusion across the stratum corneum (Roberts *et al.*, 1996). Addition of successive hydrogen bonding groups reduces the diffusion coefficient by an order of magnitude per hydrogen bonding functional group. After three are present on the molecule, the effect appears to level out. The strength of H-bonding between the permeant and the stratum corneum depends on the hydrogen bond donor and acceptor capacities of the permeant and stratum corneum (Du Plessis *et al.*, 2002). The diffusion of a series of phenols across simple silicone membrane was studied. The membrane was saturated with octanol to mimic the polar/hydrogen bonding environment of the stratum corneum lipid barrier. For octanol impregnated membrane, the diffusion coefficient decreased significantly with the number of H-bonding groups (Du Plessis *et al.*, 2001).

Hydrogen-bonding donor (α) and acceptor (β) parameters are generally derived from substructure summation and have been successfully used to predict transdermal permeability. One of the first attempts to relate log Kp values to solute structure, using hydrogen bond descriptors, was through the linear free energy relationship (Abraham *et al.*, 1997):

$$\log Kp = c + rR_2 + s\pi_2^H + a\Sigma\alpha_2^H + b\Sigma\beta_2^H + vV_x \quad \text{Equation 3-15}$$

Where log Kp is the permeability coefficient

R_2 is excess molar refraction

π_2^H is the dipolarity/polarizability

$\Sigma\alpha_2^H$ is the effective hydrogen-bond acidity

$\Sigma\beta_2^H$ is the effective hydrogen bond basicity

V_x is the McGowan characteristic volume

Pugh and coworkers also used hydrogen-bonding capability, measured by α and β , as determinants of diffusion across the stratum corneum. It was found that the stratum corneum was predominantly a hydrogen bond donor environment with donor/acceptor properties in the ratio 0.6:0.4. It was also found that certain hydrogen bonding groups reduced the diffusion coefficient more than the others and it was concluded that the number and type of pendant hydrogen bonding groups is an important factor for penetrant permeability (Pugh *et al.*, 1996).

It has also been suggested that diffusion coefficient is dependent on the adsorption of a solute by polar groups in the transport pathway of the through the stratum corneum. Accordingly, the diffusion coefficient of a solute decreases as it becomes bound to polar groups in the pathway until these groups are unable to bind to any additional hydrogen-bonding groups on the solute. Above the saturable number of groups, the diffusion coefficient of the solute appears to be relatively constant (Malan *et al.*, 2002).

3.6 Melting point

There is an inverse relationship between melting points and aqueous solubilities of drugs (Kristl *et al.*, 1999). Permeant melting point was also found to be inversely proportional to lipophilicity and therefore transdermal flux. The melting point of a substance is often considered to be an indicator of the maximum flux attainable through the skin. Attempts were made to find a correlation between flux and the reciprocal of the melting point. The ideal solubility properties increase exponentially with decreasing melting point for any given molecular mass. It is assumed that there should be an exponential increase in transdermal flux with a decreasing melting point (Guy & Hadgraft, 1989).

From the equations of Hadgraft *et al.* (1990) it is clear that the solubility parameter in the stratum corneum can be estimated more accurately if the melting point of the drug is also taken into account.

$$\log \sigma_{sc} = 1.911 \left[\frac{10^3}{mp} \right] - 2.956 \quad \text{Equation 3-16}$$

where mp= melting point (K).

In general, materials, which have low melting points, penetrate the skin more readily. A suitable method by which the melting point of a delivery system can be reduced is by eutectic formation. A binary eutectic is a mixture of two components which do not interact with each other to form a new compound but which, at certain ratios, inhibit the crystalline properties of one another resulting in a system with a lower melting point than either compounds on their own. Stott and co-workers (1998) determined the effect of melting point depression through the formation of eutectic systems between ibuprofen and several terpene skin penetration enhancers. They compared the permeation across human epidermal membranes of the eutectic systems with the flux from saturated aqueous solution of ibuprofen. The melting points of the systems ranged from 32 °C to -13 °C compared to 76 °C for pure ibuprofen. Each of the ibuprofen: terpene eutectic systems prepared, produced significant increase in flux compared to the flux of the aqueous saturated solution of ibuprofen (Stott *et al.*, 1998).

Kommuru and co-workers (1998) also indicated that enantiomers with a lower melting point might exhibit higher solubility than the racemate, and consequently have higher skin permeation profiles. For example, the flux of the pure enantiomer of nivaldipine, a calcium channel blocker, across human cadaver skin was about 7 fold higher than the racemate. The melting point difference was about 34 °C. Acyclovir, methotrexate and idoxuridine melt at 255 °C, 182-189 °C and 168-171 °C respectively (Kristl, 1999; British Pharmacopoeia; Ghosh & Mitra, 1991).

3.7 Ionisation

The pH partition theory is well documented for the general absorption of ionisable drugs across the gastro-intestinal tract but is less well described for the transdermal delivery of drugs. This is surprising given the number of drugs that are delivered to the skin and which will be ionized over the normal physiological pH range of the dermal tissues (Hadgraft & Valenta, 2000). A large number of drugs belong to the group of weak electrolytes. The nonionized moiety is usually lipid soluble, hence may dissolve in the lipid material of a membrane and may be transported by passive diffusion, whereas the ionized moiety usually is not lipid soluble enough to permit permeation.

Considerable change in degree of ionization can be expected with change of pH for acidic drugs having a pKa between 3 and 7, and for basic drugs having a pKa between 7 and 11(Ritschel, 1988). The percent of ionisation can be calculated from the Henderson-Hasselbalch equation:

$$\% \text{ ionized (for acidic compounds)} = 100 / 1 + \text{antilog (pKa- pH)} \quad \text{Equation 3-17}$$

$$\% \text{ ionized (for basic compounds)} = 100 / 1 + \text{antilog (pH-pKa)} \quad \text{Equation 3-18}$$

Experimental pKa values for methotrexate, idoxuridine and acyclovir were obtained from literature (Kristl, 1993; Dollery, 1999). pKa values were also predicted with the ACD/CHEMSKETCH™ software package. The degree of ionization (%) was calculated from equations 3-17 and 3-18. The pH of 7.4 was used for the calculations.

TABLE 3-2: pKa and degree of ionization values for methotrexate, idoxuridine and acyclovir

Permeant	pKa (Literature)	pKa (ACD™)	% ionization (Literature)	% unionised drug
Methotrexate	4.8	4.76	99.75	0.25
	5.5	5.24	98.75	1.25
Idoxuridine	8.25	7.91	87.62	12.38
Acyclovir	2.19	4.88	99.99	0.01
	9.35	8.39	98.89	1.10

The pKa and degree of ionization values for methotrexate, idoxuridine and acyclovir are shown in Table 3-2. Predicted pKa values using the ACD™ software package are also presented in the table. There is a good agreement between experimental values reported in the literature and those predicted by ACD™. For the three drugs, there is a high degree of ionization. This has significant implications for dermal delivery as it is generally believed that the unionized species of a drug permeates the stratum corneum more readily due to higher lipophilicity (Ritschel, 1988). It is therefore expected that

the skin permeabilities of methotrexate, idoxuridine and acyclovir would be low due to the high degree of ionization.

3.8 Molecular weight and size

There is a clear relationship between diffusion coefficient (D) and molecular weight (M_r): $D (M_r)^{-1/2} \approx \text{constant}$. While molecular charge is the rate determining factor in the permeability of small molecules through mucosal membranes, with increasing molecular size, the sieving effect of the pores becomes increasingly discriminating and more important than field interactions. Small molecules penetrate more rapidly than large, but within a narrow range of molecular size, there is little correlation between size and penetration rate (Malan *et al.*, 2002). Diffusion constants through the hydrated stratum corneum for many low molecular weight compounds are approximately similar. The specific effect on the penetration rate of size and shape of penetrating molecules can be separated from the influence of solubility characteristics (Idson, 1975). Potts and Guy (1992) introduced a model for compounds with a molecular mass range from 18 to > 750 and $\log K_{\text{oct}}$ from -3 to $+6$. They observed that the permeability through human skin could be predicted by Equation 3-19:

$$\text{Log} k_p (\text{cm s}^{-1}) = -6.3 + 0.71 \times \log K_{\text{oct}} - 0.0061 \times MM \quad \text{Equation 3-19}$$

Where:

- k_p is the permeability coefficient (cm/sec)
- K_{oct} is the octanol/water partition coefficient
- MM is the molecular mass.

They also found that the substitution of molecular mass for molecular volume provides an equivalent fit in the model. In conclusion they found that the apparently sigmoidal dependence of $\log k_p$ upon $\log K_{\text{oct}}$ suggests a non-linear relationship between these parameters. However, when molecular volume is taken into account, the data lies on a three-dimensional surface defined by $\log k_p$, $\log K_{\text{oct}}$ and molecular volume (Potts & Guy, 1992). The upper limit of molecular mass for permeation is still a matter of discussion. There seems to be a limit of about 5000, although some authors mention masses of not more than 3000. Nevertheless, influx of compounds into the skin does

decrease with increasing molecular mass due to parallel decrease in the diffusion coefficient in water (Idson, 1975).

Mathematical models are increasingly being applied to predict percutaneous penetration based on the physico-chemical properties of the penetrant and its vehicle. Potts & Guy (1992) proposed a two-parameter model to describe the permeability coefficient (K_p) of organic compounds through excised skin *in vitro*. This model was based upon a measure of hydrophobicity (octanol-water partition coefficient) and molecular mass. Permeability coefficient (cm h^{-1}) can be calculated using Equation 3-20 (Hadgraft & Valenta, 2000):

$$\log k_p (\text{cm h}^{-1}) = -2.7 + 0.71 \log K_{\text{oct}} - 0.0061 \text{MW} \quad \text{Equation 3-20}$$

Table 3-3 shows the molecular weights and partition coefficients of methotrexate, idoxuridine and acyclovir. Equation 3-20 was used to predict the permeability coefficients of these compounds.

Permeant	Molecular weight (MW)	LogP (literature)	$K_p (\text{cm h}^{-1})$
Methotrexate	454	-1.2	4.72×10^{-7}
Idoxuridine	354	-0.95	1.04×10^{-6}
Acyclovir	225	-1.5	7.03×10^{-6}

DERMWIN™ software from the EPIWIN™ suite was used to predict permeability coefficients for the three drugs. Even though the dataset is small ($n=3$), it can be observed that permeability coefficient of the compound with the highest molecular weight (methotrexate) is the least. The trend is not observed for idoxuridine and acyclovir probably because the low molecular weight of acyclovir is offset by a lower partition coefficient compared to idoxuridine.

Summary

Drug permeation across the stratum corneum is a complex process. The important physico-chemical determinants of transdermal drug delivery are optimal solubility in both lipoidal and aqueous systems, high diffusivity in the stratum corneum, low molecular weight and volume, little or no accumulation in the stratum corneum.

Potts-Guy equation (equation 3-19) demonstrates the relationship between partition coefficient, molecular weight and permeability. Other properties such as ionisation, hydrogen bonding and melting point are equally important. A number of algorithms has been developed to predict permeability coefficient from these parameters. Because of the complexity of these relationships, it is important to compare computationally estimated values with those determined experimentally.

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SKIN DELIVERY OF METHOTREXATE, ACYCLOVIR AND IDOXURIDINE FROM ELASTIC LIPOSOMES

CHAPTER
I

4.1 Introduction

Interest in the skin delivery of drugs from elastic liposomes has been spurred by the publication of reports demonstrating enhanced drug permeation from these vesicles (Cevc & Blume, 1992; El Maghraby *et al.*, 1999; Guo *et al.*, 2000). It was previously thought that only lipophilic substances having octanol/water partition coefficient of about 2.5 are good candidates for transdermal drug delivery (Hadgraft & Wolff, 1993). Cevc and co-workers have postulated that elastic liposomes containing so-called edge-activators can enhance the transdermal flux of even hydrophilic compounds such as insulin (Cevc *et al.*, 1998). El Maghraby *et al.* showed only 1- 3% enhancement in skin delivery of oestradiol (a lipophilic drug) from ultradeformable liposomes (El Maghraby *et al.*, 1999). El Maghraby and co-workers also investigated the delivery of a hydrophilic molecule, 5-fluorouracil, from ultradeformable liposomes prepared from sodium cholate and phosphatidylcholine (El Maghraby *et al.*, 2001). The authors reported that there was no statistically significant difference in 5- fluorouracil permeation from the liposomes and an aqueous solution (El Maghraby *et al.*, 2001). In spite of considerable scepticism, the potential of enhanced drug delivery from these vesicles remains valid (Redelmeier & Kitson, 1999; Van Kuijk-Meuwissen *et al.*, 1998; Guo *et al.*, 2000; El Maghraby *et al.*, 2001).

Due to the immunocompromised status of AIDS patients, secondary infections and malignancies are common. Conditions secondary to AIDS for which patients require treatment include Kaposi's sarcoma (treated with methotrexate), varicella-zoster (treated with antivirals such as acyclovir) and herpes simplex (also treated with antivirals like acyclovir or idoxuridine). The aim of this project was to investigate the skin delivery of methotrexate, acyclovir and idoxuridine from elastic liposomes. The objectives were to:

- validate high performance liquid chromatographic techniques for quantitative analysis of methotrexate, acyclovir and idoxuridine;
- prepare elastic liposomes of different compositions containing these drugs;
- carry out in vitro skin delivery studies of the formulations using vertical Franz diffusion cells.

