School of Pharmacy

Development and Stability of Pharmaceutical Emulsions: Influence of Physicochemical Factors on Stability of Emulsion and Bioavailability

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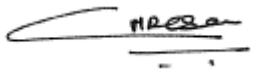
Declaration

"To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university."

Human Ethics: The research presented and reported in this thesis was conducted in accordance with the National health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Number # HR 151/2012.



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Abstract

Emulsion based drug delivery systems have been extensively used to overcome difficulties with regard to solubility, absorption and bioavailability of bioactive agents. However, emulsion based delivery systems have a limited number of technologies that are currently commercially viable, especially in relation to scale-up, the requirement for expensive specialised equipment for manufacturing and drug product stability. Thus there is a need to design innovative lipid emulsions which are safe, economic, robust and well controlled and which can be produced on a commercial scale with an adequate shelf life of at least 1-2 years.

This study was carried out to develop, optimize and scale up an emulsion based drug delivery system, characterise its stability and the physical properties of an optimised formulation; and evaluate efficacy and safety of the formulation containing an active ingredient. The drug delivery system was developed by a systematic approach through application of hydrophilic lipophilic balance (HLB), order of mixing, emulsification equipment, optimisation of a selected formulation, scale-up and clinical trials.

The HLB system was used for formulation development of an emulsion containing mixed emulsifiers. The HLB system was found to be a useful tool in the selection of surfactant ratios. Emulsions containing soybean oil as oil phase were stable at HLB $\geq$ 10. It was also evident that the HLB system was generally not reliable and required preparation and stability analysis to identify the HLB at which the selected formulation was stable.

Four different emulsification techniques were evaluated following selection of a suitable emulsifier ratio. The most stable emulsions were produced by using mixed micelle systems. The order of mixing and the type of surfactant used were found to be critical for emulsion quality and stability. Emulsions produced by mixed micelle systems were the least affected by the type of surfactant used and were stable when either lecithin or Span 80 was used as a lipophilic surfactant.

High pressure homogenisation, ultrasonication and rotor stator technologies were used to evaluate the efficiency and scalability of the emulsification process. During selection of equipment, it was observed that emulsions with mean droplet sizes D(3,2) less than 2 μm were the most stable. High pressure homogeniser,

ultrasonication and rotor stator systems, all were capable of achieving a mean droplet size D(3,2) of 2 µm or less when optimum conditions were applied. The high pressure homogenisation was found to be the most efficient process in reducing the droplets size. At the same time**,** the Diax® rotor stator was easy to scale up and validate. The rotor-stator was also capable of achieving a mean droplet size D(3,2) of 2 µm, which was an important parameter for emulsion stability. Ultrasonication was either inefficient in reduction of droplet size and/or it increased the product temperature, which can cause chemical and physical degradation. Ultrasonication also produced a wide droplet size distribution, which was the basis of emulsion instability.

The delivery system was optimised using soybean oil as an oil phase, polysorbates and lecithin as surfactant and co-surfactant and a rotor homogeniser as the emulsification technology. The lipophilic surfactant concentration was important in the formation of the emulsion. The ratio of hydrophilic and lipophilic surfactants (Ws/Wco) was critical to the stability of the emulsions and stable emulsions were produced with the Ws/Wco ratio between 0.5-4 (w/w). The mixed emulsifier system with lecithin formed multilamellar vesicles containing oil and the stability of the emulsion was due to the formation of the multilayered hydrated system.

During process optimisation it was observed that the rotational speed of the mixer was the most important factor in emulsification and reduction of droplet size. The addition of oil phase close to the agitator during mixing reduced the number of larger droplets. Mixer design was also important in the formation of the emulsion and droplet size reduction. In-line mixers were found to be efficient and can replace the overhead mixer during scale-up.

To evaluate the drug delivery system under a clinical environment, an emulsion containing 4.5% of Tween 80, 2% lecithin, 15% soybean oil and 3% lidocaine was prepared and evaluated for its efficacy and potential toxicity for postoperative analgesia for split-skin graft donor sites. The emulsion was evaluated for stability at ambient temperature (25 °C) for 30 months. The stability study at ambient temperature (25 °C) showed that the droplet size of the emulsion was stable. The peroxide value and pH changes were within an acceptable range during the stability testing period of 30 months. Lidocaine was stable beyond its expected shelf-life of 2 years. Emulsions containing lidocaine had a pharmaceutically acceptable shelf-

life of 680 ± 15 days, hence 665 days. Peroxide values of >5 mEq/kg and the presence of droplets more than 20 µm in diameter were set for an emulsion delivery system.

This study compared the safety and effectiveness of the emulsion with respect to the aqueous formulations containing lidocaine during the first dressing change of partial thickness skin graft donor sites. A double-blind randomised controlled, pilot trial was conducted in 29 patients. Subjects were randomised to either a 3% lidocaine emulsion formulation ‘‘Treatment E’’ (NOPAYNE^{TM}) or a 4% aqueous solution ‘‘Treatment A’’ (Xylocaine^{TM}). Endpoints included pain intensity measured by the numerical rating scale (NRS) up to 1 hour after dressing change commencement, sting sensation, overall satisfaction and lidocaine plasma concentration. The mean pain scores and standard error of the mean (SEM) for formulations E and A at60 min were 1.3 ± 0.3 (mean ± SEM) and 1.8 ± 0.4 ($p = 0.98$) respectively. Nearly 90% of patients were very satisfied with their treatment. The mean plasma concentrations of lidocaine for formulations A and E were 0.132 mg/l and 0.040 mg/l respectively ($p = 0.069$). A clinical trial in humans demonstrated that the emulsion delivery system containing lidocaine was as effective as an aqueous lidocaine solution; however the emulsion formulation improved the safety profile of lidocaine.

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1 General Introduction

1.1 Emulsion

An emulsion is defined as an intimate mixture of two immiscible liquids, one of which is dispersed or distributed in the form of globules. The system is stabilised by the presence of a third substance, called an emulsifying agent (emulsifier, surfactant).[1, 2] The phase that is present as fine droplets is called the dispersed phase and the phase in which the droplets are dispersed is the continuous phase.[3] Emulsions are thermodynamically unstable liquid system and understanding the theoretical factors influencing emulsion stability is critical to emulsion formulation.[4] Emulsification requires mechanical agitation, to form droplets, but without an emulsifier, when the agitation ceases, the emulsion will separate into two phase.[4] Emulsifiers are used to form an interface between the droplets and continuous phase and prevent coalescence by reducing interfacial tension or creating a physical repulsion between the droplets.[5] These properties of an emulsifier are considered important in emulsion stability, by maintaining the dispersed phase in droplet form. Overall, droplet formation and the an interfacial barrier are important features of any emulsification process.[1]

Most emulsions have droplets with diameters of 10 nm-100 μm. Smaller droplets exhibit colloidal behaviour and their stability is of a hydrophobic colloidal dispersion.[3] Emulsion based products are available in liquid and semi-solid forms.[1] Emulsions are used to deliver single or multiple active pharmaceutical ingredients (API) of differing solubility to improve the distribution, permeation and patient compliance.[4] Different types of vegetable and mineral oil can be used in lipid emulsion formulation. They can be administered by almost all available routes including topical, parenteral, oral, nasal.[6, 7]

1.1.1 Oil-water interface in emulsion

In bulk water, the water molecules are surrounded in all directions by other water molecules, attractive intermolecular forces and hydrogen bonds exist between adjacent water molecules.[8] Similarly, van der Waals attractive forces exist between adjacent oil molecules, in bulk oil. In dispersed systems, such as an emulsion, the water molecules in the interfacial region are surrounded by oil molecules and produce weak and unequal attractive forces by neighbouring oil molecules.[8] The nature of the interfacial region can be evaluated by examining the forces responsible

for surface and interfacial tension.[9] Liquids with strong intermolecular attractive forces have higher surface tension than nonpolar liquids with relatively weak intermolecular forces.[8] The interfacial tension can serve as a measure of the degree of interaction between two liquids. Interfacial tension between nonpolar oils and water increases with increase in the length of carbon chain and decreases as the number of hydroxyl groups increases.[9] The minimisation of free energy is a driving force for the droplet dispersal, mixing and formation of micelles and microemulsions and is 'directed' by lower enthalpy value (H) and higher entropy value (S) (Equation 1-1). Low free energy means that the droplet formation process is spontaneous.[10]

$$\Delta G = \Delta H - T\Delta S \quad \text{.....Equation 1-1}$$

Where ΔG is free energy, ΔH is enthalpy, ΔS is entropy and T is temperature. During droplet formation surface free energy (ΔG) is defined as the work required to increase the area by $1m^2$. The work done is therefore equivalent to interfacial tension (γ) (mN/m). The interfacial tension can be defined as the force that acts perpendicular to a the plane of the interface.[8]

$$\gamma = Force/(2L) \quad \text{.....Equation 1-2}$$

Where, γ is interfacial tension, and L is the surfactant adsorption of amphiphile molecules, which reduces surface free energy and at the interface lowers the interfacial tension.[10] The higher the surfactant adsorption, the smaller the interfacial tension produced. The degree of surfactant adsorption at the interface depends on surfactant structure and the nature of the two phases that meet at the interface and the molecule's ability to form interfacial packing.[9, 10] This is why; emulsifiers differ in their ability to lower surface tension. Low molecular weight emulsifiers (Tween, Span, sodium lauryl sulphate) are able to form tighter interfacial packing and can lower the interfacial tension to 20–25 mN/m, whereas polymers and hydrophobic gums form an interactive surface layer with higher interfacial tension (~50 mN/m).[10] Most interfaces encountered in pharmaceutical systems are curved. The Young Laplace equation (Equation 1-3) relates the pressure change across a curved interface (ΔP) to the interfacial tension γ.

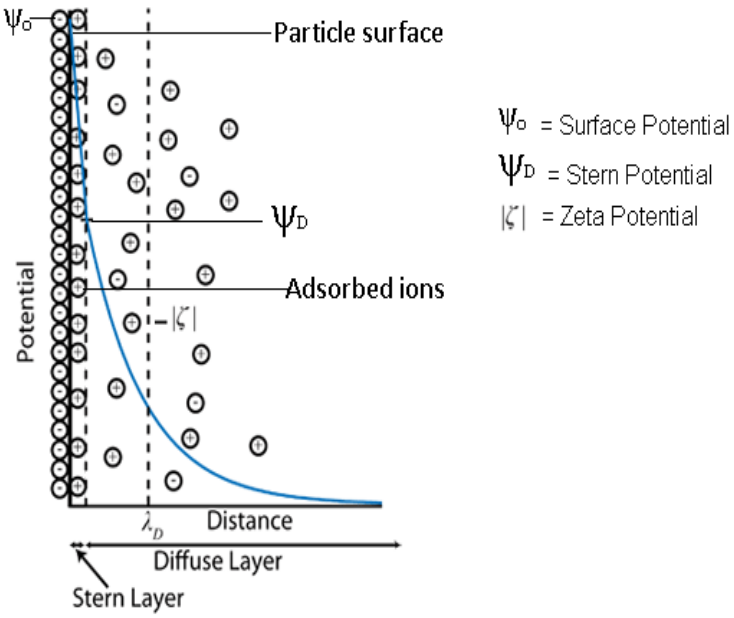
$$\Delta P = 2\gamma/r \quad \text{.....} \quad \text{Equation 1-3}$$

Where, r is the radius of the sphere and γ is interfacial tension. This equation predicts that a smaller bubble or droplet has a greater internal pressure. Since the

pressure within a small droplet of liquid is greater than that of a liquid having a flat surface, a collection of water droplets of different radii is unstable, so that the large drops grow at the expense of the smaller drops.[8]

The properties of the interfacial region are determined by the type, concentration and interaction of surfactants during emulsification and before emulsion formation through adsorption, complexation and layer-by-layer formation.[11] In oil in water (o/w) emulsions, the surface active agents adsorbed at the oil-water interface provide a layer of adsorbed species in such a way that charged hydrophilic groups form the outside surface.[8] The presence of charged particles has a profound effect on the oil-water interface.

Figure 1-1 **Schematic of electric double layer in liquid with negatively charged surface. (Adopted from Atwood et al. and Jean-Louis et al.)**



The counter-ions are attracted close to the droplets due to surface charge on the droplet.[3] The compact layer (Figure 1-1) around the core of the droplet, which contains the head groups of surfactants and the bound counter-ions, is called the Stern layer. The second layer (Figure 1-1) containing anions and cations balances the surface charge.[12] This second layer is known as the diffuse layer, where the ions are free to move with the fluid and are affected by electrical forces and random thermal motion.[3, 12] The region containing the 'stern layer' and 'diffuse layer' is called the "electric double layer". Non-ionic surfactants have a hydrophobic core surrounded by a shell of oxyethylene chains which is often termed the palisade

layer. This layer is capable of mechanically entrapping a considerable number of water molecules and is highly hydrated.[3] The adsorption of OH^- and H_3O^+ ions may result in a net charge per unit area. That is why, non-ionic surfactants often produce a negatively charged interface at neutral pH.[12]

1.1.2 Micelle and vesicle formation

Amphiphiles are anionic, cationic, and zwitterionic or non-ionic depending on the charge accumulated by the head group. In solution, they self-assemble to form variety of structures which are of the order of nano to micro size ranges. Self-assembled structures change in size and shape with concentration, pH, temperature and pressure.[13] The properties of solutions containing surface active agents change sharply over a narrow concentration range. This concentration is called the critical micelle concentration (CMC). The surface active agent has no further effect on the surface or interfacial tension at concentrations above the CMC, which suggests that a surface active agent in excess of the CMC is no longer orientating at the interface, which indicates formation of a new phase containing small structures, micelles.[8] In aqueous solution, the self-assembled structures form such that the hydrophilic region is in contact with water, and the lipophilic region is shielded from water. This phenomenon is called micellisation.[8] Micellisation is favoured by the attainment of minimum free energy of the system.[9] The driving force for micelle formation is the reduction of contact between the hydrocarbon chain and water. In the aqueous solution, the surfactant hydrophobic groups are directed towards the interior of the aggregate and the polar head groups are directed towards the solvent.[9] However, the micellisation process is opposed by the polar groups of surfactant molecules' as a consequence of being held in a fixed position. Formation of micelles depends on reaching a dynamic equilibrium of these factors between monomer molecules in solution.[3] The increase in the length of the hydrophobic chain will form micelle at low concentration or low CMC. The addition of electrolytes to ionic surfactants decreases the CMC and increases the micelle size. The effect is simply explained in terms of a reduction in the work required for the micellisation and a change in the magnitude of repulsive forces between the charged head groups in the micelle.[3]

Micelles are continuously breaking down and reforming.[8] In a more concentrated system, the equilibrium is determined by the strength of the interaction forces

between the aggregates. The forces that hold amphiphilic molecules together in micelles and bilayers are not strong covalent or ionic bonds but the weaker van der Waal's forces, hydrophobic attractions, hydrogen bonding and screened electrostatic interactions.[13] The concentration, at which micellisation become significant, is determined by the balance of these forces. A low CMC indicates that removal of the lipophilic region of the surface active agent from contact with water is the dominant factor, while a high CMC indicates that the forces opposing aggregation are significant.[8] In general, the critical micelle concentration in aqueous media decreases as the lipophilic nature of the surface active agent increases. This process may be thought of as enhancing the expulsion of the lipophilic region from water. Branching in the lipophilic region interferes with the close packing of surface active agents needed for van der Waals attraction of the hydrocarbon chains; thus, the CMC increases.[3]

Micelles are in dynamic equilibrium and the rate of exchange between a surfactant molecule and the micelle may vary by orders of magnitude, depending on the structure of the surfactant molecule.[9] The concentration of surfactant monomers in equilibrium with the micelles stays approximately constant at the CMC value when the solution concentration is increased above the CMC.[8] The number of surface active agent molecules constituting a micelle is believed to range from 50 to 100 molecules and is characterised by the aggregation number. In general, the aggregation number in aqueous solution increases with an increase in the hydrophobic region of the surface active agent.[3] The radius of the micelle will be slightly less than that of the extended hydrocarbon chain, with the interior core having the properties of a liquid hydrocarbon. Micelle formation of non-ionic surfactants depends both on the alkyl chain length and the number of ethylene oxide/hydroxyl units in the molecule.[9] With ethoxylated non-ionic surfactants, an increase in the hydrophilic oxyethylene chain length causes an increase in the CMC.[3] Ethoxylated surfactants with an average alkyl chain length of 8 to 12 carbon atoms and containing more than 5 ethylene oxide units are usually soluble in water at room temperature. However the solution gradually becomes cloudy as the temperature rises and is referred to as the cloud point of the surfactant.[9] The addition of electrolytes to ionic surfactants decreases the CMC and increases the micelle size. The effect is simply explained in terms of a reduction in the magnitude of the

forces of repulsion between the charged head groups in the micelle, allowing the micelles to grow and also reducing the work required for their formation.[3]

The interior core of a micelle can be considered as having the properties of a liquid hydrocarbon and is thus capable of dissolving materials that are not soluble in water.[9] The hydrophobic core of the micelle solubilises non-polar substances and shields them from the aqueous environment. Water soluble compounds are oriented with the polar group at the surface of the ionic head groups.[3] Slightly polar solubilizates without a distinct amphiphilic structure partition between the micelle surface and the core.[3]

Amphiphilic phospholipid molecules form a closed bilayer sphere in an attempt to shield their hydrophobic groups from an aqueous environment. The resulting sphere may consist of several layers of lipid separated from one another and called multilamellar vesicles (MLV), a single lipid bilayer, small unilamellar vesicles (SUV) surrounded or large unilamellar vesicles (LUV), a single lipid layer vesicles.[13] Vesicles formed using amphiphiles, are called Niosomes. Non-ionic based Niosomes are stabilised against the aggregation due to repulsive, steric and electrostatic forces.[13] Lipids and surfactants also exist in lamellar, cubic, hexagonal and continuous liquid crystal states, which has implications for the stability of the product.[10, 14] Lamellar lyotropic liquid crystalline systems are thermodynamically stable and formed spontaneously. Lyotropic liquid crystalline states exhibit pronounced distinct phase transitions as a function of both temperature and concentration change within a solvent.[10, 14] A lamellar phase exhibits interesting solubility properties which make it a good choice as a vehicle. Lamellar phases possess one dimensional order with hydrophobic and hydrophilic layers, so it is possible to incorporate water soluble, oil soluble as well as amphiphilic drugs within structured lamellar layers.[13]

They can be stored for long periods without phase separation. Depending on the concentration of the solvent and on the polarity of the solvated vesicles, these systems can undergo various phase transitions.[15] The formation of a lamellar liquid crystalline phase is dependent on the water content and temperature.[13]

1.1.3 Lipid emulsions as drug delivery system

Traditionally, emulsions have been used to render oily substances such as vegetable oil and liquid paraffin more palatable. Oil-soluble and water-soluble medicaments can be incorporated in emulsions.[3] Amongst pharmaceuticals, emulsions are popular due to their ability to mask the taste and/or texture of medicaments.[16] Lipid emulsions have been used as injectables for more than four decades as a life-saving treatment.[17] In recent years, emulsion-based drug delivery systems have received considerable attention.[17] Lipid formulations such as liposomes and submicron emulsions show potential to achieve desired properties of -drug delivery systems. One common approach adopted to increase the efficacy of drugs and decrease adverse effects is to deliver the necessary amount of drugs to the sites of their action.[16, 17]

The types and formulation of pharmaceutical emulsions are based on the application of the final product and medicament.[18] As an example, emulsions for intravenous (IV) administration must be of an o/w type and dispersed particles of submicron size.[19] Lipid emulsions are biocompatible, physically stable, and relatively easy to produce on a large scale.[3] Their major advantage is the solubilisation of drugs with low aqueous solubility and the stabilisation of labile drugs against hydrolysis or oxidation and toxicity.[16, 17] Therapeutic advantages of the lipid-based drug delivery systems are an improved absorption profile and a decrease in toxicity of drugs in contrast to other dosage forms.[4] Lipid emulsion based drug delivery systems are also being viewed as possible adjuvants to enhance the potency of DNA vaccines.[7]

1.1.4 Types of emulsion

Two common types of emulsion are found in lipid based drug delivery systems.[1, 18]

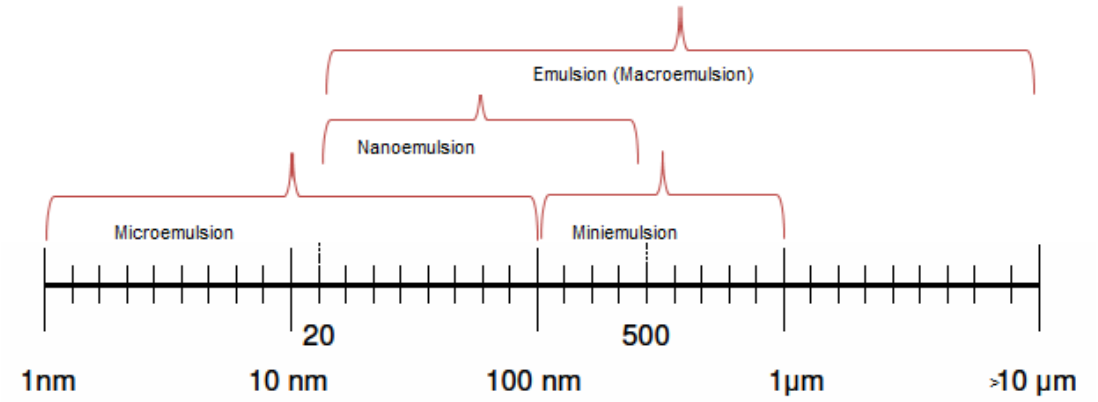
- Oil in water (o/w), in which the oil droplets are dispersed in the water. Oil in water lipid emulsions can be administered by a variety of parenteral routes including intravenously in nutrition applications.
- Water in oil (w/o), in which the water droplets are dispersed in the oil. Water in oil emulsions are used for the sustained release of steroids and vaccines by intramuscular injection.

The formation of w/o or o/w emulsion is dependent on phase volume ratio. Higher phase volumes produce larger numbers of droplets, which, in turn, increase the chances of collision. This means the phase present in greater amount usually will

act as the external phase.[3, 4] Because of the stabilising mechanism involved, polar groups are far better barriers to coalescence than their non-polar counterparts. An o/w emulsion can be made with greater than 50% of the disperse phase compared to w/o emulsions.[3, 20] The type of emulsion formed is also dependent on the polar/non-polar characteristic of the emulsifying agent and is a function of relative solubility of the emulsifying agent in each phase.[20] This helps to explain why charged surface active agents, which are highly ionized and possess strong polar groups, favour o/w emulsions.[3] An emulsion containing small water droplets within larger oil droplets, which are dispersed in water to form water-in-oil-in-water emulsion (w/o/w) or their o/w/o counterparts are termed multiple emulsions and are of interest in recent years as drug delivery systems.[19]

Emulsions are also defined based on the particle size distribution of the dispersed phase. The purpose of the dispersed phase is to increase the solubility of the active compound and increase absorption by increasing surface area.[1] The droplet size of the dispersed phase determines the appearance of an emulsion. The radius of the emulsified droplets in an opaque or white, emulsion ranges from 0.25µm to 10µm.[4] Dispersed particles with a diameter of less than 120nm, do not refract light and therefore appear transparent.[4, 6] International Union of Pure and Applied Chemistry (IUPAC) defines an emulsion as a fluid system in which liquid droplets in the form of amorphous, liquid crystalline or any mixture, whose diameters range from approximately 10 nm to 100 µm, are dispersed in a liquid.[21]

Figure 1-2 **Emulsions classifications and droplet size distribution (Adopted from Marino H et al.)**



1.1.5 Macroemulsions

The most well-known emulsion type is an opaque emulsion with particles >1μm, easily visible under a microscope. Such emulsions are also called macroemulsion, and referred to as an "emulsion".[6] A macroemulsion usually contains particles of the dispersed phase with diameter ranges from approximately 1 to 100 μm. The average diameter of droplets in macroemulsion is close to 10μm or more.[21] Macroemulsions are unstable and coalescence of the dispersed and continuous phases usually occurs within time periods from a few seconds to a few hours, depending upon the viscosity of the continuous phase and the size and density of the droplets. [6]

1.1.6 Microemulsions

Microemulsions are transparent, thermodynamically stable dispersions containing two immiscible liquids with particles of 10–100 nm (0.01–0.1 μm) diameter.[21] The term microemulsion, or submicron emulsion, has also been applied to thermodynamically unstable mini- or nanoemulsions. These two-phase o/w systems occur when the droplet size is made extremely small by physical methods,[7] whereas microemulsions are formed spontaneously when the components are mixed in appropriate ratios. Microemulsions differ markedly from both nano and miniemulsions, since these two types depend upon intense agitation for their formation.[22].

Microemulsions are homogenous, transparent or translucent and thermodynamically stable systems and exhibit the properties of hydrophobic colloids.[19] Microemulsions are frequently called solubilised systems and are complex systems of water-oil and surfactants.[4, 7] Microemulsions are considered a solution of swollen micelles containing a solubilised liquid in one-phase and therefore have no interface against either liquid as long as the micelles are capable of solubilizing more of the second liquid.[6, 22] In the case of a dispersion of tiny droplets of one liquid in a second liquid, the interfacial area is so large that an exceedingly low interfacial tension must be present to permit formation of the microemulsion with little work. An interfacial tension of the microemulsion against both of liquids is required close to zero for their formation and stability and is generally not possible to achieve with a single surfactant, instead the mixed system

of surfactant and/or surfactant and co-surfactant is usually preferred.[4] When a mixture of surfactant and co-surfactant is added to a biphasic oil–water system, a thermodynamically stable isotropic mixture spontaneously forms. The interfacial region should be highly flexible, either to permit the large curvature required to surround exceedingly small particles or to allow the easy transition from oil-continuous to water-continuous structures that is characteristic of microemulsions.[6] Long chain polar compounds are generally not desirable as co-surfactants since they tend to form liquid crystalline structures that may increase the viscosity of the system and the rigidity of the interface.[19] The mixed surfactant systems form globular interfaces such as micellar, reverse micellar or interconnected, which give a bicontinuous phase. [6] The presence of packed swollen micelle structures can solubilise large amounts of both oil and water soluble drugs within microemulsions.[7] Due to the large amount of surfactant required for their formulation, this restricts the choice of acceptable components is restricted.[3]

1.1.7 Nanoemulsions

Nanoemulsions are emulsions with mean droplet diameters ranging from 50 to 500 nm. Usually, the average droplet size is between 100 and 400 nm. The particles can exist as oil-in-water and water- in-oil forms, where the core of the particle is either oil or water, respectively. It is defined as a blue-white emulsion containing particles less than 0.5μm in diameter.[21]

1.1.8 Miniemulsions

A miniemulsions is a special case of emulsion in which the particles of the dispersed phase have diameters in the range from approximately 50 nm to 1 μm, typically a size between 300 and 1000 nm [21, 23]. A miniemulsion is obtained by shearing a mixture comprising two immiscible liquid phases, one surfactant and one co-surfactant. The shearing usually proceeds via ultrasonication of the mixture or with a high-pressure homogeniser, which induces a high-shearing process.[24] Sometimes, nanoemulsions are also referred to as miniemulsions and submicron emulsions.[21] The size of the droplets is governed by the surfactant phase structure induced by either temperature or composition.[25]

In an ideal miniemulsions system, the interface phase contains mixed stabilisers, surfactant and co-surfactant, or a water-insoluble compound.[23] Droplets are usually

stabilised against coalescence and Ostwald ripening due to the presence of the surfactant and co-surfactant.[21] Miniemulsions can be produced by using reasonably low surfactant concentrations of <10% and most preferably between 2 and 6%. A co-surfactant with a chain length of at least 12 carbons is stirred with the surfactant for an hour or more to produce a mixed micellar solution. The phase inversion temperature (PIT) method can also be employed to produce miniemulsions.[6]

Miniemulsions can substitute both liposomes and vesicles, and are suitable for efficient delivery of active ingredients through the skin.[26] The large surface area of the emulsion system, the low surface tension of the whole system and the low interfacial tension of the o/w droplets allow enhanced penetration of active agents.[26] The capacity of miniemulsions to solubilise large quantities of water insoluble compounds makes them ideal vehicles. Drugs solubilised in the hydrophobic core are protected from hydrolysis and enzymatic degradation.[25]

Advantages of miniemulsions as drug delivery:[25]

1. Provide better absorption for drugs
2. Increase solubility of drugs with inadequate water solubility
3. Can be used for drug delivery through the skin and sublingually to avoid the first pass effect and other physiologic contraindications
4. Can be used as an injectable as particle size is <1μm.
5. Increased bioavailability of drugs and increased the half life
6. Reduced skin irritation and sensitization if used for skin delivery
7. Economical to produce

1.1.9 Emulsifying agents

An emulsifier promotes emulsification by reducing interfacial tension between two phases and/or kinetically stabilises the droplet form of the dispersed phase by forming a film. There are small number of emulsifying agents used in the pharmaceutical industry out of hundreds of emulsifying agents available in the market.[27] Emulsifiers are amphiphilic in nature and consist of a non-polar hydrophobic portion containing 8–18 carbon atoms attached to a polar or ionic portion (hydrophilic).[4] As a result of their structure, they are attracted to both the oil phase and the water phase.[8, 28] They are also known as surface active agents due to their ability to change surface properties including surface or interfacial tension.[4]

Emulsifying agents, with a reasonable balance between hydrophilic and hydrophobic regions, are adsorbed at interfaces.[4, 20]

Surface active agents are orientated so that the hydrophilic region is in the aqueous phase while the lipophilic region is in the oil phase.[8] The hydrophilic portion could be non-ionic, ionic or zwitterionic, whereas the polar or ionic head group interacts strongly with water molecules via hydrogen bonds (dipole or ion–dipole interactions).[9] Hydrocarbon chains interact weakly with the water molecules in an aqueous environment and are expelled from the bulk of the water phase. Thus, a molecule containing both characteristics is concentrated at an interface and this accounts for their ability to change the surface properties.[8] The adsorption of surfactant molecules at the interface will reduce the interfacial tension. Boyd et al. proposed a distinctive pattern of surfactant molecular adsorption at the oil-water interface. They suggested that oil soluble molecules will be oriented so that each hydrocarbon chain lies on the oil side of the interface and each aqueous ring lies on the water side. Water soluble molecules exhibit strong attraction for water and are oriented at the oil-water interface in such a way that only a part of each hydrocarbon chain lies within the oil phase, the other part being located in the aqueous phase on the other side of the interface.[29] This behaviour suggests that molecules diffuse through the water until they reach the interface, where they are adsorbed to form a stable system.[3]

Most emulsifying agents may enable both types of emulsion to occur depending on the way in which the emulsion is produced.[2] However, as a general rule of thumb; the type of emulsion produced by emulsifying agents is based on polarity of the dominant group.[1] Hence, an emulsifying agent with a dominant hydrophilic group produces o/w emulsions.[1] Moreover, the concentration of the emulsifying agent determines not only its emulsifying power, but even the type of emulsion formed.[6] Almost all emulsions contain a primary emulsifying agent, known as an emulsifier and secondary emulsifying agent, called a stabiliser.[2] The stability of an emulsion is influenced by the charge at the interface and by the packing of the emulsifier molecules.[9 6]

1.1.9.1 Types of emulsifying agents and their properties

Emulsifying agents are classified as below:[1, 2]

1. Natural products
    - Vegetable source (e.g. acacia, tragacanth, soy lecithin)
    - Animal source (e.g. wool fat, beeswax, egg lecithin)
2. Surface active agents
    - Anionic surfactants
    - Cationic surfactants
    - Non-ionic surfactants
3. Finely divided solids

1.1.9.1.1 Natural emulsifying agents

The majority of natural emulsifying agents are complex carbohydrates and show batch–to-batch variation.[19] Most natural emulsifying agents are susceptible to bacterial attack and mould growth.[19] However, recent developments in separation technology have made it possible to produce refined natural emulsifying agents. They are often used as stabilisers due to their lack of surface active properties.[2]

Vegetable sources: A large number of surfactants including polysaccharides, saponins, bile salts and the phospholipids are from vegetable sources.[1, 2] They act by reducing interfacial tension and increasing viscosity. However, they cannot reduce the interfacial tension to the same extent as soaps and often require additional emulsifying agent to produce a stable emulsion.[2]

Polysaccharides such as acacia and tragacanth gums are used as agents in gel and liquid formulations.[19] Polysaccharides stabilise o/w emulsions by forming a strong multimolecular film around the oil globule.[1, 19] Polysaccharide based molecules are susceptible to hydrolysis and are precipitated by high concentrations of alcohol and electrolyte, which may result in loss of emulsifying power.[2] Emulsions made with these agents are coarse in texture and cream readily.[1] Several types of semisynthetic polysaccharides, including methylcellulose, stabilise o/w emulsions by increasing viscosity and forming a film around oil globules without problems associated with batch-to-batch variations. Agar, Irish moss, alginates, dextrin and pectin are also useful as secondary emulsifying agents and require a primary emulsifying agent to produce stable emulsion.[19] The addition of ethoxylated groups may increase water solubility and enhance chemical stability. The modified ether carboxylates are also more compatible with electrolytes and with other non-ionic, amphoteric and

sometimes even cationic surfactants. The ester carboxylates are very soluble in water, but undergo hydrolysis.[9]

Recent developments in emulsion based drug delivery systems have increased the use of natural emulsifying agents such as lecithin.[1] Lecithins are used as emulsifiers in skin creams, injectables and food products.[19] Lecithins are mixtures of several phosphatidyl esters. Lecithin is a water dispersible phosphatide in its pure form. It can undergoe oxidation, which makes it useful as an antioxidant. It is liable to bacterial growth and requires a preservative.[1] The majority of phospholipids are from egg or soya lecithin, are unsaturated, and these materials are widely used as emulsifiers for intravenous use.[30]

Phosphatides are the phosphoric acid esters of a diacylglyceride and further esterified with the hydroxyl group of amines such as choline, ethanolamine or serine. Lecithins possess two fatty acid chains, but only one amphoteric hydrophilic head, which accounts for their tendency to form micelles at low concentrations.[19, 28] The length of the acyl chain influences the transition temperature and the surface behaviour, both in bilayers and in monolayers, with longer chains causing higher melting temperatures.[31] The majority of natural lecithin has chain lengths from fourteen to twenty carbons, with sixteen to eighteen being predominant.[30] The transition temperatures are relevant for the melting of hydrated bilayers and similar phenomena have been observed in lipid monolayers. The extension of these phenomena to emulsions stabilised by solid or liquid phase phospholipids is unclear. However, it is suggested that saturated phospholipids may produce more stable emulsions.[30]

Phosphatides are amphoteric in nature. Phosphatides, and especially phosphatidyl choline, is of major interest in this study. The choline esters, major constituents of lecithin, are quaternary salts and effective emulsifiers and solubilisers.[31] Lecithin is water dispersible, oil soluble and forms micelles at low concentrations. Lecithin produces o/w type emulsions by reducing interfacial tension.[28] The predominant head groups are choline and ethanolamine, which produce phosphatides; phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) that are neutral at pH 7. Serine and glycerol head groups produce acidic lipids, which are negatively charged at pH 7. Their presence in a small concentration is sufficient to confer a

surface charge of -40 to -60 mV on the emulsion droplets, which results in long term stability of several years.[30]

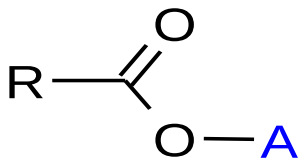
Animal sources: wool fat, wool alcohol and beeswax are the most commonly used animal based emulsifying agents in topical preparations by the pharmaceutical and cosmetic industries.[19] Sterol based natural emulsifying agents are derived from animal sources. They are commonly used as primary emulsifiers for the w/o type of emulsion and can also be used in small quantities as stabilisers for o/w emulsions.[2] Due to their instability and characteristic odour, the use of sterol based emulsifying agents is largely limited to the cosmetic industry.[1] Sterols have excellent emollient properties and absorb a considerable quantity of water.[1]

1.1.9.1.2 Anionic surfactants

Anionic surfactants form one of the largest groups of emulsifying agents.

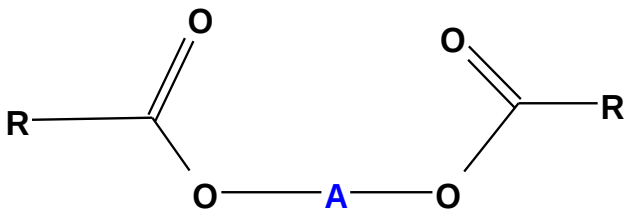
Alkali soaps: produce fairly stable oil-in-water emulsion. Alkali soaps are the sodium, potassium and ammonium salts of fatty acids (Scheme 1-1.[19] Alkali soaps are also used in disinfectant and antiseptic solutions to increase the solubility of the active ingredient.[1] Fatty acid esters containing less than 10 and more than 20 carbon atoms are not good surfactants.[2] Alkali soaps are formed *in situ*.[19] Soaps can be used at pH 9 and emulsions of alkali soaps coalesce in the presence of electrolytes, polyvalent cations and acids. They are not suitable for internal preparations or application on broken skin due to the high pH and unpleasant taste. They are resistant to microbial growth.[2]

Scheme 1-1 **Structure of alkali soaps, where R = a hydrocarbon chain and A= sodium, potassium, or ammonium group**



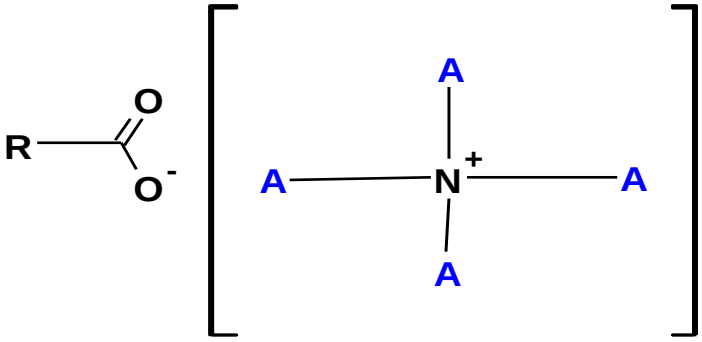
Metallic Soaps: Metallic soaps contain two fatty acid chains, which produces a dominant hydrophobic group (Scheme 1-2). This is the reason that metallic soaps produce water-in-oil emulsions.[1, 19] They have similar properties to alkali soaps but are less sensitive to acid and are less alkaline.[2]

Scheme 1-2 **Structure of metallic soaps, where R = a hydrocarbon chain and A= a metal ion such as calcium, zinc, mangesium or aluminium**



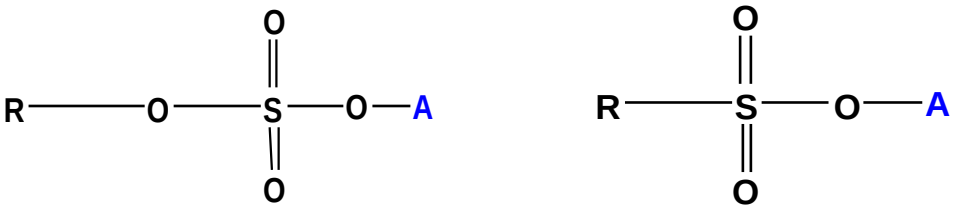
Organic soaps: commonly known as ammonium soaps are produced by replacing the hydrogen atoms in ammonia by organic groups (Scheme 1-3). Ammonium soaps are produced *in situ* by reaction of amines with fatty acids.[19] Organic soaps have well balanced hydrophilic and lipophilic properties.[1] An example of an amine soap *in situ* is triethanolamine stearate produced by the reaction of triethanolamine with stearic acid.[19] Preparations made using triethanolamine are neutral in pH and suitable for broken skin. They are not suitable for internal use.[2]

Scheme 1-3 **Structure of organic soaps. Where R = a hydrocarbon chain and A= a hydrogen or hydrocarbon chain**



Sulphated and sulphonated compounds: produced by esterification of a fatty alcohol or an unsaturated fatty acid by sulphuric acid (Scheme 1-4).[28] Sulphated compounds are widely used as primary emulsifiers to produce oil-in-water emulsions.[28] This group of emulsifying agents are unable to form strong films at the oil/water interface due to their high water solubility and are best used in conjunction with secondary emulsifying agents.[2, 19] Due to their detergent properties, sulphated compounds are extensively employed in cosmetics and household products.[19] Sulphated fatty alcohols are pH and soluble calcium or magnesium tolerant. Pharmaceutical grade sulphated compounds are considered safe for external use.[2]

Scheme 1-4 **Structure of sulphated and sulphonated compounds; where R = a hydrocarbon chain and A= Sodium or triethanolamine**



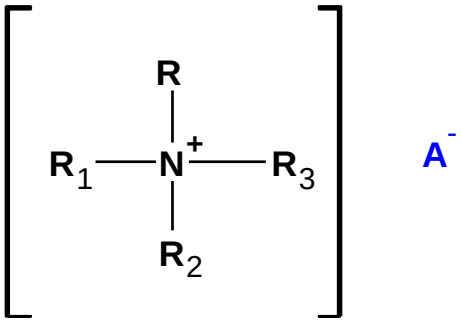
Alkyl Sulphate Alkyl Sulphonate

Sulphonated compounds are similar to sulphated compounds. They are commonly used as detergents and are good wetting agents.[19] Sulphonated compounds are less prone to hydrolysis and have excellent stability in both alkaline and acidic conditions.[1, 28]

1.1.9.1.3 Cationic surfactants

Cationic surface active agents dissociate in aqueous solutions and form positively charged cations (Scheme 1-5).[19] Due to their surface active properties, cationic surfactants readily adsorb at negatively charged substrates, including many types of bacteria and exert a bactericidal effect by interfering with their metabolic process.[1, 28]

Scheme 1-5 **Structure of cationic surfactants; where R = a hydrocarbon chain and A= halogen compound (chlorine or bromine)**



Cationic surfactants are widely used as disinfectants and preservatives.[2] They form water soluble salts, which produce oil-in-water emulsions. The presence of a secondary emulsifier or stabiliser is required to produce stable emulsions.[1] Cationic surfactants are incompatible with anionic surfactants and polyvalent anions and compatible with non-ionic and amphoteric surfactants.[28] Quaternary ammonium salts are the most commonly used cationic surfactants in topical pharmaceuticals and the cosmetic industry.[2] Quaternary salts are available in the form of

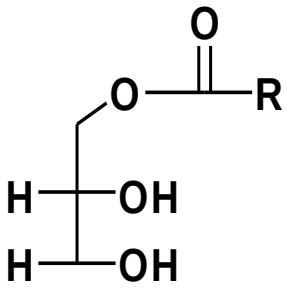
hydrochloride or hydrobromide salts and the most commonly used compound is cetrimide.

1.1.9.1.4 Non-ionic surfactants

Non-ionic surfactants are the largest surfactant group ranging from oil soluble to water soluble materials and many are commonly used in the pharmaceutical industry. These agents do not dissociate and retain their properties over a wide range of pH.[1, 19] Non-ionic surfactants contain a fatty acid or fatty alcohol as the lipophilic domain, and an alcohol and/or ethylene oxide group as the hydrophilic domain, which are connected with an ester bond.[19] They are compatible with most surfactants.[9] The type of emulsion produced will depend upon the type of the dominant group of the agent. If the hydrophobic group of the molecule is predominant, then the surfactant will be oil soluble and will be an effective w/o emulsifier.[2] However, surfactants with an equal balance of hydrophobic and hydrophilic groups produce more stable emulsions due to their concentration at the oil/water interface.[19] The most common non-ionic surfactants are glycol esters, glycerol esters, sorbitan esters, glucosides (and polyglucosides) and sucrose esters. Amine oxides and sulphinyl surfactants represent non-ionic surfactants with a small head group.[9]

Glycol and Glycerol esters: Glyceryl monostearate is a polyhydric alcohol fatty acid ester (Scheme 1-6). It is a strongly hydrophobic material and water insoluble.[1] A polyhydric alcohol with more OH groups will increase the hydrophilic nature of the ester. In the presence of fatty acid salts, glycol and glyceryl esters produce a 'self-emulsifying' o/w emulsifier.[19]

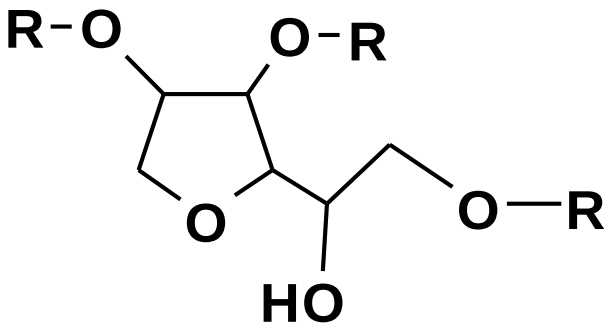
Scheme 1-6 Structure of Glyceryl monostearate (R= C17H35)



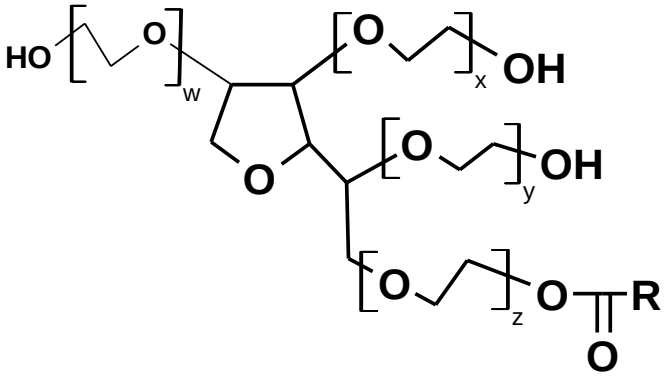
Sorbitan esters and Polysorbates: Most of these agents are derived from sorbitol by esterification of the hydroxyl groups of sorbitan with fatty acids (Scheme 1-7). They produce stable w/o emulsions.[19]

Increase in the strength of the hydrophilic group by adding fatty acid with ethylene oxide in place of polyhydric alcohol (Scheme 1-8), produces an o/w emulsifier.[1] These agents are known as polysorbates. Variations in the number of oxyethylene groups and the type of fatty acid used produce a range of products with different solubilities in oil and water.[28] Polysorbates are well known in the pharmaceutical and cosmetic industries for their ability to produce 'self-emulsifying' preparation with non-ionic oil soluble emulsifiers.[19] Polysorbates are compatible with most surfactants, stable to change in pH and electrolyte concentration.[28] They are widely used in oral and parenteral preparations due to their low toxicity. Major disadvantages of polysorbates are their unpleasant taste and complexation with preservatives.[19]

Scheme 1-7 Structure of sorbitan esters; where R= H or fatty acid radical (R-CO-)



Scheme 1-8 Strurcture of polysorbates; where R= fatty acid and w+x+y+z averages



Ethoxylated esters and ethers: a polyether, commonly referred as polyethylene glycol (PEG) and is produced by polymerisation of ethylene oxide.[28] Polyethylene

glycols are strongly hydrophilic. They are good solubilisers and weak secondary emulsifiers or stabilisers for o/w emulsions.[28] Viscosity of PEG increases by increasing the molecular weight or number of moles of ethylene oxide.[1] PEG is stable, tasteless, water soluble and has solvent properties.[1]

PEG is used for the manufacture of wide range of surfactants containing fatty acids and alcohols.[28] Ethoxylated fatty alcohols are considered to be chemically inert and useful in the compounding of strong acidic or alkaline conditions. Fatty alcohol polyglycol ethers, known as cetomacrogol, are produced by condensation of polyethylene glycol and fatty alcohols.[19] These agents are water-soluble and produce stable o/w emulsions in the presence of secondary emulsifiers such as fatty alcohols.[19]

1.1.9.1.5 Finely divided solids

Finely divided solids with suitably balanced hydrophobic and hydrophilic properties are adsorbed at an oil-water interfaces forming a coherent film that prevents coalescence of the dispersed globules.[19] If the solid particles are preferentially wetted by the oil w/o emulsions are formed.[19] In pharmaceuticals, this class of emulsifying agent is used as a secondary emulsifier. Examples include montmorillonite minerals including bentonite and colloidal silicon dioxide.[2] Finely divided solids have been shown to be good emulsifiers, especially in combination with surfactants and/or macromolecules that increase viscosity.[28] Amongst these are polar inorganic solids, such as heavy metal hydroxides, non-swelling clays and pigments and nonpolar solids.[2]

1.1.10 Formulation

Formulation of pharmaceutical emulsions is dependent on the route of administration, drug candidate and the type of emulsion designed.[1] If the emulsion is designed for ingestion or injectable, then only a limited range of emulsifying agents and oil are available.[19] Formulation variables including the type and purity of the oil, concentration and phase volume ratio of oil, emulsifier used, pH and drug concentration affect the formulation and stability of the emulsion.[2] Processing conditions that affect an emulsion during manufacture are the pressure, temperature and the number of passes used.[1]

1.1.10.1 Selection of oil phase

The materials making up the oil portion of an emulsion and their relative amounts are determined empirically. The oil phase may include a wide variety of lipids of natural or synthetic origin, from mobile liquids to fairly hard solids.[19] Emulsions are composed not only of fatty acids but also substances such as triglycerides (TG) and phosphatidylcholine (PC), in variable amounts.[7] The TG in emulsions are available as long chain triglycerides (LCT) and medium chain triglycerides (MCT). LCT contain fatty acid chains with more than 14 carbon atoms and sometimes present as saturated, mono or polyunsaturated forms.[7] A variety of fixed oils of vegetable origin are the most widely used in internal and external emulsion formulations because of their lack of toxicity.[19] The type of oil used has an effect on the viscosity, particle size of the product and absorption. Oil phases can be used individually or in combination with each other to control emulsion consistency.[19] For example the mean diameter of particles in a LCT based emulsion is greater than that in a MCT emulsion. MCT may solubilise 100 times more active ingredient than LCT.[7]

1.1.10.2 Phase volume ratio

The volume of the dispersed phase compared with the volume of the external phase greatly influences the characteristics of the emulsion.[28] The ratio of the internal phase to the external phase is frequently determined by the solubility of the active ingredient or desired consistency of the final product.[4, 28] As the volume of the dispersed phase in an emulsion increases, the interfacial film expands further to surround the droplets of dispersed material, and the basic instability of the system increases.[6] Generally speaking, the most stable emulsions have an internal phase occupying 40 percent of the emulsion. Dilute or high concentrated emulsions are less stable and more difficult to prepare.[1] When a dilute emulsion is required, it is sometimes preferable to produce a more concentrated emulsion of greater stability and to dilute this to the required concentration before use.[4]

When one phase is in large excess, the emulsification will tend towards production of an emulsion with the smaller volume in the dispersed phase.[4] If an emulsion contains equal parts of oil and water, then the type of emulsion produced depends upon the nature of the emulsifying agent added and upon the technique of

emulsification adopted.[1] As the volume of the dispersed phase increases beyond that of the continuous phase, the o/w type of emulsion becomes more and more unstable and the emulsion may invert. If the emulsifying agent strongly favours only the original type of emulsion, it may not invert, and may instead form a multiple emulsion.[6] The phase-volume ratio has a considerable effect on the viscosity of an emulsion due to close packing of dispersed droplets at high phase volume ratio.[4, 20]

1.1.10.3 Selection of emulsifier

Selection of the most suitable emulsifying agent and its correct concentration can often be quantified.[1] Several factors can be taken into account to optimise selection of emulsifiers for an emulsion. Usually, the type of emulsion is selected from the anticipated use of the product.[19] The lipophilicity of the oil phase used can also effect the selection of emulsifiers.[6]

Adsorption of the emulsifying agent at the oil-water interface and formation of a rigid interfacial film is essential for emulsification and the stability of the emulsion.[1] A reduction of the interfacial tension makes emulsification easy, but does not by itself prevent coalescence of the particles and resultant phase separation.[1] A mixture of oil soluble and water-soluble emulsifiers produces better and more stable emulsions than an individual surfactant.[6] The complex interfacial films formed by the mixed emulsifying agents produce stable and elastic interfacial films, which could reduce the coalescence of dispersed particles.[2] For example anionic sulphated compounds have high water solubility and do not produce a stable interfacial film. To improve the stability and organoleptic properties, they are widely used in combination with non-ionic surfactants or fatty alcohols.[1, 19]

1.1.10.4 HLB value

A systematic approach to emulsifier selection was developed by Griffin in 1947 as the Hydrophilic-Lipophilic Balance (HLB) value of the surfactants.[4] Griffin assigned a number (0–40) indicative of emulsification behaviour and related to the balance between the hydrophilic and lipophilic (hydrophobic) portions of the molecule to the emulsifying agents.[32] In addition, a similar range of numbers has been assigned to various substances that are frequently emulsified, such as oils, lanolin, paraffin wax, xylene, carbon tetrachloride, and so on.[6] In general, oil-

soluble components have low HLB values; those that are water soluble have high HLB values. An o/w emulsion requires an emulsifier with an HLB in the range of 8 to 18 (Table 1-1).[32] Emulsifiers with HLB values in the range of 4 to 6 (Table 1-1) are used for w/o emulsions.[32] Most of the common surfactants lie outside these ranges and require mixed emulsifiers to achieve the required HLB.[23] There are some emulsifying agents, which can be considered as complete emulsifiers as they are capable of reducing the interfacial tension, and producing a sufficiently rigid interfacial film.[3] The required HLB for oils are given in Table 1-5.[4]

Table 1-1 HLB Scale and classification of surfactant solubility and application[19]

HLB	Surfactant Solubility
0	Hydrophobic/ Oil soluble
3	
6	Water dispersible
9	
12	Hydrophilic/ Water soluble
15	
18	

HLB range	Surfactant Application
2-3	Antifoaming Agent
3-6	w/o Emulsifying agent
7-9	Wetting agents (7-9)
8-16	o/w Emulsifying agents
13-15	Detergents
15-18	Solubilizing agents

The HLB value of an emulsifier can be determined experimentally or can be computed from the structural formula of the surfactant. Griffin defined the HLB value of a surfactant as the mol% of the hydrophilic group divided by 5, where a completely hydrophilic molecule has an HLB value of 20. Davies proposed a calculation of the HLB value by algebraically adding the values assigned to a particular atomic grouping within the molecule of the emulsifier.[4, 33]

$$HLB = (E + P)/5 \quad \text{.....Equation 1-4}$$

Where *E* is the percentage by weight of oxyethylene chains and *P* is the percentage by weight of polyhydric alcohol groups in the molecule. If the surfactant contains polyoxyethylene as the hydrophilic group then the simpler equation 1-5 applies.[33]

$$HLB = E/5 \quad \text{.....Equation 1-5}$$

Alternatively, HLB values can be derived directly from the chemical formula using empirically determined groups numbers. The formula is:

$$HLB = 7 + \Sigma(\text{hydrophilic group numbers}) - \Sigma(\text{lipophilic group numbers}) \quad \text{.....Equation 1-6}$$

Table 1-2 HLB numbers of hydrophilic groups[4, 19, 34]

Groups	Group Number
-SO4 – Na+	38.7
-COO –K+	21.1
-COO –Na+	19.1
Tertiary Amine	9.4
-COO	2.4
-COOH	2.1
-OH	1.9
-O-	1.3
-OH (sorbitan ring)	0.5

Table 1-3 HLB numbers for lipophilic groups[19, 34]

Group	Group Number
-CH-, $-CH_2-$, $-CH_3$, =CH-	-0.475
$-(CH_2-CH_2-O)-$	+0.33
$-(CH_2-CH_2-CH_2-O)-$	-0.15

The HLB of polyhydric alcohol fatty acid esters such as glycerol monostearate is obtained from the saponification value of the ester and the acid number of fatty acid using:

$$HLB = 20\left(1 - \frac{S}{A}\right) \quad \text{.....Equation 1-7}$$

The HLB requirements for emulsification of a particular ingredient differ markedly, depending on whether the ingredient is in the dispersed phase or the continuous phase, and depending on the type of emulsion it will become. Thus, paraffinic

mineral oil has an HLB value of 11 for emulsification as the dispersed phase in an o/w emulsion and a value of 4 as the continuous phase in a w/o emulsion.[35]

Table 1-4 HLB values of most commonly used emulsifying agents.[19, 34]

Chemical Name	HLB
Sorbitan trioleate	1.8
Propylene glycol monostearate	3.4
Glycerol monostearate (non-self-emulsifying)	3.8
Propylene glycol monolaurate	4.5
Sorbitan monostearate	4.7
Glycerol monostearate (self-emulsifying)	5.5
Lecithin	8.0
Sorbitan monolaurate	8.6
Polyoxyethylene-4-lauryl ether	9.5
Polyethylene glycol 400 monostearate	11.6
Polyoxyethylene-4-sorbitan monolaurate	13.3
Sucrose stearate	14.5
Polyoxyethylene-20-sorbitan monopalmitate	15.6
Polyoxyethylene-40-stearate	16.9
Sodium oleate	18.0
Sodium lauryl sulphate	40.0

To determine the optimum emulsifier combination, various mixtures of different emulsifying agents with the same weighted average HLB number must then be tried to determine which structural types of emulsifying agents give the best results with this particular combination of emulsion ingredients.[35, 36] However, they are thermodynamically unstable systems and may undergo physical destabilisation such as coalescence, sedimentation and Ostwald ripening. In order to make them kinetically stable, additional polymers and solid particles are added.[37]

The HLB method can be used for calculating the relative quantities of these emulgents necessary to produce the most physically stable emulsion.[19] In addition, it has been suggested that certain emulsifying agents of a given HLB value appear to work best with a particular oil phase, and this has given rise to the concept of a required HLB value for any oil or combination of oils.[34] However, this does not necessarily mean that every surfactant having the require HLB value will produce a good emulsion. The presence or absence of any polarity in the material being

emulsified is important, because this will affect the polarity required in the emulsifier.[24, 38] The HLB of a mixture of surfactants, consisting of fraction x of A and (1-x) of B, is assumed to be an algebraic mean of the two HLB numbers:[3]

$$HLB_{mix} = xHLB_A + (1-x)HLB_B \quad \text{.....Equation 1-8}$$

Table 1-5 Required HLB values of common oils/lipid[19]

Oil/Lipid	W/O emulsion	O/W emulsion
Stearic Acid	—	17.0
Alcohol, cetyl	—	13.0
Lanolin, anhydrous	8	15.0
Soybean oil	-	7 -8.0
Mineral oil, light	4	10–12.0
Mineral oil, heavy	4	10.5
Wax, beeswax	5	10–16.0
Microcrystalline	—	9.5
Paraffin	—	9.0

1.1.10.5 Mixed emulsifying systems

Occasionally, a stable emulsion can be produced with the desired viscosity using a single emulsifier. However, a combination of lipophilic and hydrophilic surfactants appears to produce mixed interfacial phases of high surface coverage as well as of sufficient viscosity to promote stability. Almost any HLB can be obtained with greater efficiency and low concentration by appropriate blending of emulsifiers.[4]

Schulman and Cockbain showed that a mixture of an oil-soluble co-surfactant/stabiliser such as cholesterol and a surface active agent such a sodium cetyl sulphate was able to form a stable, and flexible condensed film at the oil/water interface.[39] Mixed surfactants may provide better resistance against rupture and present lower interfacial tension, compared with that produced by either component alone and also produce a stable emulsion similar to a lyophobic colloidal dispersion.[39] For complex formation at the interface the correct ‘shape’ of molecule is necessary. For o/w emulsions stabilised with non-ionic polyoxyethylene esters , emulsion stability increases with increase in the length of the polyoxyethylene esterschain; for w/o emulsions, with length of the hydrophobic group.[40] The effect of chemical structure on the type of interfacial film must be taken into account when

developing the optimum emulsifier blend. Condensed films are produced by emulgents having long, saturated hydrocarbon groups, thus providing maximum cohesion between adjacent molecules. In most cases it has been found that the most stable emulsions are formed when both emulsifying agents are of the same hydrocarbon chain length.[1, 19]

A temporary complex surfactant/co-surfactant layer helps to emulsify the oil phase. The co-surfactant diffuses towards the interior of the droplets reducing the stability of the droplets. A minimum amount of co-surfactant is needed to saturate the droplets and have enough co-surfactant to form the complex at the interface.[24] The droplets are protected against both diffusional degradation and droplet coagulation by using a water insoluble compound and an efficient surfactant.

