Chapter 2

Literature review

2.1. Introduction

Administration by the oral route remains the most popular method of drug delivery. Despite the popularity and versatility of the oral route, significant problem remains. Not all the drug molecules posses the physical, chemical or biological characteristics necessary for successful therapy by the oral route. Problems such as poor solubility or chemical stability in the environment of the gastrointestinal tract, poor permeability through biological membranes, or sensitivity to metabolism ara well known to result in the rejection of potential drug candidates as practical problems. In selected cases, formulation approaches appear to be able to provide adequate bioavailability. Lipid-based drug delivery systems have been proposed as the means of bypassing some of the more resistant chemical or physical barriers associated with poorly absorbed drugs. These potential drug delivery systems include the more conventional approaches such as emulsions and microemulsions, as well as the more recent methods such as liposome, solid lipid nanoparticles, lipid chocolates, microspheres, and cubosomes. Clear evidence exists in the literature that lipid based systems have been most successful in enhancing the bioavailability of class II molecules that are poorly water soluble but highly permeable drug molecules (Amidon GL et al, 1995) Examples of enhanced oral bioavailability of highly water soluble but poorly permeable molecules (class III) brought about by lipid-based system also exist (Cavelli R et al., 2002) Lipid based dosage forms consist minimally of an oil phase and either liquid or solid drugs (Malmsten M et al., 2002). Most formulations also include a surfactant and some a co-surfactant. When the oily formulation is dispersed in water, a variety of lipid assemblies are found. Figure 2.1 is an idealized phase diagram, showing all possible lipid aggregates. The various possible lipid assemblies arise out of a complex interplay of molecular properties of the phases, the surfactant and the relative volumes of the components. Conventional micelles and liposomes comprise surfactants and contain little to no oil phase, yet they too differ by size and stability. The relative proportions of the various aggregates are sensitive to temperature. (Malmsten M et al., 2002) Not all combinations of oil /water /surfactant will produce all of the structures listed in Figure 2.1. Nonetheless, many systems exhibit a wide variety of assemblies depending upon the composition and temperature.

In addition to the composition, the molecular structure and molecular properties of the surfactant can strongly influence the nature of the lipid assemblies observed. Surfactants are molecules that have ampiphillic character- that is, significant regions that are hydrophobic (often a hydrocarbon chain) and regions that are hydrophilic. This dual character results in a molecule that tends to accumulate at an interface, to simultaneously maximize interactions with water for the hydrophilic portions and minimize interactions with water for the hydrophobic portions.

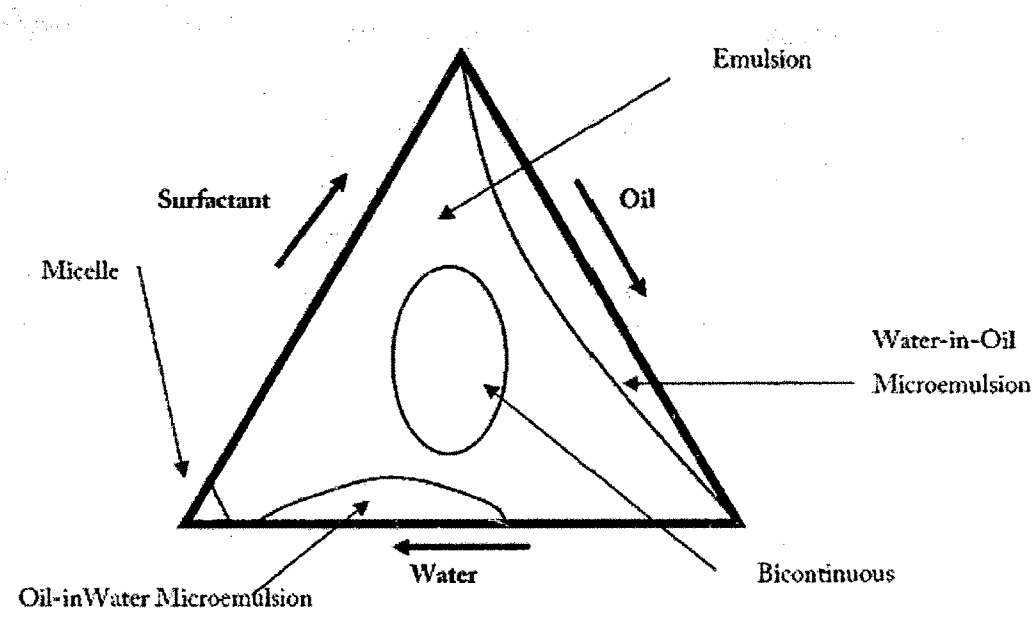

Figure 2.1: Generic phase diagram of surfactant-oil-water mixture showing the possible aggregates.

Nanosizing of a bulk material may also lead to dramatic changes of the physical properties of the substance, i.e. the depression of the melting point which results in the existence of supercooled melts (B. Siekmann et al., 1994). In case of much decreased particle sizes the properties of the material are determined by the surface properties. Therefore colloids are not trivial systems. Furthermore, different colloidal structures might coexist. Of course, nanometric systems have to fulfill the requests for safe drug delivery systems mentioned above. Most of all, precautions against aggregation,

coalescence or Ostwald ripening (L. H. Block et al., 1989) have to be attempted by optimized stabilization. Additionally, incorporation of sensitive drug molecules in some carrier matrices is claimed as protection against enzymatic degradation, hydrolysis or light (V. Jenning et al., 2000) (A. Dingler et al., 1998) (J. Kreuter et al., 1994) (V. Jenning et al., 2000) (J. Kristl et al., 2003). That is remarkable, knowing the diffusion constant for solid materials at approximately 10-15 cm^2 s^{-1} at room temperature. According to the Einstein-Smoluchowski equation (P. W. Atkins et al., 1990), within 14 h a molecule could be able to diffuse over a distance of 100 nm, what is diminished in amorphous materials to 50 s. Great attention should be paid to proposed storage stabilities of sensitive drugs in nanoparticles as far as contact to the particle surface and rapid degradation by the outer environment is possible (T. Okubo et al., 1997). Despite of their small size, colloidal carriers have to guarantee controlled drug release. Burst release (A. z. Mühlen et al., 1998) (A. Lamprecht et al., 1999) can be explained by this Einstein-Smoluchowski equation, too. Entire physicochemical characterization and evaluation is not trivial because systems in the nanometric range do not offer the whole variety of investigative methods (B. Magenheim et al., 1991). Remarkable efforts must be made to avoid artifacts due to invasive analyzing techniques (T. A. Barber et al., 1993) or only to avoid dilution which is required by many analytical methods. Special attention should be paid to other, competitive nanocompartments for the drug within a formulation (W. Mehnert et al., 2001), i.e. mixed micelles in a tenside-stabilized nanoparticle dispersion. Concerning the choice of a drug formulation, it should be remembered that none delivery system per se meets all desired requirements for overall/general problem solution. Moreover, optimal formulations have to be chosen carefully for each drug, according to the features of the nanocarriers. The aim is to achieve desired drug release profiles in vivo by minimizing undesired side effects.

2.2. Classification of lipid-based systems:

Pouton (Pouton CW et al., 2002) has devised a systemic classification of lipid based formulations based upon the presence of formulation components. In turn, it will be shown that these components influence drug solubility, colloidal stability and possibly the routes of absorption of the drug. Type I formulations are those composed of

triglycerides oil alone and lack surfactant. In this class, the oil must often be digestible so that digestion products (fatty acids and monoglycerides) can form mixed micelles with bile salts. Type II system contain surfactant, usually in concentration of 20-60% (w/w). Useful surfactants have HLB values less than 12 and so tend to act as emulsifiers at low concentration and micro- emulsifiers at high concentration. At the higher concentration of the surfactant, the ability to digest the lipid is less critical, presumably because micelles can be formed by the formulation components directly. Type III systems contains co-solvents (often alcohol or propylene glycol) to enhance the solubility of the drug in the preconcentrate but at the cost of slightly inhibiting the digestion of the oil. Type IIIa systems have a small amount of the co-solvent, and digestion of the oil will be slightly inhibited. Type IIIb systems have a greater amount of co-solvent replacing triglycerides, and lipid digestion has been eliminated. This later system is advantageous in that release is likely to be independent of the digestion kinetics. Absorption is likely to be extremely rapid from Type IIIb systems.

2.3. Overview of colloidal drug carrier systems

2.3.1. Nanosuspensions

In a narrower sense, by the term "nanosuspensions" poorly water-soluble drug crystals in the nanometric range are described (R. H. Müller et al., 1998) (K. P. Krause et al., 2001) (C. Jacobs et al., 2001) (E. Merisko-Liversidge et al., 2003) (A. T. Serajuddin et al., 19990 (R. H. Müller et al., 2000). Nanosuspensions are saturated solutions. Therefore, they represent the simplest colloidal carriers with respect to composition. Their drug payload amounts to nearly 100 %. In an aqueous environment, the drug is pearl milled, precipitated or high pressure homogenized to a particle distribution mostly below one micrometer. Despite of the use of tensides, particle growth up to micrometric drug crystals may occur when the drug molecules of small particles dissolve in the outer environment and precipitate later on the surface of larger particles (Ostwald ripening). According to the Kelvin equation (A. E. Johnson et al., 2002), the increased dissolution is an effect of strong particle curvature, so smaller particles are more affected than larger ones. Intravenous application of nanosuspensions stands for a risk (J. B. Boyett et al., 1989).

Due to tremendous interface areas between drug and environment, solubilization velocity of the drug is increased according to the Noyes-Whitney equation (M. Ghyczy et al., 1998). Corresponding to the equation of Thomson-Gibbs-Freundlich, even the solubility of active substances may be increased in nanometric carriers. Attention has to be paid on drugs with small safety margins where burst release has to be avoided. But controlled release and reproducible blood levels are not easily achievable because as a release controlling barrier only the tenside layer may serve in these nanosuspensions. Suspensions of crystals in the micrometer range are already established in the market (i.e. Prednigalen^{TM}). The only two registered nanosuspensions are Rapamune^{TM} and Emend^{TM} for immediate delivery.

Attention should be paid to the polymorphism of the drug, too. In contrast to the crystalline state, amorphous drug is solubilized fast. Sometimes, crystallization of the drug is retarded. Due to the high surface-to volume ratio and due to the presence of emulsifiers supercooled melts with different physical properties are formed (B. Siekmann et al., 1995).

2.3.2 Liposomes

Liposomes (Y. Barenholz et al., 1994) (R. Schubert et al., 1998) (M. Ghyczy et al., 1998) (D. D. Lasic et al., 1993) (M. J. Otto et al., 1987) (G. Gregoriadis et al., 1993) (D. J. A. Crommelin et al., 1994) consist of one or more lipid bilayers of amphiphilic lipids (i.e. phospholipids, cholesterol, glycolipids). The lipophilic moiety of the bilayers is turned towards each other and creates an inner hydrophobic environment in the membrane. Lipophilic or amphiphilic drugs can be associated with the non-polar parts of lipid bilayers if they fit in size and geometry (Y. Barenholz et al., 1994). The hydrophilic molecular head groups face the outer water phase and the inner aqueous core of the vesicles. Water-soluble compounds can be included within the aqueous compartments. Liposomes are classified as large multilamellar liposomes (MLV), large unilamellar vesicles (LUV), small unilamellar vesicles (SUV), oligolamellar large vesicles (OLV), and multivesicular vesicles (MVV), depending on their size, the number of bilayers and the existence of inner vesicles in a vesicle. The size of liposomes varies from 20 nm to few micrometers, with lipid membranes of approximately 5 nm (M. Ghyczy et al., 1998).

Marketed products (F. J. Martin et al., 1990) are i.e. AmBisome™, DaunoXome™, and Pevaryl™-Lipogel, indicating the parenteral and topical administration as main application routes for liposomes. Liposomes often suffer rapid degradation by the pH of the stomach, by intestinal enzymes and bile salts when they are given perorally. Further instability problems can arise during storage when the unsaturated fatty acids and the ester bindings of phospholipids are hydrolyzed and oxidized, respectively. To overcome this last mentioned drawback, niosomes (R. Schubert et al., 1998) (A. T. Florence et al., 1993) (J. A. Bouwstra et al., 1994) were invented.

Niosomes are non- ionic surfactant vesicles (NSV), resemble in their constitution to liposomes and consist of synthetic surface active molecules, i.e. alkyl polyoxyethylene ethers. Saturated hydrocarbon chains and intramolecular ether bindings increase the chemical stability of the niosomes. Whether niosomes are superior to liposomes in vivo is under investigation (A. T. Florence et al., 1993) (H. Schreier et al., 1994).

2.3.3 Mixed micelles

As long-chain phospholipids are known to form bilayers when dispersed in water, the preferred phase of short-chain analogues is the micellar phase (H. Hauser et al.,2000). The prediction of the arrangement keeps demanding, because it is related with chemical structure, temperature and water content (S. Segota et al., 2001) (P. Alexandridis et al., 1995). In general, amphiphilic ionic, anionic or ampholytic molecules, which are able to decrease the surface tension of a solvent, arrange in micelles, as Tween™ or sodium dodecylsulfate above a certain critical concentration. A micellar solution is a thermodynamically stable system formed spontaneously in water (D. M. Small et al., 1986), and also in organic solvents. The latter is of less interest in pharmaceutical technology. Micelle formation can only occur above a certain solute concentration, the critical micellar concentration (CMC), and at solution temperatures above the critical micellar temperature (CMT). The small colloidal aggregates (micelles) are in rapid thermodynamic equilibrium with a measurable concentration of monomers. The size (mostly around 5 to 10 nm (R. Voigt et al., 2000) and shape of micelles depend ultimately on the chemical structure of the detergent. According to Small (D. M. Small et al., 1986), spherical, rod-shaped, and discoidal micelles exist in water.Micellar solutions

exhibit solubilization phenomena. The micelle solubilizes host molecules (i.e. drugs) in any zone of the micelle volume, but the penetration into the micelle depends over all on the inner space of the micelle (as mentioned, diameter of entire micelle often below 10 nm), on the hydrophobicity of the drug and on the charge of the incorporated molecule (I. Capek et al., 2002) (A. Meziani et al., 1997). The interaction between micelles and lipophilic drugs leads to the formation of mixed micelles (MM), often called swollen micelles, too. The addition of salt, alcohol etc. can vary the degree of penetration into the micelle (co-solubilization). In mixed micelles, the mobility of the micellar phase was found to be decreased due to incorporated molecules (A. M. Wasserman et al., 2002). Considerably, swollen micelles are larger than the analogous "free micelles" because solubilization may result mostly from the increase in micellar size (K. Shinoda et al., 1967).

Micelles of common surfactants usually have relatively high CMC and are unstable upon strong dilution, e.g. in the blood volume. Toxic side effects of some tensides on human cells have to be considered beside bad taste of tensides in peroral liquids. Furthermore, investigations have to be focused on drug-tenside incompatibilities and on initial over-saturation (R. Voigt et al., 2000) what would lead to later drug expulsion from the micelle. The kinetics of micelles are driven by both rapid micelle-monomer exchanges and by dissolution and new formation of micelles (H. Wennerström et al., 1979), but nevertheless the extent of water-amphiphile contact is discussed controversially (H. Wennerström et al., 1979) (R. Zana et al., 1997). Newer findings indicate an extensive contact between water and methylene and methyl groups and an extreme disorder of the micelle interior. But simultaneously recent discovery excludes water penetration into the micelle although surfactants move constantly in and out of micelles (R. Zana et al., 1997). Swollen micelles are fluid systems, but sufficiently stable to be used as delivery systems for stable drugs (i.e. Valium MMTM, Konakion MMTM) (M. A. Hammad et al., 1998). The hemolytic activity of bile salts is not longer present in MM, therefore they are parenterally applicable (M. A. Hammad et al., 1997) (A. Supersaxo et al., 1991). Latest developments for mixed micelles are presented in (G. A. Husseini et al., 2000) (A. Marin et al. 2001) (V. P. Torchilin et al., 2001) (A. Krishnadas et al., 2003). Nowadays, polymeric micelles (V. P. Torchilin et al., 2001) (N. Rapoport et al., 2003) as

pharmaceutical carriers with high solubilization capacity and rather low CMC value (to refer to arising monomer toxicity after dilution) are proposed.