4.2 Materials

Methotrexate, acyclovir, idoxuridine, sodium cholate, sodium deoxycholate, Span 20 (sorbitan monolaurate), Span 40 (sorbitan monopalmitate), Span 60 (sorbitan monostearate) and Span 80 (sorbitan monooleate) were purchased from Sigma-Aldrich Corporation (Johannesburg, South Africa). Phosphoric acid and HPLC grade methanol were supplied by Merck (Johannesburg, South Africa). Dipotassium hydrogen orthophosphate, absolute ethanol (99.9%), sodium chloride (NaCl), anhydrous disodium hydrogen orthophosphate (Na_2HPO_4) and sodium dihydrogen orthophosphate hydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) were purchased from Saarchem (Johannesburg, South Africa). Trypsin was purchased from Gibco (Johannesburg, South Africa). Double distilled deionised water was prepared using a Milli-Q water purification system (Millipore, Milford, USA). Hydrogenated phosphatidylcholine 90% (Phospholipon 90 H), Phosphatidylcholine 95% (Phospholipon 90 G), Phosphatidylcholine 78.6% (Phospholipon 80), Phosphatidylcholine 50 % (Phosal PG) were kindly donated by Nattermann Phospholipids (Germany).

4.3 High-pressure liquid chromatography (HPLC) method

The HPLC analyses of methotrexate, acyclovir and idoxuridine were performed with an Agilent 1100 series HPLC equipped with a G1310A isocratic pump, G1313 autosampler, G1314 variable wavelength detector and Chemstation version 8.0x for control and data analysis. A Luna 5u (C_{18}) column (250 x 4.60 mm) from Phenomenex was used. The mobile phase for methotrexate and idoxuridine consisted of methanol and 40mM dipotassium phosphate pH 7.4 buffer (30:70) and the flow rate was 1ml/min. The eluent was monitored at 300nm. The mobile phase for acyclovir consisted of methanol and 40mM dibasic potassium phosphate pH 7.4 buffer (10:90) and the flow rate was 1ml/min. The eluent was monitored at 254nm. The retention times for methotrexate,

idoxuridine and acyclovir were ~ 6.9, 5.2 and 7.1 min respectively. The limit of detection was 10ng/ml for the three compounds.

4.3.1 Preparation of solutions

4.3.1.1 Preparation of Phosphate buffered Saline (PBS)

2.1g of sodium acid phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), 4.4g of sodium chloride (NaCl) and 9.2g of sodium phosphate $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ were accurately weighed and transferred into a 1000 ml volumetric flask. This was dissolved in and filled to volume with analytical-grade water. The solution was stirred with a magnetic stirrer until complete dissolution was achieved. Thereafter, the pH was adjusted to 7.4 with phosphoric acid.

4.3.1.2 Preparation of standard solutions.

Ten milligrams of each compound (methotrexate, idoxuridine and acyclovir) were weighed accurately and transferred into a 100ml volumetric flask. This was dissolved in the prepared phosphate buffered saline and filled to volume to produce a 100 $\mu\text{g/ml}$ solution. This solution was serially diluted to produce a final stock solution of 100ng/ml. Various injection volumes were introduced into the chromatograph to obtain solutions with concentrations of 10; 20; 50; 100; 200 and 300ng/ml.

4.3.2.1 Linearity

The assay linearity for methotrexate, idoxuridine and acyclovir was determined by performing linear regression analyses on the plot of the peak-area ratios against concentrations in the range 10ng/ml- 300ng/ml. Regression values for the three compounds were > 0.99.

4.3.2.2 Precision

The precision of the method was investigated in terms of inter-day (reproducibility) and intra-day (repeatability) variations.

4.3.2.2.1 Inter-day variability

The inter-day variability was determined by performing HPLC analyses on three samples of six known concentrations of methotrexate, idoxuridine and acyclovir (10,20,50,100,200 and 300ng/ml) on three consecutive days. Tables 4-1, 4-2 and 4-3 show the results of the inter-day variability studies for methotrexate, idoxuridine and acyclovir respectively. The result for each concentration is an average of three measurements. The relative standard deviation was less than 5%. The results complied with the criteria of the International Conference on harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH).

TABLE 4-1: Inter-day variability of methotrexate.

Concentration (ng/ml)	Peak area (mAU)				Standard deviation (SD)	Relative standard deviation (RSD)
	Calibration Day 1	Calibration Day 2	Calibration Day 3	Mean		
10	0.2566	0.2538	0.2605	0.2569	0.0027	1.0680
20	0.5070	0.5079	0.5425	0.5191	0.0165	3.1823
50	3.0031	2.9933	2.8913	2.8606	0.0718	2.5102
100	5.9119	5.9350	5.7982	5.6039	0.1512	2.6981
200	11.8407	11.5597	11.1900	11.5301	0.3263	2.8302
300	17.7507	17.2457	16.65263	17.2163	0.5496	3.1925

TABLE 4-2: Inter-day variability for acyclovir

Concentration (ng/ml)	Peak Area (mAU)				Standard deviation (SD)	Relative standard deviation (RSD)
	Calibration Day 1	Calibration Day 2	Calibration Day 3	Mean		
10	0.3049	0.3075	0.3277	0.3134	0.0102	3.2551
20	0.5377	0.5446	0.5446	0.5423	0.0032	0.5954
50	1.4909	1.3935	1.4061	1.4302	0.0432	3.0233
100	2.8015	2.8334	3.0999	2.9116	0.1337	4.5942
200	6.3370	6.3023	6.2939	6.3111	0.0186	0.2949
300	9.5205	9.5427	9.5187	9.5273	0.0109	0.1147

TABLE 4-3: Inter-day variability for idoxuridine

Concentration (ng/ml)	Peak Area (mAU)				Standard Deviation (SD)	Relative standard deviation (RSD)
	Calibration Day 1	Calibration Day 2	Calibration Day 3	Mean		
10	0.1602	0.1579	0.1560	0.1580	0.0017	1.0812
20	0.2233	0.2056	0.2122	0.2137	0.0072	3.4125
50	0.5021	0.5576	0.5273	0.5290	0.0226	4.2853
100	0.8890	0.8797	0.9025	0.8904	0.0093	1.0501
200	1.7686	1.7760	1.7600	1.7682	0.0065	0.3704
300	2.6762	2.666	2.6387	2.6604	0.0158	0.5975

4.3.2.2. Intra-day variability

The intra-day variability was determined by performing HPLC analyses on three samples of six known concentrations of methotrexate, idoxuridine and acyclovir three times during a single day. The results are given in Table 4-4, 4-5 and 4-6. The relative standard deviation was less than 5%. The results complied with the criteria of the International Conference on Harmonisation of Technical requirements for registration of pharmaceuticals for human use.

TABLE 4-4: Intra-day variability for methotrexate

Concentration (ng/ml)	Peak Area (mAU)				Standard deviatio n	Relative Standard deviation
	Calibration	Calibration	Calibration	Mean		
	A	B	C			
10	0.2566	0.2362	0.2528	0.2485	0.0088	3.5503
20	0.5070	0.5134	0.5052	0.5086	0.0035	0.6905
50	3.0031	2.9786	2.9982	2.9933	0.0129	0.4322
100	5.9119	5.9629	5.9302	5.9350	0.0258	0.4353
200	11.8397	11.8456	11.8367	11.8407	0.0044	0.0379
300	17.7583	17.8234	17.7507	17.7507	0.0767	0.4329

TABLE 4-5: Intra-day variability for acyclovir

Concentration (ng/ml)	Peak Area (mAU)				Standard Deviation (SD)	Relative standard deviation (RSD)
	Calibration A	Calibration B	Calibration C	Mean		
10	0.3114	0.3049	0.3169	0.3110	0.0049	1.5795
20	0.5446	0.5482	0.5377	0.5435	0.0043	0.8014
50	1.4061	1.3935	1.4968	1.4321	0.0460	3.2143
100	2.8582	2.8015	2.8243	2.8280	0.0232	0.8234
200	6.3370	5.7829	5.9267	6.0154	0.0234	3.9026
300	9.5205	8.7764	8.9334	9.0768	0.3202	3.5280

TABLE 4-6: Intra-day variability for idoxuridine

Concentration (ng/ml)	Peak Area (mAU)				Standard Deviation (SD)	Relative standard deviation (RSD)
	Calibration A	Calibration B	Calibration C	Mean		
10	0.160228	0.1551	0.1691	0.1615	0.0058	3.5998
20	0.2233	0.2454	0.2357	0.2348	0.0090	3.8588
50	0.5021	0.4584	0.4572	0.4726	0.0209	4.4236
100	0.8890	0.8912	0.8982	0.8928	0.0039	0.4395
200	1.7686	1.7505	1.7898	1.7696	0.0160	0.9076
300	2.6762	2.665	2.6931	2.6781	0.0114	0.4288

4.3.2.3 Selectivity

Selectivity is the capacity of a method to analyse a component in the presence of other components such as decomposition products of a drug or biological materials. Blank samples of the phosphate buffer solution used as the receptor phase in the diffusion studies as well as PBS samples containing known concentrations methotrexate, idoxuridine and acyclovir were injected into the HPLC. No interfering peaks were encountered at the retention times of ~ 6.9 , 5.2 and 7.1 min (for methotrexate, idoxuridine and acyclovir respectively).

4.3.2.4 Sensitivity

The sensitivity of a method can be determined by obtaining the limit of detection for methotrexate. The limit of detection is the smallest concentration detected by the HPLC. Based on a 10 μ l sample volume, the limit of detection of the proposed method for methotrexate, idoxuridine and acyclovir was 10ng/ml.

4.3.2.5 System repeatability

In order to evaluate the repeatability of the peak area and of the retention time, sample (100ng/ml) of each compound was injected six times. The variation in the response (% RSD) of the detection system when six determinations of each compound were made on the same day, and under the same conditions, was found to be < 1.1% for the peak area and less than 0.8% for the retention time. The system repeatability for all the compounds was well within acceptable criteria of the ICH.

4.4 Solubility determination

Saturated solutions of methotrexate, idoxuridine and acyclovir were prepared by equilibrating excess amount of each compound with PBS. A water bath with a constant temperature of 25 °C was used. Stirring was maintained for 24 hr until equilibrium was attained. Preliminary work indicated that equilibrium (no further change in concentration) was attained within 24 hours. An excess of solute was always present in the slurries. The solutions were then filtered through Millipore filters (0.45 μ m). Each filtrate was appropriately diluted with PBS prior to its assay by HPLC. All experiments were carried out in six replicates.

4.5 Preparation of elastic liposomes

Elastic liposomes were prepared using conventional rotary evaporation technique (Trotta *et al*, 2002; Du Plessis *et al*, 1994). 440 mg of phospholipid and 60 mg of surfactant was dissolved in 10ml of methanol/chloroform (1:2). The organic solvent was evaporated using a rotary evaporator and solvent traces removed by drying under vacuum overnight. The film was hydrated with phosphate buffered saline (10ml) containing 9mg/ml, 2mg/ml and 2.5mg/ml of methotrexate, idoxuridine and acyclovir respectively. Homogeneously sized liposomes were then produced following extrusion

through 100nm polycarbonate filters (Nucleopore®) using a Lipex™ Extruder (Northern Lipids, Canada).

4.5.1 Composition of liposomes- effect of type of phospholipids

Liposomes of different compositions were prepared from sodium cholate and various phospholipids. The vesicles were loaded with methotrexate, acyclovir and idoxuridine. The compositions of the liposomes are shown in Table 4.7.

TABLE 4-7: Composition of elastic liposomes prepared from sodium cholate and various phospholipids.

Phospholipid	Drug		
	Methotrexate	Acyclovir	Idoxuridine
Phosphatidylcholine 78.6% Phospholipon 80®	MP1	AP1	IP1
Phosphatidylcholine 50% Phosal PG®	MP2	AP2	IP2
Hydrogenated phosphatidylcholine Phospholipon 90H®	MP3	AP3	IP3
Phosphatidylcholine 95% Phospholipon 90G®	MP4	AP4	IP4

4.5.2 Composition of liposomes- effect of surfactants

Liposomes were prepared from 95% phosphatidylcholine (Phospholipon 90G®) and various surfactants. The vesicles were loaded with methotrexate, acyclovir and idoxuridine. The compositions of the liposomes are shown in Table 4-8.

TABLE 4-8: Composition of elastic liposomes prepared from 95% phosphatidylcholine and various surfactants.

Surfactant	Drug		
	Methotrexate	Acyclovir	Idoxuridine
Sodium deoxycholate	MS1	AS1	IS1
Sorbitan monolaurate (Span 20®)	MS2	AS2	IS2
Sorbitan monooleate (Span 80®)	MS3	AS3	IS3
Sorbitan monostearate (Span 60®)	MS4	AS4	IS4
Sodium cholate	MS5	AS5	IS5
Sorbitan monopalmitate (Span 40®)	MS6	AS6	IS6

4.5.3 Liposome Characterisation

Particle size determination and visualisation was carried out with a Phillips CM-10 Transmission electron microscope (Ganesan *et al.*, 1984; Guo J *et al.*, 2000;). 1 % Phosphotungstic acid was used for negative staining before TEM (Guo J *et al.*, 2000). The liposomes were approximately spherical with mean particle size of 120 ± 8 nm as shown in Figure 4-1.