1.1.11 Emulsification

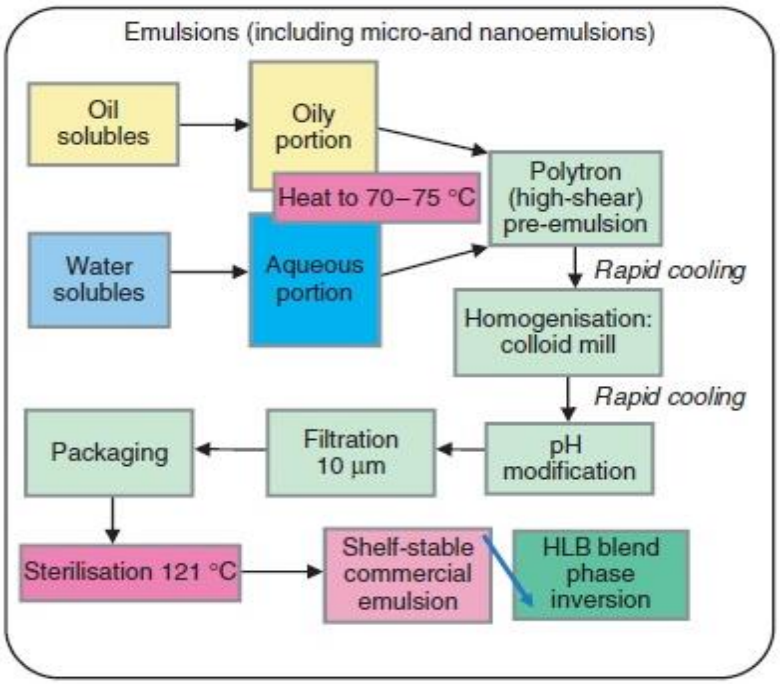
Emulsification or formation of emulsion requires energy input in the form of mechanical agitation, pressurisation, ultrasonic vibration, heat or electricity. This energy input reduces the internal phase into small droplets.[4] The size of the droplets will depend on the amount of work applied. In emulsion systems, the interfacial tension (γ) is always greater than zero, and since there is a marked increase in interfacial area (ΔA) during the process, the minimum work involved is the product of the interfacial tension and the increase in interfacial area ($W_{min} = \Delta A \times \gamma$).[6]

The amount of work required depends on the type of energy applied and duration of the energy input. Overall, formation of the emulsions will be dependent on the type of equipment used and parameters set for the emulsification process including temperature, rate of addition of internal phase and timing.[4] There are two ways of emulsification: high energy and low energy. High energy emulsification is carried out by two main types of devices: high pressure homogeniser and ultrasound generators. Low-energy emulsification methods use internal chemical energy stored in the components and by changing physical properties such as interfacial tension and the spontaneous curvature of the surfactants. These arrangements are more energy efficient and require less intense mechanical energy.[41] Almost all methods to produce emulsions use some sort of agitation or force to break up the internal phase into droplets. Factors that breakup the liquid include type of agitation, force applied or degree of shear, duration of agitation, viscosity of the

internal and external phase, and the interfacial tension between the two liquids. The latter two are dependent on the type of emulsion, phase volume ratio and concentration of emulsifier.[4] Additional factors are dependent on the type and specifications of equipment of used. The amount of work involved in producing an emulsion is dependent to a considerable extent upon the interfacial tension. If that is high, a considerable expenditure of energy is necessary to produce an emulsion; if it is low, little work may be required.[1]

An important parameter, which influences the emulsification process, is temperature. Vaporisation is an effective way of breaking almost all the bonds between the molecules of a liquid. Increase in temperature can reduce the interfacial tension as well as viscosity, which would favour emulsification. However, increased temperature raises the kinetic energy of droplets and thereby facilitates their coalescence. This phenomenon occurs during storage at elevated temperatures.[4] Changes in temperature also alter the distribution of the emulsifiers, especially polysorbates.[42] In practice, there is little to be gained and much to be lost by increasing the temperature of the mixture above the optimum temperature for the emulsifying agent or mixture of emulsifying agents.[1] It is impossible to predict if raising the temperature will favour emulsification or coalescence.[4]

Figure 1-3 Flow chart of method of preparation for pharmaceutical emulsion



Agitation time has a profound influence on the process of emulsification. During the initial period of agitation required for emulsification, droplets are formed. However, after agitation for certain period of time, the chance for collision between droplets becomes more frequent, and coalescence can occur.[4] The speed of rotor also affects the emulsion droplet size, viscosity and ultimately stability. The mechanical aspects of preparation including stirrer type, speed and agitation time need to be considered during formulation development.[4] The rate of addition of the oil phase in an o/w emulsion can affect the particle size and stability of the finished product. The cooling and heating rate of the initial stage can also influence the characteristics of the final products.[4]

1.1.11.1 Order of mixing

1.1.11.1.1 Addition of internal phase to external phase

The gradual addition of the internal phase to the external phase is the most satisfactory general method of producing an emulsion. If the external phase is water, and the internal phase oil, water soluble substances are dissolved in the water and all oil-soluble substances in the oil. The oil mixture is then added gradually in small portions to the aqueous mixer, with vigorous stirring or agitation. Once the primary emulsion is formed, it is diluted to volume with the aqueous phase. Once the emulsion is formed there will be extremely difficult in adding anything to the dispersed phase.[1]

1.1.11.1.2 Addition of the external phase to the internal phase

When the external phase is added to the internal phase in o/w emulsion, initially a w/o emulsion is formed. On subsequent addition of further quantities of water, phase inversion takes place and an o/w emulsion is formed. This is called the "dry–gum" method. Prolonging the mixing of the oil and gum could break the emulsion.[1]

1.1.11.1.3 Mixing both phase after warming each

In this method the waxes and oils are melted with the emulsifying agents and the water, and any water soluble ingredients are warmed to a temperature which is a few degrees higher than that of the oil phase. The two phases are then mixed and the mixture stirred until cold. It is important for both phases to be at the same temperature when mixed in order to avoid crystallisation of the wax. Continuous

mixing is required for slow cooling and to avoid a granular product due to crystallisation of the wax.[1]

1.1.11.1.4 Emulsification by precipitation

In this method, the oily phase is dissolved in water-miscible organic solvents, such as acetone, ethanol and ethyl methyl ketone. The organic phase is poured into an aqueous phase containing surfactant to yield an emulsion.[25] Spontaneous emulsion can occur due to displacement of solvent from the oily to the aqueous phase which induces great turbulence at the water/oil interface. The rapid transfer of hydrophilic materials from the oil to the water phase results in a dramatic increase of the interfacial area, giving rise to the metastable emulsion state.[25] The oil is precipitated in the form of fine globules, and a dilute o/w emulsion is produced. If a suitable emulsifying agent was present in the original alcoholic solution or in the water, a fairly stable dilute emulsion results.[1] In order to obtain nanometric-scaled droplets with this method a very high solvent/ oil ratio is required.[41] The solvent displacement method has been adopted for polymeric nanoparticles.

The major drawback of this method is the use of organic solvents, such as acetone, which require additional energy inputs for their removal from emulsion. The process of solvent removal can pose several difficulties during scale-up.[25]

1.1.11.1.5 Phase inversion method

This method generates emulsions without the use of any organic solvent or high energy process. Emulsions obtained from this process are not thermodynamically stable, although they might have high kinetic energy and long term colloidal stability.[25] Phase transitions occur during the emulsification process and these involve a lamellar liquid crystalline phase or D-type bicontinuous microemulsion during the process. Phase inversion in emulsions can be of two types: catastrophic phase inversion (CPI) and transitional phase inversion (TPI).[23]

In the phase inversion temperature method (PIT method), oil, water and non-ionic surfactants are all mixed together at room temperature and stirred to form an emulsion. The o/w emulsion is gradually heated.[25] Solubility of non-ionic ethoxylated surfactants is highly dependent on temperature. These surfactants are subjected to dehydration of the polyethyleneoxide chain with increasing

temperature.[25] As a consequence, the surfactants become more lipophilic and solubilised in the oily phase. At this point, the emulsion undergoes phase inversion from the initial o/w to w/o emulsion. The temperature at which this inversion occurs is termed the PIT.[23] PIT is generally considered to be the temperature at which the hydrophilic and the lipophilic properties of the emulsifier are in balance and is therefore also called the HLB temperature.[4, 41] Then the emulsion is quickly cooled down to obtain an o/w emulsion. This procedure forms a basis of nanoemulsion fabrication using the PIT method.[23]

1.1.11.2 Mechanical stirrers:

Most commonly used stirrers for emulsion preparations are overhead propeller mixers with paddle blades, counter rotating blades or planetary action blades.[2] Selection of mixer and blade will depend on quantity, viscosity, and force required for agitation. The degree of agitation is controlled by the speed of impeller rotation.[43] The particle size reduction, efficiency of mixing and force provided by agitation will be dependent on type of impeller, position in the container, type of baffles and shape of the container. Vigorous agitation may be required for producing fine droplets, which may limit the use of stirrers. High shear rates also produce foam in the system containing surface active agents.[4]

Table 1-6 Unit operations and their variables (adopted from S. Tamilvanan)[7]

Unit Operations	Independent variables	Dependent Variables
Solubilisation	Solubility, temperature, mixing speed, mixing time	Clarity of solution, transparency
Emulsification	Mixing speed, mixing time, energy input per unit volume, temperature	Viscosity, particle size, particle size distribution
Homgenisation	Temperature, process time, flow rate, volume	Particle size, particle size distribution

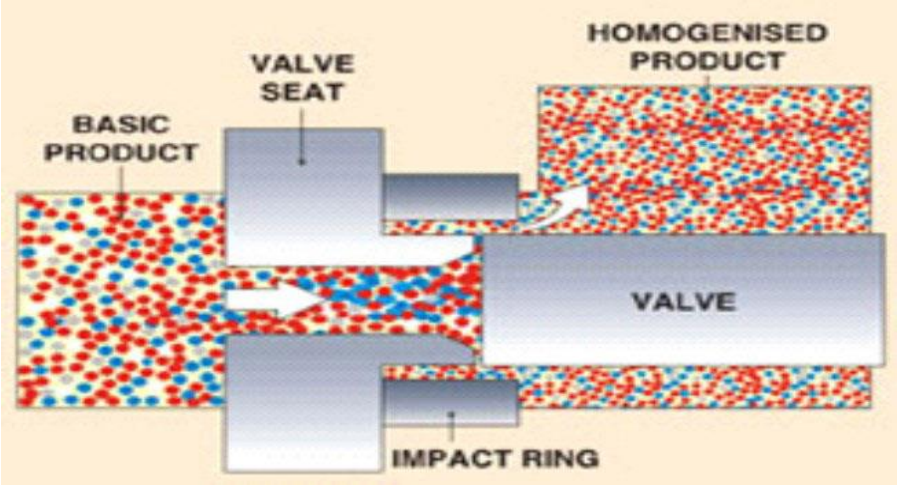
Table 1-7 Performance of different types of mechanical stirrer[43]

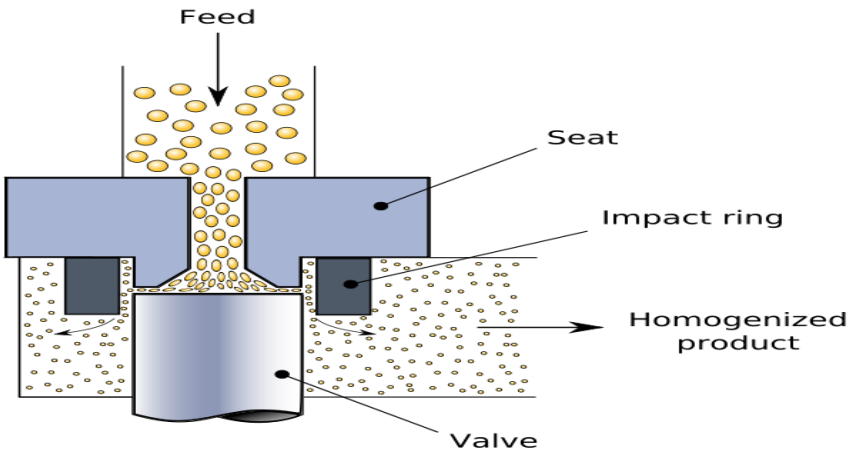
Type of Device	Energy Dissipation (m^2/s^3)	Particle size range (µm)
Static Mixers	10-1000	50-1000
Agitated vessel	0.1-100	20-500
Rotor stator	1000-100000	0.5-100
Valve homogeniser	~10^8	0.5-1
Ultrsonics	~10^9	0.2-0.5

1.1.11.3 Homogeniser

Homogenisation is also known as micro-fluidization, which operates by agitating a crude emulsion under high-pressure or using a positive displacement pump (500 - 5,000 psi). Depending on the model, homogeniser are capable of operating at up to 23,000 psi and can pump at flow rates as high as 200 gallons per minute.[25] The product is forced through the fine valve orifices called “microchannels”.[23] During this process, several forces, such as hydraulic shear, intense turbulence and cavitation (Table 1-7), act together to yield nanoemulsion.[25] Homogenised emulsion contains smaller and more uniform size globules, which have much greater stability during storage.[1] The resultant product can be resubjected to high-pressure homogenisation until an emulsion with desired droplet size and polydispersity index is obtained.[25]

Figure 1-4 Mechanism of Homogenising Valve (Adopted from homogeniser handbook 2009, APV Gaulin Homogeniser Group, SPX Corporation)





Homogenisers have been employed successfully to reduce the particle size to the nanometre range.[4] Emulsions in a high-pressure homogeniser, are pushed with high pressure (100-2000 bar) through a narrow gap in the range of a few microns, with constant velocity (Figure 1-4). Due to the instantaneous pressure at a point, the liquid ruptures giving a void filled either with the vapour of the liquid or with the gases that are dissolved in the liquid. The collapse of such voids results in a strong shock wave on a microscale. This phenomenon leads to the formation of very fine but polydispersed droplets.[23] The temperature rises as the product passes through the valve. The temperature rise (°F) through the homogeniser can be estimated by the following equation:[44]

$$\Delta T = \frac{\Delta P}{40.42\ C_v\ d} \quad \text{.....Equation 1-9}$$

where ΔP is in psi, Cv is specific heat (BTU/lb°F) and d is density (lb/ gallon). [44]

1.1.11.4 Ultrasonifiers

In ultrasonic emulsification, the energy input is provided through so called sonotrodes containing piezoelectric quartz crystals that can expand and contract in response to alternating electrical voltage. As the tip of the sonicator probe contacts the liquid, it generates a mechanical vibration.[45] Cavitation is the main phenomenon responsible for ultrasonically induced effects. Cavitation is the formation and collapse of vapour cavities in a flowing liquid. The collapses of these cavities causes powerful shock waves throughout the liquid breaking the dispersed droplets. Within the ultrasound range, the power available varies inversely with the frequency.[45, 46] A powerful ultrasound (0-200kHz) is able to produce physical and chemical changes such as emulsification. Ultrasound is the preferred method to prepare fine emulsions from coarse emulsions. Due to the small product throughput the ultrasound emulsification process is mainly applied in laboratories.[46] The commercial equipment is based on the principle of the Pohlman liquid whistle. The dispersion is forced through the orifice at pressure of 150 to 350 psi and impinges upon a vibrating blade producing an ultrasonic note.[4]

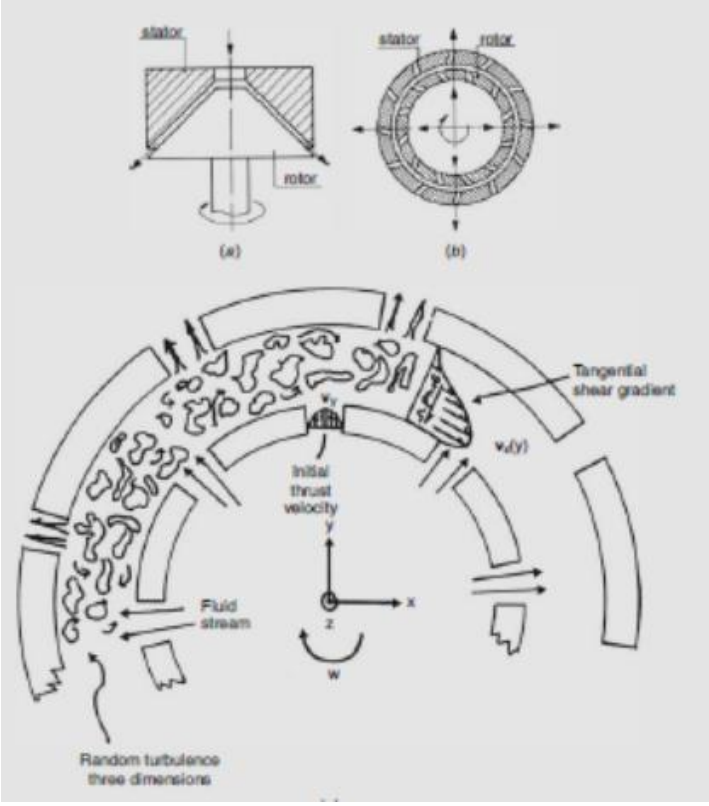
1.1.11.5 Rotor mixer/ colloid mill

A rotor mixer is a high speed rotor, typically 10 to 50m/s in close proximity to a stator. These high shear devices produce a shear rate from 20,000 to 100,000 s^{-1}.[43]

Rotor stator mills can be applied to a range of products and processes including viscous materials as high as 100-150 Pas. A conical couette type of mixer with more complex variations of teeth or slots type mixer is also known as colloid mill.[4, 43] Colloidal mills operate on the principle of high shear, which is normally generated between the rotor and the stator of the mill.[4]

In all types of rotor mixers the work on the product takes place between a stationary part (stator) and a rotating cone (rotor).[23] By decreasing the distance between the rotor and stator, shear on the product is increased (Figure 1-5).[43] More popular mixers such as the Silverson® have a radial impeller that rotates inside a stationary housing with slots.[23] The rotor moves the fluid out of the mixer head through the slots, which generates the tangential shear flow inside the stator.[43] Rotor stators are particularly suitable for producing emulsions with a continuous phase of medium to high viscosity. A narrow droplet size distribution is obtained if the energy density in the space between rotor and stator is well controlled.[23]

Figure 1-5 **Mechanism of Rotor Stirrer: (a) simple couette, (b) toothed rotor-stator (c) Hydrodynamic presentation**



1.1.12 Proposed study

In recent decades there has been a great deal of research focused on the drug delivery of pharmaceutical and biopharmaceuticals. Generally, the emulsification method has been extensively used for the encapsulation of bioactive agents, which are water-insoluble and susceptible to denaturation and degradation during manufacturing. Micro particles prepared by the emulsion technique in the form of droplets, microsphere or liposomes are excellent reservoirs for the encapsulation of a variety of pharmaceutical and biopharmaceutical compounds. The physicochemical properties of emulsion and its stability are dependent on many factors including drug properties, emulsion composition and surfactant to lipid ratio, and method of manufacture. The issue of emulsion based delivery systems is that there is a wide range of approaches being identified. In reality however, there are a limited number of technologies that are currently truly commercially viable.

The major limitations of emulsion based delivery are large scale manufacturing and physical stability. Submicron emulsions are especially difficult to scale-up and require expensive specialised equipment for manufacturing. Emulsion based delivery systems are also known for their physical instability, which further exacerbated by environmental changes such as heat, shaking, light and storage conditions. Thus there is a need to design innovative lipid emulsions which are safe, economic, robust and well-controlled to produce commercially and has prolonged shelf life of 1-2 years.

The main objective of this study was to develop and optimize an emulsion technology platform for drug delivery: and areas of investigation included process scale up and characterisation of the stability and physical properties of optimised formulation; and evaluation of the efficacy and safety of the formulation containing active ingredient. These objectives were pursued in sequential projects throughout this study and are described as follows:

Project 1: Development of an emulsion formulation

Project 2: Optimisation of emulsion formulation and process scale up

Project 3: Stability of the emulsion

Project 4: Clinical evaluation of an emulsion containing lidocaine

2 Development of a Pharmaceutical Emulsion

2.1 Introduction

Colloidal droplets, such as some emulsion droplets, liposomes and mixed micelles are potential delivery systems and have been investigated for the last two decades. There is a need for multipurpose vehicle platform systems with the increasing number of new lipophilic drug candidates'.[47] Various approaches to solubilise drugs, such as microemulsions or liposome formulations have been reported in the literature.[48] However, all of these systems have limitations due to their physicochemical nature, toxicity and pharmaceutical acceptability.[47] There is an increasing interest in emulsions as a carrier of lipophilic drugs. Emulsions are biocompatible, have longer shelf-life and may be manufactured on an industrial scale.[48, 49] Progress is being made in emulsion formulations which provide efficacy with reduced systemic toxicity. For example, reduced nephrotoxicity has been shown for amphotericin B fat emulsions. Submicron emulsions have also been investigated as carriers for transdermal drug delivery using diazepam as a model drug.[50] There are also several commercially produced and essentially non-toxic fat emulsions available including Intralipid®, Lipofundin® and Liposyn®. The relatively low toxicity coupled with their extensive use in total parenteral nutrition has made the emulsion an attractive drug delivery system.[51]

Emulsion drug delivery requires special attention to the nature of components and processing methods. Intravenously administered large colloidal droplets are rapidly taken up by the reticuloendothelial system (RES).[50] Submicron size droplets and those with hydrophilic surfaces have slow clearance rates.[52] The droplet size of emulsions for intravenous pharmaceutical drug delivery is required to be below 1 μm, to prevent the incidence of emboli and changes in blood pressure related to droplets larger than 4–6 μm .[53] Besides, emulsions containing the globules of 200–500 nm also tend to be the more physically stable.[54] This makes processing of emulsions difficult, requires specialized equipment and complex technology. The complexity of the process further increases in the preparation of sterile emulsions under aseptic condition. Therefore, emulsion formulations that are of submicron droplet size require their physicochemical properties to be used collectively with commonly accessible processing technology.

The rationale behind the development is that submicron sized, emulsified droplets, ranging from 0.5 to 1 μm, are possibly similar in structure to chylomicrons,

produced in the liver and intestine.[51] Miniemulsions are submicron sized emulsions (100-1,000nm) and are being investigated as drug carriers to improve the delivery of therapeutic agents. They effectively encapsulate hydrophobic molecules with increased solvent power.[50] Miniemulsions form a kinetically stable system in which stability is conferred by appropriate selection of surfactant and co-surfactant.[47] The most common surfactants and co-surfactants are amphiphilic molecules and are anionic, cationic or non-ionic.[47] There is no standard guide in selecting an emulsifying agent and the technique used to prepare an emulsion, which can serve the ultimate objective of the emulsion formation and the type of emulsion developed.[1, 47]

The standard process to produce o/w emulsions requires oil soluble emulsifiers to be dissolved in the oil phase and water soluble emulsifiers in the aqueous phase with a HLB value of the emulsifier mixture in the range of 8.0 -16.5.[38] An emulsions ability to incorporate a maximum amount of dispersed phase into the continuous phase is an important characteristic. The use of a single surfactant may not necessarily provide enough solubilisation capacity. Mixed micelles have been proven to enhance the solubilisation capacity in emulsion systems.[48] The micelles with a mixture of emulsifiers may improve the overall product performance and may also minimise the total amount of surfactant required for the final product or processes.[55]

Emulsifiers normally form a film at the interface of the droplets. A strong interfacial film can improve droplet stability.[56] In an o/w emulsion the hydrophilic polar portion of the emulsifier provides a better barrier towards coalescence than their non-polar counterpart.[4] Emulsion stability can be improved by use of the non-ionic emulsifiers or polymeric stabilisers in the presence of salts.[57] Sometimes, diffusion during Ostwald ripening shows an increase in the average droplet size over time at the smaller droplets' expense. Oswald ripening, in the presence of insoluble material, increases the chemical potential of the solute in the droplets, and the dispersed phase material diffuses back to the small droplets.[47, 57]

On the other hand, only a number of emulsifiers are commonly listed as safe for human use, especially for internal administration. Most commonly used synthetic and efficient emulsifiers are toxic in nature and regarded as unsafe for parenteral administration. Those already approved by the various regulatory authorities and

listed in various pharmacopeias' for internal administration and most frequently used in formulations are phospholipids, poloxamer, Spans and Tweens.[56, 58] Therefore, in this study special consideration was given to phospholipids, Tweens and Spans.

2.1.1 Phospholipids

The phospholipids are heterogeneous mixtures of phosphatides. Soy phospholipid (lecithin) is in the form of solid, yellow waxy flakes. Lecithin normally contains 20% phosphatidylcholine (PC), 15-22% phosphatidylethanolamine (PE), and ~20% phosphatidylinositol, as the major phospholipids.[59, 60] Lecithin is water insoluble, but is dispersible at room temperature or at temperatures higher than the transition temperature. Lecithin is soluble in iso-propanol and chloroform, and may require heating to dissolve in ethanol and vegetable oil.[60, 61] The use of lecithin is permitted in most countries. In the United States, lecithin is affirmed by the FDA as GRAS and is an approved ingredient in many pharmaceutical and food products. In Europe, lecithins are covered under E322 regulations (492). [60]

Commercial lecithin may be purified and yet still contains complex mixtures of phosphatides and other materials.[62] They contain PC (>70%) as the major component, which is in zwitterionic form and overall neutral over a wide pH range. PC forms an interfacial lamellar liquid crystalline phase in the presence of water.[58] The PE content in purified lecithin is <20% and it is negatively charged.[63] The PC and PE contents in commercial phospholipids differ from each other due to the dissimilarity in purification technology (Table 2-1).[56]

Table 2-1 Example of commercial lecithin PC and PE content[56]

Commercial Lecithin	PC content (%)	PE content (%)
Lipoid EPC	98	0.1
Lipoid E-80	81	8.4

Lecithin based emulsions are biodegradable, biocompatible and physically stable.[50] Lecithin is dissolved in the oil phase at higher temperatures or in the presence of a solvent such as alcohol or chloroform for the preparation of o/w emulsions.[53] In most o/w injectable emulsions, the stabilising agent is purified lecithin.[50, 53] The first approved intravenously administered emulsion, Intralipid® was an oil-in-water

emulsion of soybean oil droplets stabilised by a monolayer of egg yolk phospholipids.[62] The phospholipid based emulsions for drug delivery solubilise lipophilic drugs in their core or in the interfacial phospholipid layer. [50]

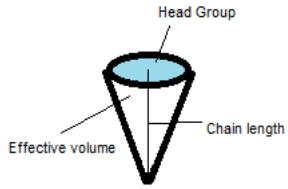
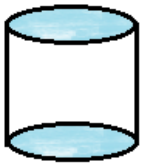
Microemulsions studied by Moreno et al. were stable after dilution, and it was suggested this was due to the interfacial activities of the surfactants employed to form microemulsions.[64] Moreno et al. also argued that short chain alcohols, as co-surfactants were not surface active agents and so they did not exert their effect at the interfacial layer but instead act by decreasing the HLB of the real surfactant and reducing the solubility of the polar head group in the aqueous phase.[64] Lecithins work in two ways as they are oil soluble and surface active amphiphiles. Being amphiphilic in nature lecithin is localised predominantly in the interfacial layer stabilising the system by creating a strong film around the globule.[64] The phospholipid monolayer in triglyceride emulsions is nearly balanced with minimum interfacial tension.[62]

Lecithin stabilises emulsion droplets, by the formation of a mechanical barrier and generating an electrical surface charge. The ionization of polar head groups of the hydrophilic group generates an electrical charge on the surface of the droplets and is pH dependent.[63] The correct proportions of PC and PE content of lecithin may improve the emulsion stability.[58, 63] The surface charge produced by the phospholipids is relatively low and dependent on the exact proportions of the charged phosphatides. The phospholipid mixture enriched with negatively charged phosphatides, including PE, does not provide stabilisation by electrical charges.[49, 65] It has been argued that the proportions of PE and PC are an important aspect for the packing, curvature, and the polarization of the emulsifier membrane, and consequently the stability of the final product. The interaction between PC and PE with each other as well as with solubilised oil within the core is yet to be investigated in the case of interfacial area changes, heating, and degradation.[56] The spontaneous curvature of natural phospholipids at the oil/water interface is close to zero. It was suggested that the required spontaneous curvature can be attained by changing the phospholipid polar head group, the length and degree of saturation of the acyl chain, and the nature of the oil.[30] PE has a small head group relative to PC, and according to the curvature model for emulsion stability, o/w emulsions stabilised by PE are expected to be less stable than PC.[50]

Phospholipids form aggregates, when dispersed in aqueous media. The aggregation is primarily dependent on the fatty acid chain length and concentration. Synthetic PC with two identical fatty acid chains of four or less carbon atoms would stay as a monomer in an aqueous medium. Whereas the PC with chains of 6-8 carbon atoms forms micelles above the critical micellar concentration (cmc), however that of PC with longer carbon chains forms a lamellar phase.[66] Most soy PC contains fatty acid chain with 16-18 carbon atoms. The transition temperatures and the surface behaviour are also influenced by the length and saturation of the fatty acyl chains.[66] Washington et al. suggested that the saturated lipids have transition temperatures above room temperature, and saturated phospholipids may produce more stable emulsions.[30] The transition temperature is reduced by introducing unsaturated bonds in the acyl chain.[30] The stability of the emulsion can be affected due to possible interference in the packing arrangements at the interface.

The long chain phospholipids exhibit several different hydrated phases, a property called lyotropic mesomorphism. With increasing water content, the molecular area increases to about 70 A^2, and the lamellar structure becomes predominant.[67] The lyotropic phases are also dependent on the lecithin composition and temperature.[66] The transition temperature (Tm), also known as a thermotropic phase transition, is associated with disordering of the hydrocarbon chains in the interior of the bilayer and concomitant fluidity transformation. A liquid crystalline to gel phase results when the temperature exceeds the phase transition temperature.[66] Furthermore, the space covered by the hydrated 'head groups' has a strong influence on molecular orientation in organised arrangements and is dependent on the amount of water present.[67] Lamellar lecithin shows an increase in inter bilayer spacing with uptake of water, and closed bilayer vesicles are formed upon dilution. Curvature of the phospholipid bilayers and packing geometry affect the distribution of the phospholipid molecules at the interface. The size of the bilayer is also dependent on the molecules packing parameter. PC forms a micellar, lamellar or hexagonal phase depending on the type of head group, fatty acid, temperature and hydration.[68] The organisational behaviour of typical amphiphilic molecules is defined by separation from the polar part to the nonpolar part of the molecule and can be demonstrated by the packing parameter concept (PPC).[47, 57] The packing geometries of surfactants including phospholipids in aqueous solution is summarised below.

Table 2-2 Molecular shapes and association structure of surfactant (adopted from Cevc et al. and Christopher Roop et al.) [47, 68]

Critical Packing Shape	Critical packing parameter (p)	Surfactant /phoshoplipid
	<0.5	Lysophospholipids, Amphiphiles such as Tweens, Pluronic F68
	0.5-1	Double chained lipids with large head groups including phospholipids such as PC,
	~1	Double chained lipids with small head groups e.g. PE
	>1	Double chained lipids with polyunsaturated chains

Surfactant molecules with p value <0.5 attain a conical shape. Such molecules are expected to form micelles due to the hydrophobic tails located into the inner core and the polar head groups at the outer shell.[47] The smaller the packing parameter, the more spherical the micelle geometry can be expected. Phosphatidylcholines (PC) have a p-value ≥0.5 and forms vesicles or micelles, when mixed with micelle forming amphiphiles.[47, 57] Under specific conditions, phospholipids form mixed micelles when being combined with suitable hydrophilic surfactants. The dissolution of a bilayer lipid membrane by the addition of surfactants also produces mixed micelles containing both phospholipids and surfactants.[66] By removing surfactant from the mixed micelles, the lipid bilayer may be reconstituted into a liposome. The hydrophilic surfactant forms a sequence of phases in water, which can solubilise lecithin to some extent. The lamellar phase of the phospholipid can readily take up surfactant, and then swells in water to a maximum of 50% water. In the two-phase system the concentration of surfactant increases until the cmc.[69] Flexible liposomes have also been prepared by combining a lipid such as PC with

surfactant typically non-ionic Tween 80 or a bile salt.[57] It is evident that mixed micelles offer a high potential to encapsulate oil and lipophilic drug. It presents a drug delivery system with many advantages over conventional delivery systems.[47] Phospholipid serves as a water insoluble swellable amphiphilic component in a mixed micelle system and water soluble surfactant is positioned around the perimeter of the phospholipid bilayer as well as incorporated within the bilayer.[57]

2.1.2 Polysorbates

Many studies have reported the use of non-ionic surfactants owing to their biocompatibility and reduced toxicity when used as surfactant or co-surfactant for lipid emulsions.[64] In addition, non-ionic surfactants such as Spans, Tweens and polyethylene oxide (Pluronic) are commonly used in parenteral emulsions.[51] Besides, lecithin is not capable by itself of producing o/w submicron emulsions because of its lipophilicity. A co-surfactant such as a short chain alcohol has traditionally been employed to reduce the interfacial tension.[53, 64] It has been suggested that the addition of a hydrophilic surfactant may improve the stability of lecithin based emulsions. A study by Benita and Levy had used a combination of phospholipids and poloxamer to stabilise medicated parenteral o/w emulsions. The presence of a hydrophilic surfactant did not affect the pharmacological activity.[70, 71] The enhanced stability was suggested to be due to the formation of a complex interfacial film of the non-ionic emulsifier and the phospholipid molecules at the oil-water interface.[70, 71] However, the mode of interaction of hydrophilic surfactants with phospholipids in the emulsification process is still not well understood.[56] Lundberg also reported that non-ionic surfactants were much more effective as emulsifiers with the purified PC compared with zwitterionic surfactants, especially Tweens® and Brij® possessed favourable properties in combination with EPC.[52] Among Tweens better outcomes were noted for polysorbate 80, in comparisionwith polysorbates 20 and 40. The pluronic series was found to have only second level effects as emulsifiers.[52]

Within polysorbates the choice of hydrophilic surfactant may have an impact on the formation and stability of the final product. For example, polysorbate 85 has a low HLB compared with polysorbate 80 and possesses the same number of polyoxyethylene esterschains as the polar head group. Polysorbate 85 differs in hydrophobic moieties in respect to the number of carbon chains and total number

of double bonds. Double bonds in the middle of the long hydrocarbon chain could increase the volume of the chain with reduced chain length. This imposes stereo chemical constraints on the system and reduces the attractive interaction between the hydrophobic chains of surfactants. This suggests that polysorbate 80 may support the formation of the o/w emulsions more readily compared with polysorbate 85.[55]

Polysorbates are soluble amphiphiles. Above the cmc, they form micelles of various sizes and shapes. They also form thermodynamically stable isotropic solutions with insoluble co-surfactant such as phospholipids. This process which is commonly denoted as solubilisation, involves the transformation of lamellar structures into mixed micelles.[66] The penetration of water molecules into the palisade layer of the surfactant interface results in more rigid surfactant chains and promotes higher oil solubilisation.[69] The surfactant molecules penetrate the interfacial film and change the curvature and fluidity of the interfacial film.[72] One of the most important outcomes from the addition of hydrophilic surfactant is the reduction in the number of droplets larger than one micron in diameter.[73] Lundberg examined the ability of polysorbate 80 to reduce the droplet size of castor oil emulsions stabilised by egg PC. Droplet sizes of the order of 50 nm were obtained at weight ratios of polysorbate 80/EPC greater than about 4.[52] The equilibrium between both surfactants may provide synergy in the formation and stabilisation of the submicron sized emulsion system. Polysorbate 80 can reduce the interfacial tension of the system and increase the HLB of lecithin due to its surface activity and chemical structure. On the other hand, being amphiphilic in nature and in the presence of long hydrocarbon chains, lecithin would increase the solubilisation of oil within the surfactant system.[64]

The ultimate goal is to achieve a stable lipid submicron sized emulsion, which remains stable for an acceptable shelf-life of 12-24 months.[50] Hydrophilic surfactants such as Tweens are adsorbed at the surface of a hydrophobic particle, and while extending into the bulk aqueous environment they provide a steric barrier to coalescence.[50] The addition of lipophilic surfactant such as lecithin may help incorporate significantly large amounts of the internal phase, with an improved solubilisation capacity.[64]

2.1.3 A study of emulsion formulation

The aim of this study was to formulate pharmaceutically acceptable submicron emulsions, with consideration for ease of preparation, and long-term stability. In this study, emulsions were prepared using a mixed emulsifier system. Further, a hydrophilic surfactant with high HLB values of 10-18 was selected as the primary surfactant and water insoluble surfactants with low HLB values of 2-8 were selected as the co-emulsifier.

Soybean oil was selected as the oil phase because of its safety profile. Refined soybean oil is within the list of permitted additives for food products intended for human intake (FDA) and been used in marketed parenteral products including total parenteral nutrition, Intralipid.[60] No carcinogenic, mutagenic or acute toxicity has been reported for soybean oil.[74]

Development and optimization of emulsion formulations involved multiple studies. A series of sequential studies was conducted to improve the emulsification efficacy of the mixed emulsifying system at each stage. The results of each study provided useful data to refine the design of each subsequent study. The study included

I. Development of a base emulsion using the HLB system

II. Selection of the order mixing for the base emulsion,

III. Selection of equipment to achieve submicron droplets

2.2 Methodology

2.2.1 Materials

Soybean oil was purchased from Pharma Scope and Sigma Life Science, USA (lot# MKBK0322V) for development and optimisation trials. Refined soybean oil USP (lot # C126935, C153626, C151193) from PCCA Pty Ltd, Australia was used for stability and clinical trial batches. Tween 80® Ph.Eur. And Tween 80® NF were purchased from Fluka Analytical, Germany ((HLB 15, lot# 1259073, 1390440) and Fagron Inc., USA (lot# 23B20-U07-008548) respectively. Sorbitan oleate (span 80®, HLB 4.3) was purchased from Sigma-Aldrich. Epikuron 200® (Soy PC), containing (>90% phosphatidylcholine, HLB 8, lot# 129047, 199060, 1-7-9027) was either donated or purchased from Cargill GmbH, Germany and used as lipophilic surfactant unless otherwise specified. Ethanol (95%) was purchased from CSR Distilleries Group. HPLC grade acetone and iso-propyl alcohol (IPA) were purchased from Fisher Scientific, UK. Viscosity standards N1.0 (lot#10101), N2 (lot#10101), N4 (lot#10101), S6 ((lot#10201) were purchased from the Canon Instrument Company, USA.

2.2.2 Formulation selection

Batches were prepared by the method specified in Section 2.2.3.1 *Addition of Internal Phase in External Phase.* The amount of oil was kept constant for all formulations at 10% w/w. The total amount of surfactants used was kept constant at 3% w/w. Different ratios of Tween 80 and Span 80 were used to achieve the selected required hydrophilic and lipophilic balance (RHLB) (Table 2-3). The RHLB of soybean oil is reported as 6 to 7.[36, 75] Formulations were prepared based on RHLB ranging between 7 and 12 (Table 2-3 and Table 2-4). The most stable formulation ratio and HLB were used for subsequent experiments to compare the effect of the order mixing / emulsification process.

2.2.2.1 Hydrophilic-Lipophilic Balance (HLB) calculation

The empirical method of HLB determination was used.[1, 36] The total amount of surfactants required for a mixture of Tween 80 and Span 80 was calculated from Equation 2-1.

$$Qs = \frac{6(\rho_s/\rho)}{10-0.5(R-HLB)} + \frac{4Q}{1{,}000} \quad \text{..... Equation 2-1}$$

Where, Qs is total amount in gram of Tween 80 and Span 80, ρ_s and ρ are the density of the surfactant mixture and density of oil phase respectively. Q is the percent of the oil phase in the desired emulsion. A maximum desired quantity of 40% oil phase was selected. RHLB represents the hydrophilic-lipophilic balance of soybean oil that is required to form an o/w emulsion. Proportions of Tween 80 and Span 80 were calculated from Equation 2-2. Percentage proportions of surfactants are given in Table 2-3.

$$Tween\ 80 = \frac{RHLB-HLB\ low}{HLB\ high-HLB\ low} \quad \text{.....Equation 2-2}$$

Table 2-3 Proportions of Tween 80 and Span 80 for Required Hydrophillic-Lipophilic Balance between 7-12.

No	RHLB	Hydrophilic surfactant	Lipophilic surfactant	Ratio of Hydrophilic Surfactant	Ratio Lipophilic Surfactant
1	7	Tween 80	Span 80	0.25	0.75
2	8	Tween 80	Span 80	0.35	0.65
3	9	Tween 80	Span 80	0.44	0.56
4	10	Tween 80	Span 80	0.53	0.47
5	11	Tween 80	Span 80	0.63	0.37
6	12	Tween 80	Span 80	0.72	0.28

Table 2-4 RHLB calculation of surfactant mixtures for the formulation selection

No	Hydrophilic surfactant	Lipophilic surfactant	Hydrophilic surfactant HLB	Lipophilic surfactant HLB	% of Hydrophilic	% of Lipophilic	HLB
1	Tween 80	Span 80	15	4.3	0.25	0.75	7.0
2	Tween 80	Span 80	15	4.3	0.33	0.67	7.9
3	Tween 80	Span 80	15	4.3	0.43	0.57	8.9
4	Tween 80	Span 80	15	4.3	0.53	0.47	10.0
5	Tween 80	Span 80	15	4.3	0.63	0.37	11.1
6	Tween 80	Span 80	15	4.3	0.73	0.27	12.1

Table 2-5 Type of surfactants and methods' parameter of emulsion preparations: Selection of Order of Mixing

No	Order of Mixing	Hydrophillic surfactant	Lipophillic surfactant	RHLB	Phase of lipophilic surfactant	Heat (90°C)	Ethanol (%w/w)
1	Solvent evaporation	Tween 80	Soy PC	12.67	Oil	No	10.00
2	Internal to External	Tween 80	Soy PC	12.67	Oil	No	0.00
3	Phase inversion	Tween 80	Soy PC	12.67	Oil	Yes	0.00
4	Micelles	Tween 80	Soy PC	12.67	Water	No	0.00
5	Solvent evaporation	Tween 80	Span80	11.43	Oil	No	10.00
6	Internal to External	Tween 80	Span80	11.43	Oil	No	0.00
7	Phase inversion	Tween 80	Span80	11.43	Oil	Yes	0.00
8	Micelles	Tween 80	Span80	11.43	Water	No	0.00
9	Solvent evaporation	-	Soy PC	8.00	Oil	No	10.00
10	Internal to External	-	Soy PC	8.00	Oil	No	0.00
11	Phase inversion	-	Soy PC	8.00	Oil	Yes	0.00
12	Micelles	-	Soy PC	8.00	Water	No	0.00

2.2.3 Selection of the emulsification process (order mixing)

To evaluate the effect of order of mixing on the emulsion quality, four different methods, three standard emulsification methods and one novel approach, were employed. Mixtures of hydrophilic and lipophilic surfactants as well as single surfactants were used. The amount of oil was kept constant for all formulations at 10% w/w (Table 2-5). The total amount of surfactants used was also kept constant at 3% w/w (Table 2-5). The blend ratio of 0.67/0.33 for hydrophilic surfactant / lipophilic surfactant was selected from the earlier experiments of formulation selection (Section 2.2.2.1 and Table 2-4). Soy PC was also employed as a single emulsifier to compare the effect of order of mixing. Types of surfactants and method parameters are given in Table 2-5.

2.2.3.1 Addition of internal phase in external phase

The hydrophilic emulsifier was weighed in a 250 ml glass beaker. Approximately 50% of the required quantity of Milli-Q water was added to the hydrophilic surfactant and stirred with a magnetic stirrer until a clear aqueous phase was produced. Soybean oil and the lipophilic surfactant were weighed in a separate glass beaker and mixed until a clear organic phase was achieved. Aqueous phase was mixed using an Unguator® high speed mixer for 5 min. The oil phase was added to the aqueous phase and was mixed at 2,000 rpm for 10 min. The remaining Milli-Q water was added to make up to weight followed by mixing at 2,000 rpm for 5 min.

2.2.3.2 Solvent evaporation method

The hydrophilic emulsifier was weighed in a 250 ml glass beaker. Approximately 50% of the required quantity of Milli-Q water was added to the hydrophilic surfactant and stirred with a magnetic stirrer until a clear aqueous phase was produced. Soybean oil, lipophilic surfactant and water miscible solvent (acetone, or ethanol) were weighed in a separate glass beaker and mixed until a clear oil/organic phase was achieved. If required, either or both phases were heated to a maximum of 40º C. The aqueous phase was mixed by an Unguator® mixer for 5 min. The oil phase was added drop-wise using a Pasteur pipette with continuous mixing at 2,000 rpm and was mixed at 2,000 rpm for 10 min using an overhead mixer. The

remaining Milli-Q water was added to make up to weight followed by mixing at 2,000 rpm.

2.2.3.3 Phase inversion method (PI)

Soybean oil, lipophilic surfactant and/or hydrophilic surfactant, and/or solvent, were weighed in a glass beaker and mixed until clear oil/organic phase was achieved. The aqueous phase was added to the oil phase drop-wise using a Pasteur pipette with continuous mixing using an overhead mixer. Initially, the ratio of oil/water was kept at 1:1 to achieve the emulsion. The remaining aqueous phase was added in incremental amounts of 5 ml and mixed at 2,000 rpm. Once all the aqueous phase was added the mixture was mixed at 2,000 rpm for a total of 10 min.

The PIT method was carried out at an oil/water ratio of 1:1 and the emulsion was heated to 90º C. Conductivity measurements were recorded after every increase of 10º C increase. Once the emulsion had reached 90º C, it was cooled quickly by placing the beaker in a larger beaker containing ice. Mixing was kept constant at 1,000 rpm. The remaining waster was added to make up to weight at 25º C.

2.2.3.4 Emulsification with mixed micelles solution

This method involved the formation of mixed micelles and their application in self-emulsifying drug delivery systems (SEDDs) and microemulsions.[76] To achieve a stronger interfacial film and zero curvature, blends of hydrophilic and hydrophobic surfactants were used. A possible interaction between the surfactant and co-surfactant at the monolayer may provide additional fluidity to the interfacial film.[76-78] The hydrophilic surfactant was weighed in a glass beaker and dissolved in approximately 50% of the required quantity of water. The lipophilic surfactant was then added to the aqueous solution containing the hydrophilic surfactant. The aqueous mixture was stirred until all the surfactant was dispersed or dissolved and produced an aqueous phase containing mixed surfactants. The aqueous mixture was further mixed at 2,000 rpm for 5 min to ensure that all the surfactant was dispersed. The oil phase was then added to the aqueous phase and mixed at 2,000 rpm for 10 min. The remaining water was added to the emulsion and made up to weight. The final emulsion was mixed at 2,000 rpm for 5 min.

2.2.4 Selection of equipment

The energy applied during emulsification is critical to produce emulsions with small and uniform droplet sizes. In order to study the effect of the mixing process, four different types of mixing equipment were used to produce the emulsions. 1. Rotor homogeniser, 2. High-pressure homogeniser, 3. Ultrasonic, 4. High speed mixer as described in Table 2-6. The process parameters including rotational speed, pressure, amplitude, and duration were selected prior to the experiments and are specified in Table 2-7. Experiment 4 (Table 2-5) and the method described in Section 2.2.3.4 of order of mixing were used for the equipment selection trials. All parameters including order of mixing, surfactant/co-surfactant ratio (0.67/0.33), amounts of oil (10% w/w) and total emulsifiers (3% w/w) were kept constant.

Table 2-6 Details of bench scale equipment and equipment specifications

Emulsification system	Rotor-stator	High pressure	Ultrasonic	High speed mixer
Equipment name	Diax® 900	Emulsiflex® C5	UP200S	Unguator®
Manufacturer	Heidolph (Germany)	Avestin (Canada)	Hielscher (Germany)	Gako International (Germany)
Batch/ continuous	Batch	Continuous	Batch	Batch
Speed/Pressure/ Frequency	8,000-26,000rpm	500- 30,000psi (204MPa)	25kHz (20-100%)	650-2100 rpm

2.2.4.1 Rotor stator

A coarse emulsion was prepared using the high-speed mixer Unguator® at 2,000 rpm for 5 min. Coarse emulsion was then homogenised at 8,000, 11,500 or 15,000 rpm by a Diax® 900 for a total of 30 min. The samples were collected at 10, 20 and 30 min for droplet size analysis (Table 2-7).

2.2.4.2 High pressure homogeniser

The EmulsiFlex®-C5 homogeniser has an air driven, high pressure pump. There are no "O"-rings or gaskets in the entire path of the equipment. The standard EmulsiFlex®-C5 has a pneumatically controlled, dynamic homogenising valve with

adjustable homogenising pressure in the range of 500-30,000 psi (3.5-2.07 MPa). The flow rate is controlled by the homogenising pressure up to 5 L/hr.

A coarse emulsion was prepared using the high-speed mixer (Unguator®) at 2,000 rpm for 5 min. The coarse emulsion was passed through the EmulsiFlex® at different pressures of 2,500 and 5,000 psi for a total of 3 cycles at each pressure (Table 2-7). Compressed nitrogen was used as the gas to generate the required pressure. For each pressure, 200 ml of emulsion was prepared and homogenised at a set pressure of 2,500 or 5,000 psi. Then 50 ml of the homogenised sample was collected for particle size analysis and the remaining 150 ml was passed through the homogeniser for the second cycle. The third, and final, cycle was repeated for the remaining 100 ml emulsion. Inlet and outlet temperatures were not controlled. A thermometer was placed in the collection beaker to monitor changes in temperature during homogenisation. Hot Milli-Q water containing 30% ethanol was flushed through the equipment for cleaning. After cleaning, residual ethanol was removed by blowing the system out with compressed nitrogen.

2.2.4.3 High speed mixer

Unguator® is a fully automatic mixer with speed range of 650-2100rpm. Unguator® was used as high speed mixer to prepare the emulsions and/or the coarse emulsion (pre-emulsion). The aqueous phase was mixed at 2,000 rpm for 5 min. The oil phase was added to the aqueous phase and mixed at 2,000 rpm for 5 min. The remaining water was added to the emulsion and made up to weight. The final emulsion was mixed at 2,000 rpm for 5 min.

Table 2-7 Process parameters for selection of emulsification process

Equipment	Speed/Pressure/ Amplitude	Duration (min)/ No of cycle
Rotor Homogeniser (rpm)*	8,000	10, 20,30 min
	11,500	10, 20,30 min
	15,000	10, 20,30 min
High pressure Homogeniser (psi)	2,500	1,2,3 cycles
	5,000	1,2,3 cycles
Sonication (% amptitude)	20.00	2,4,6 min
	40.00	2,4,6 min
	60.00	2,4,6 min

2.2.4.4 Sonication

A 24 kHz sonicator (Dr Hielscher series, Model UP 200S) was used for emulsification. The sonicator consisted of a processor, a horn, and a sonotrode. The conventional alternating electrical current of 50-60 Hz was converted to 24 kHz. The energy was transformed into mechanical energy through expansion and contraction of a lead zirconate titanate piezoelectric quartz crystal in a convertor. The vibrations produced were transmitted to the horn tip. The horn tip selected was 7 mm in diameter and could vibrate at peak-to-peak amplitude of 175 μm at full power (100%). The output can be adjusted between 20% and 100% of the maximum output. The horn tip was immersed in the coarse emulsion prepared by the high speed mixer (Unguator®) and the sonication was turned on to the predetermined output power described in Table 2-7. All experiments were performed in a 200 ml glass beaker. A thermometer was placed in the emulsion at the time of sampling for the determination of temperature rise during sonication. Each experiment was performed in duplicate.

2.2.5 Droplet size characterisation of emulsion

The size distribution of the oil droplets was determined by laser light scattering using a Mastersizer 2000 (Malvern Instruments, UK) attached to a sampling unit Hydro SM (Malvern Instruments, UK). Three measurements were carried out for each sample and their average was reported. The relative refractive indices (RI) used for the dispersant and the dispersed phase were 1.35 and 1.47 respectively. The imaginary component of the absorption index for the dispersed phase was taken as 0.001.

A screw-capped bottle containing the emulsion preparation was shaken gently by inverting before 1 g of sample was taken and placed in a 200 ml glass beaker and diluted to 100 g by adding Milli-Q water. Diluted samples were stirred gently with a glass rod to disperse the emulsion. If dilute samples appeared milky, 10 g of diluted sample was further diluted to 100 g by adding Milli-Q water. The required amount of dilute samples were added using a Pasteur pipette to the Mastersizer sampling unit containing ~100 ml Milli-Q water whilst being gently stirred at 1900 rpm and recirculated in the Mastersizer cell. For consistency, the first measurement was carried out approximately 48 hours after preparation of the emulsion.

The droplet size distribution was presented as % frequency vs. droplet diameter. During development and optimisation of the emulsions and their characterisation, the mean diameter of the droplets was expressed as mean droplet diameter (D(1,0)) (Equation 2-3) and the Sauter diameter, representing a surface mean diameter (D(3,2)). In general, the volume mean diameter (D(4,3)) over represents the existence of larger droplets while the surface mean diameter (D(3,2)) is more associated with the smaller droplets. The small droplets do not contribute to the volume as much as large droplets. Droplet size distribution based on volume mean diameter can be misinterpreted especially when more small droplets are present. However, volume diameter is a useful tool for stability evaluation and change in droplet size and/or droplet aggregation, where large droplets are important.

$$D_{10} = \sum n\,d / \sum n \quad \text{.....Equation 2-3}$$

$$D_{32} = \sum n_i\,d_i^3 / \sum n_i d_i^2 \quad \text{.....Equation 2-4}$$

$$D_{43} = \sum n_i\,d_i^4 / \sum n_i d_i^3 \quad \text{.....Equation 2-5}$$

2.2.6 Creaming

Each emulsion sample was poured into a 100 ml graduated cylinder and the cap was closed immediately after preparation. The emulsion sample was left standing for 48 hours to settle. Observation of creaming of the internal phase was made at room temperature at 48 hours. The volumes of the creamed phase and the remaining emulsion were recorded. The creaming volume (*Vcream*) was defined in this study as the relative difference in the total volume of the emulsion (*Vemulsion*) and the volume of the creamed phase (*Vcreaming*). The value of percent creaming was calculated for each emulsion using the following equation 2-6.[53, 79]

$$V_{cream} = \frac{V_{creaming}}{V_{emulsion}} \times 100\% \quad \text{.....Equation 2-6}$$

2.2.7 Centrifugation

Centrifugation methods can be used to speed up the destabilisation process of emulsions. The forced degradation under centrifugation reflects the strength of the interfacial film.[80] The process parameters can also be immediately adjusted and optimised for the emulsification process using centrifugation.[81] Becher indicates that centrifugation at 3750 rpm in a 10- cm radius centrifuge for a period of 5 hours is equivalent to the effect of gravity for about one year.[19, 36] Centrifugation was used as a tool for the forced degradation study and therefore the impact of HLB, order of

mixing and type of equipment. Emulsions were centrifuged at 4,000 rpm for 15, 30, 60 and 120 min using the Centrifuge 5702 (Eppendorf AG, Germany) attached with swing bucket rotor with a maximum capacity of eight 15 ml falcon tubes. Relative centrifugal force (rcf) or g-force of 2290 x g was calculated based on Equation 2-7.

$$rcf = 1.118\times10^{-5}\times n^2\times radius \quad \text{.....Equation 2-7}$$

Where n is rotational speed in rpm, radius is the maximum centrifuging radius in centimetre. Centrifuged samples were analysed for creaming, phase separation and droplet size.

2.2.8 Refractive index

The refractive index of oil and Tween 80 solution was measured using a MISCO PA202 digital refractometer (MISCO Refractometer, USA). This refractometer had a working range from 1.3330 to 1.5040 nD.

2.2.9 Viscosity

Viscosity was measured by an A&D SV10 vibrational viscometer (A&D Company, Limited, Tokyo, Japan). The SV10 works on a vibrational frequency of 30Hz with measurement range from 0.3 mPas to 10Pas (±1%) at 10-40 ºC. The WinCT-Viscosity (RsVisco) software was used to import the measurements of viscosity and temperature automatically from the viscometer to a computer.

The viscometer was calibrated using a 2-point calibration with the Cannon® General Purpose Viscosity Standards N1 and S6 (Table 2-8). Viscosity measurements were made at room temperature (23-25ºC). The viscosity standards listed in Table 2-8 were used for calibration before running the samples and to ensure the accuracy. The runs were carried out 48 hr after the preparation. A total of 3 measurements were carried out for each sample.

Table 2-8 Cannon® General Purpose Viscosity Standards

Viscosity Standard	Approximate Dynamic Viscosity in mPas	
	20°C	25°C
N1.0	0.92	0.85
N2	2.2	2.00
N4	5.4	4.65
S6	9.4	7.86

2.2.10 Surface and Interfacial tension

Interfacial tension (I.T.) and surface tension (S.T.) were measured by a KSV Sigma703 (Biolin Scientific, Finland) force tensiometer using the Du Nouy ring method. The equipment was calibrated during set up and every 6 months after the first calibration. The measurements were carried out immediately after preparation. The display value of surface tension was adjusted to "ZERO" with the taring knob while the ring was completely above the surface of liquid.

2.2.10.1 Surface tension

Surface tension of oil and water was measured separately following the preparation of each phase. Approximately 30 ml of sample was poured gently in to the measurement bowl. Care was taken to avoid formation of air bubbles on surface. The ring was first immersed within the sample by slowly lifting the stage. The stage was slowly lowered until the ring broke from the liquid and the surface tension was read from the display.

2.2.10.2 Interfacial tension

The aqueous phase was poured in to the measurement bowl. The oil or oil phase was added slowly using a Pasteur pipette. Approximately 15 ml of aqueous phase and 10 ml of oil phase were used. Samples were allowed to stand for a minute before immersing the ring in to the solution. The ring was wetted with aqueous phase before being immersed in the sample. The measurement was carried out as described above by slowly lowering the ring until the ring breaks from the aqueous phase but remained submerged in oil phase.

2.2.11 Conductivity

A CDM230 conductivity meter (Radiometer Analytical, France) was used. Conductivity was measured only to confirm the type of emulsion produced during the PIT method in the development phase. The cell was calibrated using 0.1 Demal (7.41913 g/l) KCl solutions in Milli-Q water.

2.2.12 Statistical analysis

A General Linear Model (GLM) was used to identify factors which appeared to be associated with the various outcome variables (droplet size, interfacial tension,

viscosity and temperature). Independent variables were generally treated as categorical variables (so that the GLM was identical to an Analysis of Variance). However, HLB was treated as a continuous independent variable when analysing its influence on viscosity (so that the GLM was a simple regression model). Where there were 2 or more independent variables, their pairwise interactions were assessed for statistical significance. Results of fitting the models are presented as p-values associated with the independent variables, and the R^2 value, which shows the proportion of variance explained by the model. The SAS version 9.2 software (SAS Institute Inc, Cary, NC, USA, 2008) was used for these analyses, and, following convention, a p-value<0.05 was taken to indicate a statistically significant association in all tests.

2.3 Results and Discussion

2.3.1 Formulation selection

Emulsions were prepared successfully at HLB values from 7 to 12. Emulsions were analysed after 48 hours of preparation. This provided sufficient time for the emulsion to settle and possibly reduce any errors during droplet size analysis. Phase separation or creaming was evident in most emulsions after 48 hours. All preparations were milky white in appearance with a cream layer on top. There was no sign of any clear aqueous serum layer separation except at HLB 7. However, phase separation was observed for the emulsion prepared at HLB 7 within 48 hours (Table 2-9) and was discarded with no further analysis. All other preparations were stable for 48 hours and had cream layer. The creaming Volume (*Vcream*) was less than 10%, which was expected given the percentage volume of dispersed phase present in the system. The *Vcream* of 10% was observed at HLB 12 (Table 2-9). The mean droplet size D(3,2) was approximately 1.5 µm to 2.0 µm for all preparations (Table 2-9 and Figure 2-1). The mean droplet size D(4,3) was reduced from 8.21 µm at HLB 8 to 3.80 µm at HLB 9. The decrease in mean droplet size was due to the significant reduction of droplets larger than 10 µm. This can be correlated with the reduction in interfacial tension from 1.0 mNm to 0.7 mNm (Table 2-9).