2.3.4 Colloidal liquid crystalline structures

Liquid crystalline phases (K. H. Bauer et al., 1999) (R. Voigt et al., 2000) (S. T. Hyde et al., 2001) (S. Kutsumizu et al., 2002) share features from both liquids and crystalline substances. Due to their intermediate state they are named "mesophases", too. On one hand, referring to crystals mesophases are viewed as defective crystals. Orientational order and periodicity are essential, common to all liquid crystalline states. For all liquid crystals, except cubic phases (K. Larsson et al., 2000) (P. Garstecki et al., 2002) (P. Garstecki et al., 2002), anisometry is given. By the crystal-related phenomena they can by characterized by differential scanning calorimetry (DSC), X-ray diffraction and polarizing microscopy, the latter in case of anisometry only. On the other hand, liquid crystals match partially self-organized melts in providing remarkable viscosity and diffusion characteristics. Two liquid crystalline trans itions have to be distinguished, the lyotropic and the thermotropic. Materials that form liquid crystals by addition of solvents are lyotropic liquid crystals, i.e. when in aqueous solutions the concentration of water-soluble amphiphiles is increased. The amphiphilic molecules must exhibit some chemical complexity, or otherwise the solvent will simply dissolve those (S. T. Hyde et al., 2001). Liquid crystals are typically organic molecules, ranging from small molecules, i.e. detergents, to polyelectrolytes, i.e. DNA, vegetable gums (S. T. Hyde et al., 2001). The formation of lyotropic mesophases is driven by the chemical structure of the organic molecule(s), the ratio of water to amphiphile(s), and the temperature. With decreasing concentration of water, firstly hexagonal (similar to many cylinder- like micelles) and then lamellar phases (similar to stacked bilayers, discoid) are formed. In case of very polar head groups of the molecules, together with high water binding capacities, cubic phases ("balls") may be formed instead of hexagonal arrangement. Drug of adequate distribution coefficient can be incorporated in between the fluid lamellar phase (K. H. Bauer et al., 1999) (M. G. Carr et al., 1997). Cubosomes are submicron particles of bicontinuous cubic phases for lipophilic or amphiphilic active ingredient incorporation (B. Siekmann et al., 2002) (M. L. Lynch et al, 2003). For these drugs, cubosomes have

been proposed as a delivery system which may provide both a solubilization benefit (increased drug payload) and also a means for controlled or sustained release (B. J. Boyd et al., 2003). Elyzol^{TM} as an in situ forming liquid crystalline dispersion is commercially available.

Beside the lyotropic mesophase a thermotropic transition exists. A chemically pure material does not show a clear melting point, but forms liquid crystals within a certain temperature range (S. T. Hyde et al., 2001). If the liquid crystalline state (i.e. of a drug) is maintained at lower temperatures as supercooled liquid crystals, pharmaceutical use seems to be interesting (J. Patterson et al.,2002). Liquid crystals as delivery systems should be able to improve the dissolution of poorly water-soluble drugs. Lyotropic liquids crystals incorporate relatively high drug amounts, but only few drugs themselves tend to build thermotropic mesophases. Disadvantageous is that the tenside concentrations are high and that colloidal dispersions of liquid crystals occur only in a thin range of parameters. Mesophases are thermodynamically stable and self-assembling, but they form reversibly the former basic micellar or molecular dispersed state by adding water.

2.3.5 Nanocapsules

Oil containing nanocapsules differ from (oil- in water) nanoemulsions in providing a barrier made from polymers between the core and the surrounding environment, but as well nanoparticles with aqueous cores in an aqueous outer phase are published (P. Couvreur et al., 1977). Often, for the preparation of nanocapsules the ways of solvent displacement (R. Alvarez-Román et al., 2001) (H. Fessi et al., 1989) and interfacial polymerization (M. Aboubakar et al., 1999) are applied. According to the lipophilicity of the capsule content, hydrophilic and lipophilic drugs, respectively, can be dissolved (T. Pitaksuteepong et al., 2002). Additionally, the polymeric particle surface may serve as compartment where hydrophilic drugs can be adsorbed (T. Pitaksuteepong et al., 2002). Some encapsulated lipophilic drugs have already shown to be released in a controlled manner (A. Lamprecht et al., 2002). Encapsulation may decrease the toxicity of drugs after peroral or parenteral application inasmuch as the exhibition to cells is diminished. Encapsulation saves sensitive drugs from rapid degradation. With the aim to reduce the

interactions with reticuloendothelial system and to alter body distribution, the surface of nanocapsules was modified by certain materials such as surfactants (i.e. length and density of PEG chains) (J. Kreuter et al., 1991). The complex biofate of nanocapsules is not entirely understood yet.

2.3.6. Nanoemulsion

In contrast to microemulsions, emulsions are heterogeneous systems comprised of two immiscible liquids in which one liquid is dispersed as droplets in another liquid (S. Klang et al., 1998) (K. Westesen et al., 1992). For the production (S. E. Tabibi et al., 1990) (M. B. Schulz et al., 2000) an energy input is necessary and the obtained liquid-in liquid dispersion is thermodynamically unstable (M. Jumaa et al., 1998) (I. B. Ivanov et al., 1997) (H. Karbstein et al., 1994) (D. Rousseau et al., 2000) (S. E. Friberg et al., 1997). Oil- in water nanoemulsions present the most important parenteral drug carrier systems where lipophilic drugs are dissolved in the inner phase of the emulsion (S. Klang et al., 1998), i.e. Diazepam Lipuro^{TM}, Disoprivan^{TM}, Stesolid^{TM} and Lipetalon^{TM} (J. Schmitt et al., 1998) (L. C. Collins-Gold et al., 2000). Once drug-free introduced as parenteral nutrition, the ingredients of nanoemulsions are known to be physiologically well tolerable in human bodies. Degradation of the droplets containing lipophilic drug occurs very fast when administered intravenously, so retarded release is not realized. After entering the blood circulation, fat emulsions are treated as naturally-occurring fat and are therefore rapidly hydrolyzed by lipases. The phagocyte system is only activated when certain lipids are recognized as foreign. Rapid degradation for oil droplets takes place in GIT, too, if given perorally. For topical use enhanced drug penetration is described (S. Amselem et al., 1998). With regard to the mobility of the oil a protection of sensitive drug molecules from hydrolysis is hindered. Moreover, sustained release and incorporation of hydrophilic components in conventional oil- in water is not realizable. Multiple emulsions (water- in oil –in-water) are proposed to resolve these problems (S. Gohla et al., 1998) (N. Garti et al., 1999) (N. Garti et al., 1996). It is aslso important to considered that these novel nanoemulsions again are fluid, unstable systems where the production is not easy to handle.

2.3.7 Microemulsions

Microemulsions are optically isotropic, transparent or translucent, low- viscous, singlephasic and thermodynamically stable liquid systems (I. Danielsson et al., 1981) (G. M. Eccleston et al., 1994) (H. Wennerström et al., 1997) (S. P. Moulik et al., 1998). Critical solution is a term microemulsions are described with, reflecting their strong optical fluctuation and their solubilization capacity. As self-forming system the manufacture keeps simple.

Microemulsions are bicontinuous systems that are essentially composed of water and oil, separated by surfactant and co-surfactant (L.H. Block et al 1989) (R. Voigt et al, 2000) (R. Cortesi wt al, 1999) (L. M. Prince et al, 1977). Microemulsions provide ultralow interfacial tensions towards zero mN/m, despite of large oil- water interfacial areas. It is often very difficult to achieve the required interfacial area with the use of a single surfactant, hence a co-surfactant is essential. The distinction between solubilized micellar systems and microemulsions is not clear-cut since there is no well-defined transition point (D. Attwood et al, 1994) between inverted micelles, followed by microemulsions with comparable amounts of oil and water, and afterwards micelles if water is added continuously. Nevertheless, in this text microemulsions will be treated as bicontinuous structures only. The concept excludes aqueous surfactant solutions without added solubilizate, liquid-crystalline systems, and normal emulsions, too. Their characterization can be done by electron microscopy and scattering methods (X-ray diffraction) (D. Attwood et al., 2001), static and dynamic light scattering although microemulsions are unable to dilute (loss of microemulsion character in favor of micelles after dilution) and therefore size determination sometimes is difficult. Nonetheless, structures below 100 nm were often found (R. Voigt et al., 2000). Due to large interfacial areas microemulsions typically show much greater solubilizing capacities for both hydrophilic and lipophilic drugs than micellar solutions. A prominent example is Sandimune Optoral™ /Neoral™ preconcentrate (A. Meinzer et al. 1998). As reasons for better bioavailability above all the highly dispersed systems with good drug accessibility and secondary a better penetration into tissues are discussed (M. Kreilgaard et al., 2002).

Microemulsions are usually limited to dermal and peroral application because of their high surfactant concentration (R. Voigt et al., 2000). They exist in narrow regions of phase diagrams; therefore they are very restricted in tolerance to quantitative formulation changes.

2.3.7.1. Structure of microemulsion:

Microemulsions are the simple spherical or cylindrical structures formed by the aggregates of micelles that are formed by surfactants at the oil/water interface. Micelles are like drops of oil in water and reverse micelles are like drops of water in oil as illustrated in Figure. 2.2.

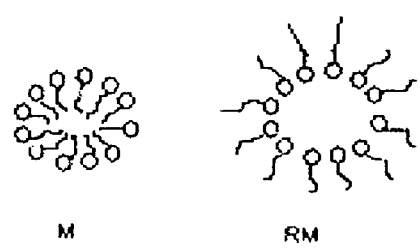

Figure 2.2: The structure of micelles. M= Micelles for o/w microemulsion, RM= Reverse micelles for w/o microemulsion.

Another microemulsion structure is the lamellae where water and oil consecutive layers are separated by surfactant layers conveniently oriented as illustrated in Figure 2.3. The lamellae structure is similar to the smectic thermotropic phase; it presents birefringence and maintains the order even at diluted concentrations. This structure is related with the spherulite structure [onion structure]. It is possible that spherulites are only out-of-equilibrium transient lamellar phases induced by mechanical work [yet to be proved] or by other stimulus.

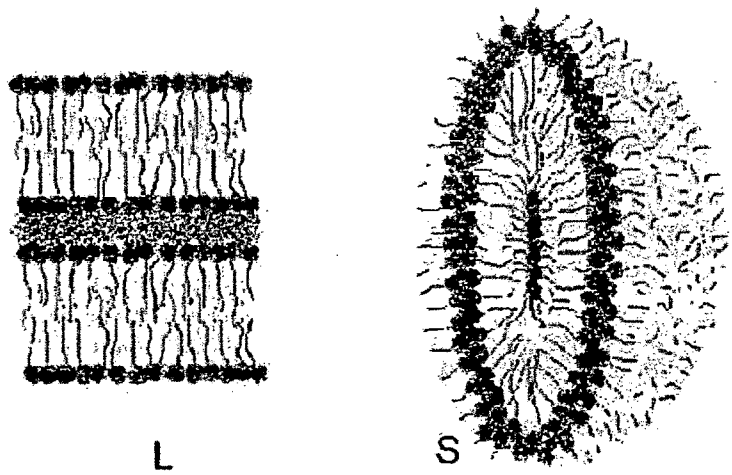

Figure 2.3: The lamellae (L) and the spherulite (S) structures. The surfactant molecules in the spherulite are arranged as onion layers

The bicontinuous structure or sponge phase is a quite intricate structure. As the name suggests, in this structure both water and oil are continuous phases. The sponge structure is a good example as the sponge has a continuous structure, but at the same time it is possible to "fill" the sponge with a liquid. In such a scenario both, material of sponge as well as liquid forms a continuous phase. Considering the sponge surface as surfactant, cartoon of a bicontinuous structure is presented in Figure 2.4.

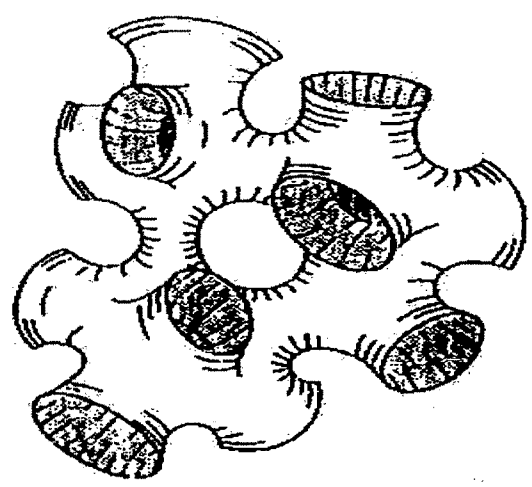

Figure 2.4: Bicontinuous structure. The 'pipeline" forms an oil continuous phase and the exterior forms a water continuous phase

Other microemulsion structures are possibly interconnected rod-like micelles, onions with an inner different structure, vesicles, etc as illustrated in Figure 2.5. It has been found that the principal factors responsible for structure changes of microemulsion are surfactant shape, entropic energy terms and solvent properties such as ionic force and pH.

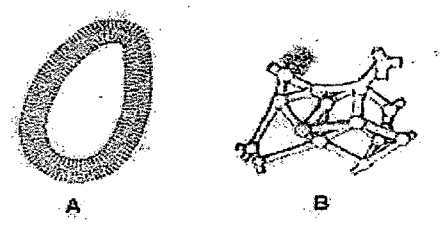

Figure 2.5.: Possible microemulsion structures: a) Vesicles b) interconnected rod-like micelles

Walde et al. has shown diagrammatically, the micellar structure changed on addition of water as well as enlargement of the micelles (Figure 2.6.) in presence of guest molecules (Cortesi, R et al. 1999).

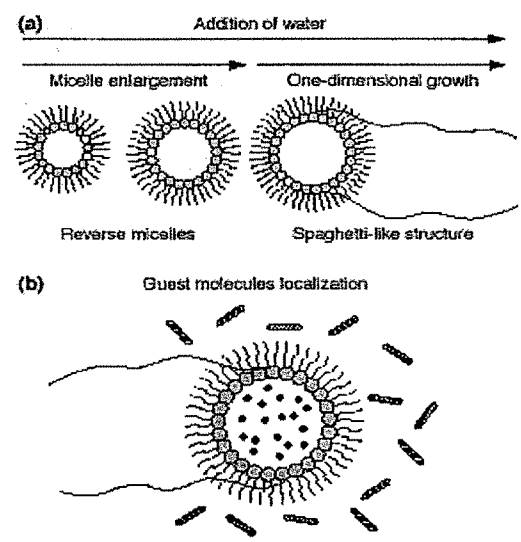

Fig: 2.6: Microemulsion base lecithin gels: (a) Schematic representation of the formation of lecithin gels upon addition of water to small phosphatidylcholine reverse micelles in apolar solvent. (b) Localisation of solubilised 'guest' molecules within lecithin gels. Lipophilic drug (stripped bar); hydrophilic drug (black circle) and ampiphillic drug (shaded head with attached tail)

2.3.7.2. Solubility studies:

The microemulsion formulations consist of one or more surfactants in combination with co-surfactant and drug dissolved in oil. The mixture is a clear, monophasic liquid at ambient temperature and possesses good solvent properties to allow presentation of the drug in solution. Oils form a distinct core in the interior of the surfactant aggregate, resulting in enhanced solubilizing capacity of the oils with improved drug loading capacities of the microemulsion. It is well established that medium chain fatty acids influence tight junctions of the epithelial cells and long chain fatty acids stimulate the lipoprotein synthesis and subsequent lymphatic absorption. In system containing comparable amount of oil and water, equilibrium bi-continuous structure is formed in which the oil and the water domain interpenetrate in a more complicated manner.

2.3.7.3. Difference from emulsion:

The term microemulsion implies a close relationship to ordinary emulsion (macro emulsion). Microemulsion state embraces a number of different microstructures, most of which have little in common with classical two phase emulsions. Microemulsions are readily distinguished from normal emulsion by their particle size, transparency, low viscosity and more fundamentally their thermodynamic stability. Although systems containing low concentration of dispersed phase (less than 25%) may, like emulsions, be composed of droplets of either oil dispersed in water (o/w) or water dispersed in oil (w/o), they are essentially stable, single phase swollen micellar solution rather than unstable two phase dispersions.

2.3.7.4. Theories for microemulsion formation:

There are different theories for the formation of microemulsion formulation, some emphasise on the formation of an interfacial film and the production of ultra flow interfacial tensions (mixed – film theories), (Daoud, M et al., 1999) (Lisiecki, I et al., 1999) (Hoar, T.P et al., 1943) whereas others emphasize on the monophasic nature of many microemulsion [solubilization theories] (Schulman, H et al., 1959). Some reports have also considered free energy of microemulsion formation and blending elasticity of the film (Shinoda, K et al., 1987) (Freiberg, S.E et al., 1983) (Rance, D.G et al., 1977)

(Ruckenstein, E et al, 1979, 1980, 1985) which is beyond scope of this article but interested readers can find related theories in the corresponding articles.

