PHARMACEUTICAL FORMULATION AND IN-VITRO TESTING OF DIOXY MP 14 (STABILISED CHLORINE DIOXIDE) AGAINST *MYCOBACTERIA TUBERCULOSIS*

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Key words

Chlorine dioxide

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Formulation development

Preformulation studies

Foam evaluation

Potentiometric acid base titrations of dioxy MP 14

Long term stability study of dioxy MP 14

Spectrophotometric method of testing disinfectant activity against TB

Mycobatericidal effect (ME) of dioxy MP 14

SUMMARY

This study was based on Dioxy MP 14 (DMP), a brand of stabilized chlorine dioxide (SCD). The active pharmaceutical ingredient (API) of DMP is chlorine dioxide (CD) which is a potent oxidant and biocide. These properties have proved invaluable for various applications. The main goals of this study were: to evaluate the effectiveness of DMP for disinfecting *Mycobateria tuberculosis* (TB) contaminated medical instruments, devices, floors and surfaces; to investigate the stability of DMP; and to explore possibilities for medical application of DMP.

Evaluation of disinfectant activity of DMP on TB was performed using the spectrophotometric method, a modification of the European suspension test, EN 14348. *M. bovis* BCG was employed as surrogate in this test. Results were as follows: The minimum inhibitory concentration (MIC_{90}) = 12.5 ppm; the minimum bactericidal concentration (MBC) = 15.4 ppm; the Mycobactericidal Effect (ME) = 8.8log reduction; and the minimum inhibitory concentration (MIC_{90}) x minimum exposure time (CT) = 12.5 ppm.s.

The long term stability study of DMP was performed by monitoring the rate of degradation of DMP stored in the fridge (2-8 ^{o}C), in the oven (40 ^{o}C), and under ambient conditions (15-30 ^{o}C). Analytical methods of assessing DMP concentration was by Iodometric titration method. The shelf life of DMP stored in a transparent bottle at room temperature was 9.8 weeks, as opposed to 52.7 weeks when stored in an amber colored reagent bottle at the same temperature. Both oven samples had an expiry date of about 20 weeks and the fridge samples about 70 weeks.

Foam formulations for a vaginal douche (VGD), mouth rinse (MRF), and foot/sit bubble bath (F/SBB)], were developed in the laboratory. DMP and the formulated concentrate were designed to be mixed just prior to administration. During foam evaluation studies, a mechanical overhead stirrer was used to generate foam. Foamability was assessed by quantifying the amount of foam generated. The stability of foams were assessed by: 1) determining the rate of foam decay and the rate of foam drainage observed concurrently from foam loaded in a measuring cylinder; and 2) determining the life span of single bubbles of each foam system i.e. the bubble breaking time (BBT). The density of each foam system was also determined. Potentiometric acid base titration was used to select suitable adjuster alkali, and to show the benefits of employing a buffer. Concentrate development was initiated by a simple mixture of all the ingredients followed by stirring and observing the deviations from desired quality attributes of the product. The subsequent five processes were improvements designed to circumvent the shortcomings of the initial procedure to arrive at the optimized method E. Prototype formulations were employed to optimize excipient quantities to eventually arrive at an optimized master formula. In foam evaluation, it was found that sodium lauryl sulphate/ammonium lauryl sulphate/cocoamidopropyl betaine/cetostearyl alcohol (SLS/ALS/CAPB/CSA) foam system was the most appropriate to use in the formulation. NaOH was selected as the adjuster solution and KHP as the buffer. The dosage formula (DF) of the VGD and F/SBB was determined to be MDF = 5 ml of 50 ppm DMP + 5 ml concentrate + 40 ml water = 50 ml and that of MRF as MDF = 19 ml diluted concentrate + 1 ml of 50 ml DMP.

In conclusion, DMP was found to be a highly effective disinfectant against *Mycobacteria*. DMP has reasonable shelf life if stored appropriately. Pharmaceutical formulation from DMP was found to be delicate due to the narrow pH window of DMP stability, but is feasible.

DEDICATION

For my son Moono Mavu who paid the highest price of all for my academic adventurism. I also dedicate this work to my daughter Sarah, and My wife Esther who waited and waited. The blessing numbers 6:24 – 26.

DECLARATION

I declare that this thesis entitled **Pharmaceutical formulation and in-vitro testing of dioxy mp 14 (stabilized chlorine dioxide) against *mycobacteria tuberculosis*** is my own work, that it has not been submitted before for any degree or examination in any other university and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Daniel Muleya Mavu November, 2011

Signed UWC, Bellville

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LIST OF ACRONYMS

ALS	Ammonium lauryl sulphate
AOAC	Association of official analytical chemists
AODC	Oleic acid albumin dextrose catalase
API	Active pharmaceutical ingredient
BBT	Bubble breaking time
BCG	Bacille Calmet Guerin
BP	British pharmacopoeia
CAPB	Cocoamidopropyl betaine
CD	Chlorine dioxide
CMC	Critical micelle concentration
CQA	Critical quality attributes
CSA	Cetostearyl alcohol
DMP	Dioxy MP (14)
F/SBB	Foot/Sit bubble bath
FA	Foaming agent
FAC	Foaming agent combinations
FD	Formulation development
FDA	Food and drug administration
GRAS	Generally regarded as safe
HLB	Hydrophilic-lipophilic balance
ICH	International committee on Harmonization
KHP	Potassium hydrogen phthalate
MAP	*Mycobacterium avium complex*

MP 9	Polyoxyethylene octyl phenyl ether
MRF	Mouth rinse formulation
MTB	*Mycobacteria tuberculosis*
NEO DAL	Ethoxylated alcohol
OD	Optical density
PP	Process parameters
Q_{10}	Temperature coefficient
SAA	Surface active agent
SCD	Stabilized chlorine dioxide
SLS	Sodium lauryl sulphate
SNLSS	Sodium N- lauryl sarcosinate
SXS	Sodium xylene sulphonate
TATM	Tuberculocidal Activity Test Method
TB	Tuberculosis
TPP	Target product profile
US EPA	United states of America environmental protection agency
VGD	Vaginal douche
WHO	World health organization
ZN stain	Zeal Neelson stain

CHAPTER 1

INTRODUCTION AND RESEARCH OBJECTIVES

1.1 History and uses of chlorine dioxide

The compound chlorine dioxide (CD), though still a subject of intense research, is not a recent discovery. The gas was first prepared by Sir Humphrey Davy in 1811 when reacting sulfuric acid with potassium chlorate (Aieta & Berg, 1986). Watt and Burgess, who invented alkaline pulp bleaching in 1834, patented CD, (US patent 11,343 of 1854) as a bleaching agent (Watt & Burgess, 1854). Since then, CD has experienced increased use in the bleaching of wood pulp in paper processing industry as well as textile bleaching industry especially from the 1920s (Jeng & Woodworth, 1990; Suess, 2010). The production of CD from chlorate is complicated and the gas is explosive. For this reason it could not be easily utilized until the discovery of efficient production methods for sodium chlorite by the Mathieson company, now called the Olin Corporation (Aieta & Berg, 1986; Alliger, 2001; Vincent, MaCmahon, & Synan, 1946). In 1944, CD was first used for primary disinfection of municipal water systems at Niagara Falls in the USA (Aieta & Berg, 1986). Shortly thereafter, it was discovered that CD also enhanced the quality of drinking water, controlling bad tastes, odors and colors, by oxidation/killing of phenolic wastes, sulfur compounds, iron, manganese, algae and other causative factors (Black and Veatch corporation, 2010; Vincent et al., 1946). CD is currently the second most important municipal water treatment agent with over 3,000 municipal water systems using CD in Europe and North America (Aieta & Berg, 1986; US EPA, 1999). CD was also found to eliminate cyanide from industrial waste (Aieta & Berg, 1986). In 1967, the US EPA first registered the liquid form of CD for use as a

disinfectant and sanitizer on a variety of sites such as animal farms, bottling plants, food processing, handling, and storage plants (Alliger, 2001; Lin, Hsieh, Liou, Lee, & Lai, 2007; Parga, Shukla, & Carrillo-Pedroza, 2003; Powis, 2005). Stabilized chlorine dioxide (SCD) was developed in the 1970s to further solve the problem of instability and volatility (Junli, Lihua, & and Zhenye, 2001). In 1978, Alliger patented an SCD gel and an accompanying lactic acid gel activator (Alliger Patents: # 4084747, # 4330531) for topical application by the individual user as a disinfectant (Alliger, 2001). In 1984 CD was first recognized as a chemosterilizing agent and was approved as a sterilant by the EPA in 1988 (Lin, Hsieh, Liou, Lee, & Lai, 2007; US EPA, 2007). In the 1990s, CD gained widespread use for small scale disinfection of water, a scenario arising from outbreaks due to inadequate disinfection or organisms resistant to the usual chlorine disinfection such as *Legionella, Cryptosporidium* etc. and for personal drinking water e.g. for military personnel (Lin, Hsieh, Liou, Lee, & Lai, 2007; Lin, Stout, & Yu, 2011; Thomas et al., 2004). To use SCD under such circumstances might require the granting of a crisis exemption (US EPA, 2007). Other uses of CD include: disinfecting flume water (Beuchat 1998), treating medical waste (US EPA, 2007), control of biofilms in pipe systems e.g. dental unit (Wirthlin, Marshall, & Rowland, 2003), fumigation treatment for inactivating sick building syndrome-related fungi and their mycotoxins (Wilson et al., 2005), national security issues such as Bio-warfare and Bio-terrorism as was the case when gaseous CD was used to disinfect anthrax virus from offices of the Hart Senate Building and Post office by the Environmental Protection Agency in 2002 etc. (Lin, Hsieh, Liou, Lee, & Lai, 2007; US EPA, 2007). Medical application of CD so far has mainly been confined to its use as a disinfectant. It is approved by the EPA for disinfection of ventilation systems and hard surfaces such as floors, walls and laboratory equipment where it is formulated as concentrates and ready to use liquid solutions. It is

applied as spray, mop, injector system, mist and fog (US EPA, 2006). No FDA approved medical formulation was found. Patents, however, exist including the Alliger gel mentioned above, various oral formulations, as well as vaginal wipes (Hughes, 2005).

1.2 Physical and thermodynamic properties of CD

Chlorine dioxide is a greenish yellow gas at room temperature. It has a pungent, distinctive, irritating smell, reminiscent of that of chlorine (Black and Veatch Corporation, 2010). According to Kaczur and Cawlfield (2000), CD has a critical temperature of 465 K; critical pressure of 8621.6 kPa; melting point of 213.55 K which is also its triple point; triple point pressure of 1.2544 kPa; boiling point of 284.05 K at 101.3 kPa; and a liquid molar volume of 4.1852x10^-2 m^3/K.mol. Its liquid density at -55^{o}C, 0^{o}C, and 10^{o}C is 1.773, 1.640, and 1.614 g/ml respectively. Kaczur and Cawlfield further states that CD has an ideal gas heat of formation of 102.5 KJ/mol; ideal gas Gibbs energy of formation of 120.5 KJ/mol; ideal gas entropy of 0.257 KJ/(mol.K); and standard net heat of combustion (gas) of -102.5 KJ/mol; Like all gases the aqueous solubility of CD can be defined in terms of Henry's law whereby the equilibrium relationship between dissolved and gaseous CD is:

$$p\mathrm{ClO_2} = [\mathrm{ClO_2}\ \mathrm{g/L}]\ e\text{^}[10.717-(3102/T)]$$

Where

$p\mathrm{ClO_2}$ = the partial pressure of CD gas in kPa

$[\mathrm{ClO_2}]$ = the CD solution concentration in grams per liter and

T = the absolute temperature in Kelvin.

This simply implies that the solubility of the gas in the liquid at a particular temperature increases as the pressure of the gas above the liquid is increased (Kaczur & Cawlfield, 2007). Various solubilities of CD in water at partial pressures of up to 20 kPa are shown in figure 1.1 below:

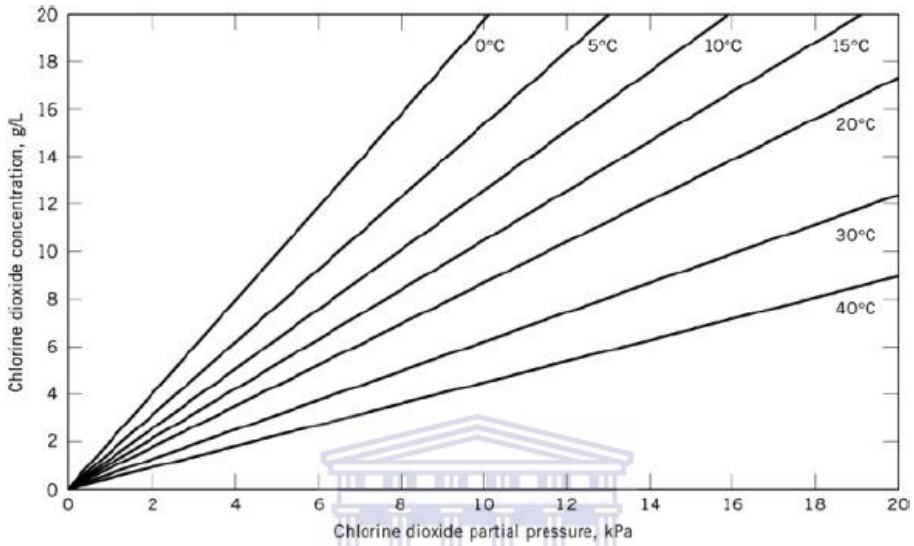


Figure 1.1: Solubility of chlorine dioxide in water (Buser & Hanisch, 1952)

Data in figure 1.1 show that CD is highly soluble in water, i.e. solubility of 3.01 g/l at 25 ^{o}C and 34.5 mm Hg (US EPA, 2006). This is higher solubility than that of comparable disinfectants such as chlorine and ozone (Black and Veatch Corporation, 2010; Emanuele, 2007). CD does not hydrolyze appreciably in water but remains as a dissolved gas (Aieta & Berg, 1986). CD forms a yellow to yellow-green color solution. The partition coefficient between water and CD gas is about 21.5 at 35 ◦C and 70.0 at 0◦C. The UV aqueous absorption spectrum of CD is the same as the gas phase spectrum and has a broad absorption band with a peak near 360 nm. Its molar extinction coefficient is 1250 $(M.cm)^{-1}$ when using high resolution, narrow bandwith spectrophotometers (Kaczur & Cawlfield, 2007).

1.3 Chemistry of CD

1.3.1 Electronic structure of CD

CD has an unusual electronic structure which chemists could not comprehend for a long time because none of the possible Lewis structures were satisfactory. The currently accepted electronic structure consists of two resonance structures involving a double bond on one side of chlorine and a single bond together with a three electron bond on the other. The O–Cl–O bonds are at an angle of about 117.5 degrees, and each chlorine–oxygen bond length is 0.147 nm (Kaczur & Cawlfield, 2007), as shown in figure 1.2 below: CD is a free radical, owing to one unpaired electron in its molecular orbital (Linus, 1988; Zsolt, 2004).

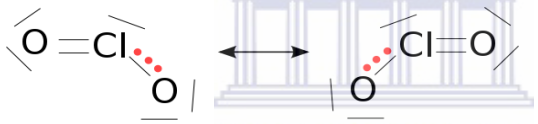


Figure 1.2: Lewis structure of CD (Linus, 1988).

1.3.2 Oxidizing properties of CD

CD gas is a strong oxidant. Its redox potentials (E) for various reactions are given in Table 1.1. In these calculations the standard reversible potentials (E°) are determined by the specific reaction chemistry and can vary depending on the pH and concentration of CD. The four redox equations below are: for an aqueous solution where chloride ion is the product ($E^\circ = -1.511$), for a reaction where chlorite is the product ($E^\circ = -1.160$), for gaseous systems where HCl gas is formed ($E^\circ = -1.436$), and a reaction where hydrogen chlorite forms ($E^\circ = -1.26$) (Kaczur & Cawlfield, 2007; Zsolt, 2004) respectively.

Table 1.1: Electrode potentials of CD for various reactions (Kaczur & Cawlfield, 2007; Zsolt, 2004).

$ClO_2(g) + 4\,H + 5e \rightleftharpoons Cl^- + {}_2\,H2O$ $\quad E = -1.511 + 0.0473\ pH - 0.0118 \log \{(P^{CO2})/ [Cl^-]\}$

$ClO_2(g) + 1e \rightleftharpoons ClO2^-$ $\quad E = -1.160 - 0.0591 \log \{(P^{ClO2})/ [ClO_2^-]\}$

$ClO2 + 5\,H+ + 5e- \rightleftharpoons HCl + 2\,H2O$ $\quad E = -1.436 + 0.0591\ pH - 0.0118 \log \{(P^{ClO2})/ (HCl)$

$ClO_2(g) + H + 1e \rightleftharpoons HClO_2$ $\quad E = -1.26 - 0.0591 \log \{(P^{ClO2})/ [HClO_2]\}$

From the above equations, it can be seen that the oxidation potential of CD depends upon the pH of the solution i.e. the more acidic the solution the higher the oxidation potential (Junli, Li, Nanqi, Fang, & Juli, 1997).

The relative oxidation efficiency of chlorine dioxide has sometimes been expressed in terms of 'available chlorine' which can be calculated as follows:

CD accepts 5 electrons when it is reduced to chloride in a reaction shown below.

$ClO_2 + 5e- \rightarrow Cl^- + 2O^{2-}$

From the above, it can be noticed that the equivalent weight of CD is 13.49 (67.45/5) g/mol and that of chlorine is 35.45 g/mol (70.90/2). This simply means that 13.49 g of CD can oxidize the same amount of material as 35.45 g of Cl_2. Thus 'available chlorine' in CD is 263 % that in Cl_2. i.e. CD has 2.6 times more capacity to oxidize than chlorine. In practice, the 263 % equivalent available chlorine is rarely attained because CD is

rarely reduced completely to chloride ion (Benarde, Israel, Olivieri, & and Granstrom, 1965; Black and Veatch corporation, 2010).

1.3.3 Reactions of CD with organic compounds

In disinfecting highly contaminated water CD is ten times more efficient than chlorine, in terms of quantities used to achieve same level of disinfection, while it is just twice more efficient than hypochlorite in disinfecting non contaminated water. Furthermore, the half-life of CD in raw sewage is higher than that of equimolar hypochlorite (Aieta & Berg, 1986; Masschelein, 1979). These observations are attributed to the selective nature of CD in its reaction with organic compound(s), which is due to its unique single electron transfer reducing to chlorite as it attacks electron rich centers (Aieta & Berg, 1986; Black and Veatch corporation, 2010; WHO, 2000). CD does not react with ammonia or primary and secondary amine(s) which are largely responsible for consumption of chlorine in contaminated water (Gagnona et al., 2005; Masschelein, 1979). CD also does not oxidize bromine to bromate as ozone does (Black and Veatch corporation, 2010) and saturated aliphatic compounds are also unreactive with CD (Rav-Acha & Choshen, 1987). Alcohols and carbonyl compounds react slowly with CD to produce carboxylic acids (Kaczur & Cawlfield, 2007). On the other hand CD rapidly oxidizes the following: tertiary amines, humic substances, cyanides and phenols such as chlorophenols (Aieta & Berg, 1986). In the reactions of CD, the formation of organohalogens such as trihalomethanes and haloacetic acid is negligible as opposed to chlorine reactions where these are present in considerable amounts (Aieta & Berg, 1986; Berg, Roberts, & Matin, 1986; Roberts, Aieta, Berg, & Chow, 1980). This is attributed to differences in reaction mechanisms where, as already stated, CD oxidizes by free radical electron abstraction as

opposed to chlorine which oxidizes by both substitution and addition. Chlorite is a well-known by product of CD reactions (Gagnona et al., 2005).

1.3.4 Thermal Decomposition of CD

Thermal decomposition of gaseous CD is characterized by a slow induction period followed by a rapid autocatalytic phase that may be explosive if the initial concentration is above a partial pressure of 10.1 kPa (76 mm Hg). During this reaction, one of the intermediates formed is the unstable Cl_2O_3. The presence of water vapour is thought to extend the duration of the induction period by reacting with this intermediate. When the water vapour concentration as well as the temperature, are both high, all decomposition takes place in the induction period hence the smooth rather than explosive decomposition. In solution, CD decomposes very slowly at ambient temperature in the dark. The thermal decomposition products of gaseous CD include chlorine, oxygen, HCl, $HClO_3$, and $HClO_4$ while in solution CD is hydrolyzed into chlorite and chlorate ions (Kaczur & Cawlfield, 2007).

1.3.5 Photochemical Reactions

In gas phase CD undergoes homolytic fission of the chlorine–oxygen bond to form ClO and O, as the primary products of photochemical reaction. Secondary products are then generated and these include chlorine peroxide (ClOO), chlorine (Cl2), oxygen (O2), chlorine trioxide (Cl2O3), chlorine hexoxide (Cl2O6), and other oxychlorine species. In aqueous solutions, CD photolyzes in a more complex manner producing chlorate (ClO_3^-), chloride (Cl^-), and hypochlorite (OCl^-) anions as the principal stable products (Kaczur & Cawlfield, 2007).

1.3.6 The hazardous nature of CD

Being such a powerful oxidizer, CD reacts violently with strong reducing agents such as hydrides, nitrites, and sulfides. Explosive reactions also occur with carbon monoxide, carbon dioxide, phosphorous, sugar, sulfur, fluorine, flouramines etc. Liquid CD explodes on contact with mercury and metal hydrides. CD is also incompatible with rubber. Though it is not combustible, CD enhances combustion of combustible material such as dust, hydrocarbons, butadiene, ethane, ethylene, methane, etc. Further to this the gas could explode at temperatures in excess of 44 ^{o}C. Explosions, even at room temperature are also likely if concentrations above 10 % v/v are exposed to light, sparks, static electricity, on impact, or may be self-initiated after an induction period. When involved in a fire CD is a source of oxygen. These incompatibility and instabilities exemplify the dangers involved in handling of CD (Pohanish & Greene, 2009).

1.4 Biocidal properties of CD

CD is a powerful and versatile biocide in both the aqueous and gas phases. This is highly advantageous for biopharmaceutical applications since it is easy to change CD from one state to the other. With regards to efficacy, CD is a broad spectrum biocide capable of inactivating bacteria, spores, cysts, viruses, yeasts, moulds and protozoa (Novak, Demirci, & Han, 2008; Ogata & Shibata, 2008). It is effective at concentrations as low as 0.1 ppm (Safeoxy, 2011). CD having a higher oxidizing capacity than comparable oxidizing disinfectants implies that it is a more efficient disinfectant on a weight by weight basis e.g. theoretically, 13.49 g of CD disinfects the same amount as 35.45 g of chlorine (Block, 2001). The selectivity of CD, alluded to in section 1.3.3 above, implies that CD is a more efficient biocide due to less wastage. Chlorine which is less selective,

for instance reacts with ammonium ions and primary amines, substances which are abundant in nature. This increases chlorine demand unnecessarily since the chlorine intended for disinfection ends up oxidizing unintended substances (Aieta & Berg, 1986; Masschelein, 1979). The disinfection efficiency of CD is also relatively independent of pH unlike biocides like chlorine whose disinfection capacity is restricted to a narrow pH range. Junli et al. (1997), states that CD is effective as a biocide in the pH range of 3 to 9. CD is approximately 10 times more soluble in water than chlorine (Black and Veatch corporation, 2010; US EPA, 1999). This could be a contributing factor for the good residual disinfectant effect of CD which is crucial for suppressing microbial growth in the long run (Black and Veatch corporation, 2010; US EPA, 1999). Since CD exists in solution as an undissociated gas, it should easily penetrate membranes and this should enhance its lethal action (Junli et al., 1997). On the safety score, CD has a relatively good disinfection and disinfection by-product profile (DBP) as it results in formation of much less harmful by-products in comparison with comparable oxidizing disinfectants (Simpson, Miller, & Laxton, G. D. and Clements, W. R., 1993; Tarquin, Rittmann, & Pino, 2002). The main undesirable byproducts of disinfection with CD are chlorite and chlorate which are discussed in section 1.5. CD also has excellent biofilms removing properties and has registered widespread use for removing biofilms in cooling towers, intra vascular catheters, small tubing water systems of dental units, pipes of municipal water supply systems etc. (Weinstein & Donlan, 2011; Wirthlin et al., 2003). One possible reason for the relatively slow re-growth of bacteria after sterilization with chlorine dioxide lies in its superior ability to penetrate and disperse a biomass (Epstein, Pokroy, Seminara, & Aizenberg, 2011). The persistent residual effect of CD seems of high value in this respect as well (Masschelein, 1979; Thomas, V.et al., 2004).

1.4.1 Mechanism of action of CD

According to Aieta, (1986), efforts to elucidate the mode of biocidal action of CD have taken two main routes: 1) identification of specific chemical reactions between CD and biomolecules and 2) evaluation of the effect of CD on physiological functions. Both approaches have been invaluable. On the first type study, it was found that CD impaired viral RNA synthesis by inhibiting further incorporation of uridine. Noss, Hauchman and Olivieri (1986) obtained contrary results finding that CD destroyed the tertiary structure of proteins such that specific attachment was no longer possible. They identified the amino acids tyrosine, cystein, and tryptophane within the protein moiety as being reactive with CD leading to viral inactivation. Fatty acids have also been implicated by some researchers as being reactive with CD and therefore this is yet another possible mode of inactivation. However, the actual target of CD remains uncertain.

Studies on CD effect on physiological functions were initiated by Bernade et al. (1967) who found that CD acted by disrupting protein synthesis. Later in 1980, it was proved that inhibition of protein synthesis, inhibition of dehydrogenase enzymes and action on nucleic acids were not the primary targets of CD inactivation of microorganisms (Roller, Olivieri, & Kawata, 1980). In 1986, Berg et al. showed that loss of permeability control by disruption of the outer membrane with efflux of potassium ions resulting in elimination of the transmembrane ionic gradient was the primary mode of action. This view is currently the popular one and has been supported by a number of other researchers (Aieta & Berg, 1986).

1.4.2 Relative efficacy of CD

Aieta et al. (1986) describes a study by Hoff and Geldreich where a number of disinfectants were ranked based on 'biocidal efficacy' and 'stability.' Biocidal efficacy measured disinfectant activity against viruses and bacteria in the pH range 6-9 while stability was meant to show disinfectant selectivity for microbes. Stability therefore measured persistence of the disinfectant in the treated system. The biocidal efficacy ranking (best to worst) was given as: ozone> CD>free chlorine>chloramines while that of stability (best to worst) was given as: chloramines > CD > free chlorine > ozone (Akin, Hoff, & Lippy, 1982). This ranking agrees with that of other investigators as explained below: Simpsom (1993) made a comparison of four oxidizing biocides, namely; ozone, CD, chlorine and bromine, in light of the criteria of an 'ideal' biocide.' He found that although each of the oxidizers studied excelled in one or more areas, when reviewed as whole, chlorine dioxide came closer to achieving the status of 'ideal' biocide than any of the others.

1.5 Toxicology of CD

According to the US EPA integrated risk information system (IRIS), CD in drinking water rapidly degrades to chlorite (ClO_2^-), chlorate (ClO_3^-), and chloride ion (Cl^-). If absorbed in the blood, CD and chlorite have similar distribution and clearance profiles and chloride ion is the major *in vivo* degradation product of CD, chlorite, and chlorate. Furthermore the US EPA is of the view that CD and chlorite have similar toxicity and potency targets. Therefore toxicity information of chlorite is applicable to that of CD.

Kaczur et al. (2007), states that the oxidative properties of sodium chlorite are directly related to its toxic effects. Sodium chlorite toxicity arises mainly from ingestion. For 31

% w/v sodium chlorite solution, the following acute lethal doses apply to rats: oral lethal dose (LD_{50}) is 284 mg/kg; dermal LD_{50} is >2000 mg/kg; and lethal concentration LC_{50} is 0.29 mg/L when inhaled for 4 hours (Arkema Inc., 2010). This means that for rats weighing about 400 mg each: consumption of about 0.4 ml each will kill about half the rat population; dermal exposure of each rat to more than 2.6 ml could be fatal to half the population; and inhalation of 0.29 ppm by each rat for four hours could decimate half the population. Sodium chlorite produces severe irritation or burns to the skin or eyes. Corneal damage and impairment of vision may occur if this material is not washed immediately from the eyes (Kaczur & Cawlfield, 2007).

Kaczur also states that sodium chlorite is not listed by any regulatory authority as a carcinogen. He points to studies conducted in mice and rats as showing that sodium chlorite is not carcinogenic although sodium chlorite has been found to have mutagenic activity in Ames *Salmonella* reverse mutation assays. The significance of these test results in regard to human health is not clear. Controlled animal and human studies in the CD/sodium chlorite concentration range of 1-1000 ppm have also been conducted. They proved the point in the opening paragraph of this section that metabolically, both CD and ClO_2^- are rapidly reduced following ingestion to chloride ions which is excreted via the renal route together with a small amount of ClO_2^-. These studies also show that the no observed effect level (NOEL), for CD and ClO_2^- generally ranges between 10 and 100 ppm. Exposures of laboratory animals to CD above 100 ppm in drinking water have shown a decrease in blood cell glutathione, red blood cell counts, and hemoglobin. Some mild effects on the thyroid and anemia were noted in younger laboratory animals. Human volunteers in one study with doses up to 24 ppm of CD or ClO_2^- showed no adverse health effects (Kaczur & Cawlfield, 2007).

1.6 Limitations/regulatory issues

Due to instability, CD gas cannot be compressed for storage in gas tanks, with or without other gases (Aieta & Berg, 1986; Gordon, Kieffer, & Rosenblatt, 1972) and by law is not permitted to be transported. It has to be produced at the site of consumption (Kaczur & Cawlfield, 2007; US EPA, 2000). After utilization, the gas is normally neutralized by sodium-bi-sulfite (Jin, Hu, Zhang, & Bo, 2009; US EPA, 2007). The instability limitations of CD gas prevent the full utilization of the superior biocidal properties of CD particularly in the medical and agricultural industries.

1.7 Stabilized chlorine dioxide (SCD)

Bio-Cide international Inc., USA, developed "stabilized chlorine dioxide (SCD)" in the early 1970s by stabilizing CD in aqueous solution using Na_2CO_3/H_2O_2 buffers. This overcame the problems of CD instability and its attendant legal restrictions on transportation. Many countries that used SCD, in a relatively wide field, have approved it. In SCD solution, CD exists as chlorite (ClO_2-) and the so-called ''stabilized chlorine dioxide'' actually is the mixture of $NaClO_2$ and $NaHCO_3$. Like CD, chlorite is a potent biocide and oxidant (Junli et al., 2001). Since its first advent in the 1970s, a number of brands of stabilized chlorine dioxide have emerged world over. Most SCD solutions are activated by adding acid and the activated solutions release CD. Dioxy MP-14 (DMP) is specifically a South African brand of SCD. DMP does not need to be activated by acids before use, but exists at very low pH where it is already in its active state. DMP solutions are normally stored cold, away from light in a tightly closed container and at concentrations of less than 10,000 ppm in order to keep the concentration of gaseous CD above the aqueous solution below the explosive limit. Where possible the storage vessel

should be filled, minimizing the headspace and therefore the accumulation of gaseous CD in the vessel (Taylor, Wohlers, & Amata, 2004).

1.8 Research objectives

The current research was based on DMP. The main goal was to explore possibilities for medical application of DMP. The effectiveness of DMP to destroy *Tuberculosis bacillus* (TB) was also determined for the possible application of disinfecting TB contaminated medical instruments, devices, floors and surfaces.

Three specific aims pertaining to the aforementioned areas have been investigated in this work:

Aim 1: To conduct in-vitro evaluation of the effectiveness of DMP for use as TB chemical disinfectant for floors, surfaces and medical instrument and devices

Aim 2: To investigate the long term stability of DMP

Aim 3: To formulate a vaginal douche (VGD), foot/sit bubble (F/SBB) bath, and mouth rinse (MRF) in foam preparations of DMP

CHAPTER 2

LITERATURE REVIEW

2.1 *In-vitro* evaluation of the effectiveness of DMP for use as chemical disinfectant (cold sterilant) against *Mycobacteria tuberculosis* (TB)

The current TB pandemic has been fueled by factors such as the HIV/AIDS epidemic, demographic and socio-economic changes, emergency of multidrug resistance *Mycobacteria* (MDR TB), inadequate sterilization of medical devices, and premature termination of workable TB programs in the case of developed countries (Erickson, Campbell, & Cerniglia, 2000; Robison et al., 1996; Sattar, Best, Springthorpe, & Sanani, 1995). Prevention of the spread of TB is one of the best strategies of overcoming this pandemic (Robison et al., 1996; Sattar et al., 1995). This aspect of the project is directed at testing disinfectant ability to preventing TB spread through disinfection of sources of contamination and chemical sterilization of exposed heat sensitive medical devices (Roup & Kelley, 2005). When considering disinfection and chemical sterilization, it should always be borne in mind that *Mycobacteria* are considerably more resistant to chemical inactivation. This is because *Mycobacteria* cell wall is made up of mycolic acids and other complex lipids which limit the uptake of biocides into the cell. Consequently, disinfectant concentrations necessary for antitubercular action must be separately established (Griffiths, Babb, & Fraise, 1998; Hernández, Martró, Matas, Jiménez, & Ausina, 2005).

2.1.1 Laboratory colour differentiation of *Mycobacteria*

There are currently three staining methods for the positive identification of *Mycobacteria* in common use: 1) the Ziehl-Neelsen (ZN), 2) the kinyoun, and 3) the fluorochrome (Truant) methods (Bollela, Sato, & Fonseca, 1999). The ZN test was used to verify the *Mycobacteria* identity of bacteria employed in this study. The wax wall of *Mycobacteria* does not allow penetration and therefore staining by aqueous based solutions such as gram's solution. As for ZN stain, the basic fuschin component contains a stain called carbofuschin, which by virtue of its lipophilicity can be taken up by the wax wall. Heating also helps in the uptake of this stain into the wax wall. Carbofuschin once taken up resists decolorization with a dilute acid rinse (Hussey & Zayaitz, 2010).

2.1.2 Official methods of testing disinfectant activity against TB (conventional plate counting methods)

The official method of testing disinfectant activity against TB in North America is the Tuberculocidal Activity Test Method (TATM) (Robison et al., 1996). The European official tests are En 14348 (suspension test) and its modified version En 14563 (carrier test). All are quantitative assays (Dauendorffer et al., 1999). EN 14563 was designed for evaluation of the mycobactericidal activity of chemical disinfectants for medical instruments under more practical conditions while the former is more general but mainly for floors and surfaces (CEN, 2008; Steinhauer et al., 2010). Official methods generally have the following disadvantages: they are laborious, expensive, and slow for clinical use; they frequently fail due to either contamination or medium dehydration; and they are time-consuming requiring at least 4 weeks to detecting the *M. tuberculosis*. Furthermore, colony counting underestimates the number of bacteria because of the formation of

aggregates and sonification bears the risk of either incomplete resolution of aggregates or of rupturing of the cells (Cousins, Wilton, Francis, & Gow, 1992; Lewin et al., 2003). In this study, the spectrophotometric method which was employed is a modification of test EN14348.

2.1.2.1 Summary of EN 14348 test methods

The EN 14348 test method involves mixing 1 ml of the test bacteria with 1 ml of soil (0.03% or 3% albumin for clean and dirty conditions respectively) and then adding 8 ml of test disinfectant. After the required contact time, 1 ml is removed to 9 ml of recovery broth (8ml neutralizer and 1ml diluent), which is then plated to detect surviving test bacteria (Hospital Infection Research Laboratory, 2007).