(a)

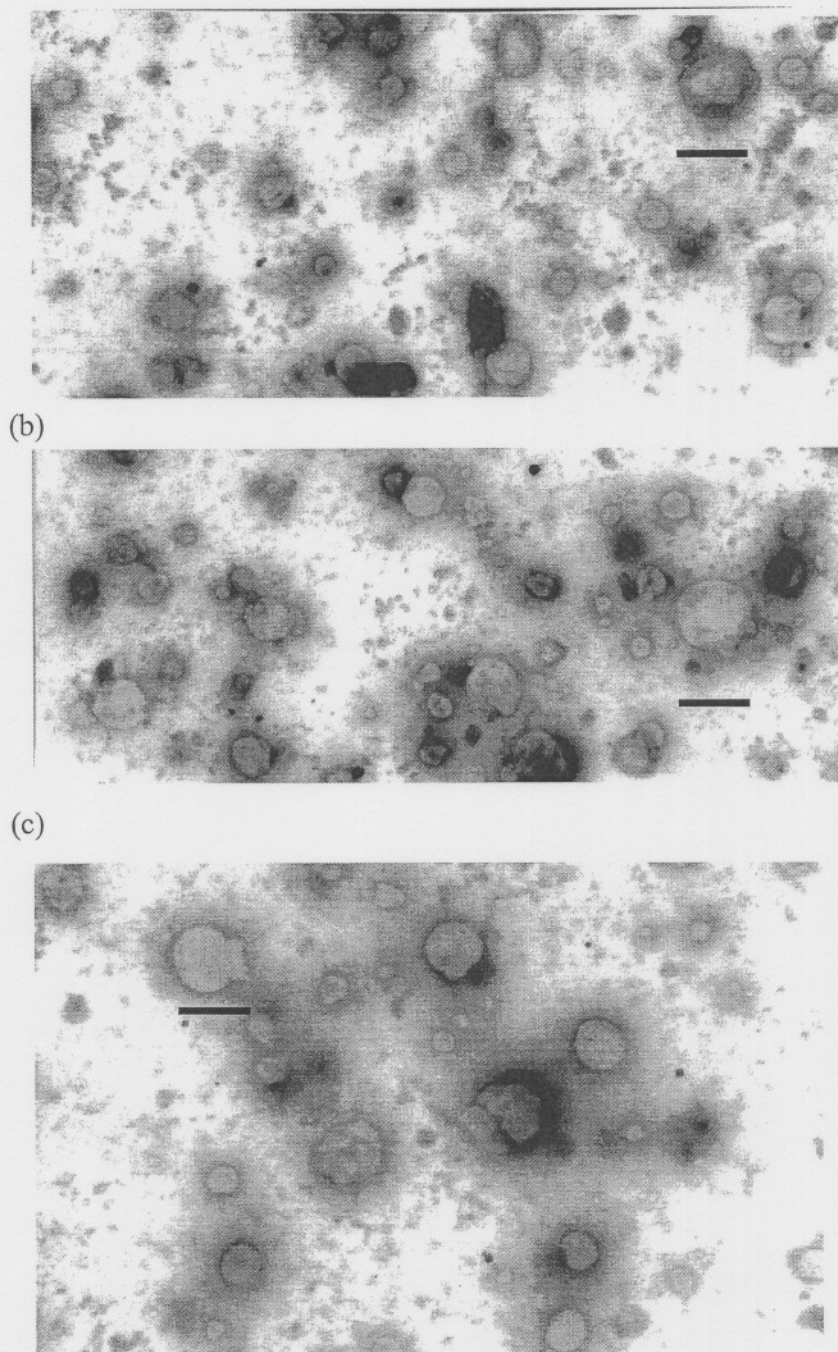


FIGURE 4-1: Transmission electron micrograph of elastic liposomes loaded with (a) methotrexate, (b) acyclovir and (c) idoxuridine

4.6 Diffusion studies

4.6.1 Skin preparation

Human epidermal membrane was used for the experiments, which were carried out under the approval of the Ethics Committee of Potchefstroom University for CHE. Female human abdominal skin tissue from cosmetic surgery was used.

Full thickness skin was thawed overnight, adipose tissue removed by blunt dissection and the skin immersed in water at 60 °C for 1 min. The epidermis was carefully peeled away from the dermis (Du Plessis *et al.*, 2002).

4.6.2 Skin permeation method

In vitro permeation studies was conducted with vertical Franz diffusion cells (Du Plessis *et al.*, 2002) with a 2.3 ml capacity receptor compartment and a 1.075 cm² diffusion area. The receptor phase was sonicated for 15 min to remove air bubbles and avoid build-up of air pockets. A loading dose of 5 µl of liposome containing 9mg/ml of methotrexate was used. Loading doses were placed in the donor compartments non-occlusively to simulate transepidermal hydration gradient (Cevc, 1998). The cells were mounted on a magnetic stirring bed in a water bath at 37 °C. 2-mm magnetic followers stirred the receptor compartments continuously at 500 rpm. At designated times up to 12 hours, the receptor phase was removed and replaced by PBS to maintain sink conditions. Samples were assayed by HPLC. A saturated solution of methotrexate (9mg/ml) in PBS served as control. The diagrammatic illustration of the Franz diffusion cell is shown in Figure 4-2.

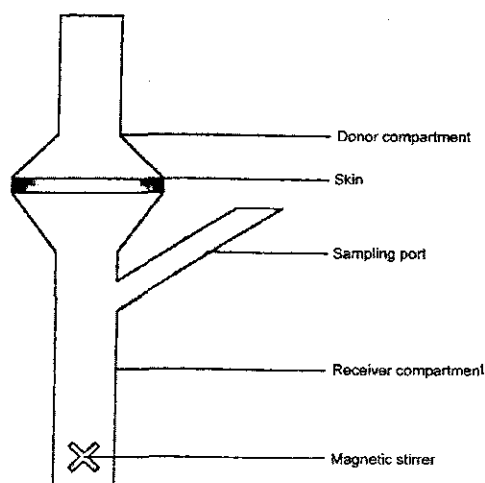


Fig 4-2: Diagrammatic illustration of the Franz diffusion cell (Bronaugh & Collier, 1993)

4.6.3 Data analysis

Diez-Sales *et al.* (1991) developed a method to analyse the diffusion equation describing the amount of drug crossing a membrane at a given time.

$$Q(t) = AKhC \left[D \frac{t}{h^2} - \frac{1}{6} - \frac{2}{\pi^2} \sum_{n=1}^{\infty} \frac{(-1)^n}{n^2} \exp\left(\frac{-Dn^2\pi^2 t}{h^2}\right) \right] \quad \text{Equation 4-1}$$

where $Q(t)$ is the quantity passed through the membrane at a given time (t), A is the actual surface diffusion area, K is the partition coefficient between the membrane and donor vehicle, h is the membrane thickness, D is the diffusion coefficient of the permeant in the membrane and C is the concentration in the donor solution. As t approaches infinity, the exponential terms become negligible and the linear steady-state expression is given by equation 4-2.

$$Q(t) = AKhC \left[D \frac{t}{h^2} - \frac{1}{6} \right] \quad \text{Equation 4-2}$$

Using the latter equation, the cumulative concentration per unit area (ng/cm^2) was plotted as a function of time (hours). Since K , D and h are unknown, the products Kh and D/h^2 can be calculated by curve-fitting the permeation data. The curve-fitting of data on Easyplot® for Windows provided α – and β – values, where:

$$\alpha = Kh$$

and

$$\beta = D / h^2$$

The product $\alpha\beta$ is equal to the permeability coefficient (k_p). The flux ($\mu\text{g} / \text{cm}^2 / \text{h}$) for methotrexate was then calculated by using Equation 4-3

$$\text{Flux } (\mu\text{g} / \text{cm}^2 / \text{h}) = k_p \times \text{saturated solubility} \quad \text{Equation 4-3}$$

Results obtained from curve-fitting of methotrexate, acyclovir and idoxuridine permeation data for the α - and β -values, permeability coefficient and transdermal flux are presented in tables 4-13, 4-14 and 4-15 respectively.

4.6.4 Statistical analysis

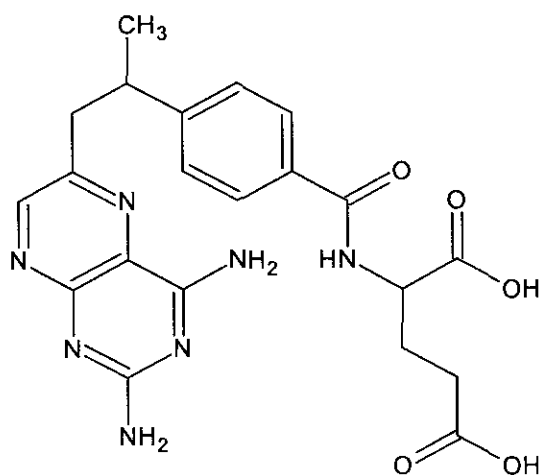
Six Franz diffusion cells were used for each drug in the diffusion studies and the mean steady-state flux was calculated. The determination of statistically significant differences between the liposomal formulations and saturated aqueous drug solutions was done using a Student's t-test software (http://www.physics.csbsju.edu/stats/t-test_bulk_form.html).

4.7 Physicochemical properties of selected drugs

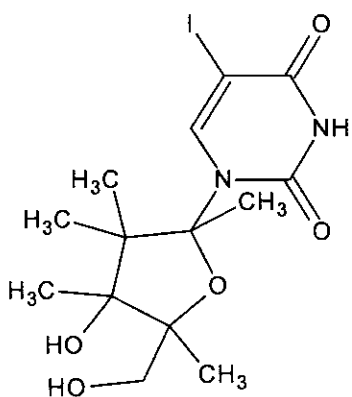
Three hydrophilic drugs were chosen for this study: methotrexate, idoxuridine and acyclovir. Since the physicochemical properties of a drug have significant effect on their skin delivery, these properties are listed in Table 4-9. The chemical structures of these drugs are shown in Figure 4-3.

Properties	Methotrexate	Idoxuridine	Acyclovir
Chemical name	4-Amino-10-deoxy 10-methylpteroyl-L- glutamic acid ¹	2'-Deoxy- 5-ioduridine ¹	9-[(2- Hydroxy ethoxy) methyl] guanine ¹
Structural formula	C ₂₀ H ₂₂ N ₈ O ₅ ¹	C ₉ H ₁₁ N ₂ O ₅ ¹	C ₈ H ₁₁ N ₅ O ₃ ¹
Molecular weight	454.5 ¹	354.1 ¹	225 ¹
Melting point	182-189°C ²	168-171 °C ³	255°C ⁴
Dissociation constant (pKa)	4.8, 5.5 ¹	8.25 ¹	2.19, 9.35 ⁴
Partition coefficient (logP)	-1.2 ⁵	-0.95 ³	-1.57 ⁴
Solubility in PBS.	9.02mg/ml ⁶	2.06mg/ml ⁶	2.51mg/ml ⁶
¹ Dollery, C. 1999. Therapeutic drugs. Edinburg: Churchill Livingstone.			
² Lund, 1994			
³ Gosh & Mitra, 1991			
⁴ Kristl, 1999			
⁵ Kristl & Vesnaver, 1995			
⁶ Experimentally determined			

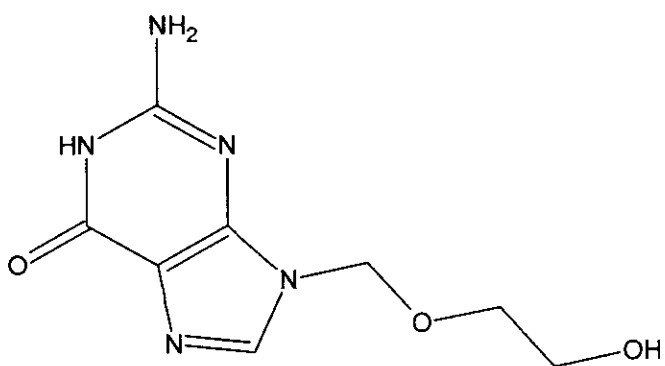
TABLE 4-9: Selected hydrophilic drugs and their various physicochemical properties.



Methotrexate



Idoxuridine



Acyclovir

Figure 4-3: Chemical structures of methotrexate, idoxuridine and acyclovir.

The influence of aqueous solubility, ionisation and molecular weight on the skin permeabilities of methotrexate, idoxuridine and acyclovir was previously discussed in Chapter 3.