2.3.7.5. Advantages of microemulsion:

(a) Improvement in oral bioavailability: Dissolution rate dependent absorption is a major factor that limits the bioavailability of poor water soluble drugs. Microemulsion is a novel approach to improve the water solubility and ultimately bioavailability of lipophilic drugs. Microemulsion presents the drug to gastrointestinal tract in solubilized and micro emulsified form; subsequent increase in specific surface area enables more efficient drug transport through intestinal aqueous boundary layer and through the absorptive brush border membrane leading to improved bioavailability (Lawrence M.J et al., 2000) (Cortesi, R et al., 1999) (Bagwe, R.P et al., 2001) (Sachdeva, A et al., 1997) (Nazzal, S et al., 2002)

(b) Reduction in inter-subject and intra-subject variability: There are several drugs which show large inter-subject and intra-subject variation in absorption leading to decreased performance of drug and patient non-compliance. Microemulsion drug delivery system is a proven approach to overcome inter and intra subject variation (Lawrence M.J et al., 2000) (Cortesi, R et al., 1999) (Bagwe, R.P et al., 2001) (Sachdeva, A et al., 1997) (Nazzal, S et al., 2002) .

(c) Reduction of food effects: Food is a major factor affecting the therapeutic performances of the drug in the body. There are several research paper specifying that performance of microemulsion is independent of food and that microemulsion offer reproducibility of plasma profile (Bagwe, R.P et al., 2001) (Nazzal, S et al., 2002) .

(d) Ease of manufacturing and scale up: This is one of the most important advantages that makes microemulsion a unique delivery system when compared to others like solid dispersions, liposome, nanoparticles etc. dealing with improvement of bioavailability. Microemulsion requires very simple and economical manufacturing facilities like simple mixer with agitator and volumetric liquid filling equipment for large scale manufacturing. This explains the interest of industry for microemulsion based drug delivery systems.

(e) Ability to deliver peptides that are prone to enzymatic hydrolysis in GIT: One unique property of microemulsion is their ability to deliver macromolecules like peptides,

hormones, enzyme substrate and inhibitors and their ability to offer protection from enzymatic hydrolysis.

(f) No influence of lipid digestion process: Unlike other lipid-based drug delivery system, the performance of microemulsion is not influenced by the lipolysis, emulsification by the bile salts, action of pancreatic lipases and mixed micellar formation. Microemulsions are not necessarily digested before the drug is absorbed as they present the drug in micro emulsified form which can easily penetrate the mucin and water unstirred layer.

(g) Improvement of bioavailability of antifungal and anti- inflammatory drug by topical microemulsion.

(h) As solid dosage form for oral administration: Microemulsion can be converted into the various solid dosage forms by adsorbing onto the solid surface (US Patent: 6309665).

2.3.7.6. Formulation consideration:

Pharmaceutical acceptability of excipients and their toxicity issues of the components used, make the formulation of microemulsion really critical. There is great restriction in selection of the components that are orally acceptable. Early studies revealed that the self micro emulsifying process is specific to the nature of the oil/ surfactant pair, the surfactant concentration, oil/surfactant ratio, the concentration and nature of the co-surfactant and surfactant/co-surfactant ratio and temperature at which micro emulsification occurs. Microemulsion formulation usually involves a combination of three to five components: an oil phase, an aqueous phase, a primary surfactant and in many cases a secondary surfactant (co-surfactant) and sometimes an electrolyte.

Although there are no strict rules for choosing the appropriate microemulsion components, there are number of general guidelines based on empirical observations. A crucial step lies in the choice of surfactant and co-surfactant for particular oil.

2.3.7.6.1. Surfactant:

The surfactant chosen must be able to:

(a) lower interfacial tension to a very small value to aid dispersion process during the preparation of the microemulsion.

(b) provide a flexible film that can readily deform round droplets.

(c) be of the appropriate lipophilic character to provide the correct curvature at the interfacial region for the desired microemulsion type i.e., for o/w, w/o or bicontinuous.
The surfactant used to stabilise the microemulsion may be i) non-ionic, ii) cationic, iii) anionic or iv) zwitterionic surfactants. Combination of anionic or cationic surfactants of high HLB (Hydrophilic Lipophilic Balance) with a co-surfactant of lower HLB, a double chained surfactant of the appropriate molecular composition or a single chained non-ionic surfactant of the polyethylene glycol alkyl ether type at appropriate temperature are generally used for the formulation of microemulsion. Combination of this, particularly ionic and non-ionic surfactant can be very effective in increasing the extent of microemulsion region. An example of non-ionic surfactant includes sorbitan monooleate (span 80) or polyoxyethylene surfactants such as Brij® 35 ($C_{12}H_{23}$). Jaykrishnan (Jayakrisnan, A et al., 1983) examined the solubilization of hydrocortisone by w/o microemulsion containing a mixture of the non-ionic surfactants Brij® 35 (Polyoxyethylene 23 lauryl ether) and Arlacel ® 186 (Glyceromonooleate- propylene glycol), isopropanol as co-surfactant, water and alkanes. The influence of the concentration of the oil soluble surfactant (Arlacel ® 186), the chain length of the oil and the alcohol concentration on the amount of water that could be incorporated in the w/o microemulsion were examined. With fixed quantities of the components, the water solubilization increased with increase of the chain length, of the alkanes between C8 to C16 and increase of the concentration of Arlacel® 186, reaching a maximum at 5:1 weight ratio of Arlacel® 186 to Brij® 35. A formulation containing 10 ml decane, 4ml isopropanol, 5gm Arlacel® 183 and 1gm of Brij® 35 was capable of incorporating about 8ml of water. Soybean lecithin and egg lecithin which contains diacyl phosphatidylcholine used as non-ionic surfactant for the preparation of microemulsion. Problem involved in the formulation of microemulsion using lecithin have been reviewed by Shinoda et al (Shinoda, K et al., 1995) (Aboofazeli, R et al., 1993). The characteristic solution properties of lecithin that must be taken into account when formulating microemulsion includes:

(a) A strong non-polar portion due to the two long hydrocarbon chains

(b) A strong polar portion due to the zwitter ionic polar head groups which are strongly hydrated.

(c) A close balance between the polar and non-polar regions, although slightly biased toward the lipophilic side; and

(d) A strong tendency to form lamellar liquid crystals.

Alteration of the HLB can be achieved by adding short chain alcohols, which make the polar solvent less hydrophilic. In addition, the incorporation of these weakly ampiphillic co-solvents in the polar parts of the lipid layers increase the area of the lipid polar head to produce the required spontaneous curvature of the lipid layers, it also decrease the stability of the lamellar liquid crystalline phase. Shinoda et al (Shinoda, K et al., 1989,) have reported the phase properties of the lecithin / propanol / water / n-hexadecane systems. About 2 – 3 w % lecithin was the minimum amount required to form microemulsion at all mixing ratios. The propanol concentration was in the range 10 -15% of the aqueous solvent, the concentration decreasing slightly with increasing in oil content. From an examination of the microstructure of the microemulsion, these authors showed a general transition from oil droplets in a water- continuous phase through a bicontinuous structure and then to water droplets in oil as the propanol concentration was decreased. Many non-ionic surfactants can produce microemulsion without the addition of a co-surfactant. Systems that have been reported have generally been produced from combinations of hydrocarbon, water and a polyoxyethylene glycol alkyl ether non-ionic surfactant [C_mE_n, where m is the hydrocarbon chain length and n is the number of oxyethylene units]. There are several research paper available using $C_{12}E_5$ for the microemulsion formation (John, W. et al., 1988) (Vinson, P.K et al., 1991).

O/W microemulsion prepared using blends of the non-ionic surfactants polysorbate 60 (Tween® 60) and sorbitan monooleate (Span® 80) together with glycerol as cosurfactant and liquid paraffin as oil (Osipow, L et al., 1963), lecithin in combination with n-butanol as co-solvent was reported (Attwood, D et al., 1992). W/O microemulsion containing egg lecithin, water, hexanol and ethyl oleate (Gasco, M.R et al., 1988), using cetyl trimethyl ammonium bromide (CTAB) (a cationic surfactant) in combination with different cosurfactant (Rees, G.D et al., 1995) (Mehta, S.K et al., 1988) and also by using sodium bis-2-ethylhexylsulphosuccinate [an ionic surfactant] used as an effective stabiliser of w/o microemulsion (Osborne, D.W et al., 1988) (Bergenholtz, J et al., 1996) (Johnston, K.A et al., 1985) (Trotta, M et al., 1990). Several researchers also reported the use of

dioctyl sodium sulphosuccinate (Aerosol ® OT, AOT) as surfactant in the preparation of microemulsions intends as drug delivery vehicles. Combination of this anionic surfactant with the non-ionic surfactant like sorbitan monooleate (Span ® 20, Arlacel® 20) results in a microemulsion capable of incorporation of large quantities of water (Johnson, K.A et al., 1985). Osborne et al. showed that the sorbitan monolaurate was functioning as a cosurfactant in a similar manner to that of an alcohol in traditional microemulsion (Osborne, D.W et al., 1990). Beside this, polyoxyethylene -40- hydrogenated castor oil, polyoxyethylene -60- hydrogenated castor oil, polyoxyethylene-10- monolauric ester, polyoxyethylene -25- monostearic ester, polyoxyethylene -40- monostearic ester, polyoxyethylene -20- sorbitan monooleic ester, polyoxyethylene -20- sorbitan coconut oil ester, polyoxyethylene -9- monolauric ether, polyoxyethylene -77-polyoxypropylene -29-polyoxyethylene -77- copolymer (Pluronic F 68), polyoxyethylene -19-polyoxypropylene -43-plyoxyethylene -19- copolymer (Pluronic P84), decaglceryl monolauric ester, hexaglyceryl monolauric ester are also used as surfactants for the preparation of microemulsion (Kawakami, K. et al., 2002). Various literature also suggest the use of Labrasol® (Saturated polyglycolised C8 – C10 glyceride, HLB: 3-4), Labrafac CM10® (saturated polyglycolised C8 – C10 glyceride HLB: 10), Labrafil M-1944 CS® (polyglycolised glycerides from apricot kernel oil, HLB: 3-4), Lauroglycol® (propylene glycol laurate HLB: 4), plurol olique® (polyglyceryl oleate, HLB: 6) used as surfactant for the formation of microemulsion (Kommuru, T.R et al., 2001) (US Patent no. 5342625) (Meinzer, A et al., 1995).

The HLB of the surfactant can be useful guide for surfactant selection. The HLB takes into account the relative contribution of hydrophilic and hydrophobic fragments of the surfactant molecule. It is generally accepted that low HLB [3-6] surfactants are favoured for the formulation of w/o microemulsion whereas surfactants having high HLB (> 12) are preferred for the formation of o/w microemulsion. Ionic surfactants having HLB greater than 20 often require the presence of co-surfactants to reduce their effective HLB a value within the range required microemulsion formation.

The HLB also determine the type of microemulsion for example o/w, w/o or bicontinuous, through its influence on molecular packing and film curvature. The analysis of film curvature for surfactant association leading to the microemulsion

formation has been explained by in terms of the packing ratio, P, defined as v/a_0lc, where v is the partial molar volume of the surfactant, a_0 is the crossectional area (i.e., size of the surfactant head group) and lc the maximum length of the surfactant chain (Israelachvili, J. N et al., 1976) (Mitchell, D. J. et al., 1981). The packing ratio provides a direct measure of HLB and is influenced by the same factors. The o/w structures are favoured if the effective polar part ($P< 1$) and the interface curves spontaneously toward water (positive curvature). The w/o structures are formed when the interface curves in the opposite direction ($P>1$, negative curvature). At zero curvature, when HLB is balanced ($P\tilde{}\ 1$), either bicontinuous or lamellar structures may form according to the rigidity of the film. The relationship between surfactant HLB, molecular packing and film curvature is illustrated in Figure 2.7.

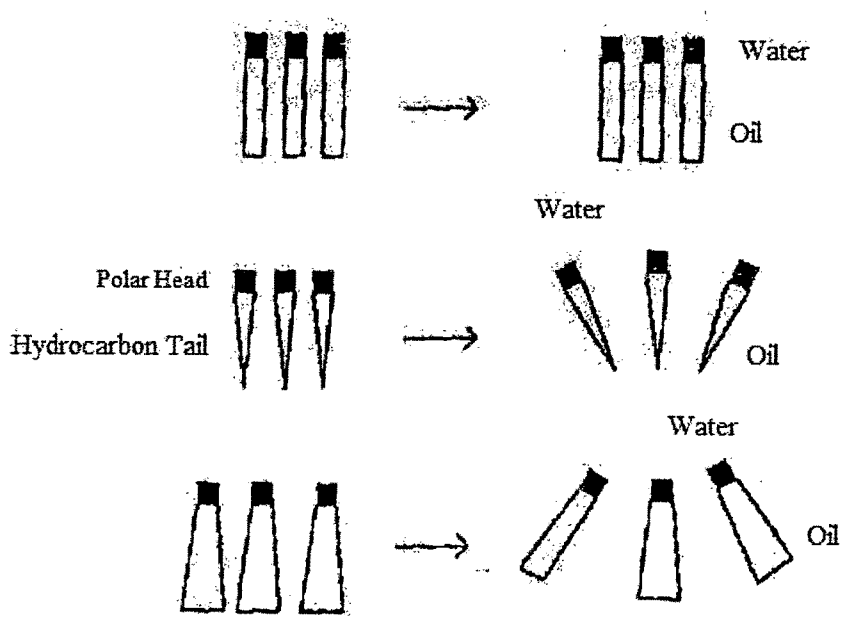

Figure 2.7.: A steric model correlating the shape of ampiphile to the spontaneous curvature of the interface. (Gennes, D et al., 1982)

2.3.7.6.2. Co-surfactant

It is generally not possible to achieve the required interfacial area with the use of single surfactants. If, however, a second ampiphillic is added to the system, the effects of the two surfactant can be additive provided that the absorption of one does not adversely affect that of the other and that mixed micelle formation does not reduce the available concentration of surfactant molecule. The second ampiphillic is referred to as the cosurfactant.

An essential requirement for the formation and stability of microemulsion is the attainment of a very low interfacial tension (?). Since microemulsions have a very large interface between oil and water because of the small droplet size, they can only be thermodynamically stable if the interfacial tension is so low that the positive interfacial energy (given by ?A, where A is the interfacial area) can be compensated by the negative free energy of mixing ?Gm. We can calculate a rough measure of the limiting ? value required as follows: ?Gm is given by ? TSm. (where ? T is the temperature), and the entropy of mixing ?Sm is of the order of the Boltzmann constant K_B; hence $K_BT = 4pr^2$? and thus for a droplet radius r of about 10nm, an interfacial tension 0.03 mN m^{-1} would be required. The role of the co-surfactant in the system is thus to reduce the interfacial tension between oil and water (typically about 50 mN m^{-1}) to this low level.

The interfacial tension between cyclohexane and water is approximately 42 mN m^{-1} in the absence of any added surfactant. The addition of the ionic surfactant sodium dodecylsulphate (SDS) in increasing amount causes a gradual reduction of ? to a value of about 2 mN m-1 at a SDS concentration of 10^{-4} g/ml. Further reduction of interfacial tension does not occur, since the cyclohexane / water interface is now saturated from micelles in the aqueous solution. Addition of 20% pentanol to the cyclohexane / water system in the absence of SDS reduces the interfacial tension to 10 mN m^{-1}. It is then theoretically possible by the addition of SDS to achieve a negative interfacial tension as SDS concentration below the level at which it forms micelles (critical micelle concentration, CMC) (Overbeek, J et al., 1978). Although pentanol is not regarded as a surfactant, it has the ability to reduce interfacial tension by virtue of its ampiphillic nature

(a short hydrophobic chain and a terminal hydrophilic hydroxyl group] and functions as the cosurfactant in this system. Its presence in this system means that the SDS is now required to produce a much lowering of the interfacial tension (10 mN m^{-1} rather than 42 mN m^{-1} in its absence] in order to produce microemulsion (Wakabayashi, T et al., 1984). Most single chain surfactant does not alter the oil-water interfacial tension sufficiently to form microemulsion, nor are they of the correct molecular structure (i.e., HLB). The cosurfactant is added to further lower the interfacial tension between the oil and water phases, fluidize the hydrocarbon region of the interfacial film and interfacial film curvature. Although free energy associated with the formation of microemulsion is negative, it is small, and therefore the order of mixing plays an important role in the time taken to reach equilibrium (Rosano, H.L et al., 1988). For example, the equilibrium is established more shortly if the surfactant is injected into the oil phase, as its greater solubility in this phase hinders its diffusion into aqueous phase. Thus the most appropriate cosurfactant is generally a small molecule, typically an alcohol of short to medium chain length (C3 –C8) which can diffuse rapidly between the bulk oil and water phases and the interface. Cosurfactant of short to medium chain-length alcohols also ensure that the interfacial film is flexible enough to deform readily around droplets, as their intercalation between the primary and surfactant molecules decreases both the polar head group interactions and the hydrocarbon chain interactions. Lamellar liquid crystalline phases rather than microemulsion phase often form with longer-chain-length cosurfactant or in the absence of cosurfactant due to rigidity of the interfacial film (Shinoda, K et al., 1984). Fletcher et al (Fletcher, D et al., 1989) reported that the length of the alcohol cosurfactant influence curvature, with bng-chain alcohols swelling the oil group region (negative curvature) more than those of shorter chain length.