Figure 2-1 Effect of HLB on mean droplet size D(3,2). Emulsions prepared using Tween 80 and Span 80

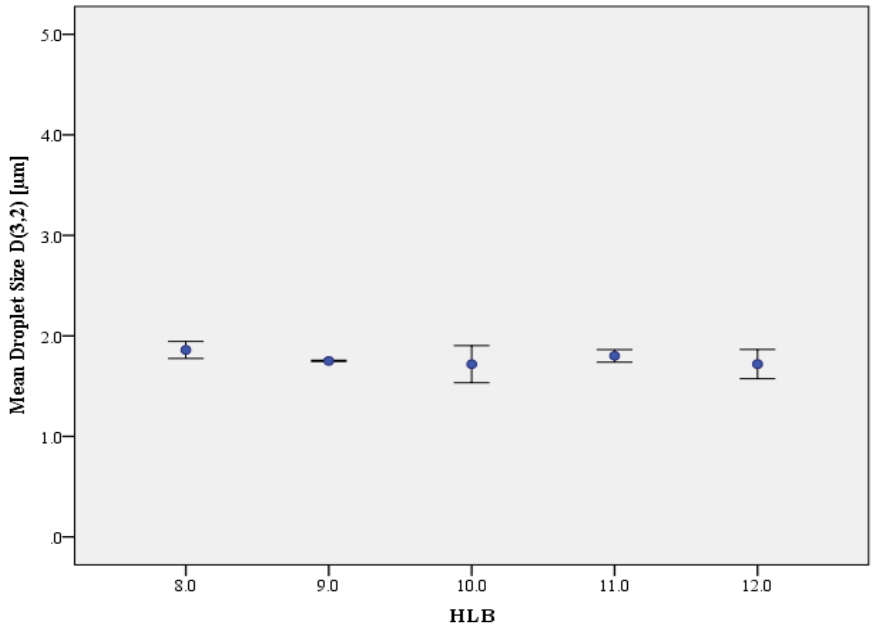


Table 2-9 Surface tension (S.T.), interfacial tension, viscosity, creaming, and droplet size of emulsion (HLB 7-12)

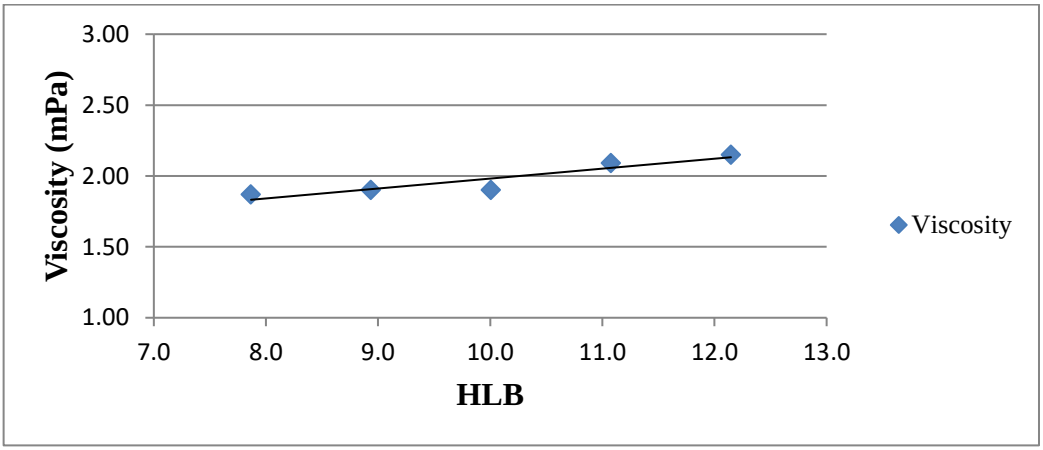
Emulsion Reference	HLB	Creaming	Particle size (μm)		Aqueous S.T.	Interfacial Tension	Viscosity
		Volume	D(3,2)	D(4,3)	mNm	mNm	mPa
1	7.0	-	-	-	44.8	1.1	-
2	7.9	0.1	1.94	8.21	44.8	1.0	1.9
3	8.9	0.1	1.76	3.80	44.0	0.7	1.9
4	10.0	0.1	1.55	4.31	43.7	0.7	1.9
5	11.1	0.1	1.74	3.76	42.7	0.7	2.1
6	12.1	0.1	1.86	5.97	41.7	0.5	2.2

Table 2-10 Droplet size distribution after centrifugation at 4,000rpm (HLB 7-12)

Emulsion Reference	HLB	Forced degradation at 4,000rpm Vs Particle size D(4,3) (μm)				
		0min	15min	30min	1hr	2hr
1	6.98	-	-	-	-	-
2	7.87	8.21	6.36	6.79	-	-
3	8.94	3.80	2.86	3.61	14.02	-
4	10.01	4.31	3.76	4.11	4.14	5.70
5	11.08	3.76	3.08	3.75	3.76	3.79
6	12.15	5.97	4.30	5.53	5.46	7.52

There was a slight increase in droplet size from HLB 11 to HLB 12. The droplet size increase at HLB 12 could be due to the increased rigidity and reduced elasticity of the interfacial film with increased amount of Tween 80.[79] The viscosity was also slightly increased with increasing HLB from 8 to 12 and the relationship between HLB and viscosity was found to be significant ($p < 0.0001$). Lin suggested that the ratio of hydrophilic to lipophilic surfactant and the initial surfactant location can affect the viscosity, emulsion stability, droplet size distribution, and emulsion type formed.[82, 83]

Figure 2-2 Viscosity of emulsions prepared at HLB 8-12

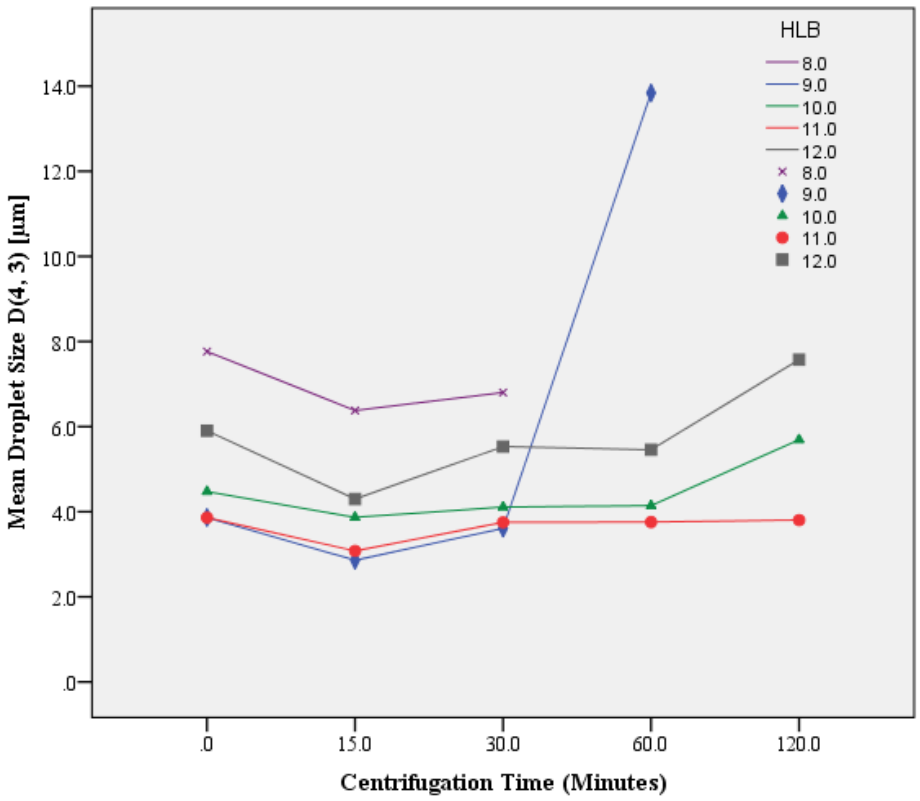


2.3.1.1 Effect of HLB on emulsion stability

All emulsions were stable after 15 min of centrifugation at a g-force of 2290 × g. There was small a decrease in the droplet size after 15 min of centrifugation. After centrifugation for 30 min, all emulsions were separated into two layers; a thick semi-rigid cream on the surface and milky white aqueous emulsion. Creaming volume was approximately 10% for all preparations. A clear layer of separated oil was visible for HLB 8 after centrifugation up to 1 hour. The emulsion at HLB 9 contained large visible oil globules on top which was evident from an increase in mean droplet size D(4,3) (Figure 2-3). There was a small increase in droplet size for preparations at HLB 10 and 12 after 2 hours of centrifugation with some visible oil globules. There were no visible droplets or droplet size change in the emulsion at HLB 11 after 2 hours of centrifugation. Garrett et al. and Tcholakova et al. have shown that the higher stability measured by centrifugation can correspond to higher long-term stability during normal shelf-storage for emulsions stabilised by similar types of emulsifier.[84-86] It was evident that stable o/w emulsions can be produced at

HLB ≥ 10 and most preferably, at emulsifier ratio at HLB 11. The above outcome can be supported by the observation from Boyd et al., who reported a minimal coalescence rate at HLB value ~12.00 for o/w emulsions. Boyd et al. also reported a sharp increase in coalescence rate with an increase in the HLB of emulsion above the optimum HLB value.[38]

Figure 2-3 **Mean droplet size D(4,3) after centrifugation at 4,000rpm for emulsions produced at HLB 8 to 12**



2.3.2 Order of mixing

Based on the above observations in Section 2.3.1 the emulsifier ratio of 0.67/0.33 for hydrophilic/lipophilic surfactant was selected for order of mixing trials. All formulation parameters were kept constant including the amount of oil (10% w/w), total amount of surfactant (3% w/w) and the emulsifier ratio.

2.3.2.1 Emulsion of Tween 80 and lecithin

Emulsions were prepared using Tween 80 as hydrophilic surfactant and lecithin as lipophilic surfactant. Emulsions were successfully prepared by solvent evaporation (Emulsion 1), addition of internal phase to external phase (Emulsion 2) and mixed micelles system (Emulsion 4) and were stable for at least 48 hours on standing at

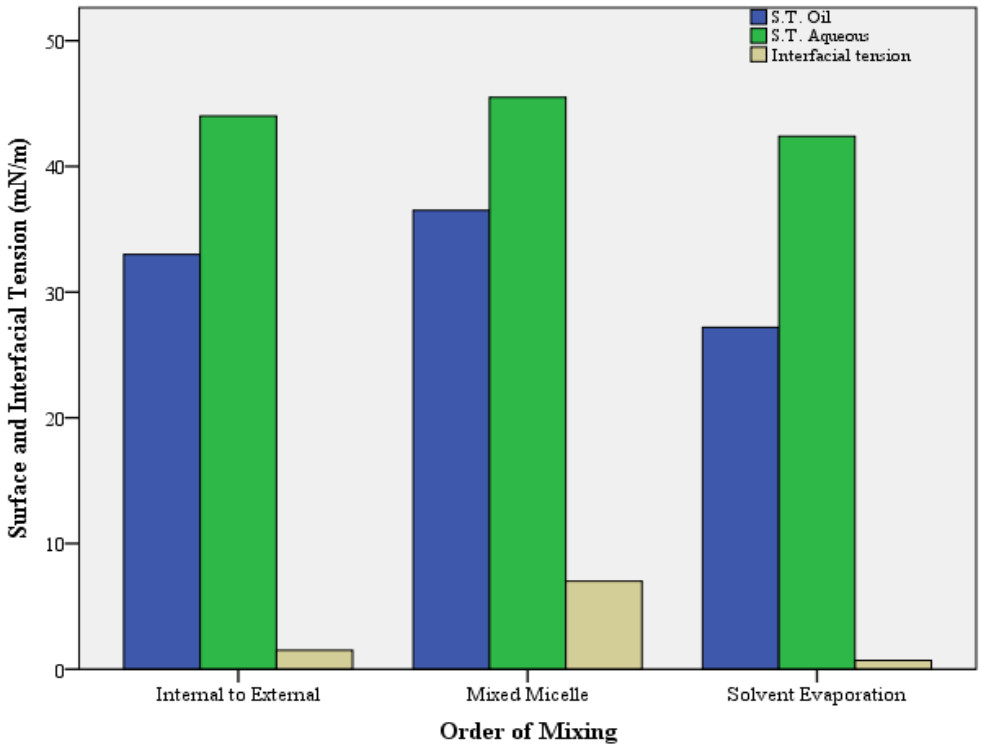
room temperature. Due to the small quantity of solvent used in the process, it was considered that the solvent would evaporate during mixing. All stable preparations were milky white in appearance with no sign of a clear aqueous layer separation. Creaming was evident for emulsions 1 and 2 and *Vcream* was approximately 10% for both preparations.

The order of mixing had an overall significant effect on surface tension of oil ($p<0.0001$; $R^2 = 92.1\%$), with all pairwise differences being significant ($p<0.006$). The order of mixing also influenced the interfacial tension ($p<0.0001$; $R^2 = 91.8\%$, with the internal to external method being similar to the solvent evaporation method ($p=0.3196$), but the interfacial tension was significantly higher for the mixed micelle method than either of these other methods ($p<0.001$ for both pairwise comparisons. The surface tension of the aqueous phase was not significantly influenced by the order of mixing ($p=0.2356$, Figure 2-4). The presence of solvent reduced the surface tension of oil and subsequently interfacial tension between oil and water. Similarly, interfacial tension was reduced by adding lipophilic emulsifier in the oil phase (Table 2-11 and Figure 2-4). There was no major difference in D(1,0) droplet size among all preparations. All emulsions had bimodal droplet distributions, especially emulsion prepared by solvent evaporation shown obvious sign of bimodal distribution with slightly more of larger droplets (Figure 2-5 and Figure 2-6).

There was a statistically significant ($p<0.0001$) difference in mean droplet size D(3,2) between emulsions prepared by mixed micelles and solvent evaporation methods. However, the results were unexpected and did not transform the lower interfacial tension into a smaller droplet size. Instead, the presence of larger droplet size for emulsions produced by the solvent evaporation method could be due to the fact that the amount of solvent used and solvent to oil ratio were far smaller than that reported in the literature.[87] This can also be supported by the fact that the interfacial tension was higher than expected for spontaneous emulsification.[88] Choi et al. also emphasized that lecithin exhibits a close balance between hydrophilic and lipophilic properties, being slightly inclined towards lipophilic properties due to two long hydrocarbon chains. The penetration of alcohols into the interfacial layer of lecithin may influence the curvature of the interface. Choi et al. reported that the

presence of alcohol supports the formation of water-in-oil emulsions compared with oil-in-water emulsions in a very limited region.[89]

Figure 2-4 Effect of order of mixing on surface and interfacial tension of oil and aqueous phase containing Tween 80 and lecithin



Phase separation was observed within 48 hours of preparation for Emulsion 3 prepared using the phase inversion technique (Table 2-11) and was discarded with no further analysis. Shinoda described that an o/w emulsions stabilised by a non-ionic polyoxyethylene derived surfactant contains oil-swollen micelles of the surfactant. Addition of oil beyond the solubilisation limit may form an o/w emulsion, being dispersed as droplets.[90] Instability of Emulsion 3 can be explained as a result of the disruption of oil swollen micelles or insufficient micellar structure during inversion and hence the size of emulsified oil droplets begins to increase. As temperature increases, the convex curvature of the adsorbed surfactant monolayer may attain flat curvature.[90] A continued rise in temperature may promote the mass transfer of both surfactants to oil and subsequently separation of the emulsion. The lipophilic nature of lecithin may not allow the transfer back at the interface upon cooling or attain the curvature required for formation of an o/w emulsion.[90]

Table 2-11 Surface tension (S.T.), interfacial tension, viscosity, creaming, and droplet size of emulsions (Order of Mixing)

Emulsion Reference	Creaming (%)	Droplet size (µm)			Oil S.T.	Aqueous S.T.	Interfacial Tension	Viscosity
		$Mean_{Avg}$	Mean (3,2)	Mean (4,3)	mNm	mNm	mNm	mPa
1	10.00	0.78	2.78	9.04	27.20	42.40	0.70	2.69
2	10.00	0.72	2.14	8.29	33.00	44.00	1.50	2.63
3	-	0.00	-	-	-	-	-	-
4	0.00	0.73	2.22	10.67	36.50	45.50	7.00	2.57
4E	0.00	0.72	2.18	9.86	37.00	45.60	7.30	2.68

Table 2-12 Droplet size distribution after centrifugation at 4,000rpm, assessment of Order of Mixing

Emulsion Reference	Forced degradation at 4000rpm Vs Particle size Mean (4,3) (µm)				
	0min	15min	30min	1hr	2hr
1	9.04	8.58	8.72	-	-
2	8.29	7.58	8.88	-	-
3	-	-	-	-	-
4	10.67	5.06	10.34	14.75	-
4E	9.86	6.27	10.47	10.35	-

Figure 2-5 **Overlay Surface Mean Diameter D(3,2) distribution of droplet size (µm) (in % frequency) for emulsion containing Tween 80 and lecithin- comparison of order mixing**

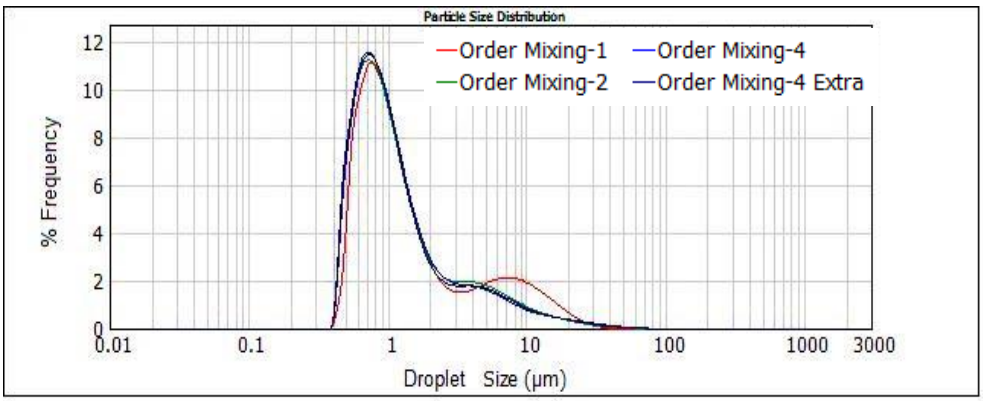
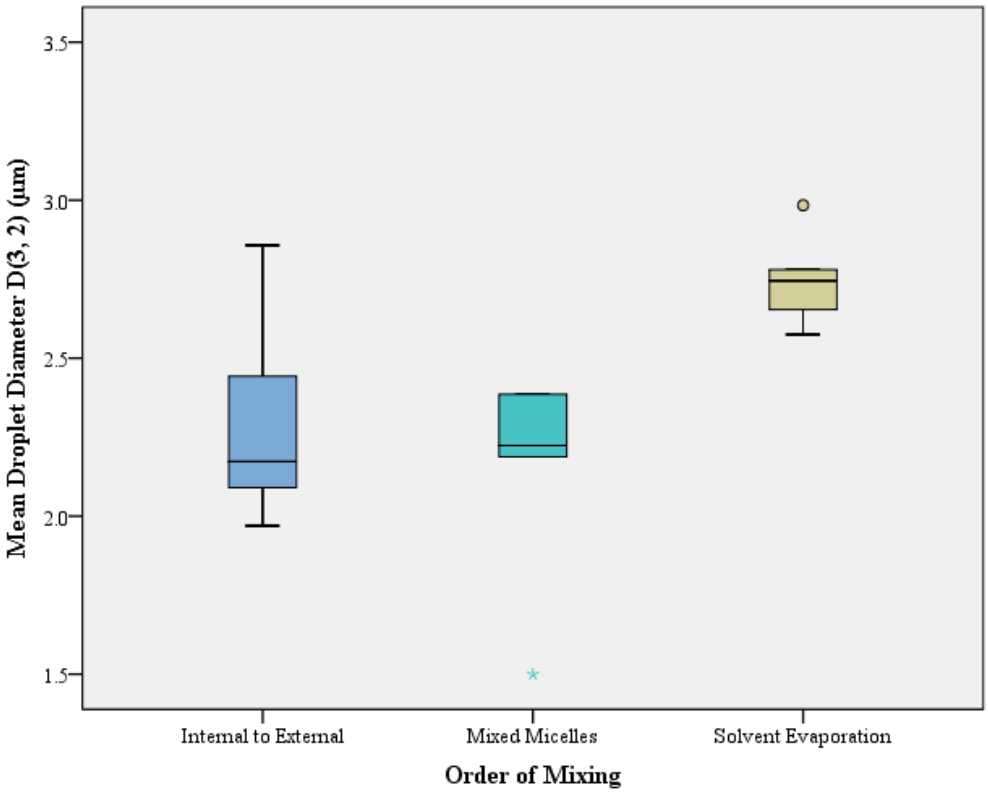


Figure 2-6 **Effect of order of mixing on mean droplet size D(3,2) for emulsions containing Tween 80 and lecithin**



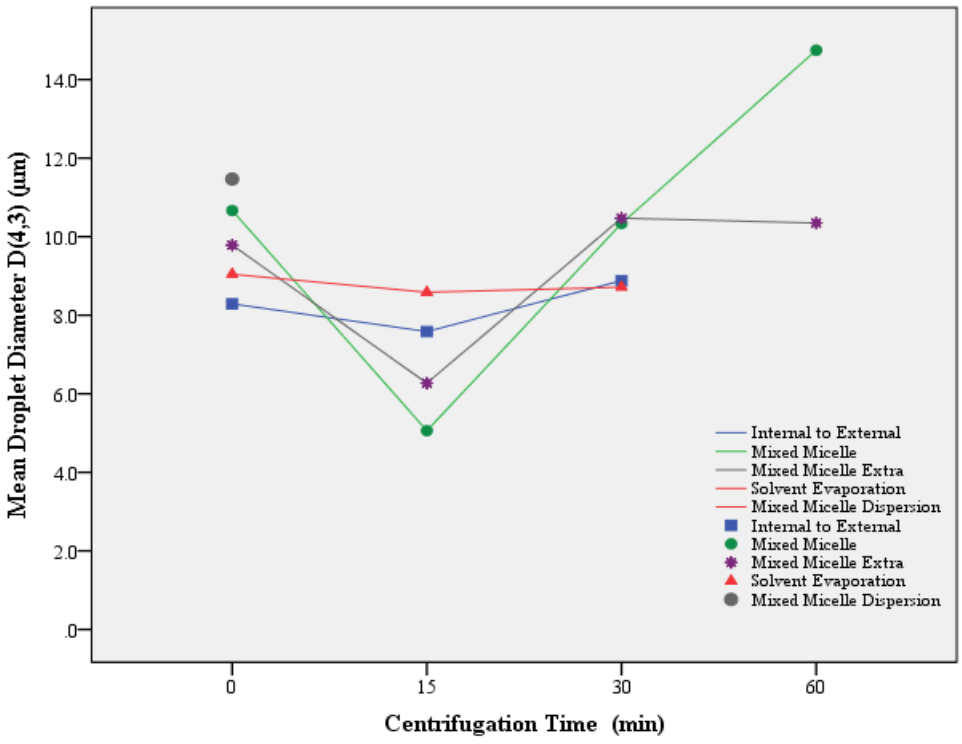
There was no creaming observed for Emulsion 4, prepared by the mixed emulsifier system. It could possibly be due to the diffusion/entrapment of oil within micellar/ vesicle structure of a mixed emulsifiers system. Matsumoto et al. described the formation of w/o/w emulsions by lipid vesicles.[91] To evaluate the impact of addition of emulsifier in a mixed emulsifier system, Emulsion 4 was reformulated as Emulsion 4E and emulsifiers; Tween 80 and lecithin were dispensed in a glass beaker before the addition of Milli-Q water and stirred by a magnetic stirrer until

dispersed. As expected, Emulsions 4 and 4E showed similar physical properties including *Vcream*, interfacial tension, and droplet size (Table 2-11). In a mixed emulsifier system, interfacial tension was recorded as higher than in the other emulsions. This could also be due to the formation of micelles and vesicles of mixed emulsifiers. Formation of larger vesicles and micelles of lecithin in the presence of Tween 80 has been reported earlier by Lim.[92]

2.3.2.1.1 Forced degradation of Lecithin and Tween 80 Emulsions

After centrifugation at 4,000 rpm for 15 min, all emulsions showed creaming of approximately 10%. There was a thick gel structure with visible large oil globules on top of the cream layer of Emulsions 1 and 2. Emulsions 4 and 4E had visible yellow sedimentation of lecithin at the bottom of tube instead. This could be explained as large vesicles formed by lecithin in the aqueous phase and can be supported by the reduction in droplet size at 15 min in Figure 2-7.

Figure 2-7 Effect of centrifugation at 4,000rpm and order of mixing on mean droplet size D(4,3) for emulsions containing Tween 80 and lecithin



Emulsion 1 prepared by a solvent evaporation method was separated after centrifugation up to 1 hour. The instability of Emulsion 1 could be due to the partitioning of alcohol at the surfactant interface. The evidence of alcohol partitioning into the bilayer head group region was presented by Ly and Longo.[93]

The partitioning was found to be dependent on the chain-length of the alcohol. The finding by Ly and Longo also supported the influence of alcohol on the reduction of interfacial tension and reduction of membrane thickness of lecithin vesicles.[93] A study by Aramaki et al. showed the effect of alcohol on the phase behaviour of aqueous non-ionic surfactant systems. They suggested that alcohol molecules tend to penetrate into the palisade layer of the aggregates, and eventually they are broken into monomers with increasing the alcohol content.[94] This suggests that the dehydration of phospholipid at the interface reduces the hydrogen bonding of phospholipid and water, subsequently weakening the interfacial film.[95]

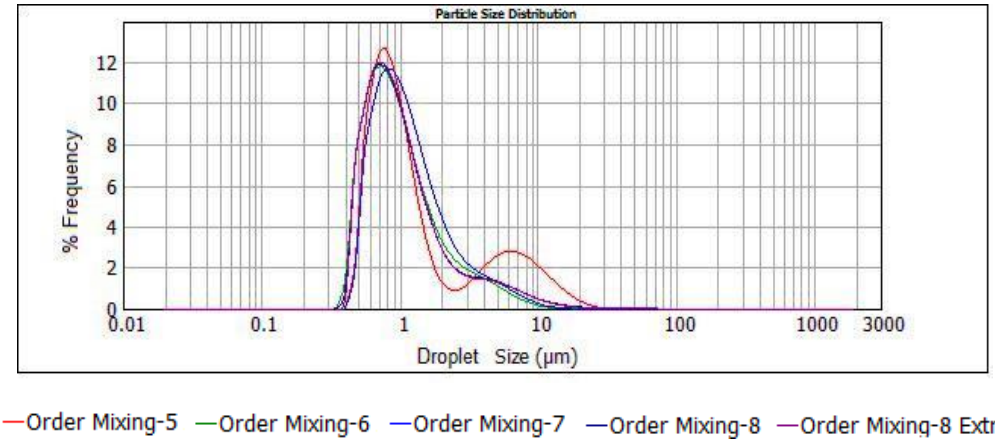
At 30 min, a separate oil layer was observed for Emulsion 2 prepared by the addition of internal phase to external phase (Figure 2-7 and Table 2-12). Emulsions 4 and 4E were found to be the most stable emulsion systems with thick gel-cream structures on top. There was a small amount of oil separation after 2 hours of centrifugation. However, sedimentation of lecithin was the most prominent for both emulsions compared with the other preparations. Lecithin dispersion in water prior to emulsification forms larger but stable droplets. The better stability of emulsions 4 and 4E compared with Emulsion 3 can be explained by improved viscoelasticity of the interfacial film in these emulsions.[27] Friberg et al. explained that lecithin forms a liquid crystalline structure when dispersed in water. The interaction of lecithin with water would exhibit increased viscoelastic behaviour of the mixed surfactant layer at the oil/water interface. The stability of Emulsions 4 and 4E could be due to the condensed and expanded monolayer of hydrated lecithin and Tween 80 system.[27] Overall, emulsions prepared by the mixed micelle system in the aqueous phase were found to be the most stable.

2.3.2.2 Emulsions of Tween 80 and Span 80

Emulsions were prepared successfully by order of mixing using a mixed emulsifying system of Tween 80 and Span 80. All emulsions were stable for 48 hours on standing at room temperature. All emulsions were milky white in appearance with no sign of clear aqueous layer separation (Table 2-13). *Vcream* for addition of internal phase to external phase (Emulsion 6) and phase inversion (emulsion 7) was lowest at 5%. *Vcream* of 10% for the mixed micelle system was

detected after careful observation and was not obvious. Emulsion 5, prepared by the solvent evaporation technique had the highest *Vcream* of about 15%.

Figure 2-8 **Overlay Surface Mean distribution of droplet size D(3,2) (µm) (in % frequency) for emulsions containing Tween 80 and Span 80- comparison of order mixing**



The trend for surface tension of oil phase and interfacial tension was almost identical when compared with emulsions, containing lecithin as lipophilic emulsifier, discussed in section 2.3.2.1 (Table 2-11 and Table 2-13). The difference in surface tension of oil was significant ($p < 0.0001$) between emulsions prepared by mixed micelles and solvent evaporation. Interfacial tension for Emulsions 8 and 8E, prepared by the mixed micelle system of Tween 80 and Span 80 was significantly higher ($p < 0.0001$) than other preparations and was similar to Emulsions 4 and 4E prepared from Tween 80 and lecithin (Figure 2-9, and Table 2-13). Aqueous phase surface tension for 8 and 8E, prepared from Tween 80 and Span 80 were significantly lower ($p < 0.001$) compared with other preparations including earlier trials of Emulsions 4 and 4E for Tween 80 and lecithin in section 2.3.2.1. The reduction in surface tension of the aqueous phase in presence of Span 80 could be described as a synergetic surface activity of both surfactants. During preparation of aqueous dispersions and emulsions foam was produced due to reduced surface tension. However, the higher interfacial tension of the mixed micelle system may indicate formation of mixed micelles in the aqueous phase, and with addition of oil may form a mixture of oil-swollen micellar solution and emulsified oil droplets.[90] The reduction of mean droplet size of Emulsions 8 and 8E compared with Emulsions 4 and 4E was considered a direct outcome of lower surface tension of the aqueous phase (Table 2-11 and Table 2-13).

Table 2-13 **Physical characteristics of emulsions containing Span 80 and Tween 80 prepared by different order of mixing**

Emulsion Reference	Creaming (%)	Droplet size (µm)			Oil S.T.	Aqueous S.T.	Interfacial Tension	Viscosity
		D(1,0)	D(3,2)	D(4,3)	mNm	mNm	mNm	mPa
5	15.00	0.72	1.47	3.49	27.70	42.50	0.70	2.20
6	5.00	0.71	1.43	2.88	33.40	42.70	0.70	2.09
7	5.00	0.81	1.49	2.86	-	-	-	2.44
8	10.00	0.74	1.57	4.60	37.50	33.80	6.00	1.86
8E	10.00	0.71	1.53	4.54	37.50	33.00	6.00	1.88

Table 2-14 **Mean droplet size D(4,3) after centrifugation at 4,000rpm, assessment of order of mixing**

Emulsion Reference	Forced stability at 4000rpm Vs Particle size D(4,3) (µm)				
	0min	15min	30min	1hr	2hr
5	3.49	3.18	3.27	3.21	-
6	2.88	2.91	2.88	2.89	2.88
7	2.86	2.38	2.77	3.08	-
8	4.60	3.36	5.28	5.05	4.40
8E	4.54	4.03	3.97	3.79	3.66

Figure 2-9 **Effect of order of mixing on surface and interfacial tension of oil phase and aqueous phase containing Tween 80 and Span 80**

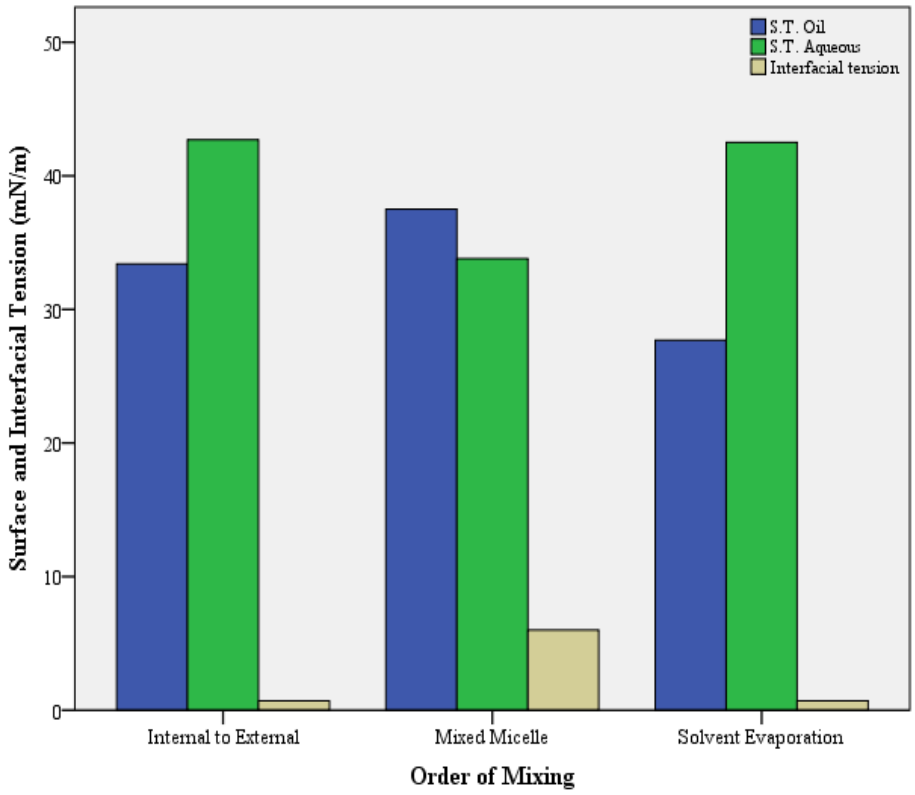
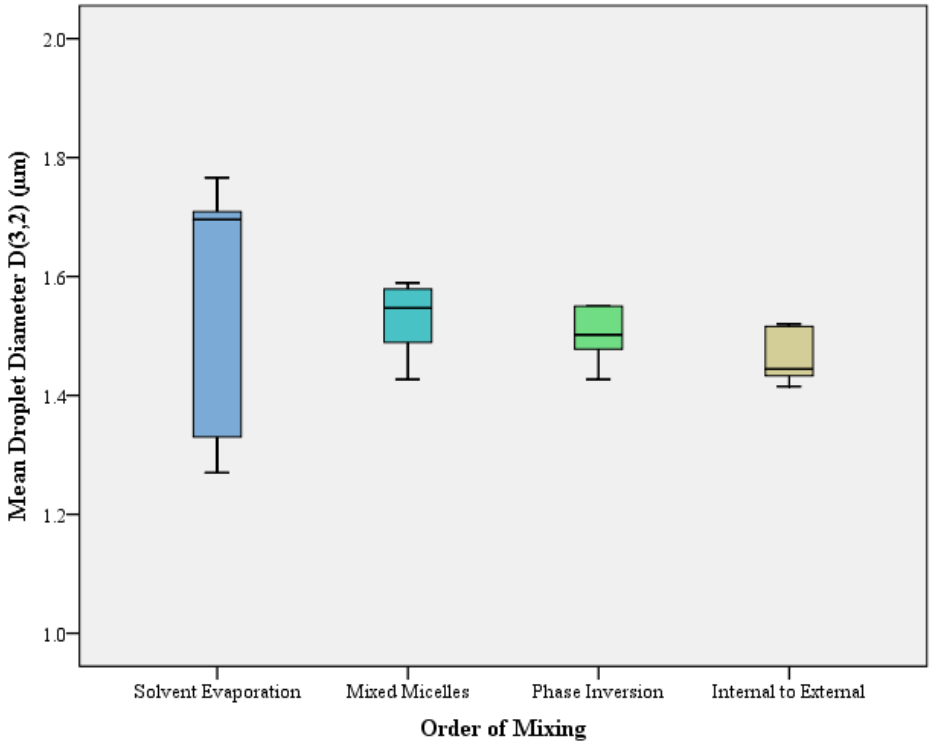


Figure 2-10 **Effect of order of mixing on mean droplet diameter D(3,2) in micrometres for emulsions containing Tween 80 and Span 80**



Overall, the mean droplet diameter for Tween 80 and Span 80 was smaller and droplet size distribution was narrower compared with emulsions prepared by Tween 80 and lecithin. The mean droplet size D(3,2) was similar for all preparations containing Tween 80 and Span 80 (Figure 2-9, Figure 2-10 and Table 2-13). There was no noticeable effect of order of mixing on droplet size, except, Emulsion 5 prepared by the solvent evaporation method which had a larger mean D(3,2) due to bimodal distribution and the higher number of large droplets was more apparent (Figure 2-8). The formation of larger droplets could be due to either smaller solvent to oil ratio or solvent diffusion to the aqueous phase. Choi et al. studied thermodynamic parameters on emulsification by the solvent diffusion method. They reported that the diffusion coefficients (Dsolvent-water, Dwater-solvents), exchange ratio (R= Dsolvent-water/Dwater-solvent) were directly proportional to the droplet size.[96]

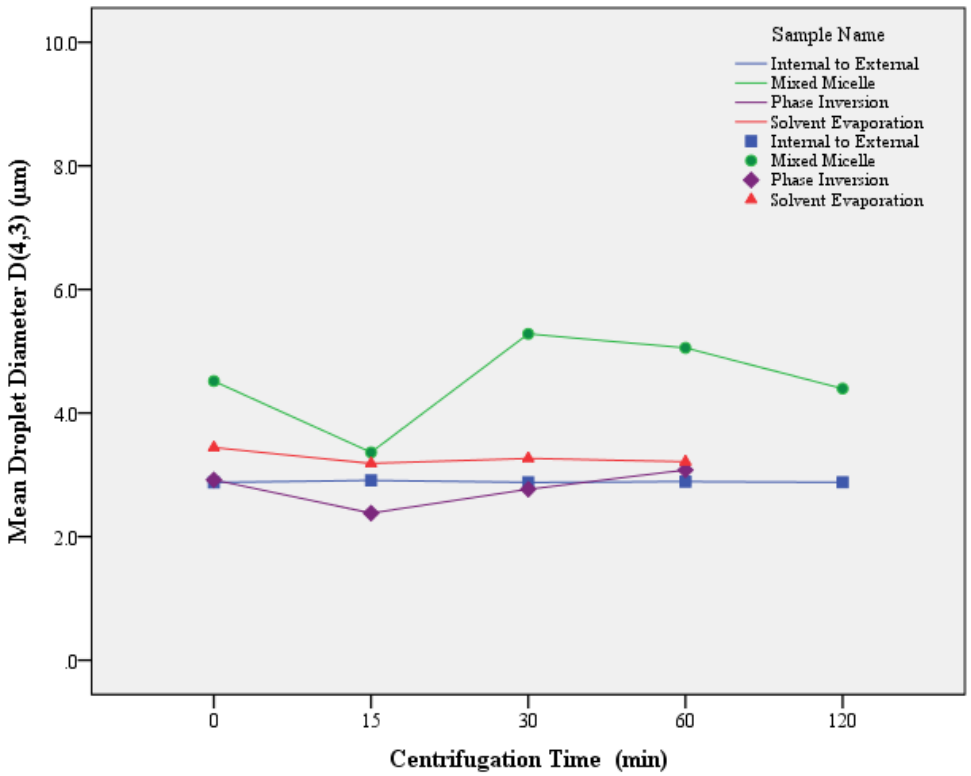
The mean droplet size for Emulsions 8 and 8E were slightly larger than for Emulsions 5, 6 and 7. A similar observation was reported for the mixed micelle system with Tween 80 and lecithin. The presence of large droplets could be due to the formation of a viscoelastic interfacial film at the oil and water interface. Based on empirical observation, the interfacial film for the mixed emulsifiers system perceived to have an enhanced elasticity compared with other emulsions including emulsions prepared in earlier HLB trials. The aqueous film of mixed micelles systems could not be easily broken during measurement of interfacial tension and stretched more than previous experiments.

Boyd et al. explained that an emulsifier system which is very effective in stabilising an emulsion may be less effective in facilitating the production of an initial small globule size and/or narrow globule size distribution.[38] In the case of a mixed micelle system the formation of viscoelastic films at the oil-water interface, may not be efficient to reduce droplet size; since an important parameter that describes droplet deformation is the Weber number (*We*), which is dependent on the viscosity of the oil. The improved viscoelasticity of the oil-water interface would require large energy input to deform a drop and subsequently reduce the droplet size.[6]

2.3.2.2.1 Forced degradation of Span 80 and Tween 80 emulsions

Overall, the emulsions containing Span 80 and Tween 80 had good stability under centrifugation at 4,000 rpm Figure 2-11. At 15 min, all emulsions had creaming of around 10%. Small oil globules were visible on the surface after 15 min centrifugation of Emulsion 7 prepared by the phase inversion technique. Oil globules were visible after 30 min for Emulsion 5 prepared by solvent evaporation. Both Emulsions 5 and 7 had an oily surface after 1 hour centrifugation followed by clear separation of oil on top when centrifuged for 2 hours. While, there was no significant change in Emulsions 6, 4 and 4E at 15, 30 and 60 min centrifugation, except for the formation of solid dry gel type cream of around 10% of total volume. Emulsions prepared by the mixed micelles method showed signs of visible globules at the end of 2 hours centrifugation. Gel structure for the mixed micelle system was thicker compared with other emulsions. Emulsion 6, prepared by addition of internal phase to external phase showed better stability compared with other emulsions for the mixed emulsifying system containing Span 80 as lipophilic surfactant.

Figure 2-11 Effect of centrifugation at 4,000rpm and order of mixing on mean droplet size D(4,3) for emulsions containing Tween 80 and Span 80



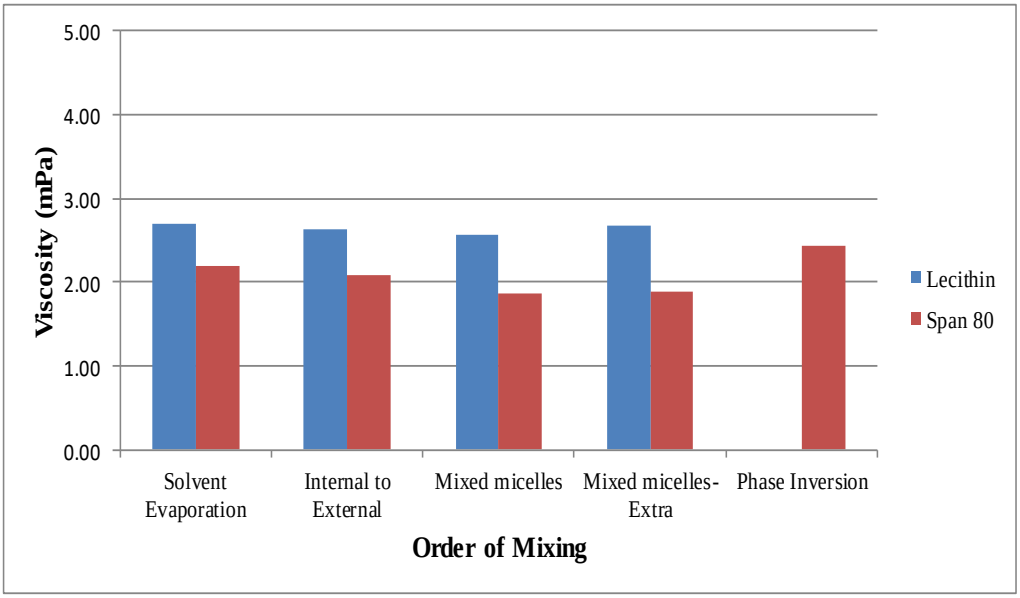
2.3.2.3 Emulsions containing lecithin

All emulsions prepared using lecithin alone had separated within 24 hours. These emulsions were discarded and no further analysis was carried out.

2.3.3 Effect of order of mixing on viscosity

Viscosity was not affected by order of mixing for emulsions containing lecithin. Viscosity of emulsions containing lecithin was slightly higher compared with emulsions containing Span 80 as lipophilic surfactant (Figure 2-12 and Table 2-13).

Figure 2-12 Viscosity of emulsions prepared by order of mixing comparison of lecithin and span 80 in combination with Tween 80



2.3.3.1 Selection of order of mixing

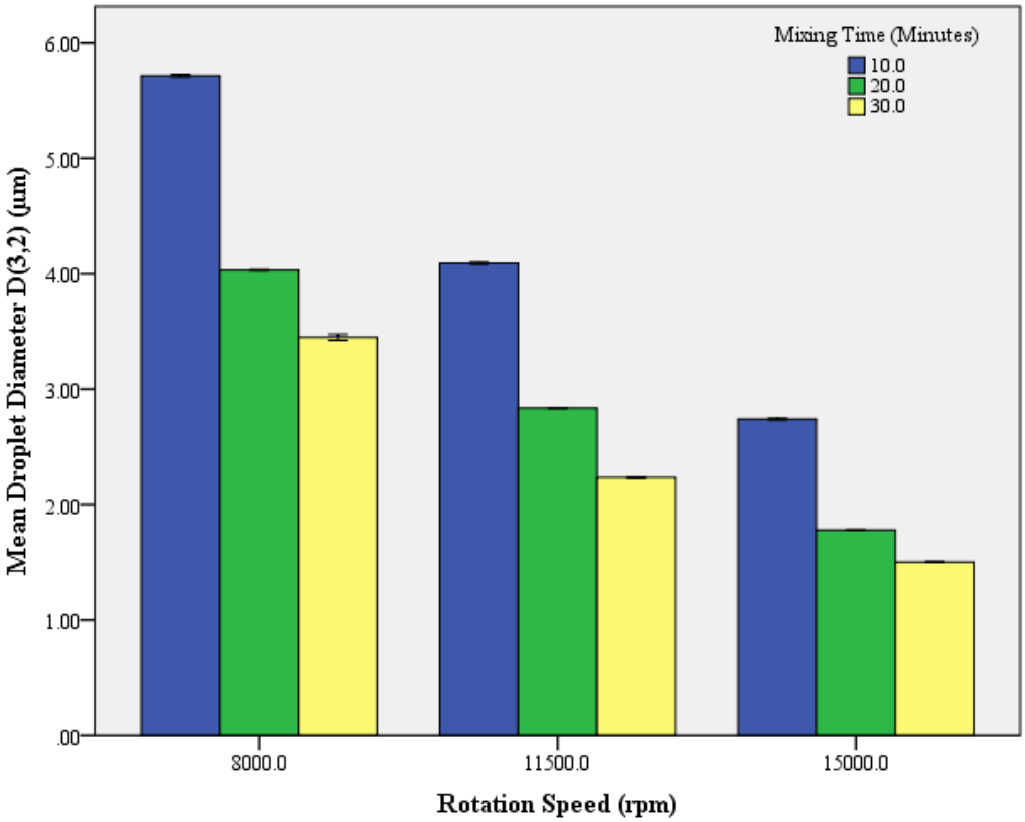
Emulsions were produced successfully by using different methods. Mixed micelle systems and addition of the internal phase to the external phase produced the most stable emulsions. The type of surfactant used was important for all techniques to form an emulsion, except in micellar rich solution. Mixed micelle systems were able to produce stable emulsions with lecithin and Span 80 as lipophilic surfactant in the presence of Tween 80 as hydrophilic surfactant. Based on the ability of the mixed micelle system to produce emulsions with different types of emulsifier mixtures and stability of emulsions under centrifugal force, the mixed micelle system was selected as the emulsification technique for the selection of the equipment for emulsification.

2.3.4 Selection of equipment

2.3.4.1 Diax® rotor-stator

The emulsions were prepared at three rotational speeds of 8,000, 11,500 and 15,000 rpm. The droplet sizes of emulsions decreased significantly ($p<0.0001$) with increase in the rotational speed (Figure 2-13 and Table 2-15). The smallest droplet size was achieved at 15,000 rpm. These observations are in agreement with the earlier reports by Hall et al., Liu et al. and Rodgers et al., [97-99] who reported that the rotor speed was an important parameter for the droplet size reduction.

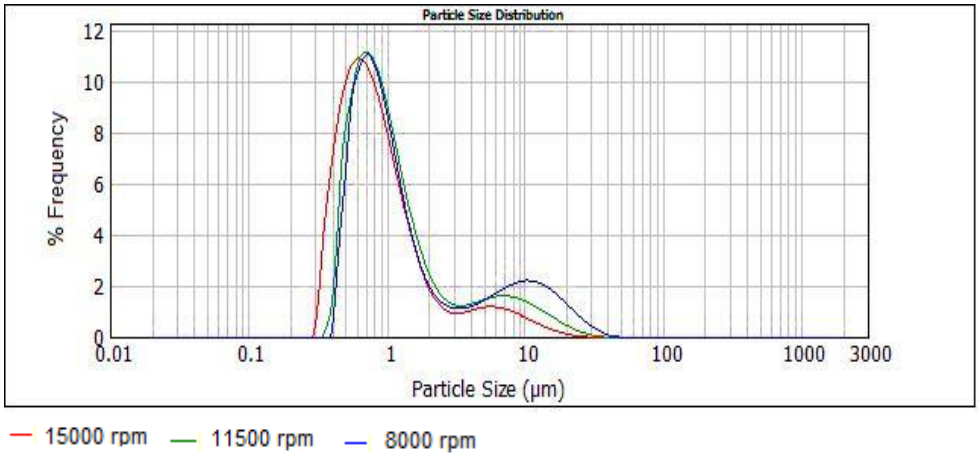
Figure 2-13 Effect of rotation speed on mean droplet size D(3,2)



The droplet size distribution was bimodal with all three rotational speeds. The larger droplets were more prominent at lower rotational speeds and were progressively reduced with increasing speed. Nearly 90% ofdroplets were less than 2 µm in diameter when the emulsion was mixed at 15,000 rpm for 30 min (Figure 2-14). The presence of large droplets at lower rotational speeds could be due to non-isotropic and intermittent turbulence produced by the mixer.[99] A large numbers of oil droplets were observed at the surface of the liquid during mixing at low rpm.

Since, the maximum shear rate produced is close to the agitator,[99] the turbulent flow produced at low rpm may not be adequate at the outer surface either to break the droplets or to pass the larger droplets through rotor stator, and the presence of larger droplets would be evident. In contrast; the turbulent flow produced at high speed would be effective to break the oil droplets and circulate the oil droplets through the rotor-stator. In addition, the shear rate is proportional to the rotor tip speed.[100] A higher shear rate would result in smaller and more consistent droplet sizes at high rotational speed. The reports from Rodgers and Cooke support the above findings and also explain the importance of the shear rate produced from the agitator, where the droplets are primarily broken.[99, 100]

Figure 2-14 Overlay Surface Mean distribution D(3,2) in % frequency of droplet size (µm) for emulsions produced at 8,000, 11,500 and 15,000 rpm for 30 min



The duration of mixing also played an important role in reduction of droplet size ($p<0.0001$). The droplet size reduced further when emulsions were mixed over the period of 30 min (Figure 2-15 and Table 2-15). Reduction in droplet size when mixing time was increased could be due to the droplets which bypassed the rotor earlier, may have passed through the agitator when processed for longer periods of 20 or 30 min. The droplet size may reach an effective equilibrium if emulsions were processed longer than 30 min at 15,000 rpm (Figure 2-15). The effective equilibrium of droplet size could be realized due to the fact that the drops had already passed through the agitator more than once and the droplets produced may have reached maximum equilibrium droplet size.[99]

Increase in both rotor speed and mixing time had significant influence on droplet size ($p<0.0001$). However, the interaction suggested that the effect of both parameters was not additive and the droplets size reduction cannot be predicted with

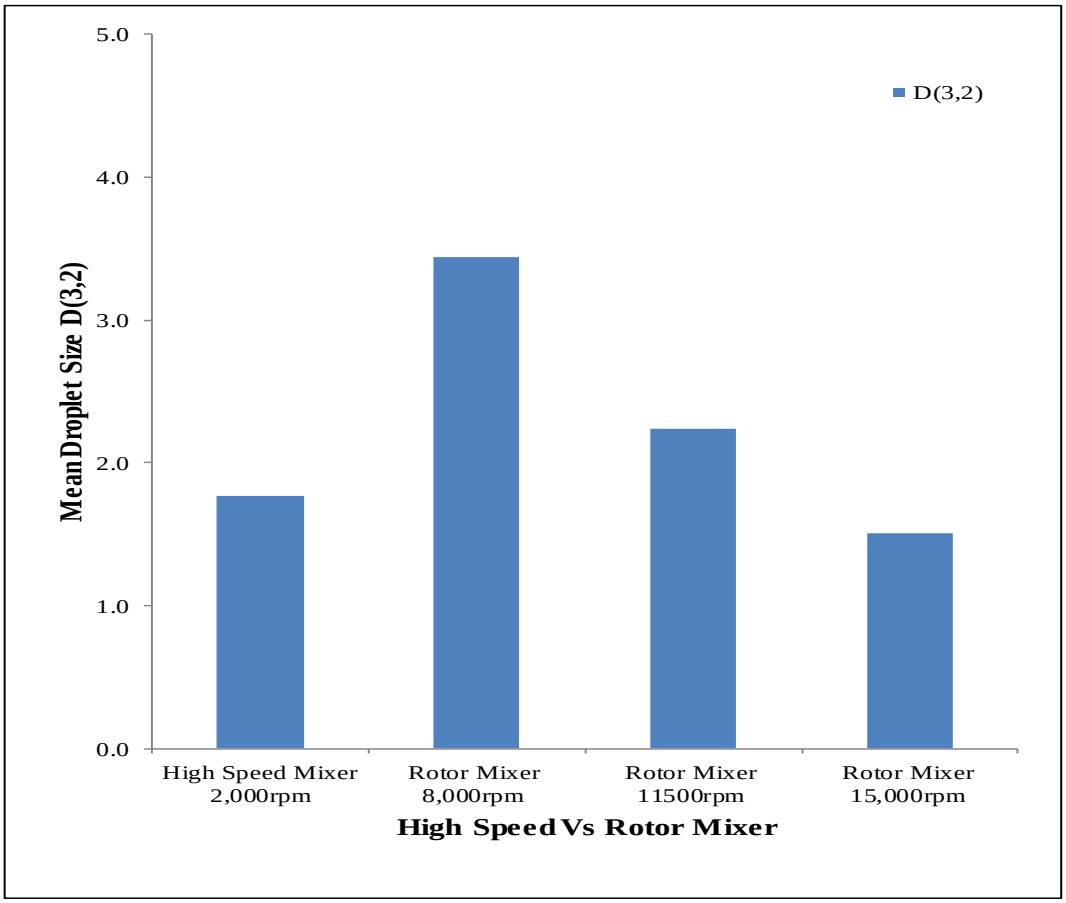
change in the either of the parameters. This outcome is central to the product quality during manufacturing and scale up.

Figure 2-15 presents a comparison of mean droplet diameters D(3,2) of emulsions produced by the Diax® rotor-stator and high speed mixer Unguator®. The mean droplet size at 8,000 and 11500 rpm using the Diax® rotor-stator were higher than the average droplet size of emulsion prepared by using the high speed mixer Unguator® at 2000 rpm (Figure 2-15). The high-speed mixer Unguator® was installed with the mechanism which moves the stirrer up and down, which brings the agitator in close contact with most of the product and produces maximum shear rate. The maximum droplet size (dmax) is related to maximum shear rate. Since, the droplet breakup occurs close to the agitator and the shear rate is proportional to the agitator tip speed.[99] The smaller droplet size even at a low rotational speed of 2,000 rpm in the Unguator® could be explained as a result of the high shear rate produced by the mixer, and the mixer mechanism which allows the agitator in contact with the entire surface of the liquid.

Table 2-15 Physical characteristics of emulsions prepared by Diax® rotor-stator for 10, 20 and 30 min (viscosity, creaming volume, droplet size distribution)

Speed	Duration	Viscosity	Creaming	Particle size (µm)		
(rpm)	(min)	(mPa)	% Volume	D(1,0)	D(3,2)	D(4,3)
8,000	10.00	2.11	0.30	0.80	5.71	16.31
8,000	20.00	1.91	0.14	0.76	4.03	13.35
8,000	30.00	2.03	0.12	0.72	3.44	12.49
11,500	10.00	1.95	0.16	0.75	4.09	11.15
11,500	20.00	2.03	0.10	0.72	2.84	9.29
11,500	30.00	2.00	0.00	0.69	2.24	7.99
15,000	10.00	1.67	0.18	0.71	2.74	9.84
15,000	20.00	1.77	0.13	0.62	1.78	6.33
15,000	30.00	2.04	0.10	0.57	1.50	5.18

Figure 2-15 **Mean droplet size D(3,2) after 30 min comparisons of high speed mixer Unguator® and Diax® rotor-stator**



2.3.4.1.1 Forced degradation of emulsion prepared by the Diax® rotor-stator

There was no apparent increase in mean diameter D(4,3) observed when emulsions were centrifuged at 4,000 rpm for up to 60 min (Table 2-16 and Figure 2-16). After the centrifugation a thick gel type of creaming was evident. The amount of creaming varied from around 30% for emulsions (Table 2-16) prepared at 8,000 rpm to less than 10% (1 ml creaming for 10 ml of sample) for emulsions prepared at 15,000 rpm.

Slightly yellow or yellowish white creaming was observed in emulsions produced at low rotor speeds. At 60 min, large visible globules were also observed on the surface for Emulsions 1 to 6. The emulsions prepared at 15,000 rpm produced white cream, when centrifuged for 30 min, which was separated into two layers; an upper white layer, and a lower yellowish-white layer after centrifugation for 60 min. The yellowish colour of creaming could suggest the presence of large lecithin vesicles containing oil. When emulsions were further centrifuged, there was a clear

separation of a free oil layer observed. None of the emulsions was found to be stable when centrifuged for 120 min at 4,000 rpm. The phase separation at 120 min could be directly related to rupture of large lecithin vesicles or diffusion of oil from vesicles after long term centrifugal force. The rupture of vesicles was evident by yellow sediments at the bottom of the centrifuged tubes especially emulsions prepared at 8,000 and 11,500 rpm. The amount of yellow sediment increased over the period of 60 min for emulsions prepared at 8,000 and 11,500 rpm. Since, the amount of oil and lecithin sediments separated from the emulsion were noted but not measured, any systematic correlation between the amounts of oil separated with lecithin sediment was not possible.