4.7.1 Melting point

There is an inverse relationship between melting points and aqueous solubilities of drugs (Kristl *et al.*, 1999). Permeant melting point was also found to be inversely proportional to lipophilicity and therefore transdermal flux. Attempts were made to find a correlation between flux and the reciprocal of the melting point. The ideal solubility properties increase exponentially with decreasing melting point for any given molecular mass. It is assumed that there should be an exponential increase in transdermal flux with a decreasing melting point (Guy & Hadgraft, 1989). Based on the melting points of methotrexate, acyclovir and idoxuridine (Table 4-9), it is expected that permeability should increase in the order acyclovir > idoxuridine > methotrexate. In other words, the least transdermal flux is expected from methotrexate.

4.7.2 Partition coefficient

The stratum corneum is lipophilic, with the intercellular lipid lamellae forming a conduit through which drugs must diffuse in order to reach the underlying vascular area and to ultimately access systemic circulation. For this reason, lipophilic molecules are better accepted by the stratum corneum. A drug must possess sufficient lipophilicity in order to transfer into the stratum corneum. Methotrexate, acyclovir and idoxuridine have very low partition coefficient values as shown in Table 4-9. Experimental partition coefficient values obtained from literature are compared with those predicted using three software packages- EPIWIN™, CHEMSKETCH/ACD™ and IALOGP™. The results are shown in Table 4-10.

TABLE 4-10: logP values for methotrexate, idoxuridine and acyclovir

Permeant	Literature	IALOGP™	EPIWIN™	CHEMSKETCH/ACD™
Methotrexate	-1.2	0.91	1.59	0.43
Idoxuridine	-0.95	0.44	2.56	1.51
Acyclovir	-1.57	-0.89	-1.00	-2.12

There is a certain degree of agreement between acyclovir partition coefficient value obtained experimentally (literature) and those predicted by the three software packages. However experimental logP values for methotrexate and idoxuridine do not correlate with values predicted by IALOGP™, EPIWIN™ and CHEMSKETCH/ACD™ packages. The reason for the discrepancies may be the inability of the predictive algorithms to include the effects of molecular conformation, proximity and hydrogen bonds into the calculation procedure (Kristl *et al.*, 1999).

The Potts-Guy Equation has been previously used to predict permeability coefficients ($\log k_p$) for the three drugs from their molecular weights and partition coefficients. The results were given in Table 3-3. Based on the results of computation, methotrexate is expected to have the least permeability coefficient compared to acyclovir and idoxuridine (See § 3-8). Pugh *et al.* (2000) used principal components analysis to examine the relationship between permeability coefficient and partition coefficient. As expected, there was a direct relationship between $\log k_p$ and $\log K_{oct}$.

4.8 Results

4.8.1 Skin delivery of methotrexate, idoxuridine and acyclovir

The aqueous solubilities (mg/ml) $\pm$ standard deviation (n=6) of methotrexate, acyclovir and idoxuridine in phosphate buffered saline (pH 7.4) were determined at 25 °C and are listed in Table 4-11.

TABLE 4-11: Solubility data of methotrexate, acyclovir and idoxuridine in phosphate buffered saline (pH 7.4).

Drug	Solubility in PBS (mg/ml) at 25°C
Methotrexate	9.0216 ± 0.05
Acyclovir	2.5134 ± 0.03
Idoxuridine	2.0638 ± 0.01

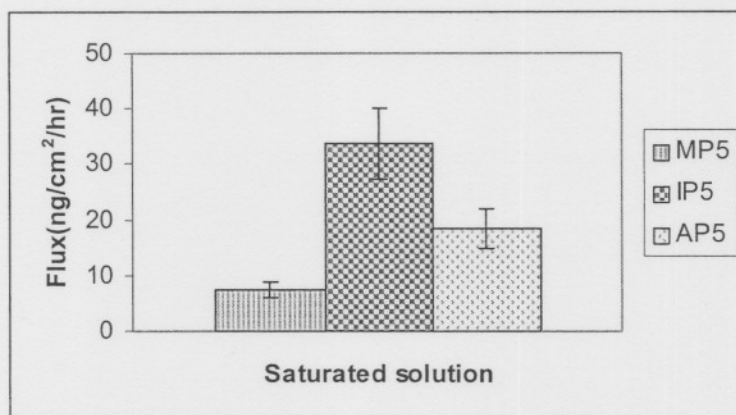


FIGURE 4-4: Flux from the saturated solutions of methotrexate, idoxuridine and acyclovir (MP5 = methotrexate; IP5 = idoxuridine and AP5 = acyclovir).

Figure 4-4 shows the flux from saturated solutions of methotrexate, idoxuridine and acyclovir. It shows that transdermal delivery increased in the order of idoxuridine > acyclovir > methotrexate.

4.8.2 Skin delivery of methotrexate, acyclovir and idoxuridine from elastic liposomes

4.8.2.1 Effect of the type of phospholipid

The mean particle size of the elastic liposomes as determined by transmission electron microscopy was 120 ± 8 nm. Mean steady-state flux data of methotrexate, acyclovir and idoxuridine prepared from sodium cholate and different phospholipids are listed in

Tables 4-4, 4-5 and 4-6 respectively. The steady-state flux was determined from the slope of the linear portion of the cumulative amount versus time plot.

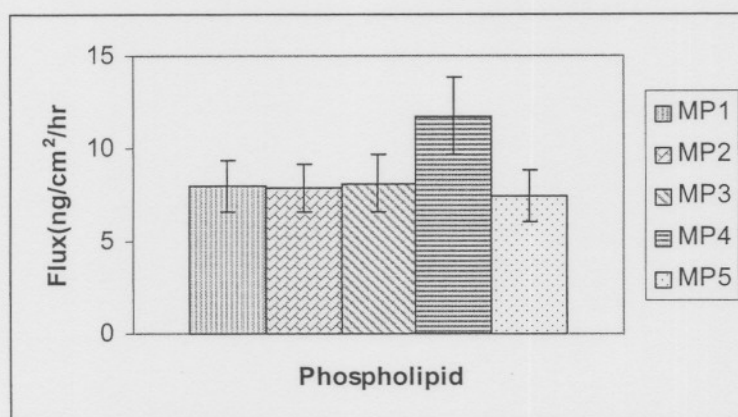


FIGURE 4-5: Effect of the type of phospholipid on the flux of methotrexate (MP1= phosphatidylcholine 78.6%; MP2 = phosphatidylcholine 50%; MP3 = phosphatidylcholine hydrogenated 90%; MP4 = phosphatidylcholine 95%; MP5 = saturated solution).

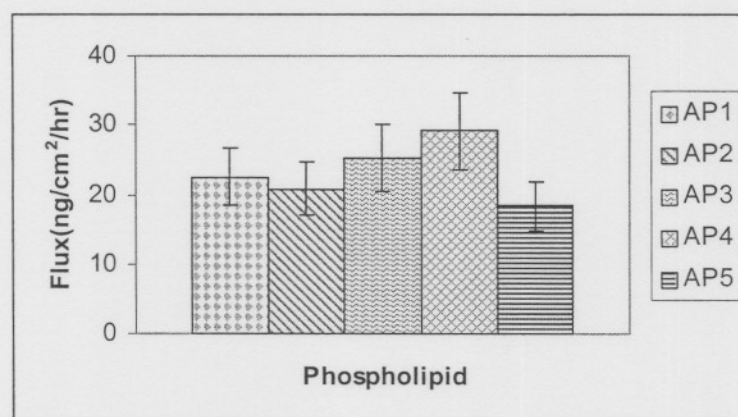


FIGURE 4-6: Effect of the type of phospholipid on the flux of acyclovir (AP1= phosphatidylcholine 78.6%; AP2 = phosphatidylcholine 50%; AP3 = phosphatidylcholine hydrogenated 90%; AP4 = phosphatidylcholine 95%; AP5 = saturated solution).

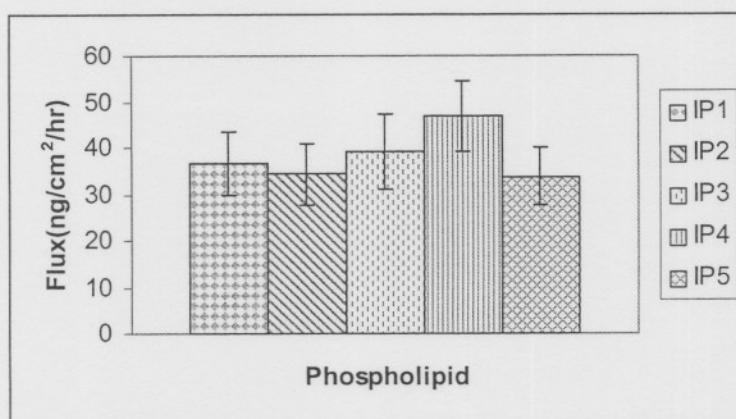


FIGURE 4-7: Effect of the type of phospholipid on the flux of idoxuridine (IP1= phosphatidylcholine 78.6%; IP2 = phosphatidylcholine 50%; IP3 = phosphatidylcholine hydrogenated 90%; IP4 = phosphatidylcholine 95%; IP5 = saturated solution).

4.8.2.2 The effect of surfactants on skin delivery of methotrexate, idoxuridine and acyclovir

Phospholipon 90G was chosen for further investigation with different surfactants. Bar plots of the mean steady-state flux and standard deviations (SD) of methotrexate, acyclovir and idoxuridine from elastic liposomes containing Phospholipon G and different surfactants are shown in Figures 4-8, 4-9 and 4-10 respectively. Cumulative amount versus time plots are shown in Figures 4-11 to 4-19.

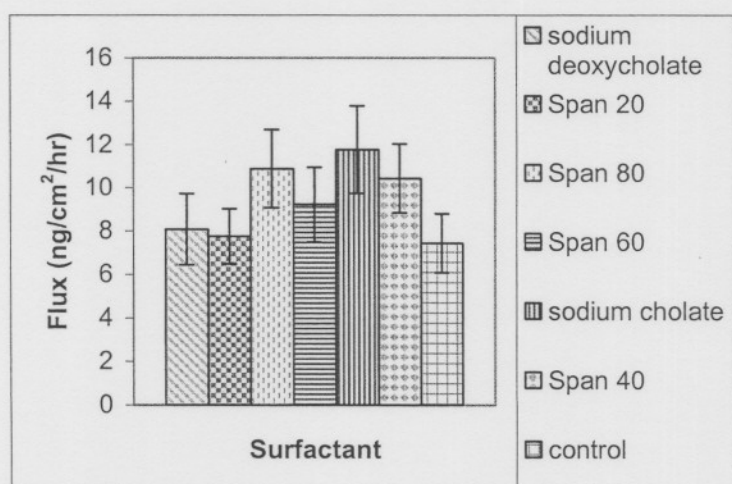


FIGURE 4-8: Effect of surfactants on permeation of methotrexate from elastic liposomes across human epidermal membrane.

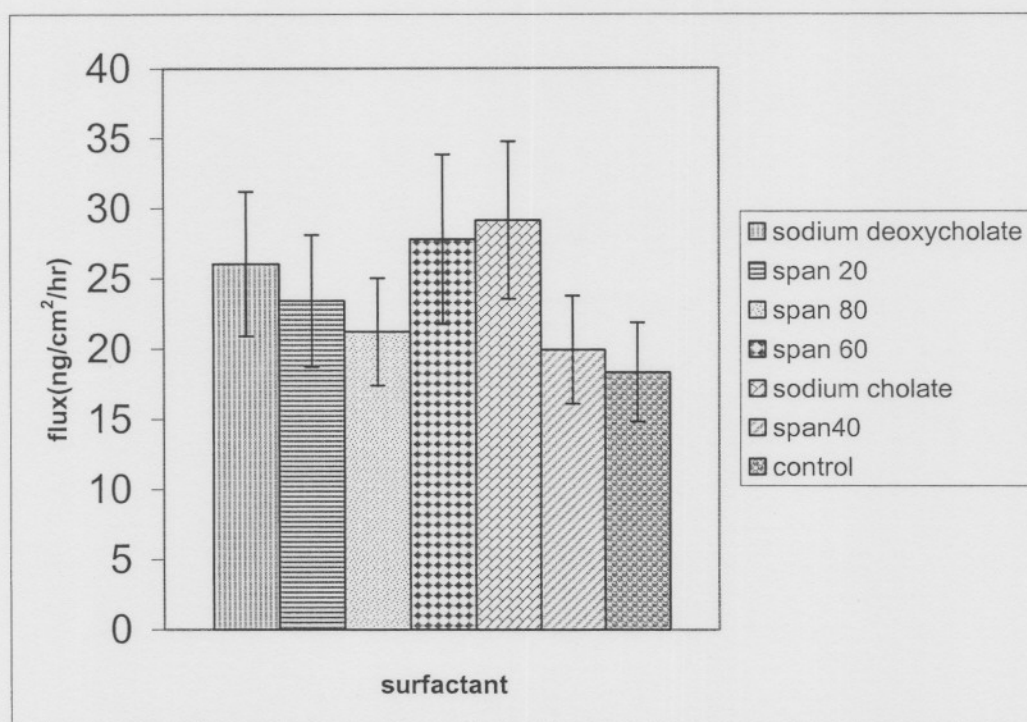


FIGURE 4-9: Effect of surfactants on permeation of acyclovir from elastic liposomes across human epidermal membrane.