Although medium chain alcohol likes pentanol, hexanol are used by many researchers because of their effectiveness form microemulsion but they are not suitable for pharmaceutical use due to their high irritant potential. Furthermore, the evaporation of the alcohol can destabilise the system. Administration of 6 – 9% propanol or 32% ethanol in water was shown to produce elongated mitochondria in rat liver after 1 month and mega-mitochondria after 2 months (Wakabayashi, T et al., 1984).

Due to this, less irritant non-ionic surfactant (polyoxyethylene alcohol esters) was investigated by many researchers for use as cosurfactant during microemulsion formulation (Johnson, K. A et al.,1985). PEG-liquid (polyethylene glycol derivative of Distearoyl phosphatidyl ethanolamine, PEG-DSPE, mean molecular weight of PEG: 2000) was used as cosurfactant for the preparation of Vincristine microemulsion [50]. Beside this ethanol (Dalmora, M.E et al., 2001), fatty acid esters of propylene glycol, oleic esters of polyglycerol, ethyldiglycol and polyethylene glycol, were also evaluated as cosurfactant for the microemulsion formulation. Alany et al (Alany, R.G·et al., 2000) shows the effect of eight different cosurfactants on the phase behaviour of the pseudo-ternary system water: ethyl oleate: nonionic surfactant blends (sorbitan mono-laurate / polyoxyethylene 20- sorbitan mono-oleate). Four aliphatic alcohols (1-propanolol, 1-butanol, 1-hexanol and 1-octanol) and four 1, 2- alkanediols (1, 2-prpanediol, 1, 2-pentadiol, 1, 2-hexanediol and 1, 2-octanediol) were used as cosurfactant. They showed how the zone of microemulsion changes in presence of different cosurfactant in compare to cosurfactant free system.

2.3.7.6.3. Oils

The oil component influence curvature by its ability to penetrate and hence swell the tail group region of the surfactant monolayer. Short chain oils penetrate the tail group region to a greater extent than long chain alkanes and hence swell this region to a greater extent, resulting in an increased negative curvature (and reduced effective HLB). Various long and medium chain triglyceride like labrafac, lauroglycol, labrafil M 1944CS and olive oil which are also reported (Kawakami, K et al., 2002) (Kommuru, T.R et al., 2001).

2.3.7.6.4. Temperature:

Temperature plays an important role in the formation of microemulsion when an non ionic surfactant is used in microemulsion formulation. At low temperature non-ionic surfactants are hydrophilic and form o/w microemulsion but at higher temperature, they are lipophilic and form w/o microemulsion. At an intermediate temperature, called the HLB temperature, hydrophilic-lipophilic interactions just balance and microemulsions may have bicontinuous structure (Warisnoicharoen, W et al., 2000).

The effect of temperature in microemulsion containing non ionic surfactant such as polyoxyethylene alkyl ethers as the polyoxyethylene group is dehydrated with increasing temperature. This has the effect of altering substantially the packing ratio and in extremity is manifested by phase separation or phase inversion. Ionic surfactants are not strongly influenced by temperature; a temperature rise does not cause an increase in positive curvature due to counter ion dissolution.

2.3.7.6.5. Surfactant-Cosurfactant ratio:

The surfactant and cosurfactant ratio is a key factor influencing the phase properties. Attwood et al showed how size and location of microemulsion is changed on changing the mass ratio of polysorbate 40 / sorbitol from 1: 1 to 1: 3.5 (Attwood, D et al., 1992). Similar studies using polysorbate 80 (Ktistis, G et al., 1990) and Polysorbate 60 (Attwood, D et al., 1989) have shown a change in the optimum polysorbate / sorbitol mass ratio (i.e., that producing the largest microemulsion region) from 1: 2.5 for polysorbate 80 to 1: 2 for polysorbate 60 to 1: 1.5 for polysorbate 40. such effects were attributed to differences in the packing of surfactant and cosurfactant at the oil/water interface, Gasco et al (Gasco, M.R et al., 1988) have used microemulsions of similar composition prepared using polysorbate 60 but with phosphate buffer rather than water, to study the in-vitro drug release of propranolol.

2.3.7.7. Phase diagram:

When oil, water and surfactants (presence or absence of cosurfactant) are mixed, microemulsions are one of a number of association structures (including emulsion, micelles, lamellar, hexagonal, and cubic and various gels and oily dispersion) that can form, depending on the chemical composition and concentration of each component. Construction of phase diagram is a useful approach to illustrate the complex series of interactions that can occur when different components are mixed. As quaternary phase diagram (for four component system) is time consuming and difficult to interpret, pseudo-tertiary phase diagram is often constructed to find the different zones including microemulsion zone in which each corner of the diagram represent 100% of the particular component. Figure 2.1 represents schematically the pseudo ternary phase diagram at

constant surfactant to cosurfactant ratio, showing microemulsion and associated lamellar and micellar structure.

Microemulsion can also exist in equilibrium with excess water, excess oil or both, which is know as Winsor Type I, Type II and Type III systems respectively (Winsor, P. A et al., 1974). The Winsor type I system consist of a lower phase o/w microemulsion coexisting with excess oil and type II system consist of an upper phase w/o microemulsion in equilibrium with excess water. The type III system forms when the surfactant (s) are concentrated in surfactant-rich bicontinuous middle phase which coexist with both oil and water which is schematically illustrated in Figure 2.8. The type of equilibrium depends on the concentration and chemical nature of the surfactant (s), oil and any solubilized substances.

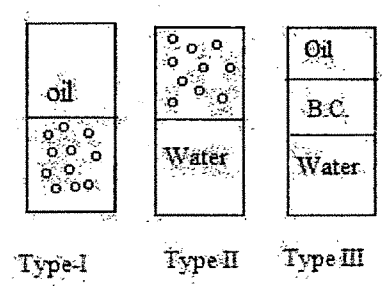

Figure 2.8.: Winsor Type I, Type II and Type III microemulsion.

2.3.7.8. Microemulsion characterisation:

It is essential to characterize the microstructure of a microemulsion as characteristic of this system which is very difficult due to its microstructure. But for successful commercial exploitation, it is essential to characterize the microstructure of a microemulsion. As reported, microstructure determines the rate of release of sodium salicylate from a lecithin based microemulsion (Khoshnevis, P et al., 1997). Different techniques are used to characterize this system. At the macroscopic level: viscosity conductivity and dielectric measurements provide useful information. Viscosity measurements can indicate the presence of rod like or worm-like reverse micelles (Yu, Z.J et al., 1995) (Angelico, R et al., 1998). Conductivity measurements provide a means of determining whether a microemulsion is oil-continuous or water-continuous, as well as

providing a means of monitoring percolation or phase inversion phenomena (D'Angelo M et al., 1996), (Yu, Z.J et 1995) (Mehta, S.K et al., 1999). Dielectric measurements provide the structure and dynamic feature of microemulsion systems (D'Angelo, M. D'Angelo, M. et al., 1996) (Feldman, Y et al., 1997) (Cirkel, P.A et al., 1998). But all the macroscopic properties depend on the microscopic structure. The size range of the microemulsion droplets and the inability to dilute many microemulsions to infinite dilution makes size determination difficult. Many techniques have been employed with varying success in the size analysis of microemulsion. It is generally true to say that, because of the limitation of this technique, it is preferable to employ a combination of technique for this system. NMR (Nuclear magnetic resonance) has become a standard technique for establishing phase diagrams of surfactant systems including both solution phase and liquid-crystalline phases. A significant advantage of the NMR method is that it is not necessary to achieve a macroscopic phase separation, and two- or three-phase character can be detected with single phase domains on the micrometer scale. Phase diagram studies by NMR are generally based on the fact that the rapid molecular dynamics causes an elimination of any spin interactions to an extent that is directly related to the degree of anisotropy of the structure. The presence of even very small quantity of isotropic phase is generally visible in ^{1}H and ^{13}C spectra. If a phase is isotropic on the relevant NMR time scale, static dipolar, quadrupolar and shift anisotropy interactions are averages to zero by molecular motion. If a phase is anisotropic, the spectrum should contain static interaction effects (Rades, T et al., 1988) (Giustini, M et al., 1996) (Olla, M et al., 1999).

a. Electron microscopy:

The macroscopic properties: viscosity, surface tension and conductivity of these systems depend on their microstructure. As these microstructures are very small (1-1000nm) and sometime several microstructure can coexist in the sane solution, it is difficult to determine their structure. Freeze Fraction Electron Microscopy (FFEM) and Transmission electron microscopy (TEM) is the most important technique for the study of microstructures because it directly produces images at high resolution and it can capture any coexistent structure and microstructure transitions (Parker Jr. W.O et al.,

1983) (Jahn, W et al, 1988) (Vinson, P.K et al., 1991) (Gulik-Krzywicki, T et al., 1984). In this technique the freezing of the microemulsion must be achieved sufficiently rapidly to avoid phase separation or crystallisation. This objective can be achieved by plunging the specimen into a liquid cryogen, by propane jetting, or by spray freezing (Jahn, W et al., 1988). The sample subsequently fractured and its visibility is enhanced by deposition of platinum-carbon layer in vacuum. The microemulsion sample, mounted on a support film and grid, is replicated by breaking apart the film and grid. The replica is then viewed by transmission electron microscopy and assumed the representative of the bulk microemulsion. Recent developments in the cryofixation technique have overcome many problems associated with artefact formation in earlier studies (Sjoblom, E et al., 1978) (Bellocq, A et al., 1979) (Biais, J et al., 1981). A complementary technique is of direct imaging, in which thin portions of the specimen are directly investigated in the frozen hydrated state by using a cryostage in the transmission electron microscope. The development of glass-forming microemulsions that do not breakdown during cooling and in which neither disperse nor matrix phase crystallizes during the cooling process has provided a way for direct studies of microemulsion structure. The first type of such systems to be reported were w/o microemulsions with a non-crystallising aqueous matrix obtained by adding propylene glycol to the water in the ratio 1 : 3 (MacFarlane, D.R et al., 1982) (Angell, C.A et al., 1984) .

b. Scattering Method:

Scattering method that have been employed in the study of microemulsions include Small Angle X-ray Scattering (SAXS) (Barnes, I.S et al., 1988) (Nakamura, N et al., 1999) (Shimobouji, T et al., 1989), Small Angle Neutron Scattering (SANS) (Bergenholtz, J et al., 1995) (Bolzinger, M.A et al., 1999) and static light scattering & dynamic light scattering or Photon Correlation Spectroscopy (PCS) (Patel, N et al., 1998) (Giustini, M et al., 1996) (Hantzschel, D et al., 1999). These techniques have a lower size limit of about 2 nm and an upper limit of about 100 nm for SANS and SAXS and a few microns for light scattering. In this case of monodisperse spheres interacting through hard sphere repulsion, the general expression for scattering intensity I(Q) is I(Q) = n P(Q) S(Q) where n is the number density of spheres and Q is the scattering vector (Q = 4 p Sin ?/ ?

with ? = scattering angle and ? = wavelength). The form factor P (Q) expresses the scattering cross-section of the particle, and the structure factor, S (Q) allows for inter-particle interference. Analytical expression may be used to calculate both P (Q) and S (Q) under favourable circumstances.

2.3.7.9. Storage Stability:

Storage stability test are much simpler and less frequently needed than for coarse dispersions, where droplet sizes and phase changes must be followed. For example, if temperature and freeze-thaw tests indicate that the system recovers quickly, the microemulsion is thermodynamically stable over these conditions and does not require frequent test on storage, unless of course chemical reaction occurs (e.g., oxidation, pH variations) which changes the nature of the components and hence of the microemulsions.

2.3.7.10. Application of Microemulsion as Drug Delivery System:

2.3.7.10. 1. Influence on Drug Release Characteristics:

Drug incorporated in microemulsions will partition between the hydrophilic and hydrophobic phase depending on their lipophilicity. The usefulness of the partition co-efficient of a drug on its release characteristics was reported by Trotta et al. (Trotta, M et al., 1989). The release of five drugs with different lipophilicity from o/w microemulsions composed of isopropyl myristate, butanol, AOT and Buffer (pH 7) was studied by determining the mass transfer constants of the drugs through a hydrophilic membrane separating the microemulsion from the receiving aqueous phase. A linear relationship between the release rate of the drug and its isopropyl myristate / water partition coefficient was noted. The rate of drug release from microemulsified drug delivery system was modified by the nature of the phase structure (Kriwet, K et al., 1995) , interaction between drug and surfactant (Gasco, M.R et al., 1988) (Pattarino, F et al., 1989), concentration of cosurfactant (Trotta, M et al., 1989). Prolonged release of propranolol was achieved by Gasco et al (Gasco, M.R et al., 1998)by increasing the partitioning of the drug using administration of octanoic acid into the dispersed oil phase.

As a result release rate through a hydrophilic membrane was decreased due to its decreased concentration in the continuous phase, providing a prolong release of drug.
The release of piroxicam- ß- cyclodextrin complex in microemulsions can be modified by inclusion of Carbopol 940® as reported by M. E. Dalmora et al. (Dalmora, M.E et al., 2001). Incorporation of an inert polymer matrix into a self-microemulsifying system which forms a microemulsion after in gestation [on contact with physiological fluid] ,from this gelled matrix continuous and uniform release of micro emulsified active agents occurs by gradual release (US Patent No. 6309665).

2.3.7.10. 2. For Topical Delivery:

Transdermal drug delivery system exhibit several advantages over other route notably oral route, by avoiding systemic side effects. But the main limiting factor for this delivery system is penetration of drug through stratum corneum, the outermost layer of the skin, which comprises keratin rich dead cells embedded in a lipid matrix. Considering the solubilizing capacity of microemulsion, these are expected to significantly affect the structure of the stratum corneum lipid self-assemblies, with obvious consequences for drug penetration. There have several studies of the penetration enhancement by microemulsion drug carriers. For example, lecithin containing w/o microemulsion for the transdermal administration of scopolamine and broxaterol and found that the transport rate obtained with the lecithin microemulsion gels was much higher than that obtained with an aqueous solution at the same concentration (Williman, H et al., 1992).
Similarly, the in vitro release of piroxicam from microemulsion provides a reservoir effect for piroxicam release and retardation of piroxicam release was obtained on incorporation of microemulsion into carboxyvinilic gel and in vitro studies showed significant inhibition of inflammation process (Dalmora, M.E et al., 2001). D. Paolino et al showed 4-5 times improvement in ketoprofen percutaneous penetration using two microemulsions as compared to conventional formulation which is due to the small particle of the internal phase droplets of microemulsion. Improvement of permeation was also due to the solubilizing effect of the drug elicited by the microemulsion lecithin matrix which having high affinity for epidermal tissue and is able to mix with the skin lipid components and permeation enhancer effect mediated by the lecithin component

(Paollino, D et al., 2002). Similar studies also suggest that the percutaneous absorption of tyrosine from o/w microemulsion was superior in compare to liquid crystal system and an emulsion (Fevrier, F et al., 1991). Enhanced delivery rate in a hairless mouse model was found in o/w microemulsion as compared to w/o microemulsions for the delivery of Prostaglandin E1. The microemulsions were based on oleic acid or Gellucire 44/14® as the oil phase and were stabilised by a mixture of Labrasol® (C8 and C1 polyglycolised glycerides) and Plurol Olique CC 497® as surfactant (Ho, H. O et al., 1998). The transdermal delivery from w/o microemulsion of diphenhydramine HCl into excised human skin was evaluated. The microemulsions were based on combination of Tween 80 and Span 20 with isopropyl myristate (Schmalfub, U et al., 1997). Skin permeation of felodipine, a calcium antagonist, from o/w microemulsions based on mixture containing Tween 20 and taurodeoxycholate (as surfactant], isopropyl myristate (as oil phase) and benzyl alcohol (as co-surfactant) was evaluated (Trotta, M et al., 1997). The transdermal penetration of glyceryl trinitrate through mouse skin was found to be enhanced by ten times (approx.) by formulating the drug in AOT micelles and reverse micelles and w/o microemulsions. Irritation studies showed that AOT reverse micellar solutions shows little or no erythema whereas normal micelles caused moderate irritation (Varshney, M et al., 1999). The use of lecithin, isopropyl myristate and water microemulsion for the transdermal transport of Indomethacin and diclofenac was reported (Dreher, F et al., 1997). Improvement of skin permeation of Diazepam from "visosized" o/w microemulsions prepared using egg lecithin, polysorbate 20, benzyl alcohol, isopropyl myristate and water / propylene glycol mixture was also reported (Trotta, M et al., 1999). Gasco et al. studied the gel like system obtained by adding the polymer Carbopol 934® to a microemulsion of decanol, dodecanol, Tween 20 and Propylene glycol containing solubilised azelaic acid. The diffusion studies on hairless mouse skin implied that the microemulsion gel was more efficient than the gel alone or cream (Gasco, M.R et al., 1988). Comparative studies have shown that an o/w microemulsion gel containing analgesic anti-inflammatory agent, flufenamic acid and stabilised by Polyethylene glycol-7glyceryl coccoate (Cetiol HE) was having higher bioavailability after topical administration as compared against macroemulsion, hydrogel and cream (El-Faham, T.H et al., 1992).

