CHAPTER II

LITERATURE REVIEW

A. Wound healing

Healing of wounds is a chain of process necessary for the removal of invaded pathogen from the damage tissue of the body and for complete or partial remodeling of injured tissue. In general, wound healing proceeds in four interrelated dynamic and overlapping phases, namely, hemostasis, inflammation, proliferation and remodeling .

First, the initial vascular response involves a vasoconstriction and hemostasis. A 5-10 minute period of intense vasoconstriction is followed by active vasodilation accompanied by increase in capillary permeability. Platelets aggregated within a fibrin clot secrete a variety of growth factor and cytokines that set the stage for an orderly series of events leading to tissue repair.

Second phase, the inflammation phase presents itself as erythema, swelling, warmth, and pain. The inflammatory response increases vascular permeability, resulting in migration of neutrophils and monocytes into the surrounding tissue. The neutrophils engulf debris and microorganisms. Neutrophil migration stops after the first few days post-injury if the wound is not contaminated. If this acute inflammatory phase persists, due to wound hypoxia, infection, nutritional deficiencies, medication use, or other factors related to the patient's immune response, it can interfere with the late inflammatory phase.

In the late inflammatory phase, monocytes converted in the tissue to

macrophages, which digest and kill bacterial pathogens, scavenge tissue debris and destroy remaining neutrophils. Macrophages begin the transition from wound inflammation to wound repair by secreting a variety of chemotactic and growth factors that stimulate cell migration, proliferation, and formation of tissue matrix.

Next, the proliferation phase is dominated by the formation of granulation tissue and epithelialization. Chemotactic and growth factors released from platelets and macrophages stimulate the migration and activation of wound fibroblasts that produce a variety of substances essential to wound repair, including glycosaminoglycans (mainly hyaluronic acid, chondroitin-4-sulfate, dermatan sulfate, and heparan sulfate) and collagen. Around the third day after wounding the growing mass of fibroblast cells begins to synthesize and secrete a measurable amount of collagen. Collagen levels rise continually for approximately three weeks. The amount of collagen secreted during this period determines the tensile strength of the wound.

The final phase of wound healing is wound remodeling, including reorganization of new collagen fibers that progressively continues to increase wound tensile strength. The remodeling process continues up to two years, achieving 40-70 percent of the strength of undamaged tissue at four weeks (Nithya, Suguna and Rose,2003; Mackay and Miller, 2003).

The objective in wound management is to heal the wound in the shortest time as possible, with minimal pain, discomfort, and scarring to the patient. There are

several reports stating that the extracts of several plants used for wound healing properties. Botanical extracts from *Centella asiatica* has been used for burns, wounds, and scars by topical or internal application. Continued use of this plant can be as healing agents has led to scientific studies. One of the primary mechanism of action of Centella manifests to be the stimulation of type-I collagen production (Bonte et al., 1994). In a similar study, Shukla et al. (1999) showed that a 0.2 percent asiaticoside solution applied topically twice daily for seven days to punch wounds in guinea pigs resulted in 56 percent increase in hydroxyproline, 57 percent increase in tensile strength, increase collagen content, and better epithelialization compared to controls. Using same punch wound model the researchers demonstrated that an oral dose of 1 mg/kg for seven days produced a 28 percent reduction in wound area and significant increase of tensile strength and hydroxyproline content of the wound. Similarly, Rao Shivakumar and Parthasarathi (1996) found that topical administration of aqueous extract of *Centella asiatica* enhanced healing in open wound in rat. Neovascularization on the fourth day and the inflammatory response up to the eight day in the test wounds indicated the entire process of inflammatory and resulted in the stimulation of fibroblasts in synthesizing collagen. Qualitative difference in the collagen on the eight day indicated a qualitative difference in the fibroblast in test wounds resulting in the faster synthesis of collagen.

B. *Centella asiatica*

Centella asiatica (Linn.) Urban is a genus of the plant family Apiaceae (Umbelliferae), the synonym *Hydrocotyle asiatica* (Linn.), locally known as Bua-bok.

It is widely distributed in open or damp shaded places in Thailand. It is a slender tailing herb, rooting at the nodes. The leaves are thin and soft, with palmate nerves, hairless or with only few hairs, and measure about 2 to 5 cm in diameter. Parts used are fresh or dried leaves, above ground herb or whole herb with root (Figure 1).
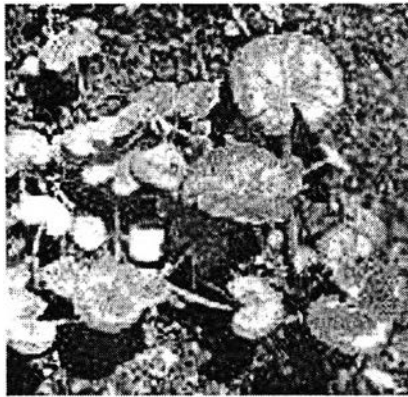

Figure 1 *Centella asiatica* (Linn.)

Centella asiatica is used in folk medicine to treat a wide range of indications such as treatment of skin diseases and wounds. It is used to support faster healing of small wounds, chaps and scratchs, surgical wounds, superficial burns and varicose ulcers. Fresh extracts of this plant have been used for many years for healing of wounds. In India and Madagascar, this plant was used to treat leprosy, lupus, syphilis, tuberculosis and improving mental function, while the Chinese prescribed the leaves in curing leukorrhea and toxic fever. In Malaysia, although this herb is commonly eaten fresh as a vegetable, it is also said to have beneficial effects in improving memory and in treating mental fatigue, anxiety, and eczema (Hamid et al., 2002; Leung and Foster, 2003).