2.1.3 Non official methods

Most companies chose to determine the efficacy of their disinfectants using non official methods. Some reasons for this are: the implementation of these alternative methods is simple, results are obtained in a short time (about a week), and the procedures enables the reduction of the cost of such studies, relative to that of official methods (Dauendorffer et al., 1999). Some of the documented non official methods of evaluating disinfectant activity against TB are: 1) the radio labeling (BATEC) method, 2) MGIT BACTEC non radiometric methods, 3) measurement of total protein content method, 4) ATP assay method, 5) quantification of the DNA content, and 6) the spectrophotometric which is also known as the turbidimetric or optical density (OD) method (Lambrecht, Carriere, & Collins, 1988; Lewin et al., 2003; Meyers, et al., 1998).

2.1.3.1 The turbidimetric/spectrophotometric/optical density (OD) method

The spectrophotometric method of evaluation of antimycobacterial activity of disinfectants is based on the Bear-Lambert law. According to this law, if a beam of monochromatic radiation of radiant power P_0, is directed at a sample solution of mycobacteria concentration c, absorption takes place and the beam of radiation leaving the sample has radiant power *P*. The absorbance, A, is proportional to the concentration c. The Beer-Lambert law is mathematically given below:

$$\mathbf{A} = log_{10}P_0/P = \boldsymbol{\varepsilon bc}$$

Where

A = absorbance

ε = the molar absorptivity (L mol^{-1} cm^{-1})

b = the path length of the cuvette in which the sample is contained express centimeters.

c = the concentration of the compound in solution, expressed in mol L^{-1}

Plotting absorbance against concentration, gives a straight line that passes through the origin (0, 0). In this manner a calibration curve could be prepared that could be useful in predicting unknown concentrations of *Mycobacteria* samples (O'Haver, T., 2010). The Beer-Lambert law has been used to estimate bacteria concentrations for a long time (Domínguez, de la Rosa, & Borobio, 2001; Lambrecht, et al., 1988).

2.1.4 Surrogates

The best organism for evaluation of disinfectant activity against TB would be MTB because of its clinical relevance. However, use of MTB in such a role is unacceptable due

to its high pathogenicity. This necessitates use of surrogates from whom accurate predictions about disinfectant activity on TB can be made (Griffiths et al., 1998). Commonly used surrogates include: *M. bovis BCG, M. smegmatis,* and *M. terrae*. *M. bovis BCG* genome is 99.9% similar to that of MTB, the difference being that the BCG genome contains several well defined deletions. The two also share physiological molecular and metabolic similarities: they have a similar growth profile; they have a similar ability to persist in the body after an infection; they share a similar resistance profile to chemical disinfection (Beste, Peters, Hooper, Avignone-Rossa, Bushell, & McFadden, 2005). *M. bovis BCG* is considered to be an ideal surrogate because of these reasons and is safer to handle (Griffiths et al., 1998). *M. bovis BCG* is the surrogate organism for the TATM (American official) method (Robison et al., 1996). It was chosen for testing disinfectant activity against TB in this study.

2.1.5 McFarland nephelometer standards

The McFarland nephelometer is a tool used to enumerate bacteria in solution (Domínguez, de la Rosa, &Borobio, 2001). It consists of McFarland standard solutions, which can easily be prepared by mixing 1 % solution of anhydrous $BaCl_2$ with 1 % H_2SO_4 solution in proportions that give standards labeled as 0.5-10. The standard solutions are stable for about 6 months from the date of preparation if stored in tightly sealed containers at 20-25 ^oC and in the dark. Each McFarland standard represents a specific concentration of $BaCl_2$ that can be used spectrophotometrically to standardize inoculums. (Bollela et al., 1999; PML microbiologicals, 2001). To avoid the hustle of preparing McFarland standards each time a procedure is to be performed, the McFarland nephelometer scale has been established as shown in table 3.1 (chapter 3).

2.1.6 *In-vitro* growth of MTB

Though *Mycobacteria* are obligate aerobes, they have the capacity to adapt to hypoxic conditions both in-vivo and in-vitro by growth termination and reversion to a dormant form (Shin, Han, Manning, & Collins, 2007; Boon, Li, Qi, & Dick, 2001). *M. bovis* and MTB grow slowly (Lewin et al., 2008) with a generation time of 12 to 18 hours and this is influenced by a number of factors including the growth medium. Meyers et al. (1998) investigated the growth of mycobacteria in 7H9 broth media, using various growth monitoring techniques of which the growth curve from the OD method, taken at 600 nm, is given in figure 2.1 below.

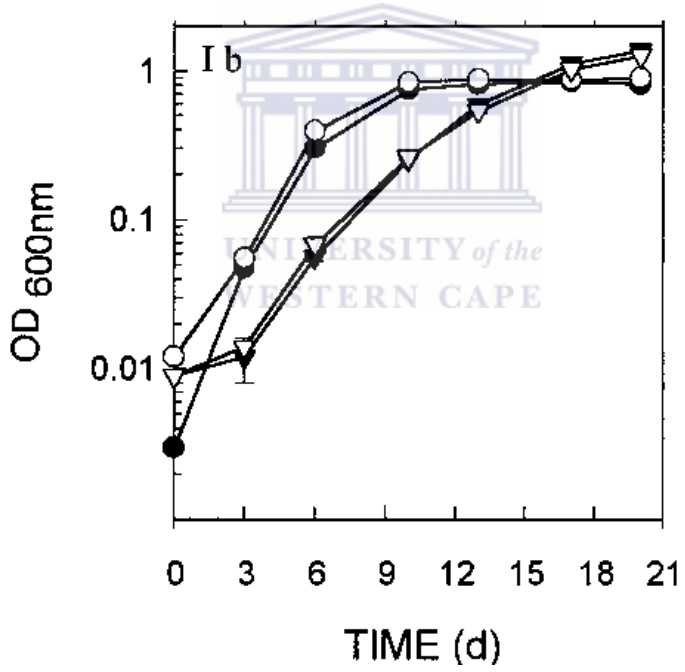


Figure 2.1: Growth curves of *M. tuberculosis* H37Rv Tokyo (II) cultures in Middlebrook 7H9 broth (Meyers et al., 1998).

The growth kinetics of mycobacteria population, like that of any bacteria population, can be divided into a lag phase, a logarithmic (exponential phase), a stationery phase and a senescence phase (Todar, 2008). From estimations based on figure 2.1 above, the lag

phase lasts from the time of inoculation to an OD of around 0.1 when the logarithmic growth commences. The logarithmic phase, in the OD range 0.1 to around 0.9, takes about 10 days. After about the tenth day mycobacteria enter the stationery phase where the cells either enter dormancy or begin to die (Meyers et al., 1998). And during the senescence phase cell death is predominant. It is normally advisable that subculture inoculums be derived from the logarithmic phase.

2.1.7 Definition of efficacy of disinfectants against TB

In Europe and the United States, antiseptics/disinfectants are required to decrease the number of viable bacilli to $1/10^3$ to $1/10^4$ of the original number of organisms to be deemed effective. Testing of instrument disinfectants against mycobacteria should be carried out with a contact of 10-20 min at 20°C. The contact time for testing environmental surface disinfectants should not be longer than 1 min (Sattar et al., 1995).

2.2 Stability study of DMP

Information from the study was used: to predict the shelf life of DMP; to recommend storage conditions; and it also helped in the selection of primary packaging materials. The information could also be useful for pharmacists in the field to offer advice where DMP has been incorrectly stored.

2.2.1 Analytical methods for measuring chlorine dioxide

There are several methods of quantifying CD and its degradation by products, chlorite and chlorate, including: the amperometric titration method; the spectrophotometric method; colorimetric methods; electrochemical methods; and the iodometric titration

method (Buser & Hanisch, 1952; Zsolt, 2004) . The latter method was employed in this study.

2.2.2 Iodometric titration method

In this method, at low pH the analyte oxidizes iodide ion to iodine as described by the equations below. The method is not really selective for CD but measures the total oxidizing power of the solution i.e. the degradation products of CD, chlorite ($ClO2^-$) and chlorate ($ClO3^-$), which inevitably are always present in all CD solutions in small quantities, are also taken in account (Zsolt, 2004).

$$2\ ClO_2 + 10\ I^- + 8\ H \rightarrow 5\ I_2 + 2\ Cl^- + 4\ H_2O$$

$$ClO_2^- + 4\ I^- + 4\ H \rightarrow 2\ I_2 + Cl^- + 2\ H_2O$$

$$ClO_3^- + 6\ I^- + 6\ H \rightarrow 3\ I_2 + Cl^- + 3\ H_2O$$

The iodine formed is titrated with a reducing titrant, commonly sodium thiosulfate ($Na_2S_2O_3$) as shown below (Zsolt, 2004). So in essence this is a back titration method.

$$2S_2O_3^{2-} + I_2 \rightarrow S_4O_6^{2-} + 2I^-$$

The full method is outlined in chapter 5 below.

2.2.3 ICH classification of stability tests

Stability test are classified into: 1) stress tests (forced degradation studies); 2) accelerated stability tests, 3) intermediate stability tests, and 4) long term stability tests (WHO, 2009). According to document Q1A (R2) of the international committee on harmonization (ICH), the long term stability test conditions for climatic zones I and II areas of the world for drug substance intended for storage in a refrigerator is 5 °C ± 3 °C

for a minimum period of 12 months. The frequency of testing are also specified as: 1) first year every three months i.e. 0, 3, 6, 9, 12; second year every six months i.e. 12, 18, 24; and third year and longer annually: 24, 36, 48, 60 (ICH, 2003; WHO, 2009). The long term stability study of DMP performed in this study was a modification of this guideline and is described in chapter 4.

2.3 Formulation development

2.3.1 Dosage form

One of the important early decisions of the drug development team is to select a suitable dosage form. This decision can be made prior to or very early in the formulation development process (FD), depending on the level of knowledge of the active pharmaceutical ingredient (API) as it relates to the disease profile, and these are the primary determinants of dosage form. Secondary determinants include market place issues such as competition and provider-patient considerations (Pavliv & cahill, 2007). Choice of dosage form should be made with a sense of finality as it is very time consuming, costly and often detrimental to change formulation in the midst of ongoing clinical trials (Pharmacelsus contract research organisation, 2004). In this project, a foam formulation was developed.

2.3.2 Topical drug delivery from a foam formulation

Compared to other topical dosage forms, foam may provide unique properties and advantages (Tanojo, Huang, & Maibach, 2007). Clinical studies indicated that foams often produce faster effects for the treatment of some dermatoses. Most commonly reported is the use of foam corticosteroid for the treatment of psoriasis and other scalp

dermatoses where foam has proved more efficient drug delivery than ointments and creams (Franz, Parsell, Myers, & Hannigan, 2000; Tanojo, Huang, & Maibach, 2007). The application of antifungal ketoconazole in foam dosage form is also reported to be more efficient than the current treatment with gels (Tanojo, Huang, & Maibach, 2007).

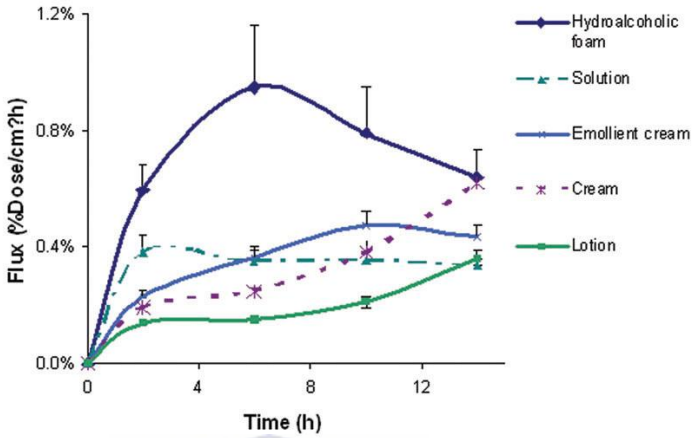


Figure 2.2: Percutaneous flux profile of clobetasol propionate from hydroalcoholic foam in comparison to solution, creams and Lotion (Rekacewicz et al., 1990)

Patients prefer foam to traditional formulations such as cream and ointments because foams are less dense and therefore easier to apply on the skin surface to achieve spread uniformity. The quick absorption of the foam with fewer residues is also found to be more acceptable because it doesn't interact with clothing or other material in contact with the applied site. The undesirable greasy feeling of many creams and ointments is less reported with foam, although the hydrating effect (especially from emollient foam) is comparable (Tanojo, Huang, & Maibach, 2007).

2.3.2.1 Mechanism of drug delivery from foam formulation

In vitro studies have proved that foam formulations are capable of a higher rate of API delivery in comparison with other dosage forms (Huang, Tanojo, Lenn, Deng, & Krochmal, 2005). These findings suggest that foam utilizes a different non-traditional

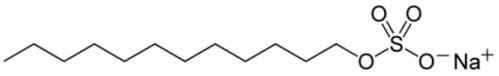
permeation pathway for drug delivery. It has been suggested that components within the foam act as penetration enhancers that reversibly alter the barrier properties of the stratum corneum thereby delivering the API across the skin via the intracellular route. In this way the API bypasses this physical barrier. The traditional topical delivery depends on, and takes place via, the hydrated intercellular spaces, which is a much slower process (Huang et al., 2005; Tanojo, Huang, & Maibach, 2007). Foam formulations also contain a significant amount of water phase, which promote extensive hydration of the intercellular spaces, especially if moisturizers are added. This maximizes drug delivery through this traditional route as well (Tanojo, Huang, & Maibach, 2007). Finally, the reservoir effect also significantly enhances API delivery i.e. during the breaking of the foam; the gaseous component evaporates with the liquid. This leaves a higher concentration of API on the surface and a concentration gradient comes into effect which contributes to delivery of the API. This study is special in that DMP will be releasing gaseous CD during application to target site, which could enhance the foaming process and at the same time improve drug delivery as the foam traps the gaseous CD drug around the site.

2.3.3 Foam theory

The physics of foams has many phenomena. Pure single-component liquids cannot be foamed. In pure water for example, bubbles rupture when they arrive at the surface. Water can only be foamed if it contains a surface-active component, also referred to as surfactants (Babcsán, Banhart, & Leitlmeier, 2003).

2.3.3.1 Surface active agents (SAA)

The surface active agents (SAA) are amphiphilic i.e. they are characterized by having two distinct regions in their chemical structure: 1) a hydrophilic head and 2) a hydrophobic tail. This dual structure is the unique feature of surfactants that is responsible for surface activity (Tichagwa, 2006).



Tail Head

Figure 2.3: Structure of a Surface active agent (SAA)

SAA are classified according to the type, or absence, of charge on the hydrophilic moiety as cationic, nonionic, anionic and amphoteric or zwitterionic (Stamatis, Xenakis, & Kolisis, 1999). The most important technical abilities of SAA and surfactant solutions are emulsifying, solubilizing, wetting, dispersing, foaming, defoaming and detergency (Centre for the Environment and the Lung - Denmark, 2002).

A classification of foaming agents employed in this study is summarized in table 2.1

Table 2.1: Classification of foaming agents (FAs) studied

SAA class	Foaming subclass	FA studied	commonly found in:
Non ionic	1. Ethoxylated sorbitan esters	- Tween 20	- Food, Skin care products, detergents
	2. Ethoxylated alcohols	- MP9 - NEO DAL	
Anionic	1. Alkyl sulfates	- SLS - ALS	- Oral care, Hair care, Skin care , Medicated products
	2. Sarcosinates	- SNLSS	
Cationic	-	-	
Amphoteric	Betaines	- CAPB	- Skin care, Hair care, Medicated products

2.3.3.2 Surface chemistry and foaming

Figure 2.4 below depicts a dynamic situation where a foaming agent (FA) is added to an aqueous medium in the absence of agitation. Initially the FA preferentially adsorbs at the air/water interface called the surface (Kanicky, Montilla, Pandey, & Shah, 2001). This lowers the surface tension but most important for a foaming agent, it increases surface elasticity. If the liquid was agitated at this point foam would be generated consisting of air spaces surrounded by elastic films. These films are stabilized by a combination of Gibbs and Marangoni effects (Babcsán, Banhart, & Leitlmeier, 2003). Further addition of FA results in a situation where the surface becomes saturated, and therefore micelles begin to form within the liquid bulk. This is the critical micelle concentration (CMC). Foam volume increases with FA concentration up to this point but addition of FA beyond

this point results in minimal increases in the volume of the generated foam (Amaral, Neves, Oliveira, & Bahia, 2008).

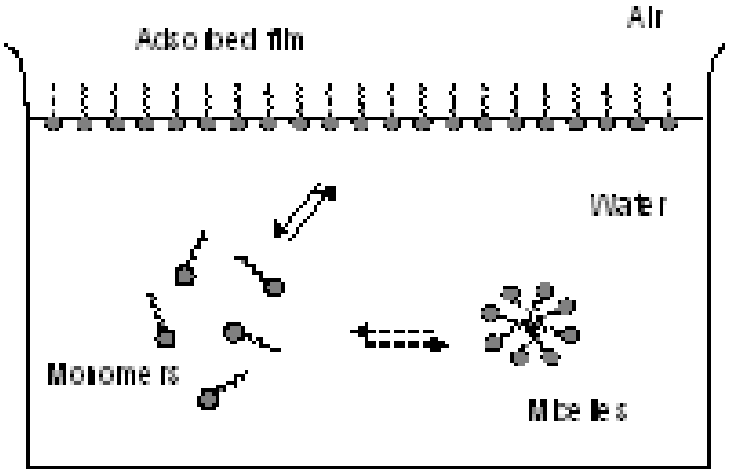


Figure 2.4: Schematic representation of surfactant molecules in water above the CMC (Kanicky et al., 2001)

2.3.3.3 Causes of foam instability

Three different processes contribute to the instability of foams i.e. drainage, coalescence and coarsening. Drainage is explained in section 2.3.4.1.3 below. Coalescence is the merging of two bubbles as a result of the rupture of the film between the bubbles. Larger bubbles appear in the foam and the number of bubbles decreases. Coarsening is interbubble gas diffusion, also called Ostwald ripening. As a result of gas diffusion larger bubbles grow at the expense of smaller bubbles. Smaller bubbles shrink and may finally disappear (Bisperink, Ronteltap, & Prins, 1992; Carey & Stubenrauch, 2009; Weairy, 2002).

2.3.3.4 Structure of Liquid foams

2.3.3.4.1 Levels of organization

Liquid foam has four levels of structural organization as depicted graphically below i.e. the molecular level (individual FA molecules), the microscopic level (film of liquid and FA molecules) and the macroscopic levels (individual bubbles making up the foam):

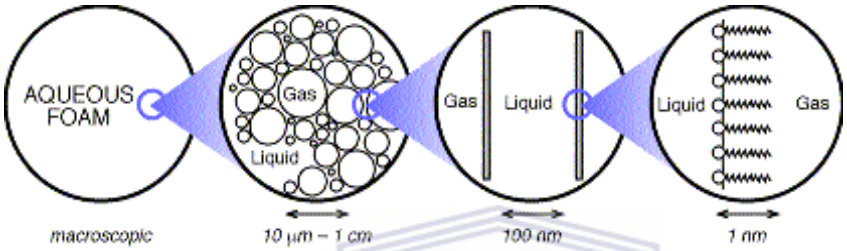


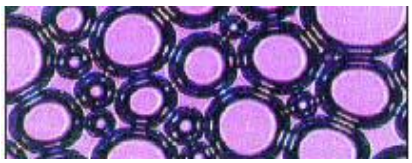
Figure 2.5: Levels of structural organization of a liquid foam (Durian, 2002).

2.3.3.4.2 Wet and dry foams

During its existence, foam loses water through drainage, which is the downward movement of the water under the influence of gravity. This reduces wet foam to dry one, with less than 1% of liquid. Wet bubbles are spherical while dry ones are polyhedral with curved faces (Bisperink et al., 1992; Weairy, 2002):

a) Wet foam

(Spherical bubbles)



b) dry foam

(Hexagonal bubbles)

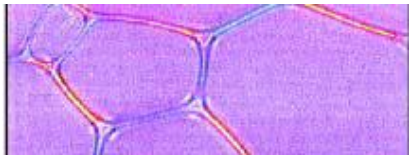


Figure 2.5: bubble structure of wet and dry foam (Morrison, 2009)

2.3.4 Preformulation studies

Early in FD, it is essential that certain fundamental properties of the API and/or proposed excipients are determined. Information from these studies enhances rational formulation development whereby the aspects learnt, decides many of the subsequent events in FD (Patel, 2009). In this study, preformulation took the form of foam evaluation studies, buffer characterization by potentiometric titrations, and compatibility studies (WHO, 2008).

2.3.4.1 Foam evaluation

Foamability and foam stability are two very important properties of any aqueous foam system (Amaral et al., 2008). In this study, the two properties have been studied with a view to optimize foaming, for patient satisfaction. Patients expect rapidly forming, lubricious, and dense foam that lasts long enough. In the absence of this the product is bound to fail (Klein, 2004). Trix, a commercial detergent, failed in the Australian market, despite being a very effective cleaning agent simply because it was a non-foaming detergent (Campbell & Campbell, 2008).

2.3.4.1.1 Evaluation of foamability

Three standard methods are popularly employed to evaluate foamability: 1) the Ross–Miles (pouring) method, 2) The Foam Scan (sparging) method, and 3) the mechanical agitation method. The last method was employed to evaluate foamability in this study by agitation of the foam solution for a fixed period of times at the same rate. The height of the generated foam, over the given period of time, could be measured and this allowed for foamability determination (Carey & Stubenrauch, 2009).

2.3.4.1.2 Evaluation of Foam Stability

Foam stability is most commonly evaluated by measuring the change in foam volume with time. This method was employed in this study and foam stability was expressed as foam height at half-life $t_{1/2}$ (Wilde & Clark, 1996). Measuring the volume of liquid that drains from the foam could also be related to foam stability and was also studied (Amaral et al., 2008; Ross, 1943) and the volume of liquid drained at foam $t_{1/2}$ was determined (Wilde & Clark, 1996). The time taken for a bubble to break was yet another characteristic studied as a measure of foam stability. Though it is a well-known and demonstrable fact that several bubbles together are more stable than each separately, experimental results demonstrate a correlation in the stability results between the single bubble observations and some foam-measuring methods for systems (Ross, 1943).

2.3.4.1.3 The role of foam stabilizers

Foam stability could be achieved by employing foam stabilizers which could be: a) wetting particles present in Plateau borders which slow down foam drainage; b) partial wetting particles which form layers on the surface of the liquid films and therefore can be considered as surfactants; c) colloidal particles in the liquid films which forms long-range non-DLVO (Derjaguin, Landau, Verwey, and Overbeek, a theory that explains the stability of colloids as being dependent mainly on the balance between attractive and repulsive forces) surface force called structural force e.g. surfactant micelles, macromolecules or solid particles. The mechanism of foam stabilizers is not fully understood yet (Amaral et al., 2008; Carey &Stubenrauch, 2009). In this research cetostearyl alcohol (CSA) was employed as a foam stabilizer (Cosmetics info.org, 2009).

CSA probably achieves foam stabilization as solid macromolecular particles as well as by micelle formation.

2.3.4.2 Potentiometric acid-base titration

A buffer for an envisaged formulation could be characterized by potentiometric acid-base titration whereby the pH changes are monitored using the pH meter. The pH meter uses an electrode whose potential depends on the concentration of H^+ ions in solution. During titration of a strong acid versus a strong base, the change in pH is initially small until the end point, where a sharp change is encountered. A major advantage of the potentiometric titration is that it is not necessary to add the titrant drop wise to obtain the equivalence point as in conventional indicator based titrations. The main requirement here is appropriate increments of added titrant which can lead to a good graph and analysis of results **(**GA/7 Potentiometric titration**,** 1999**)**.

2.3.4.2.1 Buffers

Buffers contain two functional species in solution: 1) an acidic one, which can react with any added base, and 2) a basic one, which can react with any added acid. The two species are in molar equilibrium and are referred to as conjugate acid-base pairs (Thomson, 2006). An illustration of such a pair and reactions with externally added acid or base is given below:

$$HA \underset{}{\overset{OH^-}{\rightleftharpoons}} A^-$$

(2)

Acid H+ conjugate base

The assumption here is that the concentration of buffer salts exceeds that of the acid or base being introduced to the solution.

2.3.4.2.2 Buffer Capacity

This is a measure of ability of a buffer system to resist pH change and is indicated by the buffer index (β) (Powers et al., 2005):

$\beta = \Delta B/\Delta pH$

Where: B = strong base/acid (in moles); Δ = change (delta)

Buffers with a large buffer index possess a greater buffering capacity. This means that a smaller pH change is experienced from the addition of a given amount of strong acid or base. Buffer capacity depends on the total concentration of the buffer system and on the HA/A- ratio (Powers et al., 2005; Thompson, 2004).

2.3.4.2.3 Determination of the end point

The end point of an acid base potentiometric titration could be determined in four ways:

1) Using the sigmoid curve to estimate the central part of the rise.

2) Using the first derivative plot. This is the slope of the titration curve, and can be obtained simply from equation 1 below. Each first derivative point is plotted against V' where V' is the incremental value of the added titrant. The endpoint occurs at the volume, V', where dpH/dV has the maximum value.

   $pH_2 - pH_1 = dpH$

   $V_1 - V_2 = dV'$

   $dpH/dV' = 1^{st}$ derivative plot Equation 1

3) Using the second derivative plot which is the rate of change of the slope of the titration curve, and is shown in equation 2.

$(dpH_2/d\ V')_2 - (dpH_1/d\ V')_1 = d^2\ pH$

$(dV_2 + dV_1)/2 = dV^2$

$d^2pH/\ dV^2 =$ 2^{nd} derivative plot Equation 2

Each second derivative point is plotted against V" is the increment in titrant volume. The end point occurs at the volume, V", where $D^2pH/\ dV^2$ is zero **(**GA/7 Potentiometric titration**,** 1999; Christian, 1977**)**.

4) Using the Glan's plot method which was not employed in this study (Rossotti, & Rossotti, 1965**)**.

2.3.4.2.4 Significance of a buffer

The basic function of the buffer in this study was to protect the pharmaceutical preparation from unsolicited fluctuations in pH when limited concentrations of acid or base are added (Thompson, 2004). This in turn was meant to reduce tissue irritation. Buffering was also employed to enhance stability of DMP which is pH dependent. Other common uses of buffers are to enhance solubility and absorption (DiFeo, 2003; Rossotti & Rossotti, 1965).

2.3.4.2.5 Choosing the correct pharmaceutical buffer

The following are some considerations which could be helpful in choosing the most appropriate pharmaceutical buffer: Choose a weak acid with pH >> pKa; Use buffer equation to calculate ratio of acid/base needed to give required pH; choose concentration needed to give suitable buffer capacity; choose available ingredients considering sterility,

stability, cost, toxicity; use pH meter or, at least, pH indicator paper (Thompson, 2004). In this research the phthalic acid/potassium hydrogen phthalate (KHP) buffer system was used because of its ideal buffering range of pH 2.2 to 6 (Analchem Resources, 2001).

2.3.4.3 Excipient selection

In this study sodium lauryl sulfate (SLS) was used as the primary foaming agent while cocoamidopropyl betaine (CAPB) was employed as the secondary foaming agent. Gohel, M. et al. (2007) stated that the concentration of foaming agents particularly in suspension formulations need to be 0.5 % or less as higher concentrations promote instability. In a vaginal formulation, the concern with SAA is the risk of irritation. Both SLS and CAPB are regarded as safe and are listed in Garg's compendium of vaginal excipients. Both SLS and CAPB are bitter tasting as is the case with most SAA. Glycerol was the humectant of choice in this formulation because it is readily compatible with many substances and is easy to handle (The Soap and Detergent Association, 1990). The concentration of glycerol in formulations is ideally in the range of 0-10 % w/w (Gohel, M. et al. 2007). Hydrotropes act as solubilizing agents in a formulation and without them it could be impossible to incorporate sufficient quantities of other ingredients. Recommended hydrotrope concentration in a formulation is 3-5 % (Tiger chemical company, 1997). Sodium xylene sulfonate was employed as the hydrotrope in this formulation. The MRF needed sweeteners and flavorants particularly to mask the bitters which abounded in this formulation as alluded to earlier. Bulk sweeteners such as common sugar are ideally recommended to be 15-70 % w/w in concentration, while artificial sweeteners could be the range 0-5 g/100 ml. Masking could be a complex undertaking requiring introduction of competing stimuli of specific sweetness and flavor profiles and/or blocking bitter stimuli. Improved masking can be achieved by a

combination of monoammonium glycirrhyzinate, which has an initial burst of sweetness that dissipates rapidly with sodium saccharine, whose sweetness profile is slow in onset but long lasting (Stier, 2004). Finally, it is common knowledge that most excipients perform multiple roles in a formulation. In this case in addition to the above mentioned main roles, SLS is believed to have some biocidal properties; CAPB was also acting as a thickener and as a foam booster (Cui, 2011) and glycerin acted as a thickener, sweetener, lubricant, solvent, emollient, demulcent, etc. (The Soap and Detergent Association, 1990).

2.3.4.4 Excipient compatibility testing

These are empirical tests designed to study the effects of excipients on pharmaceutical stability with the ultimate aim of excipient selection for the formulation. Compatibility monitoring in this study was mainly by physical inspection (Monkhouse & Maderich, 1989), but also by iodometric titrimetry to determine changes in concentration of CD that arose due to incompatibilities.

2.3.5 Formulation

This stage of formulation development consists of: 1) process and product design, which is essentially the initial planning aspect; 2) manufacturing process development; 3) optimization of excipient quantities in the formulation; and 4) and selection of the lead formulation (Yu, 2008). In practice, the approaches to formulation takes many and diverse paths and could be the least regulated in the drug development process i.e. the attitude of regulators is such that whatever works is acceptable so long as it can be scientifically rationalized (DiFeo, 2003).

2.3.5.1 The vaginal douche (VGD)

Desirable qualities for a vaginal formulation include: easy to use, discrete, cost effective, safe for continuous application, allow self-administration, etc. These could be achieved through judicious formulation taking into account the biology of the vagina (Das Neves & Bahia, 2006) and especially pH considerations. The pH of the normal vagina ranges 3.5-4.5 (Boskey, Cone, Whaley, & Moench, 2001). The pH of the douche needs to be around the stated vaginal pH range. Failure to this, vagina irritation or even outright acid burns could result.

2.3.5.2 Mouth rinse formulation (MRF)

Mouth rinse antiseptics control dental diseases by destroying and preventing biofilm formation. CD mouth rinse formulation (MRF) could be especially ideal for this purpose due to its powerful antimicrobial properties as discussed above. CD is known to control halitosis through a dual mechanism i.e. indirectly by killing the microorganisms responsible for halitosis and directly by oxidizing VSCs and their precursors to non-malodorous products. The later mechanism is rare among antiseptics and suggests that CD mouth rinses have a superior anti halitosis effect, which indeed has been proven in a number of studies (Shinada et al., 2010). The mouth's natural pH ranges from 6.2 – 7.0. Fortunately, the mouth is capable of tolerating acidic preparations due to the presence of effective natural buffer systems. The mouth pH can drop below 2.3 upon consuming acidic items. Saliva contains bicarbonate ions which mainly act to neutralize the acids. Saliva also contains phosphate ions and proteins that act as buffering agents to maintain oral pH (Kivela, Parkkila, Parkkila, Leinonen, & Rajaniemi, 1999). The pH of the formulated mouth rinse was designed to be around 4.0. This should be safe to take. The

pH of lemon is far lower than this and Listerine® mouth wash has a pH of 4.3 (Almeida, Poskus, Guimarães, & Silva, 2010; Emmerling, 2007).

2.3.5.3 Foot bubble/sit bath

CD is considered to be ideal for treating skin condition because it has superior antimicrobial properties, low toxicity, and is non-irritant when compared to similar commonly used skin antiseptics such as hypochlorite. In addition to this, CD does not form appreciable chlorinated hydrocarbons, which are carcinogenic. Currently, CD is commonly used in low concentration as an antiseptic for skin disinfection e.g. mastitis control (Alliger, 2001). The skin has a protective acid layer called the acid mantle. The pH of the human adult skin ranges from 4.1-5.8 with an average of about 4.8 (CP Kelco, Inc. 2008), but this tends to be higher in children (Boelsma et al., 2003). The skin also possesses potent buffering and neutralizing agents which are responsible for maintaining the normal pH range and is known to withstand exposure to low pH values without ill effects (Farage, 2010). Therefore the FB/SBB envisaged to be at around pH 4.8 should be safe. The target pH for the F/SBB was pH 4.8.

CHAPTER 3

IN-VITRO EVALUATION OF THE EFFECTIVENESS OF DMP FOR USE AS CHEMICAL DISINFECTANT (COLD STERILANT) AGAINST *MYCOBACTERIA TUBERCULOSIS* (TB)

3.1 Materials and methods

3.1.1 Equipment

Centrifuge - BeckmanJ2-21 high speed floor model - (*Beckman RIIC LTD, Great Britain*).

Autoclave – (*capacity 0.132 m^3 Albert Moore (PTY) LTD, 1981 model, Cape Town, SA*).

Biosafety lamina flow cabinet – (*Laminaire, New Jersey, USA*).

Incubator, Memmert 854 model, (*Memmert GmbH, schwabach, West Germany*).

Vortex-Genie 2, model G-560E, (*Scientific Industries, Inc. N.Y. USA*).

Spectrophotometer, Beckman DU® 640 model – (*Pegasus scientific INC. Rockville, USA*).

Fridge, model C370– (*Defy, capacity 360 L, Defy SA*)

Battery powered pipette controllers – (*Integra Biosciences, SA*)

Rubber bulb pipette fillers -

Micropipettes - (*model M1000, Gilson, INC. France*).

Micropipette fin tips 100 – 1000 ul, - (*Merk (Pty) LTD. SA*).

Sterile tissue culture flasks (25 cm^2) – (*SPL life sciences, SA*).

Appropriate glassware

3.1.2 Materials

DMP (*Rausa-Kem, Parow Valley, Cape Town, and SA*)

Absolute ethanol (*Saarchem, South Africa)*

Polysorbate (Tween) 80 (*Dynachem ltd. SA*)

Middlebrook agar 7H11 (*BD biosciences, SA*)

Middlebrook 7H9 (*BD biosciences, SA*)

Oleic acid albumin dextrose catalase (AODC) enrichment (*BD biosciences, SA*)

Basic fuschin (*Merk, KGaA, Darmstadt, Germany*)

Phenol (ELS (*PTY) limited, SA*).

Concentrated Hydrochloric acid *(SP Scientific, South Africa)*

Methylene blue (*laboratory stock*)

Immersion oil (*laboratory stock*)

3.1.3 The spectrophotometric method

The spectrophotometric method, a modification of the European quantitative suspension test EN 14348, summarized in section 2.1.2.1 of the literature review was employed to evaluate anti-tuberculoid activity of DMP. The main modifications to the EN 14348 method relate to the use of *M. bovis BCG* as surrogate in place of *M. terrae*, the use of 7H9 broth as culture media in place of agar based culture media, dilutional neutralization with sterile distilled water in place of the Tween 80/SLS/lecithin cocktail and most important, use of spectrophotometric CFU enumeration of mycobacteria technique in place of plate counts.

