In situ forming hydrogels for drug delivery to the oral mucosa

Venu Gopal Reddy Patlolla

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Supervisor:
Prof. Dr. Þórdís Kristmundsdóttir

UNIVERSITY OF ICELAND
SCHOOL OF HEALTH SCIENCES
FACULTY OF PHARMACEUTICAL SCIENCES

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LIST OF ABBREVIATIONS

HPMC	Hydroxypropyl methylcellulose
CMC	Carboxymethyl cellulose
PEG:	Polyethylene glycol
PVA:	Polyvinyl alcohol
HPβCD:	2-Hydroxypropyl-β-cyclodextrin
SBEβCD:	Sulfobutylether-β-cyclodextrin
DSC:	Differential scanning calorimetry
SD:	Standard deviation
AUC:	Area under Curve
PEO:	Polyethylene oxide
PPO:	Polypropylene oxide
HLB:	Hydrophilic-lipophilic balance
EGF:	Epidermal growth factor
BMP:	Bone morphogenic protein
FGF:	Fibrogenic growth factor
ECGF:	Endothelial cell growth factor
RAS:	Recurrent aphthous stomatitis
RAU:	Recurrent apththous ulceration
HSV:	Herpes Simplex virus
HSPs:	Heat shock proteins
MMPs:	Matrix metalloproteinases/ matrixins

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ABSTRACT

Background: Recurrent aphthous stomatitis (RAS) is a painful condition affecting 5-25 % of the general population. RAS may cause pain during eating, swallowing and talking, which in extreme cases can contribute to weight loss and thereby reducing the overall quality of life of patients. There are a few treatments available on the market today, of which Amlexanox 5 % is the most widely used treatment. A clinical trial showed that Amlexanox 5 % reduced the pain of ulcers by day 6 in majority of patients with minor RAS. Minor RAS can heal naturally in 7-14 days without leaving any scars. In an earlier clinical trial, which was based on treating the RAS by inhibiting the matrixmetalloproteinases (MMPs) with sub-antimicrobial dose of topical doxycycline containing gel, it was shown that ulcers healed completely by day 3 in majority of patients. This could be the novel treatment for treating RAS, as it has shown rapid healing time compared to Amlexanox. But the challenge here is that doxycycline is an unstable compound, it degrades rapidly in aqueous solutions and non-aqueous solvents.

Aim: The main aim of this work was to formulate an *in situ* forming hydrogel containing a sub-antimicrobial dose of doxycycline that gels at physiological conditions with primary emphasis on increasing the stability of doxycycline in aqueous formulations. Also the *in situ* hydrogels should, upon instillation onto the oral cavity, adhere to the oral mucosa with sufficient strength. For this purpose suitable mucoadhesive polymers were added to the formulations after *in vitro* mucoadhesion analysis tests. The viscosities of the formulations were analysed and priority was given to maintaining low viscosities at room temperature. Additionally the formulation release behaviour was studied with polymer non-membrane method.

Methods: A total of 40 *in situ* forming hydrogels were prepared and stability tests were carried out over 3 months, in some cases up to 23 months at 4 °C, 25 °C and 40 °C. The stability of doxycycline was analysed by HPLC. The mucoadhesive polymers were chosen after testing the mucoadhesive strengths of 14 different *in situ* hydrogels with varying concentrations and combinations of polymers using a Texture Analyser. The viscosity tests were carried out with a Brookfield DV-II cone and plate viscometer. The *in vitro* release studies were initially attempted using Franz diffusion cells and then replaced with a polymer non-membrane *in vitro* release method.

Results: The exact mechanism of how the excipients were affecting the HPLC results was identified and the HPLC method was later replaced. In the stability studies at 4 °C, the majority of formulations were 100 % stable over a period of 3 months. Also at 25 °C and 40 °C, the highest stabilities were achieved. Selection of suitable polymers and adjusting the pH of hydrogels in a right region gave 99 % stability to doxycycline at 4 °C, over a tested period of 20 months. A combination of two different mucoadhesive polymers showed enhanced mucoadhesion capability with low viscosity values at room temperature and without affecting the gel strength and gelation temperature of the poloxamers.

The results from non-membrane *in vitro* release studies showed sustained drug release behaviour from the polymer network over a period of 20 hours.

Conclusions: The results indicate that the main aim of this project of formulating a stable doxycycline *in situ* formulation that is stable for at least 2 years was achieved. At 4 °C some of the formulations were 100 % stable after 15 months and 99 % stable after 20 months, at 25 °C one of the preferred formulation was 100 % stable for up to 1 month and 91 % stable by the end of 3 months. At 40 °C, one of the formulation was 71 % stable after 3 months. All the stabilities achieved at all the 3 temperatures are highest among all the previous studies.

1 INTRODUCTION

1.1 Oral Mucosa

The oral cavity has been of interest for pharmacists as an area for drug delivery as it has large surface area, relatively high permeability for large molecular weight substances like proteins, peptides etc. and it circumvents the liver which there by avoids the first-pass metabolism[1]. The main functions of the buccal cavity are the mechanical processing of food materials, lubricating and digesting[2]. Generally the oral mucosa is referred to as the buccal mucosa but buccal mucosa specifically means the membrane lining the inside of the cheeks[3]. The entire surface area of the oral mucosa is about 100 cm^2 and the buccal mucosa makes up one third of total oral mucosa[3]. Approximately 1 litre of saliva is produced every day within the oral cavity. The salivary flow increases during food mastication and reaches up to 7ml/min. The pH of saliva can range between 6.2 – 7.4[3]. The average oral cavity pH is 6.7 and average buccal cavity pH is 6.28[4-7]. The average temperature inside the oral cavity ranges between 32-37 °C[8]. Bacteria around the teeth produce a low localised pH. The oral cavity consists of two regions, the oral vestibule and the oral cavity proper. The oral vestibule is bounded by cheeks, lips, teeth and gingiva. The oral cavity proper extends from teeth and gums back to the fauces (which leads into the pharynx) with the roof comprising of hard and soft palates. The tongue projects from the base of the cavity. The oral mucosa can be divided into 3 parts i.e. masticatory mucosa, lining mucosa and specialised mucosa. The masticatory mucosa covers the gingiva and hard palate. The lining mucosa covers the lips, cheek, floor of the mouth, undersurface of the tongue and the soft palate. The specialised mucosa covers the upper surface of the tongue and parts of the lips. All the three mucosal regions consist of a many layered thick squamous stratified epithelium[2,9-11]. The outer surface of the oral cavity is a mucous membrane consisting of epithelium, basement membrane and lamina propria overlying on submucosa which consists of blood vessels and nerves. The outer layers of masticatory mucosa are keratinised. The non-keratinised mucosa tends to be thicker than the keratinised mucosa. The buccal mucosa is about 0.5 mm thick, while the other mucosal membranes are thinner i.e. around 0.25 mm thick.

Table 1. Difference between non-keratinised and keratinised mucosa[3]

Non-keratinised	Keratinised
Thicker than keratinised mucosa	Thinner than non-keratinised mucosa
Rapid cell turn over (3-8 days)	Slow cell turnover
Consists of lower molecular weight proteins/keratins	Consists of higher molecular weight proteins/keratins
Polar lipids present on buccal mucosa and sublingual mucosa[11]	Non-polar lipids are present on gingival and palatal mucosa

There are 3 major and 1 minor salivary gland in the oral cavity. The major salivary glands produce 90 % of the total salivary production.

Major salivary glands: Parotid gland, submaxillary gland and sublingual gland

Minor salivary gland: Buccal gland.

The parotid gland opens into the oral cavity through long ducts onto the inner surface of the cheek. The submaxillary gland lies below the lower jaw and drains saliva through the ducts on either side of the floor of the mouth. The sublingual glands are situated below the tongue and empties onto the floor of the mouth through several ducts. The buccal salivary gland is located below the oral mucosa. The parotid gland produces amylase rich watery secretions, whereas the submaxillary gland produces mucin rich viscous secretions with little enzymatic activity. 70% of secretions are from the submaxillary gland, which constantly keeps the oral mucosa moist. The saliva consists of 99.5% water and 0.5% solutes. The solutes comprise ions like sodium, potassium, calcium, magnesium, phosphate, bicarbonate and chloride and other components include dissolved gases, urea, uric acid, serum albumin, globulin, mucin and enzymes lysozyme and amylase. The components of saliva are adsorbed onto the oral mucosa in the form of salivary pellicles (0.1-0.7 mm thick)[12]. The oral cavity contains a large number of micro-organisms and saliva pellicles act as determinant sites for bacterial adhesions[13]. The salivary pellicles may act as barriers for drug absorption, but they protect the mucosa from acids and enzymes[14]. The buccal mucosa is comparatively thicker than the sublingual mucosa. The buccal mucosa predominantly acts as a lipoidal barrier, which easily transports the lipophilic (less ionised) drugs[15-17]. In non-keratinised tissue the upper epithelia acts as lipoidal barrier and basal lamina propria (**Figure 1**) presents a major transport barrier for hydrophilic drugs[18]. Drug absorption predominantly takes place by the intercellular route rather than the transcellular route[19].

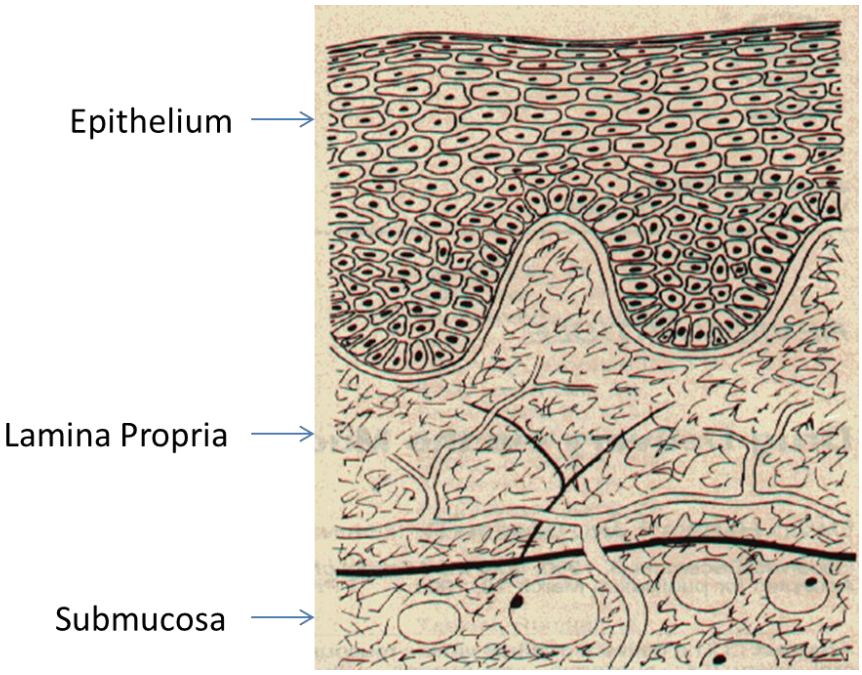


Figure 1 Structure of the Oral Mucosa[9]

1.2 Aphthous ulcers

The word aphthous comes from the Greek word “aphtha” which means ulcer. Aphthous ulcers are also known as recurrent aphthous stomatitis (RAS) or recurrent aphthous ulcerations (RAU). Oral ulcers are the most frequent cause of oral lesions which are painful inflammatory ulcerative conditions and might cause pain on eating, swallowing and talking[20-22], thereby reducing the quality of life. The frequency of prevalence of RAS in general population is 5-25 % with 50 % chances of recurrence within three months[23]. The prevalence of aphthous ulcers in HIV-seropositive patients is 2-4%[21]. The factors that could possibly contribute to recurrent aphthous ulcers are trauma, stress, foods, hormonal imbalance and tobacco smoking[24]. The etiology of ulcers is unknown[25] but there are many proposed theories.

1.2.1 Factors that might trigger aphthous ulcers

1. Trauma and stress:

Trauma and stress are considered to be the most likely causes of aphthous ulcerations. Localised trauma may include accidental self-biting, dental procedures, tooth brush bristles and sharp-edged foods[26]. Emotional and environmental stress may account for 60 % of first time aphthous ulcers and 21% of RAS[26].

2. Nutritional deficiencies:

Oral ulcers might be caused due to nutritional deficiencies like iron, folic acid, zinc, and vitamins B_1, B_2, B_6 and B_{12}[25].

3. Food allergies:

RAU is frequently reported in patients who had antibodies for cow’s milk and gluten (wheat protein)[27].

4. Infections:

Possibly RAU might be due viral infections like Herpes simplex virus and cytomegalovirus. Antibodies for HSV and cytomegalovirus were found in some patients with RAS but the results were not consistent[26,27]. Cross reactivity between streptococci heat shock proteins and oral mucosa has been suggested as possible cause of RAU in some patients[28]. The antibodies to heat shock proteins were present in these patients.

5. Genetic factors:

More than 42 % of the patients suffering from RAS have first degree relatives with RAS[29].

6. Immune disorders:

RAS is more common among patients with immune disorders like inflammatory bowel disease, Behcet’s disease, cyclic neutropenia, and HIV disease[30]. Patients with RAS have shown antibody dependent cytotoxicity and elevated serum immunoglobulins[23].

7. Drug induced:

Antineoplastic drugs like methotrexate, daunorubicin, doxorubicin, and hydroxyurea can cause RAS[31]. RAS was reported in 37 % of leukemia patients

taking antineoplastic drugs[31]. Other medications like antimicrobials, auranofin, barbiturates, didanosine, foscarnet, griseofulvin, non-steroidal anti-inflammatory drugs, penicillamine, quinidine, and sulfonamides can also cause RAS[32].

8. Other factors: sensitivity to sodium lauryl sulphate in tooth pastes might also cause RAS[33,34].

RAS can be divided into 3 types based upon size, duration and scarring.

1. Minor aphthae:

Minor aphthous ulcers account for 75-85 % of all cases of RAS[35]. Minor aphthae mostly occurs on the non-keratinised oral mucosa. Size may be lesser than 8-10mm, and heals within 7-14 days without any scarring. Lymphatic infiltrate can play a role in minor aphthous ulcers[36].

2. Major aphthae:

Major aphthous ulcers account for 10-15 % of all cases of RAS[35]. They usually tend to occur after puberty. Their appearance is round or ovoid with clearly defined margins with size more than 1 cm in diameter and usually appear on lips, soft palate and throat. They can last for weeks or months and often leave scars on healing. The condition is often painful and can accompany with fever, dysphagia and malaise[35].

3. Herpetiform ulcers:

Herpetiform ulcers account for 5-10 % of all cases of RAS[35]. They appear in crops usually 5-100 in number and 1-3 mm in size, which are round in shape and painful. They resemble herpes simplex virus but the herpes simplex virus has not been cultured from them[37]. They tend to fuse and form much larger ulcers which last for 10-14 days[35]. If they reoccur 2-4 times in a year they are called simple aphthosis or if they occur continuously with new lesions replacing the old ulcers then they are called complex aphthosis[35].

1.2.2 Treatment

1. Antimicrobial mouth washes:

Reducing the microbial population of the oral cavity with mouthwashes containing antibiotics (e.g. tetracycline) and antiseptics (e.g. chlorhexidine[38]), has been correlated with reduction of the healing times of ulcers[39]. Mouthwash containing the antiseptic chlorhexidine did not show any significant effect in reducing the pain severity or ulcer duration compared to the placebo[39-41]. In another study, mouthwash containing tetracycline or chlortetracycline significantly reduced the duration and pain of RAS, compared with the placebo, but the frequency of ulceration was unchanged[42-44]. Tetracycline mouthwashes are usually safe if their use is limited to 5 days, beyond that it can cause oral fungal infections[37]. After 2 weeks chlorhexidine mouthwashes caused brown staining to the teeth and oral mucosa[37].

2. Corticosteroids:

Topical corticosteroids: Topical corticosteroids act directly on T lymphocytes or alter the response of effector cells to precipitants of immunopathogenesis (e.g. food allergies, trauma and microorganisms)[45]. There were only two double blind, placebo controlled studies carried out to study the effectiveness of topical

corticosteroids for RAS[46,47], in one of the study the patients enrolled had minor RAS[47]. There was significant reduction in ulcer duration and pain severity but there was no change in frequency of RAS[45,47,48].

3. Amlexanox[49]:

Amlexanox is a medication used for longterm management of asthma[50]. It is a potent inhibitor of the formation and release of inflammatory mediators from mast cells, neutrophils, and mononuclear cells[37]. Topical 5 % amlexanox facilitates the healing of the aphthous ulcers but does not reduce the frequency of RAU[50]. Four randomized, double-blind, multicenter trials were carried out to assess the efficacy of amlexanox with respect to healing and pain reduction on minor RAS[51]. In one study 74 % of the patients receiving amlexanox paste reported complete resolution of ulcer and 83 % reported complete resolution of pain by day 6[51]. 2.1 % of patients using 5 % amlexanox paste reported adverse side effects[52] which included stinging, drying, bumps on the lips, and mucositis.

4. Levamisole:

Levamisol is an anthelmic and immunomodulator drug. Levamisole is an immune-potentiating agent. It showed ability to normalize CD4+ cell/CD8+ cell ratio and thereby improved the symptoms in RAS patients[53]. Seven placebo-controlled clinical trials were carried out, to study the efficacy and safety of levamisol in patients with RAS[54-58]. In 3 of the tests the patients enrolled had minor RAS, and in the rest of the tests RAS classification was not mentioned. In overall 4 tests, a reduction in duration and frequency of the RAS was observed in 43 % of the patients[55,58,59]. The side effects reported were dysgeusia(21%), nausea(16 %), and 10 % of patients reported dysosmia, headaches, diarrhea, influenza-like symptoms and rash[37].

5. Thalidomide:

Tumor necrosis factor ∝ (TNF-∝) is found in elevated levels, both systemically and locally in patients with RAS[60,61]. Thalidomide has the ability to selectively inhibit the TNF-∝, but in HIV seropositive patients it increased the levels of TNF-∝[61]. Thalidomide is reserved mainly for treatment of RAS in HIV disease[62], Behcet's syndrome[63] or a history of severe RAS[64]. Adverse side effects were reported in 6-26 % of patients[37].

6. Silvernitrate[38]:

It is a one-time topical application. In a study comprising of 97 patients, it reduced the pain in 70 % of patients by one day.

7. Debacterol[65]:

Debacterol acts by reducing the pain associated with RAS, thereby allowing the patient to resume eating and speaking. It is a one-time topical application. In a study comprising of 60 patients, 60 % of the patients reported complete resolution of ulcers by day 6, compared to 30 % placebo group[65].

8. Vitamin B12[39]:

In a study comprising of 58 patients, oral daily supplementation of Vitamin B_{12} for 6months, reduced the formation of new ulcers in 74 % of patients compared to 32 % in placebo group[39].

9. MMP inhibition by doxycycline:

Topical formulation[66]: (low dose doxycycline gel)

Matrix metalloproteinases are involved in inflammatory processes associated with RAS[67,68]. Doxycycline among all other tetracycline has the highest inhibitory action on MMPs[69,70]. By reducing the MMPs, the healing time of ulcers were halved. In a clinical trial[66] which consisted of 49 patients, doxycycline (0.15 % w/w) containing gel was applied twice a day and the test was carried out by randomized, double-blind, placebo controlled trial. In 68 % of the patients under study, the ulcers healed completely by day 3 compared to 25 % of patients in placebo group[66]. Faster reduction in pain was reported in patients applying low dose doxycycline gel, compared to placebo gel[66].

Oral doxycycline maintenance dose for MMP inhibition[71]:

In another study 20mg twice daily sub-antimicrobial dose of doxycycline, on oral administration, significantly reduced the frequency of RAS[71] compared to placebo.

1.3 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases are the extracellular proteolytic enzymes. They remain as inactive zymogens, which needs to be proteolytically activated in the body to form functional enzyme[72]. Human MMPs family consists of a 24 zinc containing endopeptidases (MMPs), which are divided into 5 sub-categories based on their (Figure 2) structure[73,74] (Table 2). Matrilysins consist of a propeptide domain and a catalytic domain with the zinc binding site. The collagenases, stromelysins and the other MMPs consist of similar structures i.e. in addition to simple matrilysin structure they contain a hemopexin-like domain connected to the catalytic domain via a proline rich hinge region. Stromelysins have a broader substrate specificity than collagenases. Gelatinases are similar to collagenases, stromelysins and other MMPs, but contain an additional region of 3 fibronectin type II repeats within their catalytic domains (Table 2). The 5th subclass of MMP is the membrane-type MMPs which are bound to the cell surface via a C-terminal transmembrane domain or glucosylphosphatidylinositol anchor. MMP-23 has a unique structure, it lacks hinge and hemopexin region instead contains a short carboxy-terminal domain containing cysteine array[75]. When activated the enzyme first loses the signal peptide and then internal bonds of the propeptide are disrupted. Disruption of Cystein-Zn^{++} bond is a prerequisite for activation of the enzyme, which allows Zn^{++} to catalyse further cleavages[76]. Doxycycline is the only compound approved by FDA as an MMP inhibitor in the treatment of periodontitis[77], it acts by inhibiting MMP-7 and MMP-8[78]. MMP-8 has been known to cause substantial connective tissue damage leading to periodontitis[79]. Increased activity of MMPs has been detected in oral ulcers[67,68]. In a study by Gracia et al. it was determined how the doxycycline attaches to the MMP-7 to form a complex[80]. Two molecules of doxycycline attached to each molecule of MMP-7 at calcium and zinc metals of the enzyme, to form a week complex[80] (K_d = 70 µM). The doxycycline may accumulate at the matrix and act in catalytic fashion by binding to both pro- and active MMP so as to disrupt the enzyme confirmation, resulting in autocleavage and loss of enzymatic activity[81,82]. The tetracyclines have the ability to inhibit already active MMPs and also pro-MMPs by downregulating their expression[83,84]. British National Formulary nos. 42 and

43 recommends that a strong concentration of tetracycline or doxycycline in mouthwashes, may be beneficial in recurrent aphthous stomatitis condition[85,86]. It has also been shown that tetracyclines in low doses do not induce the emergence of tetracycline resistance bacterial strains[87-90].

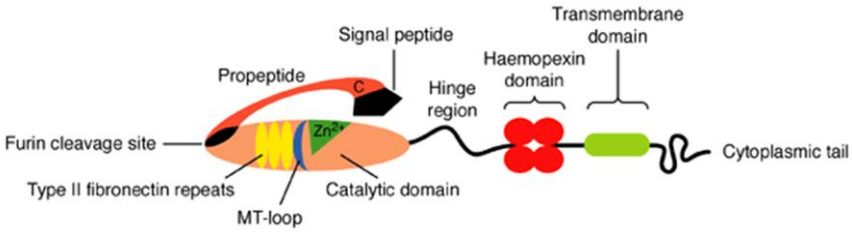


Figure 2. General structure of mammalian MMP91

1.4 Doxycycline hyclate

Synonym: Doxycycline hydrochloride[92].

Doxycycline is a long acting, semisynthetic tetracycline, which can potentially inhibit collagenase approximately 20 times greater than tetracycline[69,93]. Doxycycline in addition to antibiotic activity, also possess anti-inflammatory properties. Doxycycline is an amphoteric compound with 3 pK_a values[94,95] i.e. 3.4, 7.7 and 9.3 and a fourth de-protonation centre is likely to exist[96]. At 20 °C pK_a values of 3.5 (tricarbonyl system), 7.7 (Ketophenolic system), and 9.5 (dimethylammonium group) have been reported[97]. At pH less than pKa 3.4, doxycycline exists as positively charged ion, at pH above pKa 9.3 it exists as negatively charged ion and in between pKa's 3.4 and 9.3 it exists as zwitterion, with an isoelectric point at about pH 5.5. Doxycycline hyclate is classified as soluble[98] or freely soluble[99] in water. Doxycycline hyclate solution, containing 1% doxycycline has a pH of 2-3[100,101].

Degradation compounds: Doxycycline undergoes oxidative degradation and epimerization in aqueous solutions[102-104]. Impurities of doxycycline include 4-epidoxycycline, 6-epidoxycycline, 4,6-epidoxycycline, metacycline, and 2-acetyl-2-decarbixamidodoxycycline[102]. 4-epidoxycycline and 6-epidoxycycline lacks the anti-inflammatory and anti-proteolytic effects[102].

1.4.1 Reports of excipients used in order to stabilizing doxycycline

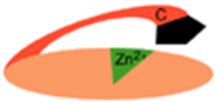
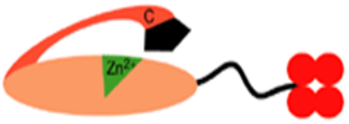
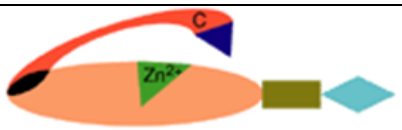
Articles & patents review:

1. Doxycycline non-ionic surfactant vesicles, prepared by solvent-injection method improved the stability of doxycycline[105].

2. Heat treating doxycycline (for a short period at 110-140 °C) at pH 4.0, increased its thermal resistance by 3 times[106].

3. Doxycycline was complexed with magnesium in the molar ratio of 1.1- 1.8[107] and the complex was added to a solution containing a non-ionic surfactant. The pH of the solution was adjusted between 5.0 and 7.0. Magnesium reacts with doxycycline to form magnesium-doxycycline chelates. Magnesium ions can be sourced from any of the following magnesium compounds, magnesium chloride, magnesium ascorbate, magnesium lactate, magnesium gluconate, etc. The

Table 2. Structural classification91 of MMPs

S.no	MMP subclass	MMP designation	Domain structures of mammalian MMPs
1	Matrilysins	MMP-7 MMP-26	
2	Collagenases	MMP-1 MMP-8 MMP-13	
3	Stromelysins	MMP-3 MMP-10 MMP-11	
4	Gelatinases	MMP-2 MMP-9	
5	Membrane-type MMPs	MMP-14 MMP-15 MMP-16 MMP-24 MMP-17 MMP-25	
6	Others	MMP-12 MMP-20 MMP-19 MMP-27 MMP-28	
		MMP-23	
N-terminal signal anchor	Cysteine array	Ig-like domain	GPI anchor

preferable concentration range for non-ionic surfactants is 7.5 to 15% w/v[107], but not more than 20 % w/v . Useful non-ionic surfactants include aryl and alkyl phenols, fatty ethers such as lauryl ether, alkyl phenol ethers, amides of fatty acids such as lauramide, polyoxypropylene glycols of molecular weight 800-900, ethoxylated compounds such as ethoxylated oleolyl ethanolamide and ethoxylated linear primary alcohols and polyoxyethylene sorbitan fatty acid

esters, partial esters of common fatty acids (lauric, palmitic, stearic and oleic) and hexitol anhydrides derived from sorbitol[107]. To preserve the color and potency of the formulation an antioxidant is incorporated. Suitable antioxidants are sodium or magnesium formaldehyde sulfoxylate (0.2-0.5 % w/v); sodium sulfite, sodium metabisulfite or sodium bisulfite (0.1-0.2 %w/v); sodium sulfide (0.002-0.004 %w/v); and thiosorbitol[107] (0.4-1.0 %w/v). The total concentration of antioxidants can be in the range 0.1-1 %[108]. The pH may be adjusted with either an organic acid and base or mineral acid and base as shown in Table 3

Table 3: Acids and bases for pH adjustment107.

Mineral acid	Hydrochloric acid
Organic acids	Citric acid, lactic acid, etc.
Inorganic bases	Ammonium hydroxide, sodium hydroxide
Organic bases	Aminomethane, dimethylaminomethanol, diethylaminoethanol, dimethylamine, diethylamine, trimethylamine, triethylamine, and 2-aminoethanol.

4. Tetracyclines were stabilized by adding the dehydrating agents[109] or any other pharmaceutical excipients in acceptable proportions to the formulation.