Centella asiatica contains a wide range of other substances. The substances of therapeutic interest are the saponin-containing triterpene acids and their sugar esters, the most important being asiatic acid, madecassic acid, asiaticoside and madecassoside (Figure 2) (Loiseau and Mercier, 2000). These are pentacyclic triterpenes, found to display chronic venous insufficiency, varicose vein and wound healing properties (Inamdar et al., 1996; Shim et al., 1996).

Rosen et al. (1967) cited in Shukla et al. (1999) have revealed wound healing activity of the plant. In primary screening an ethanolic extract of the plant showed significant wound healing activity. Repeated chromatography of the saponin mixture led to the isolation of two pure saponins identified as asiaticoside and madecassoside. Asiaticoside showed a promising wound healing activity whereas madecassoside was found to be inactive. In addition, a comparison of asiaticoside and asiatic acid showed that the sugar moiety of the molecule did not seem to be necessary for this biological activity (Bonte et al., 1993). While The European Agency for the Evaluation of Medicinal Products Veterinary Medicines Evaluation unit (1998) reported that madecassic acid, asiaticoside, and asiatic acid act on fibroblast cells and equilibrate collagen fiber synthesis. The overall effect contributes to the restoration of elastic connective tissue, a reduction in fibrosis and a short in the time necessary for wound healing.

The Centella preparations used in conventional medicine are employed in oral form (tablets and drops), topical preparations (ointments and powder) and in the form of injections (s.c., i.m. and i.v.) (Brinkhaus et al., 2000). During recent years there
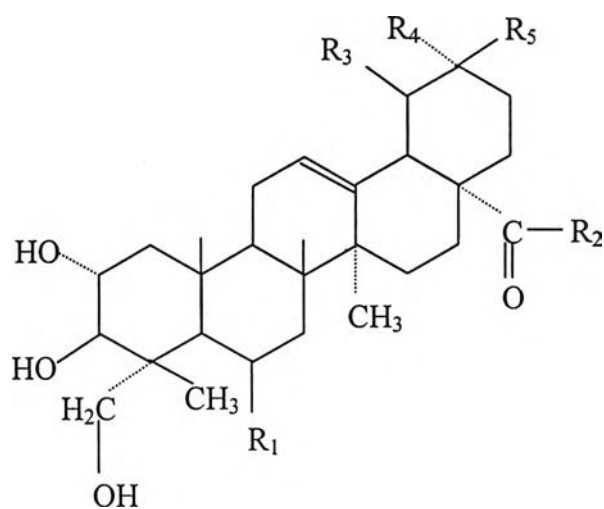
Compounds	Structure	Molecular formula	M.W.	m.p.(C°)
Asiatic acid	R_1, R_2, R_5 = H; R_3, R_4 = CH_3	$C_{30}H_{48}O_5$	488	300-306
Asiaticoside	R_1, R_5 = H; R_2 = glu-glu-rham; R_3, R_4 = CH_3	$C_{48}H_{78}O_{19}$	958	230-233
Madecassic acid	R_1 = OH; R_2, R_5 = H; R_3, R_4 = CH_3	$C_{30}H_{48}O_6$	504	262-268
Madecassoside	R_1 = OH; R_2 = glu-glu-rham; R_3,R_4 = CH_3; R_5= H	$C_{48}H_{78}O_{20}$	974	213-217

Figure 2   Structure of asiaticoside, madecassoside, asiatic acid and madecassic acid

(Brinkhaus et al., 2000).

has been interest in the development of new effective vehicle systems to modify drug penetration into human skin. Microemulsions have been included in the investigated spectrum of potential dermal therapeutics in order to obtain enhanced penetration.

C. Microemulsions

1. Terminology and Definitions

The term microemulsion was first introduced by Schulman and co-workers in 1943 to describe the clear, fluid systems obtained when normal emulsions were titrated to clarity with medium-chain length alcohol such as pentanol or hexanol. Microemulsions are isotropic, transparent or slightly opalescent, low-viscosity, and thermodynamically stable systems usually consisting of water, oil, surfactant, and cosurfactant (Alany et al., 2001). The diameter of the disperse phase of microemulsions is in the range of 10 to 100 nm. These systems show structural similarity to micelles and inverse micelles. Microemulsions have been shown to exert a high capacity for incorporating lipophilic or hydrophilic substances (Bronaugh and Maibach, 1999; Swarbrick and Boylan, 1994).

The term microemulsion implies a close relationship to ordinary emulsions. There are a number of factors for distinguish microemulsions from emulsions:

a) Microemulsion are thermodynamically stable systems, and

display stability in the absence of chemical degradation of any of its components (Kale and Allen, 1989). Emulsions, on the other hand, are merely kinetically stabilized but thermodynamically unstable, which means that emulsions will eventually phase separate between oil and water (Trotta, 1999).

b) Due to thermodynamically stable so method of preparation, microemulsions can be form with a little energy input. Emulsions, on the other hand require a large input of energy (Nielloud and Mestres, 2000).

c) Emulsions consist of relatively large droplets (typically 100 nm -10 μm in drug delivery), whereas microemulsions may consist of very small droplets (typically 10-100 nm). As a result of this, microemulsions are transparent, whereas emulsions are milky in their appearance (Kale, 1989).

d) Due to the larger droplets in emulsion systems, the surface area is generally smaller in emulsions than in microemulsions, and consequently, less surfactant is generally needed to generate an emulsion than a microemulsion system.