Figure 2-16 **Effect of centrifugation time on mean droplet size D(4,3) in micrometres, emulsions prepared by Diax® rotor-stator**

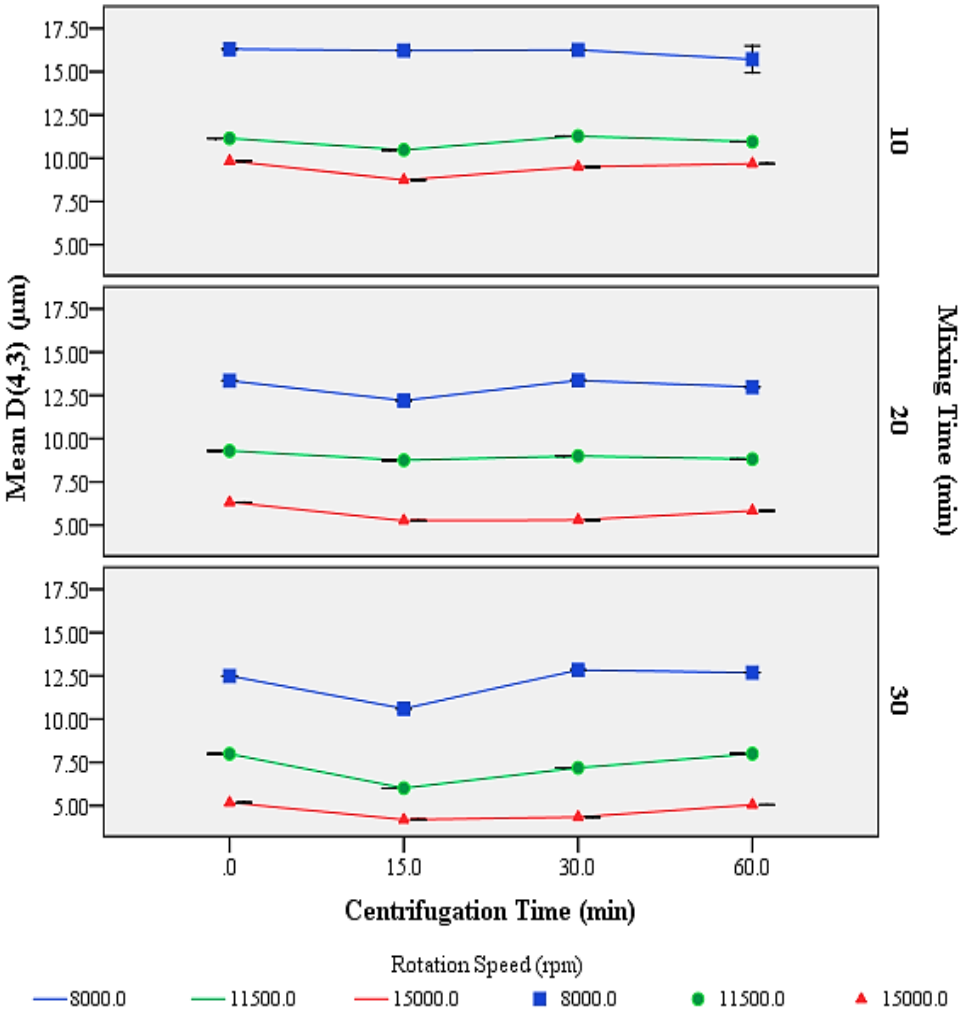


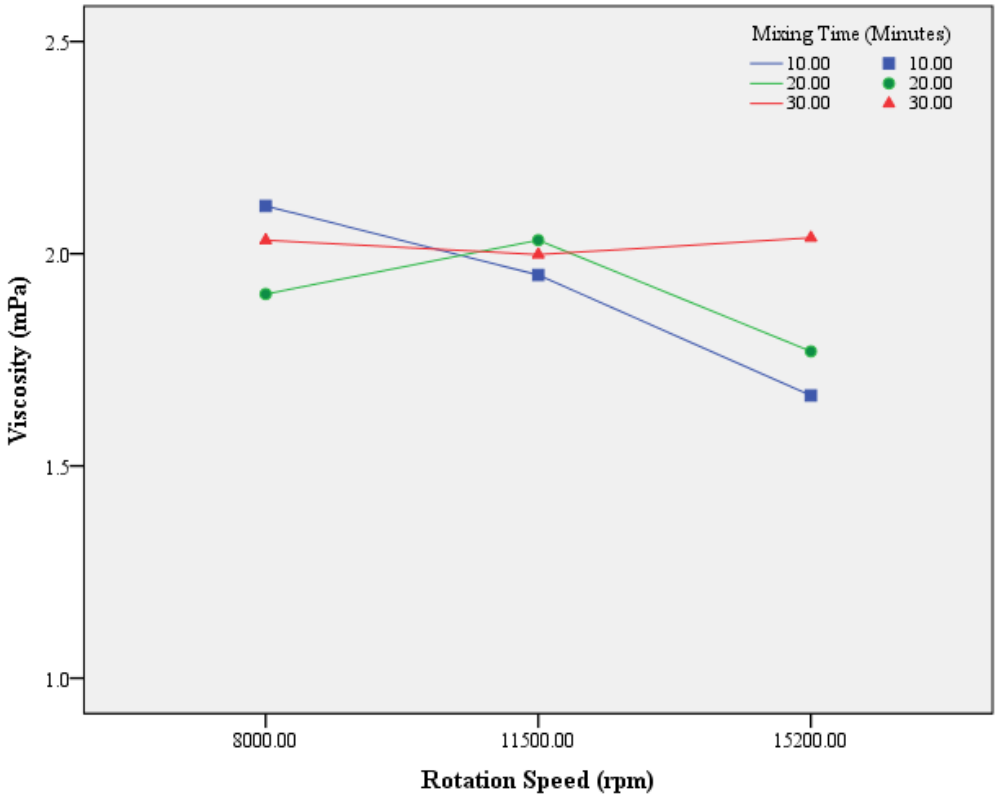
Table 2-16 Mean droplet size D(4,3) of emulsions prepared by Diax® rotor-stator, after centrifugation at 4,000rpm, for different time periods

Speed (rpm)	Duration (min)	Forced degradation at 4,000rpm Vs Particle size D(4,3) (µm)			
		0min	15min	30min	60min
8,000	10.00	16.31	16.23	16.25	14.55
8,000	20.00	13.35	12.20	13.36	12.98
8,000	30.00	12.49	10.61	12.84	12.69
11,500	10.00	11.15	10.48	11.28	10.96
11,500	20.00	9.29	8.75	8.75	8.82
11,500	30.00	7.99	6.01	7.18	8.00
15,000	10.00	9.84	8.75	9.50	9.69
15,000	20.00	6.33	5.26	5.30	5.83
15,000	30.00	5.18	4.19	4.34	5.05

2.3.4.1.2 Effect of rotor speed and duration of mixing on viscosity

Overall, there was only small changes in viscosity recorded, when the emulsions prepared at rotor speeds of 8,000, 11,500 and 15,000 rpm over 30 min (Figure 2-17 and Table 2-15). At 15,200 rpm, there was a trend of increased in viscosity over the period of 30 min (Figure 2-17 and Table 2-15). It was difficult to draw a conclusion on the direct effect of rotor speed on viscosity. An increased rotation speed was found to be statistically significant (p =0.0039) in reducing viscosity of the emulsion, which could be an outcome of a large standard deviation (Figure 2-17 and Table 2-15).

Figure 2-17 Effect of rotation speed and mixing time on emulsion viscosity



2.3.4.2 High-pressure Homogeniser

The emulsions were prepared at two different pressures of 2,500 and 5,000 psi and processed for three cycles each. The effects of system pressure and number of cycles on the droplet size of emulsions were examined. The droplet size was reduced significantly with increased pressure ($p<0.0001$) as expected. However, a small difference in D(1,0) and D(3,2) was observed due to the submicron droplet size of emulsions, when pressure was increased from 2500 psi to 5,000 psi (Figure

2-18 and Table 2-17). These results were in agreement with those obtained by Jafari et al.[45] The major effect on pressure increase was on droplets larger than 1 µm in diameter, which could also be seen by reduction in mean droplet size D(4,3). The droplet size distribution was improved by increasing homogenisation pressure. Creaming was observed only for emulsions processed through the first cycle (Table 2-17). The creaming could be associated with the presence of larger size droplets (Table 2-17). There was no major effect of homogenisation pressure and number of cycles found on viscosity (Table 2-17 and Figure 2-19).

Figure 2-18 Effect of homogeniser pressure on droplet size

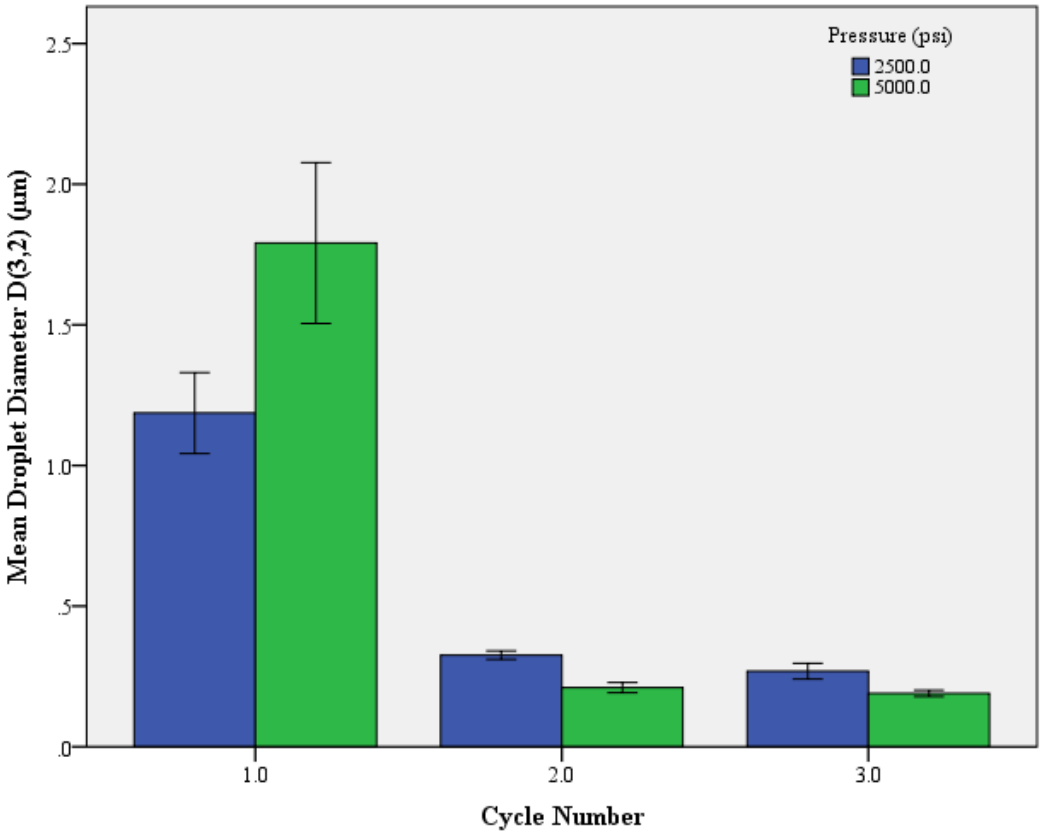


Figure 2-18 represents the effect of number of cycles on mean droplet size D(3,2). The number of cycles was found to be an important parameter in reducing droplet size of emulsions ($p<0.0001$). It was clear that when emulsions were processed for a second cycle, there was a significant ($p<0.0001$) reduction in mean droplet diameter. Pandolfe reported that pre-homogenised emulsions provided smaller mean diameter compared with emulsions with poor premix containing large droplets.[101] This can be explained by following equation from Levich.[102]

$$t = \frac{\eta r}{\sigma}$$ .....Equation 2-8

Where t is time required for droplet deformation, r is droplet radius, η is viscosity of dispersed phase and σ is interfacial tension. According to Equation 2-8, the time t required for the viscous deformation of a droplet is proportional the droplet radius r, and the viscosity of the dispersed phase. Larger radius droplets require longer residence time. When emulsions were processed through third cycle, the droplet size reduction was smaller yet significant ($p < 0.0002$). The effect of multiple passes in droplet size reduction could be appreciated due to increase in the residence time of droplet. However, further homogenisation of emulsions may not efficiently reduce the droplet size and could be due to the droplets size reduced to the size of the smallest energy-dissipating eddies, they can no longer be divided by pressure fluctuations of these eddies and efficiency of disruption is decreased.[102]

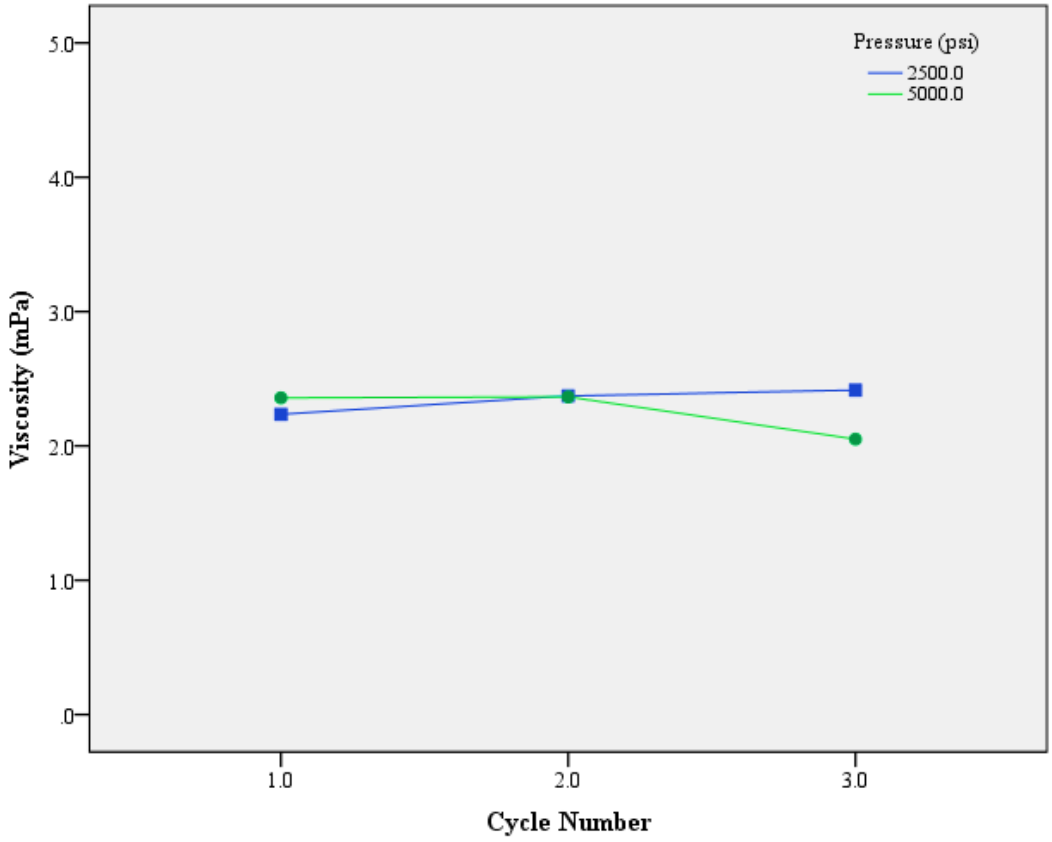
Table 2-17 Physical characteristics of emulsions prepared by high pressure homogenisation (viscosity, creaming volume, droplet size distribution)

Pressure (Psi)	Cycle Number	Viscosity (mPa)	Creaming % Volume	Particle size (µm)		
				D(1,0)	D(3,2)	D(4,3)
5,000	1	2.36	0.15	0.66	1.84	5.75
5,000	2	2.37	0.00	0.07	0.24	0.71
5,000	3	2.05	0.00	0.06	0.20	0.52
2,500	1	2.24	0.15	0.66	1.45	3.37
2,500	2	2.37	0.00	0.07	0.35	1.08
2,500	3	2.42	0.00	0.07	0.31	0.86

2.3.4.2.1 Effect of homogenisation pressure and number of cycles on viscosity

There was no apparent effect of homogenisation pressure on viscosity as observed in Table 2-17. There was a trend of increased in viscosity with number of cycles, when emulsion was processed at 2,500 psi. (Figure 2-19, and Table 2-15).

Figure 2-19 Effect of homogenisation pressure and number of cycle on emulsion viscosity



2.3.4.2.2 Forced degradation evaluation of emulsions prepared by homogenisation

During forced degradation by centrifugation, there was no creaming or yellow lecithin sediment at 5,000 psi. The thick creaming (10%) and yellow lecithin were observed for emulsions prepared at 2500 psi. All emulsions prepared by high pressure homogenisation were fairly stable during centrifugation at 4,000 rpm for 2 hours. There was no increase in mean droplet size D(4,3) for the emulsions processed through two or three homogenisation cycles (Table 2-18 and Figure 2-20). Droplet size increase was evident for the emulsions passed through only one cycle and showed some separation of oil when centrifuged for 120 min (Table 2-18 and Figure 2-20). The oil separation could be due to the coalescence of large droplets, which were absent in emulsions processed for multiple homogenisation cycles. These results correspond with earlier findings on improved emulsion quality by repeated homogenisation cycles, as measured by size distribution.[103]

Figure 2-20 **Effect of centrifugation time on mean droplet size D(4,3), emulsions prepared by high pressure homogenisation**

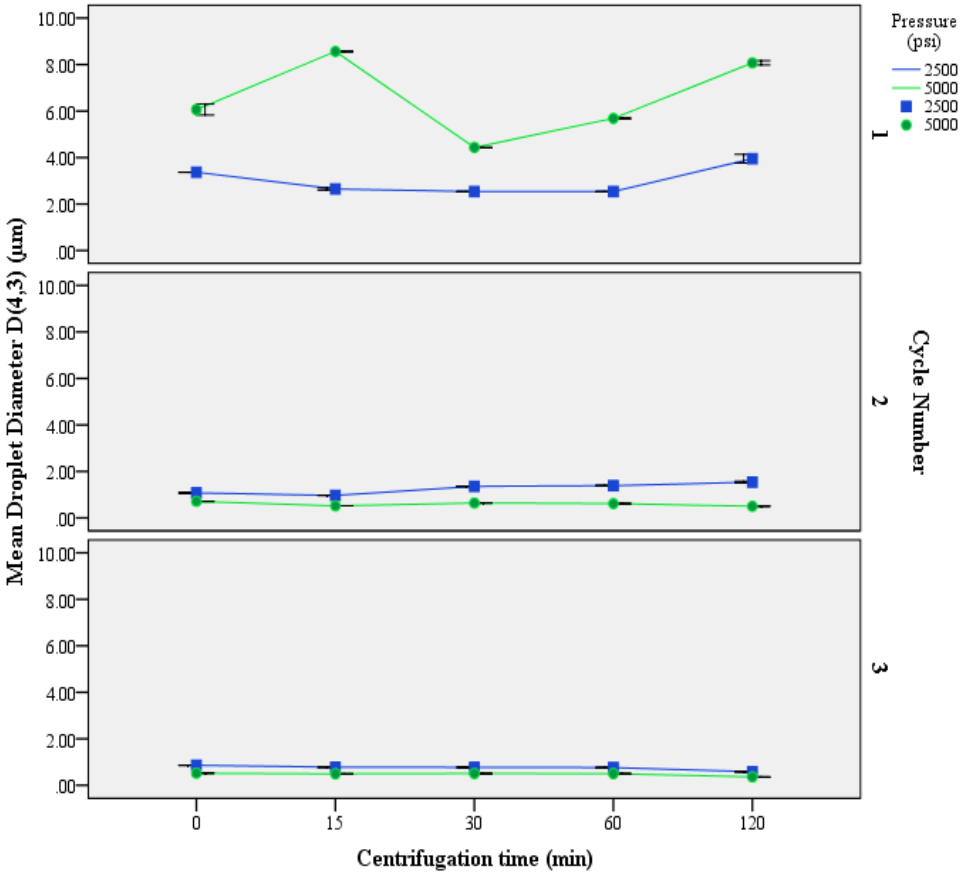


Table 2-18 **Effect of centrifugation time on mean droplet size D(4,3) of emulsions prepared by high pressure homogenisation**

Pressure (Psi)	Cycle Number	Forced degradation at 4,000rpm Vs Particle size D(4,3) (µm)				
		0min	15min	30min	60min	120min
5,000	1	5.75	8.56	4.43	5.69	7.99
5,000	2	0.71	0.52	0.64	0.61	0.50
5,000	3	0.52	0.49	0.51	0.50	0.36
2,500	1	3.37	2.64	2.54	2.54	3.98
2,500	2	1.08	0.97	1.36	1.39	1.57
2,500	3	0.86	0.78	0.77	0.76	0.58

2.3.4.3 Ultrasonication

Table 2-19 has summarised the results of emulsions prepared by ultrasonication. Creaming was recorded at all amplitudes, especially when the emulsions were sonicated for 2 or 4 min. There was no creaming observed when emulsions were sonicated for 6 min. This could be correlated with the mean droplet diameter D(3,2) of <1 µm, for emulsions produced after 6 min. The droplet breakage in the emulsion is dependent on the type and quantity of applied shear force.[104] Lower amplitude may not produce enough cavitation intensity, which translates in reduced shear force. As result, large oil droplets were observed on the surface of the emulsions produced at 20 and 40% amplitude for 2 and 4 min each. The above theory could be supported by the temperature profile in Figure 2-21. High intensity may produce the cavitational collapse, which would generate large amounts of energy and subsequently increased temperature.[105] There was a clear trend of increasing temperature with amplitude ($p<0.0001$) and sonication time ($p<0.0001$). However, there was also an association between sonication amplitude and time on temperature. There was no temperature increase when emulsions were process at 20% amplitude for 6 min. At the same time there was a temperature rise of 15 °C when the highest amplitude of 60% was applied for 6 min. The major increase in temperature was between 4 to 6 min at 40 % and 60 % amplitude.

The mean droplet diameter D(3,2) was significantly reduced ($p<0.0001$) by increasing the ultrasonication amplitude and duration of sonication (Table 2-19, and Figure 2-22 a). There was linear relationship ($r^2 = 0.9818$) between duration of sonication and droplet size reduction, when the sonication amplitude was kept at 20 to 40%. Similar findings were reported by Hashtjin and Soleiman.[106] They observed a linear relationship for mean droplet size in response to sonication time and amplitude.[106] At 60% amplitude, the mean droplet diameter reached a plateau (Figure 2-22). However, droplet size distribution for emulsions prepared was reduced with the increased sonication time. There was no effect of amplitude and duration of sonication on emulsion viscosity.

Table 2-19 Physical characteristics of emulsions prepared by ultrasonication (viscosity, creaming volume, droplet size distribution)

Amptitude (%)	Duration (min)	Viscosity (mPa)	Creaming % Volume	Particle size (µm)		
				D(1,0)	D(3,2)	D(4,3)
20	2	1.93	0.16	0.87	2.91	9.29
20	4	2.11	0.20	0.74	1.78	4.32
20	6	1.89	0.05	0.07	0.40	1.57
40	2	2.06	0.16	0.86	2.60	9.02
40	4	1.97	0.20	0.61	1.37	2.80
40	6	1.98	0.00	0.07	0.30	1.08
60	2	1.92	0.20	0.75	1.85	4.96
60	4	1.88	0.10	0.07	0.39	1.48
60	6	1.94	0.00	0.07	0.27	0.84

Figure 2-21 Effect of ultrasonication time and % amplitude on product temperature

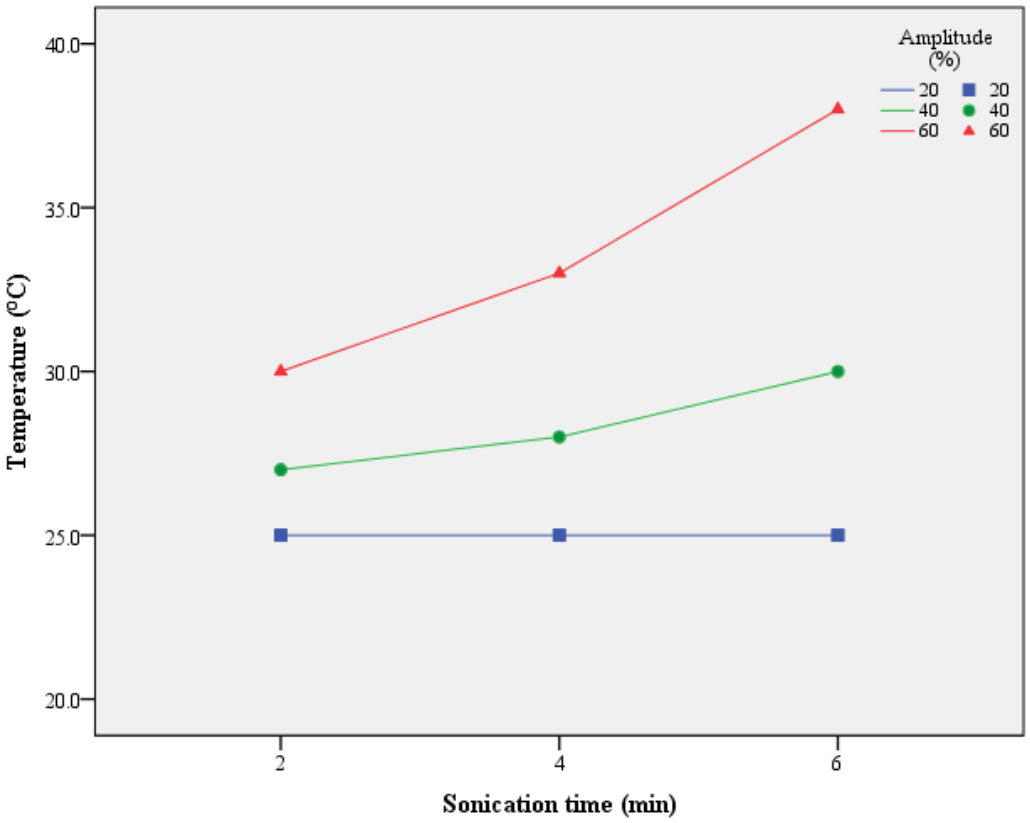
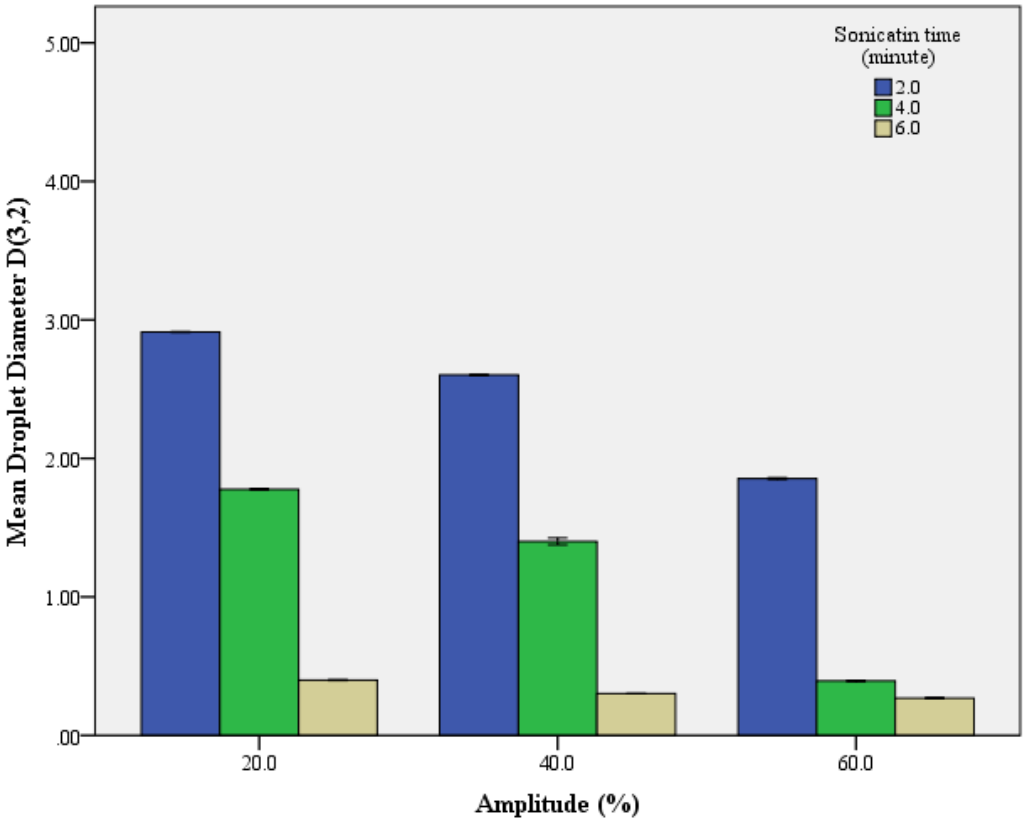


Figure 2-22 Effect of ultrasonication time on mean droplet diameter D(3,2)



2.3.4.3.1 Forced degradation evaluation of emulsion prepared by ultrasonication

When emulsions were evaluated for forced degradation, it was clear from the data shown in Figure 2-23 and Table 2-20 that the amplitude and sonication time were important parameters for producing stable emulsions. Emulsions sonicated for 2 min were unstable during centrifugation regardless of the sonication amplitude applied. The presence of yellow creaming and sedimentation were evident in emulsions 1, 4 and 6, when centrifuged for 15 min. While the emulsions processed for 4 min either separated or coalesced and formed large oil droplets when centrifuged for 2 hours.

Stable emulsions were produced, when ultrasonication time was increased to 6 min (Figure 2-23). However, the emulsions would possibly be over processed, if sonicated for more than 6 min and no significant gain of product quality would be achieved (Figure 2-22). There was no creaming and / or separation of oil observed, except for emulsions prepared at 20% amplitude that contained white creaming after 30 min of centrifugation and some visible oil droplets on the surface. The stability of emulsions could be due to the reduction in larger droplets with increasing sonication time. Jafari et al. also reported that increasing the time of ultrasonication decreased the width of the size distribution.[45] Overall, the best outcome was achieved at 40% amplitude with droplet size close to 60% amplitude at 6 min ($p=0.4369$) with minimum temperature increase ($p< 0.0001$)

Emulsions processed for 20% amplitude were separated within 60 min. While emulsions prepared by 40% amplitude showed the presence of large oil droplets on the surface and emulsions prepared at 60% amplitude had no changes except white creaming was present when centrifuged at 30 min, which turned into slightly yellow at 120 min. The above observations confirm that optimal conditions are necessary to produce a stable emulsion by ultrasonication. The longer sonication time would improve the droplet distribution, while higher amplitude would increase the droplet breakdown and reduce the droplet size, while some large droplets remained intact. This is evident as a multi-mode droplet size distribution.

Figure 2-23 **Effect of centrifugation time on mean droplet size D(4,3), emulsions prepared by ultrasonication**

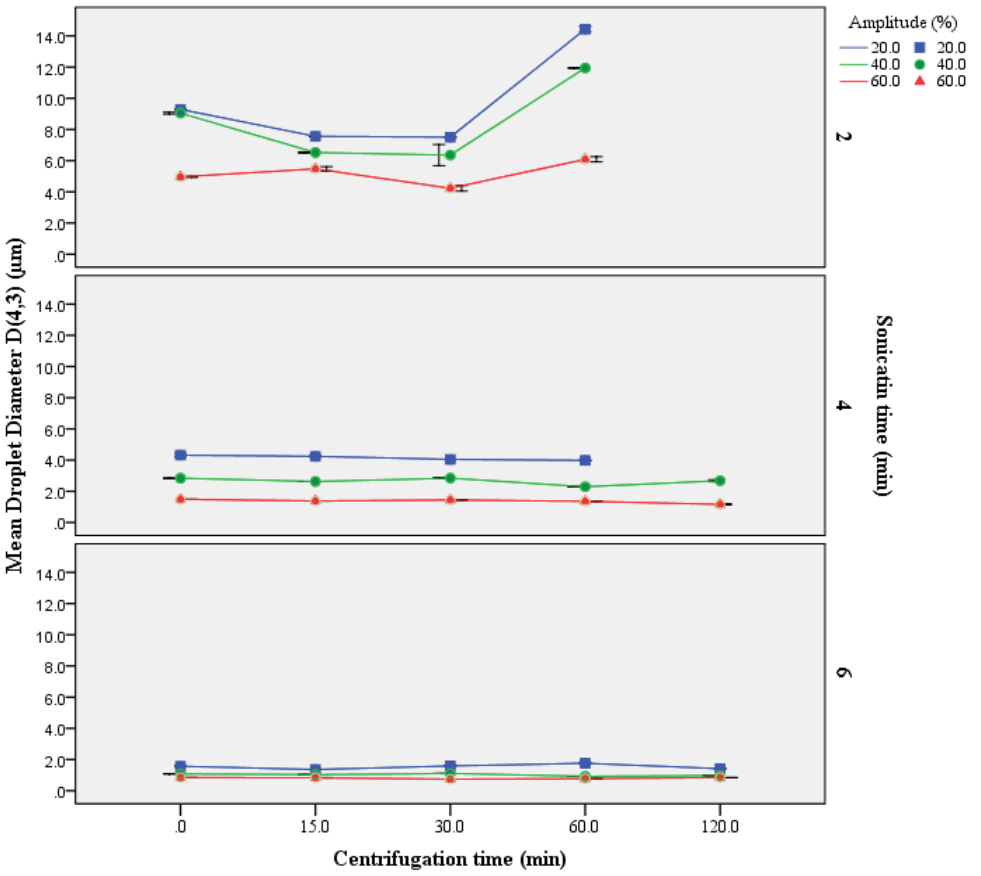


Table 2-20 **Effect of centrifugation on mean droplet size D(4,3) of emulsions prepared by ultrasonication**

Amptitude (%)	Duration (min)	Forced stability at 4,000rpm Vs Particle size D(4,3) (µm)				
		0min	15min	30min	60min	120min
20	2.00	9.29	7.56	7.51	14.37	-
20	4.00	4.32	4.24	4.04	3.97	-
20	6.00	1.57	1.36	1.59	1.76	1.42
40	2.00	9.02	6.52	7.23	11.94	-
40	4.00	2.80	2.63	2.84	2.29	2.68
40	6.00	1.08	1.03	1.11	0.92	0.97
60	2.00	4.96	5.51	4.23	6.12	-
60	4.00	1.48	1.38	1.44	1.36	1.16
60	6.00	0.84	0.83	0.74	0.77	0.84

2.3.4.4 Selection of equipment

It was clear from the forced degradation data of emulsions produced by each type of equipment - that emulsions with mean droplet sizes D(3,2) less than 2 µm were the most stable. Each equipment option was capable of achieving a mean droplet size D(3,2) of 2 µm or less when optimum conditions were applied. High pressure homogenisation was found to be the most efficient process in reducing droplet size. The crucial benefit of homogenisation was its ability to pass each droplet through a high shear zone, which provides consistent product quality.

In the case of the Diax® rotor-stator, the important factors were rotor speed, duration of mixing and the presence of droplets near the high shear zone. It may be possible to increase the probability that oil droplets passed through the high shear zone of the rotor and reduce the droplet size more efficiently. The drop wise addition or direct injection of the oil phase during emulsification may increase the efficiency of droplet breakup and improve the quality of the final product. The process using Diax® rotor-stators could easily be up scaled and validated. There are a number of inline mixers available in the market which can successfully be used in a scale-up process, without losing efficiency of droplet breakup. The high pressure homogenisation or ultrasonication could also be used more efficiently along with a Diax® rotor-stator to achieve smaller droplets with improved droplet size distribution. Emulsions produced by rotor-stators may require less energy input and shorter processing time, when homogenised or sonicated.

Ultrasonication was found impractical to produce an emulsion due to rise in temperature, scalability and uneven distribution of energy. Ultrasonication could possibly be used more efficiently along with the Diax® rotor-stator to breakdown the droplets or aggregates larger than 5 µm.

2.4 Conclusion

It was evident that o/w emulsions prepared at HLB >9 were more stable during forced degradation testing compared with emulsions prepared close to the reported HLB of the oil (7-9). The most stable o/w emulsions were produced at HLB >10 and most preferably, at emulsifier ratio at HLB 11.

Emulsions were produced using a range of different orders of mixing or emulsification techniques. The most stable emulsions were produced by using a mixed micelle system and addition of the internal phase to the external phase. The order of mixing and the type of surfactant used had an impact on emulsion quality and stability. Mixed micelle systems were the least affected by the type of surfactant used and formed stable emulsions for both lecithin and Span 80. Mixed micelle systems produced larger but stable droplets compared with other orders of mixing. The stability of emulsions produced by mixed micelle systems was presumed due to the formation of a condensed and hydrated interfacial film.

In the case of the equipment selection process, high pressure homogenisation was found to be the most efficient process in reducing droplet size. While, the process using the Diax® rotor-stator was found to be easy to scale up and validate. If the optimum conditions were provided, the rotor-stator equipment was also capable of achieving a mean droplet size D(3,2) of 2µm, which was an important parameter for emulsion stability. Ultrasonication was found impractical to produce an emulsion due to the rise in temperature, scalability and uneven distribution of energy.

3 Emulsion Optimisation and Stability

3.1 Introduction

The stability of colloidal formulations is paramount in their use as drug delivery systems. They must be sufficiently stable to be easily manufactured, sterilized and have a shelf life of at least a year, preferably more. Shelf life considerations are particularly important for emulsion systems, since they are generally stored as liquids.[107] Purely on thermodynamic grounds, emulsions are physically unstable owing to high free energy levels and are therefore subject to various processes including aggregation, flocculation, coalescence and eventually phase separation.[7]

$$\Delta G = \gamma \Delta A \quad \text{.....Equation 3-1}$$

Where γ is the interfacial tension, ΔG is the change in the free energy of the system and ΔA the surface area change of the interface at constant temperature and pressure. Thermodynamic changes during formation of emulsion can be explained in terns of free energy change according to Equation 3-1. [108, 109] Since this energy is always positive, a system always tends toward the thermodynamically stable lowest possible interfacial area of a layer of oil on water.[110] Pharmaceutical emulsions are kinetically stable and stability is reflected as shelf life, where the physicochemical properties of an emulsion are observed for change over the period of storage with respect to defined limits.[110, 111] The globule size distribution over time is a function of other factors including pH, emulsifying agents, co-additives and storage conditions.[111]

The physical properties of emulsions and external influences, and their relationship are important to emulsion stability.[19] Factors that are associated with emulsion instability are: [14]

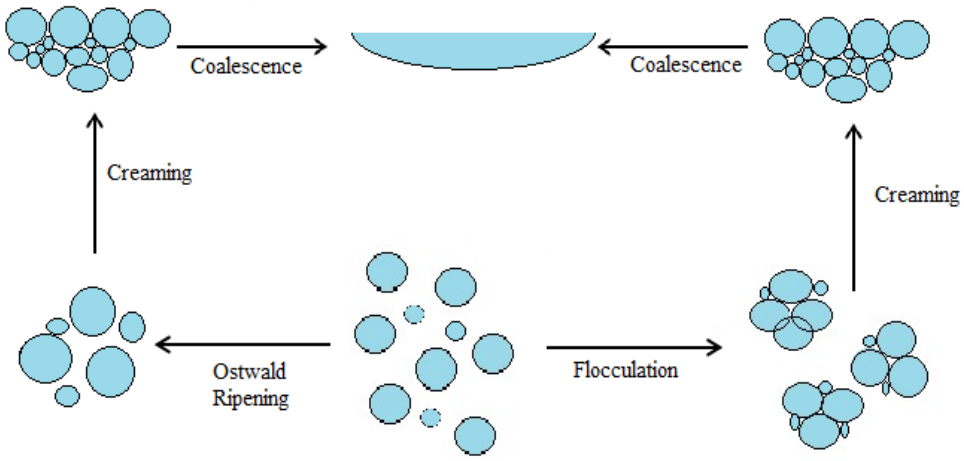
4. Temperature
5. Concentration and ratio of emulsifying agent
6. Emulsification
7. Process
8. Phase volume ratio
9. pH

Temperature increase during emulsification can cause phase separation. This is especially the case, if the emulsifying agent is susceptible to heat such as lecithin

and polyoxyethylene based non-ionic surfactants.[1, 112] Shinoda et al. reported that the relatively stable o/w type emulsions could be obtained for surfactant systems with a phase inversion temperature (PITs) of 20º - 65ºC higher than the storage temperature.[42, 113] An unoptimised formulation and the efficiency of the technique have been considered to be the most common causes for emulsions instability.[1] The physical stability of emulsions can substantially be improved with help of suitable emulsifiers that are capable of forming a mono or multilayer interfacial film around the dispersed liquid droplets.[7] The mechanical strength of the interfacial film is an important factor and it should be condensed, with strong lateral intermolecular forces, and high film elasticity.[11]

Emulsion instability is governed by four different droplet change mechanisms (Figure 3-1); flocculation, creaming, sedimentation and disproportionation. Flocculation and Ostwald ripening are major destabilisation processes in emulsion stability.[114] Compaction and compression of droplets in the floccules of a cream layer exaggerate the interparticle attraction.[14] Finally, the drops coalesce and form a separate layer of oil on top depending on the nature of the oil and its density and viscosity.[14]

Figure 3-1 Schematic representation of instability processes of emulsions



3.1.1 Flocculation and Creaming

Flocculation is defined as an aggregation of two or more droplets that become loosely attached to each other, but maintain their individual integrities without coalescence occurring.[115] Flocculation can lead to an increase in particle size that accelerates the rate of gravitational separation or creaming.[116] Flocculation of the

dispersed phase is described as reversible aggregation of droplets of the internal phase in the form of three-dimensional clusters.[115] Flocculation of emulsion droplets can occur when the mechanical or electrical barrier is not sufficient to prevent droplet coagulation.[116] Droplet flocculation in emulsions is a balance between attractive (London- van der Waals forces) and repulsive forces (steric and electrostatic).[110] The repulsive forces are dependent on the type and concentration of ionized species, ionic composition, their complexation, competitive adsorption and layer-by-layer formation of emulsifiers.[116, 117]

The droplets are prevented from aggregating by the electrical charge of adsorbed molecules on their surface and electrostatic repulsion prevents them from coming close together.[11] The presence of a charge on the dispersed droplets constitutes an electrical barrier to the close approach of two particles to each other. In o/w emulsions, the source of the charge on the dispersed droplets is the adsorbed layer of surfactant with its hydrophilic group orientated towards the water phase.[11, 20] In emulsions stabilised by non-ionic surfactants, the charge on the dispersed phase may arise either from adsorption of ions from the aqueous phase or from frictional contact between droplets and the aqueous phase.[6]

On the other hand, repulsive forces such as steric repulsion arise from the adsorbed polymeric tails of the surfactant. Repulsive forces arise as the adsorbed layers interpenetrate which is also dependent on the degree of solvation of the hydrophilic chain.[20] The hydrophilic solvated chain in the continuous phase requires space to attain all possible conformations and will repel the presence of another molecule adsorbed to another droplet.[11] Demetriades and McClements reported that the higher concentrations of Tween reduced the extent of droplet flocculation even at higher temperatures.[118]

In nano-emulsion systems, flocculation can be prevented by steric stabilisation, essentially due to the sub-micron droplet size.[115] Steric repulsion is dependent on the interfacial density, interfacial layer thickness δ, and the interactions between the interfacial layer and solvent.[108] A reduction of configurational entropy occurs when inter-droplet distance (h) becomes lower than the interfacial layer thickness (δ).[46, 108] When, the sum of the energies of interaction (G_{Total}) at $h = 2\delta$ a balanced system is produced, wherein molecules repel and particles attract each other, showing a weak minimum (Gmin).[108, 109] Below this value of h a very rapid increase in the

interaction between the two adsorbed layers occurs. At this stage, aggregation or coagulation is likely to occur and it may be steric interaction that keeps the droplets far enough apart so that van der Waals attraction is minimal.[115] The smaller sized droplets with dense adsorbed layers ensures the stabilisation of the interface and lack of thinning or disruption of liquid film, hence coalescence is also prevented.[46, 109 108]

Because of the difference in density between the two phases an increase in three dimensional volumes of floccules leads to the formation of a cream layer on the emulsion.[116] Emulsions with creaming may still be pharmaceutically acceptable as long as they can be reconstituted by a modest amount of shaking.[11] Droplet movement under gravitational force and the creaming rate at which buoyant emulsion droplets tend to rise is given by Stoke's law.[119]

$$\vartheta = 2a^2 g(\sigma - \rho)/9\eta \quad \text{.....Equation 3-2}$$

Where, v is velocity of creaming, a is radius of a spherical particle, σ is density of the particles, ρ density of dispersion medium, η viscosity, g is acceleration due to gravity.[110] Creaming rate can be reduced by reducing droplet size and increasing the viscosity of the external phase.[1, 11] Since the rate of creaming is a function of the square of the radius of the droplet, it is very important to limit droplet diameter as much as possible.[23] Small droplet sizes under the influence of Brownian motion also help the stabilisation against aggregation or creaming.[46]

There are a number of methods that have been used for the measurement of flocculation and creaming. Both creaming and flocculation measurements have limited relevance in quantitation of instability and rather provide an indication of the presence of aggregates. However, creaming volume can be an important quality control parameter of emulsions.

3.1.2 Coalescence and Ostwald ripening

Coalescence can lead to the formation of larger oil droplets and the thermodynamic stage of phase separation.[30, 110] Coalescence is an irreversible process and repeated coalescence eventually leads to emulsion breaking or phase inversion.[110] Emulsions can also break up due to Ostwald ripening. Ostwald ripening involves large droplets growing at the expense of small ones. The rate of Ostwald ripening increases with

dispersed phase solubility in the continuous phase.[120] The oil can be transported through the continuous phase from smaller droplets to larger ones; as larger droplets have a lower surface to volume ratio than the smaller ones.[23] Based on Lifshitz-Slyozov-Wagner (LSW) theory the average droplet size is reduced with decreased solubility of co-emulsifier in water.[120] Ostwald ripening occurs due to pressure inside smaller droplets. The solubility of the dispersed phase increases in the continuous phase, this later reconstituted into larger lower-pressure dispersed phase droplets.[14, 120] The net effect of this process is that only very large droplets remain, increasing the overall droplet size.[14]

Any suitable stability assessment would need to consider Ostwald ripening and coalescence and their effects on droplet size. A first order kinetics was applied to calculate the rate of coalescence by Sherman (Equation 1-3).[121] The storage time and temperature are crucial in determining stability, which could be modelled by the Arrhenius equation and the first order kinetics relationships were used to estimate the temperature dependence of globule coalescence.[38]

$$\boldsymbol{ln\, N_t = ln\, N_0 - kt} \quad \text{.....Equation 3-3}$$

Where N_0 is the number of oil globules at time 0 , N_t is the number of oil globules at time t, and k is the coalescence rate constant. Increase in the temperature of the sample increases the kinetic energy, causing increased droplet coalescence of the emulsion.[14] Droplet size increase in different storage condition would provide a quantitative measure of coalescence and make it possible to predict the shelf-life of emulsions.[110] The process by which droplet size increases, could also be predicted from the distribution profile of droplet size.[110]

The major factor which prevents coalescence in flocculated and deflocculated emulsions is the mechanical strength of the interfacial barrier. Schulman and Cockbain showed that a mixture of an oil-soluble co-surfactant/stabiliser and a surface active agent was able to form a stable, and flexible condensed film at the oil/water interface.[39] Mixed surfactants may provide better resistance against rupture and lower the interfacial tension, compared with that produced by either component alone and also produce a stable emulsion similar to a lyophobic colloidal dispersion.[39] Thus, it is widely recognized that longer shelf life and absence of coalescence can be achieved by the formation of a thick interfacial film from mixed emulsifiers. Hydrated polyoxyethylene chains of tweens provide a gel

like structure at the interface. This structure provides good stability to globule coalescence and the effect is synergised in the presence of span by the closely packed hydrocarbon chains of alternating span and tween molecules on the oil side of the interface.[38] The stabilisation of emulsions was also explained by Higuchi and Misra by retardation of interdroplet diffusion of the organic solvent due to the presence of relatively water-insoluble fatty alcohol molecules.[122] The presence of liquid crystalline structures in the aqueous phase has also been suggested to improve the stability of emulsions.[27]

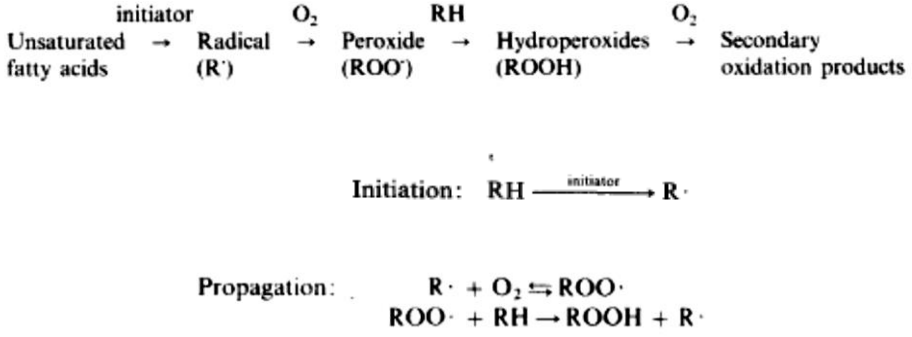
3.1.3 Oxidative stability

Lipid emulsions contain a considerable amount of polyunsaturated fatty acids (PUFAs) which can undergo auto-oxidation and produce lipid hydroperoxides. These products are unstable and are also able to interact with therapeutic agents of lipid emulsions.[123, 124] There are several mechanisms by which the lipid and surfactant components of lipid-based formations can degrade. When unsaturated fatty acids such as oleic or linoleic acids and their esters are used, lipid peroxidation is a primary route of degradation. Memoli et al. showed that vegetable lipids, because of their higher content in PUFAs, were in all cases more rapidly oxidized with respect to the saturated ones.[125] Lipids which contain unsaturated acyl chains are susceptible to autoxidation through an autocatalytic process. The reactivity of such species is partially determined by the number of double bonds in a chain.[125] Linoleic acid and other unsaturated fatty acids are much more prone to oxidation than mono-unsaturated fatty acids such as oleic acid due to the greater resonance stabilisation of the initial radical formed. The relative rates of peroxidation of oleic, linoleic and linolenic acids are 6:64:100 respectively.[126] In the case of soy lecithin (containing 90% PC) a remarkable increase of the initial oxidation index in the lipids was reported after one and two years of storage with respect to the more recently-obtained product even after storing in dry and sealed containers at a maximum temperature of 4 °C.[125]

The lipid oxidation process is free radical-initiated and driven by oxygen; it can be quite complex, as summarized in Figure 3-2. Lipid hydroperoxides are formed as intermediates, which can then decompose to a variety of degradation products, such as shorter chain ketones, alcohols, carboxylic acids, and aldehydes. The oxidation process can be monitored by a number of assays including the iodometric and the

ferric-xylenol orange assay which monitor formation of the lipid hydroperoxides intermediates.[126]

Figure 3-2 Formation of peroxides by auto-oxidation of fatty acids (chain reaction) 8



Pharmaceutical grade refined soybean oil contains a residual amount of natural photosensitisers such as porphyrins. However, refining and bleaching also remove singlet oxygen quenchers such as the carotenoids. The use of suitable packaging and/or containers that absorb energy necessary for the photosensitization, or that prevents such light from reaching the oil would be suitable approach for long term stability.[127] In a dispersed system the molecules of water may generate hydroxyl radicals which can catalyse the initiation stage of the oxidation reaction. Ambrosone et al. explained that the oxidation rate of oil in distilled water is dependent on the surface area to unit volume of the dispersed phase.[128, 129] Further, the oxidation is dependent on the amount of surface active agent in water. This makes the study of lipid oxidation in emulsified systems crucial to understand lipid stability and its impact on the stability of pharmaceutical emulsions.[128]

In the lipid oxidation process, there is usually an induction period before rapid oxidation occurs. The formation rate of hydroperoxides outweighs their rate of decomposition during the initial stage of oxidation. Therefore, the peroxide value (PV) is an essential indicator of the initial stages of oxidative change in pharmaceutical products.[130] Pharmacopoeias defined the upper PV limit of 5 mEq/kg for soybean oil. It is suggested to be an empirical value for quality control and not based on biological or toxicological limits.[131]

3.1.4 pH

pH change due to hydrolysis of lipids in emulsions was observed by Steger et al.[131] The pH drop in the course of lipid hydrolysis was suggested due to the formation

of carboxylic acids.[131] The increase in oxidation index and simultaneous decrease of pH have been found to be correlated linearly.[132] A more rapid oxidation of linoleic acid occurred in acidic pH than at higher pH values.[132] A significant inhibition of the rate of lipid peroxidation by maintaining the pH around neutral value was also noted by Misik and colleagues.[132] They attributed the observed effect to the participation of carboxyl groups in the decomposition of hydroperoxides.[126, 132]

Hydrolysis is another important degradation mechanism for lipids with fatty acids esters. Phospholipid and triglyceride hydrolysis kinetics in emulsions have been reported to be first order and follow the Arrhenius relationship. This is particularly important for lipid emulsions and liposomes, because as hydrolysis of the phospholipids proceeds, the pH will fall.[126, 131] The pH reduction arising from lipid peroxidation and hydrolysis was found to be self-promoting in emulsions and liposome formulations.[126, 133] Overall, changes in pH of emulsions could indicate lipid degradation leading to acidic products through hydrolysis.[131]

3.1.5 Proposed Study

The aim of this study was to optimise the emulsion formulation developed in Chapter 2 based on HLB and order of mixing. A series of sequential studies is planned to improve the emulsification efficiency of the mixed emulsifying system at each stage. Consideration was given to process optimisation and the optimisation of emulsification by rotor mixer before scale up trials. The results of each study were used in the design of each subsequent study.

The final acceptance of an emulsion depended on the quality and stability of the final product. The routine shelf-life determination of emulsions by storing at higher temperature for a period of time may not reflect the realistic shelf life but rather produce irrelevant results. In the present study, the intermediate periods at ambient conditions were intended to evaluate the shelf life of emulsions. There are no specific and sensitive tests or parameters for the detection of instability, which can be used to confidently predict emulsion shelf-life. The most useful parameters to assess the stability of emulsions include drug content, creaming, particle size-flocculation and coalescence process, pH, peroxide value, sterility and phase separation of free oil. The study included four parts as described below:

I. Optimisation of formulation and stability

II. Optimisation of emulsification,

III. Scale- up and stability

IV. Preparation of clinical trial batch and long-term stability at room temperature

3.2 Methodology

3.2.1 Materials

Soybean oil was purchased from Pharma Scope and Sigma Life science, USA (lot# MKBK0322V) for optimisation trials. Refined soybean oil USP (lot # C126935, C153626, C151193) from PCCA Pty Ltd, Australia was used for stability and clinical trial batches. Tween 80® Ph. Eur. and Tween 80® NF were purchased from Fluka Analytical, Germany ((lot# 1259073, 1390440) and Fagron Inc, USA (lot# 23B20-U07-008548) respectively. Epikuron 200® (soy lecithin), containing (>90% phosphatidylcholine, lot# 129047, 199060, 1-7-9027) was either donated or purchased from Cargill GbH, Germany and used as a lipophilic surfactant unless otherwise specified. APF pump spray (Lot # 74199, 76704) with manual crimper was purchased from Ing. Erich Pfeiffer GbH, Germany. Lidocaine (>98%, lot #047K0080, #074k1685, Sigma Life Science, USA), Starch indicator-Vitex (lot #241554, VMR International Ltd, UK), potassium iodide (99%, lot #2477220, Biolab Australia Ltd, Australia), sodium thiosulphate AR (lot #248785, Biolab Australia Ltd, Australia and #A809654710, BDH, UK) were used. Duotest pH strips pH: 7-10 (lot #3050809) and pH: 3-8 (lot #3042709) were purchased from Macherey-Nagel GbH & Co., Germany; Ethanol (95%) was purchased from CSR Distilleries Group. HPLC grade solvents including acetone, dichloromethane (DCM), chloroform, acetonitrile, methanol and iso-propyl alcohol (IPA) were purchased from Fisher Scientific, UK. Viscosity standards N1.0 (lot#10101), N2 (lot#10101), N4 (lot#10101), S6 ((lot#10201) were purchased from Canon Instrument Company, USA. Ultra pure water (< 6 µs) prepared from a Milli-Q purification system was used in all experiments, except for the clinical trial batch which was prepared using Water for Irrigation (WFI) (Lot # G81F0) purchased from Baxter Healthcare Pty Ltd, Australia.

3.2.2 Formulation optimisation

Once the emulsification method and equipment were selected (Sections 2.2.3 and 2.2.4), the formulation was optimised using factorial design. The amount of oil was kept constant at 10% w/w. The hydrophilic and lipophilic surfactant concentrations ranged from 0.1% - 5% w/w as shown in Table 3-1. The method of emulsification was adopted from mixed micelle systems, as described in Chapter 2, (Section 2.2.3.4) The hydrophilic surfactant was weighed and dissolved in approximately 50% of the required quantity of water. The lipophilic surfactant was then added to the aqueous solution containing the hydrophilic surfactant. The aqueous mixture was stirred until all the surfactant was dispersed and produced an aqueous phase containing mixed surfactants. The aqueous mixture was further mixed at 2,000 rpm for 5 min to ensure the all the surfactant was dispersed. The oil phase was then added to the aqueous phase and mixed at 2,000 rpm for 5 min. The coarse emulsion was homogenised at 15,000 rpm for 10 min. The remaining water was added to the emulsion and made up to weight. The final emulsion was mixed at 8,000 rpm for 5 min. The interactions between hydrophilic surfactant and lipophilic co-surfactant were investigated in terms of the effect of mass ratios of Tween 80/Lecithin (Ws/Wco) on the change in droplet size and stability. The stability evaluations of the emulsions were carried out at four time point, namely 7 days, 4 months, 8 months and 13 months.

3.2.3 Process optimisation

Based on the observations from formulation optimisation (Section 3.2.2), approximately 4.5 g of Tween 80 was weighed in a glass beaker and dissolved in approximately 50% of the required quantity of water. Then, 2 g of lecithin was added to the aqueous solution containing the hydrophilic surfactant. The aqueous mixture was stirred until all the surfactant was dispersed and produced an aqueous phase containing mixed surfactants. The aqueous mixture was further mixed at 8,000 rpm to ensure all the surfactant was dispersed. The oil phase (10 % w/w) was then added drop wise to the aqueous phase, **w**hile being mixed at ~10,000 rpm (~10 min). The coarse emulsion was homogenised at 15,000 rpm for 10 min. The remaining water was added to the emulsion and made up to weight. The final emulsion was mixed at 8,000 rpm for 5 min.

Table 3-1 Formulation optimisation study component ratios

Parameters	Form. 1	Form. 2	Form. 3	Form. 4	Form. 5	Form. 6	Form. 7	Form. 8	Form. 9	Form. 10	Form. 11	Form. 12
Lecithin (g/100 ml)	2.0%	2.0%	2.0%	2.0%	2.0%	2.0%	2.0%	0.10%	0.25%	0.50%	1.0%	4.0%
Tween 80 (g/100 ml)	0.10%	0.50%	1.0%	2.0%	3.0%	4.0%	5.0%	2.0%	2.0%	2.0%	2.0%	2.0%
Total Surfactant (g/100 ml)	2.10	2.50	3.00	4.00	5.00	6.00	7.00	2.10	2.25	2.50	3.00	6.00
Surfactant Ratio (Ws/Wco)	0.05	0.25	0.50	1.00	1.50	2.00	2.50	10.00	8.00	4.00	2.00	0.50

3.2.4 Scale up

The optimised formulation was scaled up to 1 litre which is 10 times that of the laboratory batch scale. Overhead rotor mixers from four different manufacturers and in-line mixers from two different manufacturers were selected for scale up trials. A list of equipment, their specifications and process parameters are shown in Table 3-2 and. The method of preparation and formulation were selected from earlier experiments described above in Section 3.2.2 and 3.2.3.

Table 3-2 Details of pilot scale equipment and specifications

Emulsification system	**Over Head Mixer**	**Over Head Mixer**	**Over Head Mixer**	**Over Head Mixer**	**In-line Mixer**	**In-line Mixer**
Equipment name	Dispermat® CA	Silverson® L4R	Diax® 900	Miccra® DFK	Silverson® L5	Miccra® DFK
Manufacturer	VMA Getzmann Germany	Silverson® USA	Heidolph Instrument Germany	ART Prozess Germany	Silverson® UK	ART Prozess Germany
Batch /Continuous	Batch	Batch	Batch	Batch & continuous	Batch & continuous	Batch & continuous
Speed (rpm)	2,000-20,000	3,000-8,000	8,000-26,000	8,800-33,600	3,000-11,000	8,800-33,600
Product Volume (litre)	0.05-10	0.05-12	0.01-5	2-10	0.01-10	2-45

Table 3-3 Process parameters employing the pilot equipment

Equipment	**Speed (rpm) Level 1**	**Speed (rpm) Level 2**	**Sampling Time (min)**
Dispermat® CA	2,000	4,000	5, 10, 20,30
Silverson® L4R	3,000	6,000 (Coarse / Fine screens)	5, 10,20,30,40
Diax® 900	8,000	15,000	5, 10, 20,30
Silverson® L5	3,000	6,000 (Coarse screen)	5, 10, 20,30,40
Miccra® DFK	8,800	8,800	5, 10, 20,30
Miccra® DFK	8,800	11,000	5, 10, 20,30

3.2.4.1 Emulsification by overhead and in-line mixer

The hydrophilic surfactant was weighed and dissolved in approximately 50% of the required quantity of water. The lipophilic surfactant was then added to the aqueous solution containing the hydrophilic surfactant. The aqueous mixture was stirred at speed level 1 using the overhead stirrer until all surfactants were dispersed or dissolved and produced an aqueous phase of mixed surfactants. The oil phase was added to the aqueous phase at a flow rate of approximately 10 ml/minute with continuous homogenisation by the rotor/stator homogeniser and mixed at minimum working speed limit level 1 in Table 3-3.

Once the oil phase was added, two phases were mixed at the working speed limit of level 2 for 30 min, except for the Silverson®. Emulsions prepared with the Siverson® were mixed for up to 40 min since the maximum speed limit was much lower compared with other equipment. Samples were collected at 5 and 10 min and then at 10 min time intervals to investigate droplet size reduction with duration of mixing at level 2. The emulsion was made up to weight and was mixed for 5 min.

3.2.5 Preparation of clinical trial batch and stability evaluation

The optimised formulation (1 litre) containing 3% w/w lidocaine was prepared under an aseptic environment using the Diax® 900 connected with the 25F Tool. The quantity was calculated based on a predetermined number of patients and the number of samples required for quality and stability testing. Tween 80 and lecithin concentrations in the final emulsion were 4.5% and 2% w/w respectively. The amount of soybean oil was increased to 14.5% w/w to aid lidocaine solubility in the oil phase. To ensure the improved droplet distribution, the emulsion was processed at one speed higher (~3500 rpm) than used in the optimisation process. The final product was filled into an APF pump spray (Lot # 74199, 76704) using manual crimper purchased from Ing. Erich Pfeiffer GbH, Germany. The spray bottles were packaged in sterile sleeves (Medipack®, Medipack Medical Packaging Mfg. Co., Taiwan). A total of 95 pump sprays were filled. Spray samples were stored for stability at room temperature (25 °C). A minimum three samples were taken at 0, 3, 6, 12, 18 and 30 months for stability testing. Samples were analysed for droplet size, pH, peroxide value, sterility and lidocaine content. Visual inspections of samples for colour and phase separation were also carried out. Phase separation was

observed for obvious signs of separation of oil on the surface of the emulsion. The emulsion was considered stable when phase separation was not observed or in absence of large oil droplets and the droplet size characterisation was carried out.