5. The rate of degradation of tetracyclines in aqueous formulations was slowed down by adding following excipients, chelating agents (0.1-0.5 %), antioxidant (0.1-0.5 %) and the pH of the formulation was adjusted between 4.5 and 7.5[102]. Combination of antioxidants gave more stability to doxycycline[102].

6. The presence of an impurity (degradation compound) acts as anti-aging agent (slows down the degradation) for doxycycline. The formulation was stabilized by adding an antioxidant[110].

7. Doxycycline injections were stabilized by freeze drying the doxycycline prior to adding medical excipients, lysine, sodium sulfite(1:0.01 – 1:0.05 w/w ratios of doxycycline and antioxidant respectively) and polyvinyl pyrrolidone[111] (1:0.05 – 1:0.1 w/w ratios of doxycycline and PVP respectively).

8. Doxycycline hydrochloride was stabilized by adding the following excipients i.e. a complexant, co-solvent, antioxidant, antibacterial synergist, organic solvent and triethanolamine[112].

9. Doxycycline hydrochloride injections were unstable when exposed to light. They are usually stable for 24 months at 25 °C when kept in light resistant containers[113] (protected from light).

10. At pH ≤ 6.0 doxycycline reversibly[114] converts into C4-epimer (degradation product), whereas at higher pH levels doxycycline irreversibly[114] converts into degradation products by following the first order reaction.

11. Doxycycline was fairly stable over a period of 8weeks when stored at -20 °C in sterile water for injection[115]. There was no loss in bio-potency or any change in pH or colour of formulations[115].

12. Doxycycline was complexed with HPβCD (1:24 w/w respectively), to increase the stability of doxycycline in aqueous formulation[116].

13. Doxycycline was first complexed with magnesium ions and then HPβCD is added to the solution. At 8 °C the formulation was 99.9 % stable[117] over a period of 1 month, and the pH of formulation was 5.5, compared to 92.7 % stability of the formulation containing just doxycycline. At 40 °C, after 10 days the formulation containing the complex was 90 % stable compared to 52 % stability of the formulation containing only doxycycline[117]. Unstable site of doxycycline molecule at 6-CH_3 was protected in hydrophobic cavity of HPβCD[118], whereas Mg^{2+} provided synergetic protection of the another unstable site of doxycycline at 4-$N(CH_3)_2$[118].

14. The epimerization of doxycycline into 4-epidoxycycline is reversible and the rate of formation of 4-epidoxycycline is related to kinetic equilibrium between 2 compounds. If the formulation is incorporated with small amount or 4-epidoxycycline, the rate of doxycycline epimerization into 4-epidoxycycline reduces[102]. However the rate of formation of 4-epidoxycycline appears to increase with time of storage[102].

15. Doxycycline was complexed with β-cyclodextrins to increase its stability[119]. From NMR studies, the docking results suggested the formation of inclusion complex between doxycycline and β-cyclodextrin. The “D” aromatic ring of doxycycline (**Figure 3**) was inserted into the hydrophobic cavity of the β-cyclodextrin, with a mean docked energy of -11.03 Kcal/mol[119].

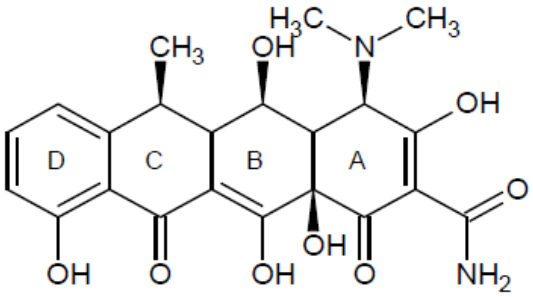


Figure 3 *Molecular structure of doxycycline*[119]

16. Acidic pH stabilized doxycycline compared to neutral pH. At room temperature after 2 weeks of storage, there was 28 % degradation at pH 5.3 compared to 51% degradation at neutral pH[102]. At acidic pH doxycycline solutions were stabilized by adding antioxidants like sodium metabisulfite, sodium thiosulfate and thiourea[102]. After 2 weeks of storage at room temperature, sodium metabiulfite solution showed a degradation of 8%, compared to 5 % with sodium thiosulfate and 20% with thiourea[102]. Addition of antioxidant sodium bisulfate did not improve the stability[102].

17. If tetracyclines are exposed to adverse conditions like light, high temperatures and humidity they are known to undergo degradation reactions. But even stomach acid can convert tetracyclines into reversible C_4 epimers like 4-epitetracycline in weak acidic conditions and anhydrotetracycline in strong acid conditions, due to conformational changes in the ring system by epimerization at carbon-4 or dehydration and aromatization of the C ring, respectively[120,121].

18. Members of the tetracycline family are subject to epimerization when they are exposed to pH of intermediate range (higher than pH 3.0), resulting in steric

rearrangement of the dimethyl amino group. The epimer of tetracycline, epitetracycline, has little or no antibacterial activity[122].

19. The tetracycline formulations were stabilized by adding bisulfites[123] like sodium metabisulfite.

20. Tetracycline family antibiotics were stabilized by selecting silicone as vehicle, an emollient ester as co-solvent and a polyethylene as gelling agent, in the preparation of a topical gel[124].

21. Doxycycline was stabilized by adding an antioxidant, caffeine and creatine[125]. The formulation pH can range between 4.5 and 8.0. The antioxidant sodium thiosulfate might be in the concentration range 0.5 – 1 % w/w, or sodium metabisulfite in preferred concentration of 0.25 % w/w. The presence of sodium metabisulfite prevents the colour change of the formulation[125]. Preferably caffeine and creatine might be in the concentration ranges between 0.05 – 2.0% w/w[125]. The pH can be adjusted by means of pharmaceutically acceptable acid like Hcl or organic base like monoethanolamine[125].

22. The stability of doxycycline was studied in aqueous solutions with pH ranging between 2.0 – 9.0, and at two different temperatures, 4 °C and 25 °C. All the solutions were protected from light and the stabilities were tested using TLC. The degradation compounds were 6-epidoxycycline and 4-epidoxycycline. The stabilities were higher at pH region 2-5 than at 5-9. After 25 days the stability of solutions at room temperatures was between 90-94.44 %, and at 4 °C the stabilities were between 98.26-94.30 %[126].

23. Molecular modelling studies confirmed that the HPβCD encapsulate the unstable site of doxycycline molecule at 6(CH_3) in its hydrobhobic cavity[118] and thereby protects from oxidation. The addition of Mg^{2+} (chelation) provided synergetic protection[118] at another unstable region of doxycycline at 4-$N(CH_3)_2$. The stability of doxycycline was improved[118] when complexed with HPβCD in both aqueous solutions and in solid states, at 25 °C.

24. All the tetracycline have the ability to arrange into up to 64 possible tautomers that can interconvert by following a complex equilibrium in solution, because of the presence of one amide and two carbonyl groups[96]. This makes tetracyclines able to adapt themselves as a result of environmental modifications. Therefore, it is difficult to predict the exact behaviour. Because of this arrangement pH plays a vital role in stability of doxycycline. Zwitterion form of doxycycline was determined to be most stable in aqueous solutions. The stability was predicted to be highest between the first and second pK_a[96]. The stability decreased as the pH approached 7.0, and further instability was observed when nearing to second pK_a(7.7).

25. Tetracyclines undergo reversible[127] epimerisation at C-4 and C-6 positions, into a mixture of degradation products.

26. Tetracyclines were stabililised by adding sodium metabisulfite and sodium thiosulfate in the concentration range of 0.001–3 %[102] (w/w or w/v).

27. Antioxidants like ascorbic acid, sodium metabisulfite, sodium sulphite and sodium formaldehyde sulfoxylate can be added in the concentration range of 0.1 – 1 % w/w[128], to stabilize doxycycline in aqueous formulations.

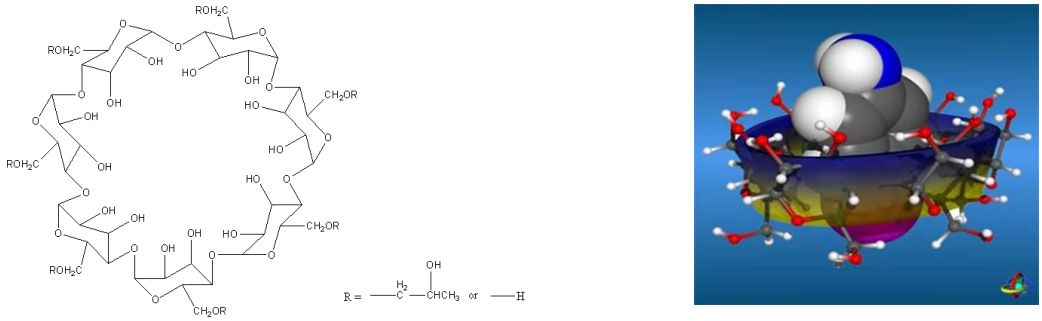
1.5 Poloxamers

The poloxamers are synthetic block co-polymers of hydrophilic poly (oxy-ethylene) and hydrophobic poly(oxy-propylene)[129,130]. They can be described as alternating copolymers, block copolymers and graft copolymers. The poloxamers are available in different forms i.e. flakes, liquids and pastes, they differ from each other with varying molecular weights of ethylene oxide and propylene oxide, ranging from 1100 – 14000 and 1:9 to 8:2, respectively[131]. Micelles are formed at critical micelle temperature, due to dehydration of PPO block[132-134]. Micellar mode of association was confirmed by ultrasonic velocity, light-scattering and small-angle neutron scattering measurements of aqueous poloxamer solutions[132,135-139]. The combination of poloxamers i.e. poloxamer 407 and poloxamer 188 or any other different molecular weight poloxamers, can yield desired gelation temperature. Poloxamer 407 alone cannot undergo gelation at body temperature (37 °C), at 20 wt% it undergoes gelation under 25 °C[131]. Poloxamers are well tolerated[140], high dosage injections can cause hypercholesterolemia and hypertriglyceridemia[131,141]. The poloxamer hydrogels are rapidly eroding gels, compared to hydrogels made of other polymers, and can only release drug for upto few days[142-146] where as other polymers have capacity to release drugs for upto few weeks. The poloxamer gels will be ideal for short-term therapies[131] like treatment of infection[144,146], pain management[143] and fertility control[145]. The poloxamers can undergo thermoreversible gelation from sol-gel and vice versa[147]. Majority of poloxamer formulations are based on PF-127/poloxamer 407, and include delivery of protein/peptide drugs like insulin, urease, interleukin-2, EGF, bone morphogenic protein, fibroblastic growth factor and endothelial cell growth factor, over a sustained release period of several hours[147]. The poloxamers can be used for solubilizing the hydrophobic drugs[148-150] in aqueous solutions and also for increasing the stability of drugs by forming stable micelles[150], specific applications in cancer and gene therapy[150]. In solid dosage forms they can be used as wetting agents, plasticizers and tablet lubricants[151]. Poloxamers have poor mechanical properties and short residence times, because of rapid dissolution nature when placed in biological environments[152]. Poloxamer aqueous solutions are very stable in the presence of acids, alkalis and metal ions[153]. Poloxamers are soluble in aqueous, polar and non-polar aqueous solvents[153]. Poloxamers are more soluble in cold water, because of increased solvation and hydrogen bonding at lower temperatures[154]. The poloxamer formulations can be administered through ocular[155,156], buccal[157-162], dental[163], intra-nasal[164-166], rectal[167-175], vaginal[176,177], ear[178], transdermal and topical[179-183], subcutaneous[163], intramuscular[145,184] and intravenous[185] and other injectable[142,143] routes.

1.6 HPβCD

Cyclodextrins are polysaccharides made up of 6-8 D-glucose units (∝, β and γ cyclodextrins respectively) connected at the C_1 and C_4 carbon atoms. They have a hydrophobic inner cavity and a hydrophilic external surface (Figure 4), which can form inclusion complexes with various guest molecules of suitable polarity

and dimensions because of their special molecular structure[186-190]. Cyclodextrins like hydroxypropyl-β-cyclodextrins and sulfobutylether-β-cyclodextrins are being increasingly utilised in pharmaceutics to increase the solubility profiles of hydrophobic drugs[191]. Cyclodextrins increases the solubility, bioavailability and over all stability of the drug molecules[192,193]. Cyclodextrins have the ability to reduce or prevent the gastric and ocular irritation, reduce or eliminate unpleasant smells or tastes that can arise due to side effects from drugs[194,195]. The cyclodextrins have a shape of truncated cone or torus rather than a perfect cylinder. The primary hydroxyl groups of the sugar residues are oriented toward the narrow edge of the cone and secondary hydroxyl groups towards the wider edge[196]. The central cavity of the cyclodextrins molecule is lined with skeletal carbons and ethereal oxygens of the glucose residue, which makes it more lipophilic[197-200]. HPβCD and SBEβCD are considered non-toxic at moderate doses upon administration through oral or intravenous routes[201,202]. HPβCD are considered toxicologically benign than natural β-cyclodextrins[203,204]. HPβCD are generally well tolerated in humans but the adverse side effects can be loose stools and diarrhoea[196,201,203].



a. HPβCD[205,206] **b.** β-cyclodextrins

Figure 4 (a). HPβCD205,206, molecular formula: (C6H10O5)7(C3H6O)5.5; (b). 3D figure showing interaction between the guest molecule and hydrophobic cavity of β-cyclodextrins207.

1.7 Chelating agents

The word “chelate” was derived from the greek term “chela”, meaning “great claw” of the lobsters or other crustecians. The word chelate describes the way in which an organic compound clamps into the cationic element which it chelates. The most commonly used synthetic chelating agent is EDTA (Figure 5).

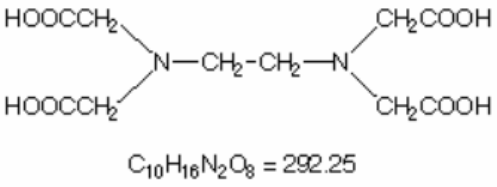


Figure 5 [208]: EDTA free acid. (Ethylene diamine-N, N, N1, N1- tertraacetic acid)

1.8 Mucus

Mucus is a viscous gel which protects the underlying tissues from environmental insults and chemical agents. Throughout the animal kingdom various species

have utilized the mucus secretions in their adaptation to the environment. In the earthworms mucus acts as a permeable barrier for the exchange of oxygen and carbon dioxide and also prevents the influx of harmful chemicals from soil. In carp fish the olfactory recesses which are studded with goblet cells and the mucus helps to prevent the passage of water to the tissue surface. In human beings the mucus has various physiological functions. The mucus covering can be found on organs which are exposed to external environments like the eyes, GI tract, respiratory tract, urinary bladder, middle ear, pancreatic tract, gall bladder and the reproductive tracts.

1.8.1 Composition of mucus

The mucus is composed of 95 % water and the rest include glycoproteins (mucin) and lipids (0.5-5%), cellular debris, mineral salts (0.5 – 1 %), and free proteins 1 %[209]. The composition varies depending on its site and in diseased conditions. The air way mucus is composed of high density glycosaminoglycan with features of both glycoproteins and proteoglycans[210].

1.8.2 Mucin

Mucin is a major constituent of the mucus. It is found in two forms soluble secretary mucin and membrane bound mucins[211-213]. The secretary mucins have the ability to form intermolecular disulphide bridges and can form thick viscous gels. The membrane bound mucins cannot form disulphide bonds but contain hydrophobic domain which anchors them to plasma membrane. The mucins consists of 10 - 30 % by weight of peptide core that is linked via *o*-glycosidic bonds to oligosaccharide chains that constitutes the remaining 70-80% of the total weight[214,215]. The carbohydrate chain length may vary from 2 to 15 sugars in length[216]. The mucins are poly-disperse[216,217] in nature and they do not contain homogenous structures and sizes even if they are collected from same organ or gland.

1.8.3 Protein component

The mucus proteins are composed of the amino acids aspartic acid, threonine, serine, proline, glutamic acid, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine and arginine[218]. The human air way proteins are produced within the airways themselves[219] which resemble serum proteins and are composed of IgA, albumin, lactoferrin and lysozyme[220].

1.8.4 Oligosaccharide component

Sugar residues are directly linked to the protein backbone of glycoprotein. Five most commomly found monosaccharides in mucin are N-acetylglucosamine, N-acetylgalactosamine, galactose, fucose and sialic acid[217]. The backbone regions of polysaccharide chains consists of a series of Gal β (1-3) units and GlcNAc β (1-4) units and are terminated by α-glycosidic-linked galactose, GalNAc, fucose, sialic acid or sulphate. The sialic and sulphate residues contribute for the overall negative charge of oligosaccharide portion of macromolecule.

1.8.5 Lipid component

The airway secretions consists of lipids like free fatty acids, triglycerides, cholesterol and phospholipids[220]. The health of the airway can be predicted by analysing the lipid components.

1.8.6 Mucin behaviour and confirmation in solutions

Mucin monomers tend to aggregate in solution forming structures like rods and threads[221]. Microscopic observation of Pig gastric mucin revealed linear structures with 5 nm in diameter and 100 to 5000 nm in length[222]. There are six different structures of mucin proposed in literature. Bhushana-Rao and Hasson have proposed tentative model for bovine cervical mucin which involves cross-linking via disulphide bridges and hydrophobic bonds[223]. Carlstedt and Sheehan have proposed a linear flexible chain for human cervical mucin with no branching[224]. Carlstedt in a follow up report proposed an additional hydro-dynamic model that consists of a glycoprotein coil around spheroidal solvent domain[224]. Meyer and Silberberg suggest that the structure of mucus gel results from labile cross-links maintained by non-covalent bonds[225]. Allen proposed bottle-brush branched structure for gastric mucus[226]. Verdugo et al. hypothesized the structure of respiratory mucus as ensemble of entangled randomly coiled macromolecules[227]. The gastric mucin at low pH ranges is seen as a gel, this confirmational change was explained by dynamic light scattering studies performed by Cao et al. [228]. They showed that at dilute mucin concentrations, the mucin molecules exists as non-associated macromolecular species and the diffusion coefficient of mucin macromolecule decreased with decrease in pH which in-turn increases the macromolecular hydrodynamic structure. The molecular confirmation was changed from coiled macromolecular state to linear confirmation. The formed gel is resistant to back-diffusion of secreted acid and maintains a pH gradient i.e., pH 2 at the lumen to pH 7 at the apical cell surface.

1.8.7 Mucus secretion, thickness, turnover

Mucus secretion takes place in stages. First the intercellular biosynthesis of mucin and other components which are stored in granules covered with lipid membrane, and then by a process called exocytosis (secretion) the storage vesicles with lipid membranes are fused with plasma membrane of the cell. Subsequently in next stages water component gets added and the glycoprotein crosslinks to form mucus. The thickness of mucus layer can be measured by observing the unfixed tissue sections mounted transversely under a light microscope[229] and electron microscope[230] usually after stabilization of the gel structure by anti-mucus anti bodies. The thickness of mucus in human stomach is 576±81 µm as stated by Bickel and Kaufman[231] whereas mean thickness was reported around 192 µm by Allen et al[232]. The general intestine and the colon of humans appears to contain a continuous mucus layer of varying thickness between 50 and 450 µm[230]. The mean thickness of mucus in airways is around 5-10 µm[233]. The mucus turnover rate in the GI tract is same as gut transit time i.e. 24-48 hours[234]. Mucus is secreted by either goblet cells or mucus glands. The secretions by mucus gland can be stimulated by cholinergic agonists, adrenergic agonists[235] and inflammatory mediators like histamine, prostaglandins (PGA_2 and $PGF_{2\alpha}$), leukotrienes, etc[236,237].

1.8.8 Mucus in disease conditions

Disease processes affect the nature and rate of mucus secretions as there can be abnormalities in the synthesis of glycoproteins resulting in functional changes to the mucus gel[238]. In case of inflammatory conditions in the intestines there is an increase in mucus production, with decrease in content of serine and threonine in the peptide core of soluble glycoprotein[239]. Goodman et al[240]. found

that the level of glucosamine synthetase was increased by 50 % in patients with Crohn's disease. Cystic fibrosis is characterised by derangements in mucus secretion and consistency associated with electrolyte disorders in the gastrointestinal and respiratory tracts[238]. In cystic fibrosis, the mucus is denser and more highly glycosylated with a higher content of fucose, galactose and N-acetyl galactosamine but with no changes in sialic acid content[238]. In case of colon cancer a marked reduction in N-acetyl galactosamine and membrane glycoproteins is reported[241]. Abnormal changes can be noted if there are any inflammatory or neoplastic conditions in the uterine-cervical regions[242].

1.9 Classification of mucoadhesive polymers

1.9.1 Classification based on source, water solubility, charge, mechanism of formation of bond

The mucoadhesive polymers can be classified according to their source (Table 4), water solubility (Table 5), charge (Table 6) and mechanism of formation of bond (Table 7).

1.9.2 First generation mucoadhesives

Also known as traditional non-specific mucoadhesives e.g. anionic, cationic and non-ionic polymers.

2.9.3 Second generation mucoadhesives

e.g. lectins, bacterial adhesions and thiolated polymers[243].

Table 4. Classification of mucoadhesive polymers based on source5

S.no	Source	Classification	Examples
1	Natural and modified natural polymers		Agarose, chitosan, gelatin, hyaluronic acid, carrageenan, pectin, sodium alginate
2	synthetic	Cellulose derivatives	Carboxymethylcellulose, thiolated carboxymethyl cellulose, sodium carboxymethyl cellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose, methylcellulose, methyhydroxyethylcellulose.
		Polymers based on poly (meth) acrylic acid	Carbopol, polycarbophil, polyacrylic acid, polyacrylates, copolymer of acrylic acid and polyethylene glycol, copolymer of methylvinyl ether and methacrylic acid, poly-2-hydroxyethylmethacrrylate, copolymer of acrylic acid and ethylhexylacrylate, polymethacrylate, polyalkylcyanoacrylates: polyisobutylcyanoacrylate, polyisohexylcyanoacrylate.
		others	Poly-N-2-hydroxypropylmethacrylamide, polyhydroxyethylene, polyvinyl alcohol, polyvinylpyrrolidone, thiolated polymers.

2. Solubility in water[5]

Table 5 Classification of mucoadhesive polymers based on solubility

S.no	Solubility	Source	Examples
1	Water-soluble	Cellulose derivatives	Carboxymethylcellulose, thiolated carboxymethyl cellulose, sodium carboxymethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose, methylcellulose, methyhydroxyethylcellulose.
		Polymers based on poly (meth) acrylic acid	Carbopol, polyacrylic acid, polyacrylates, copolymer of acrylic acid and polyethylene glycol, copolymer of methylvinyl ether and methacrylic acid, poly-2-hydroxyethylmethacrrylate, copolymer of acrylic acid and ethylhexylacrylate, polymethacrylate, polyalkylcyanoacrylates: polyisobutylcyanoacrylate, polyisohexylcyanoacrylate.
		others	Poly-N-2-hydroxypropylmethacrylamide, polyhydroxyethylene, polyvinyl alcohol, polyvinylpyrrolidone, thiolated polymers.
2	Water-insoluble		Ethyl cellulose, polycarbophil.

3. Classification based on charge[5]

Table 6 Classification of mucoadhesive polymers based on charge

S.no	Charge	Examples
1	Cationic	Aminodextran, dimethylaminoethyldextran, chitosan, trimethylated chitosan
2	Anionic	Chitosan-EDTA, PAC, carbopol, polycarbophil, pectin, sodium alginate, sodium carboxymethylcellulose, carboxymethylcellulose.
3	Uncharged	Hydroxyethyl starch, hydroxyl propyl cellulose, poly (ethylene glycol), polyvinyl alcohol, polyvinylpyrrolidone, scleroglucan.

4. Classification based on type of bond formation[5]:

Table 7 Classification of mucoadhesive polymers based on bond formation

Possible mechanism of formation of bioadhesive bond →	Covalent bond	Hydrogen bond	Electrostatic interactions
Examples →	cyanoacrylate	Acrylates, carbopol, polycarbophil, polyvinyl alcohol.	chitosan

1.9.3 Second generation mucoadhesives

1.9.3.1 Lectins

Lectins belong to a group of structurally diverse proteins and glycoproteins that bind reversibly to specific carbohydrate residues[244]. Some bacteria use these naturally occurring proteins to attach themselves to the cells of host organism. Lectins can either attach to surface of cells or enter into the cells by the receptor mediated adhesion called as endocytosis[245]. This method not only provides the mucoadhesion but also pharmaceutical macromolecules can be delivered through cell mediated uptake. Although lectins offer site specific mucoadhesion, some of their members are toxic and causes serious immunological conditions[244]. The drawback of this method is that the lectins might get prematurely activated by attaching to shed-off mucus.

1.9.3.2 Bacterial adhesions

Bacteria contain certain proteins that are capable of site specific binding. For example some of the pathogenic bacteria's like *E.coli* contain a protein called K99-fimbriae[246] which can readily attach to the gastrointestinal tract. This was even proved experimentally when this protein K99-fimbriae was covalently attached to poly(acrylic acid) networks which then showed increased adhesion *in vitro* compared to unmodified (poly-acrylic) acid[246].

1.9.3.3 Thiolated polymers

Thiolated polymers are second generation polymers with many folds increase in bioadhesive properties. These are hydrophilic polymers that have been thiolated. The presence of thiol groups allows the formation of covalent bonds with cysteine-rich domains of mucus gel layers leading to increased residence time and improved bioavailability[247]. The thiomers mimic the natural mechanism of secreted mucus glycoproteins that are covalently anchored in the mucus layer by the formation of disulphide bonds[248,249]. These interactions are less susceptible to changes in ionic strength and pH[250] as they involve covalent bonding. The presence of disulphide bonds significantly alters the mechanism of drug release due to increased rigidity and cross linking. E.g[251]. Chitosan-iminothiolane complexed polymer showed 250 folds increase in mucoadhesive properties. Poly (acrylic acid)- cysteine showed 100 fold improvement in mucoadhesive properties. Chitosan- thioglycolic acid showed 10 fold improved mucoadhesive properties. Alginate-cysteine showed four-fold increase in mucoadhesive properties. Sodium carboxymethyl cellulose-cysteine showed improvement in the mucoadhesive properties.

1.10 Barrier properties of mucus

The mucus is a barrier to the drugs from a pharmacists point of view. Earlier it was thought that only small molecules diffuse through the mucus membrane. This seemed reasonable as it would permit the end products of digestion to penetrate the mucus coat to reach the enterocytes, but the mucus coat would prevent digestive enzymes from attacking these cells. Recent work clearly demonstrated that particles much larger than digestive enzymes (even larger than 500 nm) can diffuse through mucus gels[252,253]. The thickness of individual mucin fiber, when observed biochemically was 3-10 nm. But when freshly prepared mucus is observed by electron microscopy it showed thick mesh fibres with diameter of 30-100 nm[254] which is 10 folds higher than the actual thickness. This was due to absorbing of

other constituents of the mucus gel like antibodies, lysozyme, lactoferrin, albumin, etc. Individual mucin fibre showed a thickness of 5-7 nm[222]. When the individual fibres were allowed to settle down they showed a “kinky” appearance and flexible with curvatures with uniform thickness of 15 nm[255]. When individual fibres were observed in solutions they appeared as randomly coiled structures. The diffusion speeds of particles[253] like globular proteins including bovine serum albumin, a macromolecular protein, human IGM, and two capsid viruses Norwalk virus and human papilloma virus, 500 nm polyethylene glycosylated nanoparticles were tested through the human cervical mucus. Nearly all soluble globular proteins travelled with the same speed through the mucus except for secreted antibodies. Secreted antibodies showed two folds decrease in their diffusion speeds as the antibodies formed weak low affinity bonds with the mucus fibres. The secreted antibodies are slightly mucophilic this also helps in entrapping the pathogens from the surface of the mucus[256]. When polystyrene spheres were allowed to pass through the mucus they were entrapped by the mucus network/ mesh, but when same sized capsid viruses were tested they diffused through the mucus easily[257]. This was not due to mesh pore size but the actual reason was that the polystyrene spheres were hydrophobic in nature and these inter actions reduced the speeds of polystyrene spheres through the mucus. Even though polystyrene was coated with negative charge or covalently bonded with bovine serum albumin or casein the polymer was still left entrapped in the mucus network. When the polystyrene was densely coated with polyethylene glycol even then the polymer was trapped by mucus network. 500 nm PEGylated microspheres were slowed down by four times in human cervical mucus[252]. This gave rise to predictions that the mucus mesh size is 400 nm by Amsden[258]. The nanoparticles might have been slowed down by multiple low affinity bonds with mucin fibres.