2. Structure of Microemulsions

The simplest representation of the structure of microemulsions is the droplet model in which microemulsion droplets are surrounded by an interfacial film consisting of both surfactant and cosurfactant molecules. The orientation of the amphiphiles at the interface will differ in o/w and w/o microemulsions. The hydrophobic portions of these molecules will reside in dispersed oil droplets of o/w

systems, with the hydrophilic groups protruding in the continuous phase, while the opposite situation will be true of w/o microemulsions. In general, w/o systems can be prepared at higher disperse phase concentrations than o/w systems. The oil droplets in o/w microemulsions are surrounded by the electric double layers, which can extend into the external phase for a considerably distance (up to 100 nm), depending on the electrolyte concentration as illustrated in Figure 3(a). Thus the hard sphere volume of droplets in generally considerably greater than that of the oil-core volume, which creates a strong osmotic (repulsion) force at relatively low disperse phase concentrations. In contrast, w/o systems are stabilized primarily by steric interactions between the absorbed films in such a way that the hard-sphere volume of the droplets is only slightly greater than that of the water pools as given in Figure 3 (b) (Swarbrick and Boylan, 1994).
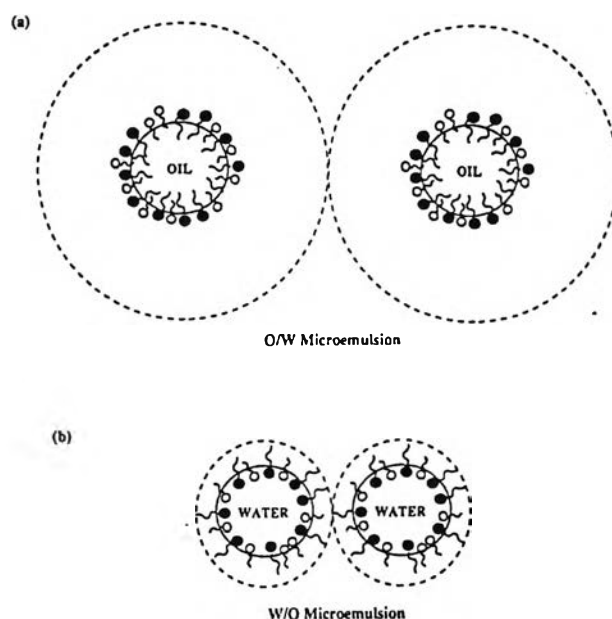

Figure 3 Diagrams illustrating the hard-sphere volume of (a) oil-in-water and (b) water-in-oil microemulsion droplets (Swarbrick and Boylan, 1994).

The HLB also determines the type of microemulsions o/w, w/o, or bicontinuous, through its influence on molecular packing and film curvature. The analysis of film curvature for surfactant associations leading to microemulsion formation has been explained in terms of the packing ratio, P, as shown in equation (1).

$$P = \frac{V}{a_o l_c} \tag{1}$$

Where V is the partial molar volume of the surfactant, a_o the cross-sectional area of the surfactant head group, and l_c the maximum length of the surfactant chain.

The o/w structures are flavored if the effective polar part is more bulky than hydrophobic part (P < 1), and the interface curves spontaneously toward water. The w/o structures are formed when the interface curved in the opposite direction (P>1). At zero curvature, when the HLB is balanced (P=1), either bicontinuous or lamellar structure may form according to the rigidity of the film. The relationship between surfactant HLB, molecular packing, and film curvature is illustrated in Figure 4 (Kreuter, 1994; Swarbrick and Boylan, 1994).

3. Microemulsion Formation and Phase Behavior

The formation of microemulsions has to be estimated using phase diagrams. A regular tetrahedron composed of four equilateral triangles can be used to plot the composition of four component systems comprising oil, water, surfactant and cosurfactant with the pure components represented by each corner of the tetrahedron and the edges presenting binary mixtures (Figure 5). Quaternary diagrams are time-
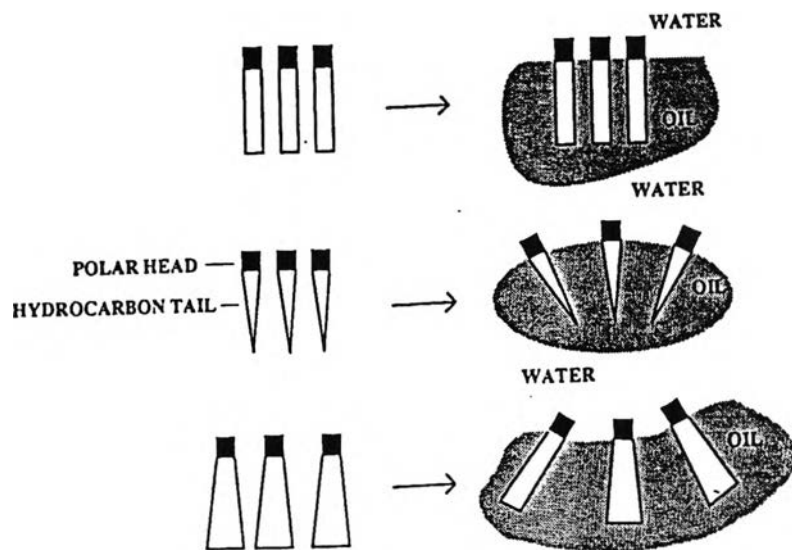
Figure 4 A steric model correlating the shape of the amphiphile to the spontaneous curvature of the interface (Swarbrick and Boylan, 1994).

consuming to prepare and often difficult to interpret. In practice it is therefore more usual to investigate planar sections of the tetrahedron by plotting a two dimentional triangular diagram (pseudoternary phase diagram), by either keeping the composition of one component fixed and varying the other three, or by using a constant ratio of two components, generally the surfactant and the cosurfactant (Swarbrick and Boylan, 1994; Aboofazeli et al, 1995).