All tests were carried out in triplicate (n=3)

3.1.3.1 Test organism

Mycobacterium bovis BCG was employed as surrogate for *Mycobacterium tuberculosis* for reasons given in the literature review. The *BCG* was obtained from the DST/NRF Centre of Excellence in Biomedical Tuberculosis Research, at the Department of Biomedical Sciences, Faculty of Health Sciences and Stellenbosch University at Tygerberg Hospital.

3.1.3.2 Culture media

Middlebrook agar 7H11 was used to stock *Mycobacteria* for storage purposes at – 80 ^{o}C while middlebrook 7H9 broth was employed to culture the *Mycobacteria* and for sub culturing purposes. Both were enriched with AODC (Flournoy & Twilley, 2001).

3.1.3.3 Ziehl-Neelsen (ZN) stain

Acid fastness of the *M. bovis BCG* was confirmed by (ZN) staining performed as follows:

3.1.3.3.1 Preparation of carbol – fuschin

0.3 g basic fuchsin was first dissolved in Ethanol, 95 % (v/v), 10 ml. The phenol crystals were heated to 45 °C to melt, then transferred to 5 ml water and dissolved. The basic fuchsin solution was then mixed with the phenol solution followed by addition of 95 ml of distilled water. The reagent was filtered prior to use as recommended to attain best results (Hussey & Zayaitz, 2010).

3.1.3.3.2 Decolorizing solvent

The decolorizing solution was prepared by mixing ethanol, 95 % (v/v), 97 ml with hydrochloric acid (concentrated), 3 ml (Hussey & Zayaitz, 2010).

3.1.3.3.3 ZN staining procedure

The smeared mycobacteria material was fixed by placing the slides on an electric hot-plate prior to staining (65 - 75 ^{o}C). This procedure was performed in the protective cabinet until the smeared material was dried and fixed. The slide was then flooded with carbofuschin followed by gentle heating until steaming. It was then incubated for 3 – 5 minutes and then rinsed with water followed by decolorization with 3 % v/v acid alcohol solution for 2 – 3 minutes. Again rinsing with water followed by replacing with fresh acid alcohol for 3 – 4 minutes until the slide retained a pink color. Again at this stage it was rinsed with water and this was followed by counter staining with 1 % w/v methylene blue. Finally, the slide was once more rinsed with water followed by drying. Once dry, immersion oil was applied and the slide was examined under a light microscope. Acid fast mycobacteria stained red (Health protection agency, 2007).

3.1.3.4 Disinfectant

The DMP was stored in the fridge (2 – 8 ^{o}C) and working dilutions were always freshly prepared for the tests. Activity was evaluated at concentrations of 2600, 1300, 650, 325, 162, 28.9, 20.2, 14.4, 11.5, 8.7, 5.7, and 3 ppm.

3.1.3.5 The pH of disinfectant

The fact that acidic DMP (pH = 0.5) was diluted with water to achieve desired concentrations followed by mixing of a small amount of this DMP with test solution

(neutral pH of water) implies that the resultant pH of the test solution was higher than that of normal DMP. The actual value was not determined.

3.1.3.6 Preparation of sterile distilled water

De-ionized water was autoclaved at 121 ^{o}C for 15 minutes in 1or 2 litre SCHOTT bottles to achieve sterility.

3.1.3.7 Glassware sterilization

Flasks and pipettes were plugged with cotton wool stoppers and/or simply wrapped in aluminum foil and sterilized by autoclaving. Pipette tips and cleaned 50 ml plastic test tubes were also sterilized by autoclaving. Cuvetts and their lids were sterilized by immersion in 70 % ethanol. 70 % ethanol was also used to sterilize any others material that needed to enter the biosafety chamber where the aseptic experiments were in progress.

3.1.3.8 Contact time

Mycobacteria were exposed to the disinfectant for 30 seconds, 1 min, 5 min, 10 min, and 20 min.

3.1.3.9 Controls

3.1.3.9.1 Positive control

Mycobacteria were exposed to sterile distilled water in place of the antiseptic at stage 2 in the schematic flow chart of the test procedure below (figure 3.1).

3.1.3.9.2 Negative control

The growth medium (7H9 middlebrook broth) was not inoculated with *mycobacteria* solution but sterile distilled water was used in place of *mycobacteria* solution at stage 8 of the same figure 3.1 schematic flow chart.

3.1.3.10 Experimental temperatures

All experiments were carried out at room temperature i.e. around 15 – 28 ^{o}C.

3.1.3.11 Preparation of the 7H9 broth culture media

Middlebrook 7H9 (4.7 g) culture media powder was suspended in 900ml of deionized water containing 0.5 g of polysorbate 80 in 1000 ml specimen bottle. This was then autoclaved at 121 ^{o}C for 15 min. 100 ml of AODC enrichment was added aseptically when the autoclaved material cooled to 45 ^{o}C.

3.1.3.12 Preparation of mycobacterium suspensions

Stock agar cultures were stored at −80 °C. Before testing, it was thawed and sub cultured by inoculating into 20 ml of 7H9 middlebrook growth medium (OADC enriched). It was then incubated for 7 days at 37 °C.

The broths were further sub-cultured every other week, by inoculating 0.2 ml of *Mycobacterium* solution in logarithmic phase of growth, into 19.8 ml 7H9 middlebrook broth in 25 cm^2 tissue culture flask(s) followed by incubation at 37 ^{o}C again for 10 days. In all such procedures aseptic techniques were strictly followed.

3.1.3.13 wavelength of OD readings

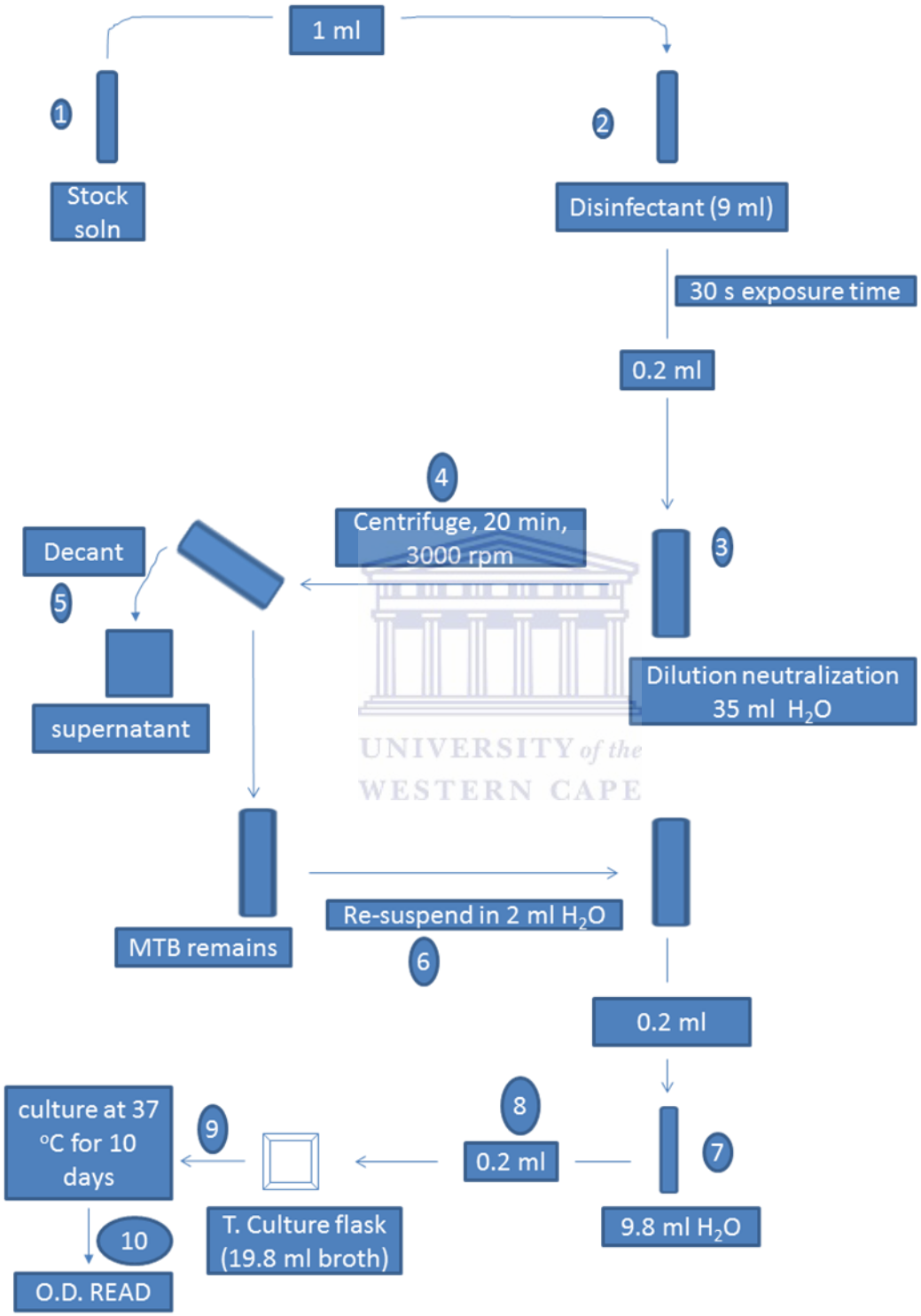
All OD readings were performed at a wavelength of 600 nm.

3.1.3.14 Tests procedure

The actual disinfectant activity test against *Mycobacteria* was performed on the 10^{th} day of incubation. The subcultures in the 25 cm^3 tissue culture flasks that were ear marked for the days experiment would be adjusted to the absorbance of reading to 0.4620 which gives a concentration of 6.25 x 10^8 CFU/ml. To achieve this, the O.D of the stock culture solution would be measured and appropriately aseptically adjusted either with more sub-culture titre or with sterile distilled water, until the O.D reading came to the desired 0.4620. Once the desired O.D reading was achieved, 1 ml of *mycobacteria* test suspension was added to 9 ml of disinfectant solutions, previously diluted to the desired concentration. After the exposure time, 0.2 ml of the solution was removed and subjected to dilution neutralization by adding it to 35 ml of sterile distilled water. The resulting suspension was then centrifuged at 3,000 rpm for 20 minutes. The supernatant was decanted and the remaining bacteria re-suspended in 2 ml sterile distilled water. 0.2 ml of the suspension was drawn and added to 9.8 ml sterile distilled water of which finally, 0.2 ml of this was inoculated to 19.8 ml 7H9 middle brook broth growth medium in a 25 cm^2 tissue culture flask. This was cultured for 10 days at 37 ^oC.

O.D. readings were performed on the 10^{th} day of incubation. The whole procedure was done in triplicate as shown in table A1 and A2 in the appendix. Averages were employed in the calculations that followed.

Figure 3.1: Schematic illustration of the TB test procedure



3.2 Results and discussion

Table A1 and table A2 in the appendix section illustrates the Mycobactericidal activity of DMP, results of the experiments done as explained in the preceding pages.

3.2.1 Problems encountered in culturing mycobacteria

Initially disinfectant activity tests at step 8 in the schematic diagram above and the subsequent culturing (step 9), were done in 10 ml sterile plastic test tubes. 4.8 ml of 7H9 broth was inoculated with 0.2 ml of *Mycobacteria,* followed by exposure to the disinfectant and processed as explained in section 3.1.3.14. It was consequently found that containers like 10 ml test tubes inhibited the growth of the BCG due to a lack of oxygen. The 5 ml column head contributed to suffocation especially since the mycobacteria settled at the bottom and thus diffusion of gases could have been inadequate. This was overcome by switching to 25 cm^2 tissue culture flasks. *Mycobacteria* are obligate aerobes as explained in the literature review. The 25 cm^2 tissue flask container closures have aeration holes which are sealed with a filter paper lining beneath. This allows for assurance of aseptic integrity, at the same time allowing for aeration. The flat 25 cm^2 base ensures an overhead column of about 1 ml, allowing for diffusion of oxygen. Daily agitation also assisted uniform distribution of oxygen.

3.2.2 Explanation of some formulas employed

3.2.2.1 Calculation of bacteria numbers as they varied due to dilution dynamics:

With reference to the schematic drawing of the methodology above (figure 3.1), an equation was developed, as shown below, to relate number of bacteria that survived exposure to disinfectant (test tube 2) if number of bacteria inoculated in the tissue culture

flask (N_0) in step 8 above was known, and vice versa. The formula was derived based on the dilution factors involved.

$P = 25{,}000N_0$

Where:

P = number of bacteria that survive exposure to disinfectant (step 2 of the schematic diagram)

N_0 = number of bacteria inoculated in the 25 cm^2 tissue culture flask (step 8)

3.2.2.2 Calculation of bacteria numbers at start and end of culturing period

The population growth of *Mycobacteria* during day 1 to day 10 of culturing, after the experiment i.e. after step 9 of figure 3.1, was logarithmic (Meyers et al., 1998) and is approximated by the equation below (Domínguez et al., 2001; Lambrecht et al., 1988):

$$N_t = N_0 e^{\lambda t} \quad (2)$$

Where N_0 and N_t are the counts at times zero and *t* respectively. λ has been used for the specific growth rate. N_t was determined by OD readings on the 10^{th} day of incubation. Since the mycobacteria population grows by binary fission (Todar, 2008), equation 2, becomes:

$$N_t = N_0 2^n \quad (3)$$

And

$$n = T/G \quad (4)$$

Where:

n = number of generations

T = duration of the incubation

G = generation time of BCG = 12 – 18 hours

7H9 middlebrook broth is specifically meant for rapid growth of mycobacteria and generation time was taken to be 12 hours (Flournoy & Twilley, 2001).

3.2.3 McFarland standards calibration curve

Table 3.1 shows the McFarland Nephelometer standards from literature sources which were used to make a standard calibration curve from plots of O.D. against bacteria concentrations (figure 3.1). O.D. readings, at the end of the incubation period, could easily be converted to concentration of bacteria, or vice versa, using the calibration curve either graphically or from the equation of the calibration curve as calculated below:

Table 3.1: McFarland Nephelometer scale (MacFarland, 1907).

McFarland Standard No.	0.5	1	2	3	4
1.0% Barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
1.0% Sulphuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density (1X10^8 CFU/ml)	1.5	3.0	6.0	9.0	12.0
% Transmittance*	74.3	55.6	35.6	26.4	21.5
Absorbance*	0.132	0.257	0.451	0.582	0.669

*at wavelength of 600 nm

The McFarland Nephelometer scale shown above is fully explained in the literature review. It is a universally acceptable tool for quantify bacteria in suspensions (Wei, Shepherd, Browne, Clark, O'Leary, 2007).

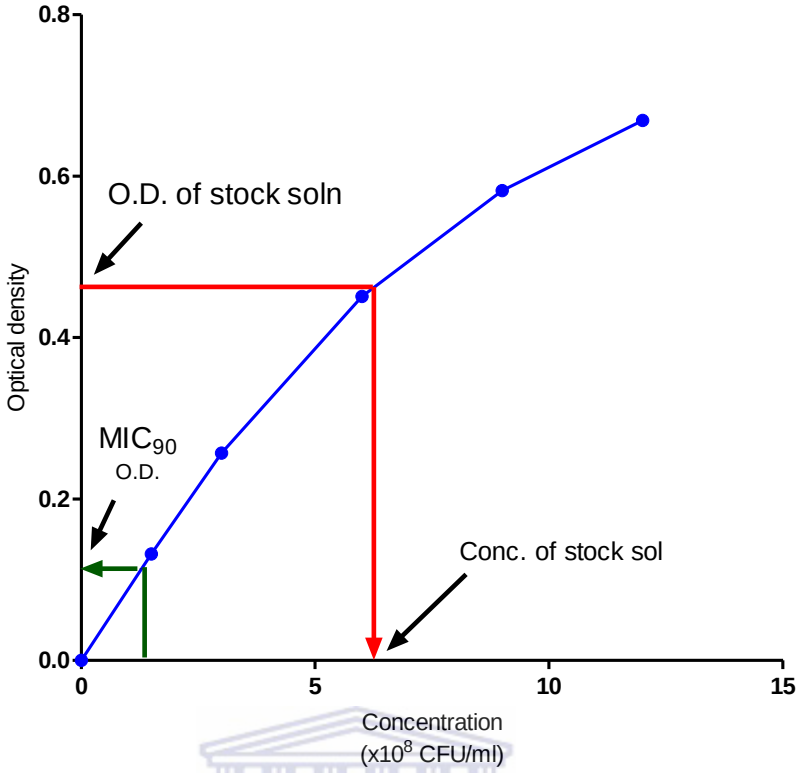


Figure 3.2: Calibration curve of opitical density against bacteria concentration Mcfarland standards

3.2.3.1 Calculation of the equation of the calibration curve

Since the curve has one minimum or maximum, its equation must be a 2 degree i.e.

General equation is:

→ $F(x) = y = ax^2 + bx + c$

→ Since intercept = 0, c = 0

→ Equation becomes: $f(x) = Y = ax^2 + bx$

→ To calculate a and b, we choose any 2 points on the curve e.g. ($3.0x10^8$,0.257) and ($6.0x10^8$, 0.451) and make two equations by substitution:

i) $0.257 = a(3)^2 + b(3)$

ii) $0.451 = a(6)^2 + b(6)$

→ From equation (ii):

→ $b = [0.451 – a(36)]/6$

→ $b = 0.0752 – a(6)$

→ Substitute b in equation (i), for example, and calculate a.

→ $0.257 = a(3)^2 + 3[0.0752 – 6(a)]$

→ $0.257 = a(9) + 0.2256 – 18(a)$

→ $– 9(a) = 0.0315$

→ $a = – 0.00346$

$b = 0.0752 – 6 (-0.0035)$

$b = 0.0962$

Therefore, the equation of the calibration curve is:

$F(x) = y = – 0.00346x^2 + 0.0962x$

This equation could be used to calculate mycobacteria concentration if O.D. was known, and vice versa, as an alternative to graphical determination. It should be remembered that the x value after such a calculation need to be multiplied by 10^8.

3.2.3.2 Quantitative evaluation of the bactericidal activity of DMP

The important data to evaluate MIC and MBC was the DMP concentrations where growth of BCG was observed (refer to table A2 in the appendix). The O.D. against DMP

concentrations (where growth was observed) were plotted and bactericidal activity determined as below.

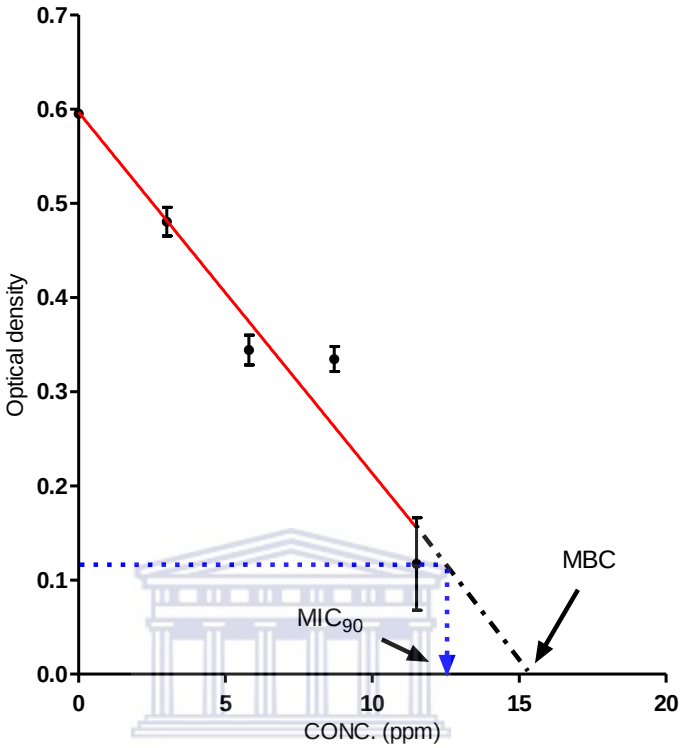


Figure 3.3: Plot of optical density against DMP concentration

3.2.3.2.1 Determination of the MIC_{90}

→ O.D. reading of stock solution was adjusted to 0.4620

→ From calibration curve 0.4620 = $6.25x10^8$ CFU/ml

→ At MIC_{90} , number of bacteria killed = 90/100 x $6.25x10^8$ =$5.62x10^8$ (i.e. 1 ml added to DMP)

→ Number of bacteria that survive exposure = P = $6.25x10^8$ – $5.62x10^8$ = $6.25X10^7$ CFU

→ Number of bacteria inoculated in the tissue culture flask = N_0 = P/25,000 = 2500 CFU

→ n = T/G = 10 days/12 hours = 240 hours/12 hours = 20 generations

→ $N = N_0x2^n = 2500 \text{ CFU} \times 2^{20} = 2.62x10^8 \text{ CFU/20ml} = 1.31 \times 10^8 \text{ CFU/ml}$

→ On the calibration curve, $1.31x10^8$ CFU/ml gives an O.D. reading of 0.115

→ This O.D. is employed in section 3.2.3.2.2 below to calculate the MIC_{90} i.e.

→ **MIC_{90} = 12.5 ppm**

3.2.3.2.2 Determination of the minimum bactericidal concentration (MBC)

The equation of the straight line in figure 3.3 can be derived as follows:

Y = MX + C

→ M = Y1 – Y2/X1 – X2 = (O.48053 – 0.213)/(30 – 100) = 0.26753/-70 = – 0.003822

→ To calculate C, point (100,0.213) is used i.e. C = Y - MX = 0.213- (-0.003822X100) = 0.5952

Note: C is actually one of the positive control results

→ The equation of the straight line: Y = -0.003822X + O.5952

At Y = 0, X = MBC = -0.5952/-0.003822 = 15.6 ppm

3.2.3.2.3 Mycobatericidal activity of DMP at higher exposure times

Only mycobactericidal activity at 30 seconds exposure time is reported because as can be seen from table A10 and A11 in the appendix, DMP is effective at low concentrations at 30 seconds exposure i.e. MIC_{90} is 12.5 ppm. This is expected to drop even further at higher exposure times.

3.2.3.2.4 Calculation of the microbiocidal effect (ME) using the Standard suspension test (SST) procedure (van Klingeren & Pullen, 1987)

ME = log Nc - log Nd

Where:

ME = Microbiocidal effect

Nc = the number of CFU per ml of the test mixture without disinfectant (+ve control)

Nd = the number of CFU per ml of the test mixture after the action of the disinfectant.

From the above results, no mycobacteria CFU survived to DMP exposure above a concentration of about 15.6 ppm. Therefore:

ME = log 5.76 X 10^ 8 - log 0

ME = 8.76

i.e. this is an 8.8 log reduction at 1 second exposure time.

3.2.3.2.5 Calculation of the $CT_{90\%}$ of DMP (Taylor et al. 2000).

$CT_{90\%}$ = concentration of CD that kills 90 % of mycobacteria X exposure time

= 12.5 ppm x 1s

$CT_{90\ \%}$ = 12.5 ppm.s

The MBC of DMP against *M. bovis BCG* was found to 15.6 ppm. This means that from this DMP concentration upward, DMP eradicates all the mycobacteria exposed to it for at least 30 s. This underscores the effectiveness of CD and shows that CD is actually more of a sterilant than a mere disinfectant. According to the standard suspension test, DMP from the initial concentration of 2900 ppm to 15.6 ppm achieved 8.8 log reduction in *Mycobacteria* concentration. This strengthens the argument that DMP is more of a sterilant than a disinfectant. A disinfectant that achieves a 4 log reduction against Mycobacteria at 10 to 20 minutes exposure time is regarded as effective by the both the European and American official disinfectant test (Griffiths et al., 1998). Since the $MIC_{90\ \%}$ is 12.5 ppm, it means that this concentration will kill 90 % of the mycobacteria exposed to it for 30 s. This also implies that at this concentration DMP has to be applied multiple times to eradicate *Mycobacteria* and will achieve complete eradication in about 3 to 4 application. This holds for any number of organisms. The CT value of 12.5 ppm.s is slightly higher than the value other researchers give for other SCD. Taylor et al. (2000), report a $CT_{99.9\%}$ value of 11± 2 ppm.s for *M. avium strain 5002*. This is expected to be more resistant than *M. bovis BCG.*

CHAPTER 4

LONG TERM STABILITY STUDY OF DMP

4.1 Materials and methods

4.1.1 Design of experiments

Six bottles containing the same initial concentration (2900 ppm) of DMP were prepared of which 3 were transparent (1 liter SCHOTT screw caped bottles) and 3 were amber colored (2 liter reagent bottles). Two bottles, one amber colored and one transparent were stored under each of the following storage conditions for the duration of the study: at room temperature on the shelves in the lab; in the oven (40 ^{o}C); and in the fridge (2 – 8 ^{o}C). The bottles were clearly labeled showing the following information: date of commencement of the experiment; storage condition of the bottle; initial concentration of DMP; and a note stating that the experiment was in progress. The containers were secured and stored under tamper proof conditions.

4.1.2 Equipment

The following equipment were employed in the course of this study:

Weighing balance *(OHAUS, Model SPU402, OHAUS Corporation, USA).*

Bunsen burner

Appropriate glassware.

Water distiller *(Analyst HP, Purite Ltd, Oxon, England).*

Electric heater (*IKA-WERKE, Germany).*

4.1.3 Materials

Materials used were as follow:

DMP– stabilized aqueous chlorine dioxide solution of approximately 2900 ppm (*Rausa-Kem, Parow Valley, Cape Town, SA)*

Concentrated sulphuric acid *(Merck, South Africa).*

Sodium thiosulphate pentahydrate *(Riedel-de Haen AG, Germany).*

Sodium hydroxide pellets AR *(B & M Scientific, South Africa).*

Concentrated hydrochloric acid *(SP Scientific, South Africa).*

Potassium iodide *(Merck, South Africa).*

Potassium bromide *(B & M Scientific, South Africa).*

4.1.3.1 Chemical reagents

Only sufficient reagents for a day's work were prepared. So most of the directions below were only used as guides to prepare just enough and avoid wastage.

4.1.3.1.1 Sodium thiosulphate 0.1 M solution

To prepare 1 L (1000 ml) solution;

Mass = molar mass x Molarity x volume (ml) / 1000

= 248.18 x 0.1 x 1000 / 1000

= 24.818 g in 1000 ml of solution

For the demands of a day's titration requirements, 1.241 g of sodium thiosulphate was weighed out and dissolved in distilled water and the volume of the solution made up to 50 ml**.**

4.1.3.1.2 Starch 0.5 % solution

Starch (0.5g) was triturated with 10 ml water to smooth paste. It was then added to 90 ml boiling water and stirred until totally dissolved.

4.1.3.1.3 Caustic soda 30 % (NaOH solution)

60 g of NaOH was dissolved in distilled water and the volume of the solution made up to 200 ml.

4.1.3.1.4 Potassium bromide 10 % (KBr) solution

25 g KBr was dissolved in distilled water and the volume of the solution made up to 250 ml.

4.1.4 Determination of concentration of DMP

The concentration was determined by the following Iodometric titration procedures (Buser & Hanisch, 1952; Kepinski & Trzeszcynski, 1964).

4.1.4.1 Procedure 1

5 ml of 10 % potassium bromide (KBr) solution and 25 ml of concentrated hydrochloric acid (HCl) solution were placed into a 50 ml Erlenmeyer flask. 20 ml of the chlorine dioxide solution (diluted 1 part biocide solution to 9 parts water to make a 1:10 solution) under examination was added and the flask closed. The flask was then exposed to light

for twenty (20) minutes. 1 g of potassium iodide (KI) was added and the flask placed in the dark for five (5) minutes. The solution was transferred into a 500 ml Erlenmeyer flask containing 30 ml of 30 % caustic soda (NaOH) solution and 100 ml distilled water. This was titrated with sodium thiosulphate ($Na_2S_2O_3$) 0.1 N, with the addition of 3 ml of starch as an indicator (volume of $Na_2S_2O_3$ used in titration = D1).

The same procedure was repeated with 20 ml water in place of the biocide sample, as a blank test (volume of sodium thiosulphate, $Na_2S_2O_3$ used in titration = D2). Titre volume was the difference between D1 and D2.

4.1.4.2 Procedure 2

2 g potassium iodide (KI) was introduced into a 250 ml glass Stoppered flask. 50 ml water, 25 ml of 25 % sulphuric acid and 20 ml biocide (diluted 1 part biocide solution in 9 parts water to make a 1:10 solution) were added and the flask contents left in the dark for five minutes. Five drops of starch indicator was added and the solution titrated with 0.1 N sodium thiosulphate solution. The titre volume was noted.

Either of procedure 1 or 2 could be used to determine titre volume. At times the two procedures were both employed in which case the average titre volume from the two results was used.

Biocide concentration was then calculated using the formula:

Chlorine dioxide, ClO_2 (mg/ml) = (Titration fig) x 0.1 x 13.49 / 20 x 10

4.1.5 Frequency of measurement of concentration of DMP during the study period

The concentrations of DMP during the stability study period were determined on a weekly basis as can be seen in table A3 of the appendix.

4.2 Results and discussion

4.2.1 Concentration of DMP at the start of the stability study

The DMP that was meant to be subjected to stability study was freshly prepared and the concentration of the stock solution was determined immediately on arrival:

Initial concentration of biocide $[A]_0$ = (vol. of $Na_2S_2O_3$ used x Normality x equiv. wt)/20 x 10

$[A]_0$ = (4.30 x 0.1 x 13.49)/20 x10 = 2900 ppm

Immediately after determining its concentration, this stock DMP solution was used to fill all the six storage containers described in section 4.1.1 above, and this marked the commencement of the stability study.

4.2.2 Changes in concentration of CD with time in the DMP solutions

The changes in concentration of CD with time in the above DMP solution as determined from the weekly Iodometric measurements, over the 53 week time span, are provided in table A3 (appendix section)

4.2.3 Graphical presentation of stability data

The table A3 (appendix section) stability study results are presented graphically in figure 4.1 below as a plot of concentration of CD in DMP, [A] against time (t).

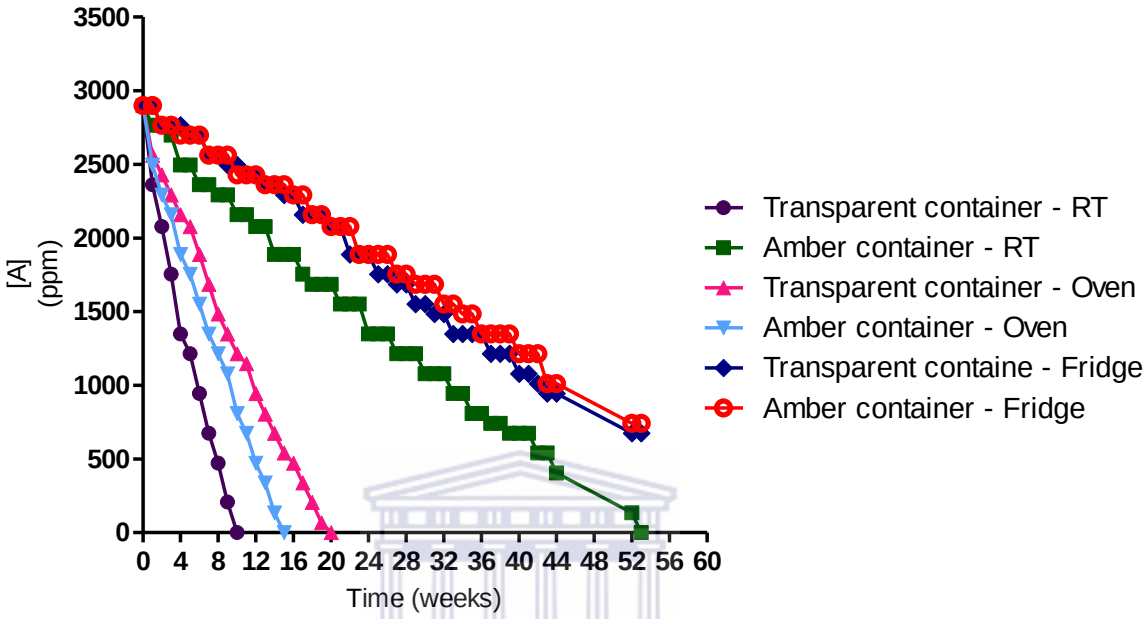


Figure 4.1: Stability profile of DMP under different storage conditions

Key

Purple and green = room (ambient) conditions; light blue and reddish pink = oven; and deep blue and reddish orange = fridge.

Note:

Average weather conditions during the study period could be summarized as follows:

Ambient temperature = 25^{O}C and

Ambient Relative humidity = 60% RH.

The above results show that a plot of the concentration of CD in DMP, [A], at any given temperature, against time (t) gives straight lines. The graph indicates that the decomposition of DMP follows zero order kinetics i.e. the rate of decay is independent of the concentration of CD in DMP. Vaidah et al. (1994) studied the thermal decomposition of CD in water by monitoring the time dependant disappearance of the absorption spectrum with diminishing CD concentration, at 294, 331, and 346 K. The decay of the absorption signal in this study revealed a first order kinetic reaction (Vaidah, Goudjil, Simon, & Flanders, 1994). This contrasts the findings of this study. A possible explanation of the discrepancy is that here we were working with DMP, which is different item from the material used in the Vaida study. DMP contains CD which has been stabilized chemically and this makes it different and more practical to be used in pharmaceutical formulations. As such, one would expect the stability data profiles to be different.

4.3.4 Application of the zero order equation to DMP degradation reactions

For the general reaction:

$$\mathbf{A \rightarrow P}$$

Where

A = chlorine dioxide

P = reaction products

The zero order rate law (Keusch, 2003; ScienTek Software I, 2006b) is written as:

$$\mathbf{r = -\frac{d[A]}{dt} = k} \qquad (1)$$

This means that the rate of the reaction never changes; it's always equal to the value of the rate constant.

Rearranging equation (1) gives

$$\mathbf{d[A] = -k \cdot dt} \qquad (2)$$

which on integration of both sides

$$\int_{[A]_0}^{[A]} \mathbf{d[A]} = -\mathbf{k}\int_{t_0}^{t} \mathbf{dt} \qquad (3)$$

leads to

$$\mathbf{[A] = -kt + C} \qquad (4)$$

When t = 0, the concentration of A is $[A]_o$ i.e. $C = [A]_0$

Now the integrated form of zero-order kinetics can be written as follows

$$\mathbf{[A] = -kt + [A]_0} \qquad (5)$$

Where,

$[A]_0$ = initial concentration of DMP

[A] = Concentration of DMP at any given time t.

k = Slope

4.3.4.1 Expiry dates of DMP stored under different conditions

To determine the expiry dates of DMP stored under different conditions, firstly the lowest effective concentration of DMP/minimum inhibitory concentration must be defined:

- The TB study below (see results section 3.2.3.2.1) suggests that the minimum inhibitory concentration of DMP on TB is 12.5 ppm.

- For microbes other than TB and spores, CD is known to be effective even at concentrations lower than 0.1 ppm e.g. in drinking water disinfection (Black and Veatch corporation, 2010; US EPA, 1999).

- In this formulation, DMP was to be diluted before use, by mixing with water and concentrate, so that after dilution, the concentration comes to about 5 ppm.

Taking the foregoing argument into account, 50 ppm was taken to be lowest effective concentration of DMP for the formulations of this study i.e. below 50 ppm, DMP was considered to be expired.