1.11 Factors affecting mucoadhesion

1. Molecular weight:

The optimum molecular weight for bioadhesion for polymer is between 10^4 and 4×10^6 Dal. The threshold required for successful bioadhesion is at least 100,000[259] molecular weight. If the molecular weight is much higher as in the case of nonlinear dextrans molecules like 19,500,000 then the adhesive groups will be shielded with its helical conformation[259]. This leads to reduced bioadhesive strengths, which is similar to linear polyethylene glycol of molecular weight of 200,000. Whereas for low molecular weight polymers inter penetration is key to having good bio-adhesive strengths[259]. Low molecular weight polymers penetrate the mucus layers better[260]. For linear molecules the mucoadhesion increases with molecular weight. Polymers with higher molecular weights will not moisten quickly to expose free groups for interaction whereas polymers with low molecular weight form loose gels and dissolve quickly[5]. High molecular weight promotes physical entangling.

2. Concentration:

The concentration of active polymers plays an important role in bioadhesion. Optimum concentration is required for the polymer to produce maximum bioadhesion. Beyond the optimum concentration of polymer the adhesive strength drops significantly because the coiled molecules become separated from the medium and the chain available for inter penetration decreases[234].

3. Polymer chain flexibility:

Polymer chain flexibility is required for diffusion of chains and their entanglement with mucin. For polymers with high levels of linkage, the mobility of individual polymer chain decreases which leads to reduced mucoadhesion strengths[261].

4. Spatial conformation:

The spatial conformation of molecule decides its mucoadhesive strengths. Despite having high molecular weight of 19,500,000 for dextrans, they have adhesive strengths similar to that of polyethylene glycol, with a molecular weight of 200,000. The helical conformation in dextrans shields the active groups necessary for bioadhesion unlike polyethylene glycol which has linear confirmation.

5. Ability to form hydrogen bonds:

Presence of hydrogen bonding functional groups like COOH, OH, affects the mucoadhesion[261].

6. Swelling:

Swelling of polymers allows mechanical entangling by exposing the polymer chains and subsequent formation of hydrogen bonds and electrostatic interactions between polymer and mucosa[262]. Over hydration results in wet slippery mucilage without adhesion, optimum water content is required for dynamic mucoadhesion. Swelling of polymers depends upon optimum concentration, ionic strength and presence of water.

7. pH:

Changes in the pH lead to differences in the extent of dissociation of functional groups in carbohydrate sequences or polypeptide amino acid sequences, as well as in the polymer[260].

8. Applied strength:

For the successful mucoadhesion of a solid bioadhesive system, it is necessary to apply a defined strength. The pressure initially applied to the mucoadhesive tissue can affect the depth of interpenetration of the polymer chains[263]. The adhesion strength increases with the applied strength and duration of application. If high pressure is applied for sufficiently long time the polymers becomes mucoadhesive even though they do not have attractive interactions with the mucin.

9. Initial contact time:

Contact time determines the extent of swelling and diffusion of polymer chains[5]. Bioadhesive strength increases with contact time.

10. Moistening:

Moistening is required to allow the mucoadhesive poymer to spread over the surface and create a macromolecular network of sufficient size for interpenetration of polymer and mucin molecules and to increase the mobility of polymer chains[262]. However critical level of hydration is required for optimum swelling and bioadhesion[5].

11. Presence of metal ions:

Interaction with charged groups of polymers and mucus can decrease the number of interaction sites and the tightness of mucoadhesive bonding[264].

12. Mucin turnover:

No matter how strong the bioadhesive interactions are, the mucin turnover dislodges the adhesive system from the surface, which results in reduced residence time. Mucin turnover varies across different types of mucosa. In buccal the cavity the mucin secretion depends on the presence of food materials. Additionally in the gastric mucosa during the early stages of fasting the mucin accumulates on the liminal surface of tissue, which moves with freshly released acid or moving food particles[265]. Lehr et al. calculated a mucin turnover of 47-270 min[265]. The ciliated cells in the nasal cavity transport the mucus to the throat at the rate of 5 mm/min and in the tracheal region mucociliary clearance was found to be 4-10 mm/min.

13. Disease state:

The physiochemical properties of the mucus are known to change during disease conditions such as common cold, gastric ulcers, ulcerative colitis, cystic fibrosis, inflammatory conditions of eye and bacterial and fungal infections of female reproductive tract. The exact structural changes are unknown but if the mucoadhesive drug delivery systems are to be used in disease conditions then the adhesive systems need to be evaluated under same conditions[5,264].

14.Tissue movement:

Mucoadhesion depends upon tissue movements like the presence or absence of liquid or food, speaking and peristaltic movements of the gastrointestinal tract[5].

1.12 Mucoadhesion:

Mucoadhesion is described as phenomenon of adhesion between two surfaces in which one is a mucous membrane. Bio-adhesion also has a similar meaning which can be described as adhesion between two materials in which one is of biological in nature. These mucoadhesive polymers are hydrophilic macromolecules containing numerous hydrogen forming groups e.g. carbomers, chitosan. Mucoadhesion can be a potential delivery system for challenging molecules like proteins and oligonucleotides. Mucoadhesion between the delivery system and mucosal membranes takes place by chemical bonds. Mucoadhesion is complex process which can be explained by some theories and broad definitions.

1.12.1 Chemical bonds

The adhesion of delivery systems to the mucosa can occur by different types of bond formations:

1. Ionic bonds: here two oppositely charged ions attract each other via electrostatic interaction to form a strong bond.
2. Covalent bonds: here electrons are shared in pairs between the bonded atoms in order to ‘fill’ the orbitals in both. These are strong bonds.

3. Hydrogen bonds: here a hydrogen atom, when covalently bonded to electronegative atoms such as oxygen, fluorine or nitrogen, carries a slightly positive charge and therefore is attracted to other electro negative atoms. Hydrogen bond formed is generally weaker than ionic or covalent bonds.
4. Van-der-Waals bonds: these are the weakest forms of interactions that arise from dipole-dipole and dipole-induced dipole attractions in polar molecules, and dispersion forces with non-polar substances.
5. Hydrophobic bonds: these bonds occur when non-polar groups are present in an aqueous solution. In order to reduce the system entropy the water molecules form hydrogen bonds with surrounding water molecules around non-polar groups and to counter this effect the non-polar groups associate with each other.

1.12.2 Theories of mucoadhesion[266]

1. Electronic theory: states that electron transfer occurs upon the contact of adhering surfaces due to differences in their electronic structure. This results in the formation of an electrical double layer at the interface and subsequent adhesion due to attractive forces.
2. Wetting theory: wetting theory deals with liquid with its surface tension spreading over a solid surface of certain surface energy and interfacial energy between them. Prerequisite is that the liquid should spread spontaneously over the solid surface for the development of adhesion. The affinity of liquid for a surface can be measured with contact angle goniometry to measure the contact angle of the liquid. The smaller the contact angle the greater is the affinity (**Figure 6**). The spreading coefficient (S_{AB}) can be calculated from the surface energies of solid and liquid using the equation

$$S_{AB} = \gamma_B - \gamma_A - \gamma_{AB}$$

Where γ_A is the surface tension of the liquid A and surface energy of solid B. S_{AB} should be positive for the liquid to spread spontaneously over the solid.
The work of adhesion (W_A) represents the energy required to separate two phases, and is given by:

$$W_A = \gamma_A + \gamma_B - \gamma_{AB}$$

The greater the individual surface energies of the solid and liquid relative to the interfacial energy, the greater is the work of adhesion.

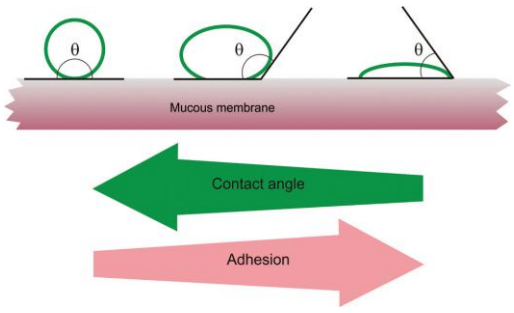


Figure 6[267]*: Showing influence of contact angle between device and mucus membrane on bioadhesion*

3. Adsorption theory: has a subsection called chemisorption theory which assumes that former theory leads to hydrogen bonding and van-der-walls interactions and the later assumes that the interaction is due to strong covalent bonding.
4. Diffusion theory: states that adhesion takes place by diffusion of polymer chains into the interface. The factors like polymer concentration, chain length, mobility and time of contact play a major role in strengthening the adhesive bond.
5. Mechanical theory: states that adhesion arises by interlocking of liquid adhesive on the rough surfaces.
6. Fracture theory: this theory relates the forces required for detachment with the adhesive strength.

1.12.3 Scenarios involved in mucoadhesion

Mucoadhesion is a complex process which can be explained by a single mechanism, hence the various scenarios which are involved in mucoadhesion are:-

1a. Dry or partially hydrated dosage forms contacting surfaces with substantial mucus layers e.g. nasal cavity.

1b. Dry or partially hydrated dosage forms contacting surfaces with thin/discontinuous mucus layers e.g. oral cavity and vagina.

2a. Fully hydrated dosage forms contacting surfaces with substantial mucus layers e.g. GI tract

2b. Fully hydrated dosage forms contacting surfaces with thin/discontinuous mucus layers e.g. Oesophagus, eye.

1.12.4 The study of adhesion involves two stages

Stage 1: Contact stage

Stage 2: Consolidation stage

1.12.4.1 Contact stage

The mucoadhesive and the mucus membrane have to become very intimate (**Figure 7**) for the adhesion to occur. In case of accessible mucous regions like oral cavity the mucoadhesive can be held together with the mucus membranes by hand for sufficient time for the adhesion to take place. But if the mucus regions are not accessible then other mechanisms have to be considered. In case of gastro intestinal tract, peristaltic movements can be utilised for bringing the mucoadhesive together with the mucus membrane. The principles of DLVO theory (described by Derjaguin, Landau, Verwey and Overbeek) was described to explain the stability of colloids but it was modified later to describe the physicochemical processes involved in adsorption of bacteria on to the surfaces. The adsorption of bacteria onto a surface is considered similar to the as adsorption of small micro-particles onto a surface. The small particles in the body will experience Brownian motion as during peristalsis and repulsive and attractive forces. The repulsive forces are due to osmotic pressure effects and the attractive forces due to van-der-Walls interactions, surface energy effects and electrostatic interactions. The smaller the particle, the greater the attractive forces due to surface-area-to-volume ratio. Attractive forces must be greater than

the repulsive forces (potential energy barrier) for a strong adhesion to occur. Weak adhesion occurs when the distance between the particle and the mucus layer is circa 10 nm and a strong bond occurs when the distance is circa 1 nm. Optimum molecular weight for mucoadhesion ranges from circa 10^4 Da to circa 4×10^6 Da. The liquid comes in close contact with the surface when the liquid layer is stationary/ unstirred and the distance between the surfaces increases with increase in flow rate. The GI mucosa is described as unstirred layer.

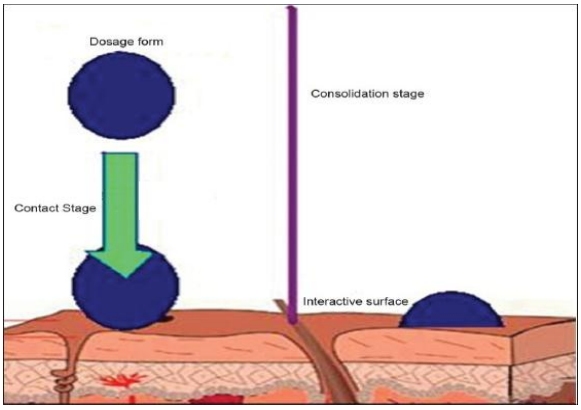


Figure 7 *showing contact stage & consolidation stage*[268]

1.12.4.2 Consolidation stage

The consolidation stage deals with making the adhesive, interface and the mucus layer into a uniform single unit (**Figure 7**). This stage is especially important if the adhesive is subjected to wear and tear forces like retinal blinking, mechanical movements of mouth. Surface energies help in adhesive bonds and also if the mucoadhesive is a dry material it tries to absorb water from the surrounding environment thus acting as suction pumps (carbomers), which help in attaching to the solid surface. In case of a cationic polymer like chitosan the electrostatic interactions with negatively charged carboxyl or sulphate groups on the mucin molecule helps strengthening the adhesive bond. Even the anionic polymers like carbopols form adhesive bonds with the mucosa with hydrophobic interactions, hydrogen bonding, and van-der-Waals interactions. If the mucus is of a single layer then the dry mucoadhesive polymer will absorb the water content from the mucus gel so that it can establish hydrogen bonds with the epithelial surface. But if the mucus is a thick covering then it is not possible to extract all the water from gel, so altering the physicochemical properties of a mucus layer is important. The consolidation or gel strengthening can be explained by two theories

a. Macromolecular interpenetration effect:-

This theory is similar to the diffusion theory. This theory states that for compatible polymeric systems the mucoadhesive molecules interpenetrate and bond by secondary interactions with the mucus glycoproteins. This theory was explained by ATR-FTR spectra and fluoresceinamine technique.

b. Dehydration theory:-

This theory states that when a dry rapidly water-absorbing polymer is placed in contact with a second gel then the water is drawn until equilibrium is achieved along with consolidation of interface. So in this theory osmotic pressure and

large swelling force play an important role in consolidating the joint e.g. poly (acrylic acid) has a high affinity to draw water.

1.12.5 Specific and nonspecific bioadhesion

The nonspecific bioadhesive synthetic polymers will adhere to most of the cell surfaces and mucus membranes. The exact mechanism of nonspecific adhesion at molecular level is still unknown. In specific bioadhesive polymers especially plant lectins also referred to second generation bioadhesives will bond to specific target chemical molecules by recognising the sugar arrays. Some other specific bioadhesives are bacterial fimbrins and invasins. Some of the examples are shown in Table 8.

Table 8 Examples[269] of specific and non-specific mucoadhesive polymers

S.no	Type of adhesion	Polymers & lectins (proteins)
1	Nonspecific	Polycarbophyll, carbopols, chitosan.
2	Specific	Tomato lectin, Ulex europaeus 1 agglutinin, wheat germ agglutinin, phaseolus vulgaris agglutinin, bacterial adhesins, bacterial invasins.

1.12.6 Mucoadhesion comparison

When different mucoadhesive polymers were tested for their mucoadhesion various interesting conclusions were drawn. For the same polymer diverse adhesion strengths were recorded at different pH ranges. Lyophilization increased the mucoadhesion of polymers[270]. The pH of the polymer and the drying methods were found to be important factors in deciding mucoadhesive potential[270]. Second generation thyolated polymers possessed many times high mucoadhesive strengths. Some of the polymers and their mucoadhesion strengths were[270]:

Chitosan-4-thiobuthylamidine pH 3 lyophilized > chitosan-4-thiobuthylamidine pH 6.5 precipitated > polycarbophil-cysteine pH 3 lyophilized > chitosan-4-thibuthylamidine pH 6.5 lyophilized > PAA_{450}-cysteine pH 3 lyophilized > PAA_{450}-cysteine pH 7 precipitated > carbopol 980 pH 7 precipitated > carbopol 974P pH 7 precipitated > polycarbophil pH 7 precipitated > carbopol 980 pH 3 lyophilized.

1.13 Sites of mucoadhesive drug delivery systems

1. The buccal cavity: The buccal cavity has a total surface area of 50 cm^2. The main advantages of buccal delivery systems are to localize the drug action and to prevent the first pass metabolism. Additionally the absence of aggressive peptidase enzymes in stomach and small intestine helps the delivery of peptides, non-keratinised mucosa is permeable to large molecular size drugs. Examples are mentioned in **Table 9**.

Table 9 Examples of some commercially available buccal mucosal delivery systems

s.no	Drug	Polymers	Duration of action
1	Triamcinolone tablet[271]	Carbopol, carboxymethyl cellulose	8 hours
2	Morphine tablet[272]		6 hours
3	Pentazocine compacts[273]	Carbopol, hydroxypropylmethyl cellulose	
4	Glucagon peptide[274]		
5	Oxytocin peptide[275]		
6	Buprenorphine[276] by 3M company matrix patch	Polymers + polymeric elastomers covered by backing	12 hours placed under upper lip

The nasal cavity:

The nasal cavity has a surface area between 150 and 200 cm^2. The main advantages of nasal drug delivery are the avoidance of first pass metabolism and that the nasal mucosa is highly vascularised and relatively permeable. The disadvantage is mucociliary action which clears the mucus at rates of 10mm/min, with a residence time between 15-30 minutes. An important aspect to consider while designing a mucoadhesive delivery system for nasal mucosa is that it should not hamper the normal mucociliary action. So bioadhesive microparticles are more suitable for nasal mucosa. Peptides (insulin), vaccines, drug can be delivered through nasal membrane. A predominantly used polymer for nasal mucoadhesion is chitosan. Even though carbopol showed longer residence times and higher bioavailability[277] than chitosan, some adverse inflammatory reactions for carbopol 971P when tested on rabbit nasal mucosa is recoded in literature. Therefore carbopol is not suitable[278] for nasal delivery systems.

The eye:

The bioavailability of drugs administered through the eye is < 5% due to rapid washing away the dosage forms with tear fluid and the frictional forces of palpebral fissure of eye. This route is utilised to treat some local conditions and not for systemic drug delivery. Carbopol formulations are often used for eye dosage formulations to enhance the viscosity rather than utilizing mucoadhesiveness. The disadvantages are gel like formulations affects the vision by blurring. Bioadhesive microparticles and inserts are promising for ocular drug delivery[279,280]. The tear turnover is between 0.5-2.2 µl/min, this results in tear turnover rate of 16%/min during waking hours[281].

The gastrointestinal tract:

The GI tract is the most important site for bioadhesion. Robinson and co-workers have pioneered in the use of bioadhesive polymers in increasing the bioavailability of drugs. Chitosans (trimethyl chitosan) and carbomers apart from mucoadhesive function also increase the intestinal permeation of drugs by opening the tight junctions between the cells. Site specific binding plant lectins

and bacterial fibrins and invasins help delivering the drug to required sites on GI mucosa. Tomato lectins specifically recognises and bind to N-acetylglucosamine-containing complexes[282]. Bioadhesive micro and nanoparticles which release the encapsulated drug only after certain degree of bioadhesion, look promising. Some of the GI mucoadhesive formulations are shown in Table 10

Table 10 Examples of mucoadhesive polymers used for enhancing bioadhesion in the GI tract

S.no	Drug	Polymers	Observation
1	chlorothiazide	Carbophil	Tested on rabbits and it showed increased bioavailability of drug
2	griseofulvin		Increased bioavailability in rabbits but results in humans were disappointing[283].
3	Radio labelled compound	Poly (acrylic acids)	Lower molecular weight materials were cleared more rapidly than the higher molecular weight substances. But the overall GI transit time was similar[284].
4	Octrotide, buserelin	Chitosan	The bioavailability of therapeutic peptides is dramatically increased[285].
5	Buserelin	Carbomer	Enhanced the bioavailability of the peptide[286].
6	Model drug	Tomatolectin conjugated with nano spheres	50 fold increase in uptake of model drug[287].
7	calcitonin	Chitosan-coated DL-lactide /glycolide copolymer nanospheres	Tested on rabbits which showed enhanced and prolonged action of calcitonin[288].

The vagina:

The vagina is highly suitable for bioadhesive formulations. This route can be used to treat local infections, delivering spermicides and hormones. Polycarbophil and carbomer containing bioadhesive formulations have shown retention time upto 72 hours. Advantage-S ™ containing the spermicide nonoxinol-9 is used as a contraceptive. Crinone® which is designed to release progesterone for atleast 48 hours after a single application, is in use[289]. The use of bioadhesive vaginal formulations can further extend horizons like in the treatment of vaginal infections e.g. *Candida albicans*[290].

1.14 *In vitro* methods for Measurement of mucoadhesion

There are many *in vitro* methods for the evaluation of mucoadhesion:

1. Measurement of tensile strength[291]: This method measures the force required to break the adhesive bond between a model membrane and test polymer. Robinson et al. used a modified tensiometer to measure the bioadhesive force. A section of tissue, having the mucus side exposed, was secured on a weighed glass vial placed in a beaker containing USP-simulated gastric fluid. Another

section of the same tissue was placed over a rubber stopper, again with the mucus side exposed, and secured with a vial cap.

2. Measurement of shear strengths[292]: Shear stress is a measure of force that causes the bioadhesive to slide with respect to the mucus layer in a direction parallel to the plane of contact. Wilhelmy plate method was reported by Smart et al. This method uses a glass plate suspended from a microbalance that is dipped in a temperature-controlled mucus sample. The force required to pull the plate out of solution is measured.

3. Adhesion weight method: This method a suspension of ion exchange resin particles flowing over the inner mucosal surface of a section of guinea-pig intestine and the weight of adherent particles was determined[293]. The drawback of this method is poor data reproducibility resulting from fairly rapid degeneration and biological variation of the tissue. It was possible to calculate the particle size and charge on adhesion after 5 min contact with averted intestine.

4. Fluorescent probe method: Park and Robinson studied the polymer interactions on conjunctival epithelial cell membrane and oral mucosa using fluorescent probes[294]. The aim of the study was to understand the structural requirements for bioadhesion. The membrane lipid bilayer and membrane proteins were labelled with pyrene and fluorescein isothiocyanate, respectively. The cells were then mixed with candidate bioadhesive, and the changes in fluorescence spectra were monitored. This gave a direct indication of polymer binding and its influence on polymer adhesion.

5. Flow channel method[295]: In this method a thin channel made of glass and filled with 2% (w/w) aqueous solution of bovine submaxillary mucin, thermostated at 37°C. Humid air at 37°C was passed through the glass channel. A particle of a bioadhesive polymer was placed on the mucin gel. The static and dynamic behaviour was monitored at frequent intervals using a camera.

6. Mechanical spectroscopic method[296]: This method used Carri-Med CSL 100 rheometer with a 4-cm parallel plate of 0.5mm gap. They studied the effect of introduction of carbopol-934P on the rheological behaviour of mucus gel. This method investigated the role of mucus glycoprotein and effect of various factors such as ionic concentration, polymer molecular weight, its concentration and the introduction of anionic, cationic, and neutral polymers on the mucoadhesive mucus interface.

7. Falling liquid film method[297]: Teng et al. developed a falling liquid film method. Small intestine segments from rats were placed at an inclination of a tygon flute. The adhesion of particles in a suspension when passed over the inclined intestine segments was recorded. The adherent strengths of different polymers can be determined.

8. Colloidal gold staining method[298]: This method uses the formation of mucin–gold conjugate. The mucin molecules get adsorbed onto the colloidal gold particles and stabilize them. The interaction between them could be easily quantified, either by measurement of the intensity of red color on the hydrogel surface or by measuring the spectroscopic changes occurring at 525 nm[298].

9. Viscometric method[299]: In this method the viscosities of 15% (w/v) porcine gastric mucin dispersion of 0.1 N HCL (pH 1) or 0.1 N acetate (pH 5.5) were measured with a Brookfield viscometer in the absence or presence of selected neutral, anionic and cationic polymers. Viscosity and bioadhesive forces were calculated[299].

10. Electrical conductance[300]: This method uses the modified rotational viscometer capable of measuring the electrical conductance. The polymer adhesion was tested on artificial bio-membrane and artificial saliva. The parameter, measured as a function of time, was found to be influenced by the sample, artificial saliva and artificial bio-membrane. The conductance was low in the presence of adhesive material. The conductance was compared with pure saliva and the values are calculated.

11. Texture analysis[301]: This is a software controlled penetrometer, TA-XT2 Texture Analyzer (Stable Micro Systems, UK). It contains a 5 Kg load cell, a force measurement accuracy of 0.0025% and distance resolution of 0.0025 mm. The mucoadhesion forces can be calculated accurately by this instrument. In this test force required to remove the formulation from a model membrane is measured which can be a disc composed of mucin, a piece of animal mucus membrane. Based on the results force–distance curve can be plotted. This method is frequently used to analyse semi-solid and solid materials.

1.15 *In situ* forming hydrogels

In situ forming hydrogels are in liquid form before administration into the body and they undergo gelation due to the physiological changes like temperature, pH, ions etc. at the site of administration. Administration of *in situ* forming hydrogel in fluid form for sustained drug delivery of drugs at the desired organ, tissue or body cavity[302] increases patient compliance and is also less invasive. The advantages of *in situ* forming hydrogels include ease of administration, reduced frequency of administration, patient compliance and comfort. Biodegradable polymers increase the safety on administration into the body e.g. gellan gum, alginic acid, xyloglucan, pectin, chitosan, poly(DL-lactic acid), poly(DL-lactide-co-glycolide) and poly-caprolactone[303]. *In situ* forming hydrogels can be classified into three types.

1.15.1 Classification of in situ forming hydrogels

1. *In situ* formation based on physiological stimuli.

2. *In situ* formation based on physical mechanism-swelling.

3. *In situ* formation based on chemical reactions.

In situ formation based on physiological stimuli

these can be further classified into thermally triggered systems and pH triggered systems.

a. Thermally triggered systems[303]:

The thermally triggered systems can be further classified into three categories: negatively thermo-sensitive, positively thermo-sensitive and thermally reversible

gels. Negative thermo-sensitive polymers have lower critical solution temperature, i.e. they undergo gelation by increase in temperature-e.g. PNIPAAm. This polymer is water soluble below LCST but hydrophobic above LCST. Positive temperature-sensitive polymers have upper critical solution temperature (UCST), such polymers contract (gel) upon cooling e.g. poly (acrylic acid), polyacrylamide, poly (acrylamide-co-butyl methacrylate. Thermo-reversible polymers can undergo reversible gelation e.g. pluronics®.

b. pH triggered systems[303]:

The pH sensitive polymers undergo gelation by changes in pH. These polymers swell in response to pH changes. pH sensitive polymers contain pendant acidic or basic group that either accept or release protons with pH changes. Swelling of the hydrogel increases with pH, in case of weakly acidic anionic drugs, and decreases in case of weakly basic cationic groups. Examples of anionic pH sensitive polymers are carbopol and carbomer. Poly (acrylic acid) solutions undergo gelation at neutral pH.

1.15.1.1 In situ formation based on physical swelling

a. Swelling: Some polymers absorb water and expand to occupy the desired space, e.g. glycerol mono-oleate (myverol18-99). This polymer is a polar cationic lipid that swells in water to form lyotropic liquid crystalline phase structures[303].

b. Diffusion: This method involves the diffusion of a solvent from the polymer solution into the surrounding tissue and results in precipitation and solidification of a polymer matrix. An example is N-methyl pyrrolidone.