The role of the surfactants in system is thus to reduce the interfacial tension between oil and water to this low level. For many nonionic surfactant systems, a sufficiently low surface tension may be obtained without any further additions. However, the possible exception to double alkyl chain surfactants and a few nonionic surfactants, it is generally not possible to achieve the required isotropic area with the
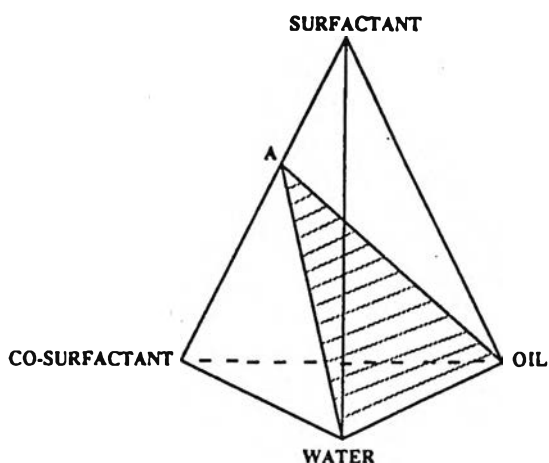
Figure 5 Multidimentional representation of the phase behavior of four-component

system; the hatched area represents a fixed surfactant/cosurfactant ratio

(Swarbrick and Boylan, 1994).

use of single surfactant.   A second amphiphile is added to the system, referred to as

the cosurfactant (Kreuter, 1994). The cosurfactant is an amphiphilic with an affinity

for both the oil and aqueous phases and partitions to an appreciable extent into the

surfactant interfacial monolayer present at the oil-water interface. A wide variety of

molecules can function as cosurfactants including non-ionic surfactants, alcohols,

alkanoic acids, alkanediols and alkyl amines (Kustrin and Alany, 2001).

Since microemulsions are thermodynamically stable, they can be prepared

simply by blending oil, water, surfactant, and cosurfactant with mild agitation. The

order of mixing the components is generally considered not to be critical.   In

pharmaceutical preparations, to constructing phase diagrams, a large number of

samples of different compositions must be prepared and time consumption. Several procedures can be used for the preparation of microemulsions. In this case, the procedure most often employed is to prepare a series an initial coarse macroemulsion by adding the oil-surfactant mixture to some of the aqueous phase in a temperature-controlled container with agitation. The system is then titrated to clarity with the cosurfactant, and further diluted with water to give a microemulsion of the desired concentration (Gi et al, 1992; Thevenin, Grossiord and Poelman, 1996; Huibers and Shah, 1997). However, time constraints impose a physical limit on the length of time systems can be lift to equilibrate and consequently the elimination of metastable can be difficult to ensure in practice, although centrifugation can be useful to speed up any separation.

Microemulsions can also exist in equilibrium with excess water, excess oil, or both. These equilibrium systems are referred to as Type I, Type II, or Type III systems after their original description by Winsor. The Winsor type I system consists of a lower phase o/w microemulsion coexisting with excess oil, and the type II system consists of an upper phase w/o microemulsion in equilibrium with excess water. Both equilibria phases are driven by bending stress of interfacial film. The type III system forms when the surfactants are concentrated in a surfactant rich bicontinuous middle phase which coexists with both oil and water (Figure 6).

4. Theory of Microemulsion Formation

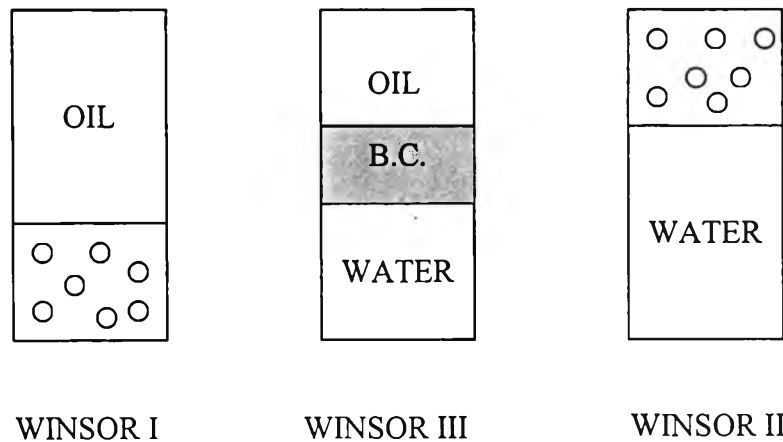Many approaches have been applied to explore the mechanisms of

Figure 6 Winsor I, II, and III systems. Type I, oil-in-water microemulsion in

equilibrium with excess oil; Type II, water-in-oil microemulsion in

equilibrium with excess water; Type III, bicontinuous structures in

equilibrium with excess oil and water (Swarbrick and Boylan, 1994).