Equation 5 above can be rearranged as follows:

$$t = ([A]_0 - [A])/k \quad (6)$$

This equation can be used to calculate the expiry dates of DMP stored under varied storage conditions once the expiry concentration is defined. Taking:

t_{50ppm} = time to for DMP degradation to 50 ppm = shelf life

$[A]_0$ = initial concentration of DMP i.e. concentration at t_0 = 2900 ppm

[A] = concentration of DMP at expiry date = 50 ppm

k = rate constant (units = ppm/week)

Table 4.1: shelf lives of DMP under different storage conditions

Storage conditions	**Container**	**$[A]_0$ in ppm**	**[A] in ppm**	**k**	**Shelf life (weeks)**
Room temperature (20-30^oC) [T_2]	Transparent	2900	50	-290	9.8
	Amber	2900	50	-54	52.7
Oven (40^oC) [T_3]	Transparent	2900	50	-145	19.7
	Amber	2900	50	-193.3	14.8
Fridge (2-8^oC) [T_1]	transparent	2900	50	-42	68
	Amber	2900	50	-40.7	70.1

4.3.5 Effect of temperature on DMP

The chemical kinetic theory suggests that increasing kinetic energy of molecules increases the likehood that a chemical reaction will occur. Raising the temperature of a system increases the kinetic energy of its molecules and therefore increases the rates of chemical reactions (LAB: Q_{10} .the effect of temperature on reaction rates, 2000). The effect of temperature on stability of DMP could best be studied by paying closer attention

to the trend in amber colored containers which were least affected by light i.e. the assumption would be that temperature is the predominant factor responsible for degradation of DMP in amber colored containers.

4.3.5.1 Rate constants at different temperatures

A convenient approximate method to estimate the effect of temperature on reaction rates is to consider the ratio of rate constants $k_{T1}:k_{T2}:k_{T3}$ at temperature T_1, T_2, and T_3 from table 4.1 above:

$k_{T1}:k_{T2}:k_{T3}$ at 5, 25 and 40 ^{o}C = 40.7:54:193.3 = 1:1.3:4.7

This means that the rate of degradation of DMP is 1.3 time faster at 25^{o}C as it is at 5^{o}C and is 4.7 times faster at 40 ^{o}C than it is at 5 ^{o}C.

4.3.5.2 Applying Arrhenius equation

The Arrhenius equation below is an equation of a straight line obtained by plotting of ln k against 1/T.

$$\ln k = \ln A - Ea/RT$$

Where:

Ea/R = slope

T = absolute temperature in K

ln A = Intercept

R = gas constant = 8.314x10^-3KJ/mol/K

Table 4.2: Effect of temperature on rate constant of DMP decomposition in amber colored containers

Rate constant (k)	**ln k**	**Temperature**	**1/T**
40.7	3.7	5 ^{o}C (278 K)	0.0036
54	4.0	25 ^{o}C (298 K)	0.00336
193.3	5.26	40 ^{o}C (313 K)	0.0032

Plotting ln k against 1/T gives the Arrhenius plot for DMP as shown below in figure 4.2:

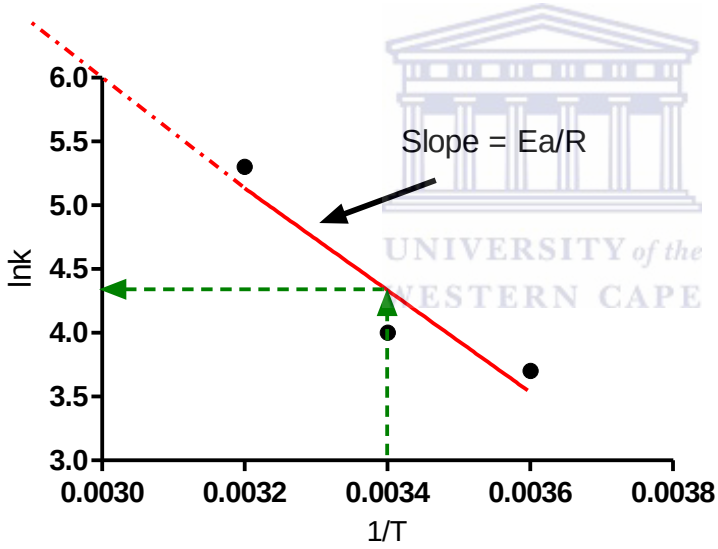


Figure 4.2: The Arrhenius plot for DMP

The Arrhenius plot can be used to calculate the rate constant of DMP degradation at any temperature and therefore, could be useful in predicting the shelf life of DMP stored at any temperature, as follows:

For example, what will be the shelf life of DMP stored at 25 ^{o}C?

Given: T = 298 K; 1/T = 0.0034; ln k = 4.34; k = 76.7

t_{50ppm} = 2850/2900x2900/k

= 0.983x2900/76.7

t_{50ppm} = 37.2 weeks

The expiry dates of DMP stored in amber containers can be predicted in this same manner at any temperature. This is the basis of prediction commonly employed in accelerated stability studies.

The observed expiry date of DMP in the amber colored container stored at room temperature was about 53 weeks. The Arrhenius plot suggests that if this DMP was stored at 25 ^{o}C consistently, the shelf life would be about 37 weeks. The difference in the two results could be explained by the fact that during the stability study temperature changes were the norm, from as low as 5 ^{o}C during winter to as high as 32 ^{o}C during summer. The 25 ^{o}C was simply the mean room temperature and the graph showing stability profiles simply portrays the general trend. The predictions of the Arrhenius plot could still hold if the temperature was constant at 25 ^{o}C.

4.3.5.3 The activation energy (Ea) of DMP

Ea of DMP = slope x R

4000 x 8.314x10^-3

Ea = 33.256 Kj/mol

The activation energy can also be calculated by setting the Arrhenius equation at two different temperatures and subtracting the second from the first:

$\ln (k_2) = \ln A - Ea/RT_2$

$-\{\ln (k_1) = \ln A - Ea/RT_1\}$

Net equation: $\ln (k_2/k_1) = -Ea/R \times (1/T_2 - 1/T_1)$

At T1 = 5^oC and T2 = $40\ ^oC$

$\ln (193.3/40.7) = Ea/R \times (0.0036 - 0.0032)$

Ea = 1.56x8.31x10^-3/0.0004

Ea = 32.4 Kj/mol

Although it is alright to estimate Ea with the Arrhenius equation by just using two rate constants at two temperatures, it is more realistic and therefore advisable to use at least three rate constants at three temperature and calculate the mean Ea as shown in table 4.3 below where all the Ea values were calculate from the Arrhenius equation as in the preceding section (ScienTek Software I., 2006a).

Table 4.3: Calculated mean Ea values from points along the straight line of the Arrhenius plot

T1	1/T1	T2	1/T2	ln K2	ln K1	ln K2 –ln K1	1/T2 – 1/T2	Ea
5	0.00360	40	0.0032	Refer to section 5.3.5.3			-4.0x10^-4	32.4
10	0.00353	30	0.0033	4.74	3.80	0.94	-2.3x10^-4	34.0
10	0.00353	40	0.0032	5.12	3.80	1.32	-3.35x10^-4	32.7
15	0.00347	40	0.0032	5.12	4.02	1.1	-2.8x10^-4	32.7
20	0.00341	30	0.0033	4.74	4.28	0.46	-2.18x10^-4	33.8
Average Ea = (32.4+34.0+32.7+32.7+33.8)/5 =								33.1 KJ/mol

The average Ea of thermal degradation of DMP has been found to be about 33.1 KJ/mol. This makes sense if we consider the work of Hu et al. (2009), who studied the kinetics of a reaction in which CD oxidizes thiocyanide. Hu used UV spectrometry to monitor the concentrations of both CD and thiocyanide. He found the Ea of the reaction to be 2.5 KJ/mol. This is a faster reaction than the degradation reaction of this study, hence a lower Ea. Wells, in his book pharmaceutical preformulation, states that Ea values for most of the drugs are in the range of 10 – 100 Kcal/mol (41.9 – 418.7 KJ/mol). This also is in agreement with the observed lower than average Ea of DMP considering that most drugs have expiry dates of more than at least two years, but that of CD is bare a year depending on the storage temperature i.e. DMP degradation is a faster reaction than the degradation reactions of most drugs.

In the Vaida et al. (1994) study mentioned above, the Ea of thermal decomposition of CD in water, study temperatures being 294, 331, and 364 K, was determined to be 0.60 eV (64.6 KJ/mol) (Vaida et al., 1994). The difference with the results of this study seems significant. It is advisable in situations like this where the true Ea value is in dispute to assume a low value, since this assumes high reaction rates and any prediction of expiry dates will be conservative (Wells, 1988).

4.3.5.4 Q10 value calculations

Q10 is the factor by which the rate constant increases for a 10 ^{o}C temperature increase. The commonly held view, particularly for biochemical reactions, is that a 10 ^{o}C increase in temperature doubles the rates of chemical reactions i.e. Q_{10} = 2. This view is not accurate but could be useful in making conservative estimates of expiry dates (Beavon, 1998; Kenneth et al., 1986). The Q10 factors for DMP can be calculated from the formula below (LAB: Q_{10} .the effect of temperature on reaction rates, 2000):

Q_{10} = (K_2/K_1)^10/(T_2-T_1) (1)

Where

T_2 = higher temperature K_2 = rate at T_2

T_1 = lower temperature K1 = rate at T_1

When T2 – T1 = 10 ^{o}C, then the expression (1) simplifies to:

$Q_{10} = (K_2/K_1)$^10/10 = K2/K1 (2)

The above model suggests that Q_{10} for a particular drug is constant. Actually, Q_{10} is not constant but decreases with increasing temperature (Beavon, 1998; Kenneth et al., 1986).

In practice, the Q_{10} value of the 20 to 30 ^{o}C temperature interval is taken as the Q10 value for any drug and this is the value that appears in monographs. Furthermore, Q_{10} values are usually rounded up to the nearest values of either 2 or 3 or 4 to represent low or average or high estimates of Ea respectively i.e. the above rounded up values are linked to three corresponding Ea values of 12.2, 19.4 and 24.5 Kcal/mol, respectively and this makes expiry date approximations easy (Kenneth et al., 1986; Shaheen, 2005).

Table 4.4: Q_{10} Values of DMP on raising temperature from 0 to 10; 10 to 20; 20 to 30; and 30 to 40 (data from Arrhenius plot figure 5.2)

T (K)	**1/T**	**ln $k_{(T+10)}$**	**$K_{(T+10)}$**	**ln k_T**	**k_T**	**Q10**
273	0.00366	3.86	47.5	3.33	27.9	1.7
283	0.00353	4.34	76.7	3.86	47.5	1.6
293	0.00341	4.71	111	4.34	76.7	1.4
303	0.0033	5.12	167	4.71	111	1.5
313	0.0032	5.58	265	5.12	167	1.6

From the above results the Q_{10} value of DMP in the 20 to 30 ^{o}C is 1.5. This must be rounded up for purposes of Q_{10} predictions of shelf life, to $Q_{10} = 2$.

For any arbitrary change in temperature $\Delta T = T_2 - T_1$, an expression that could be useful to determine the factors by which rate constants (K) changes given Q_{10} values is shown below:

$$Q_{\Delta T} = k_{(T+\Delta T)}/k_T = Q_{10}^{(\Delta T/10)} \quad (3)$$

For example the factors by which the rate constants change as the temperature is changed from 10^{o}C to 40 ^{o}C are:

$Q_{+30} = Q_{10}^{(30/10)}$

Given that the Q10 of DMP = 2

$Q_{+30} = 2\text{^}30/10 = 2^{3} = 8$

Table 4.5: Factors by which rate constant changes for a given change in temperature given Q_{10} values from table 5.3.

Temp change (ΔT)	**Q_{10}**	**$Q_{10}^{(30/10)}$**
10 – 20 ^{o}C	1.6	4.1
20 – 30 ^{o}C	1.4	2.7
30 – 40 ^{o}C	1.5	3.4

On the other hand a change from 40 ^{o}C to 10 ^{o}C would change the rate constants by the following factors:

$Q_{-30} = Q_{10}^{(-30/10)}$

Given Q10 of DMP = 2

$Q_{-30} = 2^{-3} = 0.125$

Table 4.6: Factors by which rate constant changes for a given change in temperature given Q_{10} values from table 5.3.

Temp change (ΔT)	**Q_{10}**	**$Q_{10}^{(-30/10)}$**
40 – 30^{o}C	1.6	1/4.1
30 – 20^{o}C	1.4	1/2.7
20 – 10^{o}C	1.5	1/3.4

4.3.5.4.1 Prediction of DMP shelf life using its Q10 value

If the expiry date of DMP is known at one temperature, its expiry date can be established at any other temperature using Q10 estimation. For example if the expiry date of DMP stored at 5 ^{o}C is 70 weeks. Suppose the DMP is accidentally stored at 25 ^{o}C. What will its new expiry date be?

The expiry date for both zero order and first order reactions can be written in a general as:

$t_{50\ ppm} = a/K_T$ (refer to equation 6 of section 4.3.4.1)

For T_1 and T_2 = 5 and 25 ^{o}C respectively:

$t_{50\ ppm(T1)} = a/K_{T1}$ and (1)

$t_{50\ ppm(T2)} = a/K_{T2} = a/K_{T1+\Delta T}$ (2)

Rearranging equation 1 gives

$a = t_{50\ ppm} \times K_{T1}$ (3)

From section 5.3.5.4 above:

$$Q^{\Delta T/10} = K_{(T1+\Delta T)} / K_{T1} \quad (4)$$

Rearranging this equation gives:

$$K_{(T1+\Delta T)} = Q^{\Delta T/10} \times K_{T1} \quad (5)$$

Substitute equation 4 into equation 2:

$$t_{50\ ppm(T2)} = a / Q^{\Delta T/10} \times K_{T1} \quad (6)$$

Substitute equation 3 into equation 6:

$$t_{50\ ppm(T2)} = t_{50\ ppm(T1)} \times K_{T1} / Q^{\Delta T/10} \times K_{T1} \quad (7)$$

$$t_{50\ ppm(T2)} = t_{50\ ppm(T1)} / Q^{\Delta T/10} \quad (8)$$

Equation 8 is called the Q_{10} equation and is applicable to any order of chemical reaction

$t_{50\ ppm}$ = 70weeks/2^20/10

= 70/4

$t_{50\ ppm}$ = 17.5 weeks

For storage of DMP at 25 ^{o}C, calculation in section 5.3.5.2 from the Arrhenius plot predicted an expiry date of 37.2 weeks and now Q10 prediction is giving an expiry date of 17.5 weeks for storage at the same temperature. Q_{10} calculations are never accurate but are meant to fix conservative approximate expiry dates on the side of patient safety when the actual expiry date is not known. The Q10 expiry date is usually 6 months less than the actual shelf life of the drug (Kenneth et al., 1986; Shaheen, 2005).

4.3.6 Effect of light on DMP degradation

The effect of light on degradation of DMP was found to be tremendous. DMP stored in a transparent bottle at room temperature, and therefore well exposed to light, had an expiry date of just over 9 weeks and had no trace of CD on the 10^{th} week. Transparent bottles in the fridge and oven were not well exposed to light and therefore the influence of light on these was minimal.

4.3.7 Need for tight closing of containers of DMP

Tight closing of containers of DMP was found to be probably the foremost factor in preserving DMP. This is because CD is essentially a gas with good solubility in water. Gas solubility is always limited by the equilibrium between the gas and a saturated solution of the gas. The dissolved gas will always follow Henry's law as explained in the introduction. Therefore it was found that tight closing of the containers and elimination of the head space by filling to the brim where possible prevents escape of CD.

CHAPTER 5

FORMULATION DEVELOPMENT (FD)

Preformulation studies which consisted of foam evaluation, acid-base potentiometric titration and compatibility studies were performed. The ideal foam system was determined. The appropriate alkali to adjust the pH was also determined and DMP-NaOH/KHP buffer interactions were characterized. Finally, a foam concentrate was formulated and manufactured that could be mixed to DMP prior to clinical application.

5.1 Materials and methods

5.1.1 Materials

Sodium hydroxide pellets AR *(B & M Scientific, South Africa).*

Sodium per carbonate (C*rest Chemicals, South Africa).*

Potassium hydrogen phthalate (*Merck N.T. Laboratory Supplies (Pty) Ltd., SA*)

Sodium lauryl sulphate (*Aspen Pharmacare, SA*)

Ammonium lauryl sulphate (*Sigma-Aldrich GmbH, France*)

EDTA (*BDH laboratory supplies, England*)

Polysorbate 20 (*Sigma-Aldrich Chemie, GmbH, Switzerland*)

SNLSS (*Sigma-Aldrich, Co. USA*)

Sodium xylene sulfonate (*Sigma-Aldrich chemie GmbH, France*)

Peppermint oil (*Barrs Pharmaceutical Industries, SA*)

Cocoamidopropyl betaine (*Amka Products (Pty) ltd., Pretoria, SA*)

Glycerol (*Merk KGaA, Darmstadt, Germany*)

Cetostearyl alcohol (*Kimix Chemicals, CT, SA*)

Xylitol (*pharmaceutics store room, UWC*)

Sucralose (*pharmaceutics store room, UWC*)

Monoammonium glycyrrhizinate (*pharmaceutics store room, UWC*)

Appropriate glassware

5.1.2 Preformulation studies

5.1.2.1 Foam evaluation

Foams generated by various foaming agents and foaming agent combinations (FA/FAC) were evaluated in terms of foamability, stability, elegancy and aesthetic properties as well as other relevant criteria. The foams were studied at pH 4.0 and at pH 5.5 which corresponds to the pH range appropriate for the intended application.

5.1.2.1.1 Foamability

5.1.2.1.1.1 DMP PH adjustment

400 ml of DMP, supplied by Rausa-Kem, Parow Valley, Cape Town, was dispensed into 500 ml beaker. 30 % w/v NaOH solution was used as agent for adjusting the pH of DMP solution to either pH 4.0 or 5.0. The pH of the solutions was measured using a portable laboratory pH meter (model GLP21 from Crison, Barcelona, Spain). The pH meter was calibrated before application using standard buffers at pH 7.00 and 4.01. The pH

measurements were carried out by direct immersion of the electrodes into the DMP sample. The temperature during these studies ranged between 15 and 28 ^{o}C. Once the desired pH was attained, the 400 ml DMP was transferred to a 2000 ml graduated beaker which was ideal for conducting Foamability studies.

5.1.2.1.1.2 Foam generation

Foamability studies were carried out in a fume hood. One FA/FAC-DMP system was studied at a time whereby progressively increasing amount of FA/FAC were added to the DMP solution followed by stirring, each time measuring the foam volume generated. A mechanical overhead stirrer (IKA model 20 RW, Janke and Kunkel GmbH and Co. KG, Staufen, Germany) fitted with a four axial blade impeller was employed. The mixing/agitation speed was fixed at 2000 rpm for 60 seconds immediately after which the amount of foam generated was measured (Carey & Stubenrauch, 2009; Klein, 2004).

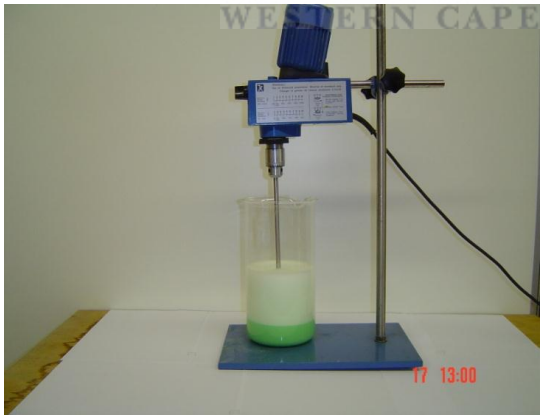


Figure 5.1: Mixer, 2000ml beaker

5.1.2.1.1.3 Foamability measurements

The amount of foam generated at each concentration of the FA/FAC was-determined by measuring the volume of the foam in the 2000 ml measuring cylinder in which the foam

was generated. A ruler was also used to confirm the reading. Foamability assessments of each foam system were done in triplicate (Azira, Tazerouti, & Canselier, J 2008; Carey & Stubenrauch, 2009; Klein, 2004). The results are in section 5.2.1.1.

5.1.2.1.2 Foam stability

The stability of the various foam systems studied was assessed using foam decay and BBT methods. Foam drainage was studied concurrently with foam decay. The density of each foam system was also determined.

5.1.2.1.2.1 Determination of foam decay and drainage

The 2% w/v or v/v solution of each of the FA/FAC, as appropriate, in DMP was stirred at 2000 rpm for 60 seconds, immediately after which the generated foam was transferred to a 100 ml measuring cylinder and its rate of collapse determined by reading the foam volume at 20 or 40 minute intervals. The foam was transferred to the measuring cylinder by scooping it off from the stirred solution. The $t_{1/2}$ of decay of each FA/FAC-DMP foam system was indicative of the stability of its foam (Azira et al., 2008; Iglesias, Anderez, Forgiarini, & Salager, 1995; Klein, 2004; Suliman, El Tinay1, Elkhalifa2, Babiker, & Elkhalil, 2006).

Determination of rate of collapse and the rate of drainage of liquid from the foam was determined simultaneously. The amount of liquid collected at $t_{1/2}$ of foam decay was taken to determine relationship between foam stability and rate of drainage (Carey & Stubenrauch, 2009; Klein, 2004; Miles, Shedlovsky, & Ross, 1945).

5.1.2.1.2.2 Bubble breaking time (BBT)

In a room where there was minimal air movement, 10 big bubbles of each foam system were blown from a pipette as shown below and time taken to break was noted. The 2 % w/v or v/v solution of each of the FA/FAC in DMP, as appropriate, was used to generate these bubbles. Shaking interferences were minimized by holding the blowing end of the pipette steadily while leaning the middle part on a wooden board during these stability measurements (Ross, 1943; Suliman1 et al., 2006).

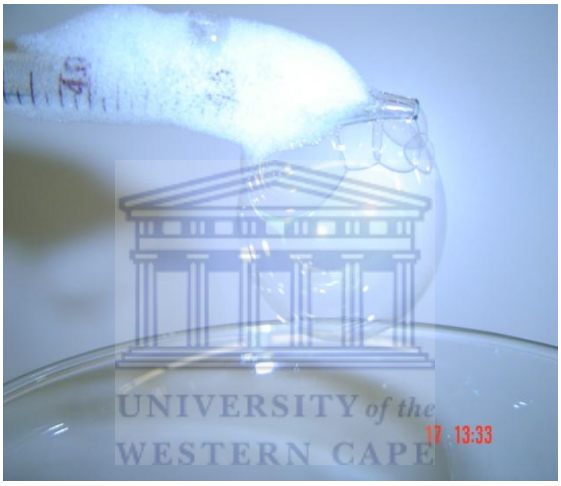


Figure 5.2: Blown bubbles

5.1.2.1.2.4 Determination of the density of the foam

The 2 % w/v or v/v solution, as appropriate, of each of the FA/FAC in DMP was agitated at 2000 rpm for 60 seconds. After 10 minutes waiting time, foam was scooped and transferred to a pre-weighed 93 ml specimen bottle to determine the weight of the foam. From these measurements the density was determined (Klein, 2004; Suliman1 et al., 2006; Wilde & Clark, 1996).

Figure 5.3: Specimen bottle (containing foam for density determinations)

5.1.2.1.3 Foam stabilizer

Cetostearyl alcohol (CSA) was used as foam stabilizer (Columbia analytical services, 2001; Oxford University, 2007). Since it is insoluble in cold water, it had to be dispersed in hot sample of FA/FAC solution drawn from the solutions to be tested. After dispersion, the CSA sample solution was added back to the test solution. The stability tests on foams containing CSA were conducted in exactly the same way as above.

5.1.2.1.4 Selection of appropriate foam system for product formulation

The final selection of foam systems appropriate for incorporation into the formulation was based on a scoring system whereby, the foam characteristics of the various FA/FACs were assigned marks according to perceived importance of their properties in relation to the target product profile (Siltech personal care, 2006). Table 5.5 in section 5.2.1.4 below gives the selection criteria.

5.1.2.2 Acid-base Potentiometric titrations

5.1.2.2.1 Determination of suitable alkali for raising the pH of DMP

Two alkalis were available to act as pH adjuster solutions of the DMP: 1) sodium hydroxide (NaOH) and 2) sodium per carbonate [$Na_2(CO_3).1/2H_2O_2$]. 5 % w/v solutions of each of these alkalis were prepared, placed in a burette and 100 ml of DMP was titrated with the alkaline solutions measuring the pH after each addition. Each experiment was carried out in triplicate. The results were plotted to produce two acid-base titration curves (figure 5.23 and figure 5.26) (GA/7 potentiometric titration. 1999; Christian, 1977; Stoog et al., 1988).

5.1.2.2.2 Demonstration of usefulness of a buffer in the formulation

In this study, the buffer system chosen was Potassium hydrogen phthalate (KHP), for reasons given in section 2.3.4.2.5. Three solutions bearing same concentration of NaOH and progressively increasing amount of KHP (i.e. 5 % w/v/0.1 % w/v KHP, 5 % w/v/1 % w/v KHP, and 5 % w/v/2 % w/v KHP), were prepared each in its own titration experiment turn. The titration experiments progressed as described in section 5.1.2.2.4 below and the raw titration data are shown in the appendix tables A19, A20, and A21 respectively for the stated solutions. This section also served to optimize the concentration of KHP in the concentrate being formulated as explained in section 5.1.3.4 and 5.2.2.2.

5.1.2.2.3 Demonstration of the usefulness of the adjuster solution

In this case the KHP was held constant while the NaOH was varied. Two solutions of: 0.25 % w/v NaOH/2.5 % w/v KHP; and 2.5 % w/v KHP without NaOH, were prepared,

again, each in its turn. The first solution was titrated as described in section 5.1.2.2.4 below. The second solution was not titrated as the trend was already very clear i.e. the low NaOH concentration demonstrated no appreciable change in pH.

5.1.2.2.4 Titration

The NaOH/KHP system as the titrant was placed in a burette, while the 100 ml of DMP as the titrand was placed in a conical flask (Thompson, 2004). DMP was titrated while carefully monitoring the pH after each addition. Each titration experiment was performed in triplicate.

5.1.3 Formulation

5.1.3.1 Product design

The product was designed to come as a two part formulation namely; DMP solution and a concentrated foam solution (concentrate). The two parts have to be mixed at the point of use to attain the desired formulation. This approach was followed to compensate for the instability of DMP. The mixing was done as follows for the different applications: to a mixture of appropriate volumes of water and concentrate, a specific amount of DMP was added to make either a VGD or an F/SBB; and an appropriate volume of pre-diluted concentrate was mixed with a specific quantity of DMP to make the MRF. If the concentrates for the VGD and F/SBB were also pre-diluted, the concentrate would be too bulky for the end user because the two formulations demand more volumes. It was envisaged that DMP would be used in its usual form. Therefore, all formulation efforts were directed at production of the foam concentrate.

5.1.3.2 Equipment for the manufacturing process

Ordinary laboratory equipment were used like beakers, thermometer, spatula, weighing scale, measuring cylinders, pH meter, Heater, crucible, stirring rod, pipette etc.

5.1.3.3 Development of the foam concentrate

Five formulas were formulated before arrival at a satisfactory one which met the desired CQAs of uniformity of content, absence of lumps, and smoothness of flow (refer to appendix table A25). In method A, which was the initial and most simplified process, all the ingredients were added to 50 ml of distilled water in a mixing vessel and made up to 100 ml followed by mixing for about 2 minutes. The resulting mixture was lumpy, separated according to phases, and was non-flowing due addition of too much SLS. The subsequent processes were designed to progressively circumvent problems identified in the initial process and are shown in table A5. Basically further addition of CAPB thickener, led to a formulation with uniformity. Lumpiness was avoided by heating to 70 ^{o}C. The addition of ingredients in a logical sequence is important and optimizing SLS leads to a formulation with ideal flow properties. An ideal foam concentrate formula was achieved in method E which is described in sections 5.2.5.1 and schematically presented in Figure 5.37 below:

From the observations made, method E was evaluated to be the best and the quantities of excipients were further optimized. Table A26 refers to the initial quantities used in method E.

5.1.3.4 Optimization of levels of NaOH/KHP buffer system in the formulation

5.1.3.4.1 Background information

Ideal pH values for the different formulations are as follows: VGD formulation must be pH 3.8 – 4.5; and MRF and F/S BB must be pH 4.5 – 5.2. The pH of the formulated concentrate was around 13.0, depending on the level of NaOH in the concentrate, while that of DMP was about 0.5. DMP and concentrate had to be mixed together with water, just prior to administration as explained in section 5.1.3.1. This mixing was meant to get a product with the desirable pH range of 3.8 to 5.2, which would be safe for patient administration. To achieve these targets depended on selecting appropriate concentrations of NaOH to raise pH of DMP to target pH and appropriate concentration of KHP to maintain the target pH.

5.1.3.4.2 Optimization of levels of KHP

As alluded to in sections 5.1.2.2.2 and 5.2.2.2, experiments that demonstrated the usefulness of the buffer in the preformulation studies also served to optimize KHP levels appropriate for employing in the final formulation. Figures 5.30, 5.31 and 5.32 demonstrated that higher concentration of KHP improved buffering. At the same time, it was noted that KHP concentration beyond 3 % w/v could not be employed because the solution became saturated. So 2.5 % w/v was deemed to be the optimal concentration. This concentration was employed at every stage of the formulation as can be attested in table A26, A27 and A28 in the appendix and in the master formula of the lead concentrate formulation (table 5.10), where the concentration of KHP is maintained at 2.5 % w/v.

5.1.3.4.3 Optimization of levels of NaOH

Six prototype foam concentrates were progressively prepared based on the concentrate development method E (section 5.2.5.1, appendix table A25). In these prototypes, quantities of all other ingredient were kept constant, as those shown in appendix table A6, while varying the concentration of NaOH (refer to table A27 in the appendix). Once prepared, each prototype at its own time was then subjected to NaOH level optimization process by mixing various volumes of concentrate as given in table A27 in the appendix**,** with 5 ml of DMP (fixed volme) and appropriate volume of water to make 50 ml of product. The pH of such a mixture was then measured and noted down as shown in the same table A27 referred to above. Noteworthy is the fact that the volume of DMP was fixed at 5 ml and that this directly determines the level of NaOH in the foam concentrates required to meet target pH values. Therefore prototypes which after mixing in the proportions given in appendix table A27 gave pH values that fell outside target range were deemed to contain either too much or too little NaOH, as the case may be, and were rejected. The raw data of the optimization process of NaOH is given in the appendix table A27. This table was summarized to table 5.9 and in figure 5.38 the pH values resulting from mixtures undertaken for each prototype were plotted against the concentrate volumes employed in respective mixtures. Once the level of NaOH was optimized, a dosage formula (DF) was derived as explained in section 5.2.5.5 in the results and discussion.

5.1.3.5 Optimization of the other excipient quantities

The initial step was to determine concentrations from literature sources, for commonly used ingredients as alluded to in section 2.3.4.3 in the literature review. In order to

determine the optimal level of the less commonly used excipients and also to refine quantities in accordance to desired quality of the final product, and therefore optimize formulation quantitatively, six different prototype formulations were progressively developed each with excipient quantities specified in Table A28 in the appendix. This means that the optimization of excipient levels were to some extent by trial and error. This is because optimization was based on evaluation of the effect of excipient quantities on the quality attributes of the product (Cho et al., 2010; Dow, 2004) i.e. excipient levels were tailored to ensure that the resultant foam concentrate had uniformity of content, absence of lumps, and smoothness of flow.

5.2 Results and discussion

5.2.1 Foam evaluation

5.2.1.1 Foamability

The foamability results of Table A5 (appendix) are graphically presented in figure 5.4 below which gives the foamability profiles of various foam systems. The result is in agreement with authors who state that foam volume increases with increasing concentration until CMC is reached (Amaral, 2008; Carey &Stubenrauch, 2009). It was observed that even after CMC was reached foam volumes continued to register but minimal increments, as also was observed by Amarah et al. (2008).

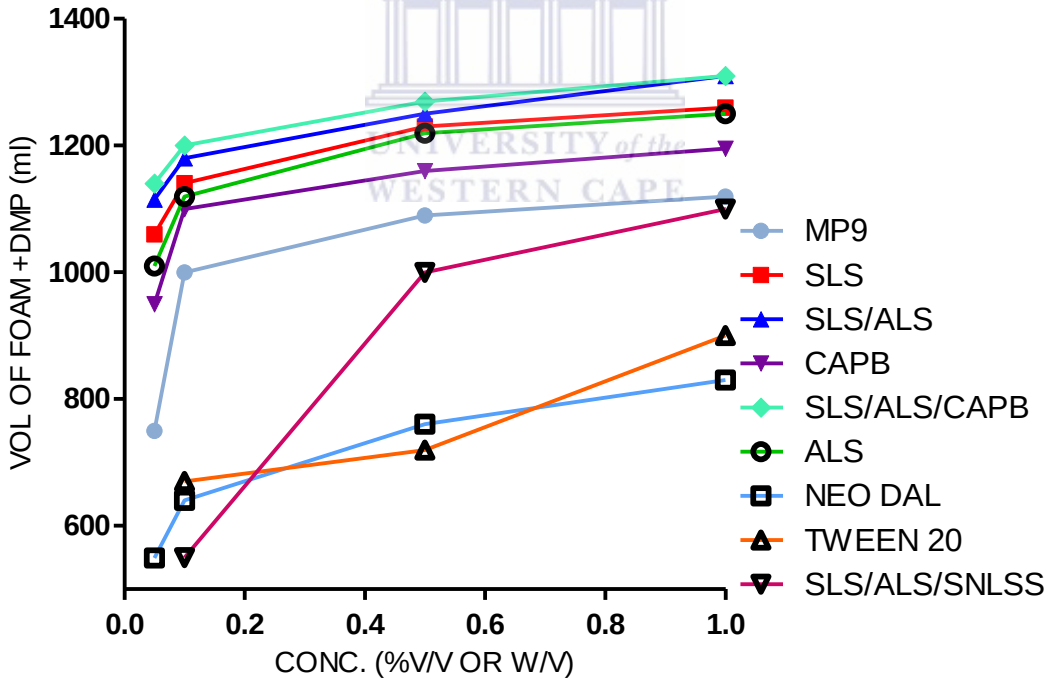


Figure 5.4: Foamability profiles of various surfactant systems at pH 4.0

From figure 5.4, it can be seen that some systems foam well at low concentrations but poor at higher concentrations and vice versa. In these instances, foamability comparisons

depend upon the time at which the comparison is made. To achieve better comparison the curves in figure 5.4 were converted into straight lines using the Lineweaver-Buck plot (figure 5.5), in which case it can be seen that the slope is linked to the variation in foam volume that accompanied the variations in concentration of FA/FAC. Iglesias et al. (1994), transformed data in various ways to obtain a numeric value for foam stability. Similar data manipulation was used for quantification of foamability.

Note:

The Lineweaver-Buck (double reciprocal) plot is a mathematical tool that is used to convert parabolic and sigmoid curves into straight lines. It is commonly employed for example in enzyme kinetic studies for calculation of the Michaelis constant (K_m) and the maximum velocity of enzyme reaction (Vmax) since these values cannot be estimated accurately using parabolic and sigmoid curves (Stryer, Tymoczko, & Berg, 2002).