2.3.7.10. 3. Oral delivery

Apart from those involving penetration enhancement in topical administration, the most interesting drug delivery application lie in the oral administration of peptide and protein drugs. Despite the many promising feature of peptide in drug delivery, many of them are difficult to administer orally without a loss in activity. The general low bioavailability of peptides administered orally means that the intra and intersubject variability is magnified and a major reason for maximising the oral peptide bioavailability is ability to control the in vivo drug concentration considering the thermodynamic stability, resulting in excellent long term storage for protein and peptide drugs. For example, SK&F- 106760 and SK&F-110679, both water soluble RGD fibrinogen receptor antagonists, were formulated in microemulsion at pharmaceutically relevant levels and their uptake after intraduodenal administration was investigated. It was found that the presence of the SK&F- 110679 peptide in varying concentration did not influence the structure of the w/o microemulsion studied. Furthermore, bioavailability of SK&F- 106760 was increased dramatically for the microemulsion formulations composed to that of the aqueous solution. (Constantinides, P.P et al., 1994).

The oral bioavailability of peptide drugs e.g., cyclosporine, a potent immunosuppressive agent when given orally, the absorption of cyclosporine is incomplete, amounting to about 30% or less. Furthermore, the absorption of cyclosporine is variable and is affected by physiological and pharmaceutical factors such as bile, food and drug delivery vehicles. This results in a high intra- and intersubject variability in pharmacokinetic parameter (Drewe, J et al., 1993). Microemulsion not only has the potential of improving the bioavailability for peptide drugs but it also have potential for oral delivery of sparingly soluble lipophilic drugs with poor bioavailability as well as fo drugs unstable at the conditions present in the stomach. For example, Novelli et al. used a microemulsion to formulate WR 2721, which is employed in the radio- and chemotherapy of cancer and needs to be protected from acid hydrolysis in the stomach in order to retain its biological activity. Using a w/o microemulsion of CTAB, isooctane and butanol, the hydrolysis was slowed down considerably in the microemulsion compared the aqueous solution (Novelli, A et al., 1992). A twofold increase in bioavailability of CoQ10 was observed when

compared to powder formulation in an in vivo rat model. The microemulsion formulation used for bioavailability studies containing Myvacet 9-45® (40%), Labrasol ® (50%) and Lauroglycol ® (10%) (Kommuru, T.R et al., 2001). An oral microemulsion formulation was reported for N-4472 [N- 2-(3, 5-di-tert-butyl-4-hydroxyphenethyl)-4, 6-difluorophenyl]-N-[4-(n-benzyl piperidyl)] urea, which is poorly water soluble drug having a lipid lowering effect, using the microemulsion technique (Itoh, K et al., 2002). The above microemulsion formulation containing the L- Ascorbic acid, Gellucire® 44/14, Hydrogenated castor oil e.g. HCO-60® and sodium dodecyl sulphate. Nitrendipine, a poorly soluble drug, has shown a remarkable enhancement of bioavailability when HCO 60® microemulsion formulation of nitrendipine was compared against suspension or an oily solution in rat model (Kawakami, K et al., 2003). The bioavailability of the several drugs including peptides using microemulsion delivery system depends not only on the nanometric particle size and thermodynamic stability but also on the composition of the microemulsion including type of oil phase, ionic nature of the surfactant etc. Recently, reported data shows that incorporation of water soluble cellulosic polymer such as hydroxypropyl methylcellulose improves the concentration time profile as well as bioavailability of PNU- 91325, which is a poorly soluble drug and used as insulin action enhancing agent, in comparison to the microemulsion having the same formulation without HPMC. This is due to generation and maintenance of high free drug concentration during the absorption phase via generation of a supersaturated state that is stabilise by precipitation inhibitors such as HPMC .

As a result the dynamic equilibrium between the microemulsion micelles and the free drug in the resulting supersaturated solution dictates drug transport and absorption kinetics (Gao, P et al., 2004). A five fold increase in the bioavailability of biphenyl dimethyl dicaroboxylate (BDD), a drug use for the treatment of liver diseases, was observed from a premicroemulsion concentrate composed of Tween 80 and NeobeeM-5® at the ratio of 2:1 and 35% of triacetin which not only increase the solubility and immediate dispersion of drug in gastrointestinal tract but also increase the bioavailability (Kim, Chong-Kook et al., 2001).

S.M Khoo et al showed improvement of oral bioavailability of halofantrine, an antimalarial drug, using microemulsion drug delivery system (52-67%) as compared to

commercially available tablet formulation (8.6 ± 5.3%) and crystalline halofantrine base alone in a hard gelatine capsule. The increase in oral bioavailability was a consequence of delivering the drug in a solubilized and rapidly dispersed manner. They also showed the difference in bioavailability when microemulsion formulation was composed of different triglyceride (medium vs. long chain length) as oil phase along with cremophor EL as surfactant and absolute alcohol as cosurfactant (Khoo, S.M et al., 1998). Commercially available drugs that use self-micro emulsifying system include cyclosporine, (Sandimmune, Neoral ®) ritonavir (Norvir®) and saquinavir (Fortovase®) (Cooney, G. F et al., 1998) (Porter, C. J. H et al., 2001)

2.3.7.10. 4. Parenteral, Pulmonary and Ocular Delivery

Gasco and his group found that prolonged action and protection of biodegradable molecules might be achieved by administering w/o microemulsion parenterally. By using the microemulsion, the plasma concentration of testosterone was similar when compared to other sustained release formulation (Gasco, M.R et al., 1990). The AUC of vincristine, an antitumor agent, was much higher when given as microemulsion formulation as compared against solution of free drug. The microemulsion formulation was composed of surfactant as polyethylene glycol-lipid and cholesterol, oil phase as vitamin E solution of oleic acid (Junping, W et al., 2003). A non-aqueous microemulsion formulation composes of lecithin/glycerol/soybean oil and contained octyl ester of Ibuprofen. Microemulsion formulation was modified by the addition of poloxamer, which alters the absorption process resulting in drug being targeted to reticuloendothelial system-rich organs (Li, M.J et al., 1995). Lecithin-stabilised microemulsion formulation has been devised for the pulmonary delivery of Salbutamol, a bronchodialating agent, as pressurised aerosol system (Evans, R. M et al., 1992). A potential ophthalmic microemulsion formulation of indomethacin, diclofenac, chloramphenicol was prepared by using triacetin, castor oil, water and propylene glycol and stabilised by Poloxamer and in vitro permeation study was evaluated (Siebenbrodt, I et al., 1993). Later on, an ocular microemulsion formulation of pilocarpine was also evaluated which was stabilised by sucrose fatty acid ester surfactants (Keipert, S et al., 1994).

2.3.8 Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC)

SLN are particles made from solid lipids (i.e. lipids solid at room temperature and also at body temperature) and stabilised by surfactant(s). By definition, the lipids can be highly purified triglycerides, complex glyceride mixtures or even waxes (R.H. Muller et al., 1996). Recently, SLN based on para-acyl-calixarenes have been prepared and studied (P. Shahgaldian et al., 2003) (A. Dubes et al., 2003). Through the work of various research groups, the carrier system SLN has been characterized intensively. The first patents have been granted in 1993 and 1996 and contain claims on different production methods of SLN (M.R. Gasco et all., 1996) The main features of SLN with regard to parenteral application are the excellent physical stability, protection of incorporated labile drugs from degradation, controlled drug release (fast or sustained) depending on the incorporation model, good tolerability and sitespecific targeting. Potential disadvantages such as insufficient loading capacity, drug expulsion after polymorphic transition during storage and relatively high water content of the dispersions (70–99.9%) have been observed. The drug loading capacity of conventional SLN is limited (generally up to approximately 25% with regard to the lipid matrix, up to 50% for special actives such as Ubidecarenone) by the solubility of drug in the lipid melt, the structure of the lipid matrix and the polymorphic state of the lipid matrix (C. Schwarz et al., 1994) ((R.H. Muller et al., 1997) (W. Mehnert et al., 1997) (H. Bunjes et al., 1996) (R.H. Muller et al., 2000) (K. Westesen et al., 2000) . If the lipid matrix consists of especially similar molecules (i.e. tristearin or tripalmitin), a perfect crystal with few imperfections is formed. Since incorporated drugs are located between fatty acid chains, between the lipid layers and also in crystal imperfections, a highly ordered crystal lattice cannot accommodate large amounts of drug (W. Mehnert et al., 1997). Therefore, the use of more complex lipids (mono-, di- triglycerides and different chain lenghths) is more sensible for higher drug loading. The transition to highly ordered lipid particles is also the reason for drug expulsion. Directly after production, lipids crystallise—partially—in higher energy modifications (a, hV) with more imperfections in the crystal lattice (C. Freitas et al., 1999) (M. Radtke et al., 2001). The preservation of the a-modification during storage and transformation after administration (e.g. by temperature changes) could lead to a triggered and controlled release and has recently been investigated for topical

formulations (V. Jenning et al., 2000). If however a polymorphic transition takes place during storage, the drug will be expelled from the lipid matrix and it can then neither be protected from degradation nor released in a controlled way.

2.3.8.1. Preparation of SLNs:

Several methods exist for preparing solid lipid nanoparticles. The first method depends upon the application of mechanical homogenization to performed microparticles. Mechanical homogenization can be further classified as "hot" or "cold", depending upon the temperature of the lipid when exposed to the sheering process. In hot homogenization, the lipid component is frst melted and the drug either dispersed or dissolved in the molten liquid. Aqueous surfactant is added at the same temperature as the melt, and the mixture is subjected to high sheer to produce small particles. Upon cooling, the lipid/drug mixture solidifies into the required particle size (usually 50-100nm). The method depends heavily on the efficiency of the surfactant to stabilize the cooled mixture against agglomeration or Ostwald ripening. This technique may be suitable to large-scale production because of the likely availability of homogenization equipment in the pharmaceutical industry. One possible concern with the hot homogenization process is the stability of the drug molecule to the elevated temperature of the lipid melt. For example, if stearic acid (m.p. 70^0C) is employed as the lipid matrix, maintaining a drug at an elevated temperature for a long period of time may result in significant degradation. It may be argued that the length of time the drug is exposed to this temperature during the homogenization step is short, and so heat-accelerated drug degradation would be expected to be low. While it might be possible to consider employing other lipids with lower melting points, the lower limit of body temperature and the possible effects of higher aqueous solubility of alternate lipids must be kept in mind.

If the drug is extremely significant to heat, the alternative "cold homogenization" process can be employed (Westesen K et al, 2000). In the cold process, the lipid is melted and drug dissolved or dispersed. The mixture is immediately cooled to room temperature and homogenized to particle size in the range of 50-100microns. These large particles then dispersed in a cold solution of aqueous surfactant and subjested to higher sheer to achive

the required particle size. Assuming that the high sheer homogenization process does not elevate the temperature of the dispersion, the cold method may offer some protection to heat-sensitive molecules. Another advantage is that the cold method has the potential of minimizing the partitioning of less hydrophobic drug out of the liid core to the aqueous phase during homogenization.

The second general method for the production of SLN avoids the use of high sheer homogenization equipment to grind the lipid dispersion to the required size. Instead, the melted lipid and drug are combined with sufficient surfactant to form a microemulsion at the melt temperature. Microemulsions are characterized by a small and uniform droplet size. The microemulsion of the lipid melts acts as a template to directly form nanoparticles of both correct size and narrow distribution of sizes upon cooling (Hsu C-H et al, 2003). Like the cold homogenization process, the microemulsion method emploes elevate dtemparature for only as long as necessary to dissolve or disperse the drug. Because there is no requirement for high sheer machinery, the method has the potential to be easily scaled from the lab scale to the industrial-sized batch. One disadvantage of the method is the need for slightly higher concentration of surfactant necessary to form a microemulsion of the lipid component at elevated temperatures. Whether this excess surfactant content promote the partitioning of drug out of the lipid aggregate to aqueous phase micelles upon cooling has yet to be adequately determined. Certainly, reducing the surfactant concentrationafter preparation of nanoparticles complicates the manufacturing process. In considering, the use of the SLN orally, the concerns about the dose of surfactant administered with the preparation are much less severe than when the preparation in intended for parenteral administration.

A third, less frequently studied method for the production of nanoparticles has been proposed. Particle size reduction of the core lipid (such as mono-acid triglyceride tripalmitin) emulsified with soy lecithin is carried out with the aid of ultrasound energy (Garcia-Fuentes M et al., 2002). The advantage of this approach is that the SLNs are produced without the aid of high concentration of surfactants. As a disadvantage, it is not clear whether it would be possible to scale up the procedure under necessary ultrasonic condition.

2.3.8.2. Drug uptake by solid lipid nanoparticles:

Three methods have been proposed to describe the localization of drug molecules in SLN (ZurMuhlen A et al., 1998). The homogenization matrix model is characterized by drug dispersed evenly through the matrix, much like a solid solution. The enriched shell model is characterized by drug selectively locating at the interface, either by fast solidification of the matrix lipid or by successful completion of the drug for the interface. Drug dispersed by such a model might exhibit a significant burst effect during release (Muller RH et al, 2002) The enriched core model is characterized by drug selectively locating in the core of the SLN, perhaps due to more rapid solidification of the drug relative to the matrix material. It has been suggested that the enriched core model would be useful to produce a membrane-controlled releasing particles. (Muller RH et al, 2002) Concern for the localization of drug within particles is of critical importance for the application of this technology. It is likely that, as with micellar solubilization, the chemical stability of the drug is largely related to localization of drug within aggregate. In addition, release kinetics of drug from the particles may be related to localization of drug within aggregate. While these proposed models are reasonable staring points for the discussion of drug localization, little experimental work directly validating the models has been presented.

Administration of hydrophilic drug molecules via SLN, particularly proteins and peptides, presents a variety of unique challenges. First, hydrophilic proteins will likely show poor solubility in the lipid matrix of the SLN. A hydrophilic protein is probably located on the surface of the particle and held by electrostatic interactions. (Cui Z et al., 2002). On one hand, the large specific surface area of the SLN suspension is well suited for such a strategy. Unfortunately, surface localization of biological macromolecules carries new challenges. Surfaces are known to disrupt the structures of proteins, and there is no guarantee that once removed form the surface the protein will regain its active structure. (Bummer PM et al., 2000). Of course, bioactive peptides, being smaller that proteins, tend to lack the tertiary structure and are thus not sensitive to surface denaturation. On the other hand, compared to proteins, peptides have fewer potential contact points with the surface and thus may not be held tightly enough to take advantage of particle-based delivery. A second concern of protein, peptide or DNS attached to the

surface is the sensitivity to either enzymatic degradation or acid catalyzed degradation in the gastrointestinal tract. Attempts heve been made to form nanoparticles from w/o/w templates to encapsulated water-soluble molecules sensitive condition to the gastro intestinal tract. (Cui Z et al., 2002). It is postulated that the water soluble drug is encapsulated within the interior water core and thus removed from ready acess by protease enzymes. In vivo success has been limited to those systems incorporating polyethylene glycol containing co-surfactants, presumably to provide "stealth" conditions protecting the drug on the surface from attack by intestinal enzymes. (Tobio M et al., 2000)

2.3.8.3. Release of drug from SLNs:

The release profile characteristics of SLN may play a significant role on the application of the technology to oral drug delivery. If the SLN is being employed to increase the apparent solubility of a poorly soluble drug, rapid and full release of the active ingredient is desirable. If the drug is formulated in SLN to take advantage of the potential to target drug to the Peyer's patches, drug release should be delayed at least until such time as the SLNs are picked up by M-cells. Peyer's patches are collections of lymphoid tissue located at regular intervals in the gastrointestinal tract with the purpose of providing immunological protection against a variety of antigens, such as viruses. Of course, enhancement of bioavailability by this approach is also likely to be modulated by physiological factors such as gastric emptying time and gastrointestinal motility.