Microemulsion formation and stability (Swarbrick and Boylan, 1994). The

spontaneous formation of microemulsion droplets was considered to be due to the

formation of a complex film at the oil-water interface by the surfactant and

cosurfactant. This caused a reduction in the oil-water interfacial tension to very low

values (from close to zero to negative). The mixed interfacial film in equilibrium with

both oil and water was considered to be liquid and duplex in nature with a two

dimentional spreading pressure, π_i, which determined the interfacial tension γ_i by

Equation. (2)

$$\gamma_i = \gamma_{o/w} - \pi_i \qquad (2)$$

where $\gamma_{o/w}$ represents the oil/water interfacial tension without the film present (Figure 7). When large amounts of surfactant and cosurfactant are adsorbed to form the interface, the spreading pressure, π_i may become larger than $\gamma_{o/w}$. A negative interfacial tension results, and energy is available to increase the interfacial area, effectively reducing droplet sizes. The interfacial film must be curved to form small droplets.
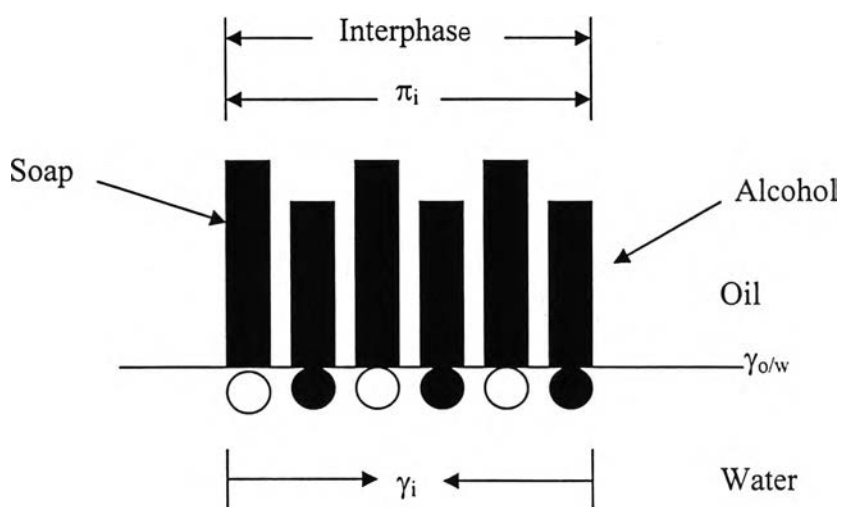
Figure 7 The two dimentional spreading pressure and interfacial tension in film soap and alcohol at oil/water interface (Krisri Umprayn, 1990).

For microemulsions to form spontaneously, the free energy involved as shown in equation 3 must be negative.

$$\Delta G_f = \gamma \Delta A - T \Delta S \qquad (3)$$

where ΔG_f is the free energy of formation, γ is the surface tension of the oil-water interface, ΔA is the change in interfacial area on microemulsification, ΔS is the change in entropy of the system which is effectively the dispersion entropy, and T is the temperature (Kreuter, 1994; Lawrence and Rees, 2000). When a microemulsion is formed the change in ΔA is very large due to the large number of very small droplets formed and the dispersion of the droplets in the continuous phase increases the entropy of the system. Thus, a negative free energy of formation is achieved when large reductions in surface tension are accompanied by significant favourable entropic change. The negative free energy changes due to the adsorption of the surfactant and cosurfactant at the oil/water interface plus the entropy of the dispersion of the droplets in the continuous phase overcome the positive product of the small interfacial tension and the large interfacial area. In such cases, microemulsification is spontaneous and the resulting dispersion is thermodynamically stable (Bronaugh and Maibach, 1999).

5. Characterization of microemulsions

Various methods used for the characterization of microemulsions are briefly described.

Microscopy : Polarization microscopy can be used to detect isotropy (ME) or anisotropy (mesogeneous phases). Electron microscopy combined with a freeze fracture technique or negative staining is applied to determine the structure and the size of colloidal phases (Alany et al, 2001.)

Light-scattering experiments: Static laser light scattering and small angle neutron scattering are used in order to measure the diameter of the pseudomicellar phase (Kale and Allen, 1989).

Spectroscopic methods: Nuclear magnetic resonance (NMR) spectroscopy is capable to characterizing the colloidal phase of microemulsion. NMR self-diffusion allows straightforward determination of whether the microemulsion consists of oil droplets in an aqueous continuum, of water droplets in an oil continuum, or bicontinuous structure. Moreover, from the oil and water self-diffusion in w/o and o/w systems, respectively, the droplet size (distribution) may be determined. Also, the self-diffusion of all components may be determined simultaneously, which is useful for understanding, e.g., the partitioning of the drug between the oil and the water phase, and hence for understanding drug release properties. However, the qualitative identification of w/o microemulsions can be done straightforwardly with as simple a method as conductivity measurements. Thus, for an o/w microemulsion, the conductivity is high, and comparable to that of the aqueous solution, whereas w/o microemulsions display a low conductivity comparable to that of an oil (Constantinides and Scalart, 1997; Baroli et al., 2000).

Other methods: The classical rheological methods are applied to determine the viscosity of microemulsion systems (Bronaugh and Maibach, 1999; Moreno, Ballesteros and Frutos, 2003).

6. Microemulsions for Topical Drug delivery

The penetration of drugs from microemulsions into human skin has been the objective of numerous studies. The drug dissolved rather than suspended in the vehicle is in a form for immediate absorption, and is generally more rapidly and more effectively absorbed. This has implications also for the release of drugs incorporated in microemulsion systems. Specifically, for o/w microemulsions, the diffusion and hence the release of hydrophobic drugs is slow, whereas that of water-soluble drugs is high. For w/o microemulsions, on the other hand, the situation is opposite (Kreilgaard, 2002).