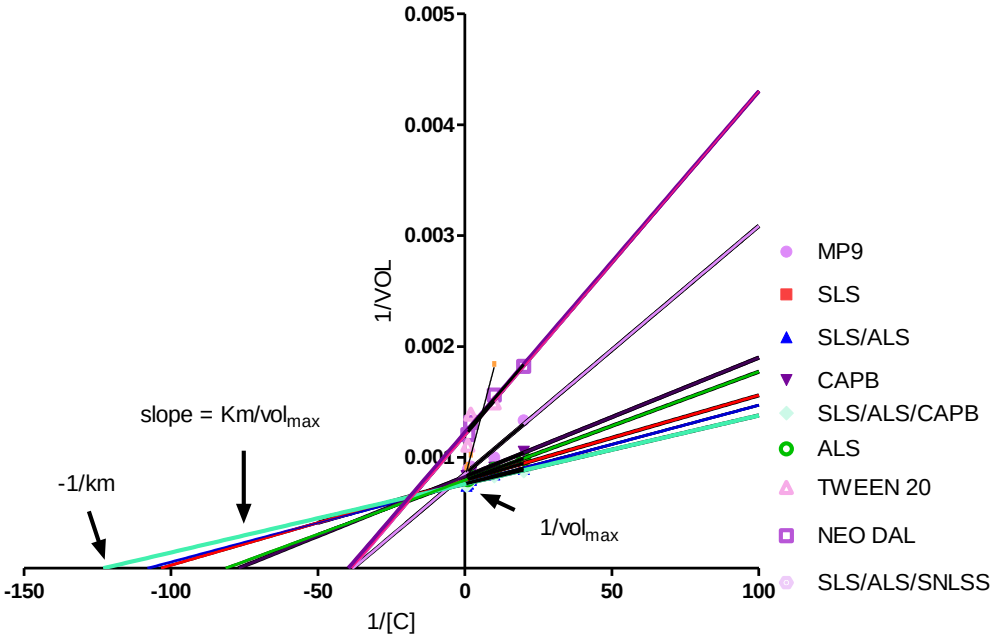


Figure 5.5: Lineweaver-Buck plot transform of foamability curves of figure 5

Foamability was quantified by comparing the inverse of the slope as expressed in table 5.1.

Table 5.1: Quantification of Foamability

FOAM SYSTEM	SLS/ALS/CAPB	SLS/ALS	SLS	ALS	CAPB	MP 9	NEO DAL	Tween 20
1/SLOPE	161,800	141,300	130,500	102,300	93,170	44710	32470	32190
1/SLOPE/32,190 (FOAMABILITY RATIO)	5	4	4	3	3	1	1	1

From table 5.1, it is clear that SLS/ALS/CAPB system has the highest capacity to foam and its Foamability is 5 times that of MP 9 or NEO DAL or Tween 20. It is followed by SLS/ALS and SLS foam system that has 4 times the foaming power of MP 9, NEO DAL or Tween 20. The rest can be interpreted in this same manner.

5.2.1.2 Foam stability

5.2.1.2.1 Foam decay and foam drainage

Figures 5.6 to 5.15 show stability profiles of various foam systems based on measurements of the rates of foam collapse. The drainage profile of each foam system is also presented as these two properties were studied concurrently. Four of these systems were measured at pH 4.0 and pH 5.5. Tween 20 foamed well at pH 4.0 but could barely foam at pH 5.5 while SNLSS foamed well at pH 5.5 but not at pH 4.0. The pH changes had little effect on the foamability of ALS, ALS/CSA and SLS, SLS/CSA foam systems which performed well at both pH values. The rest of the systems were studied at pH 4.0 only because it was noticed that DMP had stability problems at pH 5.5 and therefore could not be utilized at that pH in the final formulation. The lack of foam for SNLSS, an anionic foaming agent, at pH 4.0 did not come as a surprise since it is a well known fact that performance of ionic FAs can be affected by pH or any ionic changes for that matter (Centre for the Environment and the Lung - Denmark, 2002). The effect is such that if addition of an electrolyte causes a further reduction in surface tension, foaming is enhanced and the opposite is true if the additional electrolyte increases surface tension. This could be a possible explanation of the concepts of foam boosting and other co-surfactant enhancement of foaming capacity. The lack of foaming of Tween 20 (a non ionic FA) at pH 5.5, however was unexpected since this group is known to be least affected by pH or any ionic changes (Centre for the Environment and the Lung - Denmark, 2002; Zhang, Dalgleish, & Goff, 2004). The decay of ALS foam was a special case in that it was characterized by a dry barely visible residue of the foam sticking to the container which did not represent the true foam.

5.2.1.2.1 Foam decay/drainage graphs

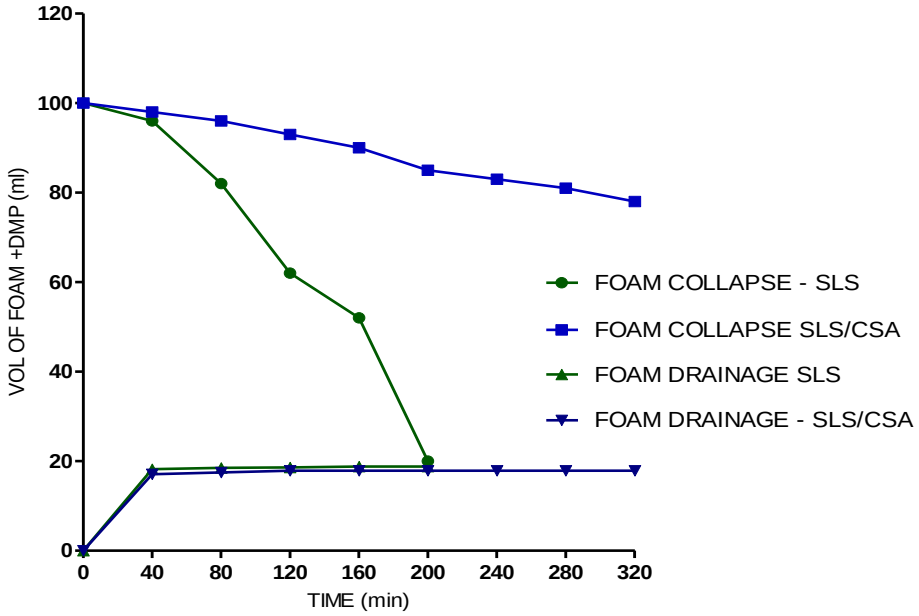


Figure 5.6: Foam collapse and drainage of SLS and SLS/CSA AT pH 5.5

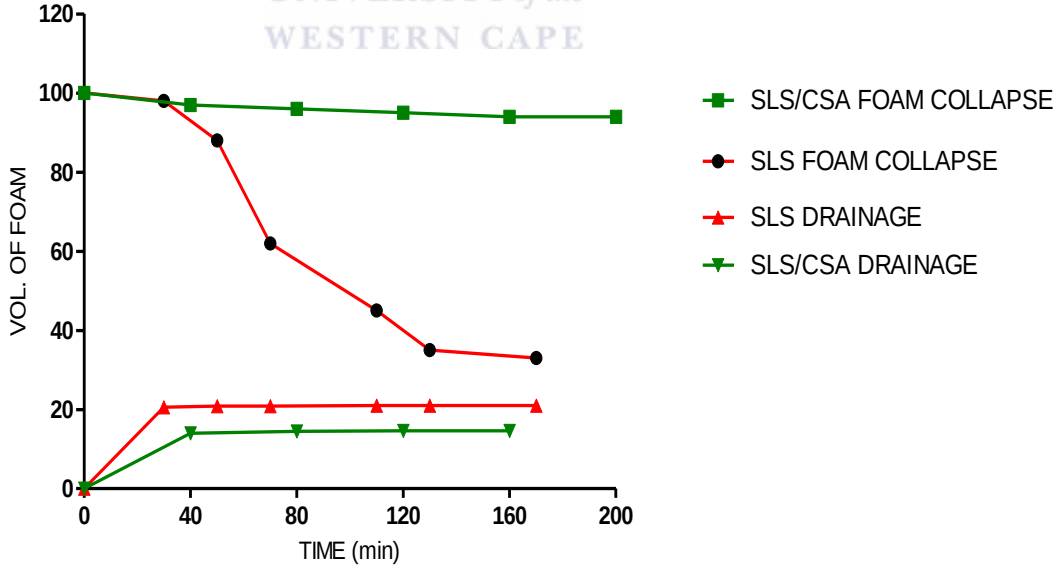


Figure 5.7: Foam collapse and drainage of SLS and SLS/CSA at pH 4.0

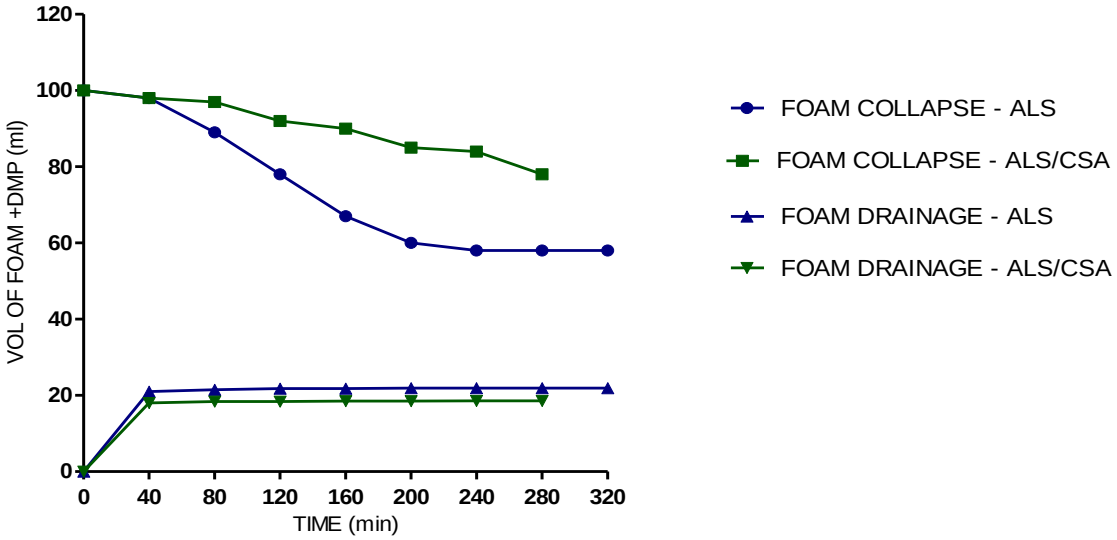


Figure 5.8: Foam collapse and drainage of ALS and ALS/CSA at pH 5.5

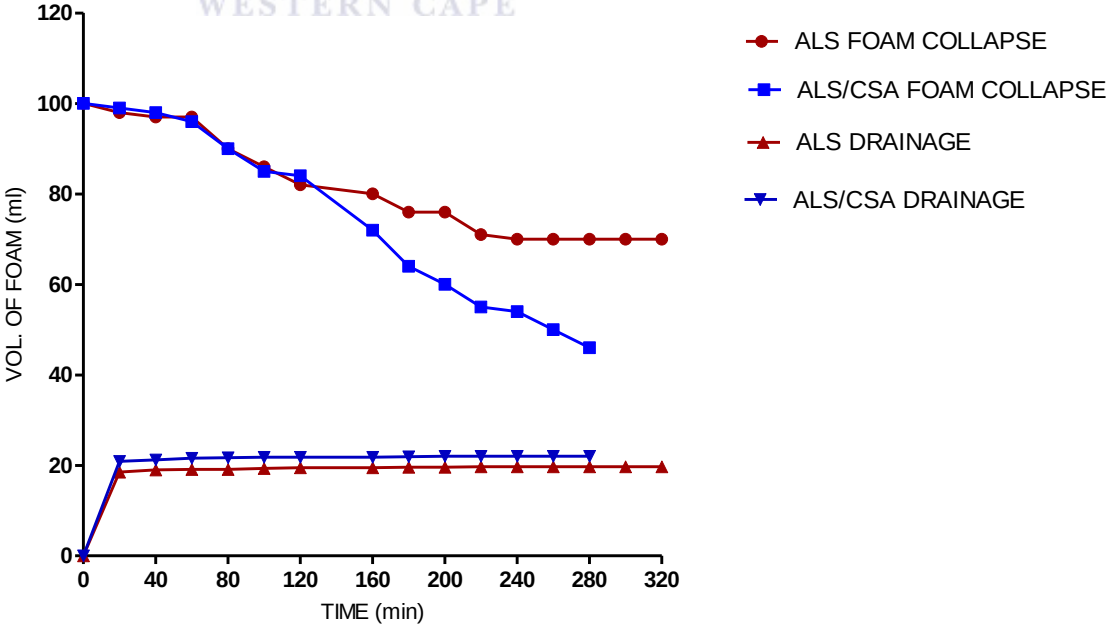


Figure 5.9: Foam collapse of ALS and ALS/CSA at pH 4.0

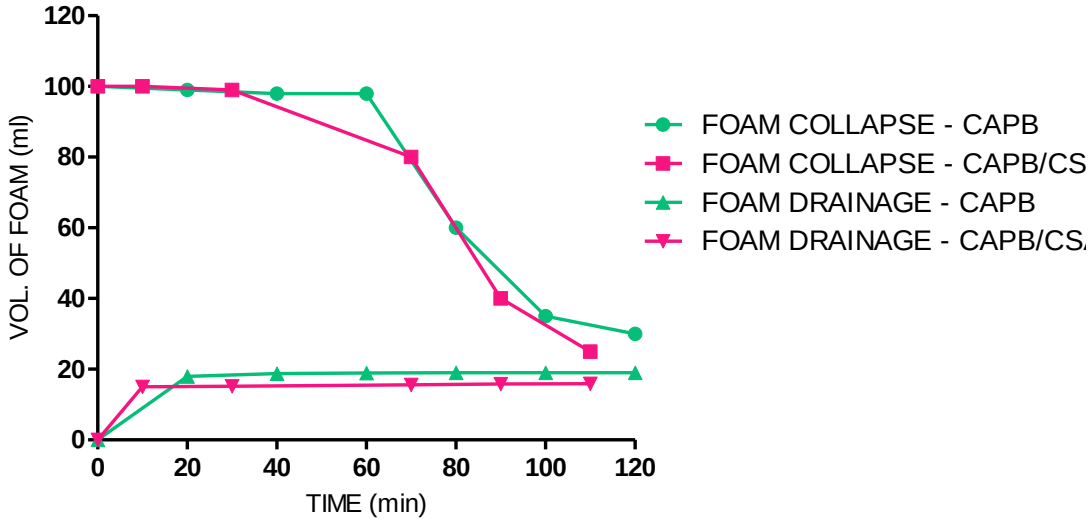


Figure 5.10: Foam collapse and drainage of CAPB and CAPB/CSA at pH 4.0

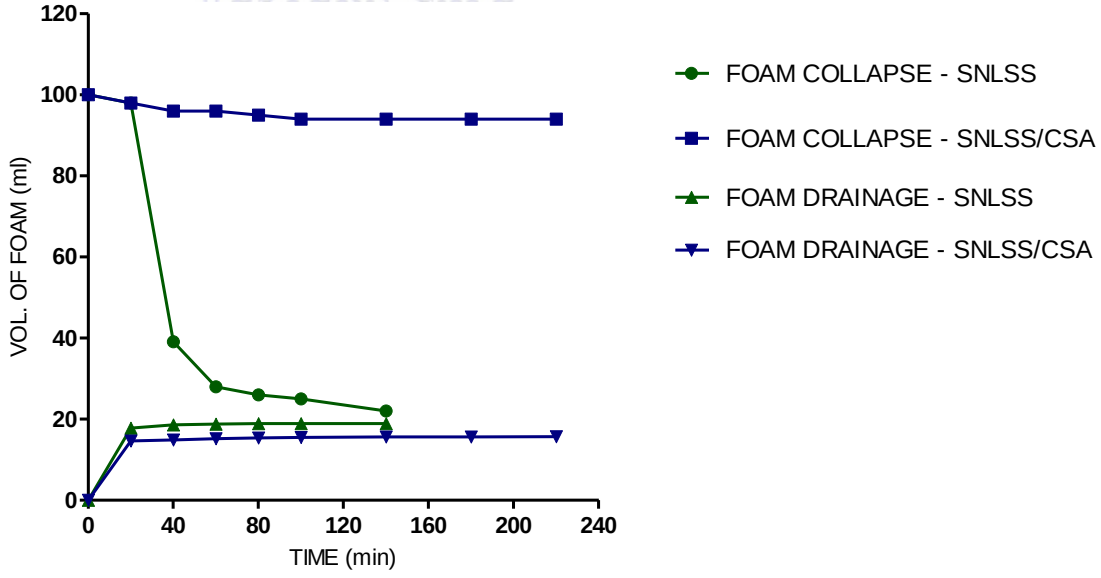


Figure 5.11: Foam collapse and drainage of SNLSS and SNLSS/CSA at pH 5.5

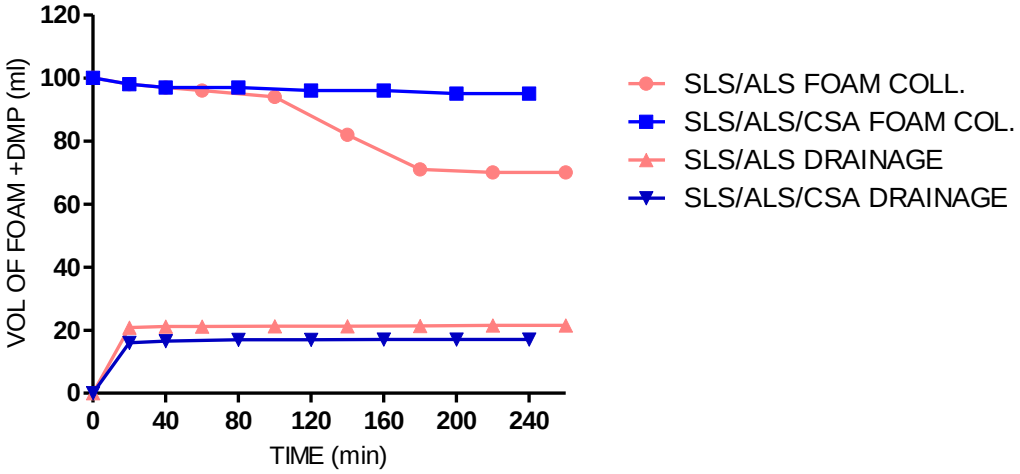


Figure 5.12: Foam collapse/drainage of SLS/ALS and SLS/ALS/CSA AT pH 4.0

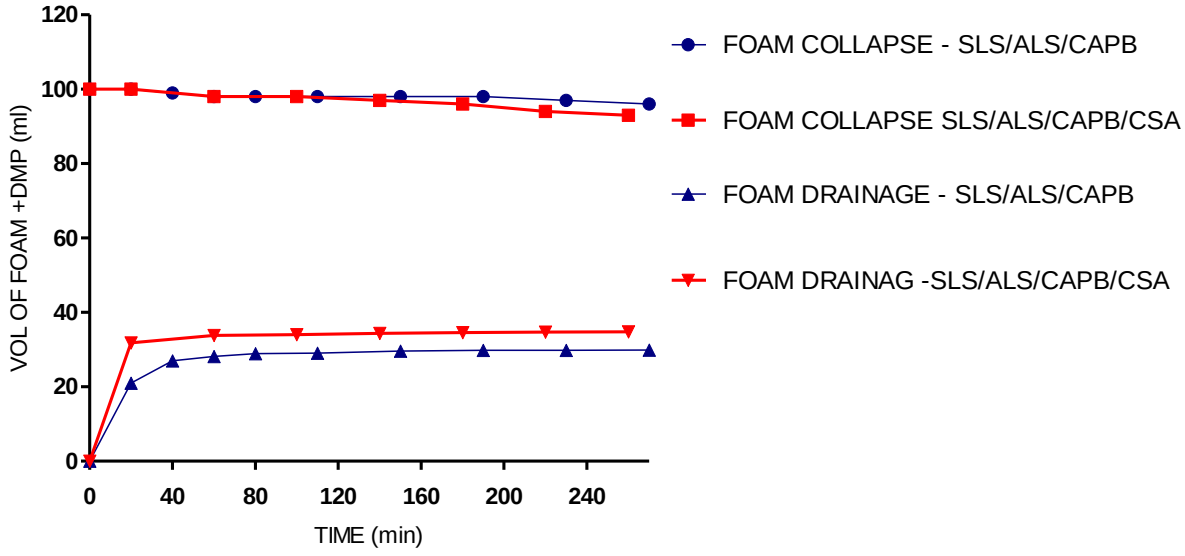


Figure 5.13: Foam collapse/drainage of SLS/ALS/CAPB and SLS/ALS/CAPB/CSA at pH 4.0

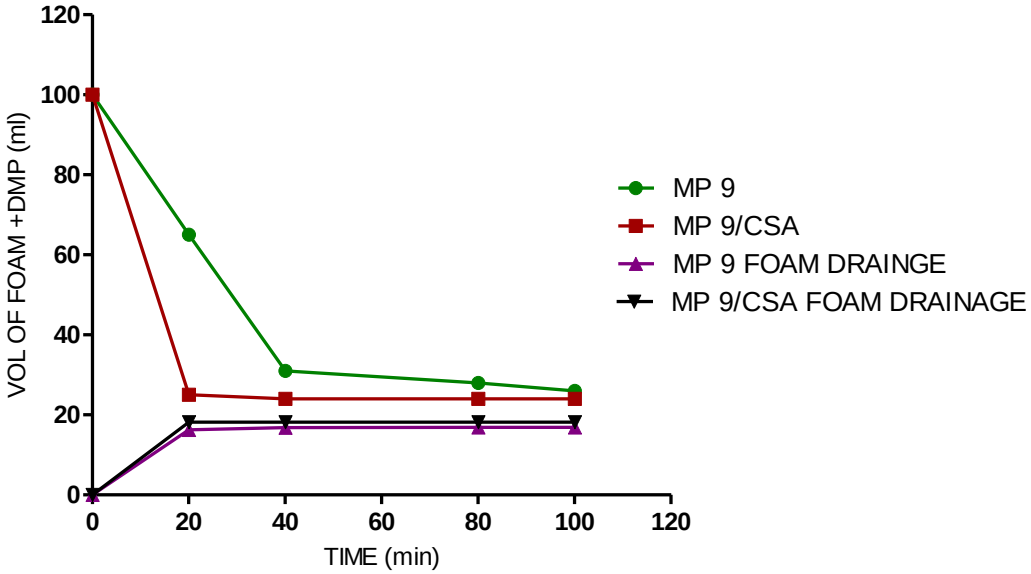


Figure 3.14: Foam collapse/drainage of MP 9 and MP 9/CSA AT pH 4.0

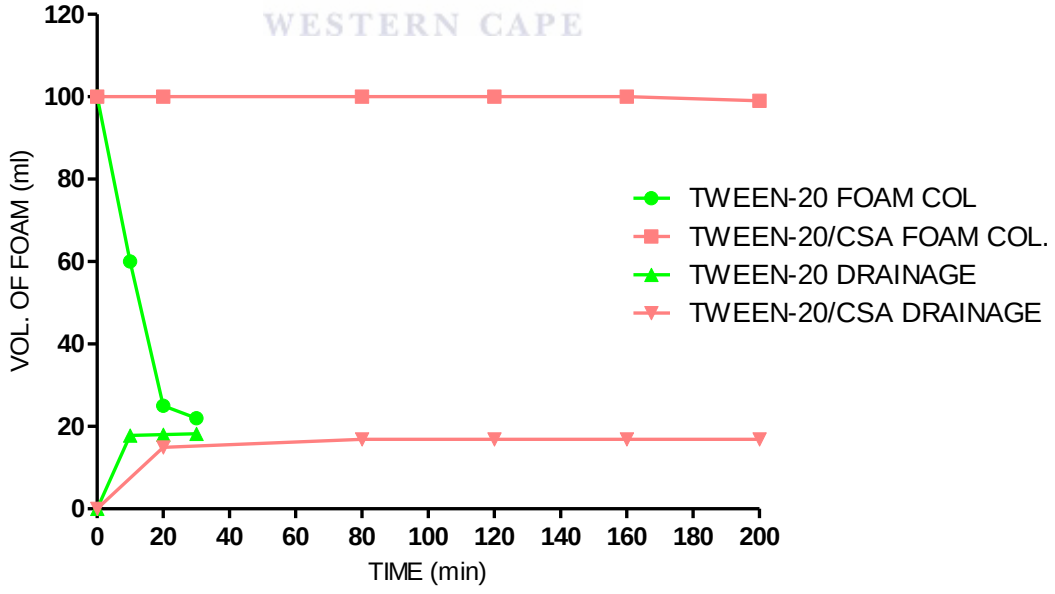


Figure 5.15: Foam collapse/drainage of TWEEN 20 and TWEEN 20/CSA at pH 4.0

5.2.1.2.2 Bubble breaking time (BBT)

Smaller bubbles if formed next to a larger bubble might disappear at the expense of the bigger one which grows bigger. This is because smaller bubbles are at a higher gas pressure than larger ones and the resulting gas diffusion leads to coalescence (Lambert, 2010). It was therefore deemed important that as far as possible only one bubble at a time was formed to avoid such type of interference and only large bubbles were studied. Table 5.2 shows the results of the BBT of 10 bubbles of each system.

Table 5.2: Bubble breaking time (BBT)

SLS pH 4.0	SLS/CSA pH 4.0	SLS pH 5.5	SLS/CSA pH 5.5	ALS pH 4.0	ALS/CSA pH 4.0	ALS pH 5.5	ALS/CSA pH 5.5	TWEEN 20 pH 4.0	TWEEN - 20/CSA pH 4.0	SNLSS pH 5.5	SNLSS/CSA pH 5.5	SLS/ALS/SNLSS pH 4.0	SLS/ALS/SNLSS/CSA pH 4.0	MP 9 pH 4.0	MP9/CSA pH 4.0	SLS/ALS pH 4.0	SLS/ALS/CSA pH 4.0	CAPB pH 4.0	CAPB/CSA pH 4.0	SLS/ALS/CAPB pH 4.0	SLS/ALS/CAPB/CSA pH 4.0
16.57	15.66	9.54	22.90	16.22	15.35	47.00	31.89	11.21	271.22	17.89	66.84	30.16	50.08	33.65	37.98	16.78	30.03	13.67	14.69	19.01	23.19
26.47	39.41	8.90	22.10	17.44	16.63	35.74	25.56	22.15	283.56	20.70	56.19	34.39	38.10	36.81	35.32	17.05	64.22	10.23	17.60	14.37	36.46
11.50	25.08	13.76	21.70	18.06	21.56	19.08	30.94	24.84	169.58	29.55	40.90	27.68	27.37	34.23	36.65	30.11	38.67	13.61	16.97	24.87	21.11
12.50	20.72	16.56	56.94	23.91	17.61	24.18	26.27	25.45	255.52	39.35	32.69	40.33	37.65	22.30	46.12	17.36	41.60	8.76	23.54	25.71	27.34
14.44	30.50	10.03	36.20	22.99	15.82	26.36	26.06	20.67	188.97	19.59	39.55	35.26	27.44	44.34	62.51	11.31	28.07	10.73	17.03	29.31	32.65
12.61	30.53	9.72	17.11	30.07	16.82	22.19	38.29	22.87	200.14	27.83	51.73	38.46	33.25	34.27	47.61	15.74	42.68	6.86	14.00	34.44	34.45
18.78	37.28	10.71	31.82	23.67	19.35	24.02	25.08	24.55	153.45	32.92	42.39	17.03	18.25	36.08	87.22	19.90	55.41	10.58	20.72	24.04	24.41
12.91	35.04	9.88	32.70	18.42	37.09	27.45	24.48	26.41	143.26	24.96	41.02	26.80	30.50	22.15	98.38	33.04	62.06	6.67	19.51	23.52	35.67
11.22	26.54	8.00	35.81	18.34	27.40	23.33	28.48	24.00	175.07	36.50	65.84	34.51	36.51	47.13	147.8	25.10	26.06	19.98	23.77	17.32	31.00
9.87	25.26	8.28	26.69	21.46	40.33	31.41	27.22	59.49	221.83	34.47	51.62	21.73	48.87	51.62	98.01	9.25	26.89	8.83	22.07	17.07	28.46

Note: time is in seconds (s)

BBT results of table 5.2 are also shown graphically as a scatter plot in figure 5.16 below

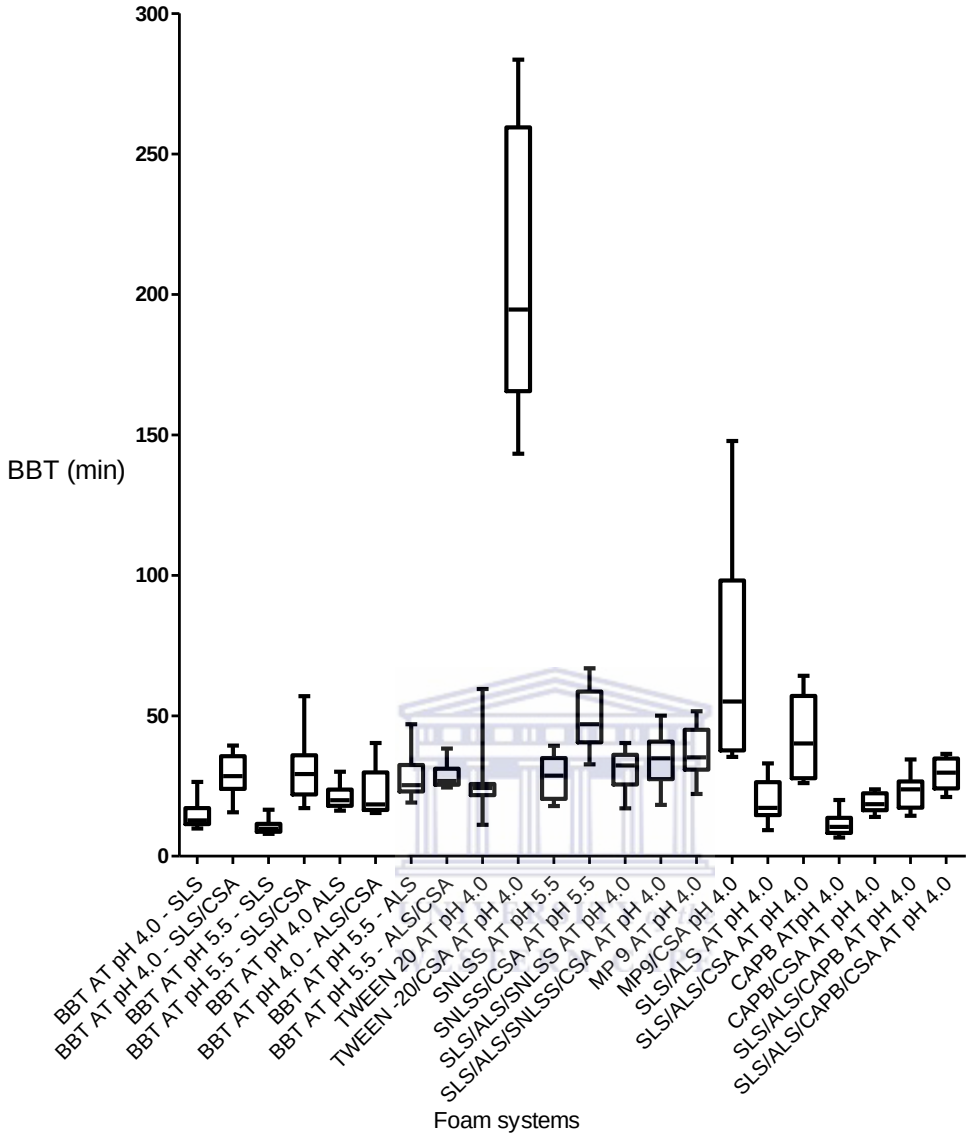


Figure 5.16: Scatter plot of mean BBTvalues

5.2.1.2.3 The significance of addition of CSA on bubble stability

An independent two sample t-test (two tailed) was applied on the data in table 5.2 above to test the significance of addition of the foam stabilizer (CSA) on the stability of bubbles and the results are shown in table 5.3 below. This can also be visually assessed in the BBT scatter plot (figure 5.16 above). The student t-test is a statistical test meant to compare two groups of related observations e.g. in this case, those taken from the same

subjects before and after a treatment (addition of foam stabilizer). The test is independent sampled because each observation in the first sample is not specifically linked to an observation in the second sample (Davis, and Mukamal, 2006). The null hypothesis was, 'there is no difference in foam stability, as measured by BBT, in the absence and in the presence of the foam stabilizer, CSA'. It was assumed that that the data was normally distributed. Graph pad prism software t-test was employed.

Table 5.3: The significance of addition of CSA on bubble stability

DMP bubble systems being compared			**Are means sig. different? (p<0.05)**		**P value**	**t**	**df**	**95% conf. interval**
			YES	NO				
SLS at pH 4.0	vs.	SLS/CSA at pH 4.0	√		p<0.0001	6.895	9	-18.48 to -9.350
SLS at pH 4.0	vs.	SLS at pH 5.5		√	p<0.0561	2.192	9	-0.1334 to 8.431
SLS /CSA at pH 4.0	vs.	SLS/CSA at pH 5.5		√	P<0.7108	0.383	9	-12.40 to 8.812
ALS at pH 4.0	vs.	ALS/CSA at pH 4.0		√	p<0.6240	0.508	9	-9.484 to 6.008
ALS at pH 4.0	vs.	ALS at pH 5.5		√	P<0.0723	2.035	9	-14.82 to 0.7811
ALS/CSA at Ph 4.0	vs.	ALS/CSA at pH 5.5		√	P<0.1465	1.589	9	-13.65 to 2.384
TWEEN-20 at pH 4.0	vs.	TWEEN-20/CSA at pH 4.0	√		0.0001	10.88	9	-217.5 to -142.6
SNLSS at pH 5.5	vs.	SNLSS/CSA at pH 5.5	√		0.0022	4.246	9	-31.42 to -9.579
SLS/ALS/SNLSS at pH 4.0	vs.	SLS/ALS/SNLSS/CSA at pH 4.0		√	0.2614	1.198	9	-12.03 to 3.699
MP 9 AT pH 4.0	vs.	MP 9/CSA at pH 4.0	√		0.0130	3.088	9	-58.04 to -8.965
SLS/ALS at pH 4.0	vs.	SLS/ALS/CSA at pH 4.0	√		0.0005	4.229	18	-32.94 to -11.07
CAPB at pH 4.0	vs.	CAPB/CSA at pH 4.0	√		0.0004	5.456	9	-11.31 to -4.682
SLS/ALS/CAPB at pH 4.0	vs.	SLS/ALS/CAPB /CSA at pH 4.0	√		0.0229	2.576	9	-12.22 -0.7931

In table 5.3, a ‘YES’ significant result implied that CSA enhanced the stability of the foam system while a ‘No’ significant result implied that CSA had no effect on foam stability of that particular system. Some writers describe CSA as a foam booster (Artec Chemical Company Limited, n.d.) and others as foam stabilizer (columbia analytical services, 2001; Oxford University, 2007). In this study, CSA was found to have minimal effect on foamability, but profoundly influenced foam stability. This is probably because CSA was always introduced in this study at FA concentrations of 2 % w/v or v/v after assessing foam decay and drainage. Such concentrations are way beyond CMC and therefore can’t be expected to yield useful foamability effect data.

5.2.1.2.4 Correlation analysis of the different measures of foam stability

Pearson’s correlation test is the most popular way of determining both the strength and the direction of the relationship between two interval variables. The correlation coefficient, r, ranges between -1 and +1. Values closer to +1 represent a positive relationship i.e. if the independent variable is increased, the values of the dependent variable also increases. Values closer to -1 indicate a negative relationship, which is the opposite of preceding relationship. Values closer to 0 represent absence of a relationship (SAMHSA, 2003).