A great number of in vitro studies have examined the rate at which drug is released from SLNs. Most frequently, biphasic release patterns are observed. Despite the abundance of work, little directly comparable work is available in the literature. Stusies have implicated a wide variety of variables influencing the rate of release. Solubility of the drug in the lipd and drug/lipid interactions appears to influence the rate of release, perhaps through the partition coefficient. Even the temperature employed during the preparation of the SLN has been suggested as influencing release by enhancing the solubility of drug in the aqueous phase during particle formation and thereby promoting drug localization at the surface region. One of the most frequently mentioned characteristics influencing release appears to be particle size. Tetracaine loaded glycerol behanate (triglycerides) SLN

showed a burst release of up to 80% of the drug from psrticles 40nm in diameter but only 40% when the particles were about 200nm in diameter. (ZurMuhlen A, Schwarz C et al., 1998) The smaller particles have a larger specific surface area. The large specific area suggests that, compared to the 200nm particles, a larger fraction of the drug may be loaded close to the surface of the 40nm particles. This might help explain why the smaller particles initially release drug at a higher rate.

Early in the microparticles, literature, it was hypothesized that the uptake of microparticles could occur via transcellular and paracellular routes in enterocytes and by the Peyer'r patches and M-cells. (Kreuter J et al, 1991). Targeting of biodegradable polymer nanoparticles to Peyer's patches continues to be studied in great detail. Surface modification of polymers such as polylactide-co-glycolide with polyethylene glycol (PEG) (Vila A et al., 2002) or Chitosan (Janes KA et al., 2001) have shown to be enhanced by mucous membranes. While lees work has been published for SLN in the gastrointestinal tract, presumably the same principles apply. PEG on the surface does appear to enhance the physical stability of polylactic acid particles toward simulated gastric conditions. (Tobio M et al., 2000) While the ability of PEG on the surface is well known to inhibit the recognition of particles by the reticulo- endothelial system, it is less understood how it might influence the surface-mediated binding of particles to M-cells.

2.3.8.4. Crystallinity of the Lipid phase:

Of importance to any dispersed system dosage form is the amount of drug that can be incorporated and the apttern of release of the active ingredient. Recent studies have suggested that both characteristics of SLN are related to the crystallinity of the dispersed lipid phase. No matter what the method of preparation of SLN, each involves melting the lipid component and subsequent rapid cooling to solidify the SLN. It is not surprising than that the lipid components can solidify with various degree of crystallinity. Triglyceride tend to form a metastable a-form upon rapid cooling from the melt, which transforms to the more stable and crystalline ß-form as a function of time. (Westesen K et al., 1997). The rate of transformation appears to be sensitive to the specific surface area of the dispersion and is accelerated in similar particles. Several types have been identified. Amorphous systems are those that show no crystal formation during storage

and are most frequently those produced with the highest melting point lipids, or those with mixtures of lipids. Imperfect lipid phases are those that exhibit crytal growth, usually slow, during the storage phase (Westesen K et al., 1997). It appears that the lower the melting point of the lipid, the better the chance of a –to-ß transformation during the storage process. Mixtures of lipids are more successful in avoiding rapid conversion to more crystalline phase structures than are pure components.

What may not ne well recognized is that not all lipid components are completely phase compatible. Some lipids will de-mix upon cooling. The data of Zimmerman and Muller (Zimmerman E et al., 2001) also reveled that the oil phase had a strong effect on pH and electrolyte sensitivity. SLN based on Pluronic F68 showed identical zeta potentials but different stability when glycerol palmitostearte, glycerol monostearate, or glycerol behanate were employed were employed as the matrix. Another group of scientists (Feitas C et al., 1999) examined the effect of electrolytes on glycerol behanate SLN stabilised with Polaxomer surfactant. Multivalent cations calcium and aluminum had a stronger tendency to promote growth of the mean diameter upon storage than did monovalent cations such as sodium. The effect of multivalent cations suggests particle aggregation as opposed to Ostwald ripening as the means of size growth. Even low concentrations of sodium, well below those expecte din vivo, did show a destabilizing effect on dispersion stability. This suggests that zeta potential may be an important parameter to control for maximal stability of SLN dispersions. Whether stability in vitro correlates to enhanced bioavailability in vivo has yet to be determined.

Stability of the SLN in th environment of the gastrointestinal tract is likely to be very importantto the intended targeting of the particles. In particular, several lipid components (triglycerides, lecithin) of the formulations are sensitive to enzymatic degradation by the lipase/co-lipase system. If lipid that make up SLN are degraded rapidly by the lipase/co-lipase system, targeting of the drug to Peyer's patches could be adversely influenced. As indicated previously, lipase/co-lipase must bind to a lipid surface to express the catalytic responses. (Staggers JA et al., 1990). In the gastrointestinal tract, this results in the formation of mono and diglycerides from triglycerides and lysophosphatidylcholine from PC. Previous studies have shown that the chemical structure of the lipid has strong influence upon the rate of digestion. For example, the rate of hydrolysis of the ester bond

in a long chain triglyceride (16 carbons long or higher) is much slower than in shorter chains. (Staggers JA et al., 1990). In combination with bile salts, the degraded lipids form mixed micelles, which are absorbed by the physiologic lipid pathway (s). Thus, SLN sensitive to lipase / co-lipase may be degraded and digested prior to targeting Peyer's patches. Because binding of these enzymes of the surface of the lipid particle is the initial step in the digestion process, preventing the binding would eliminate the digestion and diversion from Peyer's patches.

As a final note, concerns such as chemical purity of the oil phases on stability of the dispersions have yet to be examined. Although most manufacturers supply highly purified oily components, the ester linkages are sensitive to degradation during storage. Potential degradation products- for example, fatty acids and mono and diglycerols- could compete successfully with formulation surfactants for the surface. (Holstborg J et al., 1999). Fatty acids are expected to modify the zeta potentials. (Washington C et al., 1987). Fatty acids and monoglycerides can form mixed micelles that might enhance the partitioning of hydrophobic drug out of the SLN (Staggers JA et al., 1990)

NLC have been introduced at the end of the 1990s in order to overcome the potential difficulties of SLN described above (M. Radtke et al., 2001) (R.H. Mu¨ ller et al., 2002) (M. Radtke et al., 2000) . The goal was the development of a nanoparticulate lipid carrier with a certain nanostructure in order to increase the payload and prevent drug expulsion. This could be realized in three ways. In the first model, spatially different lipids, e.g. glycerides composed of different fatty acids are mixed. Using spatially different lipids leads to larger distances between the fatty acid chains of the glycerides and general imperfections in the crystal and thus to more room for the accommodation of guest molecules. The highest drug load could be achieved by mixing solid lipids with small amounts of liquid lipids (oils). This model is called "imperfect type NLC". If higher amounts of oil are mixed with the solid lipid, a different type of nanostructure is present. Here, the solubility of the oil molecules in the solid lipid is exceeded; this leads to phase separation and the formation of oily nanocompartments within the solid lipid matrix (V. Jenning et al., 2000, 2001). Many drugs show a higher solubility in oils than in solid lipids so that they can be dissolved in the oil and still be protected from degradation by

the surrounding solid lipids. This type of NLC is called "multiple type NLC" and can be regarded as an analogue to w/o/w emulsions since it is an oil-in-solid lipid-in-water dispersion. Since drug expulsion is caused by ongoing crystallization or transformation of the solid lipid, this can be prevented by the formation of a third type, the "amorphous type NLC". Here, the particles are solid but crystallisation upon cooling is avoided by mixing special lipids (e.g. hydroxyoctacosanylhydroxystearate and isopropylmyristate) (V. Jenning, et al., 2000). So far, NLC have only been exploited for the topical delivery, however their advantages over conventional SLN are also of interest for parenteral application routes.

Melt-emulsified nanoparticles based on lipids (or waxes) solid at room temperature have been developed (B. Siekmann et al., 1992) (R. H. Müller et al., 1995) (R. H. Müller et al., 1986) (K. Westesen et al., 1998). Advantages of these solid lipid nanoparticles (SLN) are the use of physiological well-tolerable lipids (R. H. Müller et al., 1996), the avoidance of organic solvents in some preparation processes, a wide potential application spectrum (dermal, peroral, intravenous) and high pressure homogenization (R. Lander et al., 2000) as an established production method which allows large scale production. To manufacture SLN, the hot high pressure homogenization above the melting point of the lipid and subsequent recrystallization (N. Rodriguez-Hornedo et al., 1990) is recommended (meltemulsification), but the cold high pressure homogenization (S. Liedtke et al. 2000) (high pressure milling of lipid suspensions) for thermo labile drugs exists, too. Other production methods for SLN as the production from microemulsions (M. R. Gasco et al., 1993), the precipitation (B. Siekmann et al., 1996) and dispersing by ultrasound (P. Speiser et al., 1990) (A. J. Domb et al., 1993) are published and differ normally in obtained particle size distribution. Noteworthy basic information concerning the structure of lipids (K. Sato et al., 1999) (F. D. Gunstone et al., 1986) (R. J. Hamilton et al., 1999) and the influence of stabilizers during production process [156-158] are provided in the literature. Additionally, for SLN improved bioavailability, protection of sensitive drug molecules from the environment (water, light) and controlled release characteristics (R. H. Müller et al., 1995) (R. H. Müller et al., 2000) (E. Ugazio et al., 2002) have been claimed. Successful incorporation not only of lipophilic, but even of

hydrophilic drug molecules has been postulated [160,161]. Common disadvantages of SLN include particle growth, particle aggregation, unpredictable gelation tendency, unexpected dynamics of polymorphic transitions, burst drug release and inherently low incorporation capacities due to the crystalline structure of the solid lipid (K. Westesen et al., 1997) (K. Westesen et al., 1997). It has been proposed that this last mentioned drawback can be overcome by oil loaded solid lipid nanoparticles (also described as nanostructured lipid carriers or NLC) (V. Jenning et al., 2000) (R. H. Müller et al., 2002). Liquid lipids solubilize drugs to a much higher extent than solid lipids. In a preferred scenario, the liquid lipids form droplets within the solid lipid particles matrix. According to this model, the NLC nanoparticles would provide a high incorporation capacity (due to the liquid lipid) and control of drug release (due to the encapsulating solid lipid). It has been postulated that medium chain triglyceride (MCT) molecules can replace glyceryl behenate (GB) molecules in the crystal lattice in a random distribution up to a MCT load of 16 % (weight % of total lipid) (V. Jenning et al., 2000) . Even higher oil loads up to 38 % have been described to be incorporated as MCT clusters inside the solid matrix. Thereby, the solid particles are described to be spherical (R. H. Müller et al., 2002) (A. Dingler et al., 1999) (A.Z. Mühlen et al., 1996) what should offer a maximal volume for oil and drug incorporation. Despite of reinforced investigation efforts, until today neither SLN nor NLC are clinically used.

2.3.9. Polymer nanoparticles

Depending on the desired properties for polymer nanoparticles, polymers can be synthesized in a wide range of chain length, type and number. Polymers suitable for the preparation of nanoparticles include cellulose derivatives, poly(alkylcyanoacrylates), oly(methylidene malonate), polyorthoesters, polyanhydrides and polyesters such as poly(lactid acid), poly(glycolic acid) and poly(e-caprolactone) and their copolymers (G. . Barratt et al., 2000).

Obviously, toxicity of the substances, of radical starters or of solvent residues (J. Kreuter et al., 1994) has to be taken into calculation. Polymers used for parenteral delivery have to be biodegradable, they mostly belong to polyesters (i.e. polylactides) (E. Allémann et

al., 1993) (L. Brannon-Peppas et al. 1995) or to the group of polyacrylates (i.e. polycyanoacrylates) (P. Couvreur et al., 1991). For peroral administration, nondegradable polymers such as acrylate- and cellulose-derivatives can be used for nanoparticles designed not be absorbed (E. Allémann etal., 1993). In analogy to nanocapsules, attempts are done to modify the surface of the carriers. But again, little is known about the detailed in vivo fate of polymer nanoparticles (J. Kreuter et al., 1991). For the production of polymer nanoparticles monomers can be built up to polymer particles or preformed polymers are reduced to nanoparticles. Various procedures are applied: the coacervation technique, the solvent evaporation (M. C. Venier-Julienne et al., 1996) and solvent diffusion methods, the production by interfacial polymerization (J. Kreuter et al., 1994), the denaturation or desolvation of natural proteins or carbohydrates (J. Kreuter et al., 1994), and the degradation by high-shear forces (i.e. by high pressure homogenization (R. Gurny et al., 1981) or by micro fluidization (R. Bodmeier et al., 1990). Often, particles based on polymers are reproducible to manufacture and some systems show remarkable storage stability (D. Lemoine et al., 1996). Colloidal particles are either left as aqueous dispersion or they are converted into solid form, usually by lyophilization (E. Allémann et al, 1993).

Nanoparticles serve as carriers for a broad variety of ingredients (i.e. conventional drugs, antigens, vaccines or enzymes). The active components may be either dissolved in the polymeric matrix or entrapped or adsorbed onto the particle surface. Depending on polymer, drug and polymer interaction and production procedure, drug release differs (L. Illum et al., 1986). Polymer erosion, drug diffusion through the matrix or desorption from the surface may occur. Whereas polymeric microparticles entered the market (e.g. Enantone Depot^{TM}), no polymeric nanocarrier is available yet.

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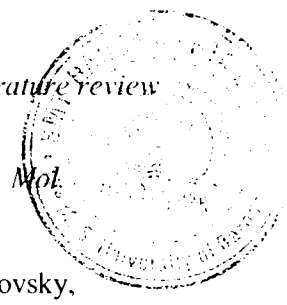

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2.4. Profile of Acyclovir

2.4.1. Introduction:

Acyclovir is a synthetic purine nucleoside analogue, which is structurally different from guanine due to the presence of an acyclic lateral chain (McEvoy G K et al 1988).

It is the most widely used antiviral agent used in its original form or as the prodrug valacyclovir in the treatment of infection caused by herpes simplex virus (HSV) mainly HSV-1, HSV-2 and verciella zoster virus (VZS). (Colloins P et al 1979) (DeClercq E et al 1980) (Frighny RJ et al 1981). This is structurally different from guanine due to the presence of an acyclic lateral chain.

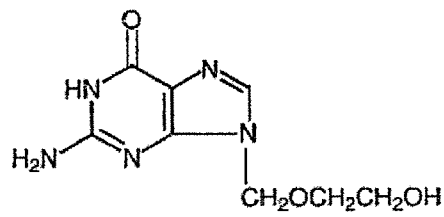
2.4.2. Description

Acyclovir is a white, crystalline powder

2.4.3. Chemical name:

2-amino-1, 9-dihydro-9- [(2-hydroxyethoxy) methyl]- guanine. (Acyclovir, AHFS drug information, 1997) (McEvoy GK. et al, 1998)

2.4. 4. Structural Formula:



2.4.5. Molecular Formula: $C_8H_{11}N_5O_3$

2.4.6. Molecular weight: 225

2.4.7. Physicochemical properties

2.4.7.1. Solubility: The maximum solubility in water at 37°C is 2.5 mg/ml.

2.4.7.2. pKa: 2.27 and 9.25.

2.4.8. Clinical pharmacology

2.4.8.1. Mechanism of action:

The inhibitory activity of acyclovir is highly selective due to its affinity for the enzyme thymidine kinase (TK) encoded by HSV and VZV (O'Brien JJ et al 1998) This viral enzyme converts acyclovir into acyclovir monophosphate, a nucleotide analogue. The monophosphate is further converted into diphosphate by cellular GMP kinase and into triphosphate by a number of cellular enzymes. In vitro, acyclovir triphosphate stops replication of herpes viral DNA. This is accomplished in 3 ways:

1) Competitive inhibition of viral DNA polymerase,
2) Incorporation into and termination of the growing viral DNA chain,
3) Inactivation of the viral DNA polymerase. The greater antiviral activity of acyclovir against HSV compared to VZV is due to its more efficient phosphorylation by the viral TK.

The mode of acyclovir phosphorylation in cytomegalovirus-infected cells is not clearly established but may involve virally induced cell kinases or an unidentified viral enzyme. Acyclovir is not efficiently activated in cytomegalovirus-infected cells, which may account for the reduced susceptibility of cytomegalovirus to acyclovir *in vitro*.