1.15.1.2 In situ formation based on chemical reactions

a. Ionic cross-linking:

Some of polysaccharide polymers undergo phase transition in the presence of ions[304]. K-carrageenan forms rigid brittle gels in the presence of K^+. I-carrageenan forms elastic gels in the presence of Ca^{2+} ions. Gellan gum is an anionic polysaccharide that undergoes gelation in the presence of monovalent and divalent cations like Ca^{2+}, Mg^{2+}, K^+ and Na^+.

b. Enzymatic cross-linking:

These are intelligent stimuli-responsive delivery systems whose gelation mechanism is catalized by natural enzymes. This method is non-toxic compared to monomers and initiators depended gelation mechanisms[303]. E.g. cationic pH-sensitive polymers containing immobilized insulin and glucose oxidase can swell in response to blood glucose level releasing the entrapped glucose in pulsatile fashion[305].

c. Photo-polymerisation:

These polymers require an electromagnetic radiation or UV or visible wavelengths for the initiation of polymerization. A solution of monomers or reactive macromer and initiator can be injected at the tissue site and appropriate application of radiation leads to formation of gel. Monomers and macromers containing acrylate fuctional groups can easily undergo photo polymerisation. A ketone is used as an intiator for UV photo-polymerisation and camphorquinone

and ethyl eosin are used as initiators for visible light systems. This method provides rapid polymerisation at the site and fibre optic cable can be used for photo-curing[303].

1.15.2 Genaral classification of commonly used in situ polymeric systems

1. Pectin:

Pectins are a family of polysaccharides with polymer backbone of α-(1-4)-D-galacturonic acid residues[303]. They readily undergo gelation in the presence of calcium ions and H^+ ions. A source of divalent ions is especially calcium ions which are suitable for drug delivery purposes. The galacturonic acid chains crosslink in the manner of egg-box model.

2. Xyloglucan:

Xyloglucan exhibit thermally reversible gelation. It is a polysaccharide derived from tamarind seeds with a backbone composed of (1-4)-β-D glucan chain, which has (1-6)-α-Dxylose branches that are partially substituted by (1-2)-β-D-galactoxylose[306]. Xyloglucan has to be partially degraded by β-galactosidase for thermo-reversible gelation properties. The sol-gel transition temperature varies with the degree of galactose elimination. These polymers can be used for oral, ocular and rectal drug delivery systems.

3. Gellan gum:

Gellan gum is an anionic deacetylated exocellular polysaccharide secreted by pseudomonas elodea with a tetrasaccharide repeating unit of one α-L-rhamnose, one β-D-glucuronic acid and two β-D-glucuronic acid residues[307]. Their gelation can be either temperature dependent or cation induced. Gellan gum can be used for formulating oral drug delivery systems.

4. Alginic acid:

Alginic acid is a linear block copolymer polysaccharide consisting of β-D-mannuronic acid and α-L-glucuronic acid residues joined by 1,4-glycosidic linkages. They undergo gelation in the presence of divalent and trivalent metal ions. They can be used for ophthalmic delivery as they are nontoxic and biodegradable[303].

5. Xanthum gum:

Xanthum gum is a high molecular weight extracellular polysaccharide produced by the fermentation of gram negative bacteria Xanthomonas campestris. It contains a cellulosic backbone β-D-glucose residues and a trisaccharide side chain of β-D-mannose-β-D-glucuronicacid-α-D-mannose attached with alternate glucose residues of main chain[303].

6. Chitosan:

Chitosan is a biodegradable, thermosensitive, pH sensitive, polycationic polymer[303] obtained by deacetylation of chitin, a natural component of shrimp and crab shell. It is pH sensitive it is soluble in solutions below pH 6.2[308], and above which it forms hydrated gel like precipitate. They also behave as thermosensitive polymers if polyol salts bearing a single anionic head such as

glycerol, sorbitol, fructose or glucose phosphate salts are added to the chitosan aqueous solutions[309].

7. Carbopol:

Carbopol is a pH dependent polymer, which stays in solution form at acidic pH but undergoes gelation at alkaline pH[303].

8. Pluronic/ Poloxamers303:

Poloxamers are non-ionic surfactants with thermo-reversible properties. They are composed of alternating polyethylene oxide-polypropylene oxide –polyethylene oxide. PPO is the hydrophobic moiety. They are available in different molecular weights. No single molecular weight poloxamers undergo gelation at physiological conditions. Different molecular weight poloxamers can be blended together for achieving gelation temperature near physiological conditions[303].

2 AIM OF THE THESIS

The aim of the study was to formulate an *in situ* gelling hydrogel which gels at physiological conditions and to incorporate sub-antimicrobial dose of doxycycline into it, for the treatment of recurrent aphthous ulcers (RAU) in the oral cavity. It is known that doxycycline has the high matrixmetalloproteinases (MMP) inhibitory effect and MMPs role in RAU has been proved. Doxycycline is unstable in aqueous formulations, and there is no reliable information regarding the stability from previous studies. Due to this stability drawback there are no readily (commercially) available doxycycline aqueous formulations. There are only 2 commercially available aqueous formulations of doxycycline named ATRIDOX® and Vibramycin®. Both the formulations are not readily usable by the customers as they come with doxycycline in powder form which needs to be added with either gel (readily supplied along with powder doxycycline) in the case of ATRIDOX® with mechanical mixing by joining two syringes, or specified quantity of water in the case of Vibramycin®, which is a syrup suspension and should be used within 15 days from the date of adding water to powdered doxycycline. ATRIDOX® is used to treat periodontal pockets. The main aim of this project was to formulate a doxycycline containing stable hydrogel with a shelf life of at least 2 years.

The aims of the thesis can be described as follows:

1. To select a non-ionic surface active polymer and preferably even the mucoadhesive polymers should be non-ionic. From a previous study it was evident that the charge on a polymer might affect the stability of doxycycline as the stability of doxycycline decreased when it was coated with carbopol (negatively charged[5]).

2. To incorporate the predominantly lipophilic[310] doxycycline into the micelles (lipophilic component) of a surface active agent, thereby protecting the doxycycline from oxidation.

3. To select a suitable pH (either weekly acidic, neutral or basic region) for the formulation, which itself might contribute to the stability of doxycycline.

4. To complex the epimerization prone sites of doxycycline with suitable complexing agents like EDTA, HPβCD, Mg^{++}, β-CD.

5. To add suitable antioxidants to the formulations, as not all antioxidants improve the stability of doxycycline.

7. To select suitable non-ionic mucoadhesive polymers which are also hydrophilic polymers, so that when they are added to poloxamer surfactants they would occupy the hydrophilic parts and leave the hydrophobic region for doxycycline. And also to evaluate the mucoadhesion strengths of formulated *in situ* forming hydrogels with the Texture analyser.

8. To measure the viscosities of the formulated *in situ* forming hydrogels.

9. To study the release behaviour of doxycycline from polymer matrices by suitable method.

3 MATERIALS AND METHODS

3.1 Material

3.1.1 Chemicals

3.1.1.1 Preparation of hydrogels in stability tests

Materials	**Manufacturer**	**Origin**
Doxycycline hyclate standard	Sigma-Aldrich	Germany
Doxycycline hyclate	HOVIONE	Macau
Metacycline	European Pharmacopoeia reference standards (Council of Europe)	Strasbourg
6-epidoxycycline	European Pharmacopoeia reference standards (Council of Europe)	Strasbourg
HPβCD	Roquette pharmaceuticals	France
Poloxamer 407	Sigma	USA
	BASF	Ludwigshafen, Germany
Poloxamer 188	Sigma	USA
	BASF	Ludwigshafen, Germany
HPMC	Norsk Medisinaldepot	Oslo
POVIDONE	Sigma	Germany
$Na_2S_2O_3$	Sigma-Aldrich	Germany
$Na_2S_2O_5$	Sigma-Aldrich	Germany
Citric acid	Merck	Germany
Tartaric acid	Sigma-Aldrich	Germany
$MgCl_2$	Merck	Germany
EDTA	Riedel-de Haën	Germany
HCl	Riedel-de Haën	Germany
NaOH	Sigma-Aldrich	Sweden
Methanol	Sigma-Aldrich	Spain
Milli-Q water	Millipore	

3.1.1.2 Preparation of mobile phase according to European & British Pharmacopoeia

Materials	Manufacturer	Origin
tert-Butanol	Riedel-de Haën	Seelze, Germany
	Sigma-Aldrich	Germany
KH_2PO_4	Fluka	Germany
NaOH	Sigma-Aldrich	Sweden
Tetrabutyl ammonium-bisulfite	Fluka	Switzerland
EDTA	Riedel-de Haën	Germany
Dilute NaOH solution	Sigma-Aldrich	Sweden
Dilute HCl solution	Riedel-de Haën	Germany
Milli-Q water	Milli-Q® Academic	

3.1.1.3 Preparation of mobile phase: Skuli's method

Materials	Manufacturer	Origin
Acetonitrile	Sigma-Aldrich	Germany
$HClO_4$	Merck	Germany
Milli-Q water (deionised)	Milli-Q® Academic	Millipore
5M NaOH solution	Sigma-Aldrich	Germany

3.1.1.4 Solubility testing of poloxamers in different buffer solutions

Buffer solution	Materials	Manufacturer	Origin
Phosphate buffered saline 7.4 (European pharmacopoeia)	NaCl	Riedel-de Haën	Germany
	Na_2HPO_4	Riedel-de Haën	Germany
	KH_2PO_4	Fluka	Germany
	Milli-Q water		
Phosphate Buffer solution 6.8 (European Pharmacopoeia)	Na_2HPO_4	Riedel-de Haën	Germany
	Citric acid	Merck	Germany
	Milli-Q water	Millipore	
Phosphate buffer 6.6 (USP)	KH_2PO_4	Fluka	Germany
	NaOH	Sigma-Aldrich	Germany
	Milli-Q water	Millipore	

3.1.1.5 Preparation of Hydrogels for Mucoadhesion analysis

Materials	Manufacturer	Origin
Poloxamer 407	BASF	Ludwigshafen, Germany
Poloxamer 188	BASF	Ludwigshafen, Germany
HPMC	Norsk Medicinal depot	Oslo
CMC	Sigma	Germany
POVIDONE	Sigma	Germany
Chitosan	Sigma	Germany
Polyethylene glycol PeG 6000	Fluka	Germany
Carbopol 974P	Noveon	Cleveland, USA
Polyvinyl alcohol	Sigma	Germany

3.1.1.6 Preparation of Artificial mucus

Materials	manufacturer	Origin
Crude mucin	Sigma	USA
Milli-Q water	Millipore	
NaOH	Sigma-Aldrich	Germany

3.1.2 Devices

3.1.2.1 Analytical scales used

Device	Name	Manufacturer
Scale	New Classic MS	Mettler Toledo
Scale	AB204-S	Mettler Toledo
Scale	AG281	Mettler Toledo
Scale	PB303-S DeltaRange®	Mettler Toledo

3.1.2.2 Manufacturing of hydrogels

Device	Name	Manufacturer
Refrigerator		Electrolux,
		Philips
Water deionizer	Milli-Q® Academic	Millipore
Magnetic stirrer	MR Hei-Standard	Heidolph Instruments
Vortex mixer	Vortex-Genie 2	Scientific Industries
Water bath with thermostat	Polystat	Cole Parmar
pH-Meter	PH 200	HM digital
pH-Meter	ORION 3 STAR PH Benchstop	Thermo electron corporation

3.1.2.3 Ovens used for accelerated stability studies

Temperature	Device	Name	Manufacturer
22°C	Oven		Heraeus instruments
25°C	Humidity chamber		NEWTRONIC
40°C	Oven	MMM-Medcentre Einrichtungen GmbH	Venticell

3.1.2.4 Mobile Phase preparation

Device	Name	Manufacturer
Ultrasound bath (degassing)	8892	Cole-Parmer
Water deionizer	Milli-Q® Academic	Millipore

3.1.2.5 Viscosity measurements

Device	Name	Manufacturer
viscometer	DV-I + Viscometer	Brookfield
Spindles	CPE-52	Brookfield
	CPE-40	Brookfield
Water heating system	Polystat	Cole Parameter

3.1.2.6 Mucoadhesion Measurements

Device	Name	Manufacturer
Texture Analyser	TA-XT2i	Stable Micro Systems
pH meter	PH-200	HM digital
Viscometer	DV-I + cone and plate viscometer	Brrokfield
DuoDerm extra thin hydrocolloide membrane	Artificial membrane	DuoDerm
Probe	Graphite probe	Stable microsystems
Oven @37°C	Humidity chamber	NEWTRONIC
Water deionizer	Milli-Q® Academic	Millipore
Scale	PB303-S DeltaRange®	Mettler Toledo

3.1.2.7 In vitro release studies

Device	Name	Manufacturer
Environ shaker	Lab-Line Orbit Environ Shaker Incubator 3527	Lab-Line
Scale	AB204-S	Mettler Toledo
oven	Humidity chamber	NEWTRONIC
Franz diffusion cells	PermeGear	USA
Semi-permeable cellophane membrane 12-1400 Da	SpectraPor®	Breda, Netherlands

3.1.2.8 HPLC

Device	Name	Manufacturer
Pump	Dual-Gradient Analytical Pump	Dionex®
Autosampler	WPS-3000SL Analytical In-Line Split Loop Autosampler	Dionex®
Degasser	SRD-3600 Solvent Rack and Degasser	Dionex®
Column Compartment	TCC-3200 2x2-6P Thermostatted column compartment	Dionex®
Detector	Ultimate 3000 Photodiode Array Detector	Dionex®
Column 1	Phenomenex® Luna 5µ C8(2) 250x4.6mm	Germany
Column 2	PLRP-S 100A 8µM 250X4.6MM	Great-Britain
Guard Catridge	Phenomenex® C8 4×10 mm I.D. guard column	Germany
syringes	BRAUN	Germany
Needles	Terumo Neolus	Belgium
Syringe filters	PHENEX GPF/CA Membrane (0.45 µm) 28mm syringe filter	Phenomenex®

3.2 Methods

3.2.1 Complexation methods

Complexes of HPβCD and doxycycline in 1:24[116] w/w were prepared by the following methods:

1. Kneading method: Doxycycline and HPβCD were triturated in a mortar with a small volume of water-methanol solution 1:2 v/v. The thick slurry was kneaded for 45 minutes and dried at 3 different temperatures, 25 °C, 40 °C for 1hour and 40°C until constant weight. The complex was then sieved (#100)[311].

2. Co-grounding method: Doxycycline was dissolved in a minimum quantity of methanol in a glass mortar and then HPβCD was added and the suspension was triturated at room temperature until the solvent evaporated[312].

3. Physical mixture method: Doxycycline and HPβCD were pulverised and sieved through(#100) and mixed in mortar and pestle without any solvent[311].

3.2.2 Gelation temperature adjustment

Gelation temperature was measured by two different methods.

3.2.2.1 Method 1: Magnetic bar method

This method was to some extent modified from the method which was mentioned in the referred article[313]. Approximately 5 g of *in situ* hydrogel were placed in a 10 ml beaker and then positioned on a magnetic stirring plate, equipped with thermostat controller. A magnetic bar was introduced into the beaker. A thermometer was held in position, manually just above the rotating magnet, without touching the bottom. The temperature of the thermostat was increased slowly (1 °C/min) until the magnet

stopped rotating. The temperature at which the magnet stopped rotating was noted from the thermometer reading and was considered as the gelation temperature[314].

3.2.2.2 Method 2: moving meniscus method

In this method the test tube containing *in situ* hydrogel was tilted at 90° angle[164], and the gelation is considered to occur if the meniscus does not move. Approximately 2 grams of *in situ* hydrogel were weighed into a test tube and the test tube was immersed in a water bath (**Figure 8**) with thermostat controller attached to it. Slowly the temperature of the water bath was increased by 1°C and the hydrogel was allowed to equilibrate for 10 minutes at that temperature. After thermostating, the test tube was tilted at 90° angle, and the procedure is repeated until the meniscus does not move. The temperature at which the meniscus does not move upon tilting was noted and considered as the gelation temperature[96,315].

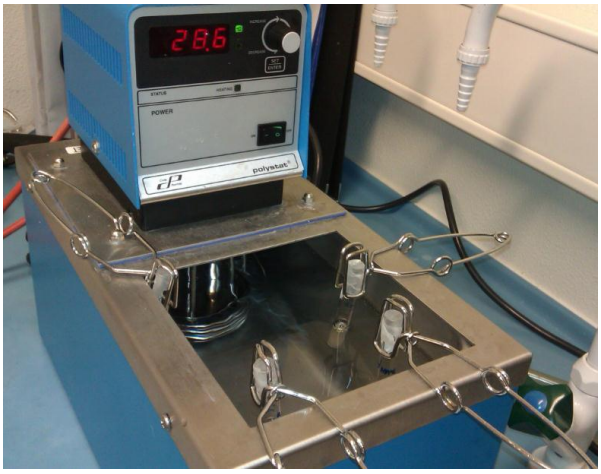


Figure 8 *Setup for gelation temperature adjustment using a water bath equipped with thermostat*

3.2.3 Hydrogels preparation by cold method

Hydrogels were prepared by cold method of preparation. There are two methods to dissolve poloxamers into the solvent:

1. Cold method[164,313,316,317]
2. Hot method

The cold method was selected, as it is the most widely used method for poloxamers and also because the active component doxycycline is unstable at high temperatures. The presence of non-ioinic surface active agent in the formulation increases the stability of doxycycline[318]. Poloxamers are non-ionic surface active agents which can be used for formulating *in-situ* hydrogels. The composition of non-ionic surfactants should not exceed 20% w/v, preferably between 7.5-15% w/v[318]. In cold method of preparation the poloxamers are dissolved in cold i.e. refrigerated solvent. The solvent used is milli q water. The solvent (water) was refrigerated for 1 hour prior to adding the poloxamers. Poloxamer407 was added in small amounts with careful stirring, manually with a glass rod. Stirring with magnetic stirrers caused excessive bubbles or foam in the dosage form which did not liquefy easily. As the poloxamers are surfactants they tend to produce foam during the process of mixing the polymer into the solvent, which usually should liquefy back after refrigeration for an hour or two. But if the excessive foam was caused due to aggressive stirring,

then the foam did not liquefy into solvent even in 1 month in one instance. After adding poloxamer 407, the formulation developed foam, which liquefied back to solvent after refrigeration for 2 hours. Then to the above solution poloxamer188 was added in small amounts with careful manual stirring with the glass rod. Again the formulation was kept in a refrigerator for 2 hours for liquefying the foam. The mucoadhesive polymers hydroxypropylmethylcellulose and povidone were added in the next step. There are two methods for adding the hydroxypropylmethyl cellulose. One method is to solubilise the HPMC in warm water and then add the viscous gel into the formulation. The second method is to add HPMC in powdered form into the formulation. When HPMC was added after solubilising in hot water, it remained as a thick viscous mass at the bottom of the hydrogel, it did not got uniformly dispersed into the formulation. When the powdered HPMC is added, it was uniformly dispersed into the formulation. So the HPMC was directly added into the formulation. The individual granules of HPMC absorbed water from surrounding hydrogel, leading to swelling of each granule, which were uniformly dispersed in the formulation. Upon leaving the formulation to stand, the swollen granules occupied the lower zone at the bottom of hydrogel, which required slight agitation to uniformly redisperse. Povidone was added in powder form. Upon leaving the formulation to stand povidone settled as white powder in the bottom of the hydrogel. By shaking the formulation the povidone was redispersed into the hydrogel. In the next step the antioxidants sodium thiosulfate and sodium metabisulfite were added. The combination of the antioxidants sodium thiosulfate and sodium metabisulfate imparted more stability to doxycycline[102]. The antioxidants concentrations can be in the range 0.1-0.5 %[102], in some cases can be 0.1-1 %[318]. Not every antioxidant improves stability of doxycycline[102]. Chelating agents were disodium edetate and magnesium chloride. Complexing with divalent metal ions (Mg, Mn, Co, Ni, Cu, Zn and Cd) increases the stability of doxycycline[117]. Magnesium ions react with doxycycline to form magnesium-doxycycline chelates[318]. The magnesium-doxycycline chelates were more stable than doxycycline alone in aqueous solutions. The preferred molar ratio for magnesium-doxycycline complex is 1:1 to 4:1 respectively[318]. Disodium edetate can be used as chelating agent[102] in the concentration range 0.1-0.5 %[102]. Citric acid and tartaric acids have antioxidant properties. Since different antioxidants can affect the stability of doxycycline citric acid and tartaric acids were added in conjugation with sodium thiosulfate and sodium metabisulfite, in one of the formulations. The antioxidant sodium bisulfite did not improve the stability of doxycycline[102]. Before adding the active component the pH was adjusted to 6.55 with 1M HCl and 1M NaOH solutions. HPβCD was added in the ratio 1:24 w/w to doxycycline to improve the overall stability of doxycycline [116]. . The active component doxycycline was added in the final step of preparing the hydrogel, and the final weight was made up by adding water.

3.2.4 HPLC methods

Two different HPLC methods were used in the experiment:

3.2.4.1 Method1: Skuli's method

Mobile phase comprised of acetonitrile: water: perchloric acid (25.75: 74: 0.25)[319] respectively, adjusted to pH 2.5 with 5 M sodium hydroxide solution. The column was Phenomenex® Luna 5 µm C_8 250 × 4.6 mm, with Phenomenex® C_8 4 × 10 mm I. D. guard column. Flow rate was 1ml/min. Injection volume for standards

and samples was 30µl. Column was maintained at 25°C. The standards and samples were maintained at 4 °C.

Preparation of standards:

Stock solution: 10mg of doxycycline, 3mg of 6-epidoxycycline and metacycline were weighed accurately into a 100 ml volumetric flask and 5 standards were prepared from a series of dilutions as shown in Table 11.

Table 11 Series of dilutions for preparing standards

S.no	Series of dilutions
Standard1	Pipette out 10 ml of stock solution and dilute to 25ml with the mobile phase.
Standard2	Pipette out 15ml of standard1 and dilute to 25ml with the mobile phase.
Standard3	Pipette out 15ml of standard2 and dilute to 25ml with the mobile phase.
Standard4	Pipette out 10ml of standard3 and dilute to 25ml with the mobile phase.
Standard5	Pipette out 5ml of standard4 and dilute to 10ml with the mobile phase.

The standards were injected in increasing order of concentration i.e. in the reverse order of above table.

3.2.4.2 Method 2: European and British pharmacopoeia's method

The mobile phase was prepared by adding 60 g of 2-methyl-2-propanol into a 1000 ml volumetric flask with the aid of 200 ml water, 400 ml of buffer solution pH 8.0, 50 ml of 10 g/L solution of tetrabutyl ammonium hydrogen sulfate adjusted to pH 8.0 with dilute sodium hydroxide solution, and 10 ml of a 40 g/L solution of sodium edetate adjusted to pH 8.0 with dilute sodium hydroxide solution, diluted to 1000 mL with water. The stationary phase was styrene-divinylbenzene copolymer 8 µm, maintained at 60 °C. The samples were diluted in 0.01 M hydrochloric acid. UV detection was set at 254 nm. Injection volume for standards and samples was 20 µl. Run time was 25 minutes. Flow rate was 1 ml/min.

Preparation of standards:

Stock solution: 10 mg of doxycycline, 3 mg of 6-epidoxycycline and metacycline were accurately weighed into a 100 ml volumetric flask and 5 standards were prepared from a series of dilutions as shown in Table 12

Table 12 Series of dilutions for preparing standards

S.no	Series of dilutions
Standard 1	Pipette out 10 ml of stock solution and dilute to 25ml with 0.01 M HCl dilution medium.
Standard 2	Pipette out 15ml of standard 1 and dilute to 25ml with 0.01 M HCl dilution medium.
Standard 3	Pipette out 15ml of standard 2 and dilute to 25ml with 0.01 M HCl dilution medium.
Standard 4	Pipette out 10ml of standard 3 and dilute to 25ml with 0.01 M HCl dilution medium.
Standard 5	Pipette out 5ml of standard 4 and dilute to 10ml with 0.01 M HCl dilution medium.

3.2.5 Robustness testing of HPLC methods

Method 1 Skuli's method:

1. Mobile phase ageing: As the mobile phase ages the peaks were gradually shifted i.e. when the mobile phase was freshly prepared (on same day of analysis) the peaks appeared at 26 minutes, after 2 weeks of ageing when freshly prepared samples were injected then the peaks appeared at 29 minutes, after 4 weeks of ageing the peaks appeared at 32 minutes, but the peak resolutions and symmetry factor remained the same.

2. Water: Pure milli-Q water in the mobile phase gave sharp peaks, if the normal distilled water was used then the peak heights were almost halved.

3. Excipients: Excipients in the formulation were interfering with the results.

Method 2 European pharmacopoeia method:

1. Mobile phase ageing: mobile phase ageing did not affect the peak appearance times, resolutions and symmetry factor.

2. Sample dilution medium ageing: Samples were diluted in 0.01M HCl. The peaks were perfect when analysed on the same day of preparation of sample dilution medium, but as 0.01M HCl solution aged the peaks were distorted and the base line was very unstable.

3. Excipients: Before starting the stability studies, the method was tested for "whether antioxidants, chelating agents, pH changes, column precision, injection volumes of samples, etc. were affecting the HPLC results." This method remained unaffected by presence of excipients in the formulation.

3.2.6 Calculation of Peak resolution according to European pharmacopoeia

Resolution (R_S) is the degree of separation between two peaks,

$$\mathrm{Rs} = 1.18\frac{(t_{R2} - t_{R1})}{W_{h1} + W_{h2}}$$

Where t_{R2} and t_{R1} are retention times of peaks 2 and 1,

W_{h1} and W_{h2} are the widths of peak 1 and 2 at half the peak height

If the peak widths are measured at the infliction points by extending the tangents at the base line (**Figure 9**) instead of at half the peak height then the following equation was used

$$R_S = 2\frac{(t_{R2} - t_{R1})}{W_1 + W_2}$$

R_S value 1 corresponds to a peak separation of 94 %. Baseline separation corresponds to R_S value 1.5. If the R_S value is greater than 1.5 then it means that the peaks are completely separated.

From the figure below, t_{R2} and t_{R1} are the joining points of tangents in upward direction.

The resolution between two impurities i.e. Metacycline (1st peak) and 6-epidoxycycline (2nd peak) should be minimum 1.25 and the resolution between 6-epidoxycycline (2nd peak) and doxycycline (3rd peak) should be a minimum of 2.0. The content of 2-methyl-2-propanol in the mobile phase[320] was adjusted to obtain peaks with desires resolution (R_S).

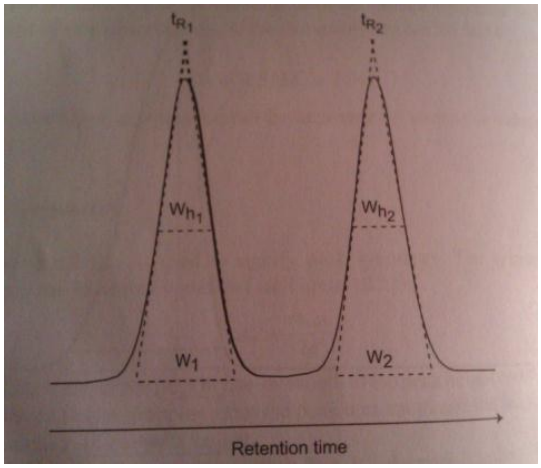


Figure 9 *parameters used for calculating Resolution (R_S)*

3.2.7 Calculation of Peak symmetry factor

The peak symmetry factor (A_S) was calculated from the following equation[321]:

$$A_S = \frac{W_{0.05}}{2d}$$

Where $W_{0.05}$ is the width of the peak at 1/20th of the peak height and d is the distance between the perpendicular dropping from the peak maximum and the leading edge of the peak at 1/20th of the peak height (**Figure 10**).

An A_S value of 1.0 signifies symmetry, A_S>1 indicates peak tailing and A_S<1 indicates peak fronting.