The data in table 5.4 below was subjected to the Pearson correlation test to determine the relationship that existed between named foaming properties. Graphpad prism software (Graphpad software, Inc., 2004) based Pearson’s correlation test was employed. The test assumption was that data was sampled from a Gaussian population. Correlation analysis revealed a positive correlation between foam decay and BBT (Pearson r = 0.5397), another positive correlation between foam density and BBT (Pearson r = 0.8842) and a

weak negative correlation between foam drainage and BBT (Pearson r = – 0.4447). This means that, the more water a foam can hold, the more stable it is, a view held by many authors (Babcsán et al., 2003; Klein, 2004). Foam density has also been linked to the amount of water a foam can hold (Hutzler, Verbist, Wenre, & Van der Stee, j. a., 1995; Klein, 2004). It is also generally agreed that BBT correlates well with foam collapse (Ross, 1943) as mentioned in the literature review. Miles et al. (1944) studied foam drainage and found a positive correlation between the rate of foam drainage and the volume of liquid in a foam, which he expressed as $y = ax^n$, where y is the rate of drainage; x is the volume of liquid in the foam; a and n are constants. On the relationship between foam drainage and foam stability, this study concludes that there is no relationship between these. This is because rates of flow of liquid through foam are influenced by the size of the bubbles and the bulk and surface viscosities. If these are not controlled, relative drainage rates do not evaluate foam stability under the same conditions. Babcsan et al. (2003), states that a limitation to foam drainage is related to foam stability of aqueous foams. He attributes the stabilizing influence of foam stabilizers such as CSA to their slowing action on liquid drainage when they are present in plateau borders.

Table 5.4: Correlation data of the different measures of foam stability

System no.	DMP foam system	Foam density (mg/ml)	Mean BBT (S)	$t_{1/2}$ of foam collapse (min)	vol. drained at $t_{1/2}$ (ml)
1	SLS at pH 4.0	5.29	14.69	98.9	21.3
2	SLS /CSA at pH 4.0	6.00	28.60	**>300**	**14.6**
3	ALS at pH 4.0	5.97	21.06	**>300**	**19.7**
4	ALS/CSA at Ph 4.0	6.54	22.08	260	22.0
5	TWEEN-20 at pH 4.0	10.2	26.16	12.6	18.6
6	TWEEN-20/CSA at pH 4.0	33.40	206.3	**>600**	**16.9**
7	SNLSS at pH 5.5	14.9	28.38	95.9	18.6
8	SNLSS/CSA at pH 5.5	22.20	48.88	**>600**	**14.9**
9	SLS/ALS/SNLSS at pH 4.0	99.20	30.64	**>600**	**26.3**
10	SLS/ALS/SNLSS/CSA at pH 4.0	106.00	34.80	**>600**	**24.0**
11	MP 9 AT pH 4.0	11.60	36.26	13	16.8
12	MP 9/CSA at pH 4.0	42.50	69.76	28	12.3
13	SLS/ALS at pH 4.0	4.92	19.56	**>300**	**21.5**
14	SLS/ALS/CSA at pH 4.0	23.40	41.57	**>600**	**17.1**
15	CAPB at pH 4.0	11.2	10.99	85	19.6
16	CAPB/CSA at pH 4.0	11.9	18.99	87	15.8
17	SLS/ALS/CAPB at pH 4.0	100.00	22.97	**>600**	**29.9**
18	SLS/ALS/CAPB /CSA at pH 4.0	100	29.47	**>600**	**34.8**
19	SLS at pH 5.5	4.70	10.54	162	18.8
20	SLS/CSA at pH 5.5	9.60	30.40	**>300**	**17.9**
21	ALS at pH 5.5	5.27	28.08	**>300**	**21.9**
22	ALS/CSA at pH 5.5	11.00	28.43	**>300**	**18.6**

5.2.1.2.4.1 Pearson correlation tests and associated Scatter plots

Below are self-explanatory scatter plots depicting correlation data graphically together with their correlation coefficients. The first scatter diagram on each page represents the initial Pearson test while the second represent the test after a few outliers were removed.

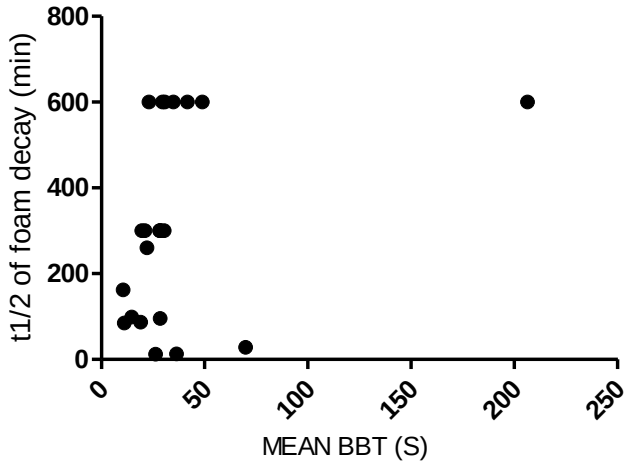


Figure 5.17: initial scatter plot correlation of $t_{1/2}$ of foam decay vs mean BBT

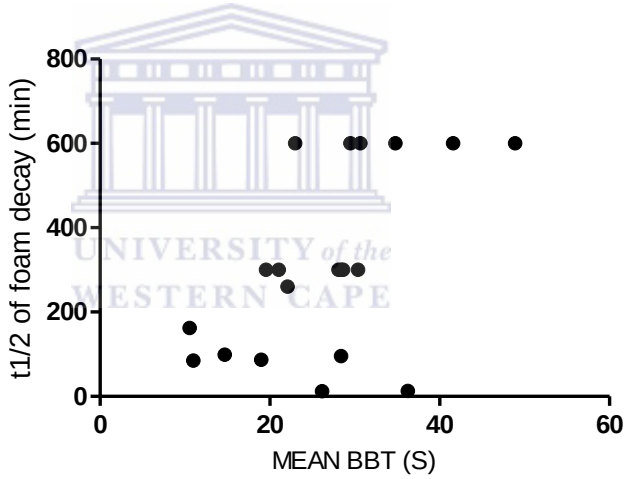


Figure 5.18: Scatter plot after removing outliers (position 6 and12)- $t_{1/2}$ of foam decay vs BBT

Number of XY Pairs	20
Pearson r	0.5397
95% confidence interval	0.1276 to 0.7929
P value (two-tailed)	0.0140
P value summary *	
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.2913

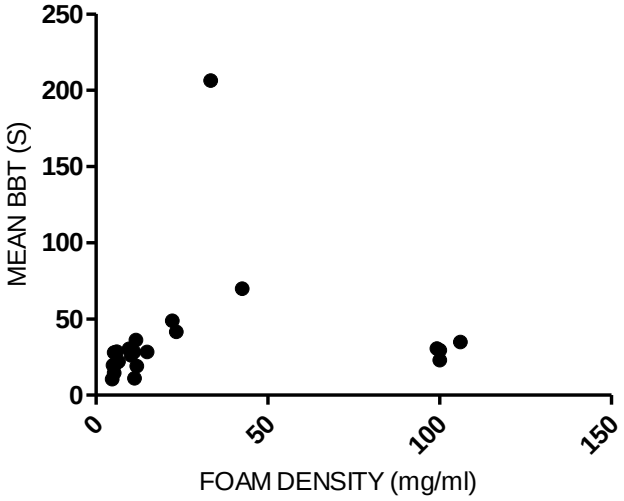


Figure 5.19: Initial scatter plot of correlatio between mean BBTvs Foam density

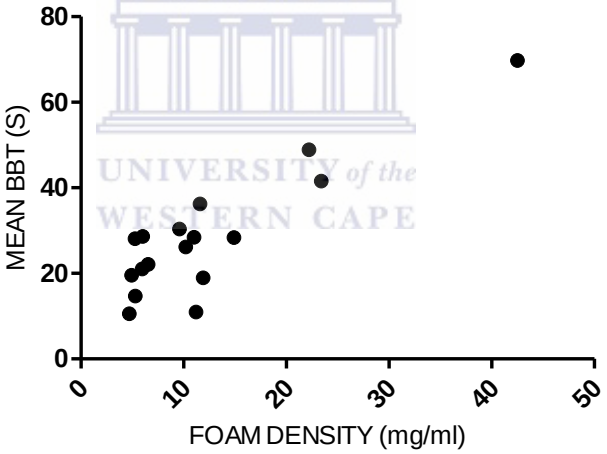


Figure 5.20: Scatter plot of correlation after removal of outliers - mean BBT vs foam density

Number of XY Pairs	17
Pearson r	0.8842
95% confidence interval	0.7018 to 0.9578
P value (two-tailed)	P<0.0001
P value summary	***
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.7819

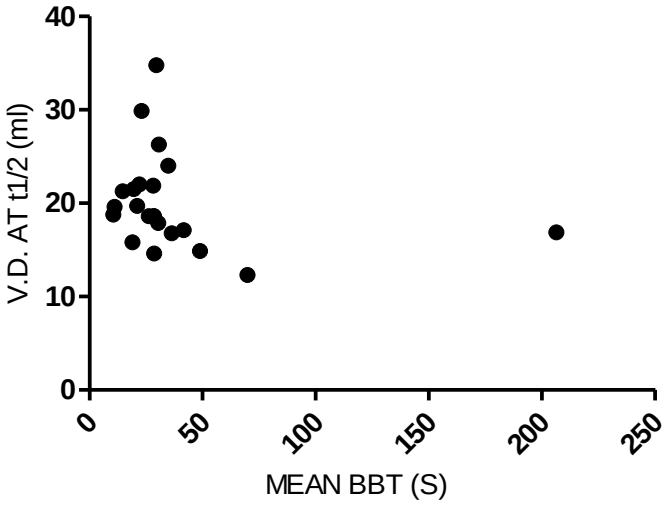


Figure 5.21: Initial scatter plot of correlation of Vol. drained at $t_{1/2}$ vs mean BBT

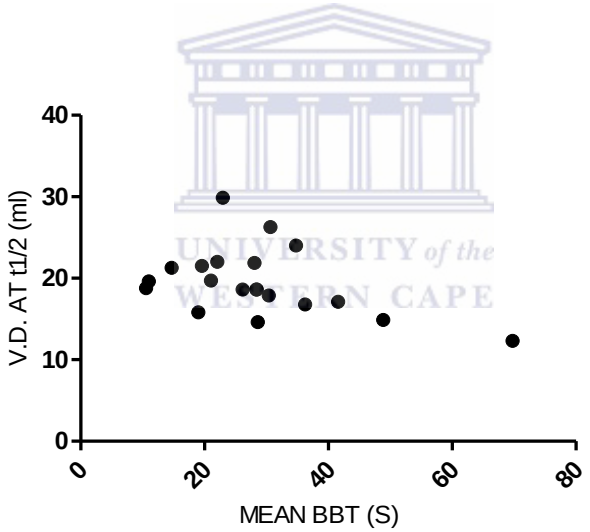


Figure 5.22: Scatter plot of correlation after removing two outliers (row 6 and 18) - Vol. drained at $t_{1/2}$ vs mean BBT

Number of XY Pairs	20
Pearson r	-0.4447
95% confidence interval	-0.7414 to -0.002649
P value (two-tailed)	0.0494
P value summary *	
Is the correlation significant? (alpha=0.05)	Yes
R squared	0.1978

5.2.1.3 The pH stability profile of DMP

An important result from the DMP-foam evaluation studies is the elucidation of the pH stability profile of DMP. DMP was found to be stable at pH ≤ 5.2. Beyond this pH DMP encounters serious stability problems such that it was no longer useful at least for formulation purposes. This meant DMP-foam systems such as SNLSS that performed well at pH 5.5 but poorly at pH 4.0, were automatically disqualified from the formulation. DMP however tended to regain some activity once the pH was dropped back from a high value to pH ≤ 5.2.

5.2.1.4 Selection of appropriate foam system for product formulation

Refer to table 5.5 below for the scoring system used to select FA/FAC appropriate for incorporation in the foam formulation (Siltech personal care, 2006). The marks were assigned as follows: Foamability = 40; stability = 40, i.e. $t_{1/2}$ of foam decay = 15, BBT = 15, drainage = 5, density = 5; color effect = 5; physical attraction = 5; and other pharmaceutical consideration = 10; Total score = 100.

Table 5.5: Selection of appropriate foam system for product formulation

Foam system → **Property¬**		**SLS**	**SLS/ CSA**	**ALS**	**ALS/ CSA**	**TWEEN 20**	**TWEEN 20/ CSA**	**SLS/ALS/ SNLSS**	**SLS/ALS/ SNLSS/CSA**	**MP 9**	**MP9/ CSA**	**SLS/ ALS**	**SLS/ALS/ CSA**	**CAPB**	**CAPB/ CSA**	**SLS/ALS/ CAPB**	**SLS/ALS/ CAPB/CSA**
Foamability (40)		35	35	30	30	10	10	10	10	10	10	35	35	25	25	40	40
Stability (40)	$T_{1/2}$ of foam decay (15)	12	15	10	15	5	15	15	15	4	6	12	15	10	15	12	15
	Mean BBT (15)	10	13	11	13	10	15	11	14	12	15	11	15	8	12	12	13
	Drainage at $t_{1/2}$ (5)	4	3	3	3	3	4	3	5	2	3	4	5	3	3	2	1
	Density of foam (5)	2	2	2	2	2	4	3	4	3	4	1	3	2	3	5	5
Colour changes (5)		5	5	5	5	5	5	5	5	5	5	5	5	4	4	4	4
Physical attraction (5)		5	5	4	4	5	5	4	4	4	4	5	5	4	4	5	5
Other pharm. factors (10)		10	10	8	8	10	10	8	8	6	7	10	10	10	10	10	10
TOTAL SCORE (100)		**83**	**88**	**73**	**80**	**50**	**68**	**59**	**65**	**46**	**54**	**83**	**93**	**66**	**76**	**90**	**93**

As seen from this table 5.5, SLS/ALS/CAPB/CSA foam system scored the highest marks and was therefore selected as the foam system of choice for incorporation into the present formulation.

5.2.2 Acid base potentiometric titrations

5.2.2.1 Determination of suitable alkali for raising the pH of DMP

In order to adjust the normal pH (acidic) of DMP to a higher value that matched the target site pH, a suitable alkali reagent had to be selected. The choice was between sodium hydroxide and sodium per carbonate.

5.2.2.1.1 Titration curve of NaOH vs DMP

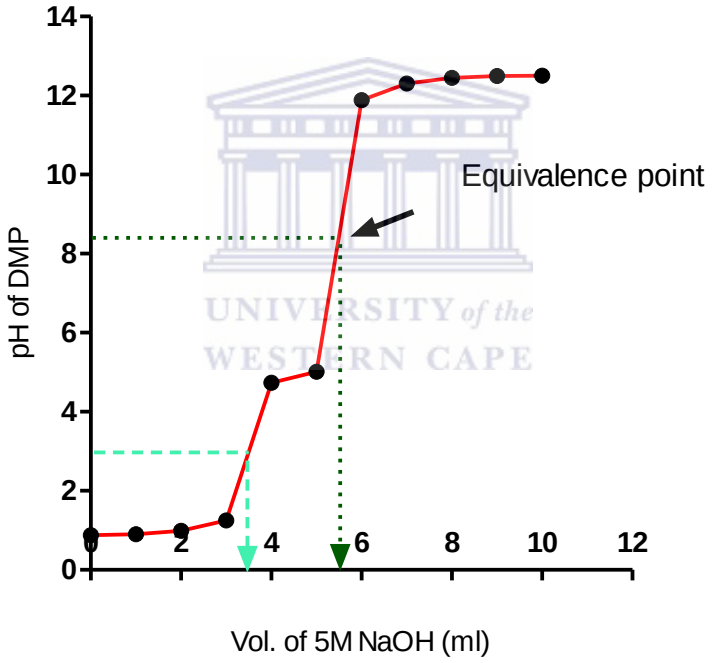


Figure 5.23: Titration curve of 5M NaOH against 100 ml of DMP

Table 5.6: First and second derivative calculations for 5 % w/v NaOH vs. 100 ml DMP titration (figure 3.24)

Vol. NaOH added (ml)	**Mean pH**	**dpH**	**dV**	**dpH/dV**	**d2pH**	**dv2**	**d2PH/Dv2**
0 (a_1)	0.88 (b_1)	0.02 (b_2- b_1)	1 (a_2-a_1)	0.02			
1 (a_2)	0.90 (b_2)	0.09 (b_3- b_2)	1 (a_3-a_2)	0.09	0.07 [(b_3-b_2)-(b_2- b_1)]	1 [(a_2-a_1)+(a_3-a_2)]/2	0.07
2 (a_3)	0.99 (b_3)	0.26 (b_4- b_3)	1 (a_4-a_3)	0.026	0.17 [(b_4-b_3)-(b_3- b_2)]	1 [(a_4-a_3)+(a_3-a_2)]/2	0.17
3 (a_4)	1.25 (b_4)	3.48 (b_5- b_4)	1(a_5 –a_4)	3.48	3.22 [(b_5-b_4)-(b_4- b_3)]	1 [(a_5 –a_4)+(a_4-a_3)]/2	3.22
4 (a_5)	4.73 (b_5)	0.28 (b_6- b_5)	1(a_6-a_5)	0.28	– 3.2 [(b_6-b_5)-(b_5- b_4)]	1 [(a_6-a_5)+(a_5 –a_4)]/2	– 3.2
5 (a_6)	5.01 (b_6)	6.88 (b_7- b_6)	1(a_7-a_6)	6.88	6.6 [(b_7-b_6)-(b_6- b_5)]	1 [(a_7-a_6)+(a_6-a_5)]/2	6.6
6 (a_7)	11.89 (b_7)	0.42 (b_8- b_7)	1(a_8-a_7)	0.42	– 6.46 [(b_8-b_7)-(b_7- b_6)]	1 [(a_8-a_7)+(a_7-a_6)]/2	– 6.46
7 (a_8)	12.31 (b_8)	0.14 (b_9- b_8)	1(a_9-a_8)	0.14	– 0.28 [(b_9-b_8)-(b_8- b_7)]	1 [(a_9-a_8)+(a_8-a_7)]/2	– 0.26
8 (a_9)	12.45 (b_9)	0.04 (b_{10}- b_9)	1(a_{10}-a_9)	0.04	– 0.10 [(b_{10}-b_9)-(b_9- b_8)]	1 [(a_{10}-a_9)+(a_9-a_8)]/2	– 0.10
9 (a_{10})	12.49 (b_{10})	0.01 (b_{11}- b_{10})	1(a_{11}-a_{10})	0.01	– 0.03 [(b_{11}-b_{10})-(b_{10}- b_9)]	1 [(a_{11}-a_{10})+(a_{10}-a_9)]/2	– 0.03
10 (a_{11})	12.50 (b_{11})						

Refer to the literature review, section 2.3.4.2.3, for definition of first and second derivative functions and for explanation of headings in this and the other related tables below.

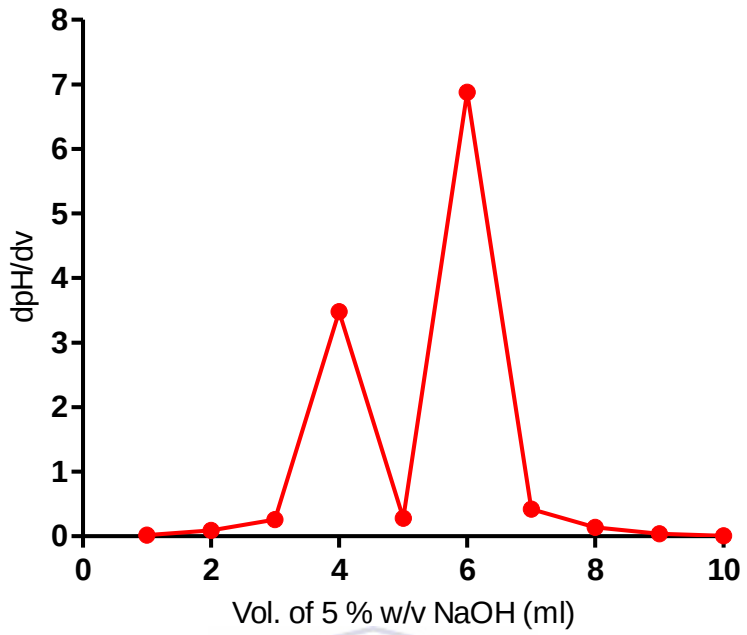


Figure 5.24: First derivative plot of potentiometric titration of 5 % w/v NaOH against 100 ml DMP

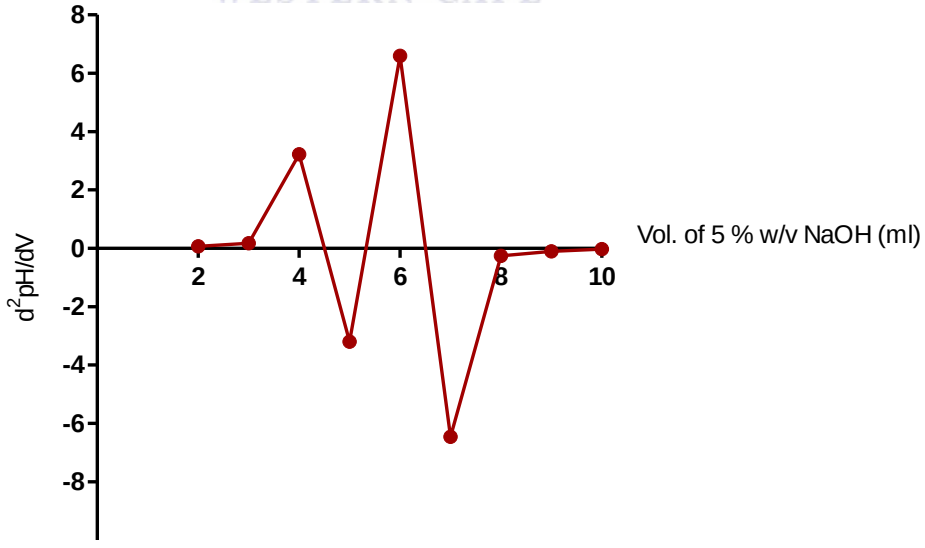


Figure 5.25: Second derivative plot of potentiometric titration of 5 % w/v NaOH against 100 ml DMP

From the above, 1st end point = 4.1 ml of NaOH and 2nd end point = 6.2 ml of NaOH. For the first derivative (figure 5.24), the end-points are the x-axis values corresponding to the two peaks while for the second derivative (figure 5.25), the end points are the x-values where lines from peaks cuts the x–axis.

5.2.2.1.2 Titration curve of $Na_2CO_3.1.5H_2O$ vs DMP

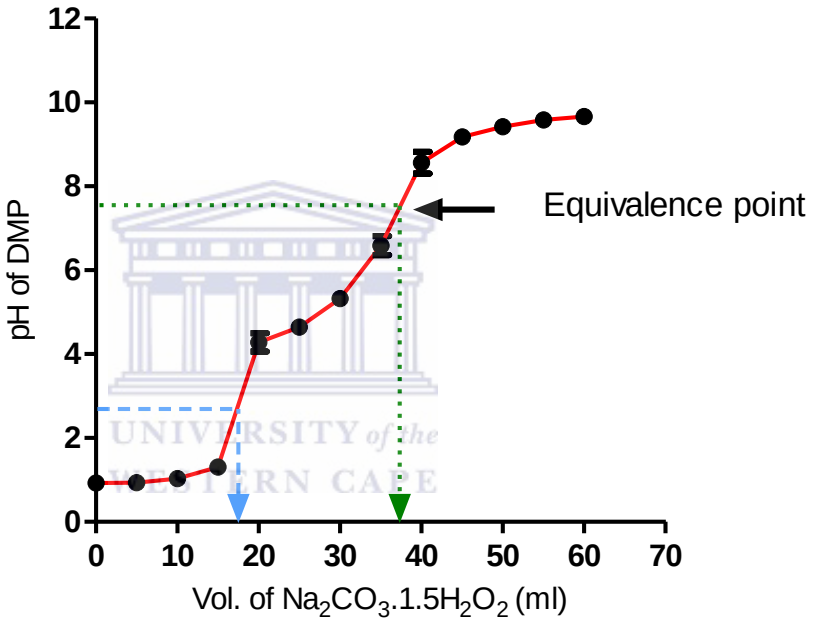


Figure 5.26: Titration curve of 5 % w/v $Na_2CO_3.1.5H_2O_2$ against 100 ml DMP

Table 5.7: First and second derivative calculations for $Na_2CO_3.1.5H_2O_2$ titration

Vol. Na2CO3.1/2H2O2 added (ml)	**Mean pH**	**dpH**	**dV**	**dpH/dV**	**d2pH**	**dv2**	**d2PH/Dv2**
0	0.93	0	5	0			
5	0.93	0.1	5	0.02	0.1	5	0.02
10	1.03	0.28	5	0.056	0.18	5	0.036
15	1.31	2.97	5	0.594	2.69	5	0.538
20	4.28	0.36	5	0.72	– 2.61	5	– 0.522
25	4.64	0.68	5	0.136	0.32	5	0.064
30	5.32	1.27	5	0.254	0.59	5	0.118
35	6.59	1.97	5	0.394	0.70	5	0.14
40	8.56	0.62	5	0.124	– 1.35	5	– 0.27
45	9.18	0.24	5	0.048	– 0.38	5	– 0.076
50	9.42	0.16	5	0.032	– 0.08	5	– 0.016
55	9.58	0.08	5	0.016	– 0.08	5	– 0.016
60	9.66		5				

Refer to table 5.6 (page 115) for the explanation of calculations in this table.

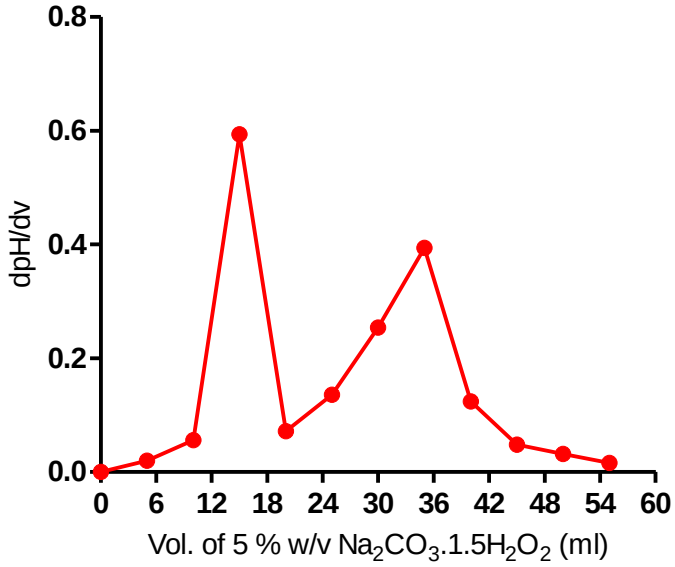


Figure 5.27: First derivative plot of potentiometric titration of 5 M $Na_2CO_3.1.5H_2O_2$ against 100 ml DMP

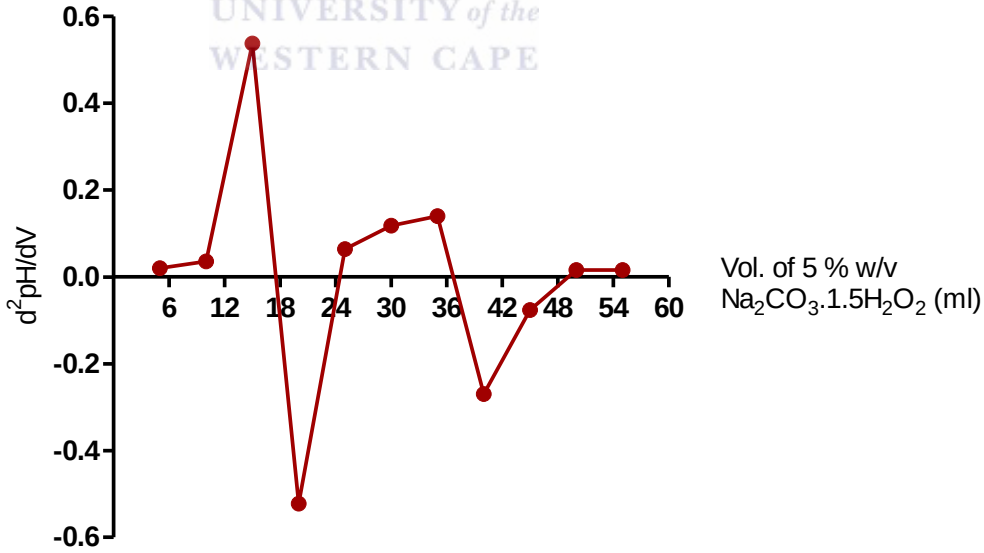


Figure 5.28: Second derivative plot of potentiometric titration of 5 % w/v $Na_2CO_3.1.5H_2O_2$ against 100 ml DMP

From the above, 1st end point is 15 ml of $Na_2CO_3.1/2H_2O_2$ and 2nd end point is 36ml of $Na_2CO_3.1/2H_2O_2$.

The titration curves for both NaOH vs. DMP (figure 5.23) and $Na_2CO_3.1/2H_2O_2$ vs. DMP (figure 5.26) have two inflection points. This implies that DMP contains either a diprotic acid or a mixture of acids. The nature of the NaOH/DMP curve also shows that the acid used to lower the pH of DMP could be a strong acid and this is confirmed by the pH of DMP (about pH 0.5).

Combining figure 5.23 and figure 5.26, gives figure 5.29 below, demonstrating the efficiency of the two alkalis in adjusting the pH of DMP.

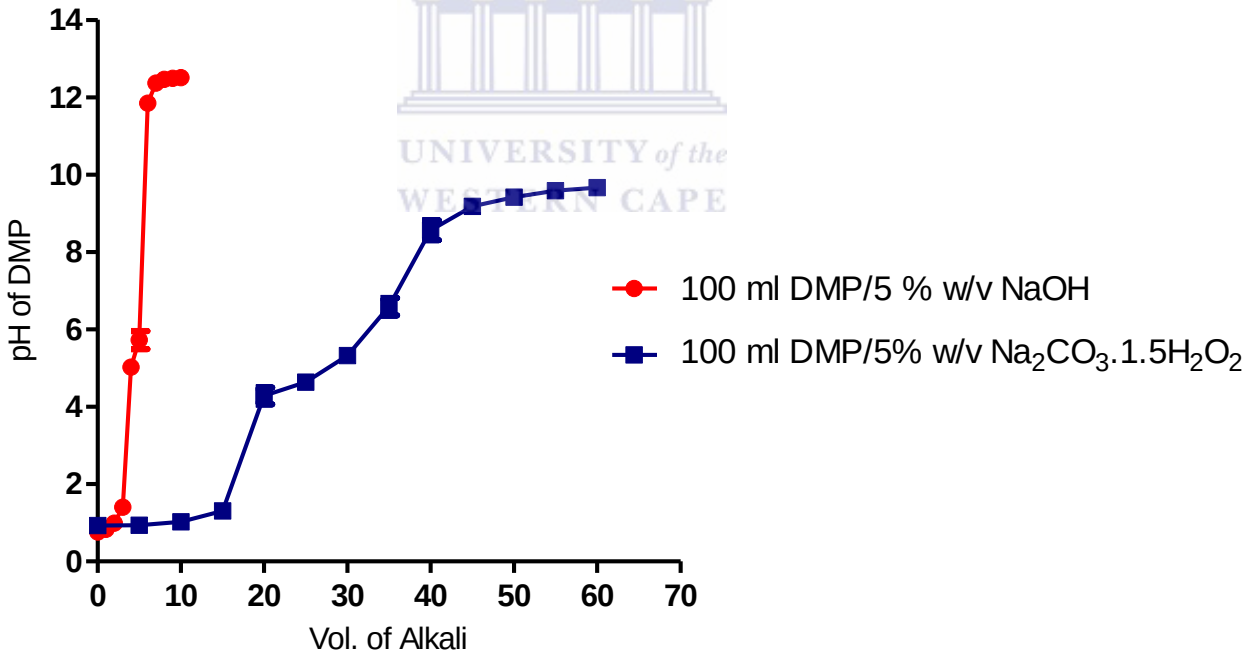


Figure 5.29: Graph comparing titration profiles of 5 % w/v of both NaOH and $Na_2CO_3.1.5H_2O_2$ against DMP

5.2.2.1.3 Selection of the most suitable alkali for the formulation

The end point of 5 % w/v NaOH vs DMP titration was found to be 4.0 ml while that of 5 % w/v $Na_2CO_3.1/2H_2O_2$ was found to be 18 ml. From this it is clear that NaOH is more efficient at adjusting the pH of DMP since just a small amount would be sufficient to adjust the pH to desired levels. Hence NaOH was selected for the concentrate formulation. The first and second derivative plots (figures 5.24, 5.25, 3.27 and 5.28) were employed because they are more accurate at determining the end point than estimations based on the inflection points of the titration curves, which is a mere graphical approximation. The first and second derivative functions are mathematical procedures and determine end point by calculation of the slope of the curve and the rate of change of the slope respectively.

5.2.2.2 Characterization of the NaOH/KHP buffer system

Potentiometric acid base titrimetry was also employed to demonstrate the resistance of a buffer to pH changes in the envisaged formulation as illustrated in figure 5.30 to 5.33. Tables A19, A20, A21 and A22 in the appendix contain the data for figures 5.30, 5.31, 5.32 and 5.33 respectively.