The human skin is vital and complex tissue, which simplistically can be seen as consisting of several layered. It is genarally subdivided into three layers: the hypodermis, the dermis and the epidermis. The epidermis can be subdivided into the strarum corneum and the remainder of the epidermis, called viable epidermis (Walters, 2002).

The hypodermis or subcutaneous fatty tissue merges with the overlying dermis. It supports the dermis and epidermis and serves as a fat storage area. This layer helps to regulate temperature, provides nutritional support and mechanic protection. It carries the principal blood vessels and nerves to the skin and may contain sensory pressure organs

The dermis is composed of a matrix of connective tissue, which contains blood vessels, lymph vessels and nerves. The blood supply keeps the dermal concentration of a permeated drug very low, and the resulting concentration

difference across the epidermis provides the essential driving force for transdermal permeation.

The viable epidermis is situated beneath the stratum corneum. It consists of various layers, characterized by differect stages of differentiation. Going inwards, the permeant cross the stratum lucidum, the stratum granulosum, the stratum spinosum and the stratum basale or basal layer. In the basal layer, mitosis of cells constantly renews the epidermis and this proliferation compensates the loss of dead horny cells from the skin surface. As the cells produced by the basal layer move outward, they alter morphologically and histochemically, undergoing keratinization to form the outermost layer the stratum corneum (Barry, 1983).

The outermost layer of the skin, the stratum corneum consists of keratin-rich dead cells embeded in a lipid matrix. It is flexible but relatively impermeable. The most important function of the stratum corneum is to act as a barrier and to limit transdermal transport, with the overall aim to protect the body from chemical and biological attack to prevent dehydration. The lipids, which only constitute about 10% of the stratum corneum, seem to be particularly important for its function. There are arranged partly in layered structures, and it has therefore been inferred that it is the structure of the lipid self-assembly which governs the barrier properties. Consequently, surfactant-based formulation, offer a way to modify this protective barrier, and specifically to reach an enhanced drug penetration over stratum comeum and result in an improved bioavailability following topical administration (Petola et al., 2003)

Drug transport from microemulsions is influenced by interactions within the vehicle as well as by interactions between vehicle and skin. In terms of the vehicle, physicochemical parameters of the drug influence the penetration process, such as the drug solubility in the formulation, the diffusion coefficient within the system, and partition coefficients (Bronaugh and Maibach, 1999).

Apart from the microemulsion structure, the drug release depends on the partitioning of the drug between the oil and water phases. The effect of the drug oil/water partitioning on the drug release rate therefore offers a possibility to control the drug release rate by changing the oil-water partitioning, there may also be simple methods available. For example, charged amphiphilic drugs may be rendered largely oil-soluble but poorly water soluble by complexation with an oppositely charged excipient.

D. Permeation study

1. Characteristics influencing skin permeation

Skin permeation is a complex process, with a variety of barriers to cross. Initially, a drug must first partition out of the dosing solution (vehicle) into the stratum corneum before diffusing across the viable epidermis and dermis from where most permeants are cleared by the circulation. For the majority of drugs, the main barrier is the stratum corneum. Fick's first law can be applied to describe the diffusion processes in equation 4 (Kriwet and Muller-Goymann, 1995).

$$J = K_p \times \Delta C = \frac{D \times K \times \Delta C}{L} \qquad (4)$$

Where:

J $\quad$ = $\quad$ steady-state flux of the permeant through the stratum corneum

K_p $\quad$ = $\quad$ permeability coefficient of the permeant in the stratum corneum

ΔC $\quad$ = $\quad$ concentration gradient of the permeant across the stratum corneum

D $\quad$ = $\quad$ diffusion coefficient of the permeant in the stratum corneum

K $\quad$ = $\quad$ apperent partition coefficient of the permeant between the stratum corneum

$\quad$ and the vehicle

L $\quad$ = $\quad$ the length of the pathway through the stratum corneum

It is apparent from this equation that the flux is constant if the permeability coefficient and concentration difference are constant. The concentration gradient over the stratum corneum will depend primarily upon chemical characteristics of the permeant including solubility, lipophilicity, ionization and stability. To obtain high levels of permeant in the first layers of the stratum corneum, the permeant should have a high tendency to leave the vehicle and migrate into the skin. As the barrier within the stratum corneum is mainly lipoidal, high lipid solubility is necessary for a maximal input of the permeant into the stratum corneum. Although for most drugs the stratum corneum is the main barrier, it should be noted that once a permeant has crossed the stratum corneum, it must partition into the underlying layers of the epidermis, dermis, and circulatory system. These tissues are more hydrophilic than the stratum corneum and can present a barrier to extremely hydrophobic permeants.

The magnitude of the partition coefficient, K, is affected by the composition of the vehicle, the chemical structure of the permeant and the charge of the permeant. For a given drug the partition coefficient may be increased by manipulating the composition of a vehicle in such way that the permeant has a higher tendency to leave it. If the permeant is ionizable, the pH of the vehicle and the permeant ionization constant, pKa, will determine the actual concentrations of ionized and non-ionized species and thus influence the partition coefficient. Generally, transport of ionized species occurs much less rapidly than transport of the non-ionized species (Walters, 2002).