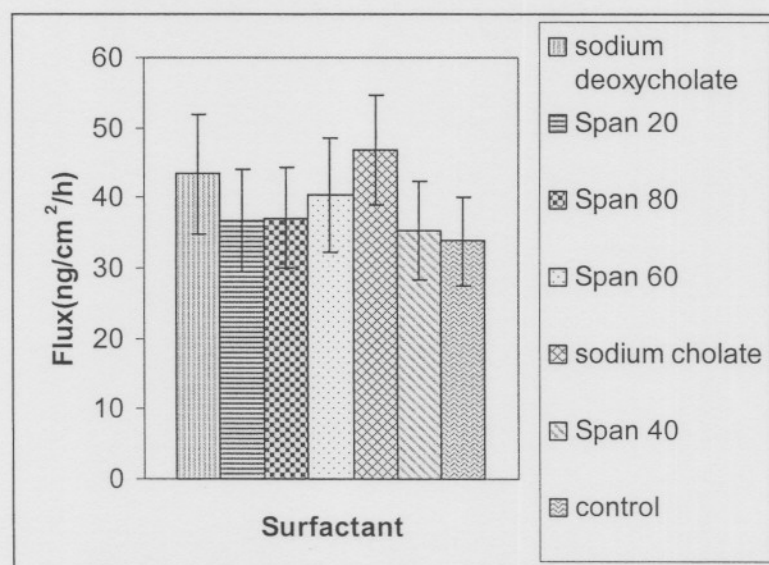


FIGURE 4-10: Effect of surfactant on permeation of idoxuridine from elastic liposomes across human epidermal membrane.

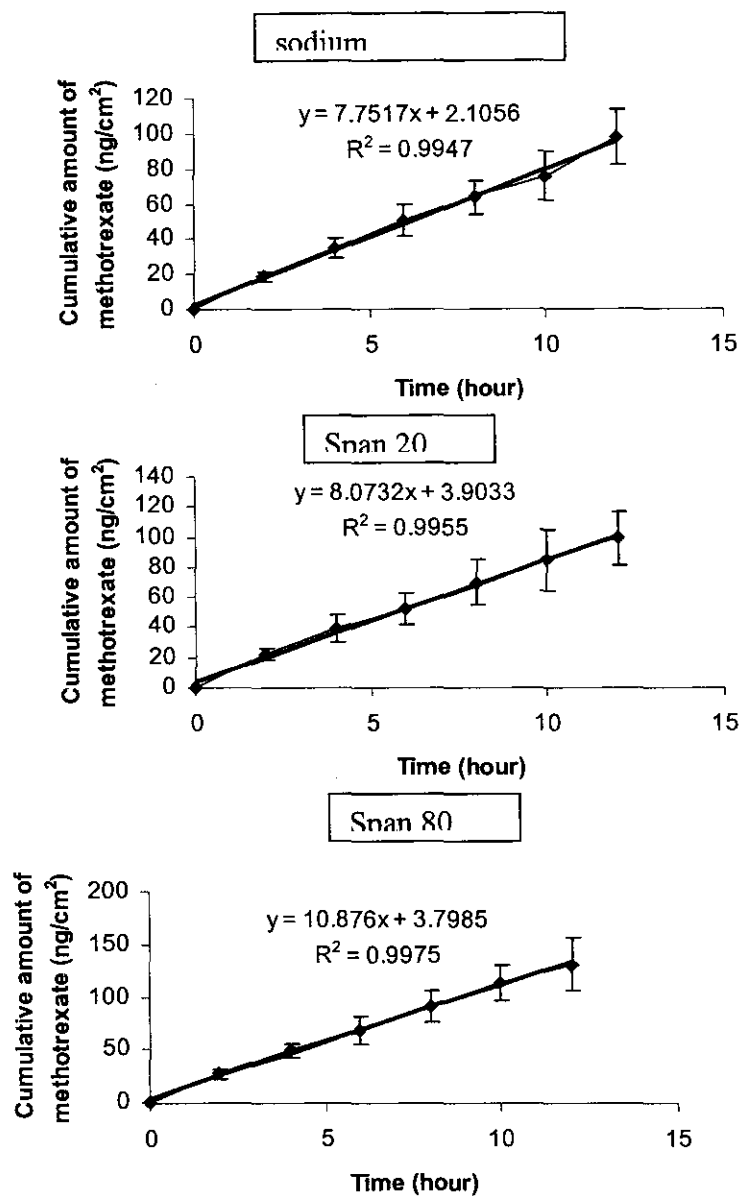


FIGURE 4-11: Skin permeation profile of methotrexate from elastic liposomes containing 95% phosphatidylcholine and different surfactants (sodium deoxycholate, Span 20 and Span 80)

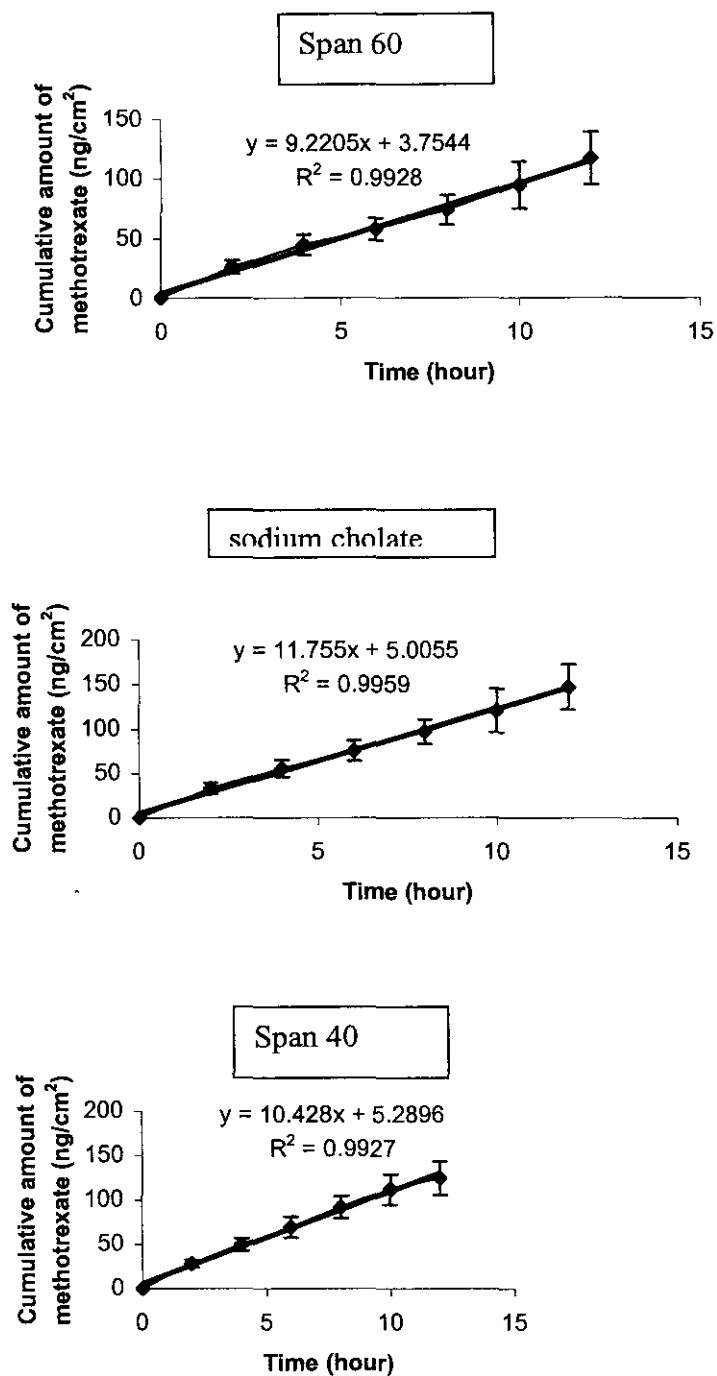


FIGURE 4-12: Skin permeation profile of methotrexate from elastic liposomes containing 95% phosphatidylcholine and different surfactants (Span 60, sodium cholate and Span 40).

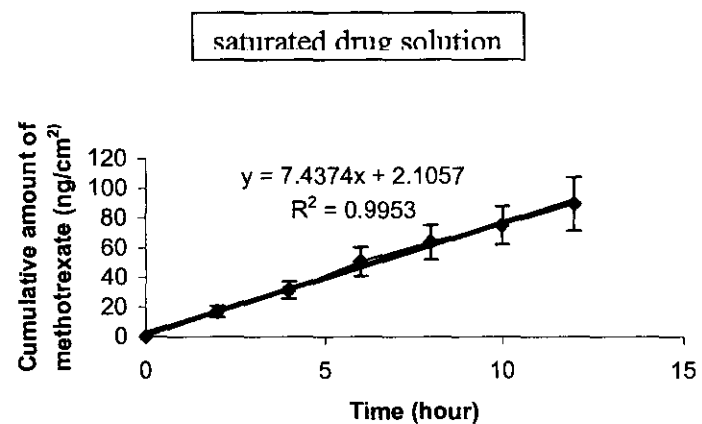


FIGURE 4-12: Skin permeation profile of methotrexate from saturated drug solution in phosphate buffered saline.

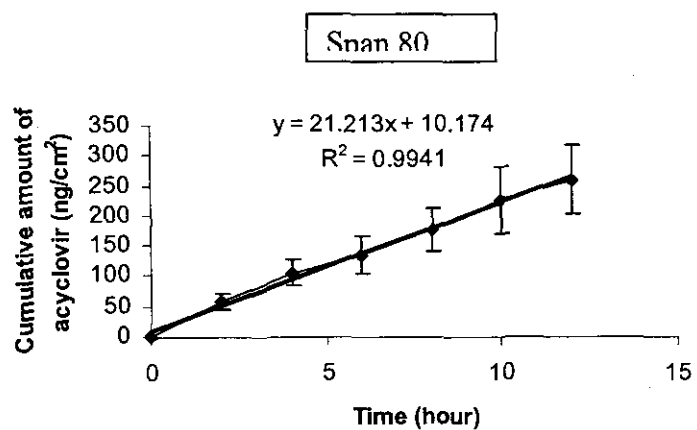
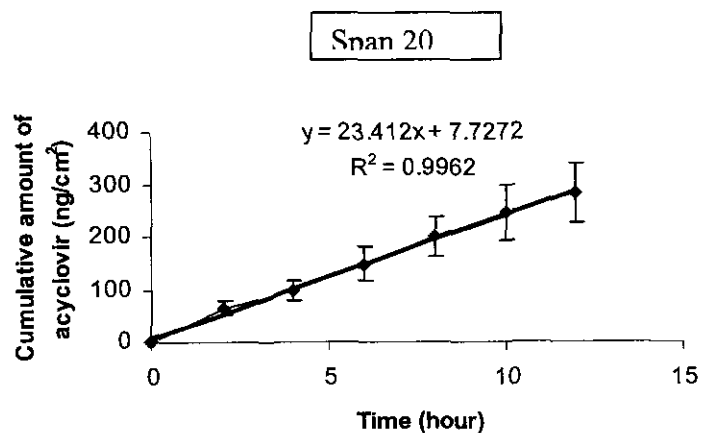
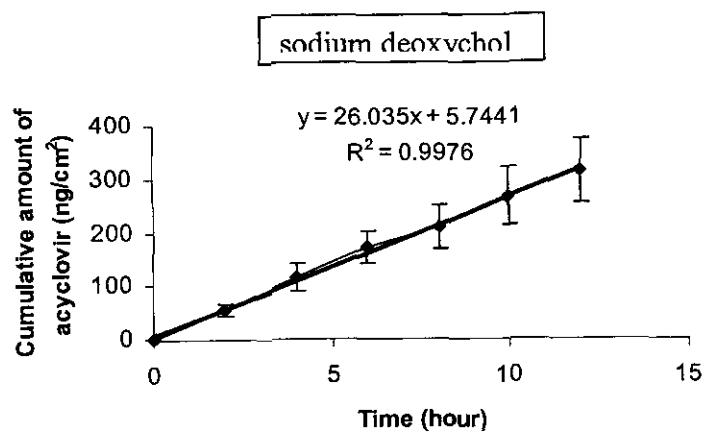


FIGURE 4-14: Skin permeation profile of acyclovir from elastic liposomes containing 95% phosphatidylcholine and different surfactants (sodium deoxycholate, Span 20 and Span 80).