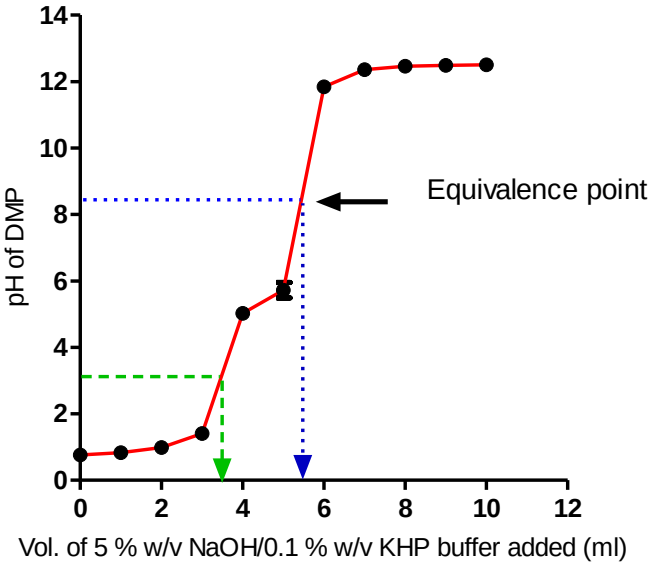


Figure 5.30: Titration curve of 5 % w/v NaOH/0.1 % w/v KHP buffer against 100ml DMP

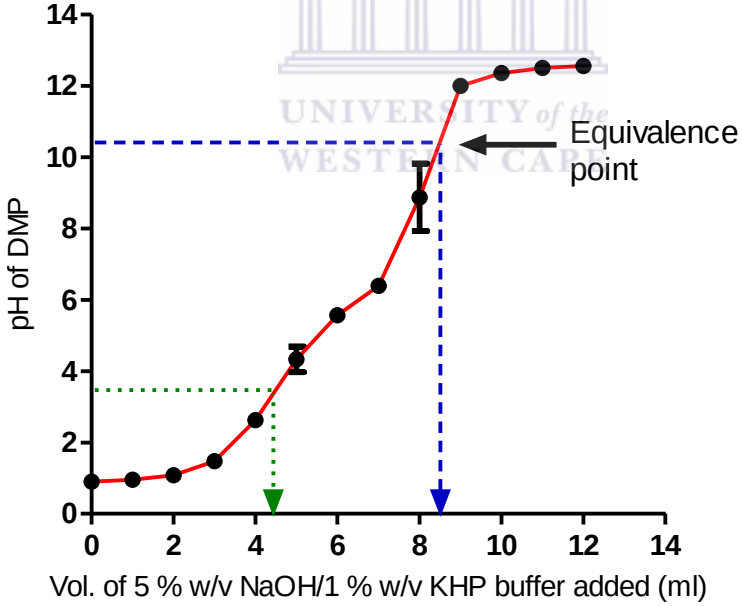


Figure 5.31: Titration curve of 5 % w/v NaOH/1 % w/v KHP against 100 ml DMP

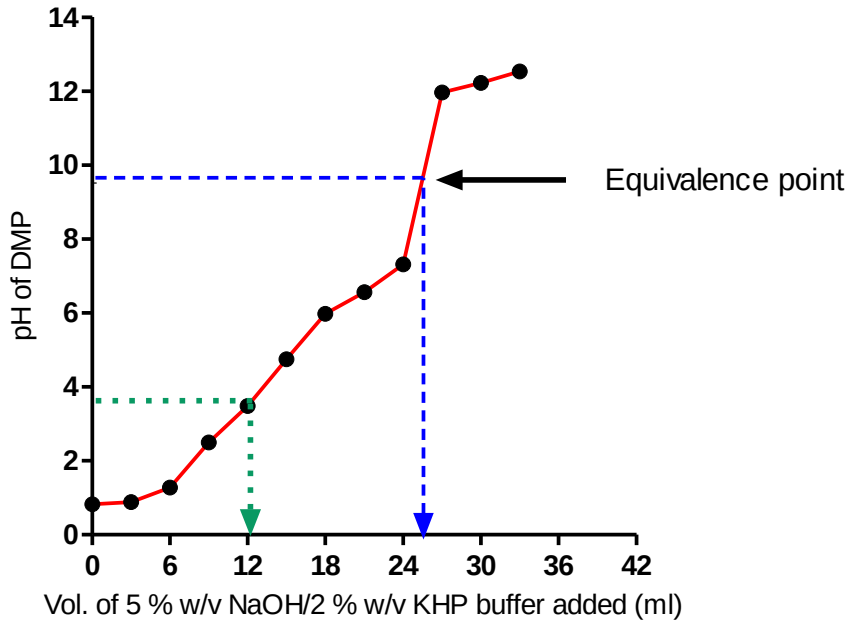


Figure 5.32: Titration curve of 5 % w/v NaOH/2 % w/v KHP buffer against 100 ml DMP

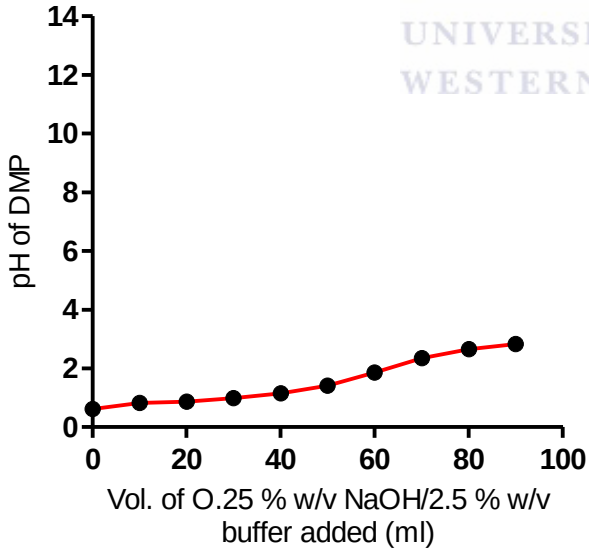


Figure 5.33: Titration curve of 0.25 % w/v NaOH/2.5 % w/v KHP against 100 ml of DMP

Table 5.8: First and second derivative calculations for 5 % w/v NaOH/2 % w/v KHP titration

Vol. 5M NaOH/2M KHP buffer added (ml)	**Mean pH**	**dpH**	**dV**	**dpH/dV**	**d2pH**	**dv2**	**d2PH/Dv2**
0	0.83	0.05	3	0.0167			
3	0.88	0.39	3	0.130	0.34	3	0.11
6	1.27	1.22	3	0.407	0.83	3	0.28
9	2.49	0.99	3	0.330	-0.23	3	-0.077
12	3.48	1.27	3	0.423	0.28	3	0.093
15	4.75	1.22	3	0.407	-0.05	3	-0.017
18	5.97	0.59	3	0.197	-0.63	3	-0.21
21	6.56	0.76	3	0.253	0.17	3	0.057
24	7.32	4.64	3	1.55	3.88	3	-1.46
27	11.96	0.26	3	0.0867	-4.38	3	0.017
30	12.22	0.31	3	0.103	0.05	3	
33	12.53						

Refer to table 5.6 (page 115) for the explanation of calculations in this table.

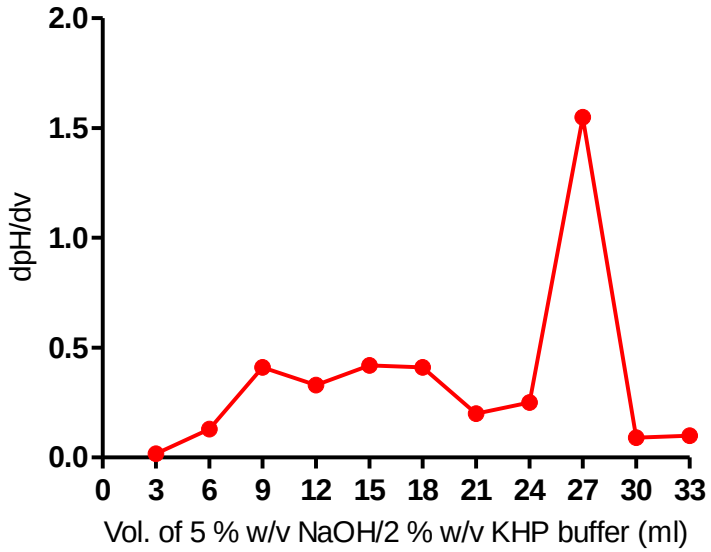


Figure 5.34: First derivative plot potentiometric titration of % w/v NaOH/2 % w/v KHP buffer system against 100 ml DMP

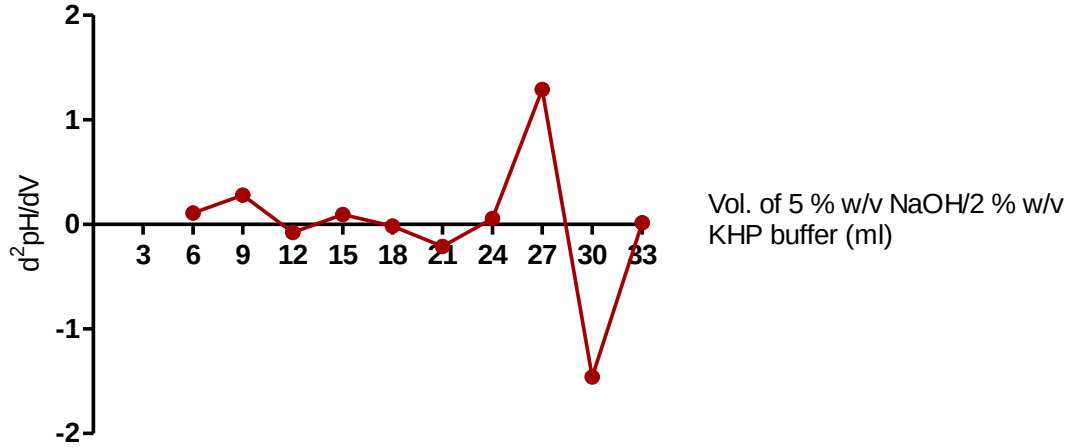


Figure 5.35: Second derivative plot of potentiometric titration of 5M NaOH/2M KHP buffer system against 100 ml DMP

From the above, 1st end point is 18 ml of 5 % w/v NaOH/2 % w/v KHP and 2nd end point is about 28 ml of 5M NaOH/2M KHP.

Figures 5.30-5.32 shows a trend in which buffering capacity increases with increasing KHP concentration. In figure 5.30 the amount of KHP is too low, buffering is negligible (buffer capacity = 3.45X10^-4) but as the amount of KHP was increased, buffering capacity also picked up as seen in figure 5.31 (buffer capacity =1.10X10^-3) and fig 5.32 shows that titration of 5 % w/v NaOH/2 % w/v KHP buffer system against DMP shifted the first end point from 4 ml (5 % w/v NaOH/0.1 M KHP vs DMP) to 12 and the buffer capacity NaOH/KHP was found to be 3.75X10^-3 as calculated in section 5.2.2.2.2. The buffer capacities of figures 5.30 and 5.31 could be calculated in a similar manner for a buffer volume change from 3 to 4 ml in appendix (tables-A19 and A20 respectively). This resistance to pH change should even be further enhanced if concentration of KHP was increased further, only that beyond KHP concentration of 3 % w/v the solutions became saturated. Consider also the fact that DMP and NaOH with which the KHP is contending in trying to maintain constant pH are very strong acid and alkali. KHP should have a higher buffer capacity under normal conditions where interference is encountered from weak acids and bases. The role of NaOH as adjuster solution in the formulation is explained in section 5.2.2.1. Without the NaOH adjuster, the KHP alone fails to raise the pH of DMP beyond pH= 1. This is illustrated in figure 3.33 where 0.25 % w/v NaOH proved to be insufficient to raise the pH of DMP beyond about 2.5. This demonstrates the need for the adjuster solution to be in sufficient strength.

Figure 5.36 is a combination of the above four graphs which gives clarity as to the effect of increasing the KHP strength in the buffer and reducing the concentration of NaOH beyond a specific limit.

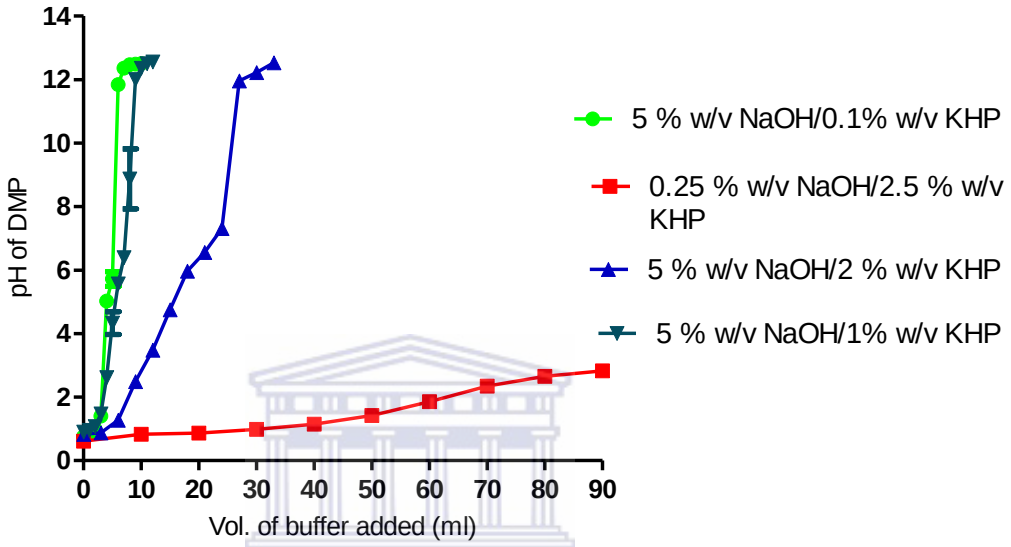


Figure 5.36: Graph showing the effect of increasing the concetration of the buffering agent KHP on DMP pH changes

5.2.2.2.1 Application of the buffer equation to this system

In the NaOH/KHP buffer as employed, the KHP exists as KP– in accordance with the equation below:

$$KHP + NaOH \rightarrow KP^- + H_2O$$

As the buffer is added to DMP, the KP– gets converted to its conjugate acid, KPH as shown below:

$$KP^- + H_3O^+ \rightarrow KHP + H_2O$$

In the initial stages of the titration almost all the KP– (conjugated base) added becomes KHP (conjugated acid). As the titration proceeds, KP– species builds up. At pH 2.2, enough KP- is available in the DMP for buffering effect to be appreciable and this buffering continue up to pH 5.9 when all the KHP in DMP gets converted to KP–.

Other buffer systems could be present in the above system e.g. in this product the possibility of HCl/$HClO^-$ exist. However, it is here assumed the KHP/KP- is the main buffer i.e. the main contributor to pH stability.

$Ka = [KP–][H30+]/[KHP]$ (1)

As the titration progresses, a point is reached where [KP–] = [KHP]

$Ka = [H3O^+]50\%/50\%$

So, $Ka = [H3O^+]$ (2)

Taking the log and multiplying on both sides gives:

$pKa = pH = 5.14$ at 25^oC (3)

$pKa = -\log Ka = 5.14 \rightarrow Ka = 7.2x10^{-6}$ (4)

$K_b = K_{water}/Ka = 1.4x10^{-9}$ (5)

Rearranging equation 1 above gives the Henderson Hasselbalch equation as shown below:

$\rightarrow pH = pKa + [KP–]/[KHP]$ (5)

From this equation, the pH of DMP can be calculated once the ratio of the acid base conjugates of KHP is known. The equation applies only to the region between pH 2.2 to 5.9 where KHP buffering action is effective (Analchem Resources, 2001).

5.2.2.2.2 Calculating the buffer capacity of NaOH/KHP buffer system against DMP

With reference to figure 3.33, when 3ml of 5 % w/v NaOH/2 % w/v KHP buffer system was added to 100ml of DMP, the pH of DMP changed from a mean 2.49 to a mean of 3.48. Refer to literature review section 2.2.2.2 for explanation of buffer capacity.

5 % w/v NaOH = 5 g of NaOH in 100 ml = 0.125 moles of NaOH in 100 ml

→ 1000 ml will have 1.25 moles

→ Molarity (M) of NaOH = 1.25 M

Number of moles of NaOH added = MV (1)

$= 1.25 \text{ moles/dm}^3 \times 0.003\text{dm}^3$

Number of moles of NaOH added = 3.75X10^-3 moles

dpH = 3.48 - 2.49 = 0.99 (2)

β = moles of NaOH added/dpH = 3.75x10^-3/0.99

β = 3.80x10^-3

5.2.3 Excipient selection

5.2.3.1 Initial excipient selection

The initial excipient selection was guided by factors such as the goals of the formulation, cosmetic considerations, route of administration, physical chemical properties and compatibility. Excipients for each category are shown in table A23, appendix section.

5.2.3.2 Final excipient selection

Safety considerations were foremost in the final selection of excipients. Only excipients regarded as safe by regulatory agencies, and therefore commonly incorporated in various registered products, were selected. Final selection was also guided by excipient availability/procurement cost considerations. So excipients deemed unsuitable based on the foregoing criteria were eliminated from the initial list to come up with the final list which is table A24 in the appendix. The selected ingredients were then subjected to drug-excipient compatibility studies.

5.2.4 Compatibility studies

Taking into account the reputably low predictive power of compatibility studies in general, to the extent that some scientists question its very worthiness (Monkhouse & Maderich, 1989), a simplified model aimed at predicting rapidly and inexpensively the short and long term stability of the mixtures, was adopted.

Literature was searched to determine documented compatibilities among the chosen excipients. Particularly, various pharmaceutical and cosmetic preparations containing excipients of interest were checked to determine if any of the chosen excipients have ever been used together before.

For the purpose of this study, binary mixtures, each consisting of 20 ml of approximately 1000 ppm DMP and each of the excipients selected were prepared. The concentrations of the excipients were in the ranges recommended for final formulation from literature sources as given in section 2.3.4.3. For multiple mixes, to 20 ml of about 1000 ppm DMP, all the selected excipients were added in the same concentrations as above. 2 % w/v or v/v was added for those excipients whose concentrations could not be determined from literature. The test mixtures were placed in glass vials which were then sealed tightly using Teflon – lined screw cap (Serajuddin et al., 1999). All tests solutions were in duplicate.

5.2.4.1 Storage

The mixtures were placed both in the fridge and at room temperature for three weeks. Vials placed at room temperature were wrapped in aluminum foil to exclude light.

5.2.4.2 Duration of study

The mixtures were stored for up to 3 weeks depending upon presence of reaction i.e. those that reacted were noted and discarded immediately.

5.2.4.3 Criteria for incompatibility judgment

The containers were physically inspected carefully during the mixing process and weekly during the time of compatibility study (Monkhouse & Maderich, 1989). Indicators which could

suggest possible interaction included: Rise in temperature during mixing which could be monitored by thermometry; color/turbidity changes which could be visually monitored; abnormal evolution of gases which could be monitored by observation of rate of bubble formation; changes in viscosity which could be monitored by changes in liquid thickness; detection of strange odors; and unexpected loss of ClO_2 determined by Iodometric titration.

5.2.4.4 Incompatibilities

1. Mixing ALS and NaOH resulted in release of a gaseous smell typical of that of ammonia and the remaining compound looked similar to SLS. It was suspected that NaOH reacted with ALS converting it to SLS. The likely reaction is shown below:

ALS **SLS**

Due to this reaction, ALS was not used it the final formulation i.e. recall that the selected foam system for the formulation under study was SLA/ALS/CAPB/CSA. The formulation ended up with SLS/CAPB/CSA which proved to work well satisfying the TPP.

2. DMP reacts with anise oil changing to a light blue color

3. DMP reacts with cinnamon oil to give light blue color

3.2.4.5 Compatibilities

All other excipients used in the experiments and formulation studies, proved to be compatible without noticeable reactions or precipitations at the concentrations used. The excipients selected after conclusion of compatibility studies were taken for the formulation studies.

5.2.5 Formulation

5.2.5.1 Development of the foam concentrate

Table A25, appendix section, gives the results of the foam concentrate development process. A narrative and a schematic presentation of the optimized development method E is given below and in figure 5.37 respectively.

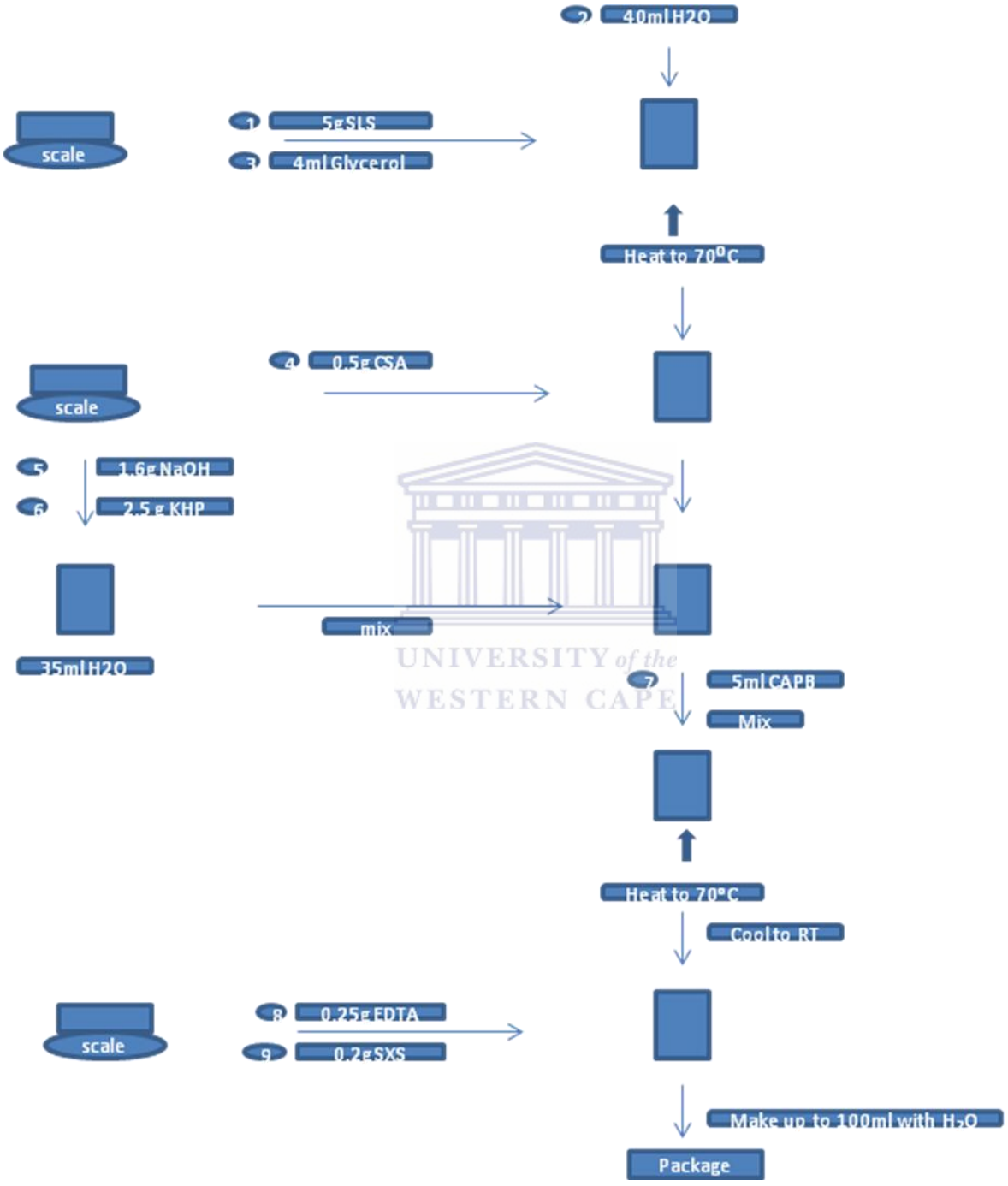
5.2.5.1.1 Preparation of the Buffer/adjuster solution

To 35 ml of 0.4 M NaOH, add 2.5 g of KHP and shake until completely dissolved. This is the NaOH/KHP buffer system. This solution was stored in the fume hood until required.

5.2.5.1.2 Preparation of foam concentrate

In a mixing vessel, 5 g of SLS was crushed to a powder, followed by addition of 40ml of distilled water and mixing by stirring. Add 5ml of glycerol and bring contents to 70 ^{o}C. Then add 0.5 g CSA with stirring to disperse the CSA. Cool the mixture to room temperature after which the NaOH/KHP buffer may be added followed by 5 ml of CAPB. Heat contents to 70 ^{o}C again, with stirring so as to dissolve contents. Cool and add 0.2 g of SXS and 0.25 g of EDTA. Make up to 100ml.

Figure 5.37: Schematic illustration of the preparation of the foam concentrate



5.2.5.2 Optimization of the quantities of NaOH and KHP (buffer system)

KHP was optimized as explained in section 5.1.3.4.2 and section 5.2.2.2. 2.5 % w/v was found to be appropriate level of KHP to be employed in this formulation. Table A27 in the appendix details the optimization of NaOH by means of six progressively prepared prototypes and was summarized to table 5.9 which is self-explanatory as shown below.

Table 5.9: Optimization of NaOH (summary of table A27)

Vol. of concentrate	**Prod. I**	**Prod. II**	**Prod.III**	**Prod. IV**	**Prod. V**	**Prod. VI**
	pH values Resulting from mixing 5 ml DMP + concentrate + water = 50 ml					
2.0	5.43	3.50				
2.5	6.10	4.22				
3.0		5.37				
4.0		6.44				
5.0	11.60	6.84	5.16	2.79	2.76	4.07
6.0			6.57			
7.5				3.43	3.41	4.98
8.0				3.65	3.85	5.30
9.0				3.87	3.92	
10.0				4.23	4.17	5.60
12.5					4.65	
15.0					4.88	

In figure 5.39 below, the pHs of the mixtures of table 5.9 were plotted against the volumes of concentrate.

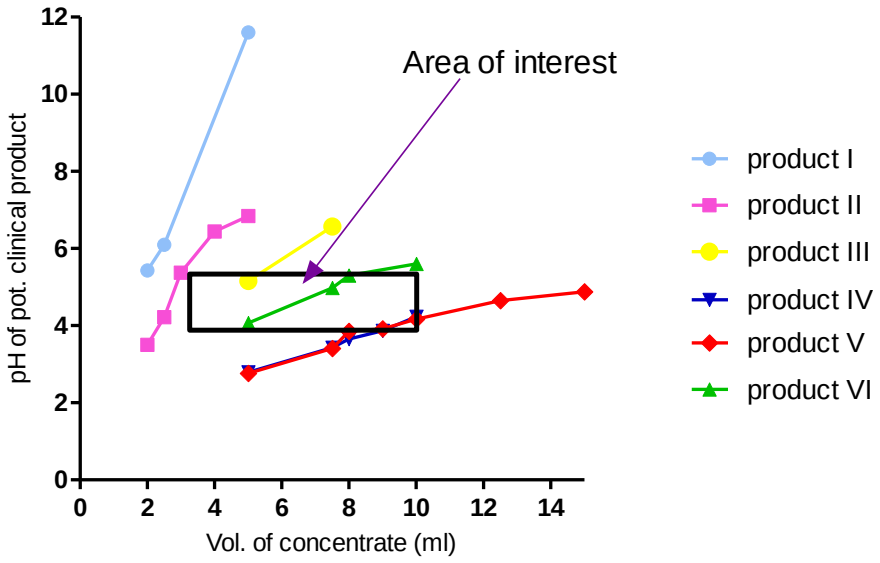


Figure 5.38: Plot of pH of the potential clinical product against volume of concentrate used

Prototype concentrate VI was found to have optimal level of NaOH at 1.6 % w/v as demonstrated in this figure.

5.2.5.3 Optimization of the quantities of the rest of excipients and selection of the lead formulation

From table A28, prototype formulation number 6 was taken and a master formula was drawn up as illustrated in table 5.10.

Table 5.10: Master formula of lead formulation

No.	Ingredient	Standard	Concentration
1	DMP		5 ppm
2	NaOH		1.6 % w/v
3	C8H5KO4 (KHP)		2.5 % w/v
4	SLS		5 % w/v
5	CAPB		5 % w/v
6	Glycerol		2 % w/v
7	EDTA		0.25 % w/v
8	CSA		0.5 % w/v
9	SXS		0.25 % w/v
10	H2O		≈ 80 % v/v
Additional ingredients added to mouth rinse concentrate only			
10	NaF		Adults = 1000 ppm Children = 500 ppm
11	Sucralose		2 – 10%
12	Monoammonium glycyrrhisinate		2 – 10%
13	peppermint		2.5%

The master formula was applicable to all three formulations namely, the VGD, F/SBB, and the MRF. The relative excipient quantities in the Master formula are graphically presented in figure 3.39 below for visual conceptualization.

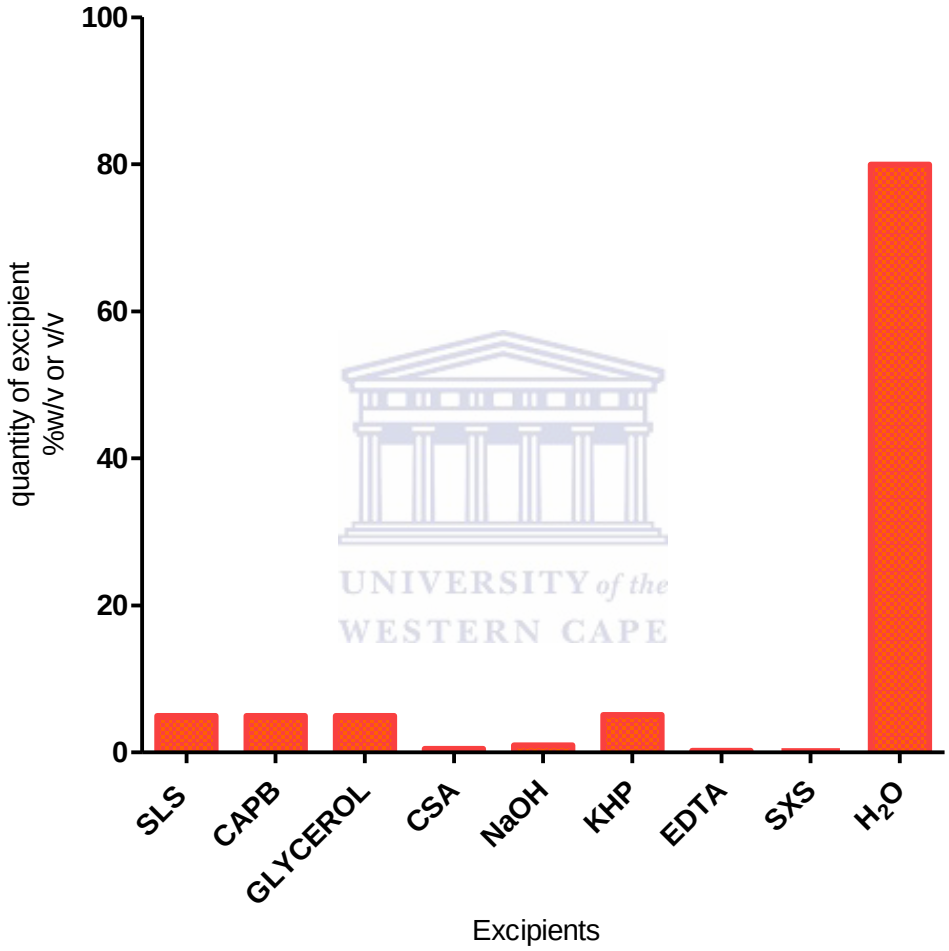


Figure 5.39: Graphical presetation of excipient quantities in the optimal formulation

The foam concentrate was clear and transparent for a day or so but then turned into a milky suspension. Most likely this signified emulsion formation. This change was undesirable

because the resulting solution was not elegant. Strategies to overcome emulsification could have been tried but for lack time.

5.2.5.4 Determination of the amount of DMP in the DMP/concentrate/water mixture

From reports in the literature the amount of DMP should be such that the final concentration does not exceed 5 ppm (Toxinet, 2004; US EPA, 1999). From the stability study results, the slopes of figure 4.1 give the rate of degradation of DMP. For DMP stored in amber colored containers in the fridge, calculations indicate that 166 ppm of CD is lost every month. Knowing the initial concentration of DMP and the rate of degradation, the concentration of DMP on a monthly basis could be predicted and from this a guide for the end user was derived that ensures that patient dosage does not exceed 5 ppm as shown in table 5.11 below. The best way to achieve 5 ppm of DMP per dose would be to first dilute DMP to 50 ppm. Then 5 ml (50 ppm) should be mixed with concentrate and water to achieve volume of 50 ml so that the final concentration of the product to be administered will always be constant at 5 ppm.

Table 5.11: Patient dosage guide for DMP

Month from date of manufacture	Conc. of DMP (ppm)	DMP:Water mixing ratio to achieve 50 ppm concentration (diluted DMP)	Concentration of DMP in: Conc./5 ml of 50ppm DMP/water = 50 ml (ppm)
Month 0	2900	1:57	5
Month 1	2734	1:54	5
Month 2	2568	1:50	5
Month 3	2402	1: 47	5
Month 4	2236	1:44	5
Month 5	2070	1:40	5
Month 6	1904	1:37	5
Month 7	1738	1:34	5
Month 8	1572	1:30	5
Month 9	1406	1:27	5

Month 10	1240	1:24	5
Month 11	1074	1:20	5
Month 12	908	1:17	5
Month 13	742	1:14	5
Month 14	576	1:10	5
Month 15	410	1:7	5
Month 16	244	1:4	5
Month 17	78	1:0.5	5

Note:

It was observed that the pH of DMP remained the same even after dilution with water to achieve 50 ppm.

5.2.5.5 Final product Dosage formula

Having determined the amount of DMP per dose of the final product as explained in section 5.2.5.4 above, the next step was to determine the amount of foam concentrate per dose.

5.2.5.5.1 Amount of concentrate in the DMP/concentrate/water = 50 ml product

It was found that when the foam concentrate volumes in excess of 10 ml were employed in the above mixtures, the resulting mixtures were too thick while foaming capacity was compromised when less than about 2 ml of concentrate was employed. Hence 2.5-10 ml was identified as the working volume range for the concentrate of which 5 ml was adopted as the actual working volume. With this information, the following final product dosage formulas were derived:

A) VDG and F/SBB

DF = 5 ml of 50 ppm DMP + 5 ml concentrate + 40 ml water = 50 ml product for application

Thus, to reconstitute the product for patient administration in the eighth (8) month of DMP storage for example, Dilute DMP as per Table 3.10 and mix according to proportions below:

DF = 5 ml of 1:30 mixture of DMP and water + 5 ml concentrate + 40 ml water = 50 ml product for application.

For other final working volumes, the volumes as stated in the DF can easily be adjusted by altering these amounts pro-rata to obtain the desired final volume.

B) MRF

For the MRF, the DMP concentration would have to be determined according to month of storage as above and then diluted to 50 ppm. 1 ml of this could then be added to 19 ml of diluted concentrate to come up with 2.5 ppm of final solution for application.

MDF = 19 ml of diluted concentrated + 1 ml of 50 ppm DMP = 20 ml product for application.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study was about exploring the possibility of employing the well known antimicrobial properties of Chlorine dioxide (CD) in the medical arena. The CD used comes as a South African brand of stabilized chlorine dioxide (SCD) known as dioxy MP 14 (DMP). The specific objectives of the study were: to evaluate the effectiveness of DMP as a tuberculosis (TB) disinfectant/cold sterilant for the possible use for floors, surfaces, and medical instruments and related devices.; to investigate the long term stability of DMP; and to formulate a vaginal douche (VGD), mouth rinse formulation (MRF), and foot/sit bubble bath (F/SBB) foam formulations. It was hypothesized that DMP could find a good niche in medical application as a biocide.

6.1.3 Evaluation of the disinfectant activity of DMP on TB

The test procedure employed was a modification the European quantitative suspension test, European Norm (EN) 14348, involving spectrophotometric evaluation of Mycobatericidal activity. *M. bovis BCG* (*Bacillus Calmette-Guérin*) was employed as surrogate for Mycobacteria tuberculosis (MTB). DMP was found to be more of a sterilizing biocide than disinfectant in that it eliminated all *Mycobacteria* from DMP concentrations of 2900 to 15.6 ppm. The minimum inhibitory concentration ($MIC_{90\ \%}$) of DMP was found to be 12.5 ppm, its

concentration x time ($CT_{90\,\%}$) value was found to be 12.5 pp.s and its mycobactericidal efficacy (ME) value was an 8.8 log reduction in *Mycobacteria* at concentrations of ≥ 15.6 ppm.

6.1.2 Long term stability study of DMP

This stability study highlighted the vulnerability of DMP to degradation by light particularly, but also to storage at higher temperatures. When stored in a fridge, DMP had an expiry date of about 70 weeks as opposed to 10 weeks when stored in a transparent bottle at room temperature and about 20 weeks if store at 40 ^{o}C. Degradation of DMP was found to follow zero order kinetics and the activation energy (Ea) of DMP was found to be 33.1 KJ/mol. Based on Arrhenius plot, the expiry date of DMP stored strictly at 25 ^{o}C was estimated to be 37.5weeks. The expiry date of DMP stored at 25 ^{o}C was predicted based on temperature coefficient (Q_{10}) estimation to be 17.5 weeks which is expected considering that Q_{10} prediction of expiry dates are very conservative for patient protection.