3.2.6 Droplet size characterisation of emulsion

The size distribution of the oil droplets was determined by laser light scattering using a Mastersizer 2,000 (Malvern Instruments, UK) attached to a sampling unit Hydro SM (Malvern Instruments, UK). Three measurements were performed for each sample and an average measurement was reported. The relative refractive index (RI) of 1.47 was used for the dispersed phase. The imaginary component of the absorption index for the dispersed phase was taken as 0.001.

1 g of sample was diluted to 100 g with Milli-Q water. If dilute samples appeared milky, 10 g of sample was further diluted to 100 g using Milli-Q water. The required amount of dilute sample was added using a Pasteur pipette to the sampling unit containing 100 ml Milli-Q water whilst being gently stirred and recirculated in the Mastersizer cell. For consistency, the first measurement was carried out within 48 hours after the experiment was completed.

Droplet size distribution was presented as area percentage vs. droplet diameter. During development and optimisation of the emulsions and their characterisation, the mean diameter of the droplets was expressed as an average mean droplet diameter (D(1,0)) (Equation 3-4) and the Sauter diameter, representing a surface average diameter. In general, volume mean diameter (D(4,3)) over represents the existence of larger droplets while the surface mean diameter (D(3,2)) is more associated with smaller droplets. The small droplets do not contribute to the volume as much as large droplets. Droplet size distribution based on volume mean diameter can be misinterpreted especially with a high proportion of small droplets. However, volume diameter is a useful tool for stability evaluation and change in droplet size and/or droplet aggregation, where large droplets are important.

$$D_{10} = \sum n\,d / \sum n \quad \text{.....Equation 3-4}$$

$$D_{32} = \sum n_i\,d_i^3 / \sum n_i d_i^2 \quad \text{.....Equation 3-5}$$

$$D_{43} = \sum n_i\,d_i^4 / \sum n_i d_i^3 \quad \text{.....Equation 3-6}$$

The effect of dilution on droplet size in distilled water was also evaluated by serial dilution. Freshly prepared emulsion samples were diluted 1,000, 2,000 and 5,000

fold. Approximately 100 ml of the diluted sample was used for analysis. Samples were gently stirred and recirculated in the Mastersizer cell for 1 minute before analysis, to avoid entrapment of air in the Mastersizer cell.

3.2.7 Creaming

Each emulsion sample was poured into a 100 ml graduated cylinder with a closure cap immediately after preparation and was left standing for 48 hours to settle. Observation of creaming of the internal phase was made at room temperature at 24 hours and 48 hours. The volumes of the creamed phase and the remaining emulsion were recorded. The creaming volume (*Vcream*) was defined in this study as the relative difference in volume of the emulsion (*Vemulsion*) and the volume of the creamed phase (*Vcreaming*). The value of percent creaming was calculated for each emulsion using the following equation 3-7.[53, 79]

$$V_{cream} = \frac{V_{creaming}}{V_{emulsion}} \times 100\% \quad \text{.....Equation 3-7}$$

3.2.8 Refractive index

The refractive index of oil and Tween 80 solution were measured using the MISCO PA202 digital refractometer (MISCO Refractometer, USA). The refractometer had a working range from 1.3330 to 1.5040 nD.

3.2.9 Microscopic observation

Microscopic evaluation was used to identify the shape and structure of the emulsion droplets including precipitation of insoluble material. A 1 g sample of emulsion was diluted to 100 g using Milli-Q water. One drop of a diluted sample was placed on 1 mm thick and 26 x 76 mm Menzel microscope slides with ground edges 90° (ISO Norm 8037/I). The drop of emulsion was covered with 0.13-0.17mm thick, 22 x 22 mm cover-slips made of transparent hydrolytic class 1 glass. Care was taken to avoid entrapment of air-bubbles. To avoid distortion of globules the cover slip was simply placed on the drop. The edges of the cover slips were sealed with wax to avoid evaporation. Microscopic analysis was carried out with optical microscopes including phase contrast, dark field and fluorescence.

A light microscope: Olympus BX41 phase contrast (Olympus Corporation, Japan) and Nikon Alphaphot2 bright-field (Nikon, Japan) microscopes were used. Both

microscopes were attached with 10x eyepiece with coaxial focusing mechanism for 10x, 20x, 40x, 100x (oil immersion) stage objectives. Total maximum magnification of 1,000x was produced by both microscopes. The phase contrast microscope was attached with a DP20-5E (Olympus Corporation, Japan) microscope digital camera.

The Olympus IX51 fluorescence microscope (Olympus Corporation, Japan) and DP71 camera (Olympus Corporation, Japan) with triple-band excitation filters was used. The microscope was attached with 4x, 10x, 20x and 40x stage objectives. DP controller software was used to set up the microscope parameters including sensitivity, magnification and background brightness. Fluorescein sodium as hydrophilic dye and Rhodamine or Oil Red O were used as lipophilic dyes.

3.2.10 Viscosity

Viscosity was measured by an A&D SV10 vibrational viscometer (A&D Company, Limited, Tokyo, Japan). SV10 works on a vibrational frequency of 30Hz with measurement range from 0.3 mPas to 10 Pas (±1%) at 10-40 ºC. WinCT-Viscosity (RsVisco) software was used to import the measurements of viscosity and temperature from the viscometer to a computer automatically.

The viscometer was calibrated using a 2-point calibration with Cannon® General Purpose Viscosity Standards N1 and S6 (Table 3-4). Viscosity measurements were made at room temperature (23-25 ºC). The viscosity standards listed in Table 3-4Table 2-8 were used for calibration before running the samples. The viscosity measurements were carried out 48 hours after the preparation. A total of 5 measurements were taken for each sample.

Table 3-4 Cannon® General Purpose Viscosity Standards

Viscosity Standard	Approximate Dynamic Viscosity in mPas	
	20 °C	25 °C
N1.0	0.92	0.85
N2	2.2	2.00
N4	5.4	4.65
S6	9.4	7.86

3.2.11 Surface and interfacial tension

Interfacial tension (IT) and surface tension (ST) were measured by a KSV Siga703 (Biolin Scientific, Finland) force tensiometer using a Du Nouy Ring method. The equipment was calibrated during set up and every 6 months thereafter. The measurements were carried out as per manufacturer's instruction immediately after preparation, by adjusting the display value of surface tension to "ZERO" with taring knob while the ring was completely above the surface of liquid.

3.2.11.1 Surface tension

Approximately 30 ml of sample was poured gently in to the measurement bowl. Care was taken to avoid formation of air bubbles on the surface. The ring was first immersed within the sample by slowly lifting the stage. The stage was slowly lowered until the ring broke from the liquid. The reading (display) immediately after the ring broke from the liquid was noted as the surface tension.

3.2.11.2 Interfacial tension

The aqueous phase was poured in to the measurement bowl. The oil or oil phase was added slowly using a Pasteur pipette. Approximately 15 ml of aqueous phase and 15 ml of oil phase were used. Samples were allowed to stand for a minute before immersing the ring in to solution. The ring was wetted with aqueous phase before being immersed in the sample. The measurement was carried out as described above by slowly lowering the ring until the ring broke from the aqueous phase but remained submerged in oil phase.

3.2.12 HPLC assay of lidocaine

A HPLC method was developed using a reversed phase Alltech Apollo C18 (lot #0601000526), 5 µm, 4.6mm x 50 mm column and a Waters HPLC system. The HPLC system was comprised of a Water 717 plus autosampler, Waters 2487 dual wavelength UV detector, Waters 1525 binary pump. The autosampler was controlled by Breeze™ software. The injection volume was 20 µl. The mobile phase was 60 % 25 mM phosphoric acid and 40% Methanol (MeOH). The flow rate was 1 ml/min. The detection wavelength was set at 210 nm.

3.2.12.1 Method validation

A lidocaine standard was prepared in 0.1 M HCl. The concentration range for standard solutions was 10 mg/l to 100 mg/l. The nominal concentration for the analysis was targeted to be 60 mg /l. 10 - 100 mg/l corresponds to 20 - 200 % of the nominal assay concentration. Linearity was measured over the range 10 - 100 mg/l, from replicate analysis of standards containing 10.00, 20.00, 40.00, 60.00, 80.00, 100.00 mg/l. Linearity was calculated by regression curves (concentration vs peak areaon at least three separate occasions. Acceptance criteria for linearity was r^2 >0.95 for each calibration curve. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated using calibration curve methods according to ICH Q2 (R1) recommendations.

$$LOD = \frac{3.3\sigma}{S} \quad \text{.....Equation 3-8}$$

$$LOQ = \frac{10\sigma}{S} \quad \text{.....Equation 3-9}$$

Where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve. The calculated limits were not validated by the analysis of a suitable number of standards at the concentration of detection or quantitation limit.

The concentration for the analysis was targeted to be 60 mg/l. Repeatability was evaluated by calculating the relative standard deviation (RSD) of the peak areas of six replicate injections for the targeted concentration of 60 mg/l. The acceptance limit of repeatability was set as a %RSD value within 2%. The recovery was measured over the range 50 - 70 mg/l, corresponding, to 80 - 120 % of 60 mg/l. Accuracy was measured by spiking the placebo emulsion and DCM at a concentration of ~60 mg/l and taken through the sample preparation procedure. Recovered concentrations were calculated for each preparation. The acceptance criterion for accuracy was 98-102 %.

3.2.12.2 Sample preparation

A 0.5 g of sample of emulsion was dissolved in IPA and the volume was made up to 25 ml with IPA. 2 ml of the sample was pipetted into a 15 ml screw capped test tube containing 4 ml of DCM. The sample in the test tube was shaken gently by inversion before 4 ml of 0.1 M HCl was added. The sample was again mixed gently

for 5 mins on a test tube rocker (Vari-Mix, Thermo Fisher Scientific, USA) followed by centrifugation for 5 mins at 1500 rpm. The aqueous upper layer was collected into a 20 ml volumetric flask. Extraction by 0.1 M HCl was carried out 3 times for total volume of 12 ml. The final volume of 20 ml was made with 0.1 M HCl and 20 µl of the sample was injected onto the HPLC column.

3.2.13 Peroxide value

The peroxide value was determined using the standardised method A from the British Pharmacopoeia.[134] Approximately, 2.50 g of the emulsion was placed in a 250 ml conical flask fitted with a ground-glass stopper. A 30 ml mixture of 2 volumes of chloroform and 3 volumes of glacial acetic acid was added and the flask shaken until the emulsion was dissolved. Then 0.5 ml of saturated potassium iodide solution was added and the flask was shaken again for exactly 1 min before 30 ml of water was added. Then 0.01 M sodium thiosulphate was titrated into the solution slowly with continuous vigorous shaking until the yellow colour was almost discharged. At that point 5 ml of starch solution was added and the titration continued, shaking vigorously, until the colour was discharged. The volume of 0.01 M sodium thiosulphate (n_1 ml) used was noted. A blank test, without sample, was then carried out under the same conditions and volume of 0.01 M sodium thiosulphate used was noted (n_2 ml). An acceptance criterion for titration was the volume of 0.01 M sodium thiosulphate used in the blank titration must not exceed 0.1 ml.

$$I_p = \frac{10(n_1 - n_2)}{m} \quad \text{.....Equation 3-10}$$

Where, Ip is peroxide value in mEq/Kg, m is weight of the sample.

3.2.14 pH

pH change was measured by a digital pH meter (Hanna Instrument, Portugal). The pH meter was calibrated using standard buffer solutions (pH 4 and 7, from Hurst Scientific Pty Ltd, Australia) before measuring emulsion samples. pH was also confirmed by Duotest pH litmus strips in addition to the digital pH meter.

3.2.15 Sterility test

The sterility testing was carried out by the PathWest laboratory at Princess Margaret Hospital. The method below is an extract from the validation document issued by the PathWest laboratory.

Half the contents was transferred into one bottle of Fluid thioglycollate medium (Medium 1) and the remainder to one bottle of Soya-bean casein digest medium (Medium 2). Both bottles containing medium 1 and 2 were shaken vigorously for 1 minute to distribute the sample throughout the media. Media 1 and 2 were incubated at 32 ºC and 23 ºC respectively for 14 days. Each test medium was examined daily, excluding weekends, for microbial growth. If turbidity, precipitate or other evidence of microbial growth appeared, the samples were analysed microscopically by Gram stain. The suspect media for single colonies using appropriate microbiological methods were subcultured. When subculture was necessary the plates were incubated for up to 7 days before discarding as no growth to identify the genus level or any organisms isolated. Identity of isolates was recorded in order to detect a pattern of recurring contaminants in the product.

3.3 Results and Discussion

3.3.1 Formulation optimisation

The formulation optimisation study was carried out using 12 different combinations of Tween 80 and lecithin in a concentration range of 0.1 to 5% w/w. Both Tween 80 and lecithin were essential in the formation of emulsions. No direct effect of Tween 80 was observed on the mean droplet size D(3,2), except for the emulsion containing 0.1% w/w Tween 80 (Table 3-5 and Figure 3-3). Mean droplet diameters D(3,2) were between 2.5 and 3.5 μm for all batches. These observations were not in agreement with earlier reports of a decrease in droplet size with an increase in Tween 80 and other high HLB surfactants.[52, 53] The study carried out by Lunderg et al. dispersed/ dissolved both hydrophilic and lipophilic surfactants in the oil phase before emulsification.[52] Krishna et al. also reported a reduction in the droplet size when the hydrophilic surfactants were added to the oil phase.[53] However, Krishna et al. reported an increase in the droplet size when the hydrophilic surfactant was added to the aqueous phase. Results from the optimisation study also suggested that the formation of a successful emulsion required a minimum quantity of the hydrophilic surfactant (Tween 80) (Table 3-5). This could be due to the fact that lecithin alone may not be capable of producing an o/w emulsion, as it is too lipophilic.[64] However, the spontaneous curvature of phospholipid can be attained by the presence of hydrophilic surfactant at different mass ratios.[50]

In relation to lecithin, an increase in concentration had a major effect on droplet size. The reduction in droplet size reached a maximum when lecithin concentration was increased to 2% w/w (Figure 3-4 and Table 3-5) and reached a plateau at 4% w/w (Figure 3-4 and Table 3-5). Levy et al. and Krishna et al. reported a similar decrease in the droplet size as the concentration of lecithin was increased.[72, 135] Krishna et al. further suggested that the minimum concentration of lecithin required for the preparation of the emulsion was 3% w/w, when lecithin was used as a primary emulsifier.[135] Non-polar oils may penetrate and introduce disorder into the lattice to form spherical micelles enclosed with oil and emulsion, which could induce droplet size reduction of emulsions.[136]

Table 3-5 Effect of the concentration ratio of Tween 80 to lecithin on emulsion properties

Surfactants Ratio (Ws/Wco)	Particle size (µm)			Surface tension (ST) of Tween 80 solution	Surface tension (ST) of Lecithin Dispersion	Viscosity
	Mean (1,0)	Mean (3,2)	Mean (4,3)	(mNm)	(mNm)	cPs
0.05	-	-	-	45.60	45.30	1.62
0.25	0.74	2.97	13.60	44.00	43.50	1.59
0.50	0.71	2.38	8.60	44.70	45.20	1.70
0.50^	0.67	2.80	10.43	43.00	44.00	1.93
1.00	0.72	2.80	11.07	44.00	44.30	1.70
1.50	0.70	3.28	12.45	42.90	48.30	1.79
2.00	0.70	2.90	10.44	43.10	49.40	1.84
2.00^	0.81	4.24	15.27	43.50	45.60	1.40
2.50	0.74	3.32	11.52	45.30	46.80	1.84
4.00^	0.74	3.50	27.68	44.00	45.30	1.32
8.00^	0.80	3.68	16.87	44.00	43.80	1.32
20.00^	0.79	4.26	15.69	44.00	42.60	1.28

^ Emulsion containing 2% w/w Tween 80 with varied amount of lecithin

Figure 3-3 **Effect of hydrophilic surfactant (Tween 80) on mean droplet diameter D(3,2)**

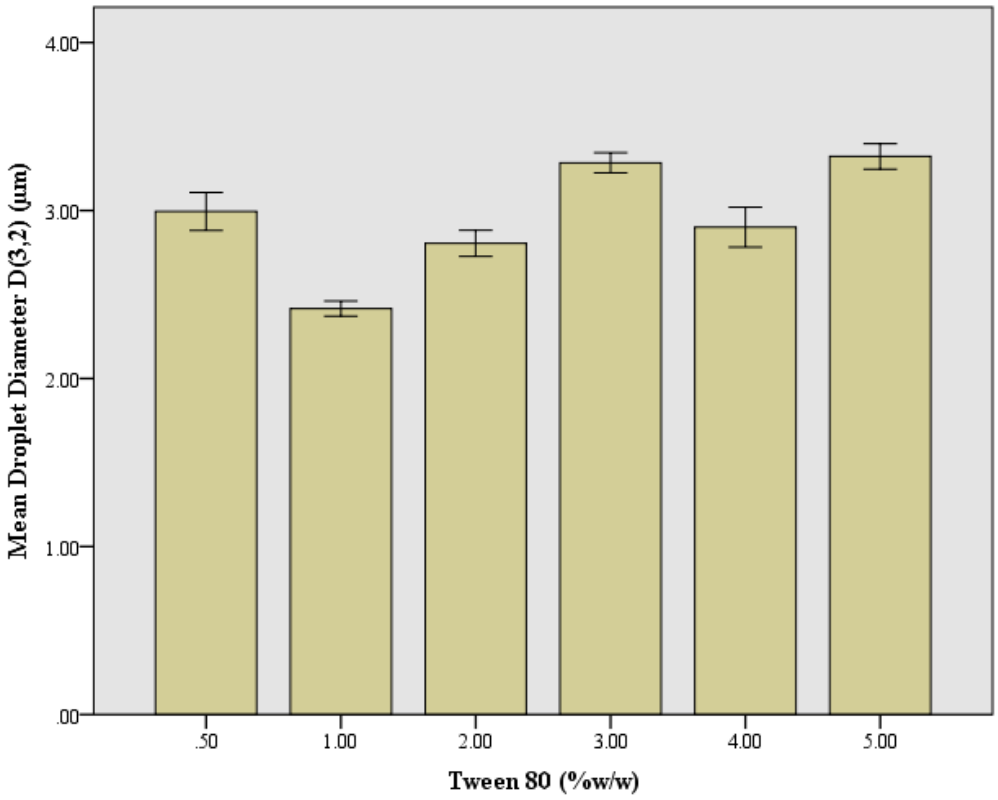


Figure 3-4 **Effect of lipophilic co-surfactant (Lecithin) on mean droplet diameters D(3,2) and D(4,3)**

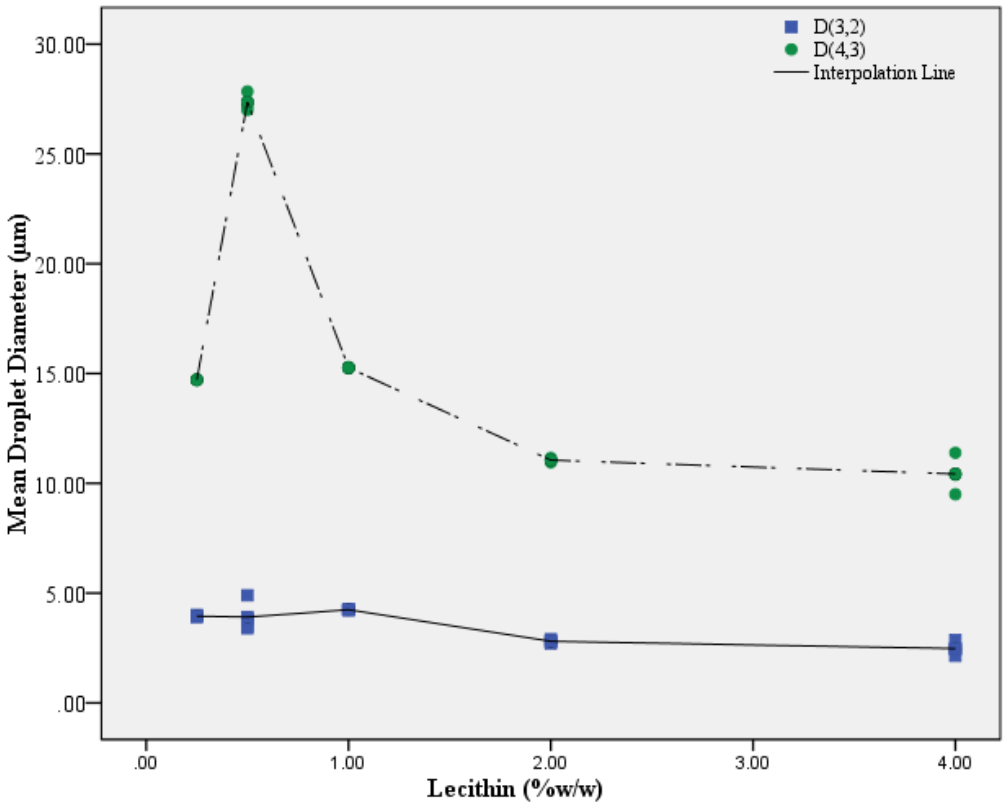
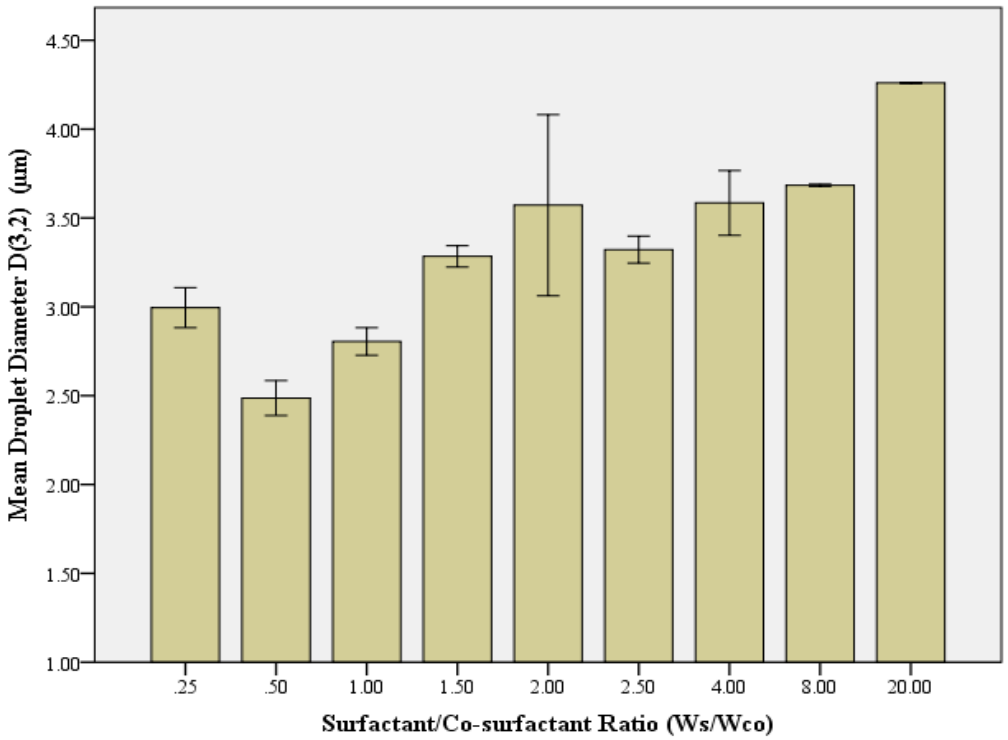


Figure 3-5 Effect of surfactant and co-surfactant ratio on mean droplet diameter D(3,2)



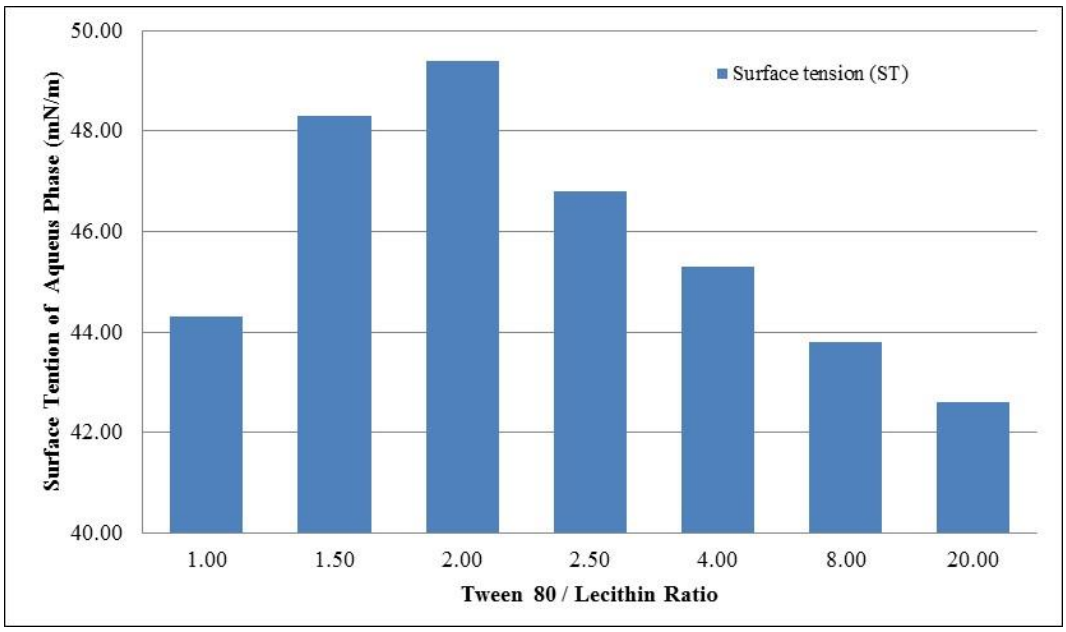
When the effect of the Ws/Wco on the mean droplet size D(3,2) was evaluated (Figure 3-5), it was found that droplet size was increased with an increase in the Ws/Wco ratio. The above results indicated the formation of lecithin vesicles with an increase in the size of vesicles with Ws/Wco to a much larger size. Edwards et al. reported the increased size of lecithin vesicles in the presence of non-ionic surfactants.[72] Lim and Lawrence specifically studied the aggregation behaviour of mixtures of Tweens and PC. They reported the formation of larger vesicles in the presence of Tween and suggested possible incorporation of Tween into the vesicle bilayers.[92] Lecithin has been well recognised for its ability to form different types of structures in water including liquid crystal, lamellar phase, micelles, mixed micelles and vesicles depending upon the concentration used and the type of surfactant present.[13, 27, 137] Kirikou et al. reported that the ratio of hydrophilic and lipophilic surfactant had a noticeable effect on the viscoelastic properties so that the viscoelasticity increased with increase in the ratio of hydrophilic to lipophilic surfactant.[138] On the other hand, droplets were disrupted by viscous and turbulent forces in a rotor-stator homogeniser.[104] Increase in viscoelasticity may have reduced the effect of turbulence on droplet break up. This might not have an impact on larger droplets. However, the smaller droplets could not be formed, where viscous stresses on the droplet are important for droplet breakage.[104] This may result in a larger

mean droplet diameter. Reduction in droplet size also increases the free energy as per Equation 3.1. Therefore, smaller droplets are more difficult to breakdown at the same applied force.

3.3.1.1 Effect of surfactants on surface tension and viscosity

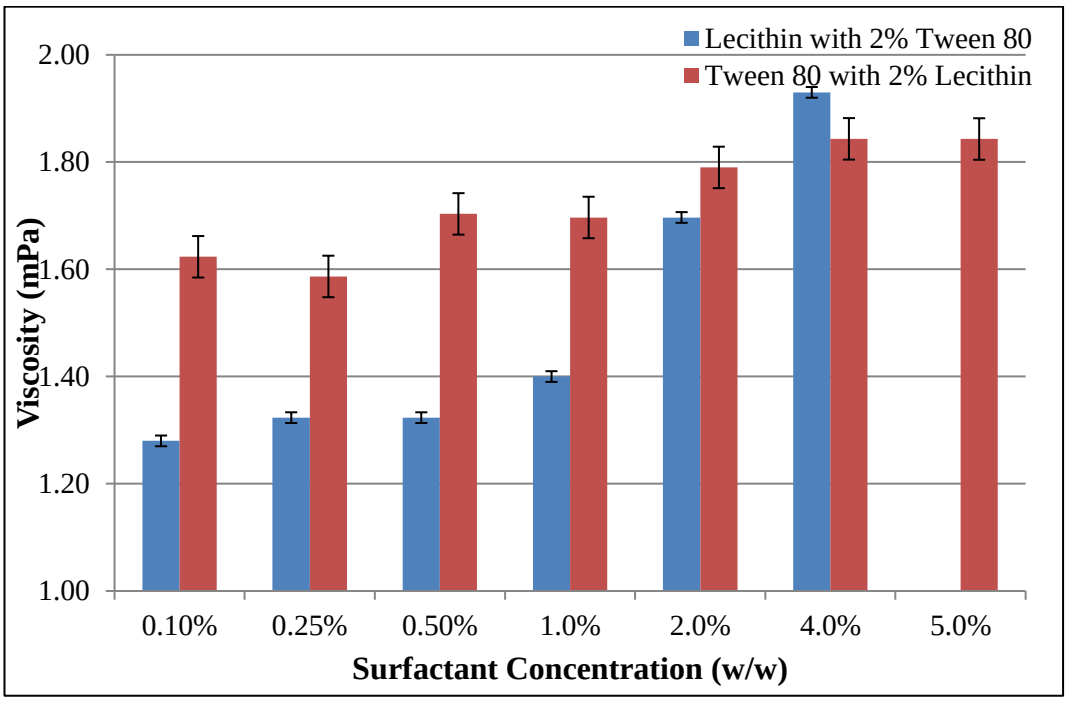
The Tween 80 concentration appeared to have no influence on the surface tension of aqueous solutions as expected for concentrations above the cmc of 15 mg/l.[139] All results were within the range of standard deviation of 3 mNm and between 42 mNm to 45 mNm. However, with the addition of lecithin the surface tension increased at first and reached a maximum of 49.40 mNm at the concentration ratio Ws/Wco of 2 (w/w) and again reduced with an increase in the concentration ratio (Figure 3-6). These results indicated the possible interactions between Tween 80 and lecithin, which was in agreement with the interaction between the blend of lipophilic and hydrophilic surfactants described in the study by Boyd et al.[38] The decrease in surface tension was apparent when the Ws/Wco ratio was increased beyond 2 (w/w). The influence of Ws/Wco on surface tension and interfacial tension due to adsorption of lipophilic surfactant and formation of a mixed monolayer needs further investigation of surface properties and possible monolayer structure formed by mixed emulsifier systems using more advanced equipment such as Langmuir trough and freeze fracture TEM to derive a plausible understanding of these interactions.

Figure 3-6 Effect of surfactant ratio of surface tension (mNm) of aqueous phase



Effect of Tween 80 concentration on viscosity was measured when lecithin concentration was held constant at 2% w/w. There was some impact of Tween 80- on viscosity of the emulsion (Figure 3-7). In particular, an increase in the amount lecithin, at constant Tween 80 concentration, apparently showed monotonic increase in the viscosity of the emulsions. The effect of lecithin on viscosity was much more pronounced when compared with Tween 80. This increase in viscosity was reflected in the droplets size increase when compared with similar Ws/Wco ratio of 2 (Table 3-5). The effect of Tween 80 concentration may be indirect and possibly correlated with the interaction between Tween 80 and lecithin at the interface and increased viscoelasticity. These results are in agreement with Moreno et al., who suggested that lecithin had more influence on the viscosity and rheological behaviour of microemulsions, While increased concentration of Tween 80 had a secondary effect on viscosity.[64]

Figure 3-7 Effect of individual surfactant concentration on viscosity*



3.3.1.2 Effect of surfactant concentration on stability

Emulsions prepared with different Ws/Wco were stored for 400 days at ambient temperature (21 ± 2 ºC). The change in droplet size, creaming and phase separation were observed at 7, 126, 260 and 400 days. There was a marked effect of surfactant and co-surfactant concentrations and Ws/Wco ratio on the overall stability of

emulsions. Creaming volume (Vcream) was found to be independent of the concentration of surfactants or ratio and varied between 25-36% (Table 3-6).

Emulsions containing 0.5% or less of either of the surfactants were found to be unstable and a separate oil layer was observed within seven days of the preparation (Table 3-1 and Table 3-6). These emulsions were discarded with no further analysis. Emulsions containing >0.5% w/w of Tween 80 and with the Ws/Wco ratio between 0.5-4 (w/w) were found to be stable. When, emulsions were prepared at a Ws/Wco ratio of 4 (w/w) or more, phase separation was observed within 4 months. This may be an effect of a reduced amount of lecithin (0.5% w/w or less). However, a separate study (Appendix 7-8) confirmed that the Ws/Wco of 3 (w/w) or more produced unstable emulsions, even at a high lecithin concentration of (1% w/w). On the other hand, if the lecithin concentration was increased to 4% or more, the stability of the emulsion was again decreased (Table 3-6 and Figure 3-8). This confirmed that lecithin played a critical role in the formation and the stability of emulsions and a minimum concentration of Tween 80 (>0.5% w/w) was required to form a stable emulsion.

Figure 3-8 **Effect of surfactant/co-surfactant ratio on stability (the bars relates to emulsions remained stable at that time point)**

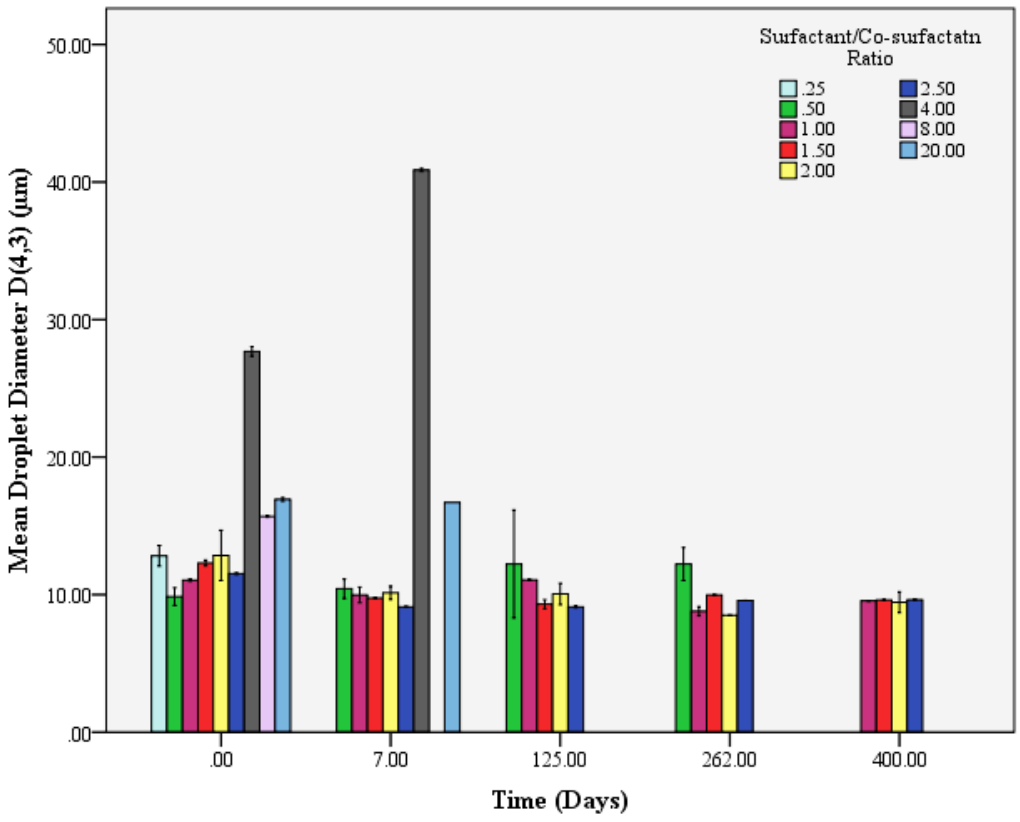


Table 3-6 **Creaming volume (%) of optimisation trials at ambient temperate for 400 days (- represent emulsions with phase separation)**

Surfactants Ratio (Ws/Wco)	% Creaming volume over 13 months (in days) (µm)				
	0.00	7.00	125.00	260.00	400.00
0.05	-	-	-	-	-
0.25	13.60	13.60	-	-	-
0.50	8.78	10.20	9.83	11.01	10.04
0.50	10.43	10.36	19.21	14.53	-
1.00	11.07	9.86	9.77	8.79	9.55
1.50	12.45	9.75	9.23	9.98	9.61
2.00	10.44	9.67	8.49	8.50	8.90
2.00	15.27	10.79	11.66	^-	10.97
2.50	11.52	9.12	9.12	9.57	9.63
4.00	27.68	40.88	-	-	-
8.00	15.69	-	-	-	-
20.00	16.87	-	-	-	-

3.3.1.3 Microscopic analysis

Earlier reports suggested that the possible formation of either mixed micelles or a lamellar phase may contribute to the stability of the emulsions.[27, 140] To investigate the presence of micellar or lamellar forms of the mixed emulsifiers system, which could possibly have contributed to emulsion formation and stability, different microscopic techniques, were used including scanning electron microscopy (SEM), confocal microscopy with Raman spectroscopy (CRM), phase contrast, dark field, fluorescence, confocal, and. SEM required dried samples and removal of water from emulsions during sample preparations caused coalescence of droplets and ultimately emulsion break-up. Confocal microscope and CRM were not suitable due to the small droplet size and Brownian motion of droplets. However, both types of microscopes showed some interesting possibilities when emulsions were prepared at low rpm. CRM showed the presence of Tween 80 at the outer layer of the droplets (data not shown). However, lecithin spectra in the presence of water were similar to those obtained by using soy bean oil and these were not distinguishable. Both techniques required further development and optimisation of the sample preparation, which was beyond of the scope for the proposed work.

Bright field, phase contrast and fluorescence microscopy (Figure 3-9, Figure 3-10 and Figure 3-11) were used after small changes in the emulsification method. An emulsion containing dyes (Figure 3-10) was prepared using a magnetic stirrer to ensure that droplets were large enough to be visible at 400x -1000x. Figure 3-9 shows the multilamellar vesicle structure formed by lecithin in the presence of Tween 80. Friberg et al. reported formation of liquid crystals of lecithin when dispersed in the aqueous phase prior to emulsification. The formation of multilamellar vesicles in the presence of non-ionic surfactant was also reported by Lim and Lawrence.[92]

Figure 3-10 and Figure 3-11 show the multilamellar vesicles containing oil. It had been reported earlier that formation of swollen lamellar structures correlates with increased stability of emulsions.[63] Friberg et al. also reported that the presence of a layered lamellar structure had a significant influence on the reduction of coalescence.[27] The formation of vesicles could possibly explain the instability due to the increased concentration ratio of hydrophilic surfactant and lipophilic surfactant. The increase in hydrophilic surfactant may have disrupted the lipid layers of vesicles and possible rupture of the lipid layers over a period of time.[72]

Figure 3-9 Photomicrograph of Tween 80- Lecithin dispersion by Olympus BX41 (1000x)

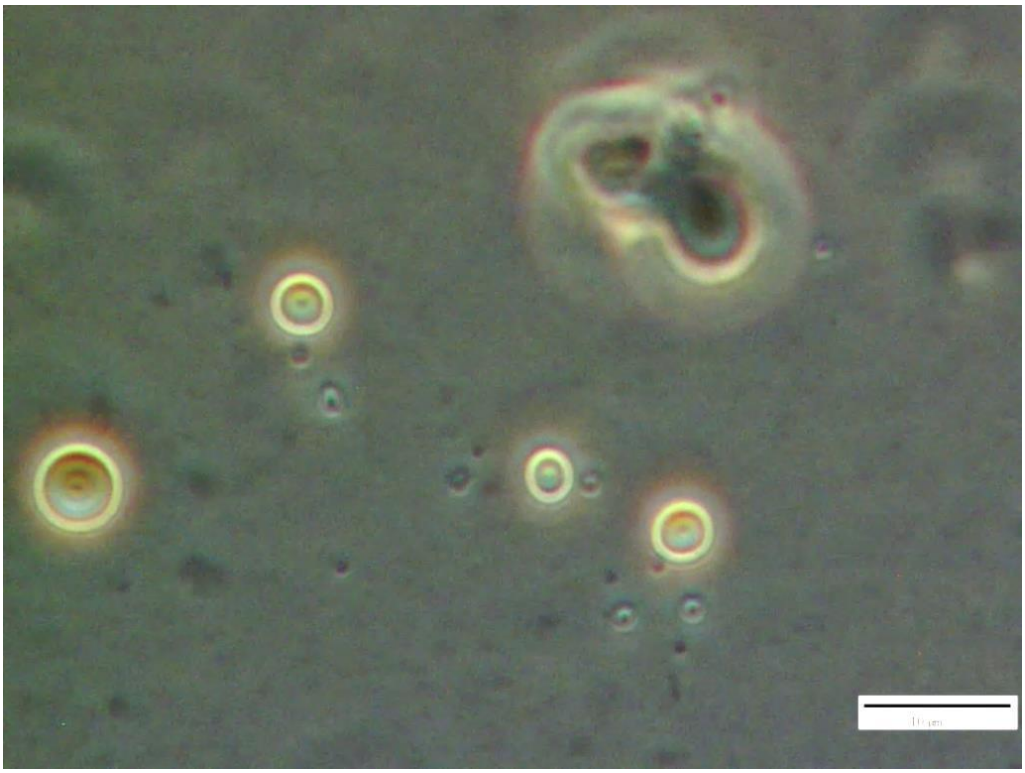


Figure 3-10 Photomicrograph of coarse emulsion containing lipophilic and hydrophilic dye using Olympus IX51 fluorescence microscope (400x)

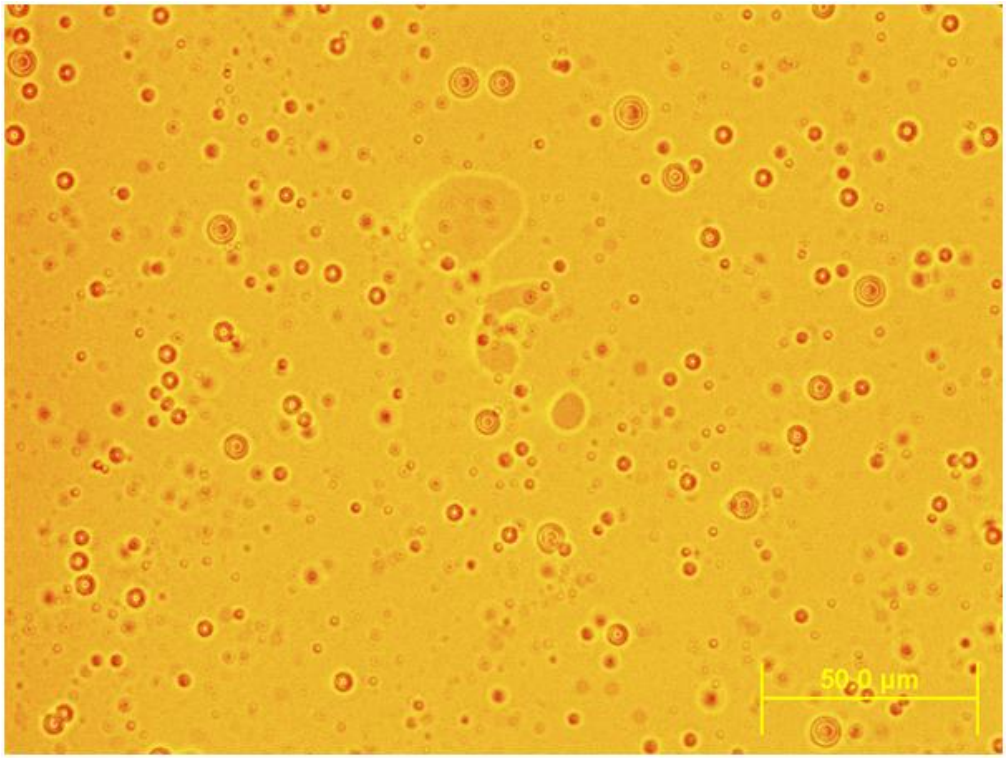
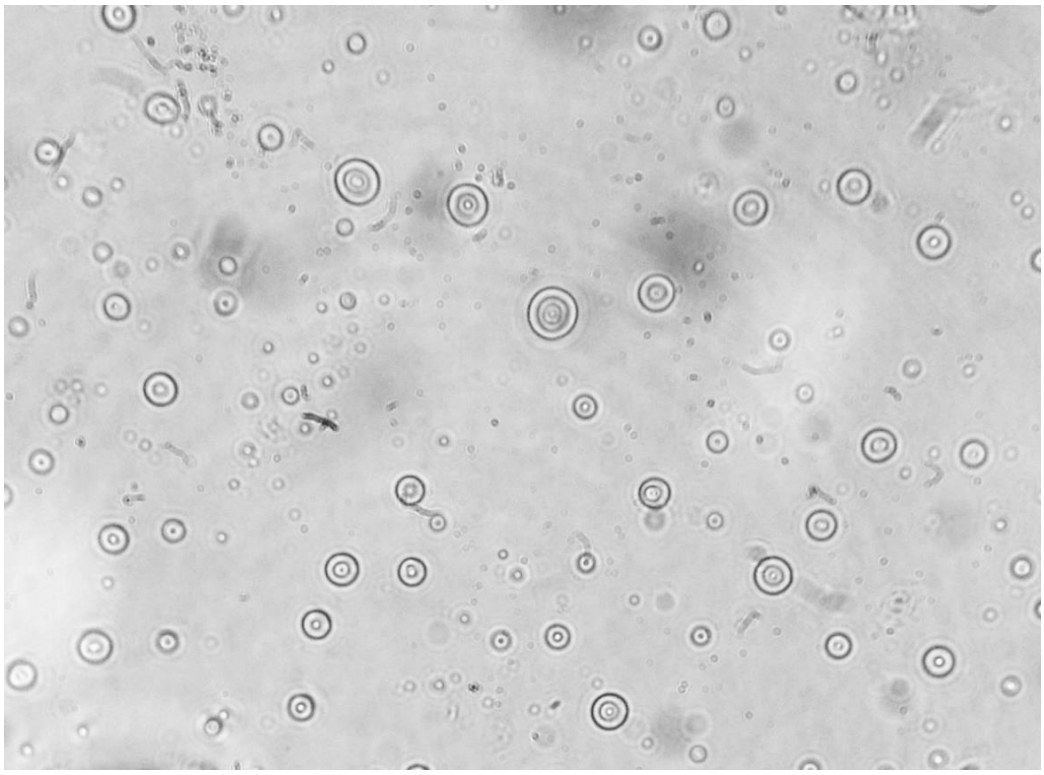


Figure 3-11 Photomicrograph of emulsion in bright-field microscope



Photograph was taken using Nikon Alphaphot2 bright-field (Nikon, Japan) microscope attached with 10x eyepiece and 100x (oil immersion) stage objectives. Total magnifications of ~1,000X. However, iPhone4S camera was used and magnification could not be calculated.

3.3.2 Process optimisation

To achieve a surface mean diameter D(3,2) less than 2 µm, the oil phase was added drop-wise during homogenisation at 8,000 rpm. Based on observations in formulation optimisation Section 3.3.1.2, the final formulation, containing 4.5% w/w Tween 80, 2% w/w Lecithin and 10% w/w soybean oil was selected. When oil phase was added slowly close to the rotor during mixing at 8,000 rpm, the D(3,2) was reduced from 1.55 µm. As expected, droplet breakup occurs close to the agitator[99] and the number of larger droplets were reduced considerably (Figure 3-12), which is evident from the apparent reduction in D(4,3) in Table 3-7. These findings suggested that the presence of dispersed phase in close proximity of the agitator and with enough energy to deform the droplets, smaller droplet size could be achieved. At the same time, the processing time was reduced to 10 min to achieve D(3,2) < 2 µm, which was three times less than that of reported earlier in Section 2.3.4.1.

Figure 3-12 Overlay Surface Mean distribution D(3,2) of droplet size (µm) (in % frequency): Effect of dropwise addition of oil phase on droplet size

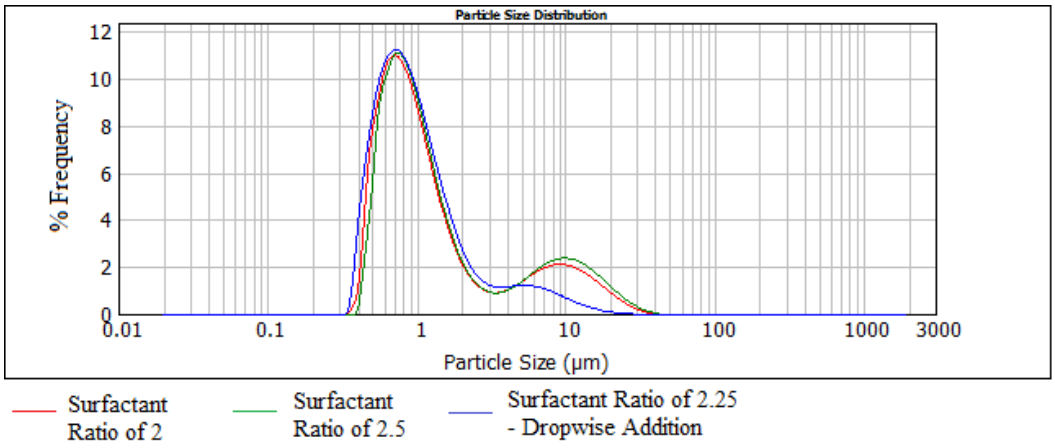


Table 3-7 Effect of drop-wise addition of oil phase on droplet size comparison

Surfactants Ratio (Ws/Wco)	Particle size (µm)		
	Mean (1,0)	Mean (3,2)	Mean (4,3)
2.00	0.70	2.90	10.44
2.50	0.74	3.32	11.52
2.25*	0.66	1.55	4.56

* Emulsion prepared by drop-wise addition of oil phase

3.3.3 Scale-up of emulsion

Scale up was carried out using in-line and overhead high speed mixers, similar to the rotor-stator type. Overhead mixers from four different manufacturers were tested. Mixer design played an important role in the emulsification process. Rotor-stator type mixers produced a large amount of foam especially Dispermat® CA from VMA Getzman (Figure 3-13). The formation of foam was reduced in the Diax® 900 by adjusting the immersion height of the stator mixer. Surprisingly, the Silverson® L4R did not produce foam during emulsification. The formation of the foam affected the emulsification process, which was reflected in the mean diameter of the emulsion (Table 3-8). The efficiency of the rotor stator for droplet break up was at the maximum near the impeller and was dependent on the shear rate produced.[98, 141] The foam production reduced the efficiency of emulsification; possibly by reducing the turbulence on the surface of the liquid and impeding the oil droplets to pass through the rotor. The smallest droplet size of emulsion was produced using Diax® 900 for 20 min and could be related to the higher rotor speed.[98]

Figure 3-13 Formation of foam during emulsification comparison of overhead mixers

VMA Getzman -Dispermat® CA

Silverson® L4R

Table 3-8 Comparison of D(3,2) for overhead mixers at different mixing time

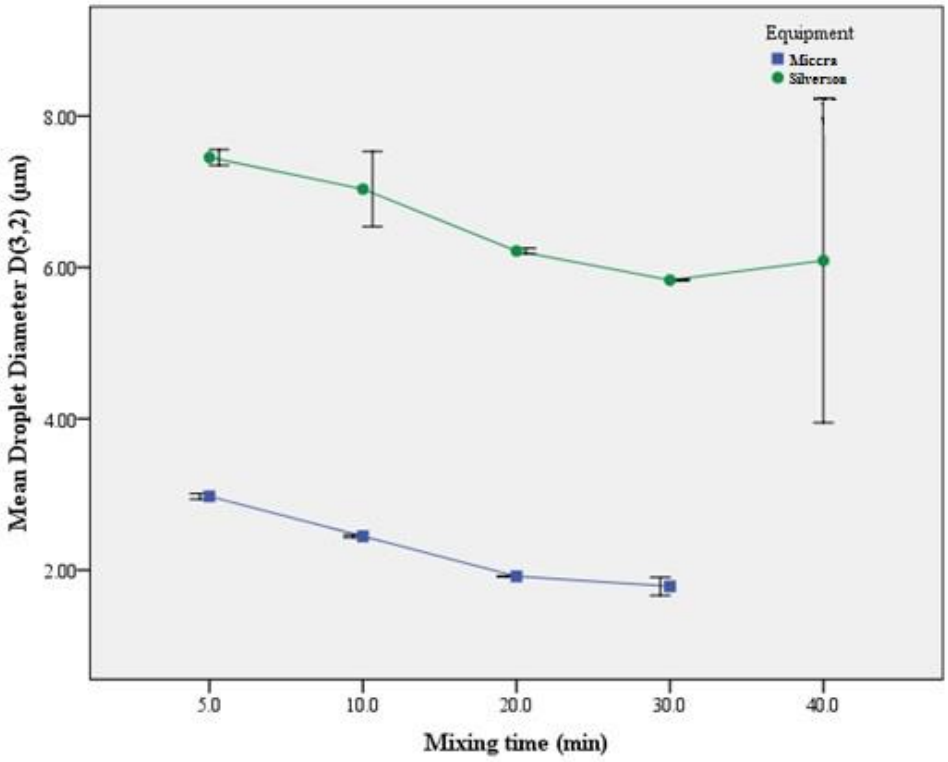
Mixing time (min)	Overhead Mixer -Mean Droplet Diameter D(3,2) (µm)				
	VMA Getzman®	Silverson®-Coarse	Silverson®-Fine	Miccra®	Diax® 900
5.0	9.82	5.49	4.20	3.95	1.91
10.0	9.29	5.39	3.92	1.96	1.54
20.0	5.20	4.50	1.75	-	1.46
30.0	-	3.69	2.54	-	-
40.0	-	3.30	1.59	-	-

The stator geometry was also critical in droplet size reduction. The fine screen attached to the stator was found to be more efficient in reducing droplet size compared with the coarse one. These results were not in agreement with an observation from Maa and Hsu.[142] They reported that to achieve minimum droplet size, a micro tip was less efficient compared with a macro tip. Rodgers and Cooke reported a reduction in droplet size when the screen over the shaft was added, which is consistent with the above results. The difference in observation by Maa and Hsu could be explained due to the flow rate produced by the micro tip which would be less than the macro tip. Also, the shear rate is proportional to tip speed and agitator diameter. The diameter of the agitator was twice the size for the macro-tip, when compared with the micro-tip. In the current study (Table 3-8 and Figure 3-14), there was no difference in the size of the shaft/agitator diameter and the only difference was the size and type of holes on the screen attached.

Figure 3-14 Screens used on Silverson® overhead mixer

Coarse Screen Fine Screen

Figure 3-15 Effect of in-line mixers and processing time on droplet size (Mean D(3,2) ± SEM)



Both the Miccra® and Silverson® in-line assemblies were used to prepare the emulsions. There was no foam produced when in-line mixers were used. Figure 3-15 shows the comparison of D(3,2) values for emulsions prepared by the Miccra® and Silverson® in-line mixers. A marked difference was observed in the droplet size of the emulsions produced by these different devices. This difference was associated with the rotation speed of the mixers.[98, 100] The difference in the droplet size could only be used for mere comparison due to difference in the equipment setup.

The oil phase was added in the vessel containing the aqueous phase, while the aqueous phase was passed through the mixer. There was no overhead stirrer used to aid the mixing of both phases in the vessel, and the mixing ability of the mixer was entirely dependent on the flow rate of the mixer. The flow rate of the mixing was directly related to the geometry and speed of the impeller.[99, 143] The geometry of the vessels was also different for both mixers.

As expected from earlier observations in Chapter 2 Section 2.3.4.1, the droplet size was reduced with mixing time for almost all mixers. Except for one unexpected reading at 20 minutes, there is a monotonous decrease in droplet size. The reason

behind unexpected increase in droplet size could be sample size. Increased residence time of droplets, obtained by increasing the mixing time or the number of cycles would reduce the droplet size till an effective equilibrium of droplet size was reached.[99]

3.3.4 HPLC validation

Retention time for lidocaine was 3.5 min. The relationship between peak area vs concentration curve in the 10 mg/l to 100mg/l was linear (R^2 = 0.9999, Table 3-9). Concentration of lidocaine for assay was calculated using Equation 3-11 derived from calibration line.

Table 3-9 Lidocaine linearity data

Concentration (mg/L)	Mean AUC (Area)	Stdev	RSD
10	778393	5079.05	0.65
20	1540993	3721.05	0.24
40	3060019	13565.38	0.44
60	4542735	32084.11	0.70
80	6068414	23467.81	0.39
100	7483952	41707.53	0.56

$$Area = (74727x\ mg\ /\ Litre) + 51538 \quad \text{.....Equation 3-11}$$

Repeatability of the method was evaluated by calculating the RSD of the peak area of six replicate injections for the standard concentration (60 mg/l) of lidocaine, which was found to be 0.62% (Table 3-10). Furthermore, the RSD of the peak area of the recovery range (50-70 mg/l) analysed in the accuracy study for each level was calculated, and it was found to be less than 2.0% for each level (Table 3-11 and Table 3-12).

Table 3-10 Repeatability

Injection No.	Area
1	4525907
2	4577504
3	4551676
4	4508037
5	4510391
6	4510460
Mean area	4530662.5
RSD	0.62%

Table 3-11 Recovery of lidocaine (50-70 mg/l) from placebo

Added (mg/l)	Measured (mg/l)	Recovery (%)	RSD %
50	49.65	99.29	0.25
60	60.5	100.85	0.15
70	71.5	102.14	0.05
Mean		100.76	0.15

Table 3-12 Recovery of lidocaine from different matrix used during extraction process

Standard	DCM/IPA	0.1 M HCl	IPA	Placebo
100 ± 2	98.83 ± 0.055	99.98 ± 0.22	100.05 ± 0.44	100.76 ± 0.15

Recovery was measured using placebo samples spiked with lidocaine corresponding to 80-120 % of 60 mg/l and analysed using a standard dilution method. No unusual peaks were detected. The lidocaine peak gave identical peak shape and retention time to that of a standard solution. Recovery was excellent (Table 3-11), with mean recovery of 100.76% of added lidocaine, over the range 50 to 70 mg/l. Lidocaine selectivity was carried out by degradation under stress conditions (acid and base hydrolysis and oxidation at 40 °C), to show that lidocaine is separated from degradation products. However, lidocaine was stable and degradation products were not detected. Stability of lidocaine solutions in different pH range and temperature was reported earlier. Lidocaine was found to be fairly stable within the pH range of this study. Results were in agreement with earlier reports. N,N-dimethyl aniline had been identified as a major degradation product of lidocaine.[144] To make sure that the degradation products were separated, a standard solution (60 mg/l) was spiked with N,N-dimethyl aniline. N,N-dimethyl aniline was identified at 2.8 minute. Soybean Oil and DCM peaks after the extraction process have also been identified at 4.3 and 6.30 min respectively.

3.3.5 Stability of Clinical Trial Batch

At first, analysis of the product was carried out by an external laboratory since it was registered under good laboratory practice (GLP). However, the results provided by the laboratory were not satisfactory due to low precision. When questioned, it was found that the project was considered as research based and method was not fully validated for recovery before analysis. Subsequently (at 3 months), all

analyses were carried out in our laboratory; the stability study data were based on the analyses carried out from 3 months onwards.