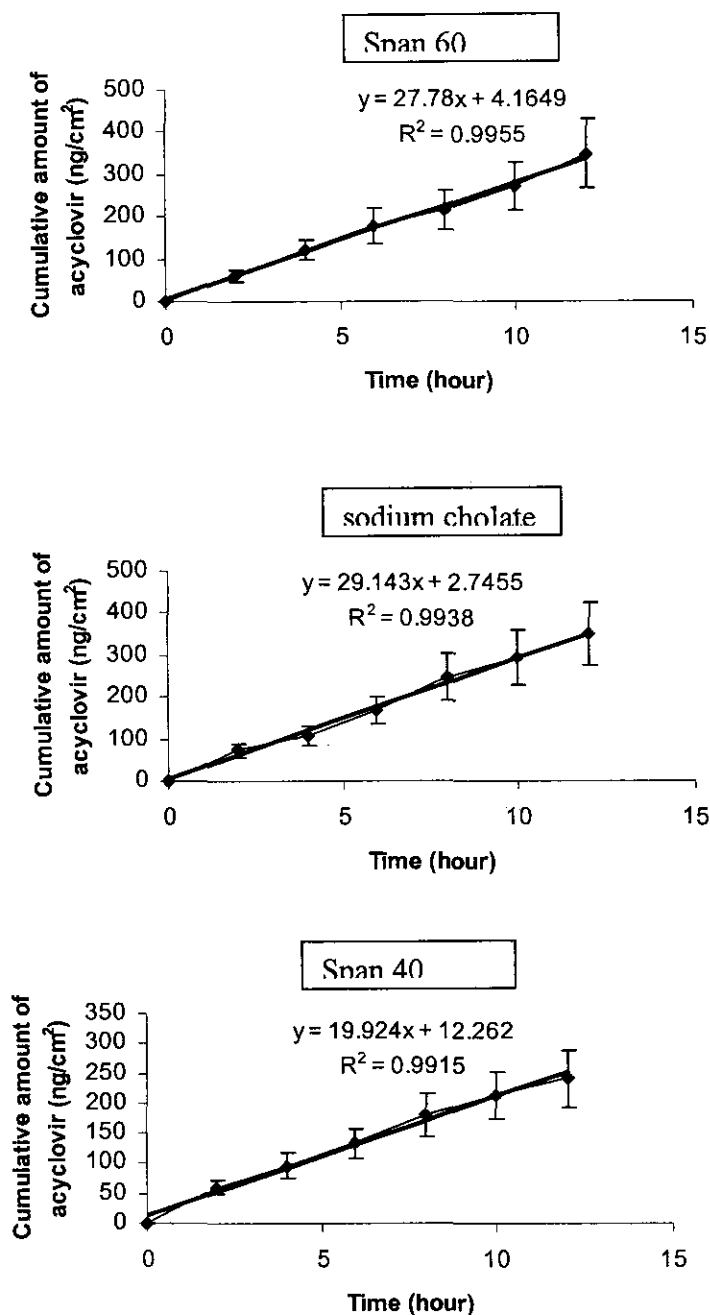


FIGURE 4-15: Skin permeation profile of acyclovir from elastic liposomes containing 95% phosphatidylcholine and different surfactants (Span 60, sodium cholate and Span 40).

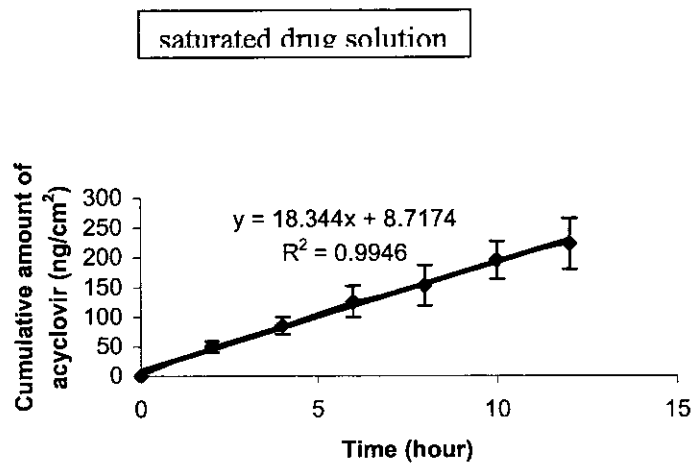


FIGURE 4-16: Skin permeation profile of acyclovir from saturated drug solution in phosphate buffered saline.

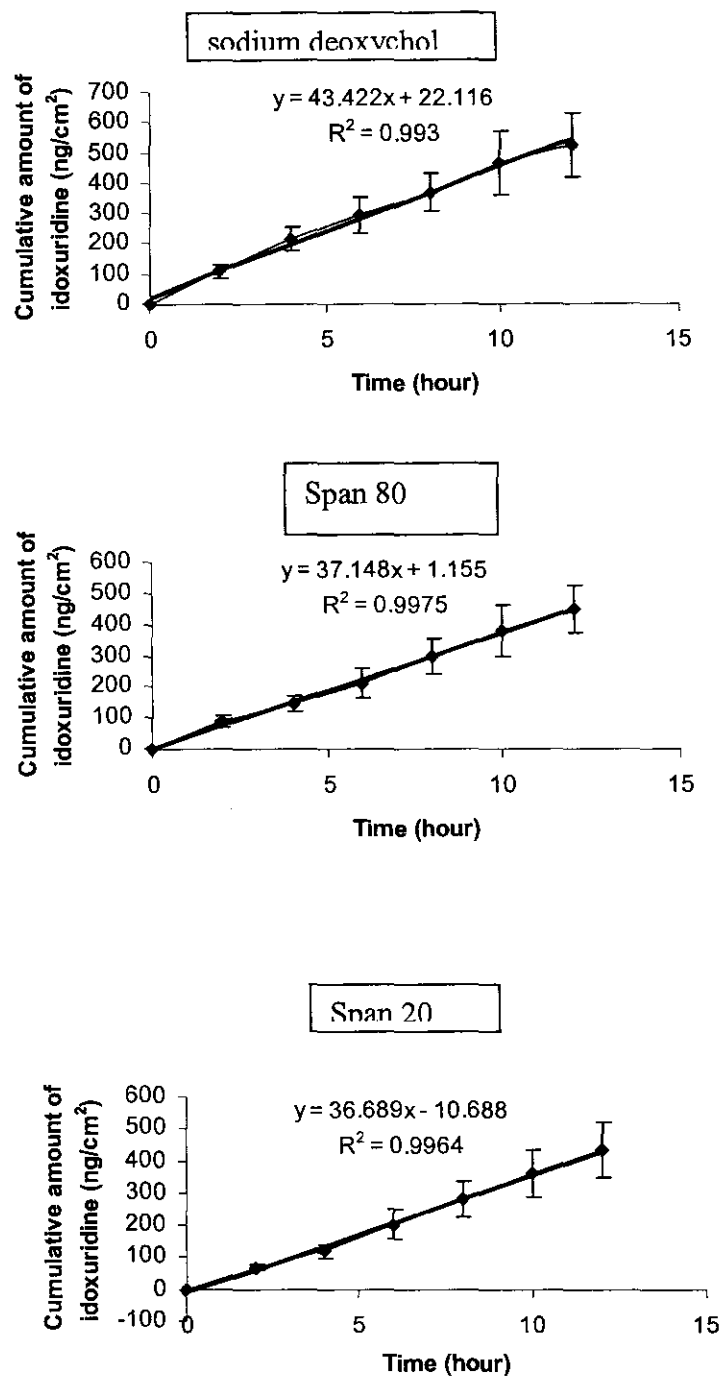


FIGURE 4-17: Skin permeation profile of idoxuridine from elastic liposomes containing 95% phosphatidylcholine and different surfactants (sodium deoxycholate, Span 80 and Span 20).

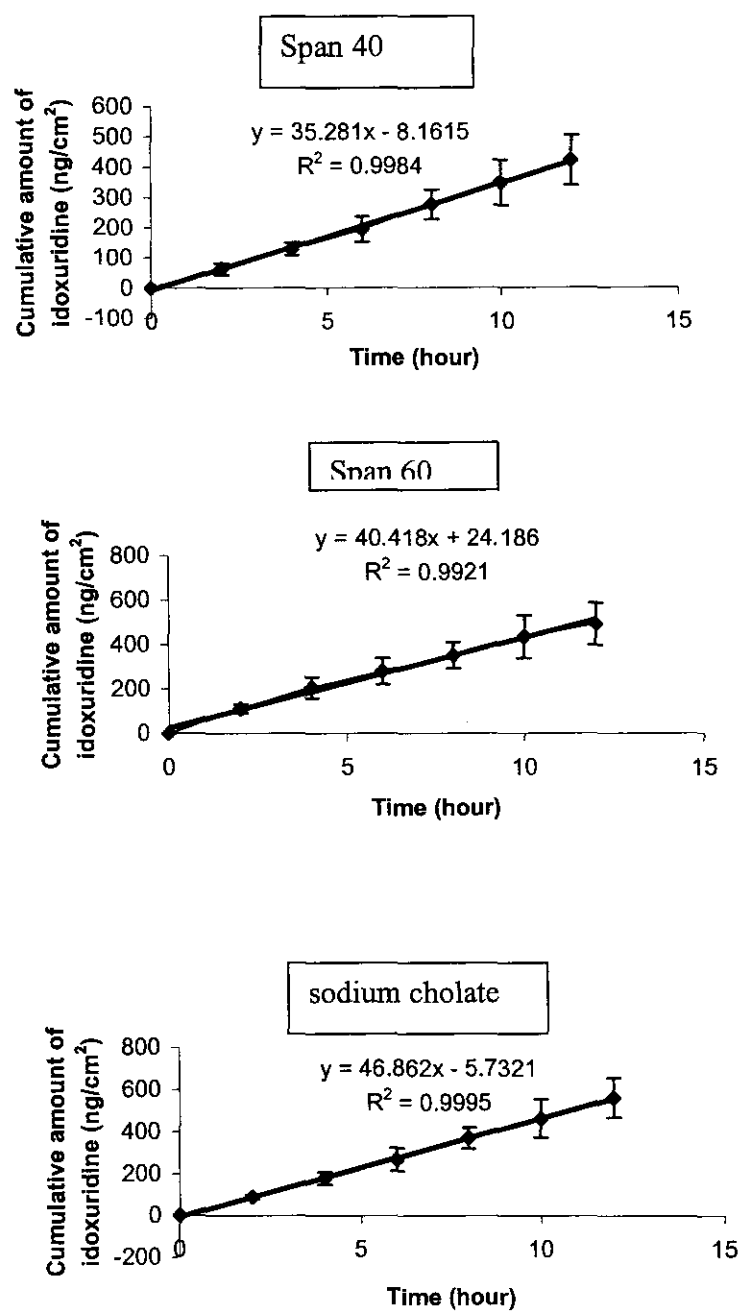


FIGURE 4-18: Skin permeation profile of idoxuridine from elastic liposomes containing 95% phosphatidylcholine and different surfactants (Span 40, Span 60, and sodium cholate).

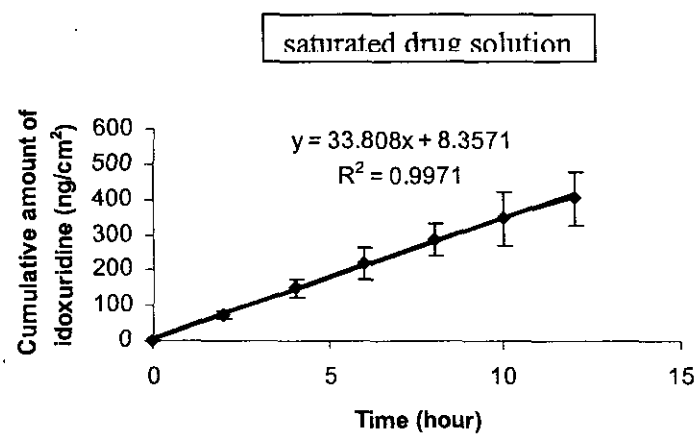


FIGURE 4-19: Skin permeation profile of idoxuridine from saturated drug solution in phosphate buffered saline.