6.1.1 Formulation development

6.1.1.1 Preformulation studies

6.1.1.1.1 Foam evaluation

Of all the foam systems studied for incorporation into the above stated formulations, sodium lauryl sulphate/ ammonium lauryl sulphate/cocoamidopropyl betaine/cetostearyl alcohol (SLS/ALS/CAPB/CSA) scored the highest mark (93).

6.1.1.1.2 Selection of pH adjustor, buffer and the rest of the ingredients.

An adjuster solution and a buffer were needed to be incorporated in the concentrate for raising the pH of DMP to match that of the target sites and to maintain the pH at these target site levels, respectively. After appropriate potentiometric acid base titrimetric evaluations, sodium hydroxide (NaOH) was selected as the pH adjuster. Potassium hydrogen phthalate (KHP) was found to be the buffer of choice with a buffering profile that matched the target site pH values. The buffer capacities of 5 % w/v NaOH/0.1 % w/v KHP, 5 % w/v NaOH/1% w/v KHP and 5 % w/v NaOH/2 % w/v KHP were found to be 3.45X10^-4, 1.10X10^-3, and 3.80X10^-3 respectively, demonstrating increased buffer capacity with increasing concentration of KHP. The rest of the other ingredients were selected for their individual pharmaceutical properties.

6.1.1.1.3 Compatibility studies

Compatibility studies revealed that NaOH reacts with ammonium lauryl sulphate (ALS), while DMP reacts with anise oil, and cinnamon oil. The rest of the ingredients proved to be compatible with no noticeable reactions or precipitations at the concentrations used.

6.1.1.2 Formulation

The design of the product was such that the DMP and the foam concentrate would come in two separate primary containers to be mixed just prior to patient administration/application. Therefore formulation efforts were directed towards the foam concentrate

6.1.1.2.2 Optimization of excipient quantities

Initial excipient concentrations were derived from literature estimations. These were refined and optimized by developing six prototype formulations with progressively varied ingredient concentrations to finally arrive at an optimized formulation that met foam concentrate expectations.

6.1.1.2.3 Dosage formula (DF)

The dosage formula (DF) for the VGD and F/SBB was found to be:

MDF = 5 ml of 50 ppm DMP + 5 ml concentrate + 40 ml water = 50 ml product for application

This could be converted to any amount of product required for patient administration/application. The DF of the MRF is different and is shown below. The difference is due to the fact that the water aspect of the above MDF would have to be added at the point of manufacture, in the MRF, and not at the point of use.

DF of MRF = 19 ml of diluted concentrated + 1 ml of 50 ppm DMP

In summary, DMP was found to be a highly effective disinfectant against Mycobacteria. DMP also has a reasonable shelf life if appropriately stored. Formulating pharmaceutical preparations of DMP is feasible but delicate in that human target site pH ranges from about 3.5 to about 7 while the pH of DMP is 0.5. Adjusting the pH to target site ranges and buffering of DMP pharmaceutical preparations is therefore essential. beyond pH of about 5.2, DMP is

unstable and formulation is not feasible. Formulating below pH 3.5 compromises patient safety. Formulation is thus only possible between pH 3.5 and 5.2.

6.2 Recommendations

There is need for a further study to assess clinical safety of the above formulations especially as far as pH values of DMP/concentrate/water mixture are concerned and also to effectively assess effectiveness of bitter taste masking for the MRF. DMP has been found to be a highly effective mycobactericidal biocide. Studies to assess its compatibility with medical instruments and devices are also recommended as well as specific formulations for disinfecting floors and surfaces. Studies on the effectiveness of a DMP aerosol for disinfecting TB wards and homes of patients particularly the multidrug resistant (MDR) patients are also needed (Lin et al. 2007).

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APPENDIX

TABLE A1: *Mycobacterium* activity of DMP at various concentrations and exposure times

CONCENTRATION OF DMP USED = 2600PPM (9ML DMP + 0ML H_2O)			
EXPOSURE TIME	**EXP 1 - O.D.**	**EXP 2 – O.D.**	**EXP3 – O.D.**
30 S	**0.0079**	**-0.0004**	**0.0015**
1 MINUTE	**0.4377**	**0.0022**	**0.0028**
5 MINUTE	**0.0051**	**0.0001**	**0.0018**
10 MINUTE	**0.0059**	**-0.0007**	**0.0012**
20 MINUTE	**0.0062**	**-0.0012**	**0.0006**
-VE CONTROL	**0.0084**	**0.0034**	**0.0025**
+VE CONTROL	**0.4373**	**0.3955**	**0.4234**
CONCENTRATION OF DMP USED = 1300PPM (4.5ML DMP + 4.5ML H_2O)			
EXPOSURE TIME	**EXP 1 – O.D.**	**EXP 2 O.D.**	**EXP 3 O.D.**
30 S	**-0.0038**	**0.0006**	**0.0002**
1 MINUTE	**0.0038**	**0.0011**	**0.0012**
5 MINUTE	**0.0029**	**-0.0015**	**0.0002**
10 MINUTE	**0.0046**	**-0.0008**	**0.0005**
20 MINUTE	**0.0038**	**0.0012**	**-0.0022**
-VE CONTROL	**0.0027**	**0.0010**	**0.0015**
+VE CONTROL	**0.3912**	**0.4106**	**0.3645**
CONCENTRATION OF DMP USED = 650PPM (2.25ML DMP + 6.75ML H_2O)			
EXPOSURE TIME	**EXP 1 – O.D.**	**EXP 2 – O.D.**	**EXP3 – O.D.**
30 S	**0.0011**	**-0.0004**	**0.0008**
1 MINUTE	**0.0013**	**0.0012**	**0.0012**
5 MINUTE	**0.0025**	**0.0007**	**0.0020**
10 MINUTE	**0.0018**	**0.0017**	**0.0016**
20 MINUTE	**0.0005**	**0.0015**	**0.0008**
-VE CONTROL	**0.0013**	**0.0022**	**0.0022**
+VE CONTROL	**0.3640**	**0.3815**	**0.6112**
CONCENTRATION OF DMP USED = 325PPM (1.125ML DMP + 7.875ML H_2O)			
EXPOSURE TIME	**EXP 1 – O.D.**	**EXP 2 – O.D.**	**EXP 3 – O.D.**
30 S	**-0.0013**	**0.0008**	**-0.0002**
1 MINUTE	**-0.0010**	**-0.0005**	**-0.0005**
5 MINUTE	**0.0006**	**0.0012**	**-0.0014**
10 MINUTE	**-0.0019**	**0.0015**	**-0.0008**
20 MINUTE	**-0.0018**	**0.0017**	**0.0003**

-Ve control	**-0.0015**	**0.0014**	**0.0010**
+Ve control	**0.3761**	**0.3657**	**0.5954**
CONCENTRATION OF DMP USED = 162.5 PPM (0.56 ML DMP + 8.44 ML H_2O)			
EXPOSURE TIME			
30S	**EXP 1 – O.D**	**EXP 2- O.D.**	**EXP 3 – O.D.**
1 Minute	**-0.0011**	**0.0003**	**-0.0007**
5 Minute	**-0.0015**	**-0.0014**	**-0.0002**
10 Minute	**-0.0017**	**-0.0008**	**-0.0016**
20 Minute	**0.0016**	**-0.0005**	**-0.0010**
-Ve control	**0.0038**	**-0.0010**	**-0.0010**
+Ve control	**0.4779**	**0.4372**	**0.4675**

The above results show that exposure at 30 s is enough to effectively kill bacteria and there is consistency in killing at higher exposure times. Therefore the rest of the study, which was meant to accurately determine the MIC, was restricted to the 30 s exposure to.

Table A2: Mycobactericidal activity of DMP at various concentrations and a fixed exposure time of 30 s

30S EXPOSURE TIME				
DISINFECTANT (DMP + H2O = 9 ML)	**DMP CONC (PPM)**	**EXP 1 - O.D.**	**EXP 2 – O.D.**	**EXP 3 – O.D.**
1.0 ML + 8.0 ML	**28.9**	**-0.0006**	**-0.0014**	**-0.0011**
0.7 ML + 8.3 ML	**20.2**	**-0.0014**	**-0.0005**	**0.0008**
0.5 ML + 8.5 ML	**14.4**	**-0.0003**	**0.0006**	**0.0006**
0.4 ML + 8.6 ML	**11.5**	**0.1102**	**0.0355**	**0.2053**
0.3 ML + 8.7 ML	**8.7**	**0.3421**	**0.3087**	**0.3536**
0.2 ML + 8.8 ML	**5.7**	**0.3132**	**0.3542**	**0.3652**
0.1 ML + 8.9 ML	**3.0**	**0.4707**	**0.4812**	**0.4797**

Table A3: Changes in concentration of CD with time in the DMP solutions

STORAGE CONDITIONS	**ROOM TEMPERATURE (20 – 30 ^{O}C)**		**OVEN (40 ^{O}C)**		**FRIDGE (2 – 8 ^{O}C)**	
TIME (WEEK)	**Transparent bottle**	**Amber bottle**	**Transparent bottle**	**Amber bottle**	**Transparent bottle**	**Amber**
0.	2900.	2900.	2900.	2900.	2900.	2900.
1.	2361.	2765.	2563.	2496.	2900.	2900.
2.	2079.	2765.	2428.	2293.	2765.	2765.
3.	1754.	2698.	2293.	2158.	2765.	2765.
4.	1349.	2496.	2158.	1889.	2765.	2698.
5.	1214.	2496.	2079.	1754.	2698.	2698.
6.	944.	2361.	1889.	1551.	2698.	2698.
7.	674.	2361.	1686.	1349.	2563.	2563.
8.	472.	2293.	1484.	1214.	2563.	2563.
9.	207.	2293.	1349.	1079.	2496.	2563.
10.	0.	2158.	1214.	809.	2496.	2428.
11.		2158.	1147.	674.	2428.	2428.

12.		2079.	944.	472.	2428.	2428.
13.		2079.	805.	337.	2361.	2361.
14.		1889.	674.	135.	2361.	2361.
15.		1889.	540.	0.	2293.	2361.
16.		1889.	472.		2293.	2293.
17.		1754.	337.		2158.	2293.
18.		1686.	205.		2158.	2158.
19.		1686.	67.4		2158.	2158.
20.		1686.	0.		2079.	2079.
21.		1551.			2079.	2079.
22.		1551.			1889.	2079.
23.		1551.			1889.	1889.
24.		1349.			1889.	1889.
25.		1349.			1754.	1889.
26.		1349.			1754.	1889.
27.		1214.			1686.	1754.
28.		1214.			1686.	1754.
29.		1214.			1551.	1686.

30.		1079.			1551.	1686.
31.		1079.			1484.	1686.
32.		1079.			1484.	1551.
33.		944.			1349.	1551.
34.		944.			1349.	1484.
35.		809.			1349.	1484.
36.		809.			1349.	1349.
37.		742.			1214.	1349.
38.		742.			1214.	1349.
39.		674.			1214.	1349.
40.		674.			1079.	1214.
41.		674.			1079.	1214.
42.		540.			1012.	1214.
43.		540.			944.	1012.
44.		405.			944.	1012.
52.		135.			674.	742.
53.		0.			674.	742.

Table A4: The target product profile for VGD, MRF, and F/SBB DMP formulations

A	TPP GENERAL STATEMENT	
i	Project name	PHARMACEUTICAL FORMULATION BASED ON DIOXY MP 14 - mouth rinse, vaginal douche and foot bubble/ sit bath
ii	Project category	Formulation of an existing drug with well studied biocide properties, but having unique challenges in terms of formulation
iii	Value to patients	Powerful oxidizing antimicrobial agent, broad spectrum of action, low probability of developing resistance
iv	Rationale for success	The stability study has shown that under ideal storage conditions, DMP can remain in effective concentrations for over a year. This proves that a reasonably stable product is assured. Furthermore, pH of DMP can be adjusted to about 5.2 without encountering stability problems. This is compatible to target site pH requirements for all the planned formulations. The chosen dosage form, which is foam formulation, is proven to have good drug delivery properties and could even be more ideal for the gaseous CD.
v	Factors for success	- Huge market for a multipurpose antiseptic drug e.g. the problem of athlete's foot among miners, footballers; multiple vaginal infections in the immune compromised, diabetics etc. - Powerful well studied biocidal properties of CD - Lower likelihood of emergence of resistance as compared to other drugs.
vi	Risk factors	- The extremely low pH of DMP is not amenable to target site application. - Upon adjustment of pH, stability problems of the DMP arise above pH 5.2
B	FORMULATION GOALS	
The intention is to develop a product that is:		- Scalable and manufacturable, - Chemically and physically stable throughout product shelf life

		- Bio-available i.e. must contain required amount of API in each dose that must reach target site - Has Excipients that are Generally regarded as safe (GRAS) and convincing rationale for inclusion of each - Meet quality standards to ensure efficacy and safety - Capable of achieving regulatory compliance		
C	CLINICAL TPP ATTRIBUTES			
	PRODUCT ATTRIBUTES	WANTS	MUST	ANNOTATION
I	Drug related			
i	Indication	Treatment and prophylaxis of:	Treatment of:	
a.	MRF	- All bacterial, fungal, viral infections of the oral cavity	- All bacterial, fungal, viral infections of the mouth cavity	{{261 Novak, J. 2008; 262 Ogata, 2008}}
b.	VGD	- All bacterial, fungal, viral and parasitic infections of the vagina and vulva	- All bacterial, fungal, viral and parasitic infections of the vagina and vulva	
c.	F/SBB	- All bacterial, fungal, viral infections afflicting the skin and those related to hemorrhoids.	- All bacterial, fungal, viral infections afflicting the skin and those related to	

			hemorrhoids	
ii	Route of administration	Oral, P.V. topical for each of the above formulations respectively	Oral, P.V. topical for each of the above formulations respectively	
iii	Dosage range			
a.	MRF	0.1 to 5 ppm		{{28 US EPA 1999}}
b.	VGD	0.1 to 5 ppm		
c.	F/SBB bath	0.1 to 5 ppm		
iv	Dosage frequency	At least BD	Not less than BD	Not studied
v	Expected duration of treatment	Till remission of symptoms; stop after 1 wk if no response	Not more than I month of continuous use	Not studied
vi	Volume per dose			Convenient volumes
a.	Mouth rinse	20 – 40 ml	20 ml	
b.	VGD	100 – 500 ml	200 ml	
c.	FBB/SB	2 – 5 liters	FBB = 5 L; SB = 5 L	
II	Product related			
i	pH			
a.	MRF	pH 4.5 – 5.2	pH 4.5	Refer to literature review
b.	VGD	pH 3.8 – 4.5	pH 4.5	
c.	FBB/SB	pH 4.5 - 5.2	pH 5.0	
ii	Excipients compedial	Yes	Yes	literature review
iii	**DILUTIONS**			
a.	Mouth rinse	DMP + diluted concentrate	DMP + diluted concentrate	Refer to section 5.2.5.5
b.	VGD	DMP + concentrate + water	DMP + concentrate + water	
c.	FBB/SB	DMP + concentrate + water	DMP + concentrate + water	

vi	Storage conditions	DMP: - Fridge 2 – 8 ^{o}C	<10 ^{o}C	Refer to Stability study
vii	Shelf life	DMP: 1 – 1.5 years Concentrate: no studies done	1 year No studies done	Stability study
viii	caution		Concentrate and DMP can cause skin burns; Not for internal use	NaOH/KHP Buffer study
III	Legal related			
i	Freedom to operate	yes	yes	Lit review
ii	Product intellectual property	Formula/process could be patented if possible		
IV	Laboratory related			
a.	Cost of ingredients	Only affordable ingredients used	affordability	Price lists
b.	Equipment needed	Common laboratory apparatus	available	Lab stock list
c	Duration of project	1 – 2 years	3 years	

Table A5: Foamability profiles of various FA/FAC

CONC.	MP9			SLS			SLS/ALS			CAPB			SLS/ALS/CAPB			ALS			NEO DAL			TWEEN 20			SLS/ALS/SNLSS		
0.05	748.	750.	752.	1057.	1063.	1060.	1115.	1115.	1115.	950.	951.	949.	1138.	1138.	1144.	1008.	1012.	1009.	548.	549.	551.						
0.10	1000.	1000.	1000.	1142.	1141.	1139.	1181.	1178.	1181.	1098.	1099.	1102.	1200.	1200.	1200.	1119.	1120.	1120.	640.	640.	640.	670.	670.	670.	549.	550.	550.
0.50	1092.	1089.	1088.	1230.	1230.	1230.	1250.	1250.	1250.	1160.	1158.	1162.	1272.	1268.	1268.	1218.	1218.	1223.	758.	761.	762.	719.	718.	722.	1000.	1000.	1000.
1.00	1120.	1120.	1119.	1260.	1260.	1260.	1310.	1310.	1310.	1195.	1194.	1197.	1310.	1310.	1310.	1250.	1250.	1250.	830.	830.	830.	900.	900.	900.	1100.	1098.	1101.
2.00							1340.	1338.	1342.	1200.	1200.	1200.	1340.	1340.	1340.										1132.	1131.	1128.

FOAM DECAY/DRAINAGE AGAINST TIME - TABLES

Table A6: CAPB and CAPB/CSA at pH 4.0

TIME (MIN)	FOAM COLLAPSE		FOAM DRAINAGE -	
	CAPB	**CAPB/CSA**	**CAPB**	**CAPB/CSA**
0.	**100.**	**100.**	**0.0**	**0.0**
10.		**100.**		**15.0**
20.	**99.**		**18.0**	
30.		**99.**		**15.2**
40.	**98.**		**18.8**	
60.	**98.**		**18.9**	
70.		**80.**		**15.6**
80.	**60.**		**19.0**	
90.		**40.**		**15.8**
100.	**35.**		**19.0**	
110.		**25.**		**15.9**
120.	**30.**		**19.0**	

Table A7: TWEEN-20 and TWEEN-20/CSA at pH 4.

TIME (MIN)	FOAM COLLAPSE		FOAM DRAINAGE	
	TWEEN-20	**TWEEN-20/CSA**	**TWEEN-20**	**TWEEN-20/CSA**
0.	**100.**	**100.**	**0.0**	**0.0**
10.	**60.**		**17.8**	
20.	**25.**	**100.**	**18.0**	**14.9**
30.	**22.**		**18.2**	
80.		**100.**		**16.9**
120.		**100.**		**16.9**
160.		**100.**		**16.9**
200.		**99.**		**16.9**

Table A8: SLS and SLS/CSA at pH 4.0

TIME (MIN)	FOAM COLLAPSE		DRAINAGE	
	SLS	SLS/CSA	SLS	SLS/CSA
0.	100.	100.	0.0	0.0
30.	98.		20.6	
40.		97.		14.0
50.	88.		20.9	
70.	62.		20.9	
80.		96.		14.5
110.	45.		21.0	
120.		95.		14.6
130.	35.		21.0	
160.		94.		14.6
170.	33.		21.0	
200.		94.		

Table A9: ALS and ALS/CSA at pH 4.0

TIME (MIN)	FOAM COLLAPSE		FOAM DRAINAGE	
	ALS		ALS/CSA	
0.	100.	100.	0.0	0.0
20.	98.	99.	18.5	20.9
40.	97.	98.	19.0	21.2
60.	97.	96.	19.1	21.6
80.	90.	90.	19.1	21.7
100.	86.	85.	19.3	21.8
120.	82.	84.	19.5	21.8
160.	80.	72.	19.5	21.8
180.	76.	64.	19.6	21.9
200.	76.	60.	19.6	22.0
220.	71.	55.	19.7	22.0
240.	70.	54.	19.7	22.0
260.	70.	50.	19.7	22.0
280.	70.	46.	19.7	22.0
300.	70.		19.7	
320.	70.		19.7	

Table A10: SLS/ALSCAPB and SLS/ALS/CAPB/CSA at pH 4.0

TIME (MIN)	FOAM COLLAPSE		FOAM DRAINAGE	
	SLS/ALS/CAPB	SLS/ALS/CAPB/CSA	SLS/ALS/CAPB	SLS/ALS/CAPB/CSA
0.	100.	100.	0.0	0.0
20.	100.	100.	21.0	31.8
40.	99.		27.0	
60.	98.	98.	28.2	33.8
80.	98.		28.9	
100.		98.		34.0
110.	98.		29.1	
140.		97.		34.4
150.	98.		29.6	
180.		96.		34.6
190.	98.		29.8	
220.		94.		34.7
230.	97.		29.8	
260.		93.		34.8
270.	96.		29.9	

Table A11: SLS/ALS and SLS/ALS/CSA at pH 4.0

TIME (MIN)	FOAM COLLAPSE		FOAM DRAINAGE	
	SLS/ALS	SLS/ALS/CSA	SLS/ALS	SLS/ALS/CSA
0.	100.	100.	0.0	0.0
20.	98.	98.	20.8	16.0
40.	97.	97.	21.2	16.5
60.	96.		21.2	
80.		97.		17.0
100.	94.		21.3	
120.		96.		17.0
140.	82.		21.3	
160.		96.		17.1
180.	71.		21.4	
200.		95.		17.1
220.	70.		21.5	
240.		95.		17.1
260.	70.		21.5	

Table A12: SNLSS and SNLSS/CSA at pH 5.5

TIME (MIN)	FOAM COLLAPSE		FOAM DRAINAGE	
	SNLSS	SNLSS/CSA	SNLSS	SNLSS/CSA
0.	100.0	100.	0.0	0.0
20.	98.0	98.	17.8	14.6
40.	39.1	96.	18.6	14.9
60.	28.0	96.	18.8	15.2
80.	26.0	95.	18.9	15.4
100.	25.0	94.	18.9	15.5
140.	22.0	94.	18.9	15.6
180.		94.		15.6
220.		94.		15.7
260.				

Table A13: MP 9 and MP9/CSA at pH 4.0

TIME (MIN)	MP 9	MP 9/CSA	MP 9 FOAM DRAINGE	MP 9/CSA FOAM DRAINAGE
0.	100.	100.	0.0	0.0
20.	65.	25.	16.3	18.2
40.	31.	24.	16.8	18.2
60.				
80.	28.	24.	16.9	18.2
100.	26.	24.	16.9	18.2

Table A14: SLS/ALS/CAPB and SLS/ALS/CAPB/CSA

TIME (MIN)	FOAM COLLAPSE -		FOAM DRAINAGE	
	SLS/ALS/CAPB	SLS/ALS/CAPB/CSA	SLS/ALS/CAPB	SLS/ALS/CAPB/CSA
0.	100.	100.	0.0	0.0
20.	100.	100.	21.0	31.8
40.	99.		27.0	
60.	98.	98.	28.2	33.8
80.	98.		28.9	
100.		98.		34.0
110.	98.		29.1	
140.		97.		34.4
150.	98.		29.6	
180.		96.		34.6
190.	98.		29.8	
220.		94.		34.7
230.	97.		29.8	
260.		93.		34.8
270.	96.		29.9	

Table A15: ALS and ALS/CSA at pH 5.5

TIME (MIN)	FOAM COLLAPSE		FOAM DRAINAGE	
	ALS	ALS/CSA	ALS	ALS/CSA
0.	100.	100.	0.0	0.0
40.	98.	98.	21.0	18.0
80.	89.	97.	21.5	18.4
120.	78.	92.	21.8	18.4
160.	67.	90.	21.8	18.5
200.	60.	85.	21.9	18.5
240.	58.	84.	21.9	18.6
280.	58.	78.	21.9	18.6
320.	58.		21.9	

Table A16: SLS and SLS at pH 5.5

TIME (MIN)	FOAM COLLAPSE		FOAM DRAINAGE	
	SLS	SLS/CSA	SLS	SLS/CSA
0.	100.	100.	0.0	0.0
40.	96.	98.	18.2	17.1
80.	82.	96.	18.5	17.5
120.	62.	93.	18.6	17.9
160.	52.	90.	18.8	17.9
200.	20.	85.	18.8	17.9
240.		83.		17.9
280.		81.		17.9
320.		78.		17.9
		78.		17.9

Table A17: Titration OF 100 ml DMP against 5 % w/v NaOH

Vol. of buffer (ml)	pH of DMP		
	1st trial	2nd trial	3rd trial
0.	0.94	0.87	0.83
1.	0.95	0.89	0.87
2.	1.01	0.99	0.96
3.	1.17	1.32	1.26
4.	4.71	4.79	4.70
5.	4.88	5.17	4.98
6.	12.02	12.10	11.54
7.	12.25	12.37	12.30
8.	12.42	12.47	12.46
9.	12.48	12.49	12.51
10.	12.50	12.49	12.52

Table A18: Titration of 5 % w/v Na_2CO_3.1/2 H_2O_2 against 100 ml DMP

Vol. of $Na_2CO_3.1.5H_2O_2$ (ml)	pH of DMP		
	1st trial	2nd trail	3rd trial
0.	0.98	0.90	0.91
5.	0.98	0.91	0.91
10.	1.06	1.01	1.02
15.	1.27	1.34	1.31
20.	3.85	4.53	4.46
25.	4.63	4.67	4.62
30.	5.01	5.52	5.44
35.	6.14	6.82	6.80
40.	8.05	8.86	8.78
45.	9.04	9.29	9.21
50.	9.32	9.50	9.44
55.	9.53	9.63	9.58
60.	9.62	9.73	9.64

Table A19: Titration of 5 % w/v NaOH/0.1 % w/v KHP against 100 ml of DMP

Vol. of buffer (ml)	pH of DMP		
	1 st trial	2nd trial	3rd trial
0.	0.90	0.500	0.88
1.	0.91	0.660	0.92
2.	1.04	0.850	1.08
3.	1.41	1.410	1.40
4.	5.00	5.060	5.02
5.	5.40	6.170	5.60
6.	11.71	11.920	11.90
7.	12.36	12.360	12.37
8.	12.48	12.460	12.46
9.	12.48	12.500	12.48
10.	12.50	12.520	12.50

Table A20: Titration of 5 % w/v NaOH/1 % w/v KHP against 100 ml of DMP

Vol. of buffer (ml)	pH of DMP		
	1 st trial	2nd trial	3rd trial
0.	0.98	0.83	0.89
1.	1.08	0.89	0.91
2.	1.17	1.04	1.03
3.	1.45	1.58	1.40
4.	2.38	2.74	2.75
5.	3.69	4.93	4.38
6.	5.39	5.74	5.58
7.	6.08	6.71	6.40
8.	7.24	10.52	8.86
9.	11.80	12.17	12.04
10.	12.35	12.38	12.35
11.	12.51	12.50	12.50
12.	12.58	12.55	12.56

Table A21: Titration of 5 % w/v NaOH/2 % w/v KHP against 100 ml of DMP

Vol. of buffer (ml)	pH 0f DMP		
	1 st trial	2nd trial	3rd trial
0.0	0.95	0.63	0.90
3.0	0.99	0.69	0.96
6.0	1.38	1.32	1.12
9.0	2.73	2.43	2.32
12.0	3.48	3.31	3.65
15.0	5.05	4.67	4.53
18.0	6.06	5.90	5.96
21.0	6.65	6.45	6.59
24.0	7.53	7.17	7.25
27.0	12.01	11.86	12.02
30.0	12.35	12.14	12.18
33.0	12.56	12.61	12.42

Table A22: Titration of 0.25 % w/v NaOH/2.5 % w/v KHP against 100ml DMP

Vol. of buffer (ml)	pH of DMP		
	1 st trial	2 nd trial	3 rd trial
0.	0.61	0.64	0.60
10.	0.76	0.92	0.80
20.	0.82	0.93	0.85
30.	0.94	1.02	1.00
40.	1.13	1.17	1.15
50.	1.41	1.42	1.42
60.	1.87	1.85	1.86
70.	2.37	2.33	2.35
80.	2.66	2.63	2.67
90.	2.83	2.82	2.84

Table A23: Initial list of excipient

No.	Functional category	Possible excipients
1	Foaming agents	SLS, ALS, CAPB (selected from pre-formulation studies)
2	Foam stabilizers	CSA, Coco amide DEA, Coco amide MEA
2	Buffer systems	KHP/NaOH, citric acid/sodium citrate
3	Moisturizer/humectants	Glycerin, urea, propylene glycol
4	Metal chelating agents	EDTA
5	hydro trope	SXS
6	sweeteners	Sucrose, sucralose, xylitol, monoammonium glycyrrhisinate
7	Flavourants	Anise oil, cinnamon oil, peppermint oil
8	Prevent tooth decay	NaF, SnF_2, Na_2PO_3F
9	Thickeners	NaCl, CAPB, PVP

Table A24: Final List of excipients

No.	Functional category	Final excipient choice
1	Foaming agents	SLS, ALS, CAPB
2	Foam stabilizers	CSA
2	Buffer systems	KHP/NaOH
3	Moisturizer/humectants	Glycerin
4	Metal chelating agents	EDTA
5	hydro trope	SXS
6	sweeteners	sucralose, monoammonium glycyrrhisinate
7	Flavourant	peppermint oil
8	Prevent tooth decay	NaF
9	Thickeners	CAPB

Table A25: Development of foam concentrate

Method	procedure	Comments	Technique to overcome challenge challenges
A	To 50 ml distilled water in the mixing vessel, add all the other ingredients with stirring. Make up to 100 ml	Phase separation, lack of uniformity, lumpy, possible chem. reaction	Prepare buffer system separate from other ingredients (OI)
B	Prepare NaOH/KHP buffer system (35 ml) separately. Then, to 40ml of distilled water, add the rest of the ingredients with stirring. Add the two systems together and make up to 100 ml	Phase separation, lack of uniformity, lumpy	Add OI preparation systematically starting with the water soluble ones first
C	Prepare NaOH/KHP buffer system (35 ml) separately, Then, to 40 ml of distilled water, add all water soluble ingredients with stirring. Mix the two systems together with stirring. Finally add the water insoluble ingredients.	Phase separation, lack of uniformity upon addition of buffer system	Heat to 70 ^{O}C to disperse water insoluble e.g CSA and again after addition of buffer system
D	Prepare NaOH/KHP buffer system (35 ml) separately, Then, to 5 g of SLS in another beaker, add 40 ml of distilled water followed by 1 ml of CAPB. Mix thoroughly followed by addition of glycerol. Heat to 70^{O}C and add by CSA. Mix the two systems together with stirring. Next Add with stirring followed by heating. After cooling, finally add SXS, EDTA and make up to 100ml with water.	Phase separation concentrate initially but (aging problems)	Add sufficient thickener (CAPB chosen because it is also a foaming agent)
E	The same as method D but with 5 ml CAPB	turns milky after a day	to be done

		of storage	
Special case of the Mouth rinse			
F	Dilute concentrate E above with distilled water to levels appropriate for a ready to use concentrate solution	Bitter taste, poor odor	Mask taste and flavor

Table A26: initial excipient quantities used during the development of the foam concentrate

No.	Ingredient	Concentration
1	NaOH	6 % w/v
2	KHP	2.5 % w/v
2	SLS	3 % w/v
3	CAPB	3 % v/v
4	Glycerol	2 % v/v
5	EDTA	0.1 % w/v
6	CSA	0.2 % w/v
7	SXS	0.2 % w/v
8	NAF	500 ppm
9	Sucralose	2 % w/v
10	Monoammonium glycyrrhisinate	2 % w/v
11	Peppermint	2 % v/v
12	Distilled water	To finally make up to 100 ml

Table A27: Optimization of the levels NaOH in the concentrate

Product I				
Conc. of NaOH = 6 % w/v			**pH of concentrate = 12.85**	
Conc. of other excipients as in Table A6			**pH of DMP = 0.5**	
Concentrate Vol. used (ml)	**Vol. of DMP used (ml)**	**Vol. of H_2O used (ml)**	**Total vol. used (ml)**	**pH of prod. I**
2	5	43	50	5.43
2.5	5	42.5	50	6.10
5	5	40	50	11.60
Product II				
Conc. of NaOH = 4 % w/v			**pH of concentrate = 12.23**	
Conc. of other excipients as in Table A6			**pH of DMP = 0.5**	
Concentrate vol. used (ml)	**Vol. of DMP used (ml)**	**Vol. of H_2O used (ml)**	**Total vol. (ml)**	**pH of prod. I**
5	5	40	50	6.84
4	5	41	50	6.44
3	5	42	50	5.37

2.5	5	42.5	50	4.22
2	5	43	50	3.5
Product III **Conc. of NaOH = 2 % w/v** **pH of concentrate = 13.2** **Conc. of other excipients as in Table A6** **pH of DMP = 0.5**				
concentrate Vol. used (ml)	**Vol. of DMP used (ml)**	**Vol. of H_2O used (ml)**	**Total vol. (ml)**	**pH of prod. I**
5	5	40	50	5.16
6	4	40	50	6.57
Product IV **Conc. of NaOH = 1.2 % w/v** **pH of concentrate = 12.25** **Conc. of other excipients as in Table A6** **pH of DMP = 0.5**				
concentrate Vol. used (ml)	**Vol. of DMP used (ml)**	**Vol of H_2O used (ml)**	**Total vol. (ml)**	**pH of prod. I**
5	5	40	50	2.79
7.5	5	37.5	50	3.43

8	5	37	50	3.65
9	5	36	50	3.87
10	5	35	50	4.23
Product V **Conc. of NaOH = 1 % w/v** **pH of concentrate = 12.90** **Conc. of other excipients as in Table A6** **pH of DMP = 0.5**				
concentrate Vol. used (ml)	**Vol. of DMP used (ml)**	**Vol. of H_2O used (ml)**	**Total vol. (ml)**	**pH of prod. I**
5	5	40	50	2.76
7.5	5	37.5	50	3.41
8	5	37	50	3.85
9	5	36	50	3.92
10	5	35	50	4.17
12.5	5	33.5	50	4.65
15	5	30	50	4.88
Product VI				

Conc. of NaOH = 1.6 % w/v			**pH of concentrate = 12.58**	
Conc. of other excipients as in Table A6			**pH of DMP = 0.5**	
concentrate Vol. used (ml)	**Vol. of DMP used (ml)**	**Vol of H_2O used (ml)**	**Total vol. (ml)**	**pH of prod. I**
5	5	40	50	4.07
7.5	5	37.5	50	4.98
8.0	5	37	50	5.3
10	5	35	50	5.6

Table A28: Optimization of the quantities of the rest of excipients using Prototype concentrates

Ingredient	**Prototype formulation 1**	**Prototype formulation 2**	**Prototype formulation 3**	**Prototype formulation 4**	**Prototype formulation 5**	**Prototype formulation 6**
	Concentration (% v/v or % w/v unless specified)					
SLS	10	7.5	2	4	3	5
CAPB	10	7.5	2	4	3	5
Glycerol	2	4	8	6	7	5
CSA	0.5	0.5	0.5	0.5	0.5	0.5
NAOH	1.6	1.6	1.6	1.6	1.6	1.6
KHP	2.5	2.5	2.5	2.5	2.5	2.5
EDTA	0.25	0.25	0.25	0.25	0.25	0.25
SXS	0.2	0.2	0.2	0.2	0.2	0.2
H_2O	67.05	73.55	80.35	79.55	79.95	80.05
comments	Too thick (paste) Glycerol not	Too thick	Too much glycerol. Foaming	Not that bad	Too much glycerol. Foaming	optimal

	enough		compromised a bit.		compromised a bit.	
Special case of mouth rinse (dilute the above **optimized concentrate 6** with distilled water in a 1:4, then add the ingredients below)						
NaF					Adult = 1000 ppm Children = 500 ppm	
sucralose					5 - 10	
Monoamm-onium glycyrrhizi-nate					5 - 10	
peppermint					0.5	