2.4.8.2. Antiviral activities

The quantitative relationship between the in vitro susceptibility of herpes viruses to antivirals and the clinical response to therapy has not been established in humans, and virus sensitivity testing has not been standardized. Sensitivity testing results, expressed as the concentration of drug required to inhibit by 50% the growth of virus in cell culture (IC50), vary greatly depending upon a number of factors. Using plaque-reduction assays, the IC50 against herpes simplex virus isolates ranges from 0.02 to 13.5 mcg/mL for HSV-1 and from 0.01 to 9.9 mcg/mL for HSV-2. The IC50 for acyclovir against most laboratory strains and clinical isolates of VZV ranges from 0.12 to 10.8 mcg/mL. Acyclovir also demonstrates activity against the Oka vaccine strain of VZV with a mean IC50 of 1.35 mcg/mL.

2.4.9. Uses:

In immunocompetent persons, the clinical benefits of acyclovir are greater in initial HSV infections than in recurrent ones, which typically are milder in severity (whitley and Gnann, 1992). Acyclovir is particularly useful in immunocompromised patients, because these individual experiences both more frequent and more severe HSV and VZV infections. Since VZV is less susceptible than HSV to acyclovir, higher doses must be use for the treating varciella or zoster cases than for HSV infections.

HSV infections: In initial genital HSV infections, oral acyclovir (200mg five times daily for 10days) is associated with significant reductions in virus shedding, symptoms, and time to healing (Fife et al 1997). Intravenous acyclovir (5mg/kg per 8 hours) has similar effects in patients hospitalized with severe primary genital HSV infections. Topical acyclovir is much less effective than systemic administration. Patient-initiated acyclovir (200mg 5 times daily for 5 days) shortens the manifestation of recurrent genital HSV episodes by 1 to 2 days. (Tyring et al, 1998). Topical acyclovir offers no significant clinical benefit in recurrent genital herpes. Frequently recurrent genital herpes can be suppressed effectively with chronic oral acyclovir 9400mg two times daily or 200mg three times daily) (Goldberg et al, 1993). During use, recurrence decreases by about 90%, and the majority of patients are free from symptomatic recurrence for periods up to 5 yeas. Asymptomatic shedding may occur during suppression, as may HSV transmission to sexual partners. Chronic suppression may be useful in those with disabling recurrence of herpetic window or HSV- related erythmea multiforme.

Oral acyclovir is effective in primary hepatic gingivostomatitis (600mg/m2 four times daily for 10 days in children) but provide modest clinical benefit in recurrent orolabial herpes. Topical acyclovir ointment is not clinically beneficial in recurrent herpes labialis. Topical acyclovir cream is not clinically beneficial in the United States, may be more effective in recurrent labial and genital herpes simplex virus infections. Pre-exposure acyclovir prophylaxis (40mg twice daily for 1 week) reduces the overall risk of recurrence by 735 in those with sun-induced recurrence of HSV infections. (Spruance et al., 1988).

In immunocompromised patient with mucocutaneous HSV infection, intravenous acyclovir (250mg/m2 per 8 hours for 7 days) shortens healing time, duration of pain and

the period of shedding (Wade et al., 1982). Oral acyclovir (800mg 5 times daily) also is effective. Recurrences are common after cessation of therapy and may require long-tem suppression. In those with very localized labial or facial HSV infections, topical acyclovir may provide some benefit. Intravenous acyclovir may be beneficial in viscerally disseminating HSV in immunocompromised patients and in HSV-infected burn wounds.

Systemic acyclovir prophylaxis is highly effective in preventing mucocutaneous HSV infections in seropositive patients undergoing immunosupression. Intravenous acyclovir (250mg/m2 every 8 to 12 hours), begun prior to transplantation and continuing for several weeks, prevents HSV disease in bone-marrow transplant recipients. For patient who can tolerate oral acyclovir (400mg five times daily) is effective and long term oral acyclovir (200 to 400mg three times daily for 6 months) also reduces the risk of VZV infection (Steer et al. 2000). Oral acyclovir prophylaxis is also effective in transplant patients and in those on chemotherapy.

In HSV encephalitis, acyclovir (10mg/kg per 8 hours for a minimum of 10 days) reduces mortality by over 50% and improves overall neurologic outcome compared to vidarabine (Whitley et al., 1986). Higher doses (15-20mg/kg per 8 hours and treatment to 21 days) are recommended by some experts. Intravenous acyclovir (20mg/kg per 8 hours for 21 days) is more effective than lower doses in neonatal HSV infections (kimberlin et al., 1999). In neonates and immunocompromised patients, and rarely, in previously healthy persons, relapses o encephalitis following acyclovir indicate that longer course of treatment are needed.

An ophthalmic formulation of acyclovir, not available, in United States, is at least as effective as topical vidarabine or trifluridine in herpic keratoconjunctivitis.

2.4.10. Pharmacokinetic:

2.4.10.1. Absorption:

The oral bioavailability of acyclovir range s form 10% to 20% and decreases with increasing dose (Wagstaff et al, 1994). The overall pharmacokinetic of acyclovir is reported in the following Table 2. 1

Table 2. 1: Acyclovir Pharmacokinetic characteristics (range)

Parameter	**Range**
Plasma protein binding	9% to 33%
Plasma elimination half-life	2.5 to 3.3 h
Average oral bioavailability	10% to 20%*

*Bioavailability decreases with increasing dose.

In one multiple-dose, cross-over study in healthy subjects (n = 23), it was shown that increases in plasma acyclovir concentrations were less than dose proportional with increasing dose, as shown in Table 2. 2. The decrease in bioavailability is a function of the dose and not the dosage form. (Fletcher C et al 1985).

Table 2.2: **Acyclovir Peak and Trough Concentrations at Steady State**

Parameter	**200mg**	**400mg**	**800mg**
C^{SS}_{max}	0.83 mcg/mL	1.21 mcg/mL	1.61 mcg/mL
C^{SS}_{trough}	0.46 mcg/mL	0.63 mcg/mL	0.83 mcg/mL

2.4.10.2. Distribution:

Acyclovir distributes widely in body fluids including vesicular fluid, aqueous humor and cerebrospinal fluid. Compared to plasma, salivary concentrations are low, and vaginal secretion concentrations vary widely. Acyclovir is concentrated in breast milk, amniotic fluid, and placenta. Newborn plasma levels are similar to maternal ones (Frenkel et al. 1991). Percutaneous absorption of acyclovir after topical administration is low.

2.4.10.3. Elimination:

The mean plasma half-life ($t_{1/2}$) of elimination of acyclovir is about 2.5 hours, with a range of 1.5 to 6 hours in adults with normal renal function. The plasma $t_{1/2}$ of elimination is about 4 hours in neonates and increases to 20urs in anuric patients (Blum et al, 1982). Renal excretion of un-metabolized acyclovir by glomelular filtration and tubular secretion is the peripheral route of elimination. Less than 15% is excreted as 9-

carboxymethoxymethylguanine or minor metabolites. The pharmacokinetic of acyclovir appear to be similar in pregnant and non-pregnant women (Kimerlin et al., 1998)

2.4.11. Indication:

Acyclovir capsules and suspension are indicated for the treatment of initial episodes and the management of recurrent episodes of genital herpes in certain patients. Acyclovir capsules, tablets, and suspension are indicated for the acute treatment of herpes zoster (shingles) and chickenpox (varicella).

2.4.12. Dosage and administration:

Acute Treatment of Herpes Zoster: 800 mg every 4 hours orally, 5 times daily for 7 to 10 days.

Genital Herpes: *Treatment of Initial Genital Herpes:* 200 mg every 4 hours, 5 times daily for 10 days.

Chronic Suppressive Therapy for Recurrent Disease: 400 mg 2 times daily for up to 12 months, followed by re-evaluation. Alternative regimens have included doses ranging from 200 mg 3 times daily to 200 mg 5 times daily. The frequency and severity of episodes of untreated genital herpes may change over time. After 1 year of therapy, the frequency and severity of the patient's genital herpes infection should be re-evaluated to assess the need for continuation of therapy with acyclovir.

Intermittent Therapy: 200 mg every 4 hours, 5 times daily for 5 days. Therapy should be initiated at the earliest sign or symptom (prodrome) of recurrence.

Treatment of Chickenpox: *Children (2 years of age and older):* 20 mg/kg per dose orally 4 times daily (80 mg/kg per day) for 5 days. Children over 40 kg should receive the adult dose for chickenpox.

Adults and Children over 40 kg: 800 mg 4 times daily for 5 days.

2.4.13. Contraindication:

Acyclovir capsules, tablets, and suspension are contraindicated for patients who develop hypersensitivity or intolerance to any component of the formulations.

2.4.14. Storage:

Store at 15° to 25°C (59° to 77°F) and protect from moisture.

2.4.15. Adverse effects:

2.4.15.1. Herpes Simplex:

Short-Term Administration: The most frequent adverse events reported during clinical trials of treatment of genital herpes with orally administered acyclovir were nausea and/or vomiting in 8 of 298 patient treatments (2.7%) and headache in 2 of 298 (0.6%). Nausea and/or vomiting occurred in 2 of 287 (0.7%) of patients who received placebo.

Less frequent adverse events, each of which occurred in 1 of 298 patient treatments with orally administered acyclovir (0.3%), included diarrhea, dizziness, anorexia, fatigue, edema, skin rash, leg pain, inguinal adenopathy, medication taste, and sore throat.

Long-Term Administration: The most frequent adverse events reported in a clinical trial for the prevention of recurrences with continuous administration of 400 mg (two 200 mg capsules) 2 times daily for 1 year in 586 patients treated with acyclovir were: nausea (4.8%), diarrhea (2.4%), headache (1.9%), and rash (1.7%). The 589 control patients receiving intermittent treatment of recurrences with acyclovir for 1 year reported diarrhea (2.7%), nausea (2.4%), headache (2.2%), and rash (1.5%).

The most frequent adverse effects reported during the second year by 390 patients who elected to continue daily administration of 400 mg (two 200 mg capsules) 2 times daily for 2 years were headache (1.5%), rash (1.3%), and paresthesia (0.8%). Adverse events reported by 329 patients during the third year include asthenia (1.2%), paresthesia (1.2%), and headache (0.9%).

2.4.15.2. Herpes Zoster:

The most frequent adverse effects reported during three clinical trials of treatment of herpes zoster (shingles) with 800 mg of acyclovir 5 times daily for 7 to 10 days in 323 patients were: malaise (11.5%), nausea (8.0%), headache (5.9%), vomiting (2.5%),

diarrhea (1.5%), and constipation (0.9%). The 323 placebo recipients reported malaise (11.1%), nausea (11.5%), headache (11.1%), vomiting (2.5%), diarrhea (0.3%), and constipation (2.4%).

2.4.15.3. Chickenpox:

The most frequent adverse events reported during three clinical trials of treatment with chickenpox with oral acyclovir in 495 patients were: diarrhea (3.2%), abdominal pain (0.6%), rash (0.6%), vomiting (0.6%), and flatulence (0.4%). The 498 patients receiving placebo reported: diarrhea (2.2%), flatulence (0.8%), and insomnia (0.4%).

2.4.15.4. Observation during clinical practice:

Based on clinical practice experience in patients treated with oral acyclovir in the U.S., spontaneously reported adverse events are uncommon. Data are insufficient to support an estimate of their incidence or to establish causation. These events may also occur as proof of the underlying disease process. Voluntary reports of adverse events, which have been received since market introduction, include:

General: fever, headache, pain, peripheral edema, and rarely, anaphylaxis

Nervous: confusion, dizziness, hallucinations, paresthesia, somnolence (These symptoms may be marked, especially in older adults.)

Digestive: diarrhea, elevated liver function tests, gastrointestinal distress, nausea

Hemic and Lymphatic: leukopenia, lymphadenopathy

Musculoskeletal: myalgia

Skin: alopecia, pruritus, rash, urticaria

Special Senses: visual abnormalities

Urogenital: Nephrotoxicity, elevated creatinine

2.4.16. Overdosage

Patients have ingested intentional overdoses of up to 100 capsules (20 g) of acyclovir, with no unexpected adverse effects.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded. Renal lesions considered to be related to obstruction of renal tubules by precipitated drug crystals occurred in the following species: rats treated with IV and IP doses of 20 mg/kg/day for 21 and 31 days, respectively, and at SC doses of 100 mg/kg/day for 10 days; rabbits and SC and IV doses of 50 mg/kg/day for 13 days; and dogs at IV doses of 100 mg/kg/day for 31 days. A 6-hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored

2.4.17. Precaution

The half-life and total body clearance of acyclovir are dependent on renal function. A dosage adjustment is recommended for patients with reduced renal function. Acyclovir has caused decreased spermatogesis at high parenteral doses in some animals and mutagenesis in some acute studies of this drug at high concentrations of drug. The recommended dosage should not be exceeded.

Exposure of herpes simplex and varicella-zoster isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. The possibility of the appearance of less sensitive viruses in humans must be borne in mind when treating patients. The relationship between the *in vitro* sensitivity of herpes simplex or varicella-zoster virus to acyclovir clinical response to therapy has yet to be established.

Because of the possibility that less sensitive virus may be selected in patients who are receiving acyclovir, all patients should be advised to take particular care to avoid potential transmission virus if active lesions are present while they are on therapy. In severely immunocompromised patients, the physician should be aware that prolonged or

repeated courses of acyclovir may result in selection of resistant viruses which may not fully respond to continued acyclovir therapy.

Caution should be exercised when administering acyclovir to patients receiving potentially nephrotoxic agents since this may increase the risk of renal dysfunction.

2.4.18. Quantitative method

2.4.18.1. HPLC:

Table 2.3 summarizes the available method for the estimation of acyclovir.

Table 2. 3: Summary of HPLC methods for analysis of acyclovir

Column used	**Mobile phase**	**Detection**	**Medium**	**Reference**
Waters Oasis HLB columns	18% acetonitrile, SDS (5m*M*) and phosphate buffer at pH 2.6	UV detection 250-260 nm	Plasma	Fernandez, M. et al 2003
C8 (150 x 4.6 mm)	0.02 M HClO4 with pH 2.0 prepared by double distilled water	Fluorescence detector. Ex: 260 nm. Em: 376 nm	Serum, saliva and urine	Testerecih et al 1998
SymmetryShield C8, 3.9x 150mm, 5µm	20mM Ammonium acetate, pH 5	UV detection, 250nm	Plasma	Waters manual
C-8 column (150 x 2.1 mm, 5 µm)	10mM acetate-citrate buffer:3.7 mM aqueous octanesulfonic acid (87.5:12.5 adjusted to pH 3.08 with phosphoric acid	UV detection, 254nm	Plasma, amniotic fluid	Stacy D. Brown et al, 2003

Cont.....

Column used	Mobile phase	Detection	Medium	Reference
C-18 column Ultrasphere, 5μm, 4.6 mm x 15 cm	0.02M K_2HPO4: Methanol (90:10)	UV detection, 250nm	Transbaccal Formulation	Amir H et al, 1998
Spherisorb ODS (250x4.6 mm)	95% aqueous phosphate buffer (pH 3.0) and 5% HPLC methanol	UV detection, 254nm	Liposome formulation	Caamano, M.M, et a; 1999
LiChrocart 250-4 RP8, 5 mm,	Acetonitrile and amonium acetate buffer 10 mM, pH 5 (2 : 98)	UV detection, 254nm	Plasma	M. Cociglio et al. 1998.
ERC-ODS-1161, 250 mm3 6.0 mm	20 mM KH_2 PO_4	Amperometric detection	Plasma	Satoshi Kishino et al,2002
Hypersil ODS, 3 mm, (15034.6 mm I.D.)	0.02 mol/ l KH_2PO_4, pH 3.5	UV detection, 254nm	Plasma	Roselyne Boulieu et al, 1997
LiChrosorb RP-8 7μm, 250 x 4 mm I.D	1% acetonitrile in 0.02 *M* disodium hydrogen orthophosphate (pH 2.5)	UV detection, 380nm	Plasma	Kok-Khiang Peh et al., 1997

2.4.18.2. Radioactivity measurements: (Choi et al.2001)

Acyclovir was determined in urine, amniotic fluid, and milk sample after administration of acyclovir into rat. The samples were mixed with Lumagel Safe (Lumac*LSC, Groningen, The Netherlands) and counted directly for radioactivity. Plasma was solubilized with Soluene-350 (Packard Instrument Co., Meriden, CT) and assayed for radioactivity following the addition of Lumagel Safe and glacial acetic acid to minimize chemiluminescence. Fetus and placenta were air-dried and combusted with a sample

oxidizer (Tri-Carb model 307, Packard) without further processing. Feces were homogenized with water and then combusted using aliquots. The resulting $^{14}CO_2$ was adsorbed on Carbo-sorb E (Packard) and then mixed with Permafluor E1 scintillation fluid (Packard). The radioactivity of sample was measured using a liquid scintillation analyzer (Tri-Carb 1500, Packard) and converted to equivalents of acyclovir based on the specific radioactivity of the administered [^{14}C] acyclovir.