TABLE 4-12: Methotrexate permeation parameters obtained from curve-fitting on Easyplot for Windows®

Surfactant	α	β	kp (cm^2/h)	J ($ng/cm^2/h$)
Sodium deoxycholate	$10^{-5} \times 1.67$	568×10^{-4}	9.48×10^{-7}	8.53
Span 20	$10^{-5} \times 2.20$	405×10^{-4}	8.91×10^{-7}	8.01
Span 80	$10^{-5} \times 2.80$	450×10^{-4}	12.6×10^{-7}	11.34
Span 60	$10^{-5} \times 2.28$	471×10^{-4}	10.7×10^{-7}	9.66
sodium cholate	$10^{-5} \times 2.72$	504×10^{-4}	13.7×10^{-7}	12.33
Span 40	$10^{-5} \times 1.93$	636×10^{-4}	12.2×10^{-7}	11.04
control	$10^{-5} \times 2.35$	363×10^{-4}	8.52×10^{-7}	7.67

TABLE 4-13: Acyclovir permeation parameters obtained from curve-fitting on Easyplot for Windows®

Surfactant	α	β	kp (cm^2/h)	J ($ng/cm^2/h$)
Sodium deoxycholate	$10^{-5} \times 1.83$	5.97×10^{-3}	10.92×10^{-6}	27.31
Span 20	$10^{-5} \times 1.99$	489×10^{-3}	9.73×10^{-6}	24.32
Span 80	$10^{-5} \times 1.19$	757×10^{-3}	9.00×10^{-6}	22.52
Span 60	$10^{-5} \times 1.41$	847×10^{-3}	11.94×10^{-6}	29.85
sodium cholate	$10^{-5} \times 5.83$	202×10^{-3}	11.77×10^{-6}	29.44
Span 40	$10^{-5} \times 1.07$	863×10^{-3}	9.23×10^{-6}	23.08
control	$10^{-5} \times 9.22$	942×10^{-3}	8.68×10^{-6}	21.71

TABLE 4-14: Idoxuridine permeation parameters obtained from curve-fitting on Easyplot for Windows®

Surfactant	α	β	kp (cm^2 / h)	J ($ng / cm^2 / h$)
Sodium deoxycholate	$10^{-5} \times 3.28$	701×10^{-3}	22.99×10^{-6}	45.98
Span 20	$10^{-5} \times 2.79$	696×10^{-3}	19.41×10^{-6}	38.13
Span 80	$10^{-5} \times 17.20$	108×10^{-3}	18.57×10^{-6}	37.15
Span 60	$10^{-5} \times 2.46$	880×10^{-3}	21.64×10^{-6}	43.29
sodium cholate	$10^{-5} \times 5.10$	475×10^{-3}	24.22×10^{-6}	48.45
Span 40	$10^{-5} \times 3.17$	585×10^{-3}	18.54×10^{-6}	37.08
control	$10^{-5} \times 4.24$	410×10^{-3}	17.38×10^{-6}	34.76

TABLE 4-15: Lag times calculated from equation 4-4 for methotrexate

Surfactant	β	Lag time (L_t) hour
Sodium deoxycholate	568×10^{-4}	2.93
Span 20	405×10^{-4}	4.11
Span 80	450×10^{-4}	3.70
Span 60	471×10^{-4}	3.53
Sodium cholate	504×10^{-4}	3.30
Span 40	636×10^{-4}	2.62
control	363×10^{-4}	4.60

TABLE 4-16: Lag times calculated from equation 4-4 for acyclovir

Surfactant	β	Lag time (L_t) hour
sodium deoxycholate	597×10^{-3}	2.79×10^{-1}
Span 20	489×10^{-3}	2.40×10^{-1}
Span 80	757×10^{-3}	2.20×10^{-1}
Span 60	847×10^{-3}	1.96×10^{-1}
sodium cholate	202×10^{-3}	8.25×10^{-1}
Span 40	863×10^{-3}	1.93×10^{-1}
control	942×10^{-3}	1.76×10^{-1}

TABLE 4-17: Lag times calculated from equation 4-4 for idoxuridine

Surfactant	β	Lag time (L_t) hour
Sodium deoxycholate	701×10^{-3}	2.37×10^{-1}
Span 20	696×10^{-3}	2.40×10^{-1}
Span 80	108×10^{-3}	1.54
Span 60	880×10^{-3}	1.90×10^{-1}
sodium cholate	475×10^{-3}	3.50×10^{-1}
Span 40	585×10^{-3}	2.80×10^{-1}
control	410×10^{-3}	4.06×10^{-1}

4.9 DISCUSSION

4.9.1 Skin delivery of methotrexate, idoxuridine and acyclovir

The partition coefficients of methotrexate, acyclovir and idoxuridine are shown in Table 4-10 while the solubilities of the drugs in phosphate buffered saline are shown in Table 4-11. The permeation of these drugs across the human stratum corneum is low. The solubility characteristics of a substance affect its ability to penetrate biological membranes. This is demonstrated by the development of topical corticosteroid preparations. Of 23 esters of betamethasone examined, bethamethasone 17-valerate possesses the highest topical activity and this coincided with the most balanced lipid-water solubility coefficient. This activity became less, as the derivatives became more lipid and less water soluble. Compounds that were more soluble in water and less soluble in lipids were similarly less active after topical application (Idson, 1975).

Goosen *et al.* (2002) also investigated thalidomide and its N-alkyl analogues. The authors concluded that not only does lipophilicity play an important role in skin permeability, but other factors such as aqueous solubility should also be taken into consideration. However, studies done on the percutaneous delivery of a lipooxygenase inhibitor, utilizing several vehicles in which these drugs have widely different solubilities showed no definite trend between drug uptake and drug solubility (Shah & Maibach, 1993).

A drug must possess both lipoidal and aqueous solubilities: if it is too hydrophilic, the molecule will be unable to transfer into the SC; if it is too lipophilic, the drug will tend to remain in the SC layers. This limitation imposes the criterion of adequate aqueous solubility ($> 1\text{mg/ml}$) for a successful transdermal drug candidate (Naik *et al.*, 2000).

Experimentally determined solubilities of methotrexate, acyclovir and idoxuridine in phosphate buffered saline were 9.02, 2.51 and 2.06 mg/ml (Table 4-11). The results are consistent with values reported in the literature (Shao *et al.*, 1994; Gosh & Mitra, 1991; Alvarez-Figuerova *et al.*, 2001). Aqueous solubility could be correlated with the high degree of ionisation (section 3-8). A comparison of experimentally determined values with those predicted by commercial software packages indicate that there is some

agreement between predicted solubility value for idoxuridine (ACD™) and our experimental value (See Table 3-1). The solubility value predicted by IALOGP™ for acyclovir is also comparable. However, there is significant variation between predicted and experimental values in all other cases. This could be as a result of inability of the software packages to incorporate the influence of molecular areas and connectivity into the calculation procedure.

4.9.2 Skin delivery of methotrexate, idoxuridine and acyclovir from elastic liposomes

The purpose of our study was to investigate the *in vitro* permeation of methotrexate, acyclovir and idoxuridine across human epidermal membrane from elastic liposomes. The intent was to establish whether formulation of these hydrophilic drugs into elastic liposomes would enhance their skin permeation parameters.

Compounds that are absorbed through the skin *in vivo* are mainly taken up and cleared systemically by blood vessels directly beneath the epidermis. Thus, compounds do not have to penetrate the full thickness of the skin (epidermis and dermis) before entering the vasculature system. Accordingly, for *in vitro* permeation studies, the epidermis (including the stratum corneum) is usually separated from the underlying dermis using a heat separation technique. This technique cannot confidently be applied to hairy skin, because hair shafts are anchored firmly in and remain in the dermis, creating holes in the epidermal membrane as the dermis is pulled away. To obviate all concern here, only skin from female cadavers has been used in this study (Goosen, 1998). Harrison *et al.* (1984) examined the integrity of the stratum corneum barrier following storage after various time periods. No differences were found by the investigators between measurements of *in vitro* percutaneous penetration of tritiated water in skin stored at –20°C for up to 466 days versus fresh skin stored at 10°C and used within 2-3 days after autopsy, indicating maintenance of barrier properties under these conditions.

Several investigators have reported enhanced skin permeation of drugs from elastic liposomes (Cevc *et al.*, 1998; El Maghraby *et al.*, 1999; Essa *et al.*, 2003). Cevc and co-workers described the skin delivery of insulin from ultraflexible vesicles. They postulated that these carriers could transport pharmacological agents, including large

polypeptides, through permeability barriers, such as intact skin. They concluded that carrier-mediated transcutaneous insulin delivery could achieve a bio-efficiency of 50% of the subcutaneous dose action (Cevc *et al.*, 1998).

El Maghraby *et al.* compared skin delivery of a lipophilic drug, oestradiol from deformable and traditional liposomes. Lipid vesicles improved skin delivery of oestradiol compared with delivery from an aqueous control. Maximum flux ($J_{\max}$) was increased 14- to 17-fold by the use of ultradeformable vesicles and 8.2- to 9.8-fold by the use of traditional liposomes. The results showed no evidence of a free drug mechanism, but revealed a possible penetration-enhancing effect for pure phosphatidylcholine vesicles, although this was not the only mechanism operating. Few reports have dealt with the delivery of low molecular weight hydrophilic drugs from these vesicles (El Maghraby *et al.*, 2000).

El Maghraby *et al.* (2000) investigated the skin delivery of 5-fluorouracil from sodium-cholate containing ultradeformable liposomes. They used an aqueous receptor and determined the cumulative amount of drug penetrating through the skin. The results showed little effect of the vesicles on the penetration of the drug compared with a saturated aqueous solution. The amount of 5-fluorouracil, which penetrated through the human epidermis after 12 hours after its open application ($5\mu\text{l}$) as ultradeformable vesicles and saturated aqueous solution (control) using an aqueous receptor after 12 hours were $0.790\mu\text{g cm}^{-2}$ and $0.733\mu\text{g cm}^{-2}$ respectively (El Maghraby *et al.*, 2001).

Bar plots of skin delivery of methotrexate, acyclovir and idoxuridine from elastic liposomes are shown in Figures 4-8, 4-9 and 4-10. The flux values of methotrexate, idoxuridine and acyclovir across human epidermal membrane from saturated aqueous solutions were 7.43 ± 1.35 , 18.34 ± 3.52 and $33.80 \pm 6.35 \text{ ng cm h}^{-1}$ respectively. Skin permeation of these drugs from elastic liposomes of different compositions did not differ significantly from these values. Statistical analyses of the results were done with the Student's t-test ($p > 0.05$). The results did not indicate any statistically significant flux enhancement. Our results are distinct from the findings of Cevc *et al.* (1998) who investigated the *in vivo* skin delivery of insulin from ultraflexible liposomes. Insulin from these vesicles was reported to lower blood glucose concentration by 20-30% within 2-4 hours. A mixture of insulin with phospholipid/bile salt micelles or a simple

liposome/insulin blend did not change glucose concentration in the serum significantly over the same time period.

4.9.2.1 The effect of type of phospholipid

The flux (J) of methotrexate, acyclovir and idoxuridine elastic liposomes prepared from sodium cholate and different phospholipids are shown in Figures 4-5, 4-6 and 4-7 respectively. The rank order of the liposomes from the highest value of J to the lowest was: MP4>MP3>MP1>MP2>MP5 respectively for methotrexate. Similar ranking for acyclovir yielded AP4>AP3>AP1>AP2>AP5. For idoxuridine the corresponding values were IP4>IP3>IP1>IP2>IP5. From the results, it is evident that flux is higher from vesicles with higher phospholipid content. The highest flux was consistently obtained from liposomes prepared from 95% phosphatidylcholine (Phospholipon G®). The trend was similar for the three permeants. The results corroborate the findings of Valenta & Janisch (2003). The authors investigated the permeation of cyproterone acetate through pig skin from different liposomal formulations. The highest cyproterone amount was released by the formulation with the highest lipid content (140mg/ml). It was suggested that the high lipid content could be responsible for increased flux. Hofland *et al.* (1995) also observed that the degree of interaction between vesicular dispersions and the skin depends on the physicochemical properties of the liposomal components. The polar moiety of the amphiphilic molecules may play an important part with respect to interaction with skin lipids. The authors investigated three types of liposomes containing various amounts of phosphatidylcholine (PC). The formulation containing the highest amount of PC (NAT 106: 85% PC) was found to have the greatest effect on the ultrastructure of the stratum corneum compared with the other two formulations (NAT89: 10% PC and NAT 50: 28% PC). It was concluded that liposomal constituents could penetrate into the stratum corneum (SC), mix with the SC lipids and induce a penetration-enhancing effect by producing ultrastructural changes in the intercellular lamellae.

Betz *et al.* (2001a) studied the penetration behaviour of heparin across human skin from liposomes prepared from Phospholipon 80®. The results showed enhanced penetration into the epidermal membrane from the liposomes while no penetration was found with a purely aqueous solution. The authors also studied the interaction of Phospholipon

80® and sphingomyelin liposomes containing heparin with the human skin using confocal laser scanning microscopy (Betz *et al.*, 2001b). The authors observed a strong and in some respects composition-dependent interaction of the phospholipids with the skin. The mechanism of stratum corneum-liposome interaction is not entirely clear although many investigations have been performed using confocal laser scanning microscopy (Cevc *et al.*, 1998), freeze-fracture electron microscopy (Hofland *et al.*, 1995) and differential scanning calorimetry (Trotta *et al.*, 2002). More studies are needed to clarify the underlying mechanisms of this interaction.

In our studies, flux of methotrexate, idoxuridine and acyclovir was consistently highest from liposomes prepared from 95% phosphatidylcholine. It was therefore chosen for further investigations with different surfactants.

4.9.2.2 The effect of surfactants

Bar plots illustrating the effect of different surfactants on permeation of methotrexate, acyclovir and idoxuridine are shown in Figures 4-8, 4-9 and 4-10 respectively. El Maghraby and coworkers have previously studied the skin delivery of a lipophilic drug from elastic liposomes containing sodium cholate, Tween 80 and Span 80. We chose sodium cholate, sodium deoxycholate and a homologous series of sorbitan monoesters for our studies.

Permeation parameters for methotrexate, acyclovir and idoxuridine obtained by Easyplot® are presented in Tables 4-10, 4-11 and 4-12 respectively. The transdermal flux values (J) obtained by curve-fitting of data using Easyplot® are comparable to those determined by linear regression. In addition, α and β values are similar suggesting that the formulations are not influencing the diffusion and partition of the drugs through the skin. We used Student's t-test to determine statistically significant differences in the flux values of the formulations. A computer program http://www.physics.csbsju.edu/stats/t-test_bulk_form.html was used for this purpose. There were no statistically significant differences between flux values from elastic liposomes and saturated aqueous solutions of the drugs.