The diffusion coefficient or diffusivity, D, is a rough measure of the ease with which a molecule can move about within a medium, in this case the stratum corneum. It is dependent on molecular weight and volume, and the degree of interaction between the permeant and stratum corneum. The larger the molecule, the more difficult it is to move about, and the lower the diffusivity. Up to a molecular weight of at least 500 dalton and perhaps 5,000 daltons, the molecular size plays no crucial role. Non-specific and specific binding may occur in both the epidermis and dermis, reducing diffusivity and thereby decreasing skin permeability.

2. Determination of the characteristics of the permeant

The solubility of a permeant is best measured by allowing an excess permeant to equilibrate in the solvent while stirring at a constant temperature. After equilibration, a sample of the liquids is filtered, diluted with solvent, and analysed. The partition cofficeint, K, can be calculated from solubility measurements, simply

as the ratio of the solubility in one solvent to that in another solvent. Alternatively, they can be measured via liquid-liquid extraction. In this case, a fixed quantity of permeant is dissolved in one liquid, and this solution is then shaken with the other liquid at a constant temperature for at least 24 hours. The ratio of concentrations of permeant in the two liquids at equilibrium is the partition coefficient (Peltora et al.,2003).

The diffusivity, D, can be determined in vitro by simply measuring the transdermal flux at early time until a steady-state flux is reached using diffusion cells. A representative plot of the cumulative amount of drug crossing the skin against time is shown in Figure 8.
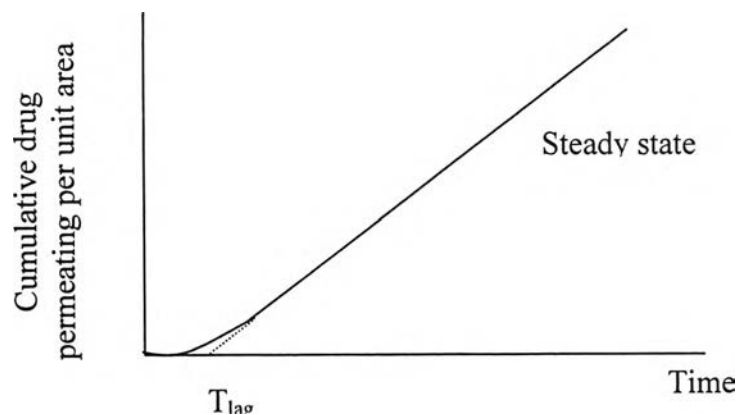

Figure 8 Typical permeation profile for a molecule diffusing across human skin.

The time before steady state is reached is characteristic for the difusivity of the permeant in the membrane, and can be used to calculate the diffusivity. The lag time,

T_{lag}, is the time obtained from extrapolation of the steady state portion of the graph to the intercept on the time axis, and is defined by the following equation 5

$$T_{lag} = \frac{L^2}{6D} \tag{5}$$

Where:

L = thickness of the membrane (cm);

D = diffusivity $(cm^2.s^{-1})$

L should represent the length of the pathway through the membrane. Thus, in practice, this method for evaluating D has several disadvantages as the exact length of the pathway through the membrane is difficult to measure. It also may vary with the constituents of the vehicle. Additionally, lag times obtained from permeation experiments with human skin tend to be very variable and may include a component arising from interactions between the stratum corneum and the permeant.

Thus, if the donor concentration and the flux of permeant are known, the permeability coefficient may be determined. The permeability coefficient is constant for a given permeant under a given set of experimental conditions and depends on the diffusion coefficient, the partition coefficient and the length of the pathway through the membrane.

3. Diffusion Cell Design

In vitro techniques to assess skin penetration and permeation are used extensively in industry and academic. Most common methods for evaluation of in vitro skin penetration use diffusion cells (Kreilgaard, 2002). The major advantage of in vitro investigations is that the experimental conditions can be controlled precisely, such that the only variables are the skin and the test material. Although a potential disadvantage is that little information on the metabolism, distribution, and effects of blood flow on permeation can be obtained.

The most commonly used solutions to diffusion equations that are applied to the in vitro situation make the following assumptions (Walters, 2002):

    a) The receptor phase is a perfect sink.

    b) Depletion of the donor phase is negligible.

    c) The membrane is a homogeneous slab.

Static diffusion cells are usually of the upright " Franz " or side-by-side type, with receptor chamber with a side arm sampling port volumes of 2-10 ml and surface areas of exposed membranes of near 0.2-2 cm^2. The jacket is positioned around the receptor compartment and heated with an external circulating bath. Excised skin is always mounted as a barrier between a donor and a receptor chamber, and the amount of compound permeating from the donor to the receptor side is determined as a function of time. During the course of an experiment, small volumes are withdrawn from the stirred receptor solution for analysis and the receptor compartment is refilled with receptor solution to keep the volume of solution constant during the experiment