The symmetry factor (A_S) can be a maximum of 1.25 for the peak due to doxycycline[320].

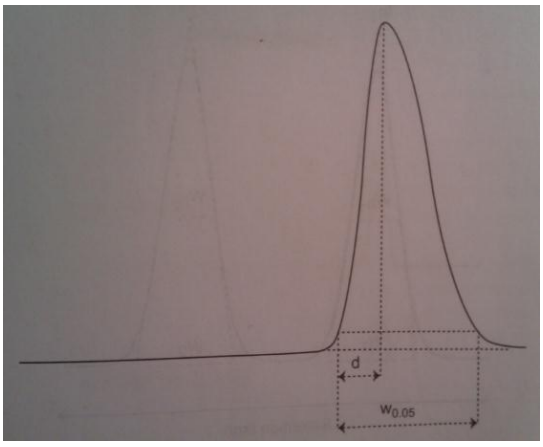


Figure 10 *parameters used for calculating symmetry factor (A_S)*

3.2.8 Quantitative analysis

The quantitative analysis of doxycycline and its degradation compounds metacycline and 6-epidoxycycline were performed on the reversed-phase HPLC component system from Dionex Sofron GmbH (Germany) Ultimate 3000 series, consisting of a p680 pump with a DG-1210 degasser, an ASI -100 autosampler, a VWD-3400 UV-Vis detector and PLRPS styrene-divinyl benzene copolymer 250 mm × 4.60 mm, and 8 µm pore size column.

3.2.9 Stability studies (w/v) method and problems encountered

The hydrogels were first prepared by w/v method in phosphate buffered saline pH 7.4 buffer solution. The hydrogels were prepared by cold method of preparation. The solvent was refrigerated prior to adding poloxamers. Poloxamer 407 was added in small amounts with slow stirring on a magnetic stirrer. While adding poloxamer 407 the solution developed bubbles, as poloxamers are surface active agents. The bubbles slowly liquefied on keeping the solution in refrigerator. Now poloxamer 188 was added. Again the solution was stored in refrigerator for liquefaction of bubbles. The bubbles did not completely liquefy even after waiting for 7-10 days. As the experiment was carried out by the w/v method, the formulations were prepared in volumetric flasks for accurate adjustment of volume, but the bubbles interrupted the volume adjustment. In the next step mucoadhesive polymer hydroxypropyl methyl cellulose was added with slow stirring and then antioxidants and chelating agents were added. In the last step either doxycycline or doxycycline – HPβCD complex was added and the final volume was made up by adding 7.4 buffer solution. The formulations were then stored in a tightly sealed containers. The bubbles interfered during the volume adjustment and this lead to improper volume adjustments and which in turn caused errors in HPLC analysis. The hydrogels containing only doxycycline stabilities were much less than 100 % even on same day of analysis and in the hydrogels which contained antioxidants and chelating agents the stability values were 1.4 times higher (130% – 150%). Improper volume adjustment because of the bubbles interfering was thought as the cause (though improper volume adjustment should give 5-10% error but here error was more than 50%) and further the experiment was continued with w/w method. Even in w/w method the same kind of error existed and this lead to further detailed analysis of how the excipients present in the hydrogel were affecting the HPLC results.

3.2.10 Investigation of why the hydrogels with antioxidants were showing 1.5 times higher drug content than what they actually contained during HPLC analysis

1. Determination of the effect of antioxidants on doxycycline peaks:

The experiment was carried out according to the w/w method. 0.5 mg of doxycycline was added to 5 g of water. 0.5 g of the above solution when diluted to 25 ml with mobile phase should contain 0.02mg/ml. There were 5 standards prepared and injected thrice. The samples concentration was in between the minimum and maximum concentration of the standards. The samples reference concentration was set at 0.020 mg/ml or 20 µg/ml i.e., if the concentration of samples will be 0.020 mg/ml then it is considered as 100 %. All the samples were injected 3 times and % yields were calculated from the average of 3 values.

2. Determination of effect of chelating agent EDTA on doxycycline peaks:

The experiment was done by w/v and v/v methods. Initially 20 mg of doxycycline was added to 20 ml volumetric flask (w/v) and diluted with mobile phase. With the help of a micropipette 0.5 ml of the above solution was diluted to 25 ml (v/v) with the mobile phase. The samples reference concentration was set at 0.020 mg/ml. All the samples and standards were injected 3 times and the percentage yields were calculated from the average of 3 values.

3. Checking the column precision between the new and old columns:

0.5 mg of doxycycline was directly added to 25 ml of mobile phase. So the reference concentration of samples was 0.020 mg/ml.

4. Assay of doxycycline from different manufacturers :

Directly 0.5 mg of doxycycline was diluted to 25 ml with the mobile phase. The reference concentration of samples was 0.020 mg/ml.

4a.Assay of doxycycline from HOVIONE Macau container, by using a different analytical scale:

Directly 0.5 mg of doxycycline was added to 25 ml volumetric flask and diluted with the mobile phase. The reference sample concentration was 0.020 mg/ml.

4b. Assay of doxycycline from HOVIONE Macau container, by using different analytical scale :

This time a different analytical scale was used. The samples were prepared in two steps involving w/v and v/v method. Some of the samples were directly prepared by w/v method. All the samples reference concentration was set at 0.020mg/ml.

5. Effect of pH of 30 µl injected solution on results :

10 mg of doxycycline was added to a 100 ml volumetric flask and diluted with mobile phase. 5 ml of the above solution was diluted to 25 ml of mobile phase. After dilution the pH of the solutions were adjusted to 2.6, 2.7, 2.8, 2.9 and 3.0 with 5 M NaOH solution. The NaOH solution was added in very minute quantities with a needle attached to syringe. The quantity of NaOH added was negligible and it should not affect the results.

6. Doxycycline assay from HOVIONE sample, with different analytical scale :

10 mg of doxycycline was weighed into a 100 ml volumetric flask and diluted with mobile phase. 5 ml of the above solution was pipetted out and diluted to 25 ml with mobile phase and analysed. The samples reference concentration was 0.020mg/ml.

3.2.11 Checking the solubility of poloxamers in different buffer solutions

As the poloxamers are surface active agents they tend to produce bubbles/foam while mixing, which should liquefy when stored in refrigerator for few hours. But in the w/v method of hydrogel preparation the bubbles did not liquefy even after 10 days and were interfering with the volume adjustments. So it was required for a detailed study on liquefaction of bubbles. The problem might be because of following reasons:

1. Incompatability between a buffer salt and poloxamers.

2. As high concentration of poloxamers (31% w/v) have to be dissolved, the problem might be due to supersaturation of the solvent.

3. Rapid mixing might have been causing excessive foam. Manual stirring with a glass rod might reduce the amount of foam and thereby reduce liquefying time.

To rule out the above possibilities, the poloxamers were dissolved in different buffer solutions Table 13 and also in pure water with careful and slow manual stirring with a glass rod.

Table 13 Solubility of poloxamers in different pH buffer solutions

s.no	Type of solvent	Ease of dissolving Poloxamer 407	Ease of dissolving Poloxamer 188	Bubbles liquefying time
1	Phosphate buffered saline pH 7.4 (European pharmacopoeia)	slightly difficult	easily soluble	48 hours (but the bubble did not liquefy completely)
2	Phosphate buffer solution pH 6.4 (European pharmacopoeia)	difficult	soluble	bubbles did not liquefy even after 10 days
3	Phosphate buffer solution pH 6.6 (US pharmacopoeia)	soluble	soluble	overnight
4	Milli-Q water	very easily soluble	very easily soluble	2 hours

1. All the hydrogels were prepared by slow (6 hours) manual stirring with a glass rod, but the formation of foam could not be minimised. It can be concluded that rapid stirring might not be the cause.

2. The phosphate buffer solution pH 6.4 contained high concentrations of buffer salts among all the solvents tested, and the liquefying time was the highest. This suggests that saturation of solvent was the actual cause of the long liquefying times. The liquefying time decreased as the concentration of buffer salts was decreased in the solvent. In pure water the bubbles/ foam liquefied very rapidly. All this suggests that supersaturation of solvents was preventing the bubbles to liquefy. The solvent had limited room to accommodate high concentration of solutes, and if already solvent accommodated high concentration of buffer salts, there might be no space left to further accommodate high concentration of poloxamers (31 % w/w).

3. So the deionised water was selected as the solvent to solve the liquefying problem and the pH was adjusted manually to 6.55 with 1 M HCL and 1 M NaOH solutions.

3.2.12 Stability studies (w/w) of hydrogels in 3 batches

The stabilities of 9 hydrogels (Table 14) were tested at 3 different temperatures, i.e. at 4 °C, 25 °C and 40 °C. So there were a total of 27 hydrogels at all 3

temperatures. The stabilities were tested over a period of 3 months. The stability tests were run during following time intervals, starting day, week 1, week 2, week 3, week 4, week 6, week 8, week 10 and week 12. There were a total of 9 runs for each batch. For convenience hydrogels at 4 °C were considered as batch 1, 25 °C as batch 2 and 40 °C as batch 3. Hydrogel 9 from the table 14 did not contained the mucoadhesive polymer povidone, as there was a previous study[322] that showed that poloxamers and povidone combination acted as a degradation pathway for hydrochlorothiazide.

Table 14 Data for the preparation of hydrogels

15 g of 0.1% w/w doxycycline containing *in situ* forming hydrogels for treatment of the aphthous ulcers												
					Mucoadhesive polymers		**Antioxidants**					
s.no	**doxycycline (0.1%w/w)**	**HPβCD (1:24 w/w with doxycycline)**	**Poloxamer407 (21%w/w)**	**Poloxamer188 (10%w/w)**	**HPMC 0.25%w/w**	**POVIDONE 0.25%w/w**	**$Na_2S_2O_3$ (0.32%w/w)**	**$Na_2S_2O_5$ (0.32%w/w)**	**EDTA (0.2%w/w)**	**$Mgcl_2$ 1:4 molar ratio with doxycycline**	**citric acid (0.2%w/w)**	**tartaric acid (0.2%w/w)**
1	15mg	----	3.15gm	1.5gm	37.5mg	37.5mg	----	----	----	----	----	----
2	15mg	----	3.15gm	1.5gm	37.5mg	37.5mg	48mg	48mg	30mg	----	----	----
3	15mg	----	3.15gm	1.5gm	37.5mg	37.5mg	48mg	48mg	30mg	24mg	----	----
4	15mg	360mg	3.15gm	1.5gm	37.5mg	37.5mg	----	----	----	----	----	----
5	15mg	360mg	3.15gm	1.5gm	37.5mg	37.5mg	48mg	48mg	----	----	----	----
6	15mg	360mg	3.15gm	1.5gm	37.5mg	37.5mg	48mg	48mg	30mg	----	----	----
7	15mg	360mg	3.15gm	1.5gm	37.5mg	37.5mg	48mg	48mg	30mg	24mg	----	----
8	15mg	360mg	3.15gm	1.5gm	37.5mg	37.5mg	48mg	48mg	30mg	24mg	30mg	30mg
9	15mg	----	3.15gm	1.5gm	75mg HPMC (0.5%w/w)	----	30mg (0.2%w/w)	30mg (0.2%w/w)	30mg	24mg	----	----

3.2.13 HPLC Sampling of hydrogels

The hydrogels contained 15 mg of doxycycline per 15 grams of hydrogel (w/w), 1 gram of *in situ* hydrogel contained 1 mg of doxycycline. For HPLC analysis the concentration of a sample should be in between the maximum and minimum concentrations of standards. The concentration of each sample was set at 20 µg/ml. 0.2 grams of hydrogel was diluted to 10 ml with 0.01 M hydrochloric acid solution. The *in situ* hydrogels stored at 4 °C and 25 °C were freely moving solutions and could be easily drawn into the syringes for weighing, but the

hydrogels at 40 °C were completely solidified. They were allowed to liquefy at room temperature for 15 minutes prior to sampling. Approximately 0.2 ml of the *in situ* hydrogels was drawn into a 1 ml syringe. The syringe tip was cleaned with tissue paper and then the needle was attached to syringe. A 10 ml volumetric flask was weighed on an analytical scale. The syringe with needle was inserted into the volumetric flask and carefully 0.2 grams of hydrogel was introduced into the volumetric flask. The 10 ml volumetric flasks with 0.2 grams of *in situ* hydrogels in it were diluted with 0.01 M hydrochloric acid. The volumetric flasks were then vortexed (shaker) for 2-3 minutes. A 3ml syringe with needle was inserted into the volumetric flask and the sample solution was drawn in. Then the needle was removed and a phenex 0.45 mm syringe filter was attached to the syringe. First few droplets were discarded and the filtered solution was introduced into the 2 ml HPLC vials. Approximately the vials were filled upto 1.5 ml. Then the vials were sealed with caps and were ready for analysis.

3.2.14 Identification of unknown impurity

The European Pharmacopoeia and the British Pharmacopoeias suggest that the main degradation compounds of doxycycline are metacycline and 6-epidoxycycline. Both pharmacopoeia's mentioned to test the above two impurities only. There are many other impurities of doxycycline but none of them except the above mentioned two are commercially available. In the stability experiment at all 3 temperatures none of the 27 hydrogels contained any noticeable amounts of metacycline or 6-epidoxycycline. But there was an unknown impurity at around 7 minutes. The US Pharmacopoeia states that 4-epidoxycycline is also a main degradation compound apart from metacycline and 6-epidoxycycline. The US pharmacopoeia mentioned the procedure to synthesize 4-epidoxycycline and 6-epidoxycycline from doxycycline. The idea was to synthesize 4-epidoxycycline and 6-epidoxycycline, and see whether the unknown peak at 7 minutes corresponds to 4-epidoxycycline. 6-epidoxycyclien and doxycycline can be easily identified, as they were already contained in standards. By synthesizing the 2 impurities, one impurity occurs at a known time and can be easily identified as 6-epidoxycycline and if unknown peak occurs at around 7 minutes it should be 4-epidoxycycline. The next step was to check how 4-epidoxycycline appears in Skuli's HPLC method.

3.2.14.1 Method to synthesize 4-epidoxycycline and 6-epidoxycycline according to USP

60 mg of doxycycline hyclate was weighed into a 10 ml volumetric flask and diluted with 0.01M HCl. 5 ml of the above solution was transferred into a 25 ml volumetric flask. A beaker was filled with water and heated to boil at around 100°C, now the 25 ml volumetric flask was immersed into the beaker and allowed to boil for 1 hour. After 1 hour the contents of a volumetric flask was poured onto a glass plate and was heated at around 70-100°C until all the solvent evaporated. Care was taken to prevent charring while heating. The left over residue was collected and diluted with 0.01M HCl and analysed with HPLC. The residue only contains 4-epidoxycycline, 6-epidoxycycline and doxycycline. The exact times of appearance for 6-epidoxycycline and doxycycline are known already and the new peak appearing should by 4-epidoxycycline.

3.2.15 Viscosity measurements

The viscosity measurements were carried out at 25 ± 1 °C using a Brookfield DV-I + cone and plate digital viscometer. The spindle used was CPE 52. Before taking the measurements the instrument was calibrated with standard viscosity solutions supplied by Brookfield. The gap was set between the bottom plate and cone to be 0.005 mm. Before introducing the sample, the cone and plate chamber was allowed to thermostat at 25 °C. Then 0.5 ml of the sample hydrogel to be tested was introduced onto the centre of plate. Then the plate was fastened to the cone and secured with a lock. Then the rpm was increased, starting with 0.3, 0.6 up to a maximum of 60-100, until the torque value was above 10. When the torque value reached above 10 the viscosity (cP) value was noted. The viscosity values were noted at low, medium and high torques (%). Medium to high torque values were recorded, to decrease the error. All the hydrogels tested were Newtonian in nature, the viscosity values did not change by rpm, shear rate and time. For testing the *in situ* gelation capacity of formulated hydrogels the viscosity values were noted from 25°C with increments of 1°C each time until 33°C. The viscometer has the upper limit testability at 30,000 cP. The formulated *in situ* hydrogels viscosity was to be noted until 37 °C, but because the instrument could only test viscosities until 31000 cPs, the values were recorded only until 33 °C. Approximately the formulated hydrogels viscosity at 37 °C would be around 1-2 lakh cPs. All measurements were performed at least 3 times.

3.2.16 In vitro mucoadhesion measurements

In vitro mucoadhesion evalution of formulated hydrogels was carried out by using TA-TX 2i Texture Analyzer (Stable Micro Systems Ltd., Surrey, UK), equipped with a 5 Kg Load cell. An artificial membrane was used instead of freshly excised porcine gastric/nasal/buccal mucosa. The artificial membrane used was DuoDerm extra thin hydrocolloide membrane, which had a textured leathery surface. The artificial membrane simulated the original porcine mucosa according to Skuli et.al[323]. Bio-Gels Pharmaceuticals. Artificial mucus was applied for the artificial membrane. The artificial mucus solution was stored at 37 °C, before applying onto the artificial membrane. A cylindrical 10 mm diameter graphite probe (P/10) was used. The artificial membrane was cut exactly to the size of probe and adhered. Same membrane was used for all the measurements. After each measurement, the mucus and hydrogel that were left adhered to the membrane, were gently cleaned with a tissue, and reapplied with the fresh artificial mucus stored at 37 °C. Approximately 2 grams of *in situ* forming hydrogel was placed in a 5 ml beaker, and then allowed to gel at 37 °C for 10 minutes. The beaker was then adhered with a double sided tape just under the moving probe (**Figure 12**). Before adhering the beaker to the floor of the Texture analyser, the probe height was calibrated on the plane bottom surface, and the 5 Kg load cell was calibrated while the 2 kg standard weight was placed on the probe head. Each measurement took around 3 minutes, during which the hydrogel remained intact as a solid. The load cell was lowered until the probe attached with artificial membrane was just few millimeters away from the surface of hydrogel. Pre-test speed was 0.1 mm/sec and contact force

was 0.005 N. The probe touched the hydrogel surface with a contact force of 0.005 N, and constantly applied 0.005 N for 90 sec onto the hydrogel surface. During the 90 sec contact time the artificial mucus and mucoadhesion polymers in the formulation were allowed to interact to establish mucoadhesion bonds. Probe withdrawal rate was 0.1 mm/s, and withdrawal height was 10 mm. The contact area was 0.79 cm^2. After 90 seconds the probe was withdrawn by moving vertically up and the force required to detach the artificial membrane which was applied with artificial mucus, from the hydrogel surface was calculated by using the software programme "Texture Exceed Expert". From the software the AUC was obtained from force-time[324,325] (N s) graph plot. The AUC values for force-time were converted (distance=speed x time) into force -distance (N mm) for the calculation of work of adhesion (**Figure 11**). The breaking force was noted. The work of mucoadhesion was calculated from the following formula[326]

$$\text{Work of mucoadhesion } \left(\frac{mJ}{cm^2}\right) = \frac{AUC}{\pi r^2}$$

Where, πr^2 = artificial mucosal surface being in contact with the gel

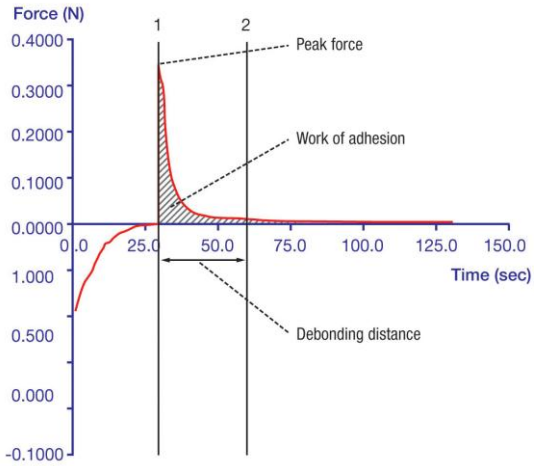


Figure 11[324]***:*** *Conversion of Force-Time plot to Force-distance values for calculation of work of adhesion*

Procedure for preparation of the artificial saliva/mucus: to simulate the natural saliva a 17 % crude mucin solution was prepared according to SOP BG02-001 by Bio-Gels ehf:

1. Accurately 3.4 g of Crude mucin (sigma/M-2378) were weighed into a glass beaker.

2. Then the mucin was hydrated by adding 12 g of purified water, and stirred until homogenised mixture was obtained.

3. The artificial saliva was stored over night at room temperature (22-25°C).

4. The pH of the solution was adjusted to about pH 6 using a 2 M hydrochloric acid solution and 2 M sodium hydroxide solution.

5. Final weight (20 g) was made up by adding purified water.

6. The viscosity of the artificial saliva was measured with a viscosity meter (Brookfield Model DV-I + and spindle No. CPE-40 at 12 rpm, 25 °C) and the viscosity was then adjusted to 39 ± 2 cPs by adding purified water and crude mucin.

The mucoadhesions of the following hydrogels were tested:

1. Hydrogel with 0.5%HPMC
2. Hydrogel with 1% HPMC
3. Hydrogel with 1.5% HPMC
4. Hydrogel with 2% HPMC
5. Hydrogel with 0.5% CMC
6. Hydrogel with 1% chitosan
7. Hydrogel with 0.5% Polyethylene Glycol PEG 6000
8. Hydrogel with 0.5% Carbopol 974P
9. Hydrogel with 0.25% HPMC + 0.25% Polyvinyl pyrrolidone(povidone) = total 0.5%
10. Hydrogel with 0.5% Povidone(K-value 29-32)
11. Hydrogel with 0.5% polyvinylalcohol
12. Hydrogel with 0.2% HPMC + 0.2% Povidone + 0.1% Polyvinyl alcohol
13. Hydrogel with 0.25% Povidone + 0.25% Polyvinyl alcohol
14. Hydrogel with 0.25% HPMC + 0.25% Carbopol 974P

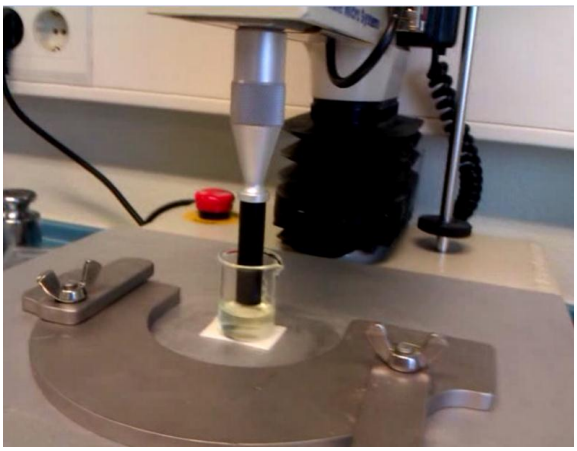


Figure 12 *Setup for mucoadhesion analysis using Texture anslyzer*

3.2.17 In vitro release studies

In vitro release studies were performed using the membrane-less dissolution model[327,328]. Membrane-less model has been widely used for poloxamer based gels[146,329-331]. The drug release from the poloxamer hydrogels is predominantly controlled by gel erosion[146,329,330]. In the membrane-less model, there is a direct contact between the poloxamers and the release medium. In the membrane-less model, two phenomena are involved: the fickian diffusion of the drug and the dissolution of the poloxamer[332]. 1.8 g of the *in situ* hydrogel was weighed into a test tube. The test tube was placed inside a small conical flask for support and the setup was then placed on the analytical scale, then TARE was pressed, exactly 1.8 grams[331] of *in situ* hydrogel was introduced with the help of a syringe

and needle. The test tube with liquid hydrogel was kept inside an oven at 37 °C for 15 minutes to induce gelation. After the gelation had occurred, the release medium was carefully added along the sides of the test tube. The release medium was 6.6 pH buffer solution, which simulates the oral environment. 10 ml of release medium[331] was carefully added from the sides of the test tube. The release medium was equilibrated at 37 °C before adding onto the hydrogel. An environ shaker equipped with temperature control was used. The environ shaker was equilibrated at 37 °C, before introducing the test tubes containing solid hydrogels with release mediums. The environ shaker was adjusted at 100 rpm[331,333]. The test tubes were placed in a test tube holder, which was fastened securely inside the environ shaker. During the sampling, entire release medium was emptied and replaced with a new release medium that was thermostated at 37 °C. The sampling was done initially at, 1 h, 2 h, 4 h, 6 h, 8 h, 20 h. The samples were diluted 10 times with 0.01 M HCl solution for HPLC analysis.

The drug release profiles from each hydrogel formulation were analysed using the zero order, First order, Higuchi, Hixson-Crowell and korsmeyer-Peppas model [332,334]

$$\frac{Mt}{M\infty} = Kt^n$$

The Korsmeyer-Peppas model was applied to study the release mechanism of the drug from the polymer matrices, where M_T and M_∞ are the absolute and cumulative amounts of doxycycline released at time t and infinite time, respectively. The K is a parameter dependent upon structural and geometric characteristic of the system[335]. The exponent "n" was calculated by fitting the experimental results of M_T and M_∞ in the time domain[336]. The "n" value provides information (**Table 15**,**Table 16**) about the drug release mechanism from the hydrogel into the release medium[337-339]. All the experiments were carried out in triplicate. The release exponent "n" was calculated from the initial 60 % of cumulative percentage drug release data[340,341]. The "n" was calculated by plotting graph of log cumulative percentage[334,342] drug release values as a function of log time values[313,] (the slope itself[343] is release exponent "n", and the slope was calculated from the linear regression curve obtained from the initial 60 % of the drug release data). The release exponent "n" in some articles was calculated from the natural log values[340,344]. Higuchi model was applied to check whether drug release was through diffusion, by plotting percentage cumulative release against square root of time[313,345]. Zero order model was applied by plotting percentage cumulative release as a function of time[313,] and first order model was applied by plotting log percentage drug remaining as a function of time[313,345]. Hixson-Crowell model was applied by plotting cube root of amount of drug remaining as a function of time, to check whether the drug release occurred through dissolution.

Table 15 Interpretation[334] of release exponent (n) values (polymeric films)

Release exponent (n)	Drug transport mechanism	Rate as a function of time
0.5	Fickian diffusion	t-0.5
0.45 < n < 0.89	Non-Fickian transport	tn-1
0.89	Case II transport	Zero order release
Higher than 0.89	Super case II transport	tn-1

Table 16 Drug transport mechanisms and diffusional exponents for hydrogel slabs[346,347]

Diffusional exponent (n)	Type of transport	Time dependence
0.5	Fickian diffusion	$t^{1/2}$
0.5 < n < 1	Anomalous diffusion	t^{n-1}
1	Case II transport	Time independent
n>1	Super case II transport	t^{n-1}

4 RESULTS

4.1 Complexation methods

The ratio 1:24 for doxycycline:HPβCD was taken from an article[116] as it stated that the stability of doxycycline was improved after adding HPβCD's. In the article the doxycycline used was monohydrate which is not soluble in water, by adding HPβCD'S, the aqueous solubility and stability of doxycycline was enhanced. There are different types of complexation methods (Table 17) to encapsulate the doxycycline with the cyclodextrins. Usually differential scanning calorimetry (DSC) is used to check the complexation efficacy by observing the changes in the thermogram[313]. As DSC was not available, HPLC was used to assay the stabilities of complexes prepared with different complexation methods with varying drying times. The aim was to evaluate if doxycycline was degrading in the process of preparation of the complex. If the complexation efficacy is good then doxycycline should be equally distributed in the complexed sample i.e. if a small quantity of sample complex is assayed then it should contain 100 % of doxycycline. If the values are not 100% then it implies that doxycycline is not equally distributed among the cyclodextrins and the complexation efficacy is not good.