The results are distinct from those of El Maghraby *et al.* (1999) who compared the skin delivery of a lipophilic drug, oestradiol, from deformable and conventional liposomes. The authors observed that deformable lipid vesicles, prepared from phosphatidylcholine (PC) and various surfactants, improved the skin delivery of oestradiol compared with aqueous control. The results showed no evidence of a free drug mechanism but revealed a possible penetration-enhancing effect for pure PC vesicles, although this was not the only mechanism operating. Positive drug uptake by the vesicles suggested increased partitioning into the skin.

Similar results to those of El Maghraby *et al.* (1999) were earlier reported by Cevc *et al.* (1997) who studied the skin delivery of hydrocortisone, dexamethasone and triamcinolone-acetonide from ultraflexible liposomes. These drugs were administered epicutaneously to mice at the dose of 1.5mg/kg, 1.5mg/kg and 1mg/kg respectively. The respective concentrations in the blood after eight hours were approximately 0.4 µg/ml, 0.75 µg/ml and 0.007µg/ml. The authors compared the results to those of a subcutaneous injection.

Cevc *et al.* (1995) also studied the skin delivery of a hydrophilic fluorescent marker, Rhodamine 123. This marker (381 Da) does not normally penetrate the skin in large quantities when applied in the form of a liposomal suspension. When used in the form of a transfersomal suspension, however, the dye was transported extensively and reached a depth of at least 30µm. The transdermal fluorescence distribution profile, however, was not uniform. The measured fluorescence intensity dropped relatively abruptly at a depth of approximately 30 µm. The authors ascribed this drop to the fact that the vesicles were facing nearly infinite sink below the stratum corneum, which resulted in extensive dye dilution and elimination through the lymphatic drainage system (Cevc *et al.*, 1995).

In spite of these impressive results, there is still considerable debate in the literature regarding the influence of elastic liposomes on skin delivery of drugs. Trotta *et al.* (2002) for instance could only detect negligible flux of dipotassium glycyrrhizinate from elastic liposomes prepared Phospholipon 100® (98% phosphatidylcholine). The authors concluded that elastic liposomes were not beneficial in the delivery of glycyrrhizinate through the skin.

Comparable findings have also been reported by El Maghraby *et al.* (2001) regarding the skin delivery of a hydrophilic drug, 5- fluorouracil from ultradeformable liposomes.

In accordance with our hypothesis, the results obtained in our project suggest that formulation of methotrexate, acyclovir and idoxuridine into these vesicles does not lead to dramatic flux enhancement. This may be explained by the fact that for reasonable flux enhancement to place three critical parameters must be optimised: solubility, partition and distribution coefficients. Potentially, skin permeation enhancement from these vesicles may occur through a free drug process, fusion effect as a result of interactions between liposome and skin phospholipids or a combination of both processes. We hypothesise that in the case of these three drugs, a combination of both processes seems to be the predominant mechanism. The deformability of liposome and the extent of skin perturbation as a result of this is insufficient to counteract the slow partitioning and diffusion which is evident from the low α - and β - values shown in tables 4-12, 4-13 and 4-14.

4.10 The effect of viable epidermis on drug delivery from elastic liposomes

4.10.1 Introduction

The present understanding of the human skin structure and composition supports the transdermal permeation of a chemical by partitioning into and transport through the cutaneous layers, which comprise the stratum corneum, the viable epidermis and the upper dermis. Evidence from in vitro experiments has demonstrated that the stratum corneum is the principal barrier to penetration, although other layers can provide some hindrance to the process of absorption depending on the nature of the permeating molecule (Lee *et al.*; 1997). The structure of the stratum corneum consists of several layers of keratinised corneocytes (10-15 μ m thickness) embedded in an extracellular matrix of lipids arranged in an ordered lamellar structure. The corneocytes are relatively impermeable, with the result that molecules diffusing through the stratum corneum primarily traverse a tortuous pathway through the extracellular lipid matrix. For lipophilic compounds, there is good evidence that this intercellular pathway

predominates over transcellular one. For polar or ionic compounds the route of transport is less well understood.

To examine the influence of the viable epidermis on idoxuridine flux from elastic liposomes, we compared diffusion across heat-separated epidermis and trypsin-isolated stratum corneum.

4.10.2 Method

The stratum corneum (SC) was isolated using the procedure described by Wagner *et al.*, (2001). Skin pieces were thawed, cleaned and transferred, dermal side down, into a petri dishes, which contained 0.15% trypsin solution in PBS buffer. The pieces were incubated for 24h at 32 °C. this procedure was repeated with fresh trypsin solution until the stratum corneum was fully isolated. The SC was washed three times with PBS buffer and distilled water and placed on metallic meshes, which were then placed on Franz diffusion cells for diffusion experiments as described in § 4.6.2. A saturated solution of idoxuridine served as control.

4.10.3 Results

Figure 4-20 shows the permeation of a model hydrophilic drug, idoxuridine, across human epidermal membrane and the stratum corneum.

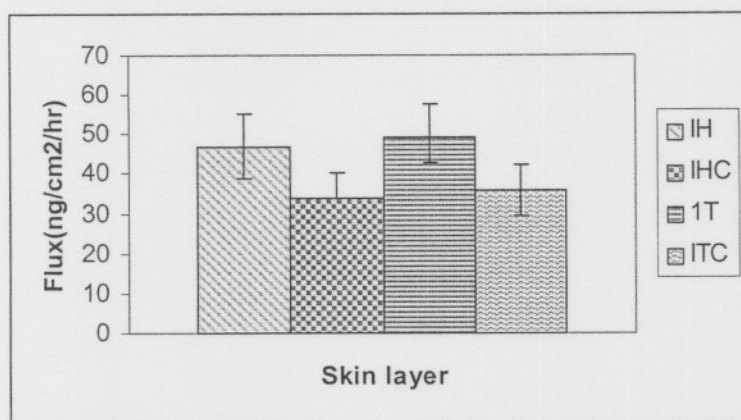


FIGURE 4-20: Permeation of idoxuridine from elastic liposomes across heat separated epidermis and trypsin-isolated stratum corneum (IH= flux of idoxuridine from elastic liposomes across heat separated epidermis; IHC= flux of saturated idoxuridine solution across heat separated epidermis; IT= flux of idoxuridine from elastic liposomes across trypsin-isolated stratum corneum; ITC = flux of saturated idoxuridine solution across trypsin isolated stratum corneum).

4.10.4 Discussion

Our results show a marginal increase in idoxuridine flux values from liposomes and control solution across the trypsin-isolated stratum corneum compared to the heat separated epidermis. But there was no statistically significant difference ($p > 0.05$) in flux (J) values between the liposomes and the control solution

Parry *et al.* (1990) studied the permeation of benzoic acid across isolated stratum corneum, stratum corneum and epidermis and split-thickness skin. The permeability coefficients for benzoic acid across stratum corneum and heat-separated epidermis were 0.031 ± 0.060 and 0.031 ± 0.089 cm/hr respectively. This was corroborated by Wagner *et al.* (2001) who studied the permeation of flufenamic acid across the epidermis and stratum corneum (SC). Steady state flux of 0.9% flufenamic acid across the epidermis and SC was 2.857 ± 0.013 and 2.9828 ± 0.649 $\mu\text{g cm}^{-1} \text{h}^{-1}$ respectively. Even though the results of several studies showing skin delivery of drugs from elastic liposomes have been published, few have studied the effect of the viable epidermis on drug permeation. El Maghraby *et al.* (2000) investigated the importance of liposome

structure on the skin delivery of oestradiol from ultradeformable vesicles. The same investigators also studied the skin delivery of oestradiol using trypsin-isolated stratum corneum (El Maghraby *et al.*, 1999). In both cases a 17-fold flux enhancement was reported.

In our study, idoxuridine flux from elastic liposomes (prepared from 95% phosphatidylcholine and sodium cholate) across heat separated epidermis was $46.86 \text{ ng/cm}^2/\text{h} \pm 8.2$ and $49.34 \text{ ng/cm}^2/\text{h} \pm 7.9$ respectively. Overall, the findings demonstrated that the stratum corneum was the rate-limiting barrier. Heat separated human epidermal membrane was used for the study.

4.11 Conclusion

The skin delivery of drugs from elastic liposomes is a subject of intense debate in the literature (Cevc *et al.*, 2002; El Maghraby *et al.*, 1999, 2001). Cevc *et al.* (1995,1997,2002) have reported flux enhancement from elastic liposomes for a wide range of drugs including corticosteroids, peptides and insulin. However the reports of El Maghraby *et al.* (1999, 2001) seem to indicate different skin permeation profiles for lipophilic and hydrophilic compounds. If ultradeformable vesicles enhance flux of oestradiol (a lipophilic drug) and fail to improve the skin delivery of 5-fluorouracil (a hydrophilic drug), it would appear that the physico-chemical properties of the permeants do play a role in the transdermal delivery of drugs from elastic liposomes. Our findings and those of El Maghraby *et al.* (2001) and Trotta *et.al* (2002) seem to support this view.

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Secondary infections and malignancies are increasingly being identified in patients suffering from the Acquired Immune Deficiency syndrome (AIDS). Some of the common skin diseases are varicella-zoster and herpes simplex infections as well as Kaposi's sarcoma. These diseases are usually treated with acyclovir, idoxuridine and methotrexate respectively. In spite of their pharmacotherapeutic utility, the clinical efficacy of these drugs is limited by their poor skin permeability.

Reports demonstrating enhanced drug permeation from elastic vesicles (Cevc & Blume, 1992; Cevc *et al.*, 1998; El Maghraby *et al.*, 1999, 2000; Guo *et al.*, 2000) have appeared in the literature. Few reports, however, have dealt with the delivery of low molecular weight hydrophilic drugs from these vesicles (El Maghraby *et al.*, 2001).

The aim of our study was to investigate *in vitro* permeation of methotrexate, acyclovir and idoxuridine across human epidermal membrane from elastic liposomes. The intent was to establish whether formulation of these hydrophilic drugs into elastic liposomes would enhance their skin permeation parameters.

The specific objectives of this study were to:

- validate high-performance liquid chromatographic techniques for quantitative analysis of methotrexate, idoxuridine and acyclovir;
- prepare and characterise elastic liposomes using various phospholipids-phosphatidylcholine 78.6%, phosphatidylcholine 50%, phosphatidylcholine 95%, hydrogenated phosphatidylcholine 90%, and surfactants -sodium cholate, sodium deoxycholate, Span 20, 40, 60, 80;
- carry out *in vitro* permeation studies through human epidermal membrane using vertical Franz diffusion cells;

- evaluate computationally predicted values of solubility and partition coefficient
- deconvolute partition and diffusion parameters from flux values;
- postulate likely mechanism of skin permeation from elastic liposomes and propose future enhancement strategies.

High- performance liquid chromatographic techniques were developed and validated for methotrexate, acyclovir and idoxuridine. Elastic liposomes containing different phospholipids and surfactants were prepared. 95% phosphatidylcholine (Phospholipon G®) was chosen for the preparation of the liposomes with different surfactants. Permeation of methotrexate, acyclovir and idoxuridine from these vesicles across human epidermal membrane was investigated.

Bar plots illustrating the effect of different surfactants on permeation of methotrexate, acyclovir and idoxuridine are shown in Figures 4-8, 4-9 and 4-10 respectively. El Maghraby and coworkers have previously studied the skin delivery of a lipophilic drug from elastic liposomes containing sodium cholate, Tween 80 and Span 80. We chose sodium cholate, sodium deoxycholate and a homologous series of sorbitan monoesters for our studies. The monoesters were chosen to investigate the possible effect of alkyl chain length on skin permeation.

Steady-state flux values for methotrexate, acyclovir and idoxuridine are presented in tables 4-10 to 4-12. The transdermal flux values (J) obtained by curve-fitting of data using Easyplot® are comparable to those obtained by linear regression. We used Student's t-test to determine statistically significant differences in the flux values of the formulations. A computer program http://www.physics.csbsju.edu/stats/t-test_bulk_form.html was used for this purpose. Our results indicate that there are no statistically significant differences between flux values from elastic liposomes and saturated aqueous solutions.

In this study:

- ❖ High-performance chromatographic techniques were developed and validated for methotrexate, acyclovir and idoxuridine.
- ❖ Elastic liposomes of different compositions were prepared and characterised

- ❖ In vitro permeation of methotrexate, acyclovir and idoxuridine from these vesicles across human epidermal membrane was investigated.
- ❖ evaluate computationally predicted values of solubility and partition coefficient
- ❖ deconvolute partition and diffusion parameters from flux values;
- ❖ postulate likely mechanism of skin permeation from elastic liposomes and propose future enhancement strategies.

Due to the hydrophilicity of these drugs their skin delivery from elastic liposomes was not significantly enhanced ($p > 0.05$).

Future recommendations

Because of the strong influence of physico-chemical parameters on transdermal delivery of methotrexate, acyclovir and idoxuridine, other means of enhancing their delivery will be explored in the future. Specifically, iontophoresis could be useful since co-application of an electric current has been known to greatly enhance transdermal permeation of compounds. This technique is equally useful since the flux can be manipulated by varying electric current. The synergy between delivery from elastic vesicles and iontophoresis will also be studied to determine the contribution of these processes to overall flux enhancement.

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