The emulsion was stored in amber coloured spray bottles. Visual inspection of the emulsion was carried out after gently pouring the emulsion into a clear glass vial and letting it settle for 2-3 hours. There was no sign of oil droplets or creaming reported once the emulsion was settled. The emulsion was found to be physically stable at around 30 months. There were no apparent changes in mean droplet diameter D(4,3) over a period of 30 months (Table 3-14). There was a reduction of less than 600 nm . However, there was also a small but consistent reduction in droplet numbers over 10 µm. The effect of the reduction in larger droplets can be seen when analysed as D(4,3). The reduction in droplets numbers larger than 10 µm size could suggest the chemical degradation of lecithin and triglycerides. Autoxidation and hydrolysis of the phospholipids and fatty acid produce free fatty acids and reduces the pH of the emulsions.[126, 133, 145, 146] Hydrolysis and autoxidation are pH dependent and synergistic.[126] The chemical degradation of lipids in the emulsion was evident by the decrease in pH and increase in peroxide value (Table 3-14). Autoxidation was found to be more controlled than expected. Higher emulsifier concentrations and the presence of Tweens have been reported to help reduce the peroxidation of PC.[147]

Table 3-13 **Droplet size distribution of clinical trial batch at 3 months**

Distribution Moments	Size (µm)
D(1,0)	0.61
D(3,2)	1.37
D(4,3)	3.24

Figure 3-16 **Surface Mean distribution D(3,2) of droplet size (µm) (in % frequency) at 3 months**

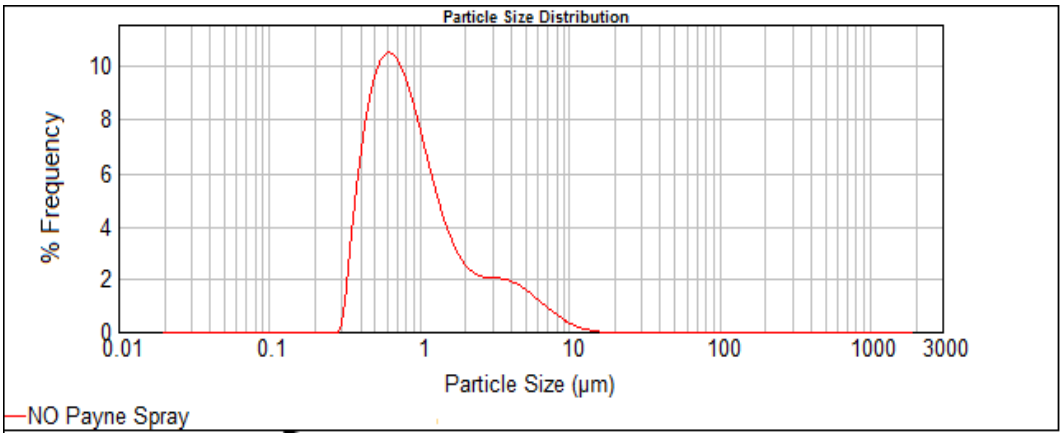


Table 3-14 **Physicochemical characterisation and stability of emulsion containing a nominal concentration of 3% w/w lidocaine for 30 months**

Test	3 Months	6 Months	9 Months	13 Months	18 Months	30 Months
Lidocaine (% w/w)	2.99 ± 0.07	2.89 ± 0.04	2.81 ± 0.01	2.81 ± 0.05	2.74 ± 0.05	2.64 ± 0.04
Assay (%)	99.7 ± 1.1	96.3 ± 1.2	93.7 ± 0.3	94.7 ± 1.3	91.3 ± 1.7	88.00 ± 1.2
Mean D(4,3)	3.24	3.55	3.21	3.21	3.10	3.28
pH	8.5 ± 0.1	8.65 ± 0.09	8.0 ± 0.1	7.75 ± 0.2	7.45 ± 0.4	7.2 ± 0.4
Peroxide Value (mEq/kg)	-	3.8 ± 0.6	2 ± 0.6	2.8 ± 1.5	1.5 ± 0.5	1.9 ± 0.4
Sterility	Pass	Pass	Pass	Pass	Pass	Pass

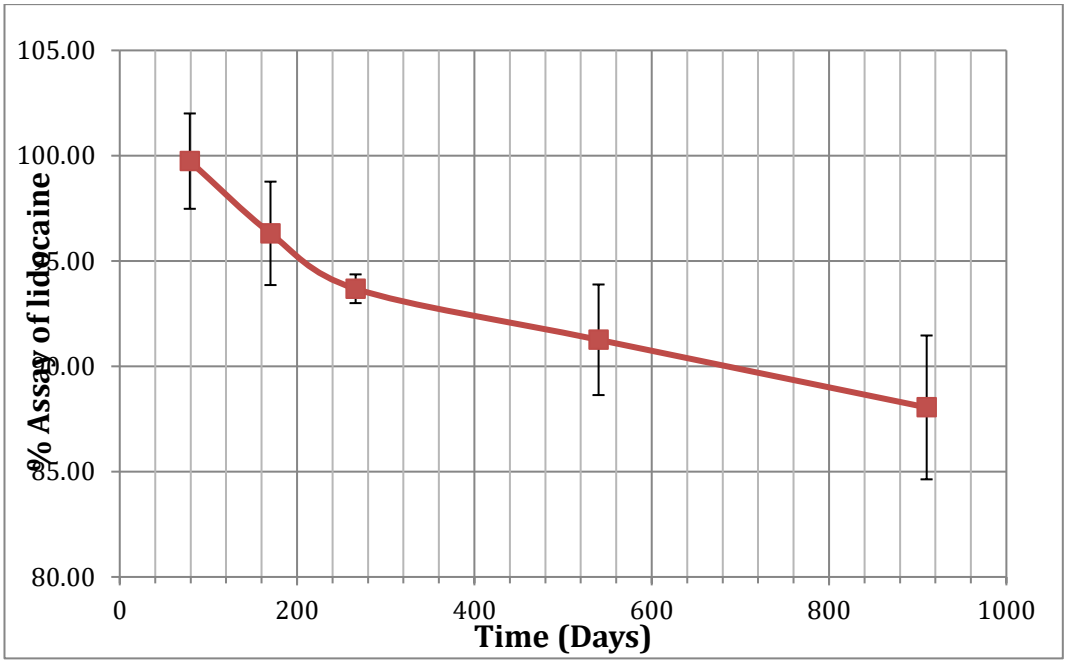
The hydrolysis and oxidation did not have an apparent influence on the physicochemical stability of the emulsion. It is possible that the multilayers around the emulsion droplets retarded the diffusion of oil molecules from the droplets to the surrounding aqueous phase and subsequently the droplet instability either by Ostwald ripening or by coalescence.[27]

The shelf-life of a drug product at the end of 30 months was mainly due to degradation of lidocaine. The major pathway of lidocaine degradation has been identified as hydrolysis in an aqueous medium.[144] The pKa of lidocaine is around 7.9.[148] Due to the hydrolysis of lipids, the pH of aqueous phase was reduced and at around 13 months the pH was less than that of the pKa of lidocaine. Lidocaine may have been released from the lipid phase in to slightly acidic aqueous phase with increasing protonation and solubility in the aqueous medium as the pH falls. The hydrolysis of phospholipid produces lyso-PC. The increasing concentration of lyso-PC may increase the bilayer fluidity and permeability.[149] The bilayers of multilamellar structure could have been destabilised due to hydrolysis and consequently lidocaine released in the aqueous phase. Wong-ekkabut et al. also suggested that presence of oxidised lipids increased the membrane permeability of water.[145]

Lidocaine degradation appeared to show zero order reaction (Figure 3-17). The reaction rate was found to be pH dependent. From kinetics, the reaction rate was essentially constant above pH 8.5.[144] Analysis of pH profile suggested that when

pH falls from 8.5 to 7.5, there would be almost a 40% reduction in the degradation rate.[144] In addition, the proportion of protonated species, which is much more aqueous soluble, also forms as pH falls below pKa 7.9. However it would appear that the stability was more controlled by reaction rate rather than the formation of the cationic species. Based on interpolation and experimental error, the shelf life concluded was approximately 680 ± 15 days (Figure 3-17).

Figure 3-17 Concentration (n=6 ± 2SD) vs time profile, degradation of lidocaine



3.4 Conclusion

The lipophilic surfactant concentration was important in the formation and the stability of the emulsion. The mixed emulsifier system of hydrophilic surfactant (Tween 80) and lipophilic surfactant (lecithin) formed multi-lamellar structures containing oil. The stability of the emulsion was improved by the multi-lamellar structure. The ratio of hydrophilic and lipophilic surfactants (Ws/Wco) was critical to the stability of the emulsions

Drop-wise or slow addition of oil phase in the aqueous phase during mixing reduced the number of larger droplets. The rotation speed of the mixer was the most important factor in emulsification and reduction of droplet size. Mixer design was important in the formation of the emulsion and droplet size reduction. Production of foam hindered the emulsification process and reduction of droplet size. In-line mixers were found to be efficient and did not produce foam. The current study also recognized that the setup of mixers was important and the best outcome could be achieved by continuous mixing, where oil phase is gradually added close to the rotor/stator work head.

An HPLC analysis method was suitable for the assay of the emulsion containing lidocaine and the stability study. Linearity was excellent, over the range 10 to 100 mg/l of lidocaine with RSD less than 1%. Mean recovery of lidocaine was 100.2 % from spiked placebo over the range 50 to 70 mg / litre.

The stability study at ambient temperature (25 °C) showed that the droplet size of the emulsion was stable over the period of 30 months. There was a small increase in peroxide value and notable pH reduction. However, over the entire product shelf-life, peroxidation and hydrolysis of lipids were within an acceptable range. Stability of lidocaine had pharmaceutically acceptable shelf-life of 680 ± 15 days.

4 A Comparison of Emulsion and an Aqueous Lidocaine Formulations for Topical - Analgesia

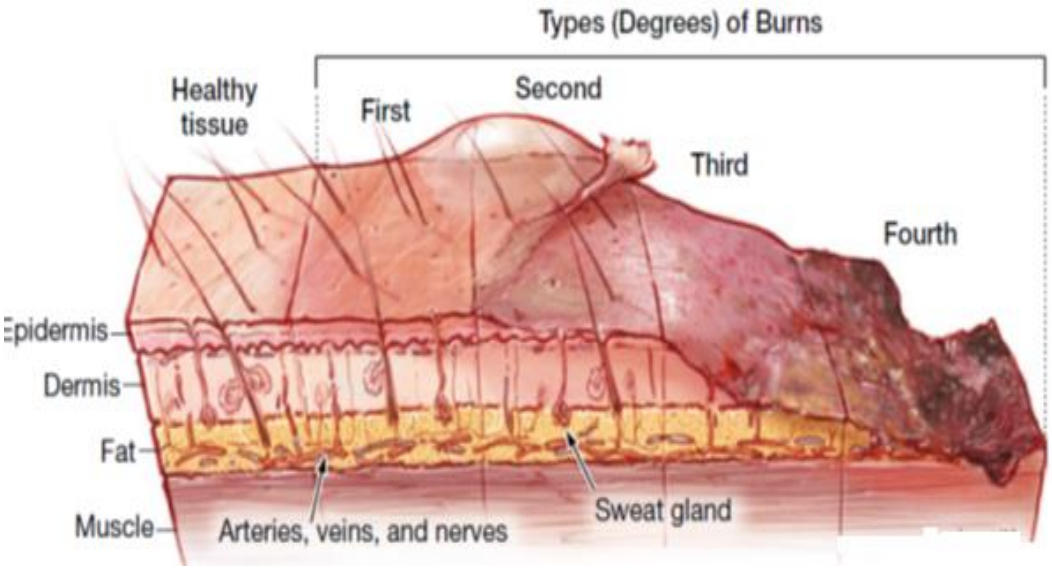
4.1 Introduction

4.1.1 Burns

A burn is damage to body tissues caused by heat, chemicals, electricity, sunlight or radiation. Scalds from hot liquids and steam, building fires and flammable liquids and gases are the most common causes of burns.[150] Burn injuries are amongst the most painful injuries affecting the human body. In the United States each year, approximately 1.25 to 2 million people are treated for burn injury and around 50,000-80,000 are hospitalized.[151, 152] Young children and elderly patients are at increasing risk of a burn injury.[152] A burn injury is classified on the basis of the extent and depth of the injury. There are three types of burns.[150, 153]

- First-degree (superficial) burns damage only the outer layer of skin
- Second-degree (partial-thickness) burns damage the outer layer and the layer underneath
- Third-degree (full-thickness) burns damage or destroy the deepest layer of skin and tissues underneath
- Fourth-degree burns extend into muscle below the skin

Figure 4-1 Pictorial presentation for type of Burns (from Torpy JM et al.)[150]



Burn treatments are dependent on the type of burn, and the amount of body tissue involved.[150] Burn treatments include fluids, pain management, surgical debridement, intensive care, and skin grafting.[153] Burns also can lead to infections because they damage the skin's protective barrier.[152] Burn dressings fall into three categories: A. Conventional, B. Biological and C. Synthetic.[153] The best cover for

a wound is skin itself, and plastic surgery makes this possible, by means of a split-thickness skin graft. Other biological dressings are listed below [153]

- Allograft Skin: Obtained from a family member or other living volunteer
- Amniotic membrane: the innermost layer of the placenta consisting of a thick basement membrane and an avascular stromal matrix,
- Xenograft: obtained from an animal of a species different from recipient
- Tissue derived type dressing such as collagen

4.1.1.1 Types of pain in burns patient

Pain caused by burn injuries and burn treatment is one of the most difficult types of suffering and has been reported as the worst pain imaginable. Burn pain is directly associated with the injury itself.[154] The immediate pain following burns is due to the stimulation of the skin nociceptors located in the epidermis and dermis.[155] The severity and nature of pain related to a burn injury is influenced by the depth of burn, stage of healing and procedures.[156] Patient characteristics such as age, sex, psychological trauma and ethnic origin have some influence on the pain related to burns.[154] Patients with a superficial burn will have pain and erythema. Partial thickness second-degree burns will cause pain because the dermis and epidermis are damaged and nerve fibres are exposed.[156]

Types of pain in burn patients:[154, 157-159]

1. Background pain: is defined as steady-state pain from wounds including steady state anxiety related to illness and immobility. It is constant and dull in nature, and often managed by an opioid regimen to maintain steady-state serum levels.
2. Procedural pain: is defined as increased pain and anxiety related to dressing changes, line insertions, physical therapy or other procedures. It is shorter in duration but much greater in intensity, occurs when procedures, e.g. dressing changes, wound cleaning, debridement and joint motion exercises are being carried out.
3. Breakthrough pain: is generally associated with incompletely controlled resting pain and is of shorter duration.

The pain from repeated therapeutic procedures is described as the most extreme and one of the most difficult to treat due to its intensity, variability and the frequent co-existence of pre-existing opioid tolerance.[160, 161] Pain from skin debridement and

grafting procedures may influence the development of psychiatric disorders and depression.[160] High levels of anxiety experienced by burn patients put them at risk for reduced pain tolerance.[160] The highest level of anxiety is usually experienced around pain producing procedures such as dressing changes and physiotherapy treatments.[154] A common clinical observation in patients experiencing postoperative pain after skin grafting procedures is that the split skin donor site is substantially more painful than the grafted site.[162] Psychological factors are also important contributors to a patient's appreciation of, and response to, pain. Many factors such as personality of the patients, presence of depression and severity of the injury may all increase the pain that the patient experiences.[158] Strong correlations have been found between pain, psychological distress, and physical as well as psychological outcomes in burn-injured children and adults. Anxiety and distress during burn dressing changes, have been found to be significantly related to overall and worst pain.[162] Burn-injured patients with high anxiety also tended to report more background and procedural pain.[162]

4.1.1.2 Procedural pain management

Procedural pain is the most intense and most likely type of burn injury pain to be undertreated. Patients describe procedural pain as having an intense burning and stinging quality and can induce excruciating, throbbing pain, thought to be caused by pressure associated with venous distension in inflamed, oedematous tissue.[158] Pain follows a surgical wound due to the stimulation of skin nociceptors. Following a partial skin grafted wound, nerve endings remain open and intact, which triggers pain throughout the time and course of the healing.[163] Primary hyperalgesia is caused by a prompt and intense inflammatory response of the chemical mediators. These mediators sensitise the active nociceptors at the site of injury to mechanical and chemical stimuli such as touch, rubbing and antiseptics or other topical applications.[158, 164] Wound debridement, dressing changes, and strenuous physiotherapy and occupational therapy that require manipulation of already inflamed tissue may contribute to increased pain and inflammation in burn wounds.[159] This may continue for min to hours after dressing changes and physiotherapy have ended.[160]

Procedural pain is a multidimensional experience that frequently induces significant anxiety and distress.[162] It is critical to aggressively manage pain and anxiety for the first dressing change. If the first dressing change evokes extreme anxiety and emotional distress it may lead to long-term pain management problems. High levels of anxiety experienced by burn patients put them at risk for reduced pain tolerance.[155 159] To address the patient's anxiety, the use of anxiolytic agents along with opioid agents is advocated during burn treatments.[154] Currently narcotics such as morphine, pethidine and fentanyl are common forms of analgesic therapy used for these patients. Even with high dosages of opioids, however, burn pain often remains quite severe because narcotic requirements are increased in burned patients.[156]

Because procedural pain is very intense, but of relatively short duration, most commonly used analgesic regimens are comprised of moderately-to highly potent opioids that have short duration of action.[159] The pharmacokinetic parameters of opioids in burn patients have been studied and the results are often inconsistent, reporting a decrease in the volume of distribution and clearance and an increase in the terminal elimination half-life of morphine.[165] In addition, the requirements increase over time so that even high doses of opioids may not totally relieve the pain in some patients. This makes procedural burn pain one of the most difficult forms of acute pain to treat, due to its intensity, variability and the frequent co-existence of pre-existing opioid tolerance.[161]

The more potent opioids may provide levels of sedation beyond that of mere analgesia. Larger or more potent doses of opioids or the concurrent use of anxiolytic sedatives may produce more pronounced sedation, respiratory depression or loss of consciousness.[159] While other patients, particularly those with a history of alcohol or drug dependence, may not respond to doses within the recommended safety and efficacy range.[159]

Sedative hypnotics such as ketamine and propofol significantly reduce pain and associated anxiety and distress during burn dressing change procedures. However, their use is usually limited to the critical care setting under supervision of an anaesthetist.[162] Ketamine has been reported for the management of burn dressing changes, producing prolonged sedation that interferes with the patient's ability to

resume oral intake.[165] Ketamine, however, seems to be a good alternative for opioid for pain management of major burn patients.[158]

Other non-opioid based approaches have recently become popular, due to the realization that narcotics may be underused by clinical staff in an effort to reduce side effects.[160] However, their use for the treatment of burn pain is currently limited. Topical application of NSAIDs on burn wounds can theoretically inhibit pain signals at the injury site with minimal systemic uptake, and does result in significant analgesia.[159] Nitrous oxide with oxygen has been used effectively for analgesia during burn wound dressing changes within an operating theatre.[159, 165] Nitrous oxide is less useful with critically ill or uncooperative patients. It is also difficult to administer outside of an operating room.[165] Nitrous oxide along with opioids, may induce profound respiratory depression.[165] It has been suggested that prolonged exposure to nitrous oxide for patients or staff may be toxic.[159]

Topical application of local anaesthetics to intact skin and surgical wounds, as a method of reducing pain has previously been evaluated.[166-168] Research studies suggest that local anaesthetics can be used safely to decrease donor site pain in the immediate postoperative period. Low blood levels and the absence of clinical signs of toxicity have provided evidence of its safety.[166, 169] Topical aerosol application when applied intra-operatively to donor sites before application of an occlusive dressing has been found to reduce opioid requirements in patients.[162] Topical local anaesthetic agents are attractive as tools for pain management because of their potential to act as both analgesic and anti-inflammatory agents without the side effects associated with opioids.[161 165] EMLA application to mucous membranes or open wounds is contradicted because of the risk of prilocaine toxicity and subsequent methaemoglobinaemia.[160]

4.1.2 Local anaesthetics

For centuries, safe and reversible anaesthesia in parts of the body has been investigated. However, a controlled manner of producing local anaesthesia was not reported until 1884.[170] Cocaine was the first local anaesthetic, discovered in 1860.[171] Unfortunately, the use of cocaine was limited by its side effects, which included corneal opacification, systemic toxicity and addiction.[170, 171] Discovery of the benzoic acid ester structure of cocaine was a significant milestone in the

development of the benzocaine, procaine, and tetracaine.[172] Procaine was introduced in 1905 and became the first local anaesthetic to gain wide acceptance.[171] The synthesis of lidocaine by Lofgren in 1943 was a major step, allowing the development of a new series of local anaesthetics. These anaesthetics have been noteworthy for their low incidence of sensitising reactions.[170] Today, lidocaine is the most widely used agent, but all local anaesthetics have comparable efficacy. They differ in potency and several pharmacokinetic parameters that account for differences in the onset and duration of anaesthesia.[15,173]

4.1.2.1 Chemistry and pharmacokinetics of local anaesthetics

Local anaesthetics are weak bases with a lipophilic region and a hydrophilic region. Typically constructed of three important components: an aromatic ring, an intermediate length ester or amide linkage, and a tertiary amine.[170] The amide group includes the most commonly used local anaesthetics such as lidocaine, prilocaine, and ropivacaine. The ester group includes cocaine, procaine, and tetracaine.[15, 173, 174]

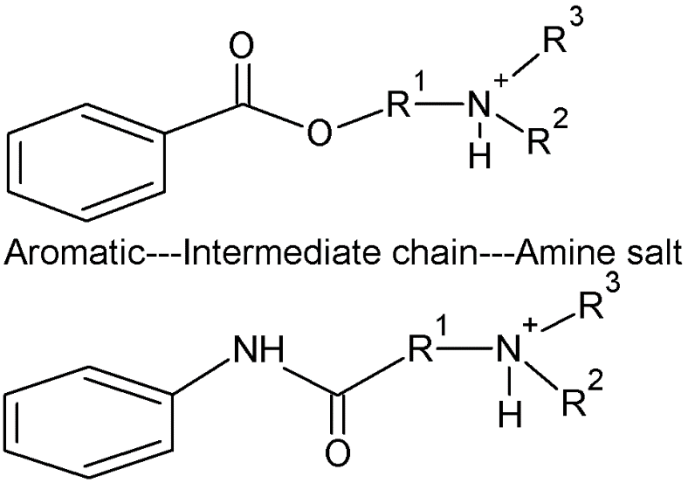
- Aromatic Ring:

The aromatic ring improves the lipid solubility of the compound, which can be enhanced further by aliphatic substitutions.[170] The increase in lipophilicity allows diffusion across the nerve cell membrane and determines the intrinsic potency of local anaesthetics.[174] Enhancing the molecular weight of a compound will enhance the potency and duration of action to a certain point. Beyond this maximum, a larger molecular weight will diminish activity.[170] Greater lipid solubility enhances onset of action by enabling faster diffusion through sheaths as well as the neural membranes of individual axons comprising a nerve trunk.[15]

- Intermediate Linkage:

The ester-type local anaesthetics are quickly hydrolysed by plasma esterase to their primary metabolite, para amino benzoic acid. Amide-type local anaesthetics, on the other hand, undergo metabolism in the liver.[15, 174]

Figure 4-2 General Structure of local anaesthetic agents [170]



- Amine salt

A secondary or tertiary amine (Figure 4-2) existing as a combination of uncharged molecules and positively charged ammonium cations is responsible for the molecule's lipophilicity, metabolism and ability to traverse the membranes.[170, 174] Local anaesthetics also vary in their lipid solubility, degree of protein binding, and dissociation constant (pKa).[175] The polar amine end is hydrophilic and responsible for water solubility and protein binding, and allows the chemical to be marketed in a salt form. This permits access to a stable compound that is easily and safely stored, solubilised, sterilised, and administered.[176] Local anaesthetics bind to alpha-1-acid glycoprotein. The variation in protein binding correlates with their affinity for protein within sodium channels and is associated with the duration of the neural blockade.[15, 174] The higher the percentage of protein binding the longer the local anaesthetics action.[15]

By manipulating these three major portions of the molecule, modifications of the lipid/water distribution coefficient (pKa), protein-binding characteristics, rapidity of onset, potency, and the duration of action can be obtained.[170] Both the aromatic and amine portions determine protein-binding characteristics, which are felt to be the primary determinants of anaesthesia duration.[173]

Because local anaesthetics are weak bases they can be solubilised and stabilised as strong conjugate acidic hydrochloride salts (pH 3-6).[172] As such, the molecules exist in a quaternary, water soluble state at the time of injection. However this form will not penetrate the neuron.[177] The lipid soluble free base crosses the axolemma and

reionizes in the acidic axoplasm to the active moiety, which blocks the sodium ionophore from within the cell or from the membrane lipid bilayer. Therefore, the non-ionized form promotes delivery into the axon and the ionized state provides activity.[172] The time of onset of local anaesthesia is therefore predicated on the proportion of molecules that convert to the tertiary, lipid-soluble structure when exposed to physiologic pH (7.4).[15, 177] The pKa of all local anaesthetics is > 7.4 and therefore a greater proportion of the molecules exist in water-soluble form when injected into tissue.[15] The different potency and several pharmacokinetic parameters account for differences in the onset, duration of anaesthesia and toxicity.[174] Toxicity developing immediately after injection is indicative of rapid absorption.[176] The maximum safe dose of local anaesthetic agents administered at one time will depend upon drug concentration, the use of vasoconstrictors, the selection of drug and the route of administration.[178]

Table 4-1 Characteristics and clinical Correlation of local anaesthetics [15] (modified from the Becker 2006)

Characteristic	Correlate	Explanation
Lipid solubility	Potency	enhances diffusion through neural coverings and cell membrane, allow a lower milligram dosage
Dissociation constant	Time of onset	Availability in the base form
Chemical linkage	Metabolism	principally hydrolysed in plasma by cholinesterase
Protein binding	Duration	Affinity for plasma proteins Affinity for protein at the receptor site within sodium channels, prolong the presence of anaesthetic at the site of action

4.1.2.2 Mode of Action and Application

The type of pain and velocity of conduction in nerve fibres are dependent on fibre diameter. The smaller fibres usually are responsible for sensing superficial pain, temperature, and autonomic activity.[170] Local anaesthetics interfere primarily in the depolarization process by preventing the membrane from reaching its threshold

potential and the action potential is not propagated.[172]. This concept of differential blockade allows inactivation of the pain fibres of complex nerves by using small doses of anaesthetic.[170] Local anaesthetic agent diffuses through the lipophilic nerve membrane in the unionized neutral form. The lower intra-cellular pH generates the ionized active form which blocks the sodium channel.[177] Sodium influx is reduced and the upsurge in the membrane potential slows.[177] The reduction of membrane permeability to sodium ions and displacement of calcium ions from their nerve membrane binding sites by the depolarization process, may trigger the opening of the sodium gates.[170] Several researchers have found evidence that local anaesthetic agents compete with calcium for a site on the membrane that controls sodium movement.[174] Local anaesthetic block is more readily achieved when the ionophore is in the activated state compared with the inactivated state and least when in the deactivated or resting state.[172] In the deactivated or resting states, the local anaesthetic can gain access only via the membrane as free base.[170] If a sufficient number of sodium channels are blocked then the threshold potential of -60mV will not be reached and impulse conduction will stop.[177]

The type and concentration of local anaesthetic affects sensory, motor, and sympathetic fibres differently.[177] For example, low concentrations of bupivacaine tend to block nerve conduction in fibres that transmit pain and temperature. Higher concentrations of bupivacaine, however, will also inhibit nerve transmission to motor fibres.[174] Ropivacaine has greater lipid solubility than lidocaine, allowing a lower milligram dose to achieve a comparable effect. Ropivacaine has a high affinity for plasma proteins, prolonging its duration of action when compared with lidocaine.[177] Lidocaine binds and dissociates rapidly from the channel, while bupivacaine binds rapidly but dissociates more slowly.[177] The speed of onset of block is related to the concentration of molecules of local anaesthetic that are in the free base or non-ionized state. This depends on the initial dose and the pH of the tissues.[172] Agents with a lower pKa, such as lidocaine and mepivacaine, have a greater proportion of molecules in the uncharged (active) form, resulting in a more rapid onset of action than an agent with a higher pKa.[174] Intravenous lidocaine has been demonstrated to have analgesic effects, reduce fatigue, and shorten the time to return of bowel function after laparoscopic colonectomy.[15, 172]

4.1.2.3 Toxicity of Local Anaesthetics

Most episodes of local anaesthetic toxicity result from high blood levels of local anaesthetic. The likelihood of CNS toxicity is proportional to local anaesthetic potency. More potent longer-acting drugs tend to be more toxic.[178] Local anaesthetics with a high degree of tissue binding or a large volume of distribution will have lower concentrations in the blood.[174] Protein binding has been shown to correlate well with duration of action and toxicity. When local anaesthetics are systemically absorbed, the plasma concentration rises slowly as it binds to non-specific protein sites.[15] Once these sites have become saturated there is a precipitous rise in the plasma concentration that may lead to toxicity. A similar situation occurs when plasma pH falls. Local anaesthetic dissociates from the protein molecules causing a sudden rise in the free fraction.[177] Initial central nervous system toxicity from local anaesthetics can manifest as numbness of the tongue and perioral area, restlessness, disorientation and slurred speech. Increase in plasma concentration produces seizures and slowed breathing may occur, which further develops cardio toxicity including dysrhythmias, respiratory arrest and/or cardiac arrest. Evidence of lidocaine toxicity may commence at concentrations >5µg/ml, but convulsive seizures generally require concentrations >8µg/ml.[179, 180] Lipid emulsions have been successfully used to resuscitate patients.[170]

The cardiovascular system may be directly affected by affinity of local anaesthetic on the sodium channel. Repeated stimulation of sodium channels allows increasing amounts of local anaesthetic to access the binding sites with a gradual increase in block.[179] There are differences in affinity between individual local anaesthetics, and this may be the main determinant of cardio-toxicity. The slow dissociation and persistent block of local anaesthetics may produce a variety of potentially lethal arrhythmias.[177] All local anaesthetics cause a dose-dependent depression in myocardial contractility and also exhibit vasodilation properties.[178] With increasing doses of lidocaine, there is prolongation of conduction time through various parts of the heart and an increase in diastolic threshold. This can cause a pronounced decrease in automaticity, resulting in bradycardia and possibly asystole. Both myocardial contractility and cardiac output are also decreased.[170] Reactions generally are seen following accidental IV injection, excessive total dosage, slow

detoxification, or poor excretion.[170] Peripheral actions of local anaesthetics can include vasodilation, which may result in hypotension after the injection.[170]

The relationship between total dose of local anaesthetic and peak plasma concentration is linear.[178] A solution's concentration may not be directly correlated with the systemic concentration, which rather depends on the total dose that produces the serum concentration.[15] For topical application, duration of exposure, surface area exposed, total dose and integrity of the skin all affect the absorption of the local anaesthetic.[15, 170] In particular, placement of the local anaesthetic on abraded skin or mucous membrane, or covering the area with an occlusive dressing can increase systemic exposure. Local anaesthetic doses should be reduced in elderly patients and patients with significant renal, hepatic or cardiac dysfunction.[15]

Ester local anaesthetics undergo rapid hydrolysis in the plasma by non-specific esterases. The speed of degradation may provide a degree of safety. However, metabolites of hydrolysis are inactive as local anaesthetics, but may be potent allergens. One of these metabolites can produce an IgE mediated hypersensitivity reaction.[181] Nevertheless, patients have occasionally experienced symptoms consistent with an allergic reaction to local anaesthetics. In some cases these episodes have been attributed to preservatives contained in the solution.[15] In a patient with a history of multiple allergies, anaphylaxis such as bronchospasm and near cardiac arrest rapidly developed after topical application of ester based local anaesthetics.[182] Preservatives such as parabens and metabisulphites can cause allergy especially amongst asthmatic or atopic patients. Procaine is representative of esters derived from para amino benzoic acid and hydrolysis liberates a moiety that is potentially immunogenic.[15, 181]

4.1.2.4 Lidocaine as a Local Anaesthetic

Lidocaine is probably the most commonly used anaesthetic in emergency medicine. It has a rapid onset and moderate potency and duration. It does not cause local tissue irritation and is a good topical agent.[171] Lidocaine increases the regional blood flow when injected intra- muscularly, but not when given subcutaneously. Epinephrine prolongs the duration of action of lidocaine four-fold, and that of prilocaine two-fold.[183] The major advantages of lidocaine are the rapid onset of anaesthesia and freedom from local irritative effects. The potency and duration of action are greater

than that of procaine, and the topical activity is seemingly not as effective as that of cocaine.[170] Lidocaine is a suitable substitute for procaine and its derivatives.[184] Concerning pharmacokinetic properties, most local anaesthetics are well absorbed after intramuscular administration. The rate of absorption depends on the site of injection as well as on the concentration of the administrated drug. About 70% of lidocaine in plasma is protein bound, mostly to α1- acid glycoprotein. Lidocaine is well absorbed from the gastrointestinal tract but undergoes a marked first-pass effect resulting in liver metabolism. The metabolites are excreted by the kidney.[174]

Metabolism includes pathways involving oxidative N-dealkylation, ring hydroxylation, cleavage of the amide linkage and conjugation.[172] Lidocaine is dealkylated to monoethyl-glycine xylidide and glycine xylidide, which can be further metabolized to monoethyl-glycine and xylidide by oxidase by cytochrome P_{450}3A2. Furthermore, lidocaine is biotransformed to 3-hydroxylidocaine by a cytochrome P_{450} isozyme belonging to the $P_{450\text{-}2D}$ subfamily.[172, 185,173] The elimination half-life following IV injection is 1.5 – 2 hours.[172] Liver dysfunction significantly increases the half-life and renal disease may increase the concentration of metabolites. Caution should be exercised in patients with epilepsy, bradycardia, cardiac condition, hepatic function and severe renal function.[179] Lidocaine is contraindicated for patients with Stokes-Adams syndrome, Wolff-Parkinson-White syndrome, sinoatrial, atrioventricular, or intra ventricular block.[170] The lidocaine dose should be reduced and patients should be monitored in those with severe renal and hepatic disease, children, and elderly patients. [170]

4.1.3 Lidocaine in pain management of partial thickness skin graft wound

Partial thickness skin wounds are painful, the exposure of the nerve endings attenuated by the inflammatory response results in ongoing pain until the surface epithelium is restored. Facilitating rapid healing is the goal of conservative wound care with dressings focused on infection control and supporting epithelial repair. Dressing changes are required and often associated with pain. Dressing change on the harvested split thickness skin graft is a routine surgical procedure and is frequently painful. The management of pain experienced by patients after surgery for acute burn wound repair by skin grafting is challenging.[159]

Current pain management regimes in burn care are mostly based on opioid analgesics which are frequently prescribed in large doses over long periods.[161] Increased opioid requirements due to a ceiling effect, may not be able to provide adequate analgesia in all patients.. Other non-opioid based approaches including nitrous oxide and ketamine have been tried with minimal success as a means of reducing pain after burn wound debridement.[160] Adequate soaking of the dressing can ease the pain of removal. Providing calm care and offering patients some control during painful procedures can facilitate comfort during therapies and wound care.[159] Local application of lidocaine improves analgesic efficacy and minimises opioid dose levels in patients during dressing changes.[186]

Topical lidocaine applied to a fresh split thickness skin graft donor site has been shown to reduce postoperative pain and analgesic requirements.[186] Lidocaine is an obvious choice in regional blockade for wound care procedures, it has also been used for burn pain analgesia as a topical gel or IV infusion.[156] Topical lidocaine applied at 1mg/cm^2 offered analgesic benefit without associated side effects. Topical lidocaine use may enhance systemic absorption at an open wound site. Administration of local anaesthetics via an epidural catheter would seem to be of benefit in patients with lower extremity burns, resulting in analgesia; specifically in procedural burn care and sympathectomy. A major drawback of this technique is that the use of an indwelling catheter in patients densely colonized with infectious organisms at the wound site is likely to increase the risk for epidural abscess formation.[159] High concentrations of lidocaine in creams (up to 30%) have been used to produce adequate anaesthesia for lumbar punctures, bone marrow punctures and minor excisions.[170] Different dosage forms of lidocaine reduce pain to different levels using the same dose of lidocaine administered as an ointment, spray, or liquid.[187]

Fear of toxicity, allergic reactions, and a presumed potential for decreased epithelialization has limited its use in the treatment of burns. However, the initially reported high incidence of allergic reactions has been found to be caused by epinephrine and not by lidocaine itself. [188] In fact, true allergic reactions to lidocaine are extremely rare.[157, 189] Reports of toxicity caused by local anaesthetics being used topically have been mainly associated with its application to mucous membranes, resulting in very rapid absorption.[157] Application of lidocaine gel to a burn wound

had been reported to cause seizures in children.[190] The effective utilization of local anaesthesia often requires careful timing and many a block has been declared a failure because time was not allowed for it to become effective. The successful employment of many techniques requires dosages of local anaesthetics which are in excess of recommendations and close to toxic levels.[191]

Interestingly, Brofeldt et al. have shown that topical 5 percent lidocaine cream applied to the burn wound at a concentration of 1 mg/cm^2, with a maximum area of 28 percent of total body surface area has been used to treat a partial thickness burn. This offered significant pain relief of up to 4-6 hours without associated systemic side effects.[156, 186] Application of up to 2 gm of lidocaine to an area as large as 2000 cm^2 was associated with an average plasma lidocaine level of 1.0 µg/ml or less. It was noted that with application of 0.7 to 4.5 gm of lidocaine, the plasma level remained fairly constant during the 4 hours of investigation, with the mean plasma level being about 2.7 µg/ml.[186] No studies to date have examined the analgesic effect of the topical application of local anaesthetics to areas where split-thickness skin grafts have been harvested or burn wounds debrided.[160]

4.1.4 Pain assessment

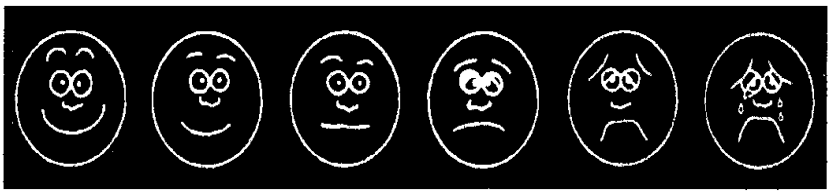
Pain rating scales have a fundamental place in clinical practice. The evidence suggests that patients are able to use them to communicate their pain experience and their response to treatment. Pain intensity is probably the easiest dimension of pain to assess. Before collecting any pain ratings for inclusion in the study, the patients should first be familiarized with the measurement instruments.

Types of rating scale are as below.

- Visual Analogue Scale (VAS)
- Visual Numeric Analogue Scale
- Verbal Rating Scale (VRS)
- Verbal Numeric Analogue Scale
- Numerical Rating Scale (NRS)

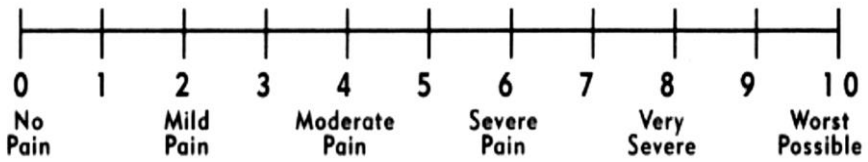
Figure 4-3 0—10 Numeric Pain Intensity Scales (from Montgomery RK.)[154]

V. Visual Numeric Analogue Scale



0 2 4 6 8 10

VI. Verbal Numeric Analogue



4.1.4.1 Numerical Rating Scale (NRS)

The pain intensity 0–100 millimetre visual analogue scale and the 0–10 numeric rating scale (PI-NRS) are commonly used metrics. The PI-NRS has become a common choice because of its ease of use for a broader range of methods of administration. Evidence also supports the results across a wide range of languages and cultures.[192] The NRS is a 11, 21 or 101 point scale where the end points are the extremes of no pain and pain as bad as it could be, or the "worst" pain. The NRS can be graphically or verbally delivered. When presented graphically the numbers are often enclosed in boxes. [193]

Bijur et al. (2003) found a significant correlation between the VAS and the NRS (r =0.94, 95% CI=0.93–0.95). They also found a strong level of agreement between the two tools.[194] DeLoach et al. also identified a high correlation in postoperative patients between the VAS and the NRS. However, the regression line slopes were 0.86 and 0.95 suggesting that the two scales do not agree. This tells us that at best the VAS and the NRS provide similar information about pain, but a direct conversion cannot be made between one and the other.[192, 195] The VAS is considered more difficult to use in the postoperative period because of residual anaesthesia, blurred vision, or nausea and several subjects have required additional instructions to complete the VAS. An 11-point verbal scale does overcome most of these difficulties. Also, the repeatability coefficients have been shown to indicate that any

single VAS score may not be a true measure of pain but are probably within 20 mm. In conclusion, the VAS has been shown to be a valid measure of pain in the immediate postoperative period.[195] However, the success rate has been significantly higher for the Numeric Rating Scale compared with Verbal Descriptor Scale and Visual Analogue Scale.[193]

4.1.5 APF pump spray

The advanced preservative free (APF) spray system is a technology patented by Aptar®. The APF pump tip seal mechanism acts as a spring loaded physical barrier thereby preventing the formulation from contamination via the orifice. The air required to compensate the volume loss in the bottle after actuation is filtered through a polytetrafluoroethylene (PTFE) multilayer membrane. The pump spray has undergone rigorous microbiological challenge tests including tip seal integrity testing (TSIT) and the closure ventilation integrity test (CVIT). TSIT was carried out by submersed actuation in a liquid containing Pseudomonas Aeruginosa and CVIT was carried out using *Bacillus atrophaeus* over multiple cycles of contamination and incubation.

Conventional pump actuation systems leave the residue of the product on the actuator channel and orifice. Due to evaporation of the solvent, the formulation crystallises and blocks the actuator channel and orifice. The APF system closes the orifice immediately when releasing the actuator. This mechanism avoids the residual product contamination and contaminated air/liquid being sucked back. This mechanism helps to deliver the full dose volume and provide accurate and consistent dose volume even after a few days in the resting state.[196]

Characteristics of APF spray pattern **[196]**

- Homogeneous droplet size distribution
- Fine distribution
- Ring shaped distribution
- Large droplets within the centre
- Sectors with a higher droplet concentration

4.1.6 Proposed Study

Lidocaine is a lipophilic molecule and used in a salt form in marketed products, much effort is made to provide quicker analgesia using different types of delivery systems and more potent local anaesthetics. There is a need for a lignocaine based disperse system to produce safe analgesia on open wounds. A formulation of lidocaine which would remain in the wound environment has the potential to reduce the risks of systemic toxicity. An emulsion was formulated as a mini-emulsion containing soybean oil and lecithin. This formulation contained no solvents, acidic salts or preservatives. The aim of this study was to evaluate the emulsion formulation of lidocaine base sprayed on the site against an aqueous solution of the salt form. The study concerns the first dressing change of post-split-thickness skin graft. The major factors assessed were pain level and systemic absorption.

4.2 Methods

4.2.1 Clinical Trials

The study was a single centre, randomised, double-blind, active-controlled, parallel pilot trial to compare a topical 4% lidocaine as a lidocaine hydrochloride aqueous solution "Treatment A" (Xylocaine™ as standard) with a 3% lidocaine base emulsion formulation "Treatment E" (NOPAYNE™). Subjects were randomised to receive either treatment A or E. The study was conducted in the State Adult Burns Unit and Burns Outpatient Dressing Clinic at Royal Perth Hospital of Western Australia. Ethics approval was given by the Royal Perth Hospital Ethics Committee (EC 2008/183, Appendix 3-1)) and was sponsored by N S Technologies Pty Ltd to be conducted in the Burns Unit and Burns Dressing Clinic at Royal Perth Hospital. Reciprocal approval was given by Curtin Human Research Ethics Committee (HR 151/2012, Appendix 3-2).

The study interventions were implemented by registered medical and nursing staff employed by the hospital and at the time of the trial, working in the Burns Service. The Clinical Trials Pharmacy was responsible for the dispensing of trial drugs and is a centralised facility catering for the hospital as a whole. The clinicians were blinded to the product administered. Plasma concentrations were measured by a central facility (Chemcentre WA) accredited by the *National Association of Testing Authorities* (NATA).

Subject suitability was determined by age and medical history. The 18 to 55 year age eligibility targeted subjects of a healthy population, and participants with major renal or hepatic dysfunction or females, who were pregnant, were excluded. Subject eligibility was based on the following inclusion and exclusion criteria.

4.2.1.1 Inclusion Criteria:

- Patients undergoing skin grafting of an area less than 2 % of total body surface area.
- Written informed consent.
- Age 18-55 years
- Able to self-assess and report their pain level.

4.2.1.2 Exclusion Criteria

- Pregnancy
- Known hypersensitivity to lidocaine
- Major renal or hepatic dysfunction
- Participation in another clinical trial.
- History of allergy to sulfites, lidocaine and mepivacaine

All split grafts were performed using an air driven dermatome. The trial evaluated the first donor site dressing removal only and subsequent dressing removals were managed according to normal clinical practice. For the reproducibility of responses of the participants the trial was undertaken at the standard first donor site dressing take down only and no repeat dressing was done for the purposes of the study. Follow up swabs of the donor site for microbiology was only done when clinically indicated.

Due to the painful nature of burns, patients received background use of regularly administered analgesia which included oral paracetamol, oxycodone sustained release and tramadol hydrochloride sustained release. In addition, immediate release oxycodone, immediate release tramadol hydrochloride and anti-inflammatory agents were administered as required in the immediate pre and post dressing period. No participants required the administration of intravenous analgesia.

4.2.1.3 Randomisation

The trial participants were allocated by the central Clinical Trials Pharmacy at RPH to receive either the Treatment A or Treatment E spray by use of a computer generated random number. The randomisation was organised using a randomly permuted blocking system (block length was fixed at 10) to ensure that numbers of patients accrued to the different treatments at approximately equal rates.. The allocation sequence was held exclusively by the Clinical Trials Pharmacy which had no clinical involvement in the implementation of the trial treatment.

The treatment was prescribed by the Burns Unit resident doctor on a standardised medication chart as “Lidocaine 3% or 4% spray-emulsion Study”. The prescription was then filled by the Clinical Trials Pharmacy. The trial and control sprays were dispensed in identical opaque pump action bottles labelled “Lidocaine 3% or 4% spray-NOPAYNE™ Study”. Patients were unaware of their treatment allocation.

4.2.1.4 Blinding

All clinical staff involved in managing the study participants (nursing staff, medical staff), the study investigator and trial coordinator (responsible for data handling and storage), as well as the participants themselves, were blinded to the treatment allocation. The trial and control sprays were dispensed in identical amber spray bottles labelled identically ("Lidocaine 3% or 4% spray-NOPAYNE™ Study") by the clinical trial pharmacy staff.

Assessments of pain and collection of other trial data including blood pressure and heart rate were performed by nursing staff. The procedure for administering both treatments was identical. The dataset was un-blinded only at the conclusion of the study, when all data had been checked and entered into a database. The statistical analysis was undertaken only once, at the conclusion of the trial.

4.2.1.5 Interventions

Venus blood was sampled and time recorded immediately prior to the application of the spray to determine baseline concentration for lidocaine absorption. Blood pressure and heart rate were also recorded to determine any pre-existing hypotension or bradycardia.

Standard donor site dressings applied in surgery consisted of a primary dressing of calcium alginate and a secondary retention tape dressing with outer reinforcement as necessary. Donor sites from both trial groups were dressed following this standard. The time the dressing commenced was recorded. Any outer dressings such as gauze and bandaging were removed from the donor site area and the primary dressing was moistened with normal saline prior to the application of the spray.

The trial treatment was applied at the commencement of the dressing change. Initially 1 spray was applied per 3 cm^2 of donor site area as the primary dressing was slowly removed. If pain was clearly evident a further 2 sprays were applied without exceeding the maximum dosage per patient weight. The maximum dosage of lidocaine determined for the study was 3 mg/kg, and maximum dosages (numbers of sprays) for a range of patient weights were tabulated as a reference guide for the nurses using the spray, to ensure that this dose was not exceeded. It was recognised that this level was conservative and chosen specifically to minimise the risk of toxic-effects.

On application of the spray the participant was asked to answer yes or no if they felt any stinging. Pain scores were measured by using a verbal self-report with the NRS on a scale of 0 to 10 (where 0 meant no pain and 10 indicated extreme pain). The pain scores were measured at intervals of 2 min, 5 min, 10 min and a final pain score assessment at 60 min followed by an overall pain score assessment on completion of dressing change. Blood pressure and heart rate were measured before and after completion of redressing. Patient satisfaction was determined using a scale of 1 to 5 where 1 indicated 'very satisfied' and 5 'very unsatisfied'. The length of time taken to complete the re-dressing was recorded. The donor site was redressed with retention tape as per standard practice. The used spray bottle was returned to the Clinical Trials Pharmacy for weighing to determine the accurate dosing and to perform sterility testing. The sterility testing was carried out according to British Pharmacopoeia Guidelines at a TGA accredited facility of PathWest Laboratory. Blood samples, to determine lidocaine absorption, were taken 1 hour post procedure, or as soon after as patients were able. The wound was photographed at the time of second dressing change.

Adverse events were recorded from the time of trial intervention to the healing of the treated donor site. The events were collected from the medical notes of the participants as recorded by nursing and medical staff following observation of the event or of reporting of the events by the participants.

4.2.2 Patient outcomes

The primary outcome was pain as measured by the final pain score at 60 min. Secondary outcomes included pain scores over time, patient satisfaction and systemic absorption of lidocaine.

4.2.3 Lidocaine plasma analysis by LC-MS-MS method

Lidocaine plasma analysis was carried out by Chemcentre WA, a National Association of Testing Authorities, Australia (NATA) accredited laboratory. The method described below was a part of validation report provided by Chemcentre WA.

4.2.3.1 Materials

10 mM ammonium formate pH 3, methanol(MeOH), mixture of methanol (MeOH)/0.1% formic acid Captiva ND lipids 96-well plate (1ml) 0.2 µm from Agilent, 1 ml 96 well collection plate, auto pipettes (range 25 µl to 1000 µl), vacuum manifold for 96-well plates, were used for the sample preparation.

4.2.3.2 Sample preparation methods

Lidocaine standard was prepared in MeOH. The concentration range for standard solutions was 20 ng/ml to 2000 ng/ml. Bupivacaine was used as internal standard (IS) at a concentration of 1000 ng/ml.

250 µl of plasma was added to a 1 ml well followed by 750 µl MeOH /0.1% formic acid. It was mixed by 5 strokes of an auto-pipette. Vacuum was applied and eluent was collected into a 96-well collection plate (1 ml per well). The vacuum was continued for a further 5 min after the filter cake appeared to have dried. 25 µl of IS was added to the eluent. Spiked plasma samples for quality control were prepared in the following manner. 250 µl of spiked material was used for the sample preparation procedure as shown in Table 4-2.

Table 4-2 Preparation of spiked QC samples for validation

Sample No	Concentration of lidocaine standard to add (ng/ml)	Volume of standard to add (µl)	Volume of blank plasma (µl)	Spiked plasma concentration (ng/ml)	Concentration after protein precipitation (ng/ml)
QC-5	50	100	900	5	1.25
QC-50	500	100	900	50	12.5
QC-200	2000	100	900	200	50
QC-400	2000	200	800	400	100

4.2.3.2.1 Matrix matched standards

8 aliquots of blank plasma (250 µl each) were used to prepare matrix matched standards by the sample preparation procedure. The eluents were spiked with internal standard and also with lidocaine standard as described below to generate matrix-matched standards as shown in Table 4-3.

Table 4-3 Preparation of QC samples for matrix matched standards

Sample No	Concentration after protein precipitation achieved (ng/ml)	Concentration of lidocaine standard added (ng/ml)	Volume of standard added (µl)	Equivalent spiked plasma concentration (ng/ml)
MM1	0.50	20	25	2
MM2	1.25	50	25	5
MM3	2.5	100	25	10
MM4	5	200	25	20
MM5	12.5	500	25	50
MM6	25	1000	25	100
MM7	50	2000	25	200
MM8	100	2000	50	400

4.2.3.3 Instrumental methods

Zorbax Extend – C18, 4.6 x 50 mm, 1.8 µm (Agilent P/N 727975-902) column was used. The LC/MS/MS used was Agilent 1200 LC as Liquid chromatography equipped with Applied Biosystems 4000 QTrap QQQ. Injection volume was 40 µl. The mobile phase was 10 mM ammonium formate at pH 3 and MeOH. The proportion of MeOH and buffer was maintained as described in Table 4-4. Flow rate was 0.5 ml/min.

Table 4-4 Instrument condition: Mobile phase ratio and time of LC-MS

Time (min)	%A (pH3 Buffer)	%B (MeOH)
0	80	20
0.5	80	20
3	5	95
5	5	95
5.5	80	20
7.5	80	20

4.2.3.4 Mass Spectrometer (MS) conditions

- Mode: Electrospray Ionization (ESI; Turbospray)
- Polarity: Positive
- Multiple reaction monitoring (MRM) as described in Table 4-5

Table 4-5 Compound-dependent parameters for MS/MS

Analyte	Transition (Q1 to Q3) (amu)	Declustering Potential (DP) (V)	Collision Energy (CE) (eV)	CXP (V)
Lidocaine	235.216 > 86.0	11	57	20
Lidocaine	235.216 > 58.2	11	47	6
bupivicaine	289.03 > 98.2	126	55	8
bupivicaine	289.03 > 84.0	126	57	16

4.2.3.5 Method validation

Linearity was measured by producing regression curves (concentration vs peak area ratio) for a minimum of n=6 matrix-matched calibration standards, prepared on at least three separate occasions. Acceptance criteria for linearity was r>0.95 for each calibration curve

Intraday reproducibility was measured by spiking blank plasma at concentrations of 2 µg/L, and 50 µg/L, and 200 µg/L, each in replicates of n >7, and taken through the sample preparation procedure. Matrix-matched standards were prepared in parallel. Recovered concentrations were calculated for each preparation. Subsequent analyses suggested that sensitivity was not robust at 2µg/L. Therefore, intra-assay reproducibility tests were repeated at 5 µg/L to establish a lower limit of quantitation (LLOQ).

Inter-assay reproducibility was examined by determining back-calculated concentrations and recoveries for plasma samples spiked at 2 µg/L, 5 µg/L, 50 µg/L, and 200 µg/L. Spiked blank materials were prepared at each level on n≥4 separate days. Back-calculated concentrations and recoveries were determined using matrix-matched calibration standards prepared with each batch. Acceptance criteria were:

- accuracy 80-120 % and precision, RSD <20% at LLOQ
- accuracy 85-115 % and precision, RSD <15% at Mid QC levels

4.2.4 Sterility testing of spray content

The sterility testing was carried out by PathWest laboratory at Princess Margaret Hospital. The method below is an extract from the validation document issued by the PathWest laboratory.

Half the contents was transferred into one bottle of Fluid thioglycollate medium (Medium 1) and the remainder to one bottle of Soya-bean casein digest medium (Medium 2). Shaken vigorously for 1 minute to distribute sample throughout the medium. Media 1 and 2 were incubated at 32 ºC and 23 ºC respectively for 14 days. Each test medium was examined daily, excluding weekends, for microbial growth. If turbidity, precipitate or other evidence of microbial growth the samples were analysed microscopically by Gram stain. The suspect media for single colonies using appropriate microbiological methods were subcultured. When subculture was necessary the plates were incubated for up to 7 days before discarding as no growth

to identify the genus level, any organisms isolated; Identity of isolates were recorded in order to detect a pattern of recurring contaminants in the product.

4.2.5 Data and statistical analysis

Standard descriptive statistics (mean and standard error of mean (SEM) for variables measured on a continuous scale, numbers and percentages for categorical variables) were used to summarise the profile of study participants. Chi-square or t-tests (as appropriate) were used to compare the profiles between the two treatment arms of the study.

4.2.5.1 Analysis of pain score data:

A Chi-square statistic was used to compare the presence of pain at different time points between the two treatment groups (primary objective). Pain scores were compared between groups using a Student's t-test (based on either the raw scores, or the log-transformed data as appropriate), or a non-parametric Wilcoxon 2-sample test. If any imbalance in demographic or baseline characteristics appeared between treatment groups, a logistic regression model would be used to assess difference in pain (presence or absence) between treatment groups after adjustment for other independent variables.

A General Estimating Equation (GEE) model was used to examine differences in pain (presence or absence) between treatments, based on all pain score measurements taken over the whole course of the study. This model takes into account the correlations between measurements made on the same individual.

4.2.5.2 Analysis of standard lidocaine concentration

The plasma concentration of lidocaine was standardised to an adult weight of 70kg and referred to as "Standardised Lidocaine Plasma Concentration" (SLPC). The SLPC at 60 min was determined from the plasma concentration at 60 min employing the following models. The total absorption time for an individual patient was calculated from patient record data. Plasma half-life of 2 hours for lidocaine was adopted from the Australian Pharmaceutical Formulary. [197, 198]

$$k = 0.693/t^{1/2} \quad \text{.....Equation 4-1}$$

$$ln\,Ct = ln\,Co - kt \quad \text{.....Equation 4-2}$$

Where; k=Elimination rate constant, t½= Half life, Ct = Plasma concentration of lidocaine at 60 min, Co = Pre-plasma concentration of lidocaine, t = Total absorption time.

Hence samples not collected at exactly 60 min were adjusted for concentration according to Equation 2. Linear pharmacokinetics were applied to adjust concentrations to a normal 70 kg of body weight. A regression model was used with the SLPC as dependent variable and combinations of absorption time, amount of sample used and/or the treatment as independent variables. Student's t-test was used to compare the mean SLPC between the two group treatments. For all statistical tests, a p-value ≤ 0.05 was taken to indicate a statistically significant association.

4.3 Results

4.3.1 Clinical trial

Participants were enrolled with informed signed consent from the inpatient population of the State Adult Burns Unit. There were 34 participants randomised into the trial and 29 completed all trial treatments and assessments. The five participants who did not complete the trial were excluded and not included in the analysis. Grounds for exclusion were;

- Too young
- Participation in another trial not known at the time of randomisation
- Inadvertent and premature removal of target dressing prior to receiving trial treatment
- Postoperative dressing inappropriate for trial treatment
- Target area was too large
- Accidental breakage of used treatment bottle in transit after the treatment

The period of recruitment extended for 6 months. Treatment was implemented on the second day post-surgery and the participant was followed up over a period of 2-4 weeks, the time frame in which burn donor sites were expected to heal without complication. A total of 29 participants received either Treatment A or E. Fourteen of the study participants were allocated to receive treatment A and 15 received treatment E. An error occurred in the recording of the drug dosage for one participant (randomised to the treatment group A), so all analyses were based on the remaining 28 subjects. The demographic and baseline clinical data for all participants are shown in Table 4-6. The p-values compare the parameters between treatment groups.

Data collected for the study included: the amount of spray used; the subject weight, dressing change time, size of skin harvest (donor site) and treatment group, presence of stinging and blood plasma concentration. The size of the donor site skin harvest was not recorded for one participant from the treatment group E and was not included in the regression analyses where wound area was included. Background pain was managed by background use of regularly administered analgesia including oral paracetamol, oxycodone, tramadol hydrochloride and anti-inflammatory agents. No participant requested the administration of analgesics for

procedural pain other than the prescribed treatment. Clinical outcomes for the study, including p-values to compare treatments are shown in Table 4-7.

Table 4-6 Demographic distribution of participants (Mean ± SEM)

Variables	Treatment group A (n=13)	Treatment group E (n=15)	p-value
Gender (Male, Female)	(9,4)	(10,5)	1.0*
Age (years)	37.0 ± 3.7	35.9 ± 3.6	0.84
Total Absorption Time (min)	60.7 ± 3.4	80.1 ± 10.6	0.14^
Subject Weight (Kg)	81.8 ± 6.5	81.6 ± 4.1	0.98
Donor Size (cm^2)	73.7 ± 12.4	80.3 ± 23.4	0.52^
Pre-dressing Heart rate (beats/min)	81.1 ± 4.0	68.4 ± 2.4	0.0088
Post-dressing Heart rate (beats/min))	79.9 ± 3.6	69.7 ± 2.0	0.0150
Pre-dressing Blood Pressure-Systolic (mmHg)	125.8 ± 4.7	127.1 ± 6.2	0.87
Post-dressing Blood Pressure-Systolic (mmHg)	128.6 ± 4.1	123.5 ± 5.1	0.46
Pre-dressing Blood Pressure-Diastolic (mmHg)	78.5 ± 3.5	75.3 ± 3.6	0.55
Post-dressing Blood Pressure-Diastolic (mmHg)	79.6 ± 2.9	74.7± 3.3	0.28

- *Fisher's Exact Test; ^Wilcoxon 2-sample test
- P-values are calculated using the t-test unless otherwise marked

Table 4-7 Comparisons of treatments, on the basis of dose of lidocaine received and detected in the serum (Mean ± SEM)

Observations	Treatment A	Treatment E	p-value
Amount of Sample Used (g)	1.8 ± 0.58	1.6 ± 0.5	0.87
Amount of Lidocaine Used (mg)	70.3 ± 23.3	49.0 ± 13.70	0.42
Lidocaine (mg) /100 cm^2	108.3 ± 33.6	112.5 ± 34.3	0.95
Lidocaine (mg)/ 70Kg person	61.5 ± 18.4	41.1 ± 9.0	0.82^
Lidocaine (mg)/70Kg/100cm^2 Dressing Site	97.0 ± 27.4	94.4 ± 26.1	0.95
No of Sprays Used per Dressing Change	13.1 ± 4.3	12.1 ± 3.4	0.86
No of Sprays/70Kg person	11.4 ± 3.4	10.3 ± 2.2	0.78
No of Sprays/100cm^2	19.9 ± 6.2	27.7 ± 8.8	0.48
Pre -dressing Lidocaine concentration (mg/l)	Not detected	Not detected	
Post-dressing Lidocaine concentration (mg/l)	0.11 ± 0.05	0.030 ± 0.04	0.044^

Standardised Lidocaine Plasma Concentration (SLPC) (mg/l)	0.13 ± 0.06	0.04 ± 0.02	0.069^
Standardised Lidocaine Plasma Concentration (SLPC) at 60 min (mg/l)	0.12 ± 0.05	0.05 ± 0.03	0.099^

- ^ Wilcoxon 2-sample test
- P-values are calculated using student's t-test unless otherwise marked

4.3.2 Pain score

The analysis of pain scores showed no significant association between pain and treatment group at any time during the study. Table 4-8 shows the numbers and percentage of subjects experiencing some pain at the given time point. At 2 min and 10 min, the number of patients experiencing some pain and pain score for treatment E was slightly higher than treatment A (Table 4-8), but this difference was not statistically significant. P-values were calculated using the Chi-square test, unless otherwise specified. A multivariate logistic regression model was also used to examine if there was any difference in pain scores between groups after adjustment for dose, but no associations appeared.