Table 17 Doxycycline – HPβCD complexation methods with percentage yields

S.no	Complexation method	Drying temperature in oven	Drying time (hours)	% Yields
1	Kneading method	25 °C	3 hours	92.75%
		40 °C	3-5 hours (until constant weight)	100.30%
			24 hours	96.75%
2	Co-grounding method	-----	-----	90.30%
3	Physical mixture method	-----	-----	88.62%

In the kneading method for trituration a 1:2 v/v water:methanol solution was used. After drying for 3 hours at 25 °C the percentage yield was 92.75 %, this low value was because of insufficient drying temperature and drying time to completely remove the water molecules. At 40 °C after drying until constant weight the percentage yield was 100.3 %, which indicates that doxycycline is equally distributed among the complex and complexation efficacy is good. After drying at 40 °C for 24 hours the percentage yield was 96.75%, this was because of high temperature and long drying time, which caused slight degradation. When using the co-grounding method the percentage yield was 90.3 % which signified that the complexation efficacy was not good. When using the physical mixture method the percentage yield was 88.62 %, which explains the importance of solvent during trituration, and the complexation efficacy was poor.

So out of all the methods tested the kneading method was selected with drying temperature 40 °C for 3-5 hours (until constant weight).

- In the aqueous solutions there is no need for pre-complexation, i.e. the drug and cyclodextrins (HPβCd) can be directly added to the solvent (water) and the complexation takes place. Pre-complexation is mostly used for solid dosage forms like tablets. Here in the experiment pre-complexation was needed because:

1. The viscosity of hydrogels at room temperature was around 200-300 cP, at this viscosity whatever solutes are added will be suspended in the hydrogel and there will not be free movement unlike pure water. So if the doxycycline and HPβCD are added separately the cyclodextrins might not reach drug molecule because of high viscosity. There were few instances to support this point.

- Initially in the preparation of hydrogels the mixing was done by either glass rod or by magnetic stirrer. During this time when the hydrogels were assayed the values were always less than 100%, this was because of doxycycline was not equally dispersed within the formulation. Irrespective of how patiently the hydrogels were mixed the results were never 100 %. But when the hydrogel was vortexed for 5 minutes, and assayed the values were 100 %. Vortexing the hydrogel has solved the uniform dispersion issue but the point here is doxycycline even after weeks was not equally dispersed in the hydrogel. This explains the lack of movement of solutes to equilibrate its concentration, because of high viscosity of the hydrogel. So doxycycline and cyclodextrins might not be able to approach each other to form complexes if added to viscous solutions, so pre-complexation was necessary.

- Another idea was to add doxycycline and cyclodextrins to distilled water before adding polymers. But the problem here was, the polymer poloxamer. Poloxamers are surfactants and while adding them into solvent they first develop foam and later liquefies into clear solutions. The foam produced during stirring contains air bubbles which might oxidise the doxycycline. So for all the above reasons pre-complexation was necessary.

4.2 Gelation temperature adjustment

The gelation temperature of the poloxamers was tested by 2 methods

1. Magnetic bar method

2. Moving meniscus method

Both methods gave almost similar results with ± 2°C differences. Poloxamers showed similar gelation behaviour irrespective of the medium in which they were dissolved either pure water or buffer solutions. Gelation temperature in phosphate buffered saline 7.4 pH was also tested simultaneously and the results were the same as when dissolved in pure water. The presence of some of the buffer salts might affect the gelation temperature of poloxamers[348]. The minimum concentration of poloxamer 407 required for gelation is 18 %, and poloxamer 407 alone gels below the room temperature[131]. Poloxamer 407 can be mixed with poloxamer 188 in appropriate concentrations[349] to achieve the gelation temperature matching with that of the body temperature. The presence

of HPMC in the formulation did not affect the gelation temperature of the poloxamers. The HPMC gelation temperature is around 75-90 °C. The gelation temperature was adjusted to 35°C, less than the actual 37°C body temperature. The presence of HPβCD increased the gelation temperature (Table 19,Table 20) of poloxamers[86].

Table 18 Gelation temperature adjustment of hydrogels containing only poloxamers with moving meniscus method

contents	Poloxamer 407 (% w/w)	Poloxamer 188 (% w/w)	Gelation temperature
Poloxamers in water	22	3.5	20
Poloxamers in water	18	10	40
Poloxamers in water	19	10	37
Poloxamers in water	20	10	34
Poloxamers in water	21	10	33

Table 19 Gelation temperature adjustment of hydrogels containing poloxamers and HPβCD with moving meniscus method

Contents	Poloxamer 407 (%w/w)	Poloxamer 188 (%w/w)	Gelation temperature
Poloxamers+ HPβCD in water	22	3.5	24
Poloxamers+ HPβCD in water	18	10	44
Poloxamers+ HPβCD in water	19	10	41
Poloxamers+ HPβCD in water	20	10	38
Poloxamers+ HPβCD in water	21	10	36

Table 20 Gelation temperature adjustment of hydrogels containing poloxamers and HPβCD with magnetic bar method

Contents	Poloxamer 407 (%w/w)	Poloxamer 188 (%w/w)	Gelation temperature (°C)
Poloxamers in water	22	3.5	20
Poloxamers in water	22	10	31
Poloxamers in water	22	11	32
Poloxamers in water	18	10	No gelation was seen
Poloxamers in water	20	10	36
Poloxamers in water	21	10	34
Poloxamers in water	21.25	10	33.3

Table 21 Gelation temperature adjustment of hydrogels containing only poloxamers with magnetic bar method

Contents	Poloxamer 407 (%w/w)	Poloxamer 188 (%w/w)	Gelation temperature (°C)
Poloxamers+ HPβCD in water	22	3.5	24
Poloxamers+ HPβCD in water	22	10	35
Poloxamers+ HPβCD in water	22	11	35.5
Poloxamers+ HPβCD in water	18	10	No gelation occurred
Poloxamers+ HPβCD in water	20	10	39
Poloxamers+ HPβCD in water	21	10	37
Poloxamers+ HPβCD in water	21.25	10	36

The hydrogel containing 21 % poloxamer 407 and 10 % poloxamer 188 was selected. At this concentration the hydrogel gelled at 34-35°C (**Table 18** and **Table 21**) in formulations without cyclodextrins and with cyclodextrins the gelation temperature was 36-37°C. The concentration of poloxamers was not corrected when cyclodextrins were added to the formulation because in the stability studies it was planned to incorporate the doxycycline into lipophilic region of poloxamers, and if the concentration of poloxamers varies, then HLB values will also vary and the stability of doxycycline might have been affected. To avoid the differences in HLB values, the concentration of poloxamers were kept the same in formulations with and without cyclodextrins. The concentration of cyclodextrins added was 1:24 w/w with the doxycycline, so 15 grams of hydrogel containing 0.1 % (15mg) of doxycycline will contain 360 mg of HPβCD. With the increase in the concentration of poloxamer 407, while the poloxamer 188 concentration was kept constant, the gelation temperature decreased. With the increase in concentration of poloxamers 188, the gelation temperature increased. The poloxamer hydrogels were rapidly losing solvent due to evaporation, so the formulations were tightly sealed with a parafilm, until they were transferred into actual container.

4.3 Identification of 4-epidoxycycline

Skuli's HPLC method

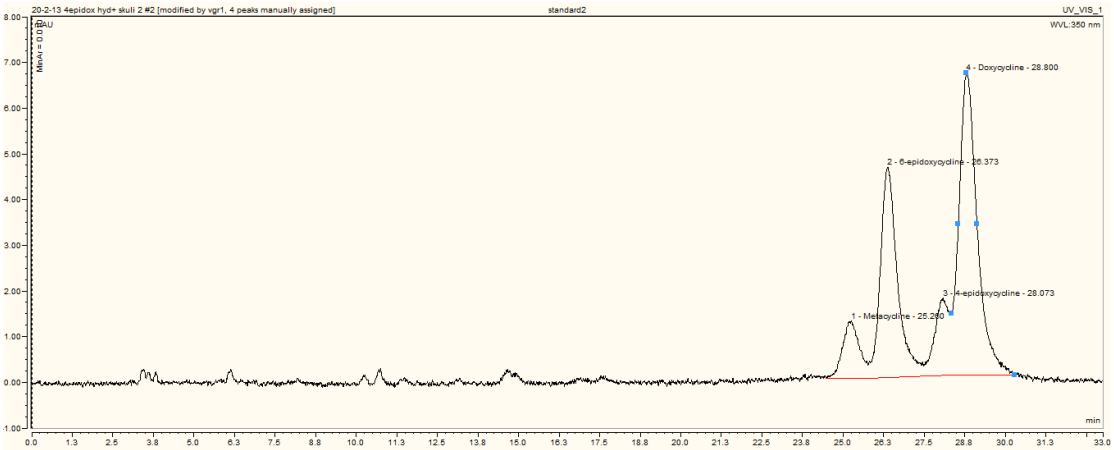


Figure 13 *Appearance of 4-epidoxycycline in Skuli's method*

4-epidoxycycline in Skuli's method appeared between the 6-epidoxycycline and doxycycline peaks (**Figure 13**). There were some instances in which 4-epidoxycycline was mistakenly considered as 6-epidoxycycline.

European and British Pharmacopoeia method:

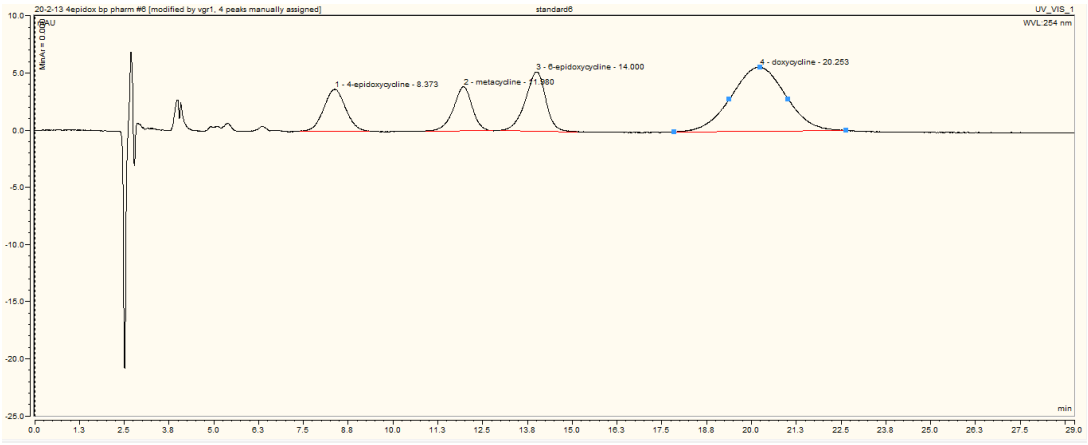


Figure 14 *Appearance of 4-epidoxycycline in the European Pharmacopoeia method*

The only degradation that was seen in the stability tests was 4-epidoxycycline. It was not available commercially to quantify. The "relative retention time" mentioned in the European Pharmacopoeia for impurity "c", i.e. 4-epidoxycycline is 0.5. The relative retention time was calculated by dividing the retention time of interest (4-epidoxycycline, retention time from the above graph is 8.0 minutes) with the main peak (doxycycline, retention time is 19.1 from above **Figure 14**) (i.e. 8.5/19.1= 0.5). So relative retention time 0.5 corresponds to 4-epidoxycycline according to European Pharmacopoeia. The unknown peak occurring at around 8.5 minutes was confirmed as 4-epidoxycycline according to European Pharmacopoeia and United States Pharmacopoeia.

4.4 Adjustment of resolution and peak symmetry

Effect of concentration of 2-methyl-2-propanol or *tert*-Butanol on peak appearance times, resolution and symmetry.

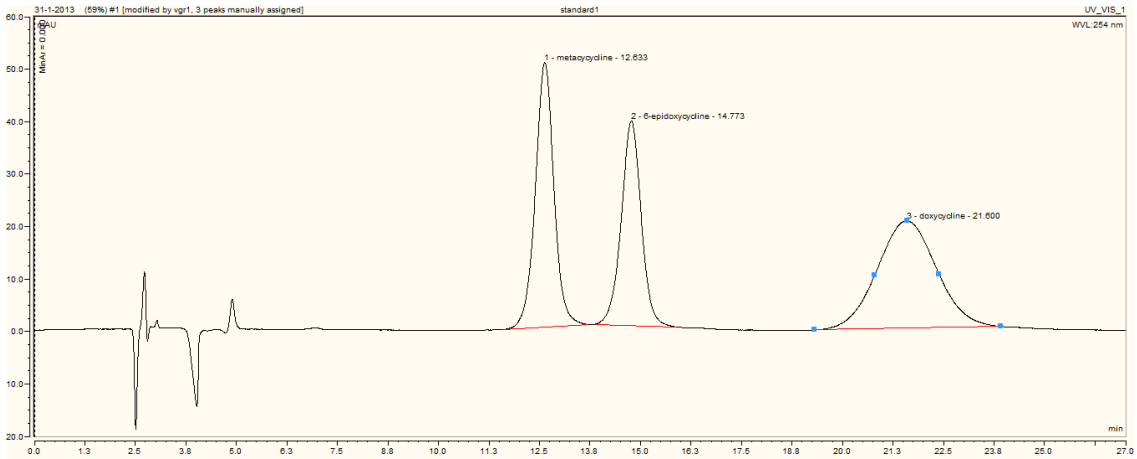


Figure 15 *Effect of concentration of 2-methyl-2-propanol on peak resolutions and symmetry factor (59 grams of 2-methyl-2-propanol in 1 litre of mobile phase)*

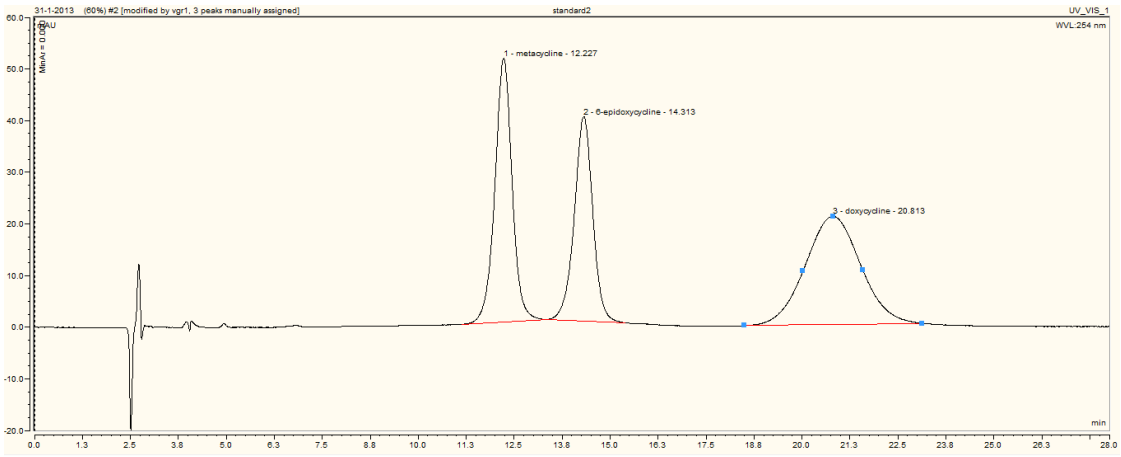


Figure 16 *Effect of concentration of 2-methyl-2-propanol on peak resolutions and symmetry factor (60 grams of 2-methyl-2-propanol in 1 litre of mobile phase)*

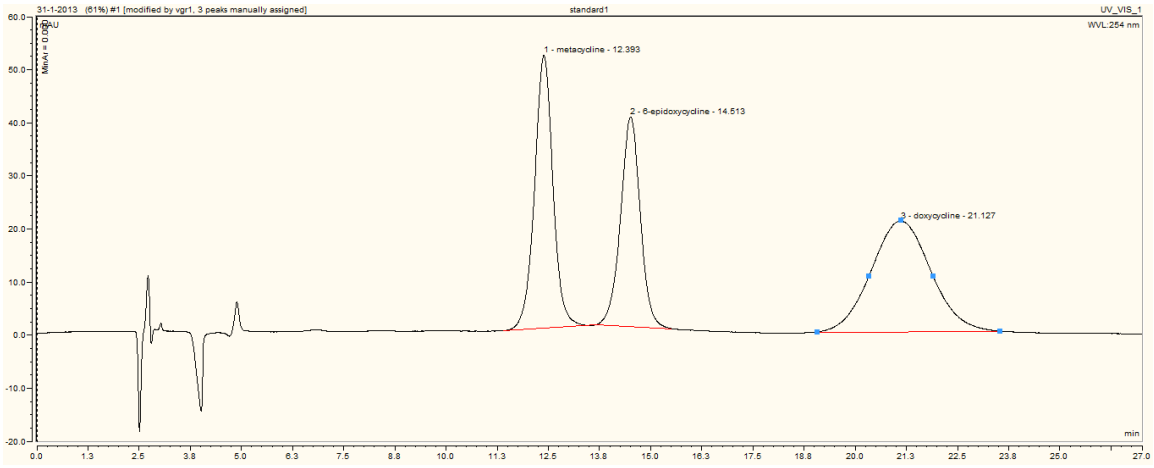


Figure 17 *Effect of concentration of 2-methyl-2-propanol on peak resolutions and symmetry factor (61 grams of 2-methyl-2-propanol in 1 litre of mobile phase)*

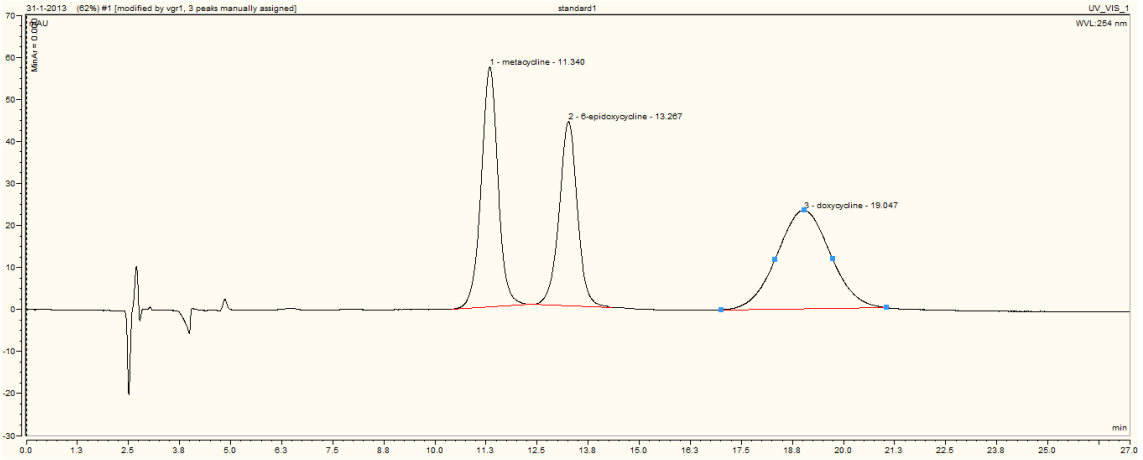


Figure 18 *Effect of concentration of 2-methyl-2-propanol on peak resolutions and symmetry factor (62 grams of 2-methyl-2-propanol in 1 litre of mobile phase)*

61 g of *tert*-Butanol in the mobile phase gave required resolutions and peak symmetry's as mentioned in the European Pharmacopoeia. So approximately, 61 g

of *tert*-Butanol was added for every 1 litre of mobile phase. The concentration adjustment for *tert*-Butanol was suggested in pharmacopoeia to obtain suitable resolution. And also *tert*-Butanol was commercially available in different purity concentrations and the concentration was needed to be adjusted accordingly.

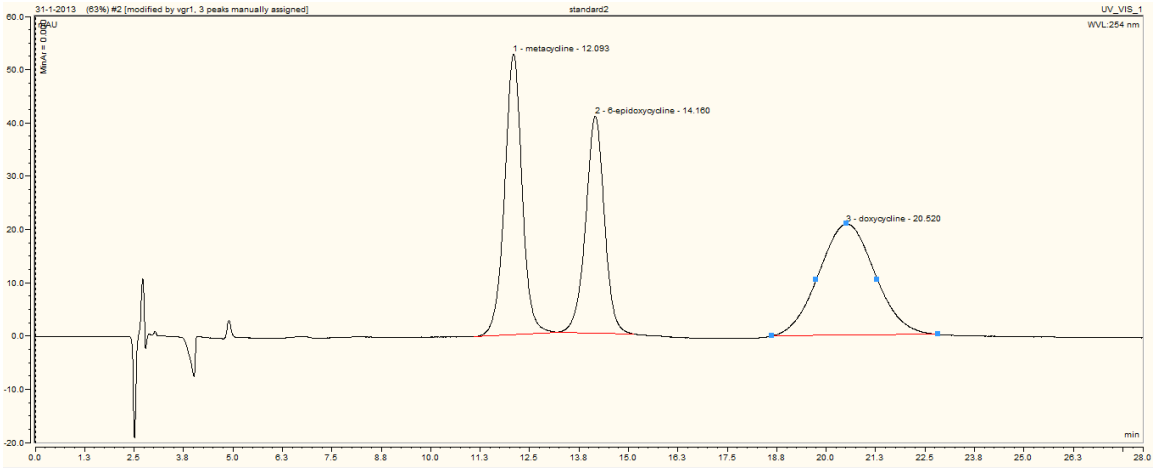


Figure 19 *Effect of concentration of 2-methyl-2-propanol on peak resolutions and symmetry factor (63grams of 2-methyl-2-propanol in 1 litre of mobile phase)*

4.5 Effect of excipients on HPLC (Skuli's method) results

There were many hypotheses made and the idea was to rule out one by one possibility until the final cause will be known:

1. Might be calculation errors (human errors)
2. Might be due to aging of doxycycline hyclate, it might have different stability within the different regions of the container.
3. Might be due to errors in micropipette used
4. Might be due to analytical scale inaccuracy
5. Might be due to faulty stationary phase (column).
6. Excipients present in the hydrogel might be interfering with the results
7. Antioxidants might be interfering with the peak resolutions or might be getting absorbed in the same region as doxycycline.
8. Chelating agents might be interfering with the peaks or might be getting absorbed in the same regions as doxycycline.

1. Determination of the effect of antioxidants on doxycycline peaks: (see HPLC results table from appendix 28-06-2012)

The % yields should be ideally 100 % if the solutions were prepared on same day, but the solutions with only doxycycline and no antioxidants and chelating agents were showing low stabilities (**Table 22**) and the ones with antioxidants and chelating agents were showing high values. This could be because of 3 reasons.

Table 22 Effect of antioxidants on doxycycline percentage yields in HPLC analysis

S.no	Solutions containing	HPLC sample no's	% Yields
1	Only doxycycline	1a,1b,1c	82 %
2	Only antioxidants	2a,2b,2c	No peaks
3	Doxycycline+ antioxidants	3a,3b,3c	102 %
4	Doxycycline+antioxidants+ chelating agent	4a,4b,4c	111 %

1. Presence of strong oxidising agent in the mobile phase. Perchloric acid is a strong oxidising agent, it contains an oxygen atom in the anionic ring, which further potentiates its oxidising capacity.

2. Antioxidants and chelating agents might be interfering with the results (solutions containing only antioxidants were injected to see if they were getting absorbed near the same time as doxycycline, but there were no peaks when only antioxidants containing solution was injected).

2. Determination of effect of chelating agent EDTA on doxycycline peaks: (see HPLC results table from appendix 03-07-2012 & 5-07-2012)

Table 23 Effect of chelating agents on doxycycline percentage yields in HPLC analysis

S.no	Solutions containing	HPLC sample no's	% Yields(3-07-2012) 1st run	% Yields (5-07-2012) 2nd run
1	Only doxycycline (w/v) & (v/v)	1A,1B,1C	124 %	129 %
2	Only EDTA (w/v) & (v/v)	2A,2B,2C	No peaks	No peaks
3	Doxycycline+EDTA (w/v) & (v/v)	3A,3B,3C	147 %	142 %
4	Doxycycline+EDTA+Antioxidants (w/v) & (v/v)	4A,4B,4C	143 %	135 %
5	Only doxycycline (0.5 g of 0.5mg/5g solution was diluted to 25 ml) (w/w) & (w/v)	5A,5B,5C	101 %	93.5 %
6	Doxycycline+EDTA+Antioxidants (0.5 g of 0.5 mg/5g solution was diluted to 25 ml) (w/w) & (w/v)	6A,6B,6C	104 %	96 %

The solution containing only doxycycline showed less values when compared to solutions with antioxidants and chelating agents (**Table 23**). It was tested whether chelating agents were getting absorbed around the same time as doxycycline, but no peaks were seen when a solution containing only EDTA was injected. When the solutions from the previous experiment were injected the values for a solution containing only doxycycline increased whereas the stability of a solution containing antioxidants and chelating agents decreased from111 % to 96 %.

3. Checking the column precision between the new and old columns: (see HPLC results table from appendix 11-07-2012 & 12-07-2012)

Table 24 Column precision testing

S.no	Solutions containing	HPLC sample no's	% Yields (new column) (11-07-2012)	% Yields (old column) (12-07-2012)
1	Only doxycycline (w/v)	1	81.5 %	84 %
2	Only doxycycline (w/v)	2	98.5 %	96 %

There were 2 columns, it was thought that the error might be due to a fault in new stationary phase bought during that time and so the same solutions were injected and percentage yields were compared. From the results (Table 24) there was not much difference in the percentage yield and the variations were of an acceptable level. So it was concluded that the stationary phase did not have any defects.

4. Assay of doxycycline from different manufacturers (see HPLC results table from appendix 12-07-2012)

Table 25 Assay of doxycycline from different manufacturers, to check the effect of ageing on peaks

S.no	Solutions containing	HPLC sample no's	%Yields
1	0.5mg of doxycycline (HOVIONE Macau) diluted to 25 ml of mobile phase	1A,1B	76.5%
2	0.5mg of doxycycline (HOVIONE Macau) diluted to 25 ml of mobile phase	2A,2B	99.5%
3	0.5mg of doxycycline (HOVIONE Macau) diluted to 25 ml of mobile phase	3A,3B	92%
4	0.5mg of doxycycline (HOVIONE Macau) diluted to 25 ml of mobile phase	4A,4B	89%
5	0.5mg of doxycycline (Sigma-aldrich Standard) diluted to 25 ml of mobile phase	5A,5B	77%
6	0.5mg of doxycycline (Sigma-aldrich Standard) diluted to 25 ml of mobile phase	6A,6B	89.5%
7	0.5mg of doxycycline (Nordisk) diluted to 25 ml of mobile phase	7A,7B	77.5%
8	0.5mg of doxycycline (Nordisk) diluted to 25 ml of mobile phase	8A,8B	79.5%
9	Previous column precision vials from previous day were injected again	9A,9B	85%
10	Previous column precision vials from previous day were injected again	10A,10B	97%

It was thought that the doxycycline in containers was degrading due to ageing and so it might have different stabilities in different regions within the container. When 0.5 g of doxycycline was mixed in the mobile phase and analysed the results were

not consistent. There were different doxycycline samples analysed and the values were fluctuating (**Table 25**). This can be because of following reasons:

1. Errors in the analytical scale. Also as a small quantity (0.5mg) was weighed, at this level the analytical scales had 10-15 % error. Minimum weight required for accurate measurement for majority of analytical scales was 10 mg.

2. Doxycycline might be oxidising due to presence of strong oxidizing agent ($HClO_4$) in the mobile phase.

4a.Assay of doxycycline from HOVIONE Macau container, by using a different analytical scale (see HPLC results table from appendix 14-08-2012):

Table 26 Assay of doxycycline Hovione samples

S.no	Solution containing	HPLC sample no's	%Yields
1	0.5mg of doxycycline (HOVIONE Macau) diluted to 25 ml of mobile phase	1,2,3	117 %
2	0.5mg of doxycycline (HOVIONE Macau) diluted to 25 ml of mobile phase	4,5,6	122 %
3	0.5mg of doxycycline (HOVIONE Macau) diluted to 25 ml of mobile phase	7,8,9	86 %

This time the analytical scale was changed and the results were still fluctuating (Table 26).