2.4.18.3. LC–MS–MS system (Chin-chung Lin et al., 2002)

The HPLC system consisted of a Perkin-Elmer Series 200 Micro LC pump and a Perkin-Elmer Series 200 autosampler (Perkin-Elmer Instruments, Shekton, CT, USA) fitted with Peltier cooling trays (maintained at 5 8C). Separation was accomplished on an Intertsil Silica column, 5033.0-mm, 5-mm (Keystone Scientific, Bellefonte, PA, USA), maintainedat 40 8C. The mobile phase solution consisted of acetonitrile and 0.05% trifluoroacetic acid (TFA) in water (95:5, v/v) delivered at 0.5 ml/min. The effluent from the HPLC system was connected 2 .5 directly to a Perkin-Elmer Sciex API 3000 MS System (PE SCIEX, Foster City, CA, USA) equipped with standard positive electrospray ionization (ESI) in the multiple reaction monitoring (MRM) mode

2.4.18.4. High-performance capillary electrophoresis (Hung C. Vo et al., 2002)

Estimation of acyclovir in plasma sample was also done by high=performance capillary electrophoresis. Separations were performed on a Beckman MDQ automated capillary electrophoresis system (Beckman Instruments, Missisauga, ON, Canada). Un- coated capillaries were used with internal diameters of 75 mm, external diameters of 360 mm and lengths of 60.2 cm (50 cm to detector). New capillaries were first rinsed with 1.0 *M* NaOH (10 min, 20 p.s.i.), followed by rinsing with the separation buffer (20min, 20 p.s.i.). The new capillary was then left to Fisher equilibrate overnight in the separation buffer prior to operation. Each separation was preceded by a 1 min rinse with 1.0 *M* NaOH, a 1 min rinse with deionized extraction water, followed by a 3 min rinse with the separation buffer. The sample was introduced using low-pres- (0.5 p.s.i.) injection for 100 s. All separations were carried out at 22 8C using a voltage of 20 kV throughout the experiment. Detection of acyclovir was monitored at 254 nm using UV. Stock borate

from buffer (600 m*M*) was adjusted to pH 8.8 using HCl(aq) and was used to dissolve SDS and HPbCD powders to make 90 m*M* borate pH 8.8 containing 175 m*M* SDS and 100 m*M* HP ßCD with 0.2% NaCl, which is the separation buffer. The buffer was passed through a 0.22 mm filter (Sartorius, Gottingen, Germany) before analysis.

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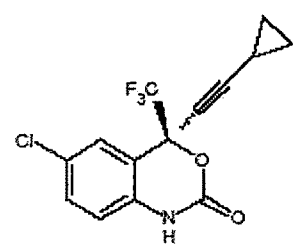
2.5. Profile of Efavirenz

2.5.1. Introduction: Efavirenz is a non-nucleoside reverse transcriptase inhibitor that has been approved by the U.S. Food and Drug Administration for the treatment of HIV, which causes the acquired immunodeficiency syndrome (AIDS). It is the first HIV drug granted traditional FDA approval based on the exceptional length of the treatment response

2.5.2. Description Efavirenz is a white to slightly pink crystalline powder

2.5.2.1. Chemical name: (S) -6- chloro-4- (cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3, 1-benzoxazin-2-one.

2.5.2.2. Structural Formula:



2.5.2.3. Molecular Formula: $C_{14}H_9ClF_3NO_2$

2.5.2.4. Molecular weight: 315.68

2.5.2.5. **Solubility**: It is practically insoluble in water (<10 µg/mL).

2.5.3. Clinical pharmacology

Mechanism of action: Efavirenz is a non-nucleoside reverse transcriptase (RT) inhibitor of human immunodeficiency virus type 1 (HIV-1). Efavirenz activity is mediated predominantly by non-competitive inhibition of HIV-1 RT. HIV-2 RT and human cellular DNA polymerase alpha, beta, gamma, and delta are not inhibited by efavirenz.

***In vitro* HIV Susceptibility:** The clinical significance of *in vitro* susceptibility of HIV-1 to efavirenz has not been established. The *in vitro* antiviral activity of efavirenz was

assessed in lymphoblastoid cell lines, peripheral blood mononuclear cells (PBMCs) and macrophage/ monocyte cultures. The 90-95% inhibitory concentration (IC90-95) of efavirenz for wild type laboratory adapted strains and clinical isolates ranged from 1.7 to 25 nM. Efavirenz demonstrated synergistic activity against HIV-1 in cell culture when combined with zidovudine (ZDV), didanosine or indinavir (IDV).

2.5.4. Uses:

Efavirenz in combination with other antiretroviral agents is indicated for the treatment of HIV-1infection. This indication is based on two clinical trials of at least one-year duration that demonstrated prolonged suppression of HIV-RNA.

2.5.5. Pharmacokinetic:

2.5.5.1. Absorption:

Peak efavirenz plasma concentrations of 1.6-9.1 μM were attained by 5 hours following single oral doses of 100 mg to 1600 mg administered to uninfected volunteers. Dose-related increases in Cmax and AUC were seen for doses up to 1600 mg; the increases were less than proportional suggesting diminished absorption at higher doses.

In HIV-infected patients at steady state, mean Cmax, mean Cmin, and mean AUC were dose proportional following 200 mg, 400 mg, and 600 mg daily doses. Time-to-peak plasma concentrations were approximately 3-5 hours and steady-state plasma concentrations were reached in 6-10 days. In 35 patients receiving efavirenz 600 mg once daily, steady-state Cmax was 12.9-±3.7 μM (mean ±S.D.), steady-state Cmin was 5.6±3.2 μM, and AUC was 184-±73 μM.h.

2.5.5.2. Effect of Food on Oral Absorption:

Administration of a single 600-mg dose of efavirenz capsules with a high fat/high caloric meal (894 kcal, 54 g fat, 54% calories from fat) or a reduced fat/normal caloric meal (440 kcal, 2 g fat, 4% calories from fat) was associated with a mean increase of 22% and 17% in efavirenz AUC and a mean increase of 39% and 51% in efavirenz Cmax, respectively, relative to the exposures achieved when given under fasted conditions. Administration of a single 600-mg efavirenz tablet with a high fat/high caloric meal (approximately 1000

kcal, 500-600 kcal from fat) was associated with a 28% increase in mean AUC of efavirenz and a 79% increase in mean Cmax of efavirenz relative to the exposures achieved under fasted conditions.

2.5.5.3 Distribution:

Efavirenz is highly bound (approximately 99.5-99.75%) to human plasma proteins, predominantly albumin. In HIV-1 infected patients (N=9) who received efavirenz 200 to 600 mg once daily for at least one month, cerebrospinal fluid concentrations ranged from 0.26 to 1.19% (mean 0.69%) of the corresponding plasma concentration. This proportion is approximately 3-fold higher than the nonprotein-bound (free) fraction of efavirenz in plasma.

2.5.5.4. Metabolism:

Studies in humans and *in vitro* studies using human liver microsomes have demonstrated that efavirenz is principally metabolized by the cytochrome P450 system to hydroxylated metabolites with 3 subsequent glucuronidation of these hydroxylated metabolites. These metabolites are essentially inactive against HIV-1. The *in vitro* studies suggest that CYP3A4 and CYP2B6 are the major isozymes responsible for efavirenz metabolism. Efavirenz has been shown to induce P450 enzymes, resulting in the induction of its own metabolism. Multiple doses of 200-400 mg per day for 10 days resulted in a lower than predicted extent of accumulation (22-42% lower) and a shorter terminal half-life of 40-55 hours (single dose half-life 52-76 hours).

2.5.5.5. Elimination:

Efavirenz has a terminal half-life of 52-76 hours after single doses and 40-55 hours after multiple doses. A one-month mass balance/excretion study was conducted using 400 mg per day with a 14C-labeled dose administered on Day 8. Approximately 14-34% of the radiolabel was recovered in the urine and 16-61% was recovered in the feces. Nearly all of the urinary excretion of the radio labeled drug was in the form of metabolites. Efavirenz accounted for the majority of the total radioactivity measured in feces.

2.5.6. Indication:

Efavirenz in combination with other antiretroviral agents is indicated for the treatment of HIV-1 infection. This indication is based on two clinical trials of at least one-year duration that demonstrated prolonged suppression of HIV-RNA.

2.5.7. Dosage and administration:

The recommended dosage of efavirenz is 600 mg orally, once daily, in combination with a protease inhibitor and/or nucleoside analogue reverse transcriptase inhibitors. It is recommended that efavirenz be taken on an empty stomach, preferably at bedtime. The increased efavirenz concentrations observed following administration of efavirenz with food might lead to an increase in frequency of adverse events. Dosing at bedtime may improve the tolerability of nervous system symptoms.

2.5.8. Contraindication:

Efavirenz is contraindicated in patients with clinically significant hypersensitivity to any of its components. Efavirenz should not be administered concurrently with astemizole, cisapride, midazolam, triazolam, or ergot derivatives because competition for CYP3A4 by efavirenz could result in inhibition of metabolism of these drugs and create the potential for serious and/or life-threatening adverse events (e.g., cardiac arrhythmias, prolonged sedation or respiratory depression).

2.5.9. Storage:

Store at room temperature in a tightly sealed container away from heat, moisture, and direct light.

2.5.10. Adverse effects:

The most significant adverse events observed in patients treated with efavirenz are nervous system symptoms, psychiatric symptoms, and rash.

Treatment with efavirenz has been associated with the development of serious psychiatric side effects including severe depression, suicidal thoughts, aggressive behavior, paranoid

reactions and manic reactions. The less serious side effects may be more likely to occur includes dizziness, drowsiness, insomnia, abnormal dreams, impaired concentration, agitation or nervousness, headache, mild nausea, vomiting, diarrhea, changes in body fat (e.g., increased fat in the upper back, neck, breast and trunk and loss of fat from the legs, arms, and face. It was also reported that, It causes an allergic reaction (difficulty in breathing, closing of the throat), swelling of the lips, tongue, or faces or hives. As the liver metabolizes efavirenz, hepatotoxicity is also a serious problem on its uses.

2.5.11. Overdose:

Symptoms of an efavirenz overdose may include confusion, poor coordination, headache, numbness or tingling, tremor, dizziness, muscle cramps, and seizures

2.5.12. Precaution:

Because the liver metabolizes efavirenz, there can be adverse interaction between this drug and other drugs which use the liver. Efavirenz lowers blood levels of most protease inhibitors. Because of this, dosages of indinavir and amprenavir should be increased. Efavirenz lowers blood levels of methadone and so this will need to be increased as well. It also lowers blood levels of methadone and so this will need to be increased as well. Efavirenz and saquinavir should not be taken together. Studies in monkeys showed that efavirenz is likely to cause birth defects. Pregnant women should not take it

2.5.13. Quantitative method

2.5.13.1. HPLC: Table 2.4.summarizes the available method for the estimation of efavirenz.

Table 2.4.: Summary of HPLC methods for analysis of efavirenz

Column used	**Mobile phase**	**Detection**	**Medium**	**Reference**
Zorbax C-18 (12.5mm x 4.6mm, 3.5µm, Agilent)	50mM phosphate monobasic (pH 4.5): Methanol (85:15)	UV detection, 220nm	Plasma	Naser L Rezk et al 2004
Eclipse XDB C-8 (150mm x 4.6mm, 5µm)	50mM sodium phosphate buffer (pH 4.8): Acetonitrile (20:80)	UV detection, 220nm	Plasma	Naser L Rezk et al 2002
C8 Lichrospher 60 RP-Select B (EM Separations) column (4 x 250 mm)	55% acetonitrile and 45% ammonium phosphate at pH 3.5.	UV detection, 247nm	Plasma	Bruce J. Aungst et al 2002
Alltech Spherisorb column (4.6 x 250mm)	58% acetonitrile in 0.1% NH_4HCO_3, pH 7.4,	UV detection, 247nm	Plasma	Suresh K. Balani et al 1998
C18 column (12.5 x 4.6 mm)	-	Fluorescence detector	Plasma	Matthews, C.Z et al 2002
C-8 (150mm x 4.6mm, 5µm)	Phosphate buffer (pH 5.75)-acetonitrile	UV detection, 250nm	Plasma	Saras-Nacenta M et al 2001
Nova-Pak C18 column (4 mm, 83100 mm, Waters)	A solution of acetonitrile, methanol and tetramethy ammonium perchlorate (TMAP) in dilute aqueous trifluoroacetic acid (TFA)	UV detection, 205nm	Plasma	Yoshiko Usami et al 2003.
Luna 5 µcolumn	sodium phosphate buffer, acetonitrile and methanol	UV detection, 254 nm	Plasma	Boffito M et al, 2002

4.6 mm × 25 cm reverse-phase Zorbax R_x-C_8 column	Acetonitrile : water (50:50, v/v) with 0.05% (v/v) trifluoroacetic acid	UV detection, 250 nm	Plasma	Minli Xie et al, 2003
Microsorb MV C8 column	Phosphate buffer and acetonitrile.	UV detection, 210nm	Plasma	Marc Pfister et al, 2003
100 RP-8 (5µ)	Uunbuffered water-acetonitrile (52:48)	UV detection, 210nm	Plasma	Cociglio M et al., 2003
Luna C18 (5µ) .	Acetonitrile and water	UV detection, 247nm	Plasma	Villani, Paola et al, 1999

2.5.13.2. LC/MS/MS method (K. M. L. Crommentuyn et al 2003)

An Agilent (Palo Alto, CA, USA) HPLC system was used, consisting of an 1100 Series pump and cooled well plate autosampler (4 °C) that accommodates two 96-well plates. The separation was carried out in a reversed-phase system with an Inertsil ODS3 column (50 ð 2.0 mm i.d., particle size 5 µm) (Chrompack, Middelburg, The Netherlands) protected with a Chromguard minibore reversed-phase precolumn (10 ð 2.0 mm i.d.) (Chrompack) and an in-line filter (0.5 µm), (Upchurch Scientific, Oak Harbor, WA, USA). A quick, stepwise gradient was used to elute the compounds from the column. At time zero, 100% eluent A (methanol–10 mM ammonium acetate buffer, pH 5.0 (35 : 65, v/v)) was flushed through the column. After 0.2 min, 15% of eluent A were mixed with 85% of methanol and this mobile phase composition was maintained for 1.6 min.

In 0.1 min the mobile phase consisted of eluent A again and the column was reconditioned in 3.5 min before the next injection. The flow-rate was 0.5 ml min_1. The column outlet was connected to the electrospray sample inlet (Sciex, Thornhill, ON, Canada) through a post-column splitter (1 : 4) (ICP-04-20-CR, LC Packings, Amsterdam, The Netherlands) and a divert valve (Valco Instruments, Houston, TX, USA). The latter was used to direct the first 1.5 min of the eluent flow to waste to prevent the introduction of endogenous compounds into the mass spectrometer. The source temperature was held constant at 350 °C. Ions were created at atmospheric pressure and were transferred to an API 3000 triple-quadrupole mass spectrometer (Sciex). The curtain (1.3 ml min¯1) and turbo (7.0 l min¯1) gases were zero air, while the nebulizer (1.8 l min ¯1) and collision

activated dissociation gas (240 x 10^{12} molecules cm^{-}2) consisted of nitrogen (grade 5.0). The electrospray voltage was +4 kV and the dwell time was 50 ms with a 5 ms pause between scans. Q1 and Q3 were operating at unit mass resolution. Multiple reaction monitoring (MRM) was used for drug quantification. Precursor ions of analytes and internal standards were determined from spectra obtained during the infusion of standard solutions using an infusion pump connected directly to the electrospray source. As a result of the very soft ionization, provided by the electrospray ion source, only singly charged molecular ions were observed.

2.5.13.3. Radioactivity Assay. (Suresh K. Balani et al 1999)

Aliquots (1 ml) of urine samples were mixed with 15 ml of Beckman Ready-Safe liquid scintillation cocktail and analyzed on a Packard CA1600 counter. The feces were homogenized in water, and 1-ml aliquots of the homogenates were air-dried and combusted using a Packard Sample Oxidizer. The resulting carbon dioxide was trapped using Carbosorb/ Permafluor (Packard) and analyzed on the liquid scintillation counter.

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