Table 4-8 The Chi-Square test data for relationship between pain scores at various time points, and the treatments (* Fisher Exact Test), Numbers in the table are the number (percentage) of patients experiencing some pain (pain score > 0).

Pain timing (min)	Treatment E (No of Patients)	Treatment A (No of Patients)	p-value
2	10 (67%)	8 (54%)	1.0 *
5-10	10 (67%)	6 (46%)	0.488
60	8 (53%)	7 (54%)	0.254*
On completion	7 (47%)	7 (54%)	0.390

Both treatments were proven to be very effective in reducing pain during the dressing change. There was a small non-significant decrease in the number of patients experiencing some pain at 60 min and final pain score for treatment E compared with standard treatment (Table 4-8, Figure 4-4 and Figure 4-5). Treatment E appeared to have longer lasting anaesthetic effect compared with the standard treatment A. On completion, 47% patients experiencing some pain in treatment E compared with 54% in the standard treatment.

Figure 4-4 **Frequency distribution of Pain Score on completion for the two different treatment groups**

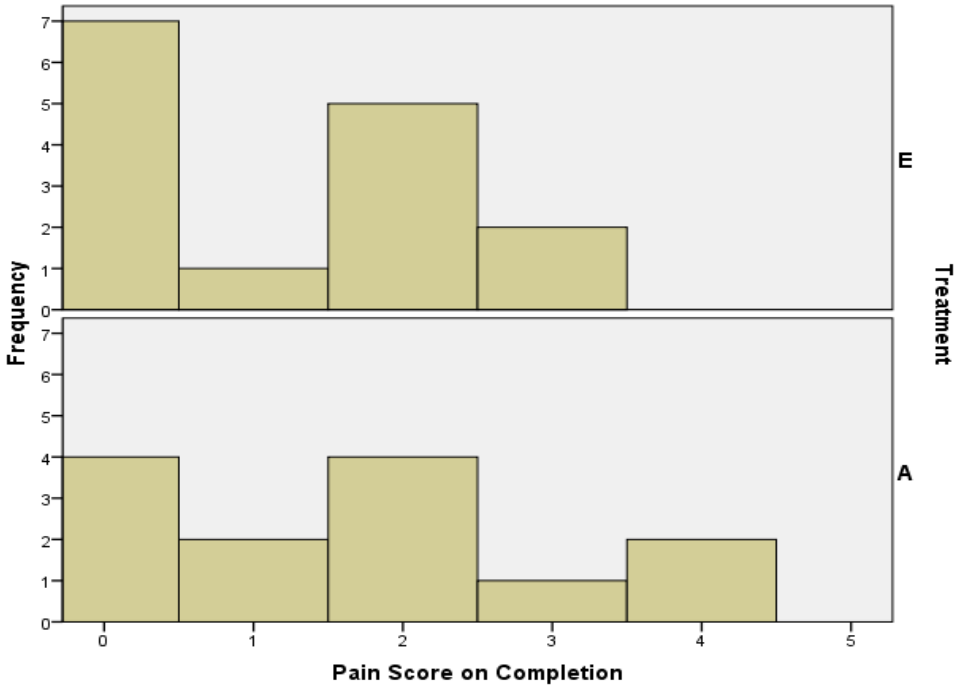
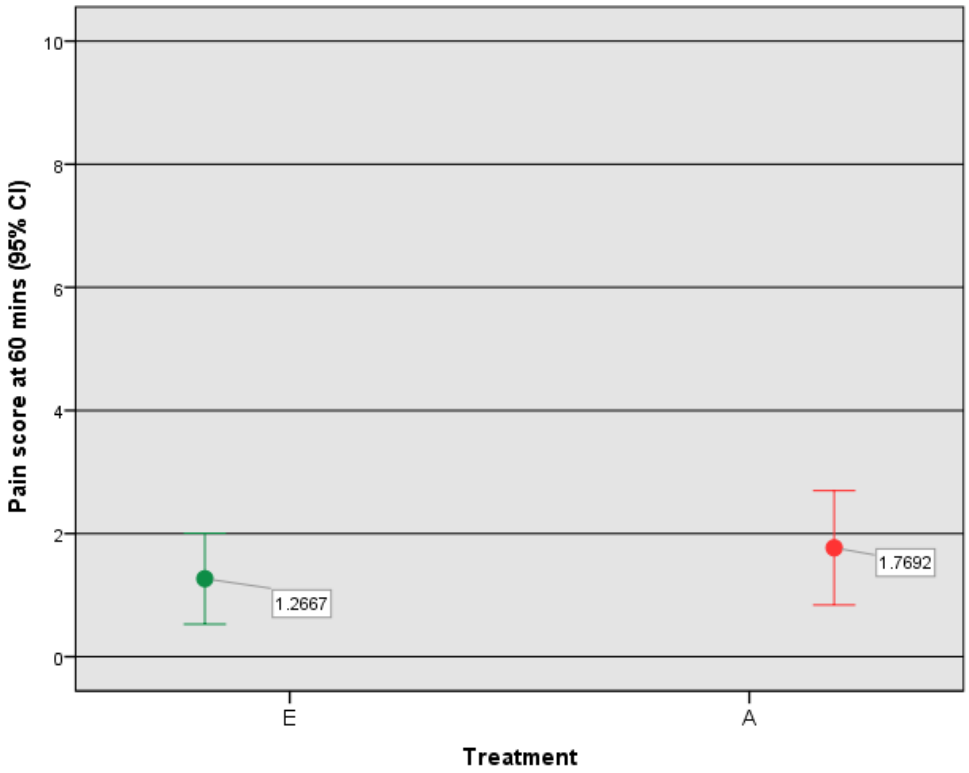


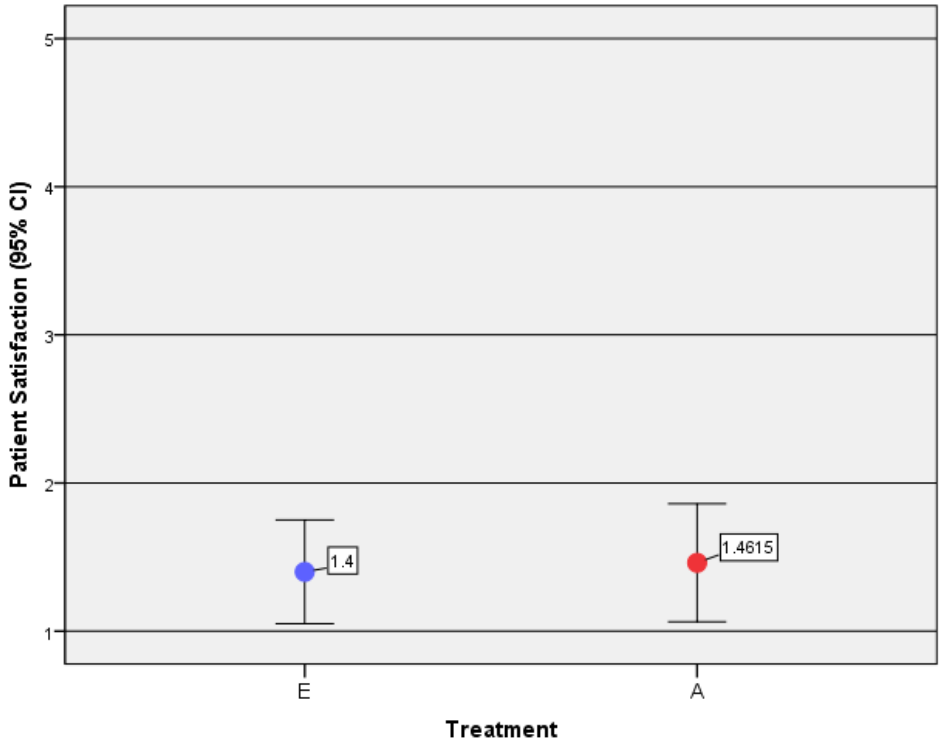
Figure 4-5 **Comparison of mean Pain score at 60 min (95% CI) between treatments (where, 0 is no pain and 10 is extreme pain)**



4.3.3 Patient satisfaction

The overall patient satisfaction with the level of pain control during the procedure were similar among the treatments E and A. Patients were very satisfied with both treatments with average satisfaction score of 1.4 (where, 1 is very satisfied and 5 is very unsatisfied).

Figure 4-6 Comparison of Patient Satisfaction (95% CI) between treatments (where, 1 is very satisfied and 5 is very unsatisfied)



4.3.4 LC/MS/MS validation

Retention time for lidocaine was 3.1 and IS was 3.7 min. Concentration range from 5 µg/L-200 µg/L found to be linear (Table 4-9). The method LLOQ was 5 µg/L. Method accuracy was 93–100% across the validated concentrations range (5 µg/L - 200 µg/L). Intra-assay accuracy and precision were within the acceptable range (Table 4-9, Table 4-10 and Table 4-11).

No significant matrix interference was detected. Mean peak area for matrix blanks was <20% of mean peak area for internal standard peak area. There was no trend for increasing instrument response corresponding to the analyte or the internal standard peak for matrix blanks in the injection sequence. Separate preparations of blank plasma were spiked with mixtures containing common pharmaceuticals at

100 µg/L, and individual concomitant medications tramadol, lorazepam, meloxicam, oxycodone and celecoxib. Spiked plasma was taken through the sample preparation procedure in parallel with blank plasma spiked at the LLOQ and matrix-matched calibration standards. Blank-subtracted responses to the analyte and internal standard were compared with responses for the practical limit of quantitation (LOR) spike (QC5).

Table 4-9 Linearity data

Analysis date	Calibration range (µg/L)	Number of calibration standards used	Regression	Weighting	R
09/09/10	5-200	6	Linear	1/x	0.9944
01/12/10	5-200	7	Linear	1/x	0.9988
20/12/10	5-200	6	Linear	1/x	0.9968

Table 4-10 Intra-assay reproducibility

Spiked concentration (µg/L)	Replicates	Mean accuracy (% recovery)	Mean calculated concentration (µg/L)	Standard Deviation	RSD (%)
blanks†	8*	N/A	<LOR (0.25)	0.7	280
5	10	95	4.8	0.52	11
50	9*	93	46	2.0	4.2
200	10	100	201	28	14

*Note: only n=9 replicates were prepared for the experiment at 50 µg/L and n=8 replicates were prepared for blank experiment †Note:lidocaine concentration was not detected for n=7 blanks, and calculated at <5 µg/L (2.0 µg/L; <LOR) for n=1 blank.

Table 4-11 Inter-assay reproducibility

Spiked concentration (µg/L)	Replicates (batches)	Mean Accuracy (%)	Mean calculated concentration (µg/L)	Standard Deviation	% RSD
5	7	96	4.8	0.40	8.4
50	7	88	44	1.5	3.4
200	6	89	177	19	11

Table 4-12 Stability of standard solutions

	First set of QC spikes		Second set of QC spikes	
Spiking level	**Calculated concentration (µg/L)**	**Accuracy (%)**	**Calculated concentration (µg/L)**	**Accuracy (%)**
QC-5	4.5	91	4.4	89
QC-50	57	110	44	89
QC-200	170	86	180	90

Table4-13 Long term stability of samples

Spike label	Calculated Concentration (µg/L)	Accuracy (%)
QC50-LT1	52.3	110
QC50-LT2	49	98
QC50-LT3	56	110
QC200-LT1	230	110
QC200-LT2	230	110
QC200-LT3	240	120
	Mean accuracy	110
	SD	9.1
	% RSD	8.0

Table 4-14 Pre-preparative stability

Spike label	Analyte Concentration (µg/L)	Calculated Concentration (µg/L)	Accuracy (%)
QC-50-BTS1	50.00	47	93
QC-50-BTS2	50.00	43	85
QC-50-BTS3	50.00	39	78
	Mean	43	86
	SD	3.9	7.8
	%RSD	9.1	9.1

Table 4-15 Post-preparative stability

First set of QC spikes			Second set of QC spikes			
Spike label	**Calculated Concentration (ng/ml)**	**Accuracy (%)**	**Calculated Concentration (ng/ml)**	**Accuracy (%)**	**Acquisition Time**	**Time (h)**
QC-5ng/ml	4.8	96	5.1	100	14:36	2.8
QC-50ng/ml	44	88	47	95	14:45	2.8
QC-200ng/ml	170	83	180	89	14:53	2.8

4.3.5 Lidocaine plasma concentration

The Drug Absorption Study included 28 observations; 13 participants who received standard treatment A and 15 participants who received treatment E. Donor site size from one participant from the treatment E group was not retrievable and was not included in the regression analyses. The data from the 28 participants were used in determining the amount of sample used relative to the subject weight, absorption time, donor size or treatment.

The regression analysis of SLPC at 60 min on treatment group and dose of treatment drug administered, showed a statistically significant interaction between these two independent variables (p = <0.0001). The interpretation from this finding is that the SLPC of lidocaine depended on the dose administered, but this relationship was different for the different treatment groups. The R^2 for the overall model was 32.7 %. The slopes of the regression lines for each treatment are given in Table 4-16. These data and their confidence intervals were found from the regression model including an intercept and the interaction between treatment and dose as the only independent variable (no main effects).

Table 4-16 The regression data for Standard plasma concentration at 60 min association with the amount of sample used and the treatment

Independent variable	Slope	95% CI for slope	p-value
Dose (Treatment E)	0.000306	-0.001 to 0.002	0.65
Dose (Treatment A)	0.002093	0.0013 to 0.0029	<0.0001

Subjects that received treatment E showed a significantly lower SLPC compared with the Treatment group A (Figure 4-9). Treatment E also showed slightly better distribution of post-plasma lidocaine concentrations compared with standard treatment (Figure 4-7). There appeared to be no relationship between dose and plasma concentration for the treatment group E (p = 0.65), but a strong positive relationship between these variables for the treatment group A (p <0.0001). This strong relationship can be seen as a graphical presentation in Figure 4-7 and Figure 4-9. Exclusion of outliers did not affect the relationship between dose and plasma concentration for treatment E. However, the slope was slightly reduced after exclusion of outliers in the Treatment A treatment group. The detailed data

regarding the spray administered and patient plasma concentrations are given in Table 4-7.

The relationship of the SLPC with absorption time, dose or treatment was evaluated. There appeared to be no association between absorption time and plasma concentration (p = 0.37), However, there was a significant interaction between SLPC and dose or treatment group (p <0.0001). The R^2 for the overall model was 67.8%. The estimated slopes of the regression lines are given in the Table 4-17:

Table 4-17 The regression data for Standard plasma concentration association with absorption time, the amount of sample used or the treatment E or A

Independent variable	Slope	95% CI for slope	p-value
Absorption time	0.00057	-0.0007 to 0.0019	0.37
Dose (for treatment E)	0.00061	-0.0070 to 0.0019	0.35
Dose (for Treatment A)	0.00267	0.0018 to 0.0035	<0.0001

Figure 4-7 Relationship of Dose Vs Plasma concentration for different treatments

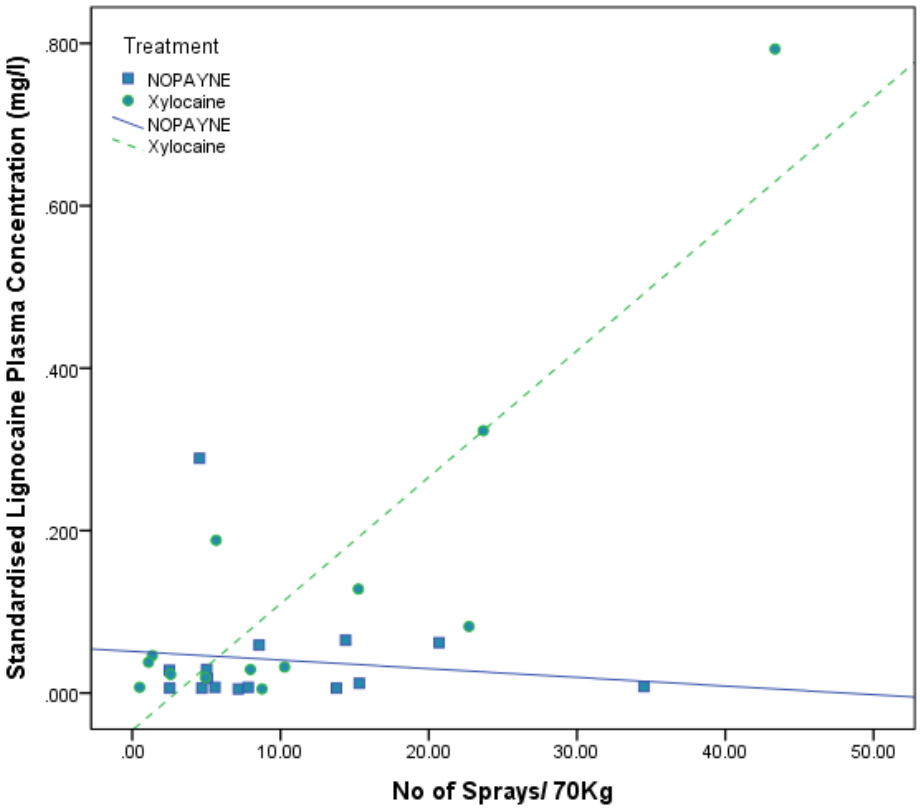


Figure 4-8 **Pre and post dressing lidocaine plasma concentrations (mg/L) for each participant in the two different treatment groups (the bars are mean ± SEM)**

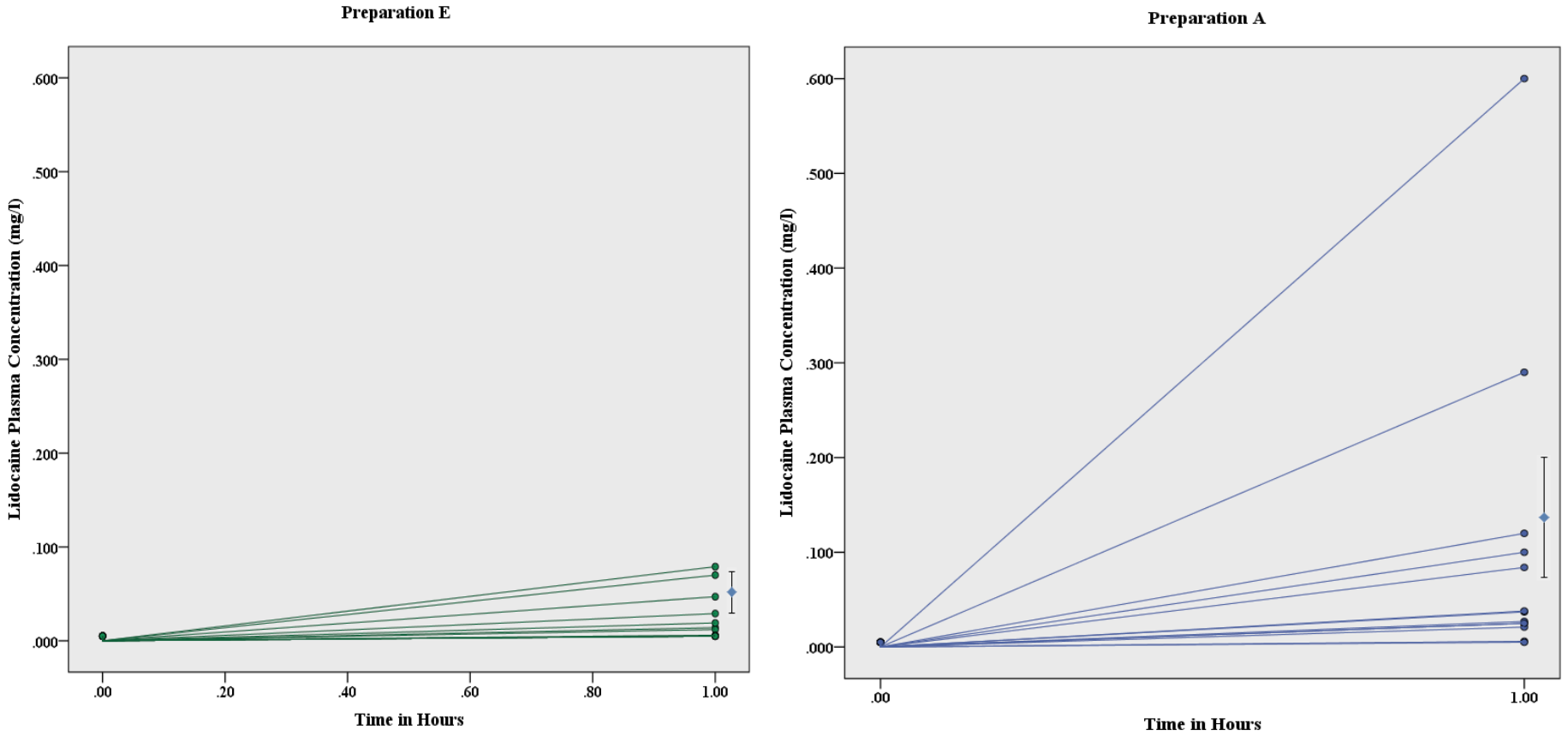
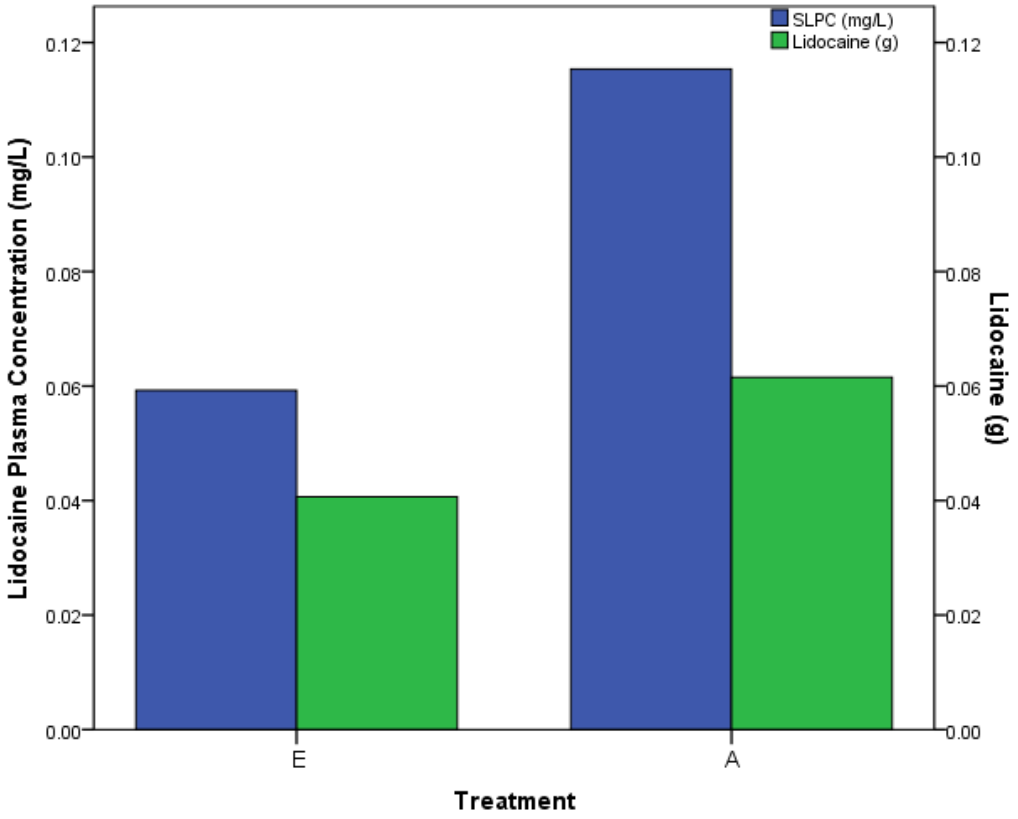


Figure 4-9 **Average distribution of Standardised lidocaine plasma concentrations (SLPC) and lidocaine dose (mg) Treatment A Vs treatment E**



4.3.6 Relationship between donor size and sample used

In the relationship of amount of sample used with subject weight, absorption time, donor size or treatment only Donor size appeared to show a significant association with amount of sample used (p = 0.0025), and all other variables showed no association. The R^2 for the model was 30.0%. The positive slope simply means as donor size increases, so does the amount of sample used.

Table 4-18 **The regression data for donor size and amount of sample**

Independent variable	Slope	95% confidence interval for slope	p-value
Donor size	0.014	0.006 to 0.023	0.0025

4.3.7 Sting rate analysis

There was no significant difference found between the two treatments for occurrence of stinging sensation at the donor site (p=0.98). The following Table 4-19 shows the numbers (and percentages) of people in the 2 groups who

experienced a ‘sting’ (0=No, 1=Yes). It appears that the percentages were essentially the same for both treatments (both about 46%).

Table 4-19 The regression data for sting rate and treatment group

Patient Score	Treatment E	Treatment A	Total
0	8 (53.33%)	7 (53.85%)	15
1	7 (46.67%)	6 (46.15%)	13
Total	15	13	28

4.3.8 Adverse events

Blood pressure and heart rate remained stable and within normal limits in all participants through the study. A total of 8 adverse events were recorded none of which was serious, unexpected or related to the lidocaine. The distribution of the events was 4 from the control group and 3 from the treatment group E. Due to the small sample this was not conclusive. All of the adverse events listed resolved with no residual effects:

- Positive microbiology from donor sites of 3 participants (Staph Aureus)
- Left lateral rib pain
- Failure of skin graft to burn area in 2 participants
- 2 episodes of small amount of blood in stools in one participant
- Mild allergic response to food

The product content of all spray bottles was sterile after application during the trial. The sterility of the product content was also examined for use in unclean environment and found to be sterile.

4.4 Discussion

Pain occurring during recovery from burns and dressing changes is an important factor for patients with pain being rated as the highest treatment priority by many patients.[199] Procedural pain is an intense burning and stinging that may continue for min to hours after dressing changes, which frequently induces significant anxiety and stress.[162] Procedural pain is also one of the most difficult forms of acute pain to treat, due to its intensity, variability and the frequent co-existence of opioid tolerance. Topical local anaesthetics agents such as lidocaine can be used for pain management without the side effects associated with opioids.[161]

This study was a randomised double–blind controlled trial comparing the effect of two different products containing lidocaine in patients undergoing a painful dressing change. A topical 4% lidocaine aqueous solution "Xylocaine™" was commercially available and a 3% lidocaine base emulsion formulation "NOPAYNE™" expected to be more efficacious.

4.4.1 Pain rating

Effective pain management is a major challenge to physicians. Accurate assessment of the patient's pain status is one of the most important steps in effective management of pain. Without accurate and reliable instruments any true effect of treatment can be obscured by measurement error, or ineffective treatments may be erroneously considered therapeutic.[200] The standard for pain assessment is the patient's self-report.[154] Pain intensity is commonly reported using a 0-10 numeric rating scale in clinical trials.[192] Researchers have supported the improvement in the consistency of a relationship between the percentage change in the analysis of the pain intensity numerical rating scale (PI-NRS) and the clinically important changes measured on the global scale and as an appropriate primary outcome for such clinical trials.[192] Given the inherently subjective nature of the symptom, measurements of pain rely primarily on the verbal reports of patients. The multiple dimensions of pain such as intensity, characteristics, pain relief, and the global impressions of change are considered important additional endpoints for pain clinical trials. However, for studies of pain-specific therapies change in pain intensity over time should always be the primary outcome.

Using pain rating scales can be challenging and list of problems are mentioned as below:[201]

- The patient doesn't use the pain rating scale correctly
- The patient expect a 0 pain rating
- Nurses have difficulty with facial expression or when patient is sleeping
- The patient rates pain at higher than 10 on the 0- to-10 rating scale
- Patient and nurse communications

Assessment tools are essential to the diagnosis of underlying pain syndromes and the effectiveness of their treatment. The sensitivity of a pain rating scales is the ability of the scale to detect change in pain. The lack of sensitivity can lead over or under-estimation of pain changes. The VAS and the NRS are superior in this respect because they have greater sensitivity to change. Compelling evidence exists that 11 or 21 point scales are more than adequate for the assessment of pain.[193]

Three different rating scales (VAS, VRS and NRS) were compared for their suitability in a clinical environment. Researchers reported that failure rate with the NRS and VRS are lower than failure rates with VAS. The VAS is the most robust and difficult of the three scales to use in clinical practice. The VRS is the least sensitive tool of the three, but it is easy to use. The NRS provides interval level data and is as sensitive as the VAS. The scale is easy to administer, record and allows patients to use either 11 or 21 points of intensity. As a tool for pain assessment the NRS is probably more useful than the VRS and the VAS.[193 195]

4.4.2 Primary outcome

We found that both treatments containing lidocaine were equally effective in managing procedural pain related to dressing change. All patients had low pain scores for both treatments. The pain scores of all patients were below 5 on the standard rating scale of 0-10. For the Sting prevalence analysis there was no significant difference found between two treatments. All patients were satisfied with the treatment received. All the donor sites healed with no signs of clinical infection within this period. .

Our results showed agreement with those of Jellish et al., who reported that the topical application of lidocaine at a skin harvest site reduced the perception of pain.[160] Derek et al. also reported lidocaine gel 2% applied to split skin donor sites before dressing was an effective analgesia. Previous reports described lidocaine

based topical application to be safe and effective analgesic for donor sites that reduces the use of postoperative opiates.[160, 191]

At 2 min and 10 min, the number of patients that experienced some pain and pain score for treatment E was similar to treatment A solution (Table 4-8). At 60 min, the number of patients experiencing some pain and pain score for treatment E was insignificantly lower than standard treatment A. There was a slight decrease in the number of patients experiencing some pain on completion for treatment E compared to treatment A (Figure 4-4). The emulsion appeared to provide slower release and consistent release of the insoluble base from emulsion formulation, which could also be supported by blood plasma concentration (Table 4-7, Figure 4-8 and Figure 4-9). However, the wide variation in plasma concentration limits the power (27.4%) to detect the difference. Slightly improved outcome of treatment E at 60mins and during final pain scores showing a longer lasting anaesthetic effect compared with treatment A. Sinclaire et al. used lidocaine based aerosol application. Sinclair et al. also reported significantly lower pain scores and reduced opioid requirement during the first postoperative day.[168] They also found lidocaine aerosol used as topical anaesthetic in the surgical wound was simple to use, and resulted in long lasting reduction of pain after a single administration. This effect was suggested to be induced by topical micro droplets of lidocaine.[168] The use of topical lidocaine for procedures on wounds is tempered by concerns relating to the unpredictability of absorption and therefore the potential for systemic toxicity.

4.4.3 Plasma concentration

The concentrations of lidocaine in plasma were measured to understand the systemic exposure of lidocaine and related possible toxicity from the product after application. The duration of 60 minutes was decided after discussion with clinicians. The half-life of lidocaine is 90 minutes and it takes up to 60 minutes for the procedure to complete. It was concluded that blood plasma at 0 minutes and at 60 minutes provided the best outcome for the lignocaine toxicity related incidence.

Lidocaine plasma concentration were standardised per adult weight and absorption time of 60 min. The difference in lidocaine plasma concentration for both treatment groups was statistically significant and thus the rate of absorption. The relationship between the SLPC at 60 min with the amount of sample used for the treatment

showed that there appeared no relationship between dose and plasma concentration for treatment E, but a strong positive relationship was found between these variables for treatment A (Table 4-17). With an increase in the dose or number of sprays of Treatment A, there was an increase in the lidocaine plasma concentration (Figure 4-8 & Figure 4-9). This increase was not observed with treatment group E; possibly due to the low rate of absorption of lidocaine from the emulsion formulation, indicating a greater margin of safety.

The correlation between surface area, dose and plasma concentration further showed that the emulsion formulation of lidocaine base may provide safe local analgesia on wound size greater than 600 cm^2. Due to the lack of no of samples and similar strength of lidocaine, it was difficult to demonstrate the differences in pain scores. However, the difference lidocaine plasma concentration was statistically significant. A study by Jellish et al. using topical lidocaine spray found blood concentrations to be independent of the dose of lidocaine used or the type of membrane sprayed.[160] Research studies with dispersed systems such as lidocaine gel 2% applied to split skin donor sites before they were dressed was effective analgesia and it was reported that plasma concentration was well below the toxic threshold after lidocaine gel application on a split skin donor site.[202, 203]

Lidocaine has potent analgesic, anaesthetic, and anti-inflammatory effects, which has demonstrated benefit at wound sites during dressing change. However, fear of toxicity such as cardiac arrhythmias, seizures, allergic reactions and a presumed potential for decreased epithelialization have limited its use in this for the treatment of burns. Reports of toxicity associated with topical use of a local anaesthetic have mainly been observed with its application to mucosal membranes leading to rapid absorption.[156, 162] The maximum dose of lidocaine is dependent upon the size and physical status of patient and the rate of absorption. The maximum dosage of lidocaine determined to be safe was 3 mg/kg, It was recognised that this level was conservative specifically so to minimise the risk potential. No patients were compromised during the trial related to any toxic effects of lidocaine. No adverse reactions to lidocaine were reported by aerosol application of lidocaine.[168] On an average, patient undergoing a split-skin graft dressing change received 1 $mg/cm^2/kg$. The concentrations measured in all patients were well below those associated with systemic toxicity of 3-5 µg/ml. Bulmer et al. reported a sterile 1%

lidocaine gel applied to an area of the donor site equal to 3 cm^2/kg achieved the highest plasma concentration of 0.52 µg/ml. The study concluded that 1% lidocaine gel provides useful short-term postoperative analgesia for split-skin donor sites, and systemic absorption was small.[203] Local anaesthetics are usually administered in aqueous solution directly from the ampoule or via a syringe onto an alginate dressing such as Kaltostat®. This tends to produce a patchy and uneven distribution throughout the dressing and, therefore, over the donor site.[204]

In this study, a spray application of lidocaine proved an effective delivery system within a high safety margin and provided analgesia immediately after application. The CPS spray unit combined with an emulsion formulation provided a novel delivery system for open wound and procedural pain management. This study has established a successful application of spray form of local anaesthetic for drug delivery to donor sites. A similar study by Brofedlt et al. showed that an atomiser attached to any standard syringe delivered fine particles (30 µm) of lidocaine over The target area produced plasma lidocaine levels of 1.0 µg/ml or less was achieved after an application of 2 gm of lidocaine to an area as large as 2000 cm^2.[204] Brofedlt et al. also reported that the lidocaine level remained fairly constant during the 4 hours of investigation, with the mean plasma level being about 2.7 µg/ml for an lidocaine application range of 1.0 to 4.5 g.[186]

The CPS spray unit also maintained microbial integrity during and after use by creating a microbiological seal directly below the spray orifice. This mechanism along with the tip design avoided a residual drop of product at the dispensing tip and subsequently, microbial contamination after multiple use. This specialised unit allowed preservative free sterile product to maintain sterility during and after the trial period. The CPS spray unit provided accurate delivery of the product content with mean dose (±SD) of 0.135 g ±0.002 per spray. The data on spray angle (57º ± 3.5º) and diameter (32.7 mm ± 2.5) was provided by Ing. Erich Pfeiffer GmbH (Pfeiffer). The spray diameter data (40.1 mm ± 2.1) was also assessed manually in our laboratory, which corresponded with data generated by Pfeiffer.

4.4.4 Limiting factors

Limiting factors in this study include that some variation may have occurred in the pre-treatment of dressings prior to the administration of either lidocaine

formulations. A variation in the pre-treatment dressings prior to the administration of both lidocaine formulations was potentially equivalent between the 2 groups. The confounding effect of the interference in the assessment of pain and stinging by the use of oral analgesics in the acute management of post-operative burns patients' needs to be considered. All participants except one had received oral non opioid analgesia. However the pain of the procedure was the focus of the topical therapy and pain scoring. All the wounds were minor to reduce the impact of concurrent analgesia.

Donor sites bleed in the post-operative period requiring the use of calcium alginate dressings. There is variation in the amount of bleeding between patients and subsequent differences in the soiling of this dressing. Theoretically the amount of normal saline required to moisten the dressing prior to the application of the lidocaine sprays had the potential to dilute the spray especially the aqueous treatment A.

In regard to the on higher plasma levels in aqueous solution, the wound bed was considered to be consistent. The wound depth was controlled as they were all taken with an air driven dermatome. The difference could be the rate of epithelialization or the level of drying at the wound surface. In addition, there is little difference in patient demographic data and the size of the wound in the two treatment groups. This would give strength to the conclusion that the difference in outcomes can be due to the nature of formulation used in the patients rather than other factors.

In summary the formulation was seen to be as effective as standard lidocaine solution with an improved risk profile with respect to systemic absorption of the active agent lidocaine.

4.5 Conclusion

A double-blind randomised controlled, pilot trial was conducted in 29 patients undergoing split thickness skin graft surgery. The study compared the effectiveness of two different formulations of topical local anaesthetic for dressing changes of partial thickness skin graft donor sites. Both formulations showed a significant analgesia for procedural pain during dressing changes and provided similar outcomes in pain management. Almost all patients were very satisfied with the analgesia provided in the form of aerosol. The plasma concentrations of lidocaine for aqueous treatment were significantly higher than the emulsion treatment group. In a clinical trial set up, the mini-emulsion based topical local anaesthetic provided an improved safety profile.

4.6 Acknowledgement and Funding

The trial was funded by the sponsor N S Technologies Pty Ltd. We appreciate ChemCentre team for their professional collaborative work along with N S Technologies Pty Ltd. We also appreciate Ing. Erich Pfeiffer GmbH, Germany for their support for collaborative research data and production of CPS pump spray. We are thankful to Cristina Farrar at PathWest Laboratory for her valuable guidance.

5 General Discussion

5.1 General Discussion

This study was undertaken to develop and optimise an emulsion based drug delivery system, which is safe, can be commercially up-scaled and has a shelf life of 1-2 years. Soybean oil was used as the oil phase. Tweens, Spans and lecithin were evaluated as emulsifiers. The selection of lipid and surfactants for emulsions were based on safety and toxicological clearances of the lipid and surfactants for application to numerous delivery systems. Refined soybean oil is one of most commonly used triglycerides in pharmaceutical products with no reported carcinogenic, mutagenic and acute toxicity.[60, 74] Since the 1960s, parenteral products, including total parenteral nutrition Intralipid®, containing refined soybean oil, have been marketed.[54] While surfactants were selected from the approved additives listed by the various regulatory authorities and pharmacopeias' for internal administration, the most commonly used surfactants in formulations were phospholipids, Spans and Tweens.[56, 58] A mixed emulsifiers system has long been recommended producing stable emulsions due to the formation of a condensed and viscoelastic interfacial film.[38, 53] The synergistic effect of a mixed emulsifier system on solubilisation and stabilisation have been well established, especially Tweens with other non-ionic or ionic surfactants including Spans and lecithin.[205-207] To produce o/w emulsions the hydrophilic surfactant as a primary emulsifier and the lipophilic surfactant as secondary emulsifier were used.

Emulsions were evaluated for the effect of surface tension, interfacial tension, and viscosity on droplets size. The formation of an emulsion is a complex process which requires a balance between deformation for droplet break up and stabilisation of droplets to reduce coalescence. The interfacial tension is directly proportional to the external energy required during droplet break up and subsequently formation of smaller droplet sizes. A system with lower interfacial tension requires less energy to deform the droplets. [208] On the other hand the adsorption of emulsifier at the interface and the production of a stable interfacial film prevent the coalescence and enhances the viscoelasticity of interfacial film.[104] The change in viscosity of the continuous phase has also been reported to be correlated with the energy required to reduce the droplet size.[20] Increase in interfacial viscosity and dispersed phase viscosity would also retard the rate of droplet break up, and prevent the formation

of smaller size especially smaller size droplets size could not be formed. This results in a larger mean droplet size.[209]

The stability of the emulsions was evaluated through droplet size, creaming, and phase separation. Simple measurement of creaming is not a quantitative assessment of in emulsion stability. The degree of creaming can be also correlated with aggregation and coalescence. All emulsions prepared during the study were milky white with no separation of a clear aqueous phase at any time. This indicates the presence of polydispersed droplets of the oil phase with partly flocculated droplets present in the cream layer. The creaming and coalescence stability during centrifugation can help predict the long term stability of the emulsion and lead to selection of a stable formulation at an early development stage.[110] Accelerated stability, especially under centrifugal force, has been demonstrated to be an efficient tool for selection of formulations at early development stage. The centrifugation technique is prevalent and the particular force of force and time on creaming and droplet size could also be assessed during an early stage of development.[80] A low g-force has been reported to be helpful to predict of accelerated physical degradation such as distribution of droplets and coalescence.[80] In the current study, centrifugation at a g-force of 2290 × g was used for up to 2 hours to evaluate accelerated stability of emulsions and selection of the formulation and process parameters during the development phase. However, accelerated tests could not be used to predict the shelf-life of an emulsion due to the lack of any model suitable to extrapolate the experimental values to reflect storage conditions under gravity and hence a realistic long term stability program is necessary to assess the normal storage shelf life is required.[58]

The HLB system is a well-established method which permits a more systematic approach to emulsion formulation including selection of emulsifiers and their blend for the specific oil phase.[32] A stable emulsion system can be formed by selecting a correct blend ratio of surfactant and co-surfactant (Ws/Wco) with an optimum hydrophilic–lipophilic balance (HLB).[38] When emulsions were prepared using soybean oil (RHLB 7) and an emulsifier blend with HLB values ranging from 7 to 12, it was found that the emulsions with HLB <10 were unstable during the accelerated stability study. These observations showed that the HLB system may not be accurate and the published RHLB of oil is an estimated value. The HLB of

oils containing complex mixtures of fatty acids and their esters such as soybean oil, may vary and may not apply to all surfactant blends. However, the HLB system is a useful tool for the selection of specific surfactant blends with respect to fixed oil. We found that the droplet sizes of the emulsions after 2 hours of centrifugation were most stable at HLB 11 and with Ws/Wco ratio of 0.67/0.33. A similar trend of increase in coalescence with an increase or a decrease in the HLB of the emulsion at optimum HLB was observed by Boyd et al.[38], The emulsifier ratio Ws/Wco of 0.67/0.33 was selected for order of mixing and equipment selection trials considering the improved accelerated stability of these emulsions.[84, 86]

Mechanical devices and the chemical potentials of emulsion components are two factors which have a major influence on the production of stable emulsions, and are generally applied using three mixing techniques; 1.solvent evaporation, 2.addition of internal phase to external phase, 3.phase inversion.[210] The fourth method used was based on the stabilisation of emulsions by formation of oil swollen micelles and/or lamellar structures. The application of lamellar liquid crystalline structures and swollen micelles in the formation and stability of emulsions has been reported previously.[24, 27, 90] HLB does not account for processing parameters and any interaction between the emulsifiers and the aqueous and oil phases.[38] On the other hand, PIT and solvent evaporation have been reported to produce submicron sized stable droplets due to the extremely low interfacial tension produced by altering the physical and chemical profiles of the three phase system.[210]

Bouchemal et al. has described the application of solvent in the formation of nano sized droplets. The presence of solvent significantly reduces the surface and interfacial tension of the lipid phase. However, we observed that the reduction in interfacial tension alone was not enough to stabilise the system. Similar results were observed for the PIT where emulsions were found to be unstable. Multiple factors could be considered for emulsion instability and increase in the droplet size, including the type and amount of solvent used, solubility of the lipophilic surfactant and coalescence of droplets.[87-89] When the solvent evaporation or the PIT methods were employed, the type of lipophilic surfactant was found to play an important role in the formation and stability of emulsions. The inter-molecular complexes are of importance in the formation and stability of oil in water emulsions.[39] The solvent penetration and interaction with lipophilic surfactant of solvent have been reported

to reduce the interfacial film thickness and lead to instability.[93, 95] The instability of emulsions prepared by phase inversion was thought to be caused by the inability of the lipophilic surfactant to attain the curvature required for an o/w emulsion once phase inversion had taken place.[90] This is a complex system because heating the hydrophilic Tween (usually above its cloud point) markedly reduces its hydrophilicity which leads to phase inversion.

The mixed micelle system produced the most stable system regardless of the type of surfactant used. The stability of the mixed micelles system was thought to be due to the interaction of surfactant and co-surfactant and hydration of the lipophilic surfactant in the hydrophilic surfactant rich aqueous phase.[27, 38] An increased interfacial tension was observed in the mixed micelle system during order of mixing trials. The lipophilic surfactant could solubilise in micelles of hydrophilic surfactant, depending on the ratio, various types of micellar structures are formed.[47] The change in interfacial properties indicated the formation of micelles and/ or vesicles of mixed emulsifiers in the aqueous phase.[211] The interaction between lipophilic and hydrophilic surfactants in the aqueous phase was again evident during formulation optimisation, where the surface tension of the mixed micelle system initially increased with the increase of the Ws/Wco ratio and then decreased as the ratio increased further. The lipophilic co-surfactant dispersion in water prior to emulsification formed stable droplets compared with other emulsification techniques. However, emulsions containing lecithin had noticeable amounts of sediments of lecithin vesicles at the bottom of centrifugation tube. The formation of larger vesicles and micelles of lecithin in the presence of Tween 80 has also been reported earlier by Lim.[92] A noticeable effect of surfactant and co-surfactant concentrations and Ws/Wco ratio on the stability of emulsions, especially the improved the stability at the Ws/Wco ratio between 0.5-4 (w/w) was considered to be due to the interaction of lipophilic co-surfactant with hydrophilic surfactant rich aqueous phase. The interaction between surfactants was resulted in an increased viscoelastic of the mixed surfactant layer at the oil/water interface.[27, 38] Emulsion characterisation through microscopy also revealed the formation of multilamellar vesicles containing oil. This confirmed that stability of emulsions was due to the formation of the condensed and expanded multilayer of the hydrated surfactant system.[27] The instability with increased or decreased Ws/Wco ratio could be

explained as the disruption of lipid bilayers corresponding with change in Ws/Wco ratio in the aqueous system.[72]

Three types of high shear mechanisms including high pressure homogeniser, rotor stator and ultrasonication are considered the most efficient processes to produce emulsions containing fine droplets, and were evaluated for their effectiveness in droplet size reduction and feasibility for scale up.[212] High pressure homogenisation was found to be the most efficient process in reducing droplet size with mean droplet diameters D(3,2) of <0.5 μm. The efficiency of high pressure homogenisation was due to reduction of larger droplets, especially droplets larger than 1 μm in diameter.[101] The crucial benefit of homogenisation was its ability to pass each droplet through a high shear zone and increase the residence time of droplets by multiple passes of emulsions.[102]

Emulsions produced by sonication had a wider droplet size distribution containing large droplets and were unstable during centrifugation. Other researchers also suggested the wider droplet size distributions were produced by sonication and optimal conditions were required to produce a stable emulsion by ultrasonication.[45] Maa and Hsu also reported the reduced efficiency of sonication with increased viscosity.[212] On the other hand, high intensity and cavitational collapse were reported to increase the temperature during sonication, which was observed at a high amplitude of 60%.[105] This would affect the HLB of the mixed emulsifier and could influence optimum emulsification. Of the three mechanisms, sonication was the least suitable emulsification process for up-scaling due to the significant increase in temperature and uneven distribution of energy.

Emulsions with mean droplet sizes D(3,2) of ≤ 2 μm showed enhanced stability during accelerated studies. For small droplets the aggregation was expected to reverse due to Brownian motion, considering the energy minimum is of the same order as the mean thermal energy.[115] At the same time a lower secondary minimum could be expected with increased ratio (δ/r) of adsorbed layer δ and droplet radius r, which reduces droplet coagulation and subsequently droplets coalescence.[46] In the case of the rotor-stator, the rotor speed of 15,000 rpm produced emulsions with a mean droplet size of 1.50 μm, where the surface area distribution showed that more than 85% of droplets were ≤ 2 μm diameter. Also, there are number of inline and overhead mixers available for reasonably up scaled size without losing the

equipment efficiency.[142, 212] The high pressure homogenisation or ultrasonication along with rotor mixers could also be used more effectively to reduce the droplets size, especially the droplets or aggregates larger than 1 µm.[101] The rotor mixers were selected for a scale up process, since they offered better efficiency and an ability to upscale the process in conjunction with other technology.

The quality of emulsions and the droplets size of emulsions was based on the speed and duration of mixing. The droplet breakup was also found to take place close to the agitator and was reported to be dependent on the shear rate through the agitator tip speed.[99] Considering the effect of rotor speed on the quality of emulsions and to evaluate the efficiency of the mixer at the production scale with time, the rotor speed of all scale up equipment was kept at the maximum allowable speed for the production scale and duration of mixing was kept in increments of 10 min and a maximum up to 30 min.

To increase the probability of the oil droplets would pass through the high shear zone of the rotor and subsequently improve the efficiency of their break up, the drop wise addition of the oil phase during emulsification was adopted. As reported earlier, the addition of oil close to agitator was found to be effective in droplets break-up.[99] However, the presence of larger droplets was obvious from the bimodal distribution of the log normal droplet distribution curves. The presence of large droplets could be due to the escape of some droplets away from the agitator path and loss of the shear force over increasing distance. The in-line mixers were also evaluated during scale up, which was designed increase the droplet break up and probability of their passing through the high shear zone. At the same time, the small tail of larger droplets always existed as an inherent outcome of homogenisation including high pressure homogenisation. There was also a marked difference in the mean droplet diameter D(3,2) among the mixers due to significant differences in rotor speed and the stator geometry. The smallest mean D(3,2) of ~1.5 µm was achieved by the Diax 900 and Silverson L4R (fine screen). During optimisation trials formulations containing 4-5% w/w Tween 80 and 2% w/w lecithin were established to be the most stable. Based on the observations made during development, optimisation and scale-up trials, the final emulsion was prepared using 4.5% of Tween 80 and 2% lecithin for clinical evaluation of the emulsion

formulation containing local anaesthetic. This was to evaluate its use as a platform delivery vehicle.

To evaluate the drug delivery system for clinical applicability, an emulsion containing 4.5% of Tween 80, 2% lecithin, 15% soybean oil and 3% lidocaine was prepared and evaluated for its efficacy and toxicity for spray on postoperative analgesia for split-skin donor sites. Pain during the dressing change of skin graft site is intense and an important factor for patients, which induces significant anxiety and stress.[162, 199] Topical lidocaine, a potent local anaesthetic, can be used for pain management without the side effects associated with opioids.[161] Reports of toxicity associated with topical use of a lidocaine to mucosal membranes leading to rapid absorption have limited its use in the treatment of burns and open wounds.[156, 162, 204] Bulmer et al. showed that the use of a dispersed system containing lidocaine for short-term postoperative analgesia at split-skin donor sites was safe.[203] Sinclair et al. reported significantly lower pain scores and long lasting reduction of pain after lidocaine based aerosol administration.[168] The CPS spray unit was selected as the primary packaging and delivery device. The CPS spray is designed to deliver an accurate dose of 0.135g ±0.002 with an evenly distributed spray pattern (diameter of 40.1mm ± 2.1), while maintaining microbial integrity during and after use. This allowed the development of a preservative free sterile product for use on open wounds during the clinical study.

Emulsions stability was evaluated for peroxide value, pH, droplet size, phase separation and assay of lidocaine, and was found to be stable for close to 2 years at room temperature (25 °C). The droplet stability of the emulsion was considered to be due to the formation of the finer droplet size and multilayer structure of adsorbed emulsifier surrounding the droplets.[27, 46] Creaming and viscosity were found to be qualitative parameters and were did not directly influence emulsion stability during the development and optimisation stage. The increase in peroxide value and decrease in pH were obvious signs of oxidation and hydrolysis of the lipids emulsions during the period of the stability study and required monitoring for the period of the shelf-life[58] There is no specific limit for the peroxide value of emulsion formulations. However, the peroxide value of the emulsion was well below the pharmacopoeial (BP) limit of 10 mEq/kg for refined soybean oil and 5 mEq/kg for soybean oil intended for use in parenteral preparations.[213] The linolenic

and linoleic contents of soybean oil render it susceptible to oxidation. Lidocaine was reported to undergo pH dependent hydrolysis in an aqueous medium.[144] In this study lidocaine degradation was also pH dependent with possible initially zero order reaction. The shelf-life of 680 ± 15 days was derived from interpolation of the lidocaine concentration data and inclusion of experimental error of the lidocaine assay over 910 days. The shelf=life is based upon lidocaine chemical instability as the emulsion physical parameters were still acceptable after 910 days.

The clinical trial was a randomised double–blind controlled trial comparing the efficacy of the emulsion and aqueous solution containing lidocaine in patients undergoing a painful dressing change. The emulsion and aqueous solutions contained lidocaine base and lidocaine HCl respectively. The rating of pain is commonly evaluated by VAS, VRS and NRS rating scale.[193] The NRS provides interval level data and was reported to be as sensitive as the VAS, easy to administer, record and allows different intensity levels from 0 to 10 points. As a tool for pain assessment the NRS was considered to be more useful for quantitative analysis of procedural pain due to its sensitivity and ability to report intensity of pain.[193, 195]

Previous reports by Jellish et al. and Derek et al. described lidocaine based topical application to be a safe and effective analgesic for donor sites.[160, 191] In the clinical study we found that both treatments containing lidocaine were equally effective in managing procedural pain related to dressing change with no reported toxicity of lidocaine and measured plasma concentration was well below 3-5 µg/ml. The emulsion appeared to provide slower release and a slightly longer lasting anaesthetic effect compared with aqueous treatment. The dispersed systems containing lidocaine applied to burns and split skin donor sites were reported to be effective analgesia with plasma concentrations well below the toxic threshold.[186, 202, 203] A study by Jellish et al. using topical lidocaine spray found blood concentrations to be independent of the dose of lidocaine used.[160] In the clinical study the wound size of 73 cm^2 and maximum dose of 3 mg/kg was used to minimise the risk of toxic effects. The clinical study found that the emulsion spray formulation containing lidocaine provided a safe local analgesia potentially on much larger wound areas than the one used in the trial. This finding can be supported by Bredfolt et al. who reported the safe lidocaine plasma concentration

of 1.0 µg/ml or less after an application of lidocaine cream to an area as large as 2000 cm^2.[186]

5.2 Conclusions

A submicron sized emulsion was developed, optimised and evaluated for use in a clinical environment. The HLB system was found to be a useful tool for the formulation and development of the emulsions. It was evident that the HLB system o/w emulsions prepared at HLB higher than that of the RHLB reported for soybean oil had improved stability. The most stable o/w emulsion for soybean oil (HLB 7) was produced at HLB 11 at an emulsifier ratio of 0.67/0.33. This could indicate error in the RHLB value recorded for this oil.

The order of mixing (emulsification method) and the type of surfactant used were critical in the formation of a stable emulsion. Emulsions stability was also influenced by the type and amount of solvent used, solubility of the lipophilic surfactant and coalescence of droplets. Mixed micelle systems produced the most stable emulsion system and were the least affected by the type of surfactant used based on an accelerated stability under centrifugal force. The stability of emulsions produced by mixed micelle systems was probably due to the formation of condensed and hydrated multi-lamellar structures containing oil. The lipophilic surfactant concentration was of important in the formation and stability of the emulsions formed because of the formation of multi-lamellar structures containing oil. The ratio of hydrophilic and lipophilic surfactants was critical for the stability of emulsions with stable emulsions produced using a Ws/Wco ratios between 0.5-4.

Emulsions were produced most efficiently using high pressure homogenisation; while ultrasonication was found impractical for the emulsification process. The emulsification using a rotor mixer was easy to scale up and can be used in conjunction with other technology. The rotor-stator was selected for the scale up due to its capability to achieve a mean droplet sizes D(3,2) of <2μm. Drop-wise or slow addition of the oil phase to the aqueous phase during mixing further reduced the droplet size. The size of the holes on the screen was also important for maximum droplet size reduction. The rotation speed and mixer design were found to be the most important parameters in the formation of stable emulsions and reduction of the droplet size. In-line rotor mixers can also be used efficiently by continuous mixing, where the oil phase is meter fed close to the rotor/stator work head. When emulsions were prepared using in-line mixers, the effect of rotation speed on droplet

size was significant. The experiment also recognized that the setup of in-line mixers was important and the best outcome could possibly be achieved by continuous mixing, where the oil phase is meter fed just adjacent to the rotor/stator work head and the final product being collected in the vessel. The best outcomes for up scaled batches were achieved by Diax 900 and Silverson L4R (fine screen) homogenisers.

During the stability study at ambient temperature (25 °C), emulsions produced by the mixed micelles methodology were stable over the period of 30 months with small changes in peroxide value and pH reduction. Droplet size of the emulsions was not affected over a period of 30 months. At the same time, droplet size analysis alone was not sufficient to establish the quality of the emulsion for the purpose of shelf-life. The instability of the final emulsion containing lidocaine was due to hydrolysis of lidocaine. The emulsion containing lidocaine had a pharmaceutically acceptable shelf-life of just over 2 years.

A double-blind randomised controlled, pilot trial in 29 patients showed a significant analgesia for procedural pain during dressing changes and provided similar outcomes in pain management. In the clinical study, a spray application of lidocaine proved an effective delivery system which provided analgesia immediately after application. The plasma concentrations of lidocaine for the aqueous control treatment were significantly higher than the emulsion treatment group. In a clinical trial set up, the emulsion based topical local anaesthetic provided effective analgesia and a higher margin of safety due to the low rate of absorption of lidocaine. Hence the emulsion was found to be a safe and effective delivery system for lidocaine to painful wounds.

5.3 Future Studies

Considering the interaction between lecithin and Tween 80 in the aqueous phase, it would be important to analyse the interfacial behaviour of mixed emulsifier systems for their film forming ability at different concentration ratios (Ws/Wco). The amount of lecithin and surfactant ratio Ws/Wco was also found to be important in the stability of the emulsion. The ability of surfactants to alter the surface properties such as surface and interfacial tension, elasticity of the film and the force required to break the film would be an essential part of the interfacial study using a the Langmuir trough.

The formation of a multi-lamellar structure was evident from the microscopic analyses. An in-depth microscopic study using Cryo TEM along with Confocal Raman Microscopy (CRM), and a light microscope would provide greater understanding of the multilamellar structure created by the mixed emulsifier system and the effect of the surfactant concentration and their ratios on multilamellar structures.

The hydration of lecithin is central to emulsion stability. The effect of Tween 80 on the uptake of water by lecithin, the swelling behaviour of the mixed emulsifier system and the overall stability of the emulsions need to be addressed. This study can be expanded using cryo TEM along with x-ray diffraction.

The change in pH and peroxide value was observed during the stability study. The effect of oxidation and hydrolysis of fatty acids and fatty acids triglycerides from the oil phase and surfactants used is of importance. The current study did not focus on the direct relationship between chemical degradation and physical stability. These correlations will help in the understanding of factors impacting on these parameters. At the same time, there are no specific pharmacopoeial limits set for peroxide values and pH changes due to hydrolysis for pharmaceutical emulsions. Both parameters are critical to overall performance of the emulsion as drug delivery systems including drug release, especially relating to stability of active ingredient and toxicity.

6 Appendix

Appendix 6-1 Clinical Trial Approval from Human Research Ethics Committee of Royal Perth Hospital

Department of Health
Government of Western Australia
South Metropolitan Area Health Service

Royal Perth Hospital

ETHICS COMMITTEE

Prof F M van Bockxmeer PhD MHGSA, ARPCA, FAHA
PathWest Laboratory Medicine
Tel: 9224 2322 Fax: 9224 2491
Email Frank.VB@health.wa.gov.au

Room 4112 Level 4, Kirkman House
Tel: 9224 2292

Ref: EC 2008/183 **2nd April 2009**
(This number must be quoted on all correspondence)

Prof Stephan Schug Pain
Medicine
Royal Perth Hospital

Dear Stephan

EC 2008/183 Double-blind randomised pilot trial to compare effects and patient acceptance of NS NOPAYNE lignocaine spray with standard lignocaine spray for pain relief in donor-site dressing changes of burns patients

Thank you for your responses to the queries raised by the Ethics Committee and the DTC. I am pleased to advise that the above study is now **APPROVED.**

> Please note that Principal Investigators conducting clinical trials at RPH require approval letters from both the Ethics Committee and the Governance Unit (CTBU) *before a trial can commence*.