4b. Assay of doxycycline from HOVIONE Macau container, by using different analytical scale (see HPLC results table from appendix 16-08-2012)

Table 27 Assay of doxycycline from Hovione container, by avoiding error due to analytical scale

S.no	Solution containing	HPLC sample no's	%Yields
1	Doxycycline+EDTA+Antioxidants (5 ml of 10 mg/100 ml water was diluted to 25 ml with mobile phase) (w/v) & (v/v)	1,2,3	110.5 %
2	Directly 1 mg doxycycline was diluted to 50 ml with mobile phase	4,5,6	123 %
3	Directly 2 mg doxycycline was diluted to 100 ml with mobile phase	7,8,9	105 %
4	Only doxycycline (5 ml of 10 mg/100ml stock solution which was used to prepare standards was diluted to 25 ml with mobile phase) (w/v) & (v/v)	10,11,12	108 %
5	Only doxycycline (10 ml of 10 mg/100ml stock solution which was used to prepare standards was diluted to 50 ml with mobile phase) (w/v) & (v/v)	13,14,15	102 %

This time to avoid the error due to the analytical scale, directly 10 mg of doxycycline was weighed into a 100 ml volumetric flask and diluted with mobile phase. From this solution 5 ml was pipetted out and diluted to 25 ml. So the error due to analytical

scale was avoided. But even then the solutions containing only doxycycline were showing lower values whereas the solutions containing antioxidants and chelating agents were showing high values (**Table 27**). In some cases when doxycycline was directly weighed into volumetric flask and analysed the results were some instances below 100% and some times above 100 %. Over all these values suggested that there might be error in the HPLC method itself.

5. Effect of pH of 30 µl injected solution on results (see HPLC results table from appendix 23-08-2012)

Table 28 Study of effect of pH on doxycycline HPLC assay

S.no	Solution containing	HPLC sample no's	% Yields
1	pH 2.5	1,2	83 %
2	pH 2.6	3,4	110 %
3	pH 2.7	5,6	103 %
4	pH 2.8	7,8	98 %
5	pH 2.9	9,10	70 %
6	pH 3.0	11,12	90 %
7	Doxycycline + antioxidants + chelating agent (pH was 2.6)	13,14	110 %

10 mg of doxycycline was added to a 100 ml volumetric flask and diluted with mobile phase. 5 ml of the above solution was diluted to 25 ml of mobile phase. 6 such solutions were prepared and the pH was adjusted to 2.6, 2.7, 2.8, 2.9, 3.0 with a 5M NaOH solution, the quantity of NaOH added was negligible. Even though concentrations might not be affected, while adjusting the pH, when NaOH was added, the concentration should decrease, as the solution was getting more diluted but the values increased. This showed that the pH was affecting the results (Table 28). When antioxidants and chelating agents were added the pH of the mobile phase was changing as the buffer capacity was not good. It is known that for acidic and basic compounds, pH can affect the UV absorbance. When the same amount of antioxidants and chelating agents contained in the sample of hydrogel were added to the mobile phase, the pH of the mobile phase has shifted to 2.6. So at pH 2.6 the values were higher. And it did not follow any pattern while the pH was further increased. This might be only one of the reasons to affect the HPLC peaks. In the absence of antioxidants and chelating agents the reason why the values were fluctuating is unexplainable, but there was a pattern observed in these fluctuations like, in the absence of antioxidants and chelating agents the values were fluctuating below 100 % (70 – 100 %) and in the presence of antioxidants and chelating agents the values were fluctuating between 120 – 145 %. The reason for fluctuating values in the absence of antioxidants and chelating agents might be doxycycline might be instantly degrading on exposure to water molecules and strong oxidising agents in the mobile phase .

6. Doxycycline assay from HOVIONE sample, with different analytical scale (see HPLC results table from appendix 27-08-2012)

Table 29 Effect of quality of analytical scale on HPLC results

S.no	Solution containing	HPLC sample no's	% Yields
1	5 ml of 10 mg/ml solution was diluted to 25 ml with mobile phase	1	100.5 %
2	5 ml of 10 mg/ml solution was diluted to 25 ml with mobile phase	2	101 %
3	5 ml of 10 mg/ml solution was diluted to 25 ml with mobile phase	3	100 %
4	5 ml of 10 mg/ml solution was diluted to 25ml with mobile phase	4	100 %
5	5 ml of 10 mg/ml solution was diluted to 25ml with mobile phase	5	100 %

The analytical scale was changed again and the solution was prepared by adding 10 mg of doxycycline into a 100 ml volumetric flask and diluted with mobile phase. 5 ml of the above solution was pipetted out and diluted to 25ml with mobile phase and analysed. All the 5 samples showed 100 % yields (**Table 29**).

Same effect of excipients was observed even when hydrogels containing doxycycline were quantitatively analysed by Skuli's method:

Table 30 Stabilities of hydrogels at 4 °C from starting day to week 5

S.no	At Temperatures	1 Run(150612) % Yields	2 Run (After 9 days)(240612) % Yields	3 Run (After 5 weeks) (130712) % Yields
Only doxycycline	4 °C	61 %	91.50 %	102.50 %
	23 °C	62.70 %	89 %	90 %
	40 °C	67.50 %	74.50 %	37.50 %
Docycycline + Na2S2O3 + Na2S2O5 + EDTA	4 °C	107 %	131 %	116.50 %
	23 °C	115.50 %	128 %	107.50 %
	40 °C	111.90 %	107.50 %	84.50 %

The hydrogels were prepared by w/w method even then same kind of error existed as like when the hydrogels were prepared with w/v method. Initially when the hydrogels were prepared by w/v method the foam interfered with the accurate volume adjustment and this was thought to be the cause of high percentage yield in the presence of antioxidants and chelating agents. From the above table, at 4 °C the stabilities of hydrogels when measured on the same day of preparation, the values were 61 % for hydrogel containing only doxycycline and in the presence of antioxidants and chelating agents the stability was 107 %. After 10 days when the same hydrogels were analysed the values in the hydrogel containing only doxycycline increased to 91.5 % and in the hydrogel containing antioxidants and chelating agents the stability increased to 130 %. Again after 5 weeks when the same hydrogels were analysed, the stability of the

hydrogel containing only doxycycline was 102.5 % whereas hydrogel containing antioxidants and chelating agents showed 116 % stability. From the above Table 30 important conclusions can be drawn.

1. In the hydrogel containing only doxycycline the stability appears to be increasing with time but the actual phenomenon behind this was that, the drug was not uniformly dissolved in the formulation. While preparing the formulation to minimise the foam formation, the hydrogels were prepared by manual stirring with a glass rod. The hydrogels were manually stirred very patiently for upto 1-2 h even then the drug was not uniformly dispersed in the formulation. As the formulation had high viscosity due to high concentration of poloxamers, the solute particles were trapped in the high viscous formulation and the movement of solute particles was highly restricted and the solute particles were not able to equilibrate their concentration in the solvent rapidly. The above values prove that solute particles slowly equilibrating and finally it took nearly 5 weeks to uniformly disperse into the formulation. Manual stirring with a glass-rod or by using a magnetic bar could not equilibrate the solute particles into the high viscosity solvent.

2. Vortexing the hydrogel for 5 minutes after adding all the excipients equally distributed the drug into the formulation.

3. This slow movement of solute particles in the high viscous solvent was considered even while deciding whether to add doxycycline and HPβCD separately or to add them after pre-complexation into the formulation. Doxycycline and HPβCD have the ability to form complex even if they are directly dissolved as individual components into the solvent. But pre-complexation was preferred because the free movement of doxycycline and HPβCD in the high viscous solvent is doubtful.

4. On the initial day and after equilibration of the concentration the hydrogels with antioxidants and chelating agents was consistently showing higher percentage yields. This was because, the presence of antioxidants and chelating agents in the hydrogel were causing an increase in pH of mobile phase from 2.5 to 2.6 and at this pH region the percentage yields were higher as the HPLC method was sensitive to change in pH and also because the buffer capacity of mobile phase is not good as it was unable to resist the change in pH. Because of this reason the HPLC method was changed to European Pharmacopoeia method.

5. When the drug is not equally dispersed into the formulation the stability values should be random i.e. once it should be less than 100 % and sometimes it should be greater than 100 %. But there is a consistent pattern that the hydrogel containing only doxycycline always the stability values were less than 100 % and in the hydrogels containing the antioxidants and chelating agents the values were always above 100 %. It can be confirmed that the Skuli's HPLC method was sensitive to changes in pH.

6. After switching to European Pharmacopoeia method the results were never over 100 %, if insolubility was the case then the values were below 100 % which would equilibrate after vortexing for 5 min. The buffer capacity of mobile phase in the European Pharmacopoeia's was good as it contained high concentration of buffer salts.

→ In a more recent stability studies of doxycycline in water[350], it was mentioned by the author that the stabilities were much higher in solutions which consisted of antioxidant citric acid[350] in comparison to solutions which did not contained any antioxidant. This supports the present conclusion in this study of how excipients like antioxidants can affect the HPLC peaks and thereby causing high percentage yields. The author also mentioned that the commercially available veterinary oral powder Presoldox™, when assayed gave high yields, by mentioning that this high yields might be due to contents in formulation. All this information clearly supports the current study of effect off excipients on doxycycline peaks.

4.6 Accelerated stability studies

All the 27 hydrogels in 3 batches (Table 31) contained 0.1 % doxycycline. For HPLC analysis 0.2 g of the hydrogel was weighed into a 10 ml volumetric flask and diluted with 0.01 M HCl. The concentration set for samples was 20 µg/ml. All the samples were injected 3 times, and the standard deviations were calculated. The summary graphs for all the HPLC runs in stability studies, over a 3 months period is shown below.

Table 31 Labelling for accelerated stability experiment

Labelling↓	Indicates↓
Batch I	Hydrogels stored at 4°C
Batch II	Hydrogels stored at 25°C
Batch III	Hydrogels stored at 40°C
Week 0	Starting day of analysis of hydrogel

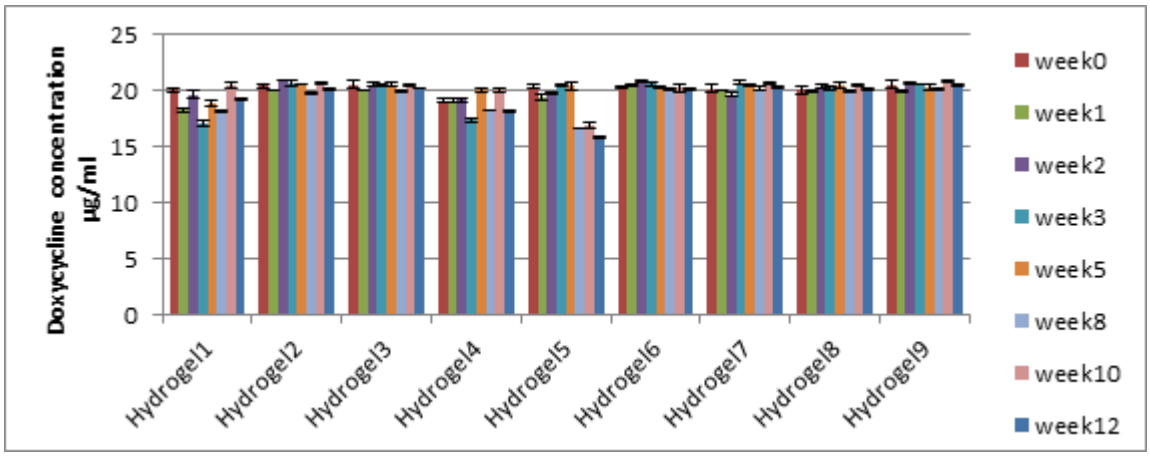


Diagram 1 *Graph showing the average and standard deviations of 9 hydrogels stored at 4 °C from week 0 to week 12*

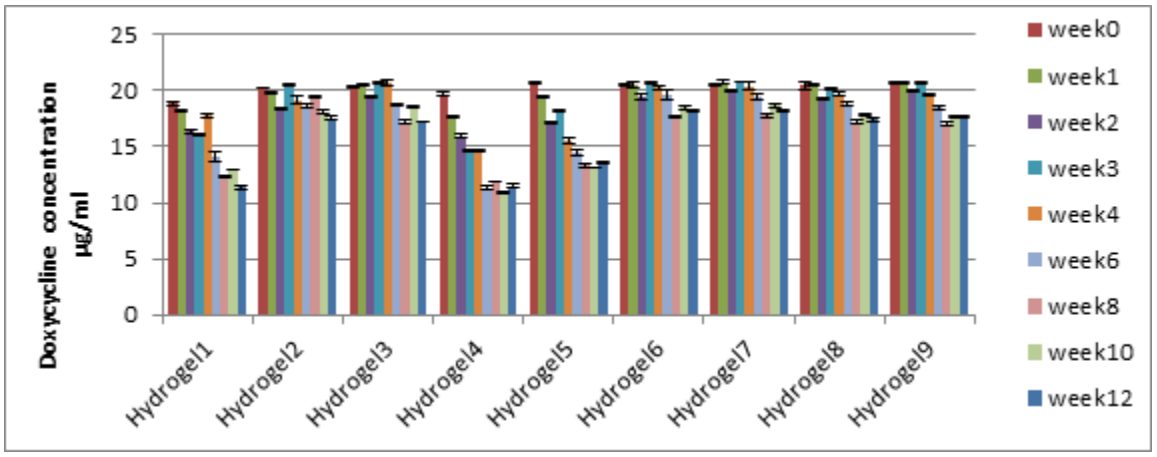


Diagram 2 *Graph showing the average and standard deviations of 9 hydrogels stored at 25 °C from week 0 to week 12*

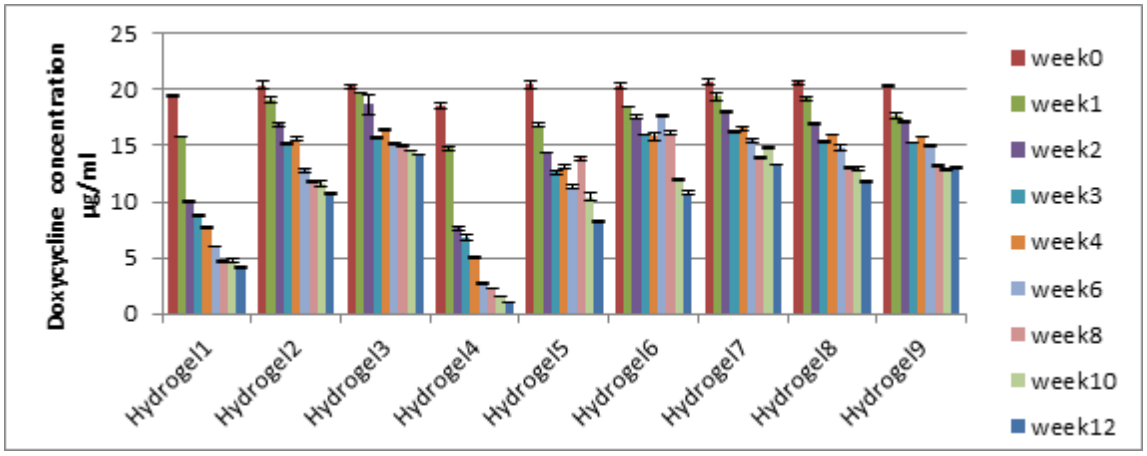


Diagram 3 *Graph showing the average and standard deviations of 9 hydrogels stored at 40 °C from week 0 to week 12*

Comparison of Results:

Mucoadhesive polymer povidone was excluded in hydrogel 9 because, the combination of poloxamer and povidone have acted as a degradation pathway to hydrochlorothiazide. Though hydrochlorothiazide is not related to the current project and the effect was seen in solid dosage form i.e. tablets, it was evident that combination of poloxamer and povidone might affect the stability of doxycycline. It was hypothesized that hydrochlorothiazide in the presence of poloxamers and povidone was exposed to moisture content within the tablet[322] and thereby degradation occurred. Here in the experiment doxycycline was expected to occupy the hydrophobic part (micelles) of surfactant i.e poloxamer and thereby protecting it from oxidation. By adding povidone in the presence of poloxamers, doxycycline might be exposed to water molecules and might degrade. Also hydrogel 9 contained less concentration of antioxidants.

Table 32 Hydrogels and ingredients

15 g of 0.1 %w/w doxycycline hyclate *in situ* forming hydrogels for treatment of the aphthous ulcers												
S.no	doxycycline(0.1%w/w)	HPβCD(1:24 w/w with doxycycline)	poloxamer407(21%w/w)	poloxamer188(10%w/w)	Mucoadhesive polymers		Antioxidants		EDTA (0.2%w/w)	$Mgcl_2$ 1:4 molar ratio with doxycycline	citric acid(0.2%w/w)	tartaric acid(0.2%w/w)
					HPMC 0.25%w/w	POVIDONE 0.25%w/w	$Na_2S_2O_3$ (0.32%w/w)	$Na_2S_2O_5$ (0.32%w/w)				
1	15mg		3.15gm	1.5gm	37.5mg	37.5mg						
2	15mg		3.15gm	1.5gm	37.5mg	37.5mg	48mg	48mg	30mg			
3	15mg		3.15gm	1.5gm	37.5mg	37.5mg	48mg	48mg	30mg	24mg		
4	15mg	360mg	3.15gm	1.5gm	37.5mg	37.5mg						
5	15mg	360mg	3.15gm	1.5gm	37.5mg	37.5mg	48mg	48mg				
6	15mg	360mg	3.15gm	1.5gm	37.5mg	37.5mg	48mg	48mg	30mg			
7	15mg	360mg	3.15gm	1.5gm	37.5mg	37.5mg	48mg	48mg	30mg	24mg		
8	15mg	360mg	3.15gm	1.5gm	37.5mg	37.5mg	48mg	48mg	30mg	24mg	30mg	30mg
9	15mg		3.15gm	1.5gm	75mg HPMC (0.5%w/w)		30mg (0.2%w/w)	30mg (0.2%w/w)	30mg	24mg		

→ **Note: Hydrogel 1, refers to S.no 1 from above table, hydrogel 2 refers to S.no 2 and so on**.

1. Hydrogel 1 at 4, 25 & 40°C.

Hydrogel 1 contained no antioxidants, chelating agents and cyclodextrins. The pH of the formulation was adjusted to 6.55 with 1M HCl and 1M NaOH solutions. At 4 °C the formulation was stable over a 3 month period (Diagram 4), but the stability was not consistently 100 %, and it was fluctuating between 90-100 %. This might be due to insolubility, but when compared to stabilities of other hydrogels tested, it was evident that the stability values were fluctuating in hydrogels that did not contain any antioxidants and chelating agents. Other possibility might be that doxycycline was degrading when exposed to HCl in the dilution medium. There were no degradation peaks at 4 °C. To rule this factor out, a small amount of antioxidants was added to the dilution medium and the results were analysed. At 25°C the degradation was fast, there were no metacycline or 6-epidoxycycline peaks seen, only 4-epidoxycycline was evident. At 40 °C the degradation was very rapid. From the above results the following conclusions can be hypothesized:

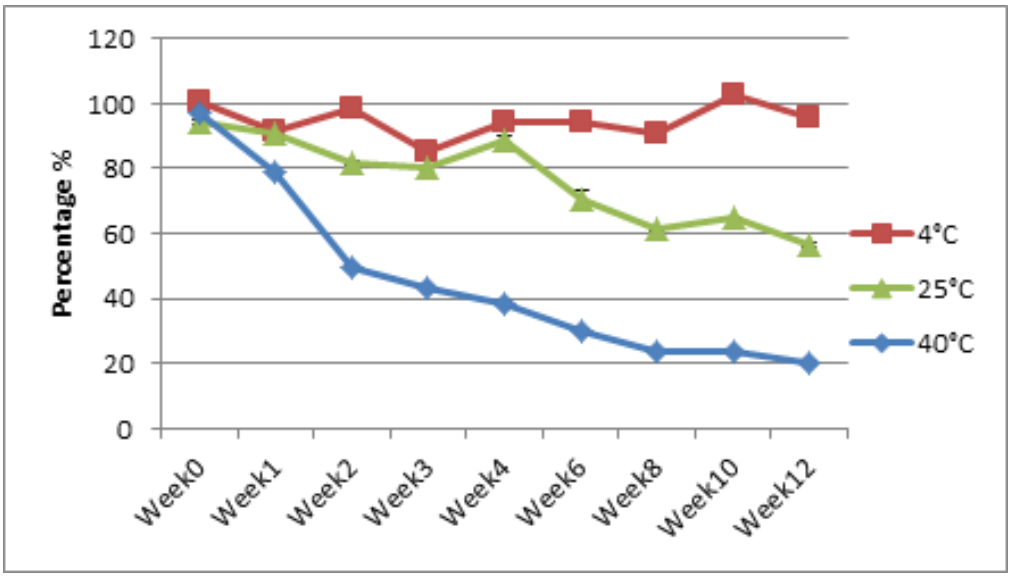


Diagram 4 *Graph showing the stabilities of hydrogel 1 at 4, 25 & 40 °C*

1. Doxycycline undergoes oxidation when in aqueous solutions, even at 4°C, but here at 4°C the formulation did not show any degradation compounds indicating that the drug is protected from the water molecules. The surfactant poloxamers contains 70% hydrophilic component and 30% lipophilic component. The doxycycline hyclate is hydrophilic and lipophilic, but it is considered more lipophilic. The chances of doxycycline hyclate to occupy lipophilic component of the surfactant will be 30%. The results indicate that doxycycline might have occupied the lipophilic regions of the surfactant and was protected from water molecules.

2. pH was one of the important factor considered while designing the experiment. Literature review suggested that doxycycline was unstable at all 3 regions i.e. acidic region, neutral region and basic region. Initially when the experiment was started, the pH of the formulation was 7.4, and a rapid degradation was observed even at 4°C. Taking this into account, the pH was adjusted to a weakly acidic region. Within the oral cavity the pH keeps changing between 6.2 and 7.5, depending upon the type of food being masticated. So the formulation can have pH anywhere between 6.2 and 7.5. Some literature suggested that pH below 6.4 can cause tooth demineralisation, so the formulation pH should be above 6.4. Finally pH was adjusted at 6.55, which is far from neutral region and safe for oral administration. At this pH the formulation was stable compared to 7.4 pH. This indicates that pH might not be the direct pathway for oxidation but it might act as a catalyst.

3. At 25°C the degradation might by due to temperature.

4. At 40°C the degradation might be due to temperature. And also hot water is more lipophilic, so the water molecules might have penetrated into the lipophilic regions of surfactant and oxidised the doxycycline.

2. Hydrogel 2 at 4, 25 and 40 °C.

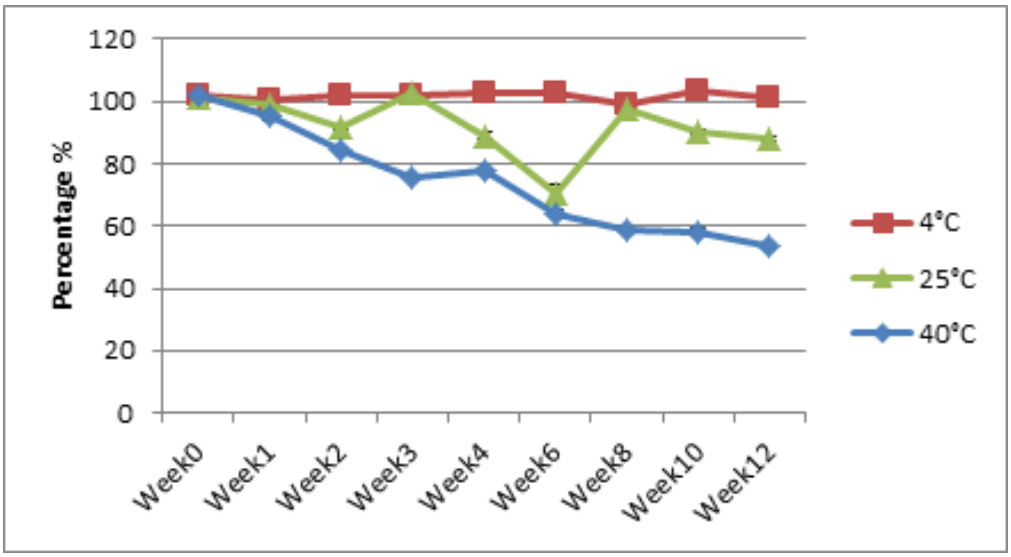


Diagram 5 *Graph showing the stability of Hydrogel 2 at 4, 25 & 40°C, from week 0-week 12*

The hydrogel 2 consisted of a combination of two antioxidants and a chelating agent. It can be seen that the values at 4°C are consistently stable unlike for hydrogel 1 (**Diagram 5**). At 25°C, and 40°C the degradation was very slow, because of the presence of antioxidants and chelating agent. Antioxidants, generally protects the compounds from oxidation, here the degradation was due to temperature and the addition of antioxidants and chelating agents reduced the degradation due to temperature. Increase in temperature causes degradation through oxidizing the compound into epimers and the presence of antioxidants helped to minimise this phenomena.

3. Hydrogel 3 at 4, 25 & 40 °C.

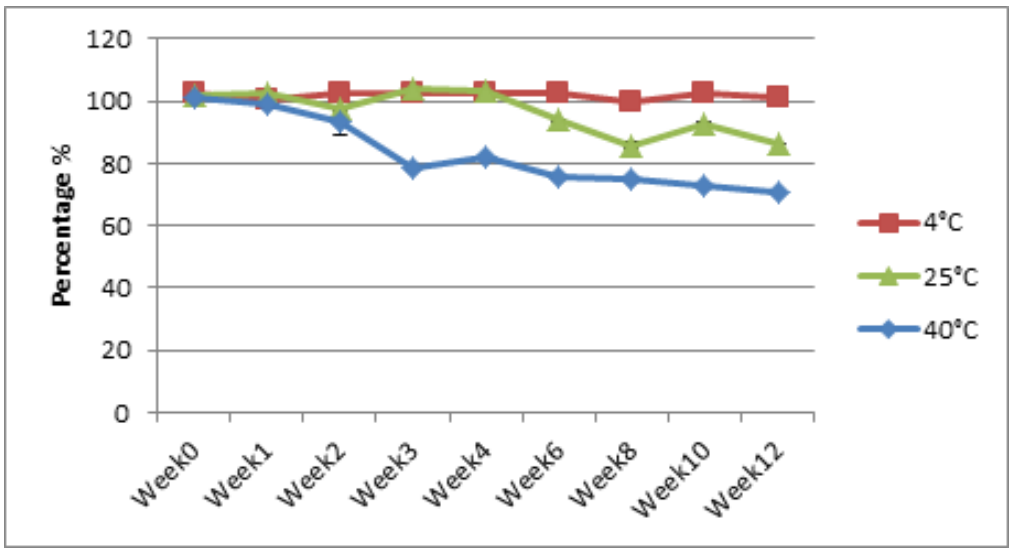


Diagram 6 *Graph showing the stability of Hydrogel 3 at 4, 25 & 40°C, from week0-week 12*

Hydrogel 3 consisted of combination of 2 antioxidants and 2 chelating agents. 2 chelating agents were added to check whether the synergetic action of chelating agents has any effect on stability of doxycycline. And this effect was evident at 40°C, the hydrogels with 2 chelating agents were 20% more stable (**Diagram 6**) than the hydrogels containing single chelating agent. At 4°C there were no fluctuations in the stability. At 25°C the fluctuations in values were minimised compared to hydrogel1. And at 40°C, the stability at the end of 3 months is 71%, this is the highest stability achieved till date.