(Chien, 1987; Hadgraft and Guy, 1989 and Walters, 2002). A modified Franz diffusion apparatus is illustrated in Figure 9.
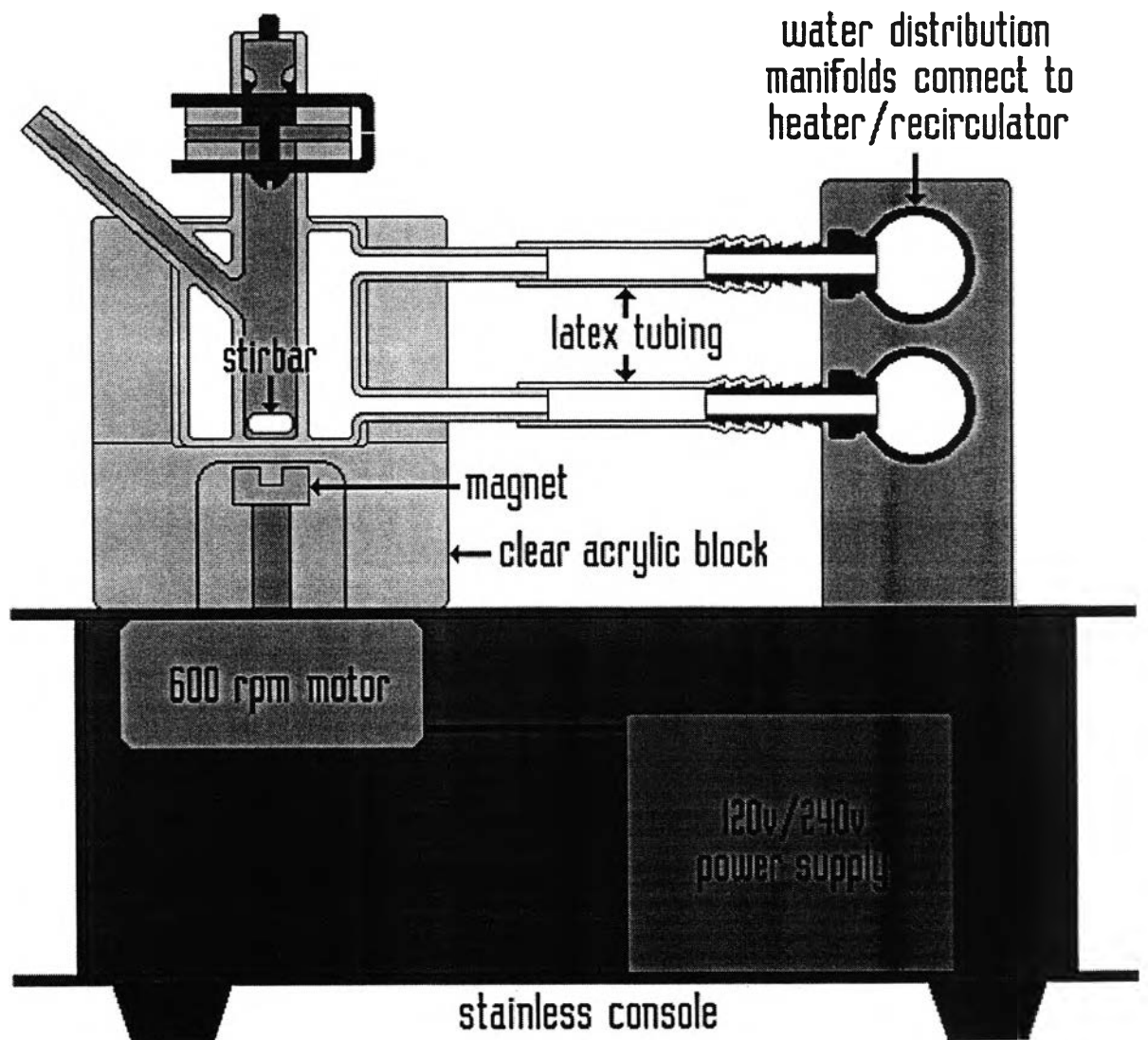

Figure 9 Schematic illustration of modified Franz diffusion apparatus.

3.1 Receptor Chamber and Medium

The receptor phase can act as a sink, while ensuring that sample dilution does not preclude analysis. As a general rule the concentration of the permeant in the receptor fluid should not be allowed to exceed approximately 10 % of saturation solubility (Osborne and Amann, 1990). The most commonly used receptor fluid is pH 7.4 phosphate buffered saline (PBS), although this is not always the most appropriate material. It has been shown that if a compound has a water solubility of less than about 10 µg/ml, then a wholly aqueous receptor phase is unsuitable, and the addition of solubilizers becomes necessary.

One of particularly useful fluids is 25% (v/v) aqueous ethanol, which provides a reasonable "sink" for many permeants, while removing the need for other antimicrobial constituents. Other examples of modified receptor phases include 1.5-20% Volpo N20 rabbit serum, 3% bovine serum albumin, 50% aqueous methanol, 1.5-6% triton-X100, and 6% Poloxamer 188. It is important to recognize the possibility that solubilizers may interfere with the barrier function of the skin itself.

3.2 Selection of Skin Membranes

Animal skins are widely used as substitutes for human skin, primarily owing difficulties in obtaining human tissue. (Gibson, 2001; Walters,2002). Recently there has been considerable interest in the use of shed snake skin as a model membrane for in vitro diffusion studies monitoring the release of a number of drugs from semisolid formulations. Although shed snake skin is not a mammalian integument, it has been reported that some compounds penetrate snake skin and human stratum corneum at similar rate. Snake skins can be obtained without injury to

the animal and do not have to be subjected to chemical or heat stress prior to use. The epidermal is shed as a large intact sheet, thus a single snake skin can provide multiple samples. Shed snake skin is not a living tissue, can be stored for a long period at room temperature and is easily transported. Stored and fresh snake skins appear to show no difference in permeability.

Shed snake skin is composed of two very different regions, scales which are separated by hinges. The scales are rigid, whereas the hinge region is elastic. The scales on the dorsal surface are much smaller than the scales on the ventral surface and the size of the scales varies considerably between species. However, some studies report that species of snake was utilised and wheter dorsal or ventral skin was used in the experiments (Haigh et al., 1998).