The following general conditions apply to all approvals by this Committee, and starting a trial or research project following the issue of ethics approval will be deemed to be an acceptance of them by all investigators:

1. The submission of an application for Ethics Committee approval will be deemed to indicate that the investigator and any sponsor recognises the Committee as a registered (with AHEC) Health Research Ethics Committee and that it complies in all respects with the National Statement on Ethical Conduct Research Involving Humans and all other national and international ethical requirements. **The Committee will not enter into further correspondence on this point.**

2. All income arising from the study must be lodged in a hospital special purposes account. Performance of a clinical trial for a sponsor is a service for tax purposes and all GST obligations must be met.

3. The investigator will report adverse events accompanied by a statement as to whether or not the trial should continue. The Committee reserves the right to not receive reports whose complexity or level of detail requires the expenditure of unreasonable time and effort. The Committee receives voluminous paperwork relating to adverse event reporting. From time to time the Committee chairman may require these reports to be summarised and approval is granted subject to the agreement of the investigator that he or she will prepare such a summary on request.

4. The Committee has decided that, as the responsibility for the conduct of trials lies with the investigator, all correspondence should be signed by the investigator.

5. All trial drugs must be dispensed by the Pharmacy Department. A fee is levied for this service and investigators must regard this fee as an item requiring a budget

allocation. Alternatively, if a sponsor agrees, separate direct funding of pharmacy services may be undertaken. There are provisions for this fee to be waived for locally-inspired unfunded studies not having an external sponsor.

1. Though state institutions are outside the jurisdiction of the Privacy Act and related legislation, the Committee will assume that the privacy provisions of that Act will be the minimum standards applying during the conduct of a trial at Royal Perth Hospital. Traditional standards of patient confidentiality will apply.

2. The Committee will not acknowledge trial communications as a matter of course, unless they relate to a matter requiring Committee approval. Evidence of dispatch of a letter will be deemed to be evidence of receipt. This rule may be waived at the Committee's discretion on provision of a *pro forma* receipt by the investigator for the Chairman's signature and return. However, trivial correspondence (as judged by the Committee) will not be acknowledged even if a *pro forma* receipt is provided. Where an investigator requests written approval or written record of a matter for special purposes (say at the request of a sponsor), the investigator should prepare the required letter for the chairman's signature rather than expect the Committee secretary to prepare it. This mechanism increases the probability that the trial details in the letter are correct.

3. The Committee will provide the names and representative affiliation of members on request, but will not provide personal details or voting records.

4. A brief annual report on each project approved will be required at the end of each fiscal year, in default of which approval for the study may be suspended. Ethics approvals at RPH do not carry an expiry date so the annual report is an important part of Ethics Committee procedure.

5. The Committee has the authority to audit the conduct of any trial without notice. Exercise of this authority will only be considered if there are grounds to believe that some irregularity has occurred or if a complaint is received from a third party, or the Committee wishes to undertake an audit for QA purposes.

6. Complaints relating to the conduct of a clinical trial should be directed to the Chairman and will be promptly investigated. Complaints about the Ethics Committee decisions or policies that cannot be resolved by discussion with the Chairman or about any actions of a particular member including the Chairman, should be directed to the Director of Clinical Services. Only written complaints (not e-mail) will be accepted for investigation.

Investigators of sponsored studies are advised to draw the above conditions to the attention of the sponsor. Investigators are reminded that records of consent or authorisation for participation in special studies (including clinical trials) form part of the Acute Hospital Patient Record and should be stored with that record in accordance with the WA *Health Patient Information Retention and Disposal Schedule (Version 2) 2000.* A copy of the 'Patient Information Sheet' should also be included in the medical records as part of informed consent documentation.

Yours sincerely

Prof Frank van Bockxmeer
Chairman, Royal Perth Hospital Ethics Committee

Appendix 6-2 Clinical Trial Approval from Human Research Ethics Committee of Curtin University

Curtin University

Memorandum

To	Mr Chiragkumar Desai, School of Pharmacy,
From	Professor Stephan Millett, Chair, Human Research Ethics Committee
Subject	Protocol Approval **HR 151/2012**
Date	18 December 2012
Copy	Professor Stephan Schug, Anaesthesia and Pain Medicine, RPH, Prof. Fiona Wood, RPH,

Office of Research and Development
Human Research Ethics Committee

TELEPHONE 9266 2784
FACSIMILE 9266 3793
EMAIL hrec@curtin.edu.au

Thank you for your application submitted to the Human Research Ethics Committee (HREC) for the project titled "*Double-Blind Randomised Pilot Trial to Compare Effects and Patient Acceptance of NS NOPAYNE Lignocaine Spray with Standard Lignocaine Spray for Pain Relief in Donor Site Dressing Changes of Burns Patients*". The Committee notes the prior approval by Royal Perth Hospital Ethics Committee (EC2008/183) and has reviewed your application consistent with Chapter 5.3 of the *National Statement on Ethical Conduct in Human Research*.

- You have ethics clearance to undertake the research as stated in your proposal.
- The approval number for your project is **HR 151/2012.** *Please quote this number in any future correspondence*.
- Approval of this project is for a period of twelve months **18-12-2012** to **18-12-2013**. To renew this approval a completed Form B (attached) must be submitted before the expiry date **18-12-2013**.
- If you are a Higher Degree by Research student, data collection must not begin before your Application for Candidacy is approved by your Faculty Graduate Studies Committee.
- The following standard statement **must be** included in the information sheet to participants:

  This study has been approved by the Curtin University Human Research Ethics Committee (Approval Number HR 151/2012). The Committee is comprised of members of the public, academics, lawyers, doctors and pastoral carers. If needed, verification of approval can be obtained either by writing to the Curtin University Human Research Ethics Committee, c/- Office of Research and Development, Curtin University, GPO Box U1987, Perth, 6845 or by telephoning 9266 2784 or by emailing hrec@curtin.edu.au.

Applicants should note the following:

It is the policy of the HREC to conduct random audits on a percentage of approved projects. These audits may be conducted at any time after the project starts. In cases where the HREC considers that there may be a risk of adverse events, or where participants may be especially vulnerable, the HREC may request the chief investigator to provide an outcomes report, including information on follow-up of participants.

The attached **FORM B** should be completed and returned to the Secretary, HREC, C/- Office of Research & Development:
When the project has finished, or

- If at any time during the twelve months changes/amendments occur, or
- If a serious or unexpected adverse event occurs, or
- 14 days prior to the expiry date if renewal is required.
- An application for renewal may be made with a Form B three years running, after which a new application form (Form A), providing comprehensive details, must be submitted.

Yours sincerely

Professor Stephan Millett
Chair Human Research Ethics Committee

Appendix 6-3 Individual patient treatment, absorption time, weight, wound size and amount of lidocaine used

Subject Number	Treatment	Total Absorption time (min)	Subject Weight (Kg)	Donor Size (cm2)	Amount of lignocaine (mg)	Lidocaine Dose(mg)/ 70Kg
1	NOPAYNE	70	87.6	24	25.68	20.52
3	NOPAYNE	49.5	69	36	61.14	62.03
4	NOPAYNE	68	78	32	25.17	22.59
9	NOPAYNE	75	68	0	9.84	10.13
11	NOPAYNE	72.5	75.7	200	60.33	55.79
12	NOPAYNE	71	65	85	54.12	58.28
14	NOPAYNE	169	82.5	45	22.41	19.01
17	NOPAYNE	47.5	87.2	40	43.17	34.65
18	NOPAYNE	170	108	40	31.35	20.32
19	NOPAYNE	66.5	55	225	65.85	83.81
24	NOPAYNE	47.5	115	300	229.62	139.77
25	NOPAYNE	125	87.9	120	23.1	18.4
26	NOPAYNE	68	70	15	28.98	28.98
30	NOPAYNE	39	80.4	35	11.76	10.24
32	NOPAYNE	62.5	95	8	42.99	31.68
2	Xylocaine	55	60	100	47.56	55.49
5	Xylocaine	63	74.8	84	88.04	82.39
6	Xylocaine	65	82.5	15	3.2	2.72
8	Xylocaine	61	68	80	119.12	122.62
10	Xylocaine	65	85	15	8.8	7.25
13	Xylocaine	42.5	78	120	142.48	127.87
15	Xylocaine	82	54	40	20.52	26.6
16	Xylocaine	71	75	15	46.12	43.05
22	Xylocaine	32.5	92.5	75	309.36	234.11
28	Xylocaine	66.5	131.45	144	57.36	30.55
29	Xylocaine	65.5	69.7	70	47.08	47.28
33	Xylocaine	59	126.9	140	10.8	5.96
34	Xylocaine	61.5	65.7	60	13.12	13.98

Appendix 6-4 Primary outcomes: sting rating and pain scores

Patient Number	Treatment	Sting Rating	Pain score 2 mins	Pain score 5-10 mins	Pain score 60 mins	Patient Satisfaction	Final Pain Score
1	NOPAYNE	1	3	4	2	1	0
3	NOPAYNE	1	2	2	4	1	2
4	NOPAYNE	0	0	0	0	1	0
9	NOPAYNE	1	2	0	0	1	0
11	NOPAYNE	0	0	4	2	2	2
12	NOPAYNE	1	4	4	2	2	2
14	NOPAYNE	0	0	1	0	2	1
17	NOPAYNE	0	0	0	0	1	0
18	NOPAYNE	0	2	0	0	1	0
19	NOPAYNE	1	3	3	2	2	3
24	NOPAYNE	1	4	3	0	3	0
25	NOPAYNE	0	2	4	2	1	2
26	NOPAYNE	0	3	3	2	1	2
30	NOPAYNE	0	0	0	0	1	0
32	NOPAYNE	1	4	4	3	1	3
2	Xylocaine	1	0	0	1	1	0
5	Xylocaine	1	2	0	0	1	0
6	Xylocaine	0	0	0	0	1	0
8	Xylocaine	1	3	4	5	2	4
10	Xylocaine	0	0	0	0	2	0
13	Xylocaine	1	0	2	4	3	4
15	Xylocaine	0	3	3	1	1	1
16	Xylocaine	1	2	1	1	1	1
22	Xylocaine	0	1	0	2	1	2
28	Xylocaine	1	0	0	3	1	3
29	Xylocaine	0	2	2	2	2	2
33	Xylocaine	0	4	5	2	2	2
34	Xylocaine	0	2	3	2	1	2

Appendix 6-5 Lidocaine delivered, number of sprays and plasma concentration per 70kg adult weight

Patient Number	Treatment	Amount of lignocaine (mg)	Lidocaine Dose (mg)/ 70Kg	No of Sprays Used	No of Sprays/ 70Kg	Post-plasma concentration (mg/L)	SLPC (mg/L)	SLPC conc/ 60 min (mg/L)
1	NOPAYNE	25.68	20.52	6	5	0.014	0.018	0.019
3	NOPAYNE	61.14	62.03	15	15	0.012	0.012	0.011
4	NOPAYNE	25.17	22.59	6	6	0.006	0.007	0.007
9	NOPAYNE	9.84	10.13	2	3	0.029	0.028	0.031
11	NOPAYNE	60.33	55.79	15	14	0.005	0.006	0.006
12	NOPAYNE	54.12	58.28	13	14	0.07	0.065	0.069
14	NOPAYNE	22.41	19.01	6	5	0.005	0.006	0
17	NOPAYNE	43.17	34.65	11	9	0.047	0.059	0.054
18	NOPAYNE	31.35	20.32	8	5	0.019	0.029	0.055
19	NOPAYNE	65.85	83.81	16	21	0.079	0.062	0.064
24	NOPAYNE	229.62	139.77	57	35	0.005	0.008	0.008
25	NOPAYNE	23.1	18.4	6	5	0.23	0.289	0.42
26	NOPAYNE	28.98	28.98	7	7	0.005	0.005	0.005
30	NOPAYNE	11.76	10.24	3	3	0.005	0.006	0.005
32	NOPAYNE	42.99	31.68	11	8	0.005	0.007	0.007
2	Xylocaine	47.56	55.49	9	10	0.037	0.032	0.031
5	Xylocaine	88.04	82.39	16	15	0.12	0.128	0.13
6	Xylocaine	3.2	2.72	1	1	0.006	0.007	0.007
8	Xylocaine	119.12	122.62	22	23	0.084	0.082	0.082
10	Xylocaine	8.8	7.25	2	1	0.038	0.046	0.047
13	Xylocaine	142.48	127.87	26	24	0.29	0.323	0.292
15	Xylocaine	20.52	26.6	4	5	0.025	0.019	0.022
16	Xylocaine	46.12	43.05	9	8	0.027	0.029	0.031
22	Xylocaine	309.36	234.11	57	43	0.6	0.793	0.677
28	Xylocaine	57.36	30.55	11	6	0.1	0.188	0.195
29	Xylocaine	47.08	47.28	9	9	0.005	0.005	0.005
33	Xylocaine	10.8	5.96	2	1	0.021	0.038	0.038
34	Xylocaine	13.12	13.98	2	3	0.025	0.023	0.024

Appendix 6-6 Age, sex, microbiology swab, container sterility and infection rate

Patient Number	Age	Sex	Treatment	Swab Microbiology	Bottle sterility post use	Infection
1	22	M	NOPAYNE	Healed	pass	No
3	26	M	NOPAYNE	Moderate	pass	No
4	49	F	NOPAYNE	Healed	pass	No
9	56	M	NOPAYNE	Healed	pass	No
11	33	F	NOPAYNE	Healed	pass	No
12	47	F	NOPAYNE	Healed	pass	No
14	23	M	NOPAYNE	Healed	pass	No
17	46	M	NOPAYNE	Healed	pass	No
18	43	F	NOPAYNE	Healed	pass	No
19	19	M	NOPAYNE	Healed	pass	No
24	17	M	NOPAYNE	Healed	pass	No
25	41	M	NOPAYNE	Healed	pass	No
26	57	M	NOPAYNE	Healed	pass	No
30	40	F	NOPAYNE	Healed	pass	No
32	20	M	NOPAYNE	Healed	pass	No
2	37	M	Xylocaine	Infected	pass	Yes
5	49	M	Xylocaine	Healed	pass	No
6	20	F	Xylocaine	Healed	pass	No
8	54	M	Xylocaine	Healed	pass	No
10	19	M	Xylocaine	Infected	pass	Yes
13	50	F	Xylocaine	Healed	pass	No
15	22	M	Xylocaine	Healed	pass	No
16	43	M	Xylocaine	Healed	pass	No
22	24	M	Xylocaine	Healed	pass	No
28	30	M	Xylocaine	Healed	pass	No
29	34	M	Xylocaine	Healed	pass	No
33	42	F	Xylocaine	Healed	Bottle Broken	No
34	57	F	Xylocaine	Healed	pass	No

List of award, patent, presentation and publications

VII. **Curtin Commercial Innovation Awards 2012:** Chiragkumar Desai, Miniemulsions for Drug Delivery, 2012; Curtin University, Perth.

VIII. **Patent:** Chiragkumar Desai, Method of forming miniemulsions and use thereof for delivering bioactive agents, 2010.

IX. **Poster presentation:** Desai C, Wood FM, Schug SA, Parsons RW, Fridlender C, Sunderland VB. Development of a Novel Mini-emulsion Based Topical Formulation of Lignocaine for Application to Open Wounds, 2012; FIP Congress, Amsterdam.

X. **Publication:** Desai C, Wood FM, Schug SA, Parsons RW, Fridlender C, Sunderland VB. Effectiveness of a topical local anaesthetic spray as analgesia for dressing changes: A double-blinded randomised pilot trial comparing an emulsion with an aqueous lidocaine formulation. Burns. 2014; 40(1):106-112.

XI. **Oral Presentation:** Desai C, Sunderland VB. A Novel Mini-emulsion as drug delivery: Development and Topical Application for Anaesthesia, 2013; The Society of Hospital Pharmacists of Australia, Perth.

XII. **Oral Presentation:** Desai C, Parsons R, Sunderland VB. Development of a Novel Emulsion Based Formulations and its Application, The Mark Liveris Health Sciences Research Seminar, 2014; Curtin University, Perth

Appendix 6-7 Patent: Method of forming miniemulsions and use thereof for delivering bioactive agents

1. (WO2012075534) METHODS FOR FORMING MINIEMULSIONS AND USE THEREOF FOR DELIVERING BIOACTIVE AGENTS

PCT Biblio. Data | Description | Claims | National Phase | Notices | Drawings | Documents

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Title **(EN)** METHODS FOR FORMING MINIEMULSIONS AND USE THEREOF FOR DELIVERING BIOACTIVE AGENTS
(FR) PROCÉDÉS DE FORMATION DE MINIÉMULSIONS ET UTILISATION ASSOCIÉE POUR L'ADMINISTRATION D'AGENTS BIOACTIFS

Abstract:

(EN) The present invention relates to methods of forming miniemulsions and use of the miniemulsions as a delivery system for bioactive agents. In particular, the present invention relates to methods for forming a miniemulsion comprising providing a first phase comprising a hydrophilic surfactant, lipophillic surfactant and water and a second phase comprising a lipid, wherein said miniemulsion comprises emulsified particles having a mean diameter of 1µm or less.
(FR) La présente invention concerne des procédés de formation de miniémulsions et l'utilisation des miniémulsions en tant que système d'administration d'agents bioactifs. La présente invention concerne en particulier des procédés de formation d'une miniémulsion comprenant la fourniture d'une première phase comprenant un surfactant hydrophile, un surfactant lipophile et de l'eau et d'une seconde phase comprenant un lipide, ladite miniémulsion comprenant des particules émulsifiées ayant un diamètre moyen de 1 µm ou moins.

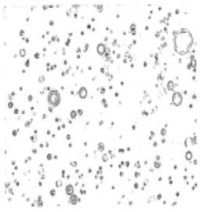

FIGURE 1

Designated States: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
African Regional Intellectual Property Organization (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW)
Eurasian Patent Organization (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM)
European Patent Office (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR)
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Appendix 6-8 Poster presentation: Development of a novel mini-emulsion based formulation for the topical delivery of lidocaine

Curtin University

Development of a Novel Mini-emulsion Based Formulation for the Topical Delivery of Lidocaine

Authors: Chiragkumar Desai[1,4], Fiona M. Wood[2], Stephen A. Schug[3], Richard W Parsons[1], Charles Fridlender[4], Vivian Bruce Sunderland[1]

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OBJECTIVE

To develop an emulsion based topical formulation for local anaesthetic delivery, which is stable for at least 1 year and potentially reduces systemic absorption of drugs.

INTRODUCTION

Emulsions containing mixed emulsifier systems (surfactant and co-surfactant) with a droplet size in the range of less than 1µm, typically of a size between 100 and 700 nm are often referred to as miniemulsions.[1] Topically applied lidocaine has been used for analgesia in several clinical settings. Local anaesthetic use on open wounds carries the potential risk of enhanced systemic absorption. Also, there has been a lack of sterile topical formulations containing local anaesthetic for use on open wounds.[2]

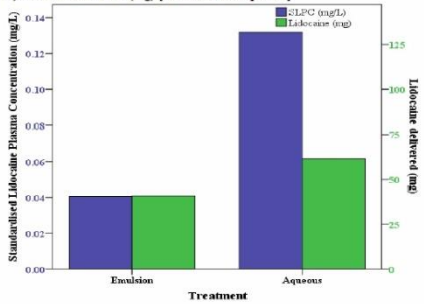
Table 1: Optimisation of mini-emulsion formulations in different ratios of surfactant and co-surfactant

Parameters	Form.1	Form.2	Form.3	Form.4	Form.5	Form.6	Form.7
Lecithin (% w/w)	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Tween 80 (%w/w)	0.5	0.75	1.0	1.50	2.25	3.0	4.5
Mean Particle size (µm)	0.70	0.72	0.68	0.65	0.66	0.68	0.73
Phase separation	Stable	Stable	Stable	Stable	Stable	Less Stable	Less stable
% Particle (<1 µm)	77.72	77.01	79.69	81.43	81.45	77.72	79.41

Table 2: Stability study of a Clinical Trial Batch (formulation 4) at 25° ± 2° C temperature up to 30months

Months→ Test ↓	3	6	9	14	15	18	30
% Assay	99.74	96.39	92.24	94.94	99.70	91.26	89.00
Mean Particle Size (µm)	0.62	0.62	0.65	0.67	0.67	0.64	0.67
pH	8.40	8.65	8.00	7.75	7.75	7.45	7.10
Peroxide Value (mEq/kg)	-	3.00	2.00	1.55	4.00	1.50	1.89

Figure 1: Average distributions of Standardised lidocaine plasma concentration (SLPC) and lidocaine dose (mg) [Emulsion Vs Aqueous]



METHODS

Emulsion formulations were developed using soy oil as an oil phase, polysorbates and lecithin as surfactant and co-surfactant. Different concentration ratios of Tween 80® and lecithin were used. The effect of homogenisation on particle size reduction was determined for the final optimised formulation. Particle size (Mastersizer), pH, phase separation, microscopy, peroxide value and HPLC assay of lidocaine were used as primary parameters for stability evaluation. All the stability samples were stored at ambient temperature of 25 ± 2° C.
A randomised, double-blind, active-controlled, parallel pilot trial was carried out to evaluate safety and efficacy of the optimised product compared to a topical 4% lidocaine hydrochloride aqueous solution. Plasma concentrations of lidocaine were analysed by LC-MS-MS.

RESULTS

The ratio of co-surfactant/surfactant was critical to produce stable mini-emulsions. A ratio of 1:3 or more reduced emulsion stability significantly (Table 1). Homogeniser rotor speed had significant effect on particle size of mini-emulsion. Relationship of rotor speed and reduction in particles size was in agreement with the following relationship. [3] $d_{max} = \rho_c^{-0.6} \sigma^{0.6} N^{-1.2} D^{-0.8}$

The mini-emulsion based formulation was stable for up to 18 months without refrigeration (Table 2). It was a significant breakthrough for a pharmaceutical based emulsion technology.
Both formulations showed a significant analgesia for procedural pain during dressing changes and provided similar outcomes in pain management. The pain scores for mini-emulsion and aqueous were 1.3 ± 0.3 (mean ± SEM) and 1.8 ± 0.4 (p=0.98) respectively. Nearly 90% of patients were very satisfied with their treatment. The mean plasma concentrations of lidocaine for Aqueous and Emulsion were 0.132mg/L and 0.040mg/L respectively (p=0.0694, Figure1).

CONCLUSIONS

Mini-emulsion based formulations produced mean particle size of less than 1µm and significantly increased stability without refrigeration. In a clinical trial set up, the mini-emulsion based topical local anaesthetic provided an improved safety profile.

REFERENCE

[1] Lieberman HA, Rieger MM, Banker GS. Pharmaceutical dosage forms--disperse systems : in three volumes. 2nd ed. New York: M. Dekker; 1996; p. 91-152
[2] Patterson DR, Hoflund H, Espey K, Sharar S. Pain management. Burns. 2004; 30(8):A10-A15.
[3] Sprow FB. Drop size distributions in strongly coalescing agitated liquid-liquid systems. AIChE Journal. 1967; 13(5):995-998.

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Appendix 6-9 Publication (Journal Format): Effectiveness of a topical local anaesthetic spray as analgesia for dressing changes: A double-blinded randomised pilot trial comparing an emulsion with an aqueous lidocaine formulation

7 References

1. Spalton LM. Pharmaceutical emulsions and emulsifying agent. London: Chemist and Druggist; 1953.

2. Cooper JW. Emulsions and Creams. In: Cooper and Gunn's dispensing for pharmaceutical student. Tunbridge Wells: Tunbridge Wells : Pitman Medical; 1975. p. 120-167.

3. Attwood D. Disperse Systems. In: Aulton ME, editor. Pharmaceutics : the science of dosage form design. 2nd ed.. ed. Edinburgh: Edinburgh : Churchill Livingstone; 2002. p. 70-100.

4. Rieger MM. Emulsions. In: Lachman L, Liebermann HA, Kanig JL, editors. The Theory and practice of industrial pharmacy 3rd ed. Philadelphia: Lea & Febiger; 1986. p. 502-533.

5. Davis SS, Washington C, West P, Illum L, Liversidge G, Sternson L, et al. Lipid emulsions as drug delivery systems. Ann N Y Acad Sci. 1987; 507:75.

6. Rosen MJ, Kunjappu JT. Emulsification by Surfactants. In: Rosen MJ, Kunjappu JT, editors. Surfactants and Interfacial Phenomena. Fourth ed: John Wiley & Sons, Inc; 2012. p. 336-366.

7. Tamilvanan S. Oil-in-water lipid emulsions: implications for parenteral and ocular delivering systems. Prog Lipid Res. 2004; 43(6):489-533.

8. Hem SL, Feldkamp JR, White JL. Basic Chemical Principles Related to Emulsion and Suspension. In: Lachman L, Liebermann HA, Kanig JL, editors. The Theory and practice of industrial pharmacy. 3rd ed. Philadelphia: Lea & Febiger; 1986. p. 100-122.

9. Tadros TF. Applied surfactants : principles and applications Weinheim, Great Britain: Wiley; 2005.

10. Sarker DK. What is an emulsion? In: Pharmaceutical Emulsions: John Wiley & Sons, Ltd; 2013. p. 15-48.

11. McClements DJ, Decker EA, Weiss J. Emulsion-based delivery systems for lipophilic bioactive components. J Food Sci. 2007; 72(8):R109-R124.

12. Jean-Louis S. Emulsion Properties and Related Know-how to Attain Them. In: Pharmaceutical Emulsions and Suspensions: CRC Press; 2000. p. 73-125.

13. Giddi HS, Arunagirinathan MA, Bellare JR. Self-assembled surfactant nano-structures important in drug delivery: a review. Indian J Exp Biol [Review]. 2007; 45(2):133-59.

14. Sarker DK. Stability, Metastability and Instability. In: Pharmaceutical Emulsions: John Wiley & Sons, Ltd; 2013. p. 49-62.

15. Becker DE, Reed KL. Essentials of local anesthetic pharmacology. Anesth Prog. 2006; 53(3):98-108; quiz 109-10.

16. Mizushima Y. Lipid microspheres (lipid emulsions) as a drug carrier — An overview. Adv Drug Del Rev. 1996; 20(2):113-115.

17. Tomii Y. Lipid formulation as a drug carrier for drug delivery. Curr Pharm Des. 2002; 8(6):467-474.

18. Stevens J, Mims P, Colkes N. Lipid Emulsins as Drug Delivery Systems. Pharmatech. 2003;

19. Billany M. Suspensions and Emulsions. In: Aulton ME, editor. Pharmaceutics : the science of dosage form design. 2nd ed. Edinburgh: Churchill Livingstone; 2002. p. 334-359.

20. Friberg SE, Quencer LG, Hilton ML. Theory of Emulsions. In: Lieberman HA, Rieger MM, Banker GS, editors. Pharmaceutical dosage forms: disperse systems. 2nd ed., rev. and expanded.. ed. New York: Marcel Dekker; 1996. p. 53-90.

21. Slomkowski S, Aleman JV, Gilbert RG, Hess M, Horie K, Jones RG, et al. Terminology of polymers and polymerization processes in dispersed systems (IUPAC Recommendations 2011). Pure Appl Chem. 2011; 83(12):2229-2259.

22. Mishra BK, Valaulikar BS, Kunjappu JT, Manohar C. Influence of microemulsion structure on reaction rates. J Colloid Interface Sci. 1989; 127(2):373-376.

23. Marino H. Phase inversion temperature emulsification: from batch to continuous process. Bath: University of Bath; 2010.

24. Brouwer WM, El-Aasser MS, Vanderhoff JW. The role of water solubility of the oil phase in the formation of miniemulsions. Colloids Surf. 1986; 21:69-86.

25. Charles L, Anthony AA. Current State of Nanoemulsions in Drug Delivery. J Biomater Nanotech. 2011; 02(05):626.

26. Sarker DK. Engineering of nanoemulsions for drug delivery. Curr Drug Del. 2005; 2(4):297.

27. Friberg S, Jansson PO, Cederberg E. Surfactant association structure and emulsion stability. J Colloid Interface Sci. 1976; 55(3):614-623.

28. Rieger MM. Surfactants. In: Lieberman HA, Rieger MM, Banker GS, editors. Pharmaceutical dosage forms disperse systems Second ed. New York: New York : M. Dekker; 1996. p. 211-286.

29. Boyd JV. Rheological properties of two dimensional surface films. Rheologica Acta. 1971; 10(1):153-157.

30. Washington C. Stability of lipid emulsions for drug delivery. Adv Drug Del Rev. 1996; 20(2-3):131-145.

31. Pichot R, Watson RL, Norton IT. Phospholipids at the Interface: Current Trends and Challenges. Int J Mol Sci. 2013; 14:11767-11794.

32. Griffin WC. Classification of surface-active agents by "HLB". J Soc Cosmet Chem. 1949; 1(5):311-26.

33. Pasquali RC, Taurozzi MP, Bregni C. Some considerations about the hydrophilic-lipophilic balance system. Int J Pharm. 2008; 356(1-2):44-51.

34. James Swarbrick, Rubino JT, Rubino OP. Coarse Dispersion. In: Troy DB, Remington JP, editors. Remington : the science and practice of pharmacy. Philadelphia: Pharmaceutical Press; 2013.

35. Griffin WC. Calculation of HLB values of non-ionic surfactants. J Soc Cosmet Chem. 1954; 5:249.

36. Becher P. Emulsions : theory and practice / Paul Becher. Washington, D.C.: Washington, D.C. : American Chemical Society : Oxford University Press; 2001.

37. Lv GJ, Wang FM, Cai WF, Zhang XB. Colloid Surf. A-Physicochem. Eng. Asp. Characterization of the addition of lipophilic Span 80 to the hydrophilic Tween 80-stabilized emulsions, 2014

38. Boyd J, Parkinson C, Sherman P. Factors affecting emulsion stability, and the HLB concept. J Colloid Interface Sci. 1972; 41(2):359-370.

39. Schulman JH, Cockbain EG. Molecular interactions at oil/water interfaces. Part I. Molecular complex formation and the stability of oil in water emulsions. Transactions of the Faraday Society. 1940; 35:651-661.

40. Shinoda K, Saito H, Arai H. The effect of the size and the distribution of the oxyethylene chain lengths of nonionic emulsifiers on the stability of emulsions. J Colloid Interface Sci. 1971; 35(4):624-630.

41. Anton N, Vandamme TF. The universality of low-energy nano-emulsification. Int J Pharm. 2009; 377(1–2):142-147.

42. Shinoda K, Saito H. The Stability of O/W type emulsions as functions of temperature and the HLB of emulsifiers: The emulsification by PIT-method. J Colloid Interface Sci. 1969; 30(2):258-263.

43. Atiemo-Obeng VA, Calabrese RV. Rotor Stator Mixing Devices. In: Paul EL, Atiemo-Obeng VA, Kresta SM, editors. Handbook of industrial mixing : science and practice. New Jersey: Wiley; 2004. p. 479-505.

44. Pandolfe W. Homogenizers. In: Francis FJ, editor. Wiley encyclopedia of food science and technology. New York: New York : Wiley; 2000.

45. Jafari SM, He YH, Bhandari B. Nano-emulsion production by sonication and microfluidization - A comparison. Int J Food Prop. 2006; 9(3):475-485.

46. Anton N, Benoit J-P, Saulnier P. Design and production of nanoparticles formulated from nano-emulsion templates—A review. J Controlled Release. 2008; 128(3):185-199.

47. Rupp C, Steckel H, Muller BW. Mixed micelle formation with phosphatidylcholines: the influence of surfactants with different molecule structures. Int J Pharm. 2010; 387(1-2):120-8.

48. Takino T, Konishi K, Takakura Y, Hashida M. Long circulating emulsion carrier systems for highly lipophilic drugs. Biol Pharm Bull. 1994; 17(1):121-5.

49. Katrin B, Bernd WM. Emulsions as Drug Delivery Systems. In: Pharmaceutical Emulsions and Suspensions: CRC Press; 2000. p. 191-228.

50. Riess JG, Weers JG. Emulsions for biomedical uses. Curr Opin Colloid Interface Sci. 1996; 1(5):652-659.

51. Collins-Gold LC, Lyons RT, Bartholow LC. Parenteral emulsions for drug delivery. Adv Drug Del Rev. 1990; 5(3):189-208.

52. Lundberg B. Preparation of drug-carrier emulsions stabilized with phosphatidylcholine-surfactant mixtures. J Pharm Sci. 1994; 83(1):72-5.

53. Krishna G, Wood GC, Sheth BB. Improving emulsification efficacy of lecithin by formulation design. I: Effect of adding a secondary surfactant. PDA J Pharm Sci Technol. 1998; 52(6):331.

54. Floyd AG. Top ten considerations in the development of parenteral emulsions. Pharmaceutical Science & Technology Today. 1999; 2(4):134-143.

55. Kundu K, Paul BK. Physicochemical investigation of mixed surfactant reverse micelles: Water solubilization and conductometric studies. Colloids Surf Physicochem Eng Aspects. 2013; 433(0):154-165.

56. Yang R, Zhang X, Li F, Ding L, Li B, Sun H, et al. Role of phospholipids and copolymers in enhancing stability and controlling degradation of intravenous lipid emulsions. Colloids Surf Physicochem Eng Aspects. 2013; 436:434-442.

57. Ogunsola OA, Raghavan SR, Kraeling ME, Zhong S, Pochan DJ, Bronaugh RL. Structural analysis of "flexible" liposome formulations: New insights into the skin-penetrating ability of soft nanostructures. Soft Matter. 2012; 8(40):10226-10232.

58. Benita S, Levy MY. Submicron emulsions as colloidal drug carriers for intravenous administration: comprehensive physicochemical characterization. J Pharm Sci. 1993; 82(11):1069-79.

59. Nieuwenhuyzen W. Lecithin production and properties. J Am Oil Chem Soc. 1976; 53(6):425-427.

60. Handbook of pharmaceutical excipients / edited by Raymond C. Rowe, Paul J. Sheskey, Marian E. Quinn. 6th ed. ed. London: Pharmaceutical Press : American Pharmacists Association; 2009.

61. Szuhaj BF. Lecithins. In: Shahidi F, editor. Bailey's Industrial Oil and Fat Products, Edible Oil and Fat Products: Specialty Oils and Oil Products: John Wiley & Sons, Inc.; 2005. p. 361-456.

62. Yeadon D, Goldblatt L, Altschul A. Lecithin in oil-in-water emulsions. J Am Oil Chem Soc. 1958; 35(8):435-438.

63. Rydhag L, Wilton I. The function of phospholipids of soybean lecithin in emulsions. J Am Oil Chem Soc. 1981; 58(8):830-837.

64. Moreno MA, Ballesteros MP, Frutos P. Lecithin-based oil-in-water microemulsions for parenteral use: pseudoternary phase diagrams, characterization and toxicity studies. J Pharm Sci. 2003; 92(7):1428-37.

65. Yamaguchi T, Nishizaki K, Itai S, Hayashi H, Ohshima H. Physicochemical characterization of parenteral lipid emulsion: Influence of cosurfactants on flocculation and coalescence. Pharm Res. 1995; 12(9):1273-1278.

66. Lichtenberg D, Robson RJ, Dennis EA. Solubilization of phospholipids by detergents. Structural and kinetic aspects. Biochim Biophys Acta. 1983; 737(2):285-304.

67. Janiak MJ, Loomis CR, Shipley GG, Small DM. The ternary phase diagram of lecithin, cholesteryl linolenate and water: phase behavior and structure. J Mol Biol. 1974; 86(2):325-39.

68. Gennis R. The Structures and Properties of Membrane Lipids. In: Biomembranes: Springer New York; 1989. p. 36-84.

69. Almgren M. Mixed micelles and other structures in the solubilization of bilayer lipid membranes by surfactants. Biochim Biophys Acta. 2000; 1508(1-2):146-63.

70. Benita S, Friedman D, Weinstock M. Pharmacological evaluation of an injectable prolonged release emulsion of physostigmine in rabbits. J Pharm Pharmacol. 1986; 38(9):653-8.

71. Levy MY, Benita S, Baszkin A. Interactions of a non-ionic surfactant with mixed phospholipid—oleic acid monolayers. Studies under dynamic conditions. Colloids Surf. 1991; 59:225-241.

72. Edwards K, Almgren M. Solubilization of lecithin vesicles by C12E8: Structural transitions and temperature effects. J Colloid Interface Sci. 1991; 147(1):1-21.

73. Geyer R, Olsen F, Andrus S, Waddell W, Stare F. Preparation of fat emulsions for intravenous alimentation. J Am Oil Chem Soc. 1955; 32(6):365-370.

74. McNiff BL. Clinical use of 10% soybean oil emulsion. Am J Hosp Pharm. 1977; 34(10):1080-6.

75. Lieberman HA, Rieger MM, Banker GS. Pharmaceutical dosage forms--disperse systems : in three volumes. 2nd ed. New York: M. Dekker; 1996; p. 91-152.

76. Date AA, Patravale VB. Microemulsions: applications in transdermal and dermal delivery. Crit Rev Ther Drug Carrier Syst. 2007; 24(6):547-96.

77. Pouton CW. Formulation of self-emulsifying drug delivery systems. Adv Drug Del Rev. 1997; 25(1):47-58.

78. Narang AS, Delmarre D, Gao D. Stable drug encapsulation in micelles and microemulsions. Int J Pharm. 2007; 345(1–2):9-25.

79. Jiao J, Burgess DJ. Rheology and stability of water-in-oil-in-water multiple emulsions containing Span 83 and Tween 80. AAPS Pharmsci. 2003; 5(1)

80. André V, Willenbacher N, Debus H, Börger L, Fernandez P, Frechen T, et al. Prediction of emulsion stability: facts and myth. Cosmetics and Toiletries Manufacture Worldwide. 2003; 102

81. Küchler S, Schneider C, Lerche D, Sobisch T. Process optimisation for making stable emul-sions using accelerated dispersion analysis by multisample analytical centrifugation. LabPlus Int. 2006; 20(4):14.

82. Lin TJ. Surfactant Location and Required HLB. J. Soc. Cosmet. Chem. 1970; 21:365-375.

83. Lin TJ. Effect of initial surfactant locations on the viscosity of emulsions. J Soc Cosmet Chem. 1968; 19(10):683-697.

84. Tcholakova S, Marinov R, Denkov N, Ivanov I. Evaluation of short-term and long-term stability of emulsions by centrifugation and NMR. Bulg. J. Phys. 2004; 31(3-4):96-110.

85. Latreille B, Paquin P. Evaluation of Emulsion Stability by Centrifugation with Conductivity Measurements. J Food Sci. 1990; 55(6):1666-1668.

86. Garrett ER. Prediction of stability in pharmaceutical preparations. VIII. Oil-in-water emulsion stability and the analytical ultracentrifuge. J Pharm Sci. 1962; 51:35-42.

87. Bouchemal K, Briançon S, Perrier E, Fessi H. Nano-emulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimisation. Int J Pharm. 2004; 280(1–2):241-251.

88. Mason TG, Wilking J, Meleson K, Chang C, Graves S. Nanoemulsions: formation, structure, and physical properties. J Phys Condens Matter. 2006; 18(41):R635.

89. Choi S-Y, Oh S-G, Bae S-Y, Moon S-K. Effect of short-chain alcohols as co-surfactants on pseudo-ternary phase diagrams containing lecithin. Korean J Chem Eng. 1999; 16(3):377-381.

90. Shinoda K. The correlation between the dissolution state of nonionic surfactant and the type of dispersion stabilized with the surfactant. J Colloid Interface Sci. 1967; 24(1):4-9.

91. Matsumoto S, Kohda M, Murata S-I. Preparation of lipid vesicles on the basis of a technique for providing W/O/W emulsions. J Colloid Interface Sci. 1977; 62(1):149-157.

92. Lim WH, Lawrence MJ. Aggregation behaviour of mixtures of phosphatidylcholine and polyoxyethylene sorbitan monoesters in aqueous solution. Physical Chemistry Chemical Physics. 2004; 6(7):1380-1387.

93. Ly HV, Longo ML. The Influence of Short-Chain Alcohols on Interfacial Tension, Mechanical Properties, Area/Molecule, and Permeability of Fluid Lipid Bilayers. Biophys J. 2004 [cited 2015/07/12]; 87(2):1013-1033.

94. Aramaki K, Olsson U, Yamaguchi Y, Kunieda H. Effect of Water-Soluble Alcohols on Surfactant Aggregation in the C12EO8 System. Langmuir. 1999; 15(19):6226-6232.

95. Chiou J-S, Krishna PR, Kamaya H, Ueda I. Alcohols dehydrate lipid membranes: an infrared study on hydrogen bonding. Biochimica et Biophysica Acta (BBA) - Biomembranes. 1992; 1110(2):225-233.

96. Choi S-W, Kwon H-Y, Kim W-S, Kim J-H. Thermodynamic parameters on poly (d, l-lactide-co-glycolide) particle size in emulsification–diffusion process. Colloids Surf Physicochem Eng Aspects. 2002; 201(1):283-289.

97. Liu C, Li M, Liang C, Wang W. Measurement and analysis of bimodal drop size distribution in a rotor–stator homogenizer. Chem Eng Sci. 2013; 102:622-631.

98. Hall S, Cooke M, El-Hamouz A, Kowalski AJ. Droplet break-up by in-line Silverson rotor-stator mixer. Chem Eng Sci. 2011; 66(10):2068-2079.

99. Rodgers TL, Cooke M. Rotor–stator devices: The role of shear and the stator. Chem Eng Res Des. 2012; 90(3):323-327.

100. Rodgers T, Cooke M Correlation of drop size with shear tip speed. 14th European Conference on Mixing, Warszawa; 2012.

101. Pandolfe WD. Effect of premix condition, surfactant concentration, and oil level on the formation of oil-in-water emulsions by homogenization. J Dispers Sci Technol. 1995; 16(7):633-650.

102. Pandolfe WD. Effect of dispersed and continuous phase viscosity on droplet size of emulsions generated by homogenisatoin. J Dispers Sci Technol. 1981; 2(4):459-474.

103. Floury J, Desrumaux A, Lardières J. Effect of high-pressure homogenization on droplet size distributions and rheological properties of model oil-in-water emulsions. Innovative Food Science & Emerging Technologies. 2000; 1(2):127-134.

104. Walstra P. Principles of emulsion formation. Chem Eng Sci. 1993; 48(2):333-349.

105. Hielscher T Ultrasonic production of nano-size dispersions and emulsions. 1st Workshop on Nano Technology Transfer; 2005; Paris, Frace: Dans European Nano Systems Worshop – ENS 2005 p. 138-143.

106. Hashtjin AM, Abbasi S. Nano-emulsification of orange peel essential oil using sonication and native gums. Food Hydrocolloids. 2015; 44:40-48.

107. Washington C, Davis SS. Ageing effects in patenterai fat emulsions: the role of fatty acids. Int J Pharm. 1987; 39(1–2):33-37.

108. Tadros T, Izquierdo P, Esquena J, Solans C. Formation and stability of nano-emulsions. Adv Colloid Interface Sci. 2004; 108–109(0):303-318.

109. Tadros TF. Emulsion formation, stability and rheology. In: Tadros TF, editor. Emulsion formation and stability. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA; 2013. p. 1-75.

110. McClements DJ. Critical Review of Techniques and Methodologies for Characterization of Emulsion Stability. Crit Rev Food Sci Nutr. 2007; 47(7):611-649.

111. Gonyon T, Patel P, Owen H, Dunham AJ, Carter PW. Physicochemical stability of lipid injectable emulsions: correlating changes in large globule distributions with phase separation behavior. Int J Pharm. 2007; 343(1-2):208-19.

112. Shinoda K, Arai H. The effect of phase volume on the phase inversion temperature of emulsions stabilized with nonionic surfactants. J Colloid Interface Sci. 1967; 25(3):429-431.

113. Shinoda K, Saito H. The effect of temperature on the phase equilibria and the types of dispersions of the ternary system composed of water, cyclohexane, and nonionic surfactant. J Colloid Interface Sci. 1968; 26(1):70-74.

114. Reddy SR, Fogler HS. Emulsion stability: Delineation of different particle loss mechanisms. J Colloid Interface Sci. 1981; 79(1):105-113.

115. Tadros TF, Vincent B. Emulsion Stability. In: Becher P, editor. Encyclopedia of emulsion technology. New York: New York : M. Dekker; 1983. p. 129-286.

116. Chanamai R, McClements DJ. Creaming Stability of Flocculated Monodisperse Oil-in-Water Emulsions. J Colloid Interface Sci. 2000; 225(1):214-218.

117. Dickinson E. Hydrocolloids at interfaces and the influence on the properties of dispersed systems. Food Hydrocolloids. 2003; 17(1):25-39.

118. Demetriades K, McClements DJ. Influence of pH and Heating on Physicochemical Properties of Whey Protein-Stabilized Emulsions Containing a Nonionic Surfactant. J Agric Food Chem. 1998; 46(10):3936-3942.

119. Robins MM. Emulsions — creaming phenomena. Curr Opin Colloid Interface Sci. 2000; 5(5–6):265-272.

120. Capek I. Degradation of kinetically-stable o/w emulsions. Adv Colloid Interface Sci. 2004; 107(2–3):125-155.

121. Sherman P. Colloidal stability of ice cream mix*. Journal of Texture Studies. 1969; 1(1):43-51.

122. Higuchi WI, Misra J. Physical degradation of emulsions via the molecular diffusion route and the possible prevention thereof. J Pharm Sci. 1962; 51:459-66.

123. Muhlebach SF, Steger PJ. Lipid peroxidation of intravenous fat emulsions: a pharmaceutical issue with clinical impact? Nutrition. 1998; 14(9):720-1.

124. Steger PJ, Muhlebach SF. Lipid peroxidation of intravenous lipid emulsions and all-in-one admixtures in total parenteral nutrition bags: the influence of trace elements. JPEN J Parenter Enteral Nutr. 2000; 24(1):37-41.

125. Memoli A, Palermiti L, Travagli V, Alhaique F. Studies of differently inudced peroxidation phenomena in lecithins. J Agric Food Chem. 1996; 44:2814-2817.

126. Cannon JB. Chemical and physical stability considerations for lipid-based drug formulations. American Pharmaceutical Review. 2008; 11(1):132-138.

127. Halbaut L, Barbe C, Aroztegui M, de la Torre C. Oxidative stability of semi-solid excipient mixtures with corn oil and its implication in the degradation of vitamin A. Int J Pharm. 1997; 147(1):31-40.

128. Ambrosone L, Mosca M, Ceglie A. Oxidation of water emulsified olive oils. Food Hydrocolloids. 2006; 20(7):1080-1086.

129. Ambrosone L, Mosca M, Ceglie A. Impact of edible surfactants on the oxidation of olive oil in water-in-oil emulsions. Food Hydrocolloids. 2007; 21(7):1163-1171.

130. Frankel EN. Lipid oxidation. Prog Lipid Res. 1980; 19(1-2):1-22.

131. Steger PJ, Muhlebach SF. In vitro oxidation of i.v. lipid emulsions in different all-in-one admixture bags assessed by an iodometric assay and gas-liquid chromatography. Nutrition. 1997; 13(2):133-40.

132. Misik VV. Inhibition of lipid peroxidation of lecithin liposomes kept in a pH-stat system near neutral pH. Free Radic Res Commun. 1991; 15(3):159.

133. Samuni AM, Lipman A, Barenholz Y. Damage to liposomal lipids: protection by antioxidants and cholesterol-mediated dehydration. Chem Phys Lipids. 2000; 105(2):121-34.

134. Appendix X F: Peroxide Value. British Pharmacopoeia, 2007. London, UK: Stationery Office;

135. Levy MY, Benita S. Design and characterization of a submicronized o/w emulsion of diazepam for parenteral use. Int J Pharm. 1989; 54(2):103-112.

136. Schulman JH, Friend JA. Penetration and complex-formation in monolayers. J Soc Cosmet Chem. 1949; 1(5):381-393.

137. Li J, Wang X, Zhang T, Wang C, Huang Z, Luo X, et al. A review on phospholipids and their main applications in drug delivery systems. Asian Journal of Pharmaceutical Sciences. 2015; 10(2):81-98.

138. Kirikou M, Sherman P. The influence of tween 40/span 80 ratio on the viscoelastic properties of concentrated oil—water emulsions. J Colloid Interface Sci. 1979; 71(1):51-54.

139. Product information sheet: Polysorbate 80. Sigma Aldrich; 2010.

140. Zhang W, Liu L. Study on the Formation and Properties of Liquid Crystal Emulsion in Cosmetic. Journal of Cosmetics, Dermatological Sciences and Applications. 2013; 03(02):139-144.

141. Sprow FB. Drop size distributions in strongly coalescing agitated liquid-liquid systems. AIChE Journal. 1967; 13(5):995-998.

142. Maa Y-F, Hsu C. Liquid-liquid emulsification by rotor/stator homogenization. J Controlled Release. 1996; 38(2–3):219-228.

143. Rodgers TL, Cooke M, Hall S, Pacek A, Kowalski A. Rotor-stator mixers. Chem Eng Trans. 2011; 24:1411-1416.

144. Powell MF. Stability of lidocaine in aqueous solution: effect of temperature, pH, buffer, and metal ions on amide hydrolysis. Pharm Res. 1987; 4(1):42-5.

145. Wong-ekkabut J, Xu Z, Triampo W, Tang IM, Tieleman DP, Monticelli L. Effect of Lipid Peroxidation on the Properties of Lipid Bilayers: A Molecular Dynamics Study. Biophys J. 2007; 93(12):4225-4236.

146. Tirosh O, Kohen R, Katzhendler J, Alon A, Barenholz Y. Oxidative stress effect on the integrity of lipid bilayers is modulated by cholesterol level of bilayers. Chem Phys Lipids. 1997; 87(1):17-22.

147. Coupland JN, McClements DJ. Lipid oxidation in food emulsions. 0924-2244. 1996; 7(3):83-91.

148. Donnelly RF. Stability of buffered lidocaine in glass vials. Can J Hosp Pharm. 2011; 64(4):289-90.

149. Grit M, Crommelin DJA. Chemical stability of liposomes: implications for their physical stability. Chem Phys Lipids. 1993; 64(1):3-18.

150. Torpy JM, Lynm C, Glass RM. JAMA patient page. Burn injuries. JAMA [Patient Education Handout]. 2009; 302(16):1828.

151. Brigham PA, McLoughlin E. Burn incidence and medical care use in the United States: Estimates, trends, and data sources. J Burn Care Rehabil. 1996; 17(2):95-107.

152. Sheridan RL. Comprehensive treatment of burns. Curr Probl Surg [Review]. 2001; 38(9):657-756.

153. Quinn KJ. Principles of burn dressings. Biomaterials. 1985; 6(6):369-377.

154. Montgomery RK. Pain management in burn injury. Crit Care Nurs Clin North Am. 2004; 16(1):39-49.

155. Latarjet J, Choinere M. Pain in burn patients. Burns. 1995; 21(5):344-8.

156. Pal SK, Cortiella J, Herndon D. Adjunctive methods of pain control in burns. Burns. 1997; 23(5):404-412.

157. Bryant JA. Local and topical anesthetics in ophthalmology. Surv Ophthalmol [Review]. 1969; 13(5):263-83.

158. Zor F, Ozturk S, Bilgin F, Isik S, Cosar A. Pain relief during dressing changes of major adult burns: Ideal analgesic combination with ketamine. Burns. 2010; 36(4):501-505.

159. Patterson DR, Hoflund H, Espey K, Sharar S. Pain management. Burns. 2004; 30(8):A10-A15.

160. Jellish WS, Gamelli RL, Furry PA, McGill VL, Fluder EM. Effect of topical local anesthetic application to skin harvest sites for pain management in burn patients undergoing skin-grafting procedures. Ann Surg. 1999; 229(1):115-20.

161. Wasiak J, Spinks A, Costello V, Ferraro F, Paul E, Konstantatos A, et al. Adjuvant use of intravenous lidocaine for procedural burn pain relief: a randomized double-blind, placebo-controlled, cross-over trial. Burns. 2011; 37(6):951-7.

162. Summer GJ, Puntillo KA, Miaskowski C, Green PG, Levine JD. Burn Injury Pain: The Continuing Challenge. J Pain. 2007; 8(7):533-548.

163. Atchison NE, Osgood PF, Carr DB, Szyfelbein SK. Pain during burn dressing change in children: relationship to burn area, depth and analgesic regimens. Pain. 1991; 47(1):41-45.

164. Godfrey H. Understanding pain, part 1: physiology of pain. Br J Nurs [Review]. 2005; 14(16):846-52.

165. MacLennan N, Heimbach DM, Cullen BF. Anesthesia for major thermal injury. Anesthesiol [Review]. 1998; 89(3):749-70.

166. Chen CS, Tunng MC, Yang CL, Wang CT. Topical application of local anesthetics for postoperative analgesia in children undergoing circumcision. Ma zui xue za zhi = Anaesthesiologica Sinica [Clinical Trial Randomized Controlled Trial]. 1992; 30(2):113-7.

167. Joshi GP. Multimodal analgesia techniques and postoperative rehabilitation. Anesthesiol Clin North America [Review]. 2005; 23(1):185-202.

168. Sinclair R, Cassuto J, Hogstrom S, Linden I, Faxen A, Hedner T, et al. Topical anesthesia with lidocaine aerosol in the control of postoperative pain. Anesthesiol. 1988; 68(6):895-901.

169. Buvanendran A, Kroin JS. Multimodal analgesia for controlling acute postoperative pain. Curr Opin Anaesthesiol [Review]. 2009; 22(5):588-93.

170. Altman RS, Smith-Coggins R, Ampel LL. Local anesthetics. Ann Emerg Med. 1985; 14(12):1209-17.

171. Barbour HG. Local Anesthetics. Science. 1920; 51(1325):497-504.

172. Columb MO, MacLennan K. Local anaesthetic agents. Anaesth Intensive Care Med. 2007; 8(4):159-162.

173. Friedman PM, Mafong EA, Friedman ES, Geronemus RG. Topical anesthetics update: EMLA and beyond. Dermatol Surg. 2001; 27(12):1019-26.

174. Conklin KA. Pharmacology of local anesthetics. AANA J. 1987; 55(1):36-44.

175. Covino BG. Pharmacology of local anaesthetic agents. Br J Anaesth [Review]. 1986; 58(7):701-16.

176. Tucker GT, Mather LE. Clinical Pharmacokinetics of Local Anaesthetics. Clin Pharmacokinet. 1979; 4(4):241-278.

177. Lagan G, McLure HA. Review of local anaesthetic agents. Current Anaesthesia & Critical Care. 2004; 15(4–5):247-254.

178. Osman HA. Toxicity of Local Anesthetics. AJAIC. 2005; 8(4)

179. Cox B, Durieux ME, Marcus MA. Toxicity of local anaesthetics. Best Pract Res Clin Anaesthesiol [Review]. 2003; 17(1):111-36.

180. Holstege CP. Lidocaine. In: Editor-in-Chief: Philip W, editor. Encyclopedia of Toxicology (Second Edition). New York: Elsevier; 2005. p. 714-715.

181. Vandam LD. Current concepts in therapy: local anesthetics. III. N Engl J Med. 1960; 263:1188-9.

182. Vandam LD. Current concepts in therapy: local anesthetics. III. The New England journal of medicine. 1960; 263:1188-9.

183. Albert J, Lofstrom B. Effects of epinephrine in solutions of local anaesthetic agents. Acta Anaesthesiol Scand Suppl [Comparative Study]. 1965; 16:71-7.

184. Vandam LD. Current concepts in therapy: Local anesthetics. I. N Engl J Med. 1960; 263(15):748-750.

185. Saranteas T, Mourouzis C, Koumoura F, Tesseromatis C. Effects of propranolol or paracetamol on lidocaine concentrations in serum and tissues. J Oral Maxillofac Surg. 2003; 61(5):604-7.

186. Brofeldt BT, Cornwell P, Doherty D, Batra K, Gunther RA. Topical lidocaine in the treatment of partial-thickness burns. J Burn Care Rehabil. 1989; 10(1):63-8.

187. Giddon DB, Quadland M, Rachwall PC, Springer J, Tursky B. Development of a method for comparing topical anesthetics in different application and dosage forms. J Oral Ther Pharmacol [Clinical Trial Comparative Study]. 1968; 4(4):270-4.

188. Giovannitti JA. Evaluation of local anesthetic hypersensitivity reactions. Clin Prev Dent. 1981; 3(1):20-2.

189. Steinhaus JE. Toxic reactions to local anesthetics. J Med Assoc State Ala. 1962; 32:168-72.

190. Wehner D, Hamilton GC. Seizures following topical application of local anesthetics to burn patients. Ann Emerg Med [Case Reports]. 1984; 13(6):456-8.

191. Derek J D. Local and regional anaesthesia. Baillieres Clin Anaesthesiol. 1987; 1(3):715-728.

192. Farrar JT, Polomano RC, Berlin JA, Strom BL. A comparison of change in the 0-10 numeric rating scale to a pain relief scale and global medication performance scale in a short-term clinical trial of breakthrough pain intensity. Anesthesiol. 2010; 112(6):1464-72.

193. Williamson A, Hoggart B. Pain: a review of three commonly used pain rating scales. J Clin Nurs. 2005; 14(7):798-804.

194. Bijur PE, Latimer CT, Gallagher EJ. Validation of a verbally administered numerical rating scale of acute pain for use in the emergency department. Academic emergency medicine : official journal of the Society for Academic Emergency Medicine [Validation Studies]. 2003; 10(4):390-2.

195. DeLoach LJ, Higgins MS, Caplan AB, Stiff JL. The visual analog scale in the immediate postoperative period: intrasubject variability and correlation with a numeric scale. Anesth Analg. 1998; 86(1):102-6.

196. Aptar Consumer Health Care Division APF Plus: Advanced Preservative Free Plus. Germany:

197. Sansom L. Australian Pharmaceutical Formulary and Handbook. 19th ed. Canberra: Pharmaceutical Society of Australia; 2010.

198. Birkett DJ. Pharmacokinetics Made Easy. 2nd ed. Sydney: McGraw-Hill Australia Pty Ltd; 2010.

199. Bell C, McCarthy G. The assessment and treatment of wound pain at dressing change. Br J Nurs. 2010; 19(11):4-8.

200. Bijur PE, Silver W, Gallagher EJ. Reliability of the visual analog scale for measurement of acute pain. Acad Emerg Med. 2001; 8(12):1153-7.

201. McCaffery M. Using the 0-to-10 pain rating scale. Am J Nurs. 2001; 101(10):81-2.

202. Read JM, Bach PH. Sterile topical lignocaine jelly in plastic surgery: an assessment of its systemic toxicity. S Afr Med J. 1980; 57(17):704-6.

203. Bulmer JN, Duckett AC. Absorption of lignocaine through split-skin donor sites. Anaesthesia. 1985; 40(8):808-809.

204. Mills L, Durrani AJ, McGregor JC, Kent J. Refinement in application of local anaesthetic to graft donor sites. Br J Plast Surg. 2005; 58(5):753.

205. Kundu K, Bardhan S, Saha SK, Paul BK. Water solubilization, conductivity and structural characteristics of single and mixed surfactant water-in-oil microemulsions in absence and presence of ionic liquids. Fluid Phase Equilibria. 2014; 361(0):237-249.

206. Liang H, Yang Q, Deng L, Lu J, Chen J. Phospholipid-Tween 80 mixed micelles as an intravenous delivery carrier for paclitaxel. Drug Dev Ind Pharm. 2011; 37(5):597-605.

207. Opawale FO, Burgess DJ. Influence of Interfacial Rheological Properties of Mixed Emulsifier Films on the Stability of Water-in-Oil-in-Water Emulsions. J Pharm Pharmacol. 1998; 50(9):965-973.

208. Walstra P. Formation of Emulsions. In: Becher P, editor. Encyclopedia of emulsion technology. New York: New York : M. Dekker; 1983. p. 57-129.

209. Lucassen-Reynders EH. Dynamics interfacial properties in emulsification. In: Becher P, editor. Encyclopedia of emulsion technology. New York: New York : M. Dekker; 1996. p. 63-90.

210. Solans C, Izquierdo P, Nolla J, Azemar N, Garcia-Celma MJ. Nano-emulsions. Curr Opin Colloid Interface Sci. 2005; 10(3–4):102-110.

211. Clint JH. Micellization of mixed nonionic surface active agents. Journal of the Chemical Society, Faraday Transactions 1: Physical Chemistry in Condensed Phases [10.1039/F19757101327]. 1975; 71(0):1327-1334.

212. Maa Y-F, Hsu CC. Performance of Sonication and Microfluidization for Liquid–Liquid Emulsification. Pharm Dev Technol. 1999; 4(2):233-240.

213. Appendix X F. Peroxide Value. British Pharmacopoeia 2015. London, UK: Stationery Office;