4. Hydrogel 4 at 4, 25 & 40 °C.

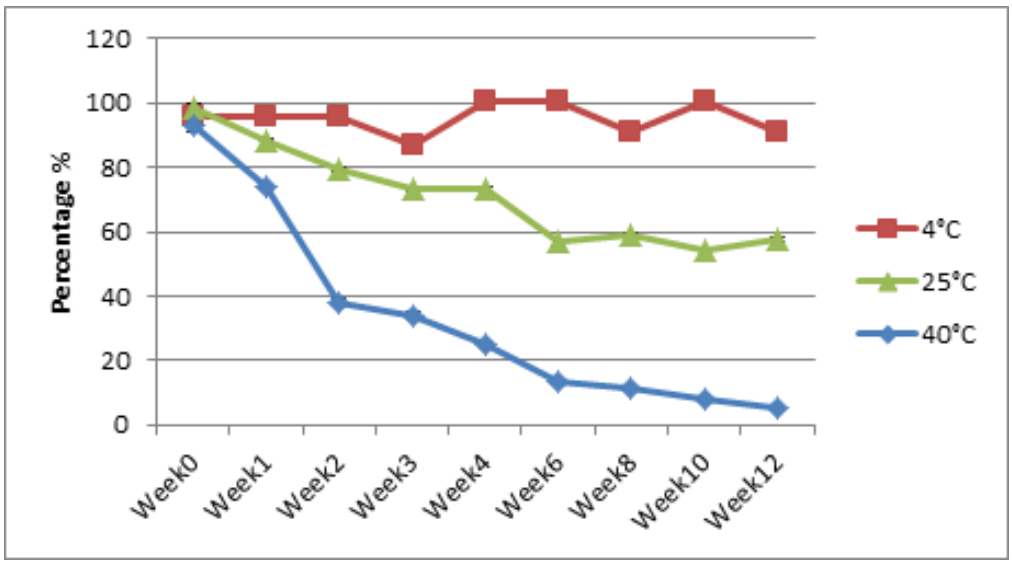


Diagram 7 *Graph showing the stability of hydrogel 4 at 4, 25 & 40°C, from week 0-week 12*

Hydrogel 4 comprised of only HPβCD. With the addition of cyclodextrin the stability of hydrogel decreased at all 3 temperatures (**Diagram 7**). HPβCD when complexed with the any molecule, it increases the hydrophilicity of the compound. Doxycycline which is more lipophilic was expected to occupy the lipophilic region of the surfactant but when it was complexed with the cyclodextrin the hydrophilicity of the compound was increased and the compound was more readily exposed to water molecules and hence more degradation. HPβCD was expected to attach to the $6CH_3$position of the doxycycline molecule. HPβCD might slightly increase the stability of doxycycline in water, but here surfactants are giving almost 100 % stability and when cyclodextrins were added the stability actually decreased.

5. Hydrogel 5 at 4, 25 & 40 °C.

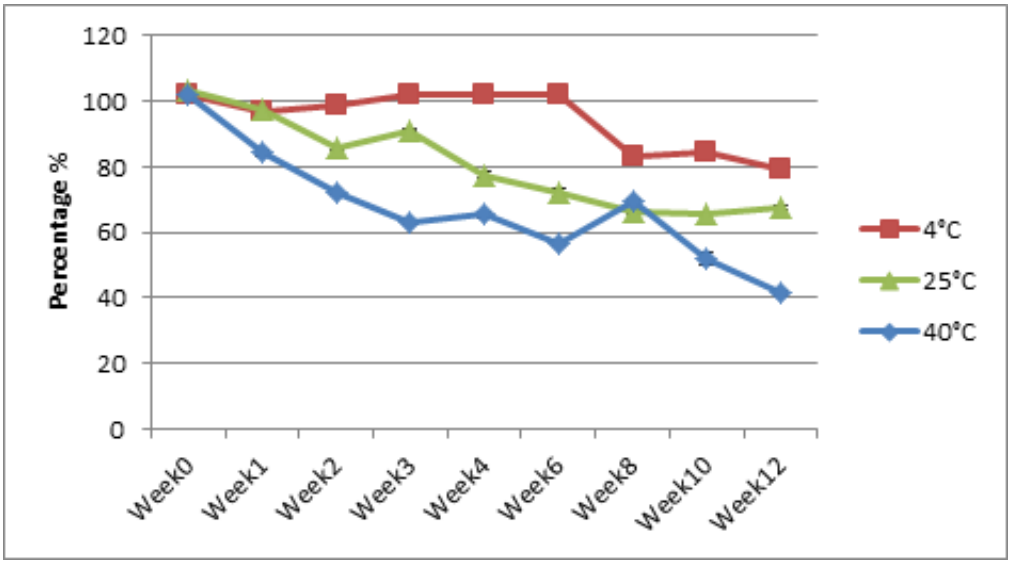


Diagram 8 *Graph showing the stability of Hydrogel 5 at 4, 25 and 40°C, from week 0-week 12*

Hydrogel 5 consisted of HPβCD and antioxidants. At 4 °C it is the only hydrogel which was unstable (**Diagram 8**). The cyclodextrins might be acting as a barrier between the antioxidants and doxycycline or cyclodextrins might have increased the hydrophilicity of doxycycline which in turn is exposed it to water molecules within the surfactant.

6. Hydrogel 6 at 4, 25 & 40 °C.

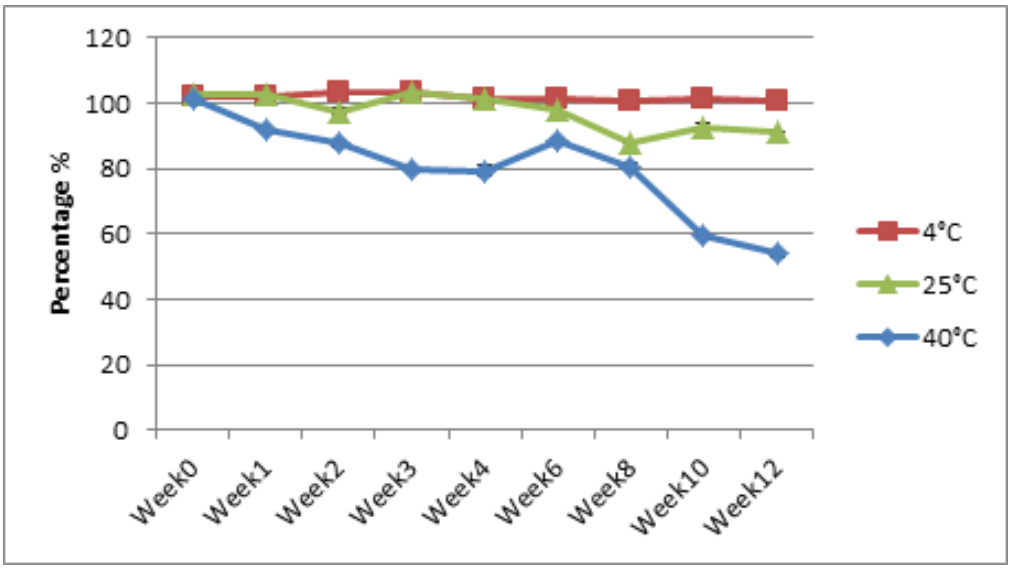


Diagram 9 *Graph showing the stability of Hydrogel 6 at 4, 25 & 40 °C, from week 0-week 12*

Hydrogel 6 consisted of HPβCD, 2 antioxidants and a chelating agent. Addition of chelating agent greatly improved the stability of doxycycline at all 3 temperatures (**Diagram 9**).

7. Hydrogel 7 at 4, 25 & 40 °C.

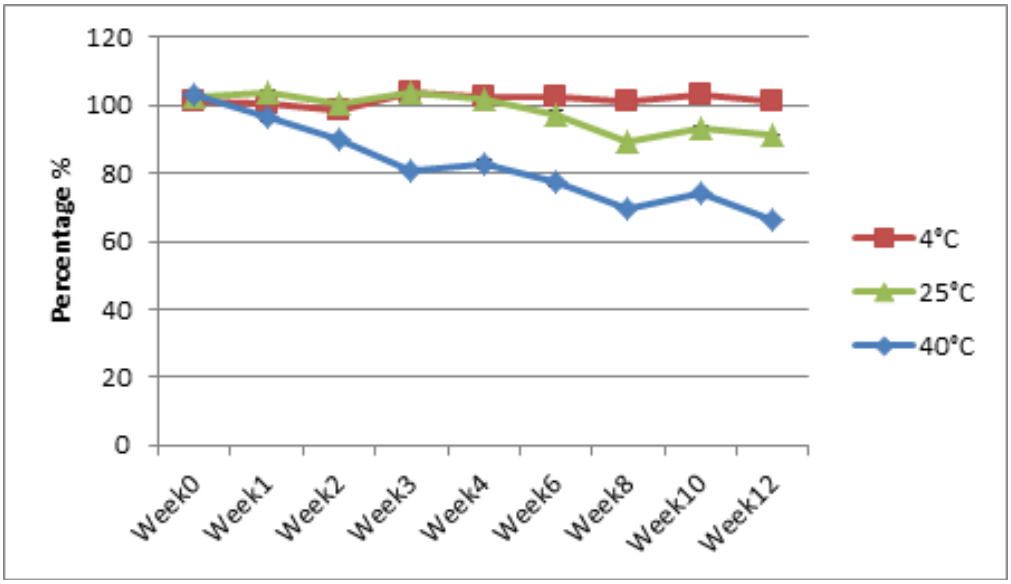


Diagram 10 *Graph showing the stability of hydrogel 7 at 4, 25 and 40 °C, from week 0-week 12*

Hydrogel 7 consisted of HPβCD, 2 antioxidants and 2 chelating agents. Because of the synergetic action of chelating agents better stabilities were achieved at 25 and 40°C. At 25°C the hydrogel was 91% stable (**Diagram 10**).

8. Hydrogel 8 at 4, 25 & 40 °C.

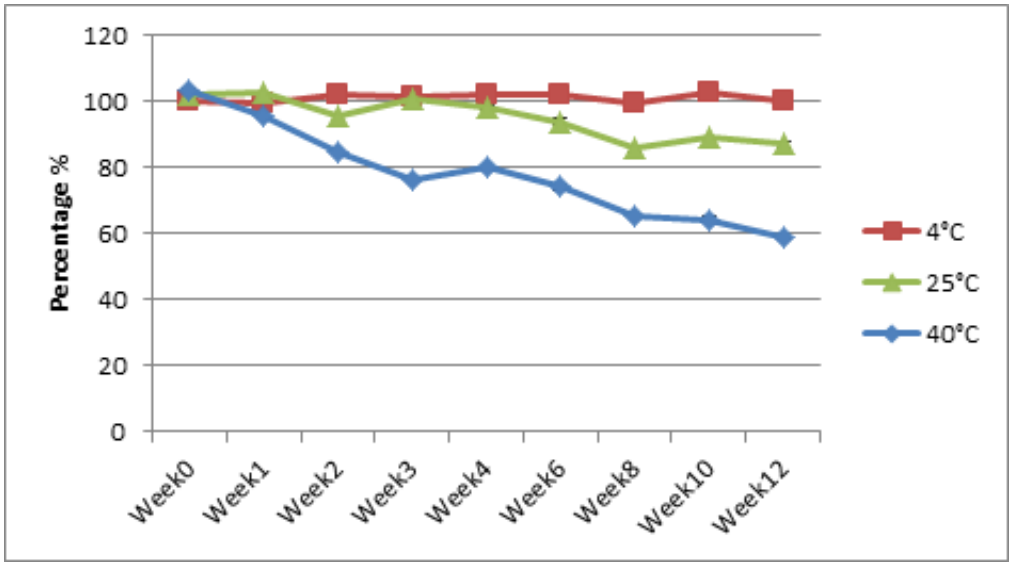


Diagram 11 *Graph showing the stability of Hydrogel 8 at 4, 25 & 40 °C, from week 0-week 12*

Hydrogel 8 consisted of HPβCD, 2 chelating agents and 4 antioxidants. From the literature review it was evident that not all the antioxidants gave stability to doxycycline. So, 2 new antioxidants were added to the formulation to improve the stabilities at 25 and 40°C (**Diagram 11**). But the stabilities were similar to that of hydrogel 7.

9. Hydrogel 9 at 4, 25 & 40 °C.

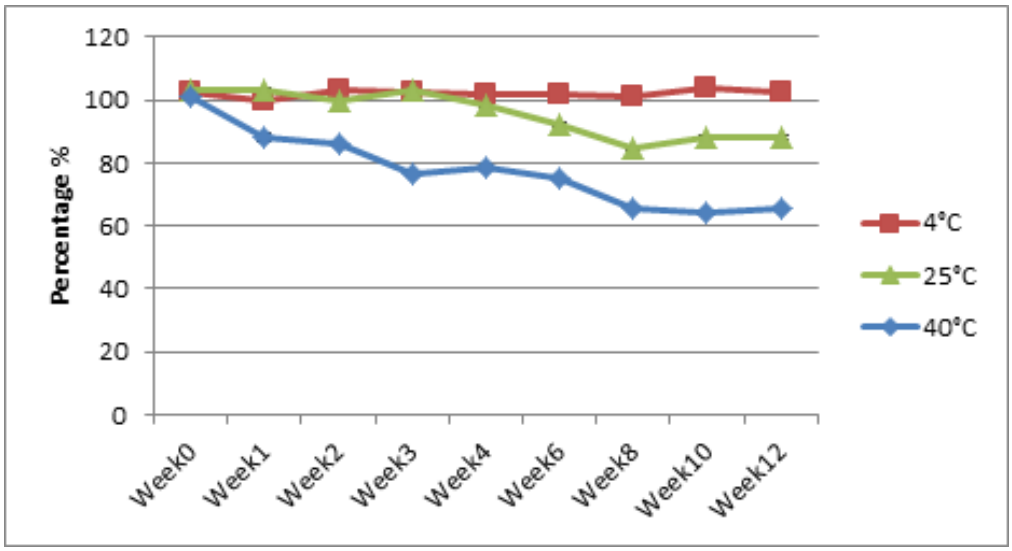


Diagram 12 *Graph showing the stability of hydrogel 9 at 4, 25 & 40 °C, from week 0-week 12*

Hydrogel 9 consisted of 2 antioxidants and 2 chelating agents. There was only one mucoadhesive polymer HPMC in the formulation. Povidone was not included in this formulation, to check whether it has an effect on the stability of doxycycline. The composition of this hydrogel was similar to that of hydrogel 3 but the concentrations of antioxidants were much lower. At 40°C, hydrogel 3 was more stable when compared with hydrogel 9 (**Diagram 12**). This was because of increase in concentration of antioxidants.

<u>Comparisons of stability between 9 hydrogels at 4, 25 & 40 °C, from week 1 to week 12.</u>

1. Comparisons of stability of Hydrogel 1 vs Hydrogel 4 at 4°C.

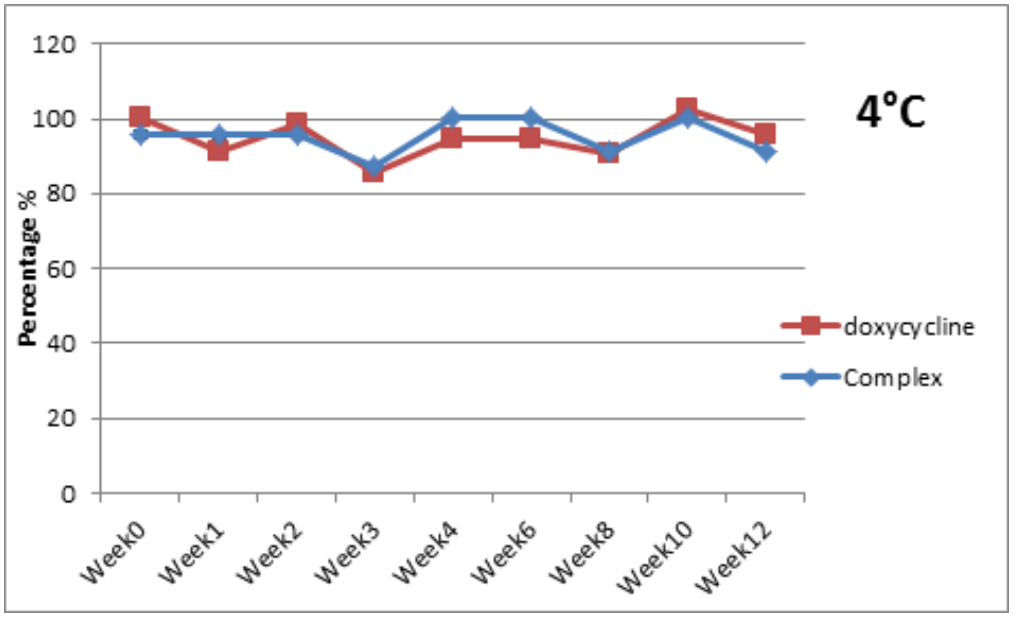


Diagram 13 *Graph comparing the stabilities of Hydrogel 1 and Hydrogel 4 at 4 °C*

Hydrogel 1 comprised of only doxycycline whereas hydrogel 4 was contained doxycycline-HPβCD complex. The effect of cyclodextrins on the stability of doxycycline was observed. There is not much difference in stabilities with or without adding cyclodextrins (HPβCD) especially at 4 °C. Both the formulations had similar stabilities (**Diagram 13**) at 4°C by the end of 3 months.

2. Comparisons of stability of Hydrogel 1 vs Hydrogel 4 at 25°C.

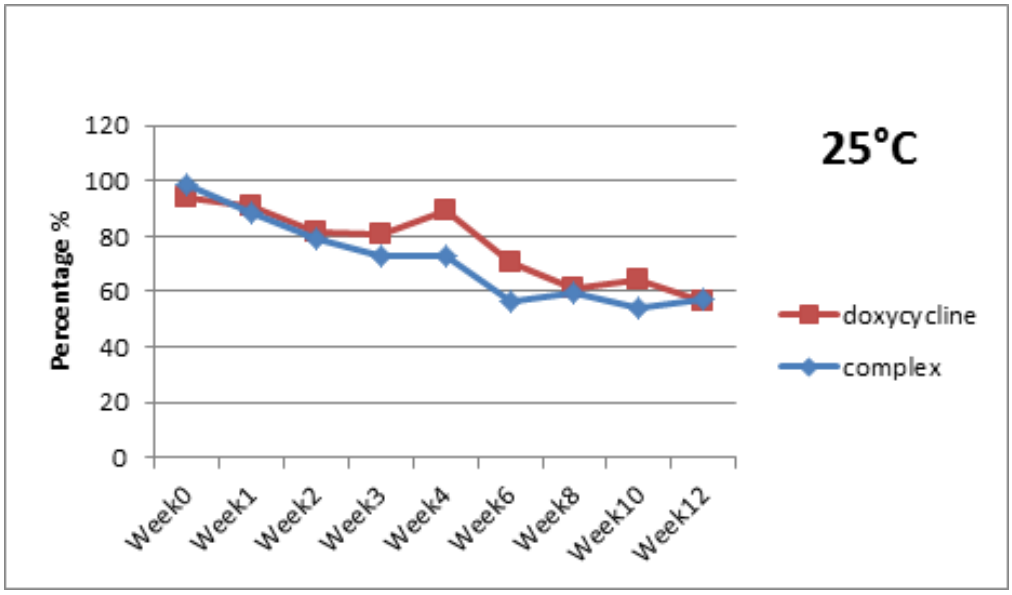


Diagram 14 *Graph comparing the stabilities of Hydrogel 1 and Hydrogel 4 at 25 °C*

Hydrogel 1 comprised of only doxycycline whereas hydrogel 4 was comprised of doxycycline-HPβCD complex. The effect of cyclodextrins on stability of doxycycline was observed at 25 °C. It can be seen from the **Diagram 14** that, cyclodextrins have reduced the stability in the intermediate time periods but overall stability at the end of 12 weeks was similar for both the hydrogels at 25 °C. This might be because of two reasons. Before the complexation with cyclodextrins, the doxycycline might be occupying the lipophilic regions (micelles) of the surfactant because of relatively more lipophilic nature, which might be protecting it from exposure to water molecules with in the surfactant and thereby preventing it from oxidising. It is known that HPβCD's increases the solubility profiles of lipophilic drugs in aqueous solutions. So after complexation, doxycycline might have become more hydrophilic and exposed to water molecules with in the surfactant and oxidised. Heat might act as a catalyst for degradation of doxycycline, when exposed to water molecules. Also hot water will disrupt lipophilic components, so when the temperature was increased the water molecules might have penetrated the lipophilic regions of the surfactant and might have oxidised the doxycycline.

3. Comparisons of stability of Hydrogel1 vs Hydrogel 4 at 40 °C.

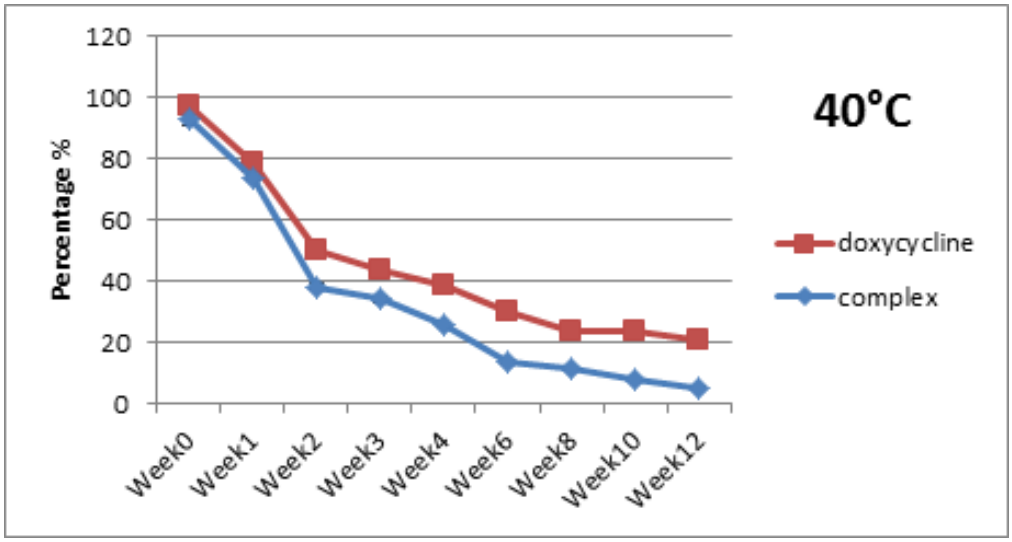


Diagram 15 *Graph comparing the stabilities of Hydrogel 1 and Hydrogel 4 at 40°C*

At 40°C the hydrogels with cyclodextins were less stable (**Diagram 15**). This also proves the hypothesis that after complexation, doxycycline became more hydrophilic and exposed to water molecules within the surfactant and got oxidised.

4. Comparisons of stability of Hydrogel 1 vs Hydrogel 2 at 4°C.

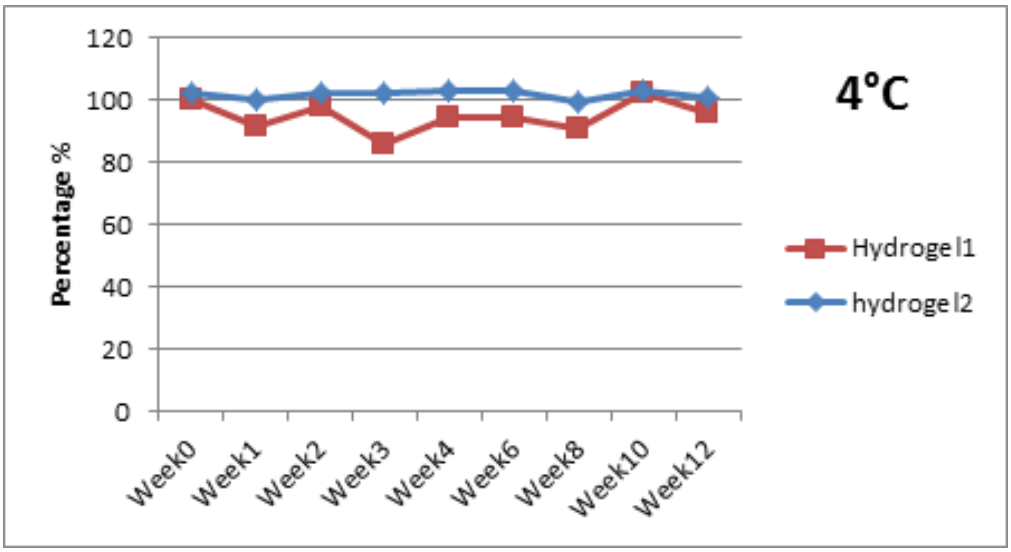


Diagram 16 *Graph comparing the stabilities of Hydrogel 1 and Hydrogel 2 at 4 °C*

Hydrogel 1 was comprised of only doxycycline, whereas hydrogel 2 was comprised of 2 antioxidants and chelating agent. The chelating agent was expected to bind at 4-N$(CH_3)_2$ site of doxycycline. From the **Diagram 16** it is evident that the presence of antioxidants and chelating agents have increased the stability of doxycycline. Hydrogel 1 was stable but the results were not consistent. This might be because of 2 reasons. In the absence of antioxidants the doxycycline stability might be constantly fluctuating depending upon the factors, and it is known that at pH less than 7.0 doxycycline reversibly converts

into epimers (degradation compounds). When the factors favour the doxycycline might again become 100% stable. And also the dilution medium for HPLC analysis consisted of strong oxidising agent and this might be even causing the results to fluctuate.

5. Comparisons of stability of Hydrogel1 vs Hydrogel 2 at 25°C.

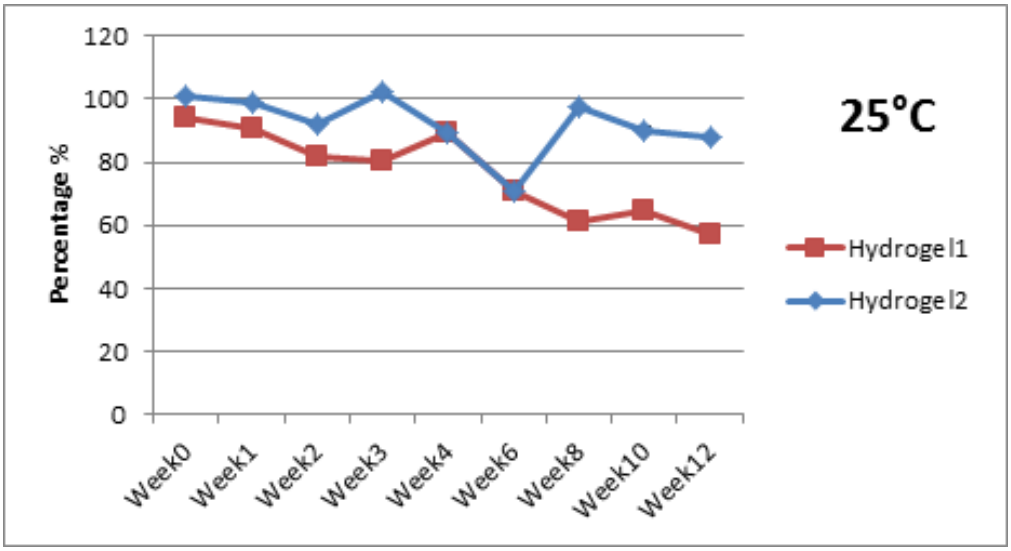


Diagram 17 *Graph comparing the stabilities of Hydrogel1 and Hydrogel 2 at 25 °C*

From the **Diagram 17**, it is evident that the, hydrogel 2 containing antioxidants and chelating agents is more stable than hydrogel 1, which contained only doxycycline.

6. Comparisons of stability of Hydrogel 1 vs Hydrogel 2 at 40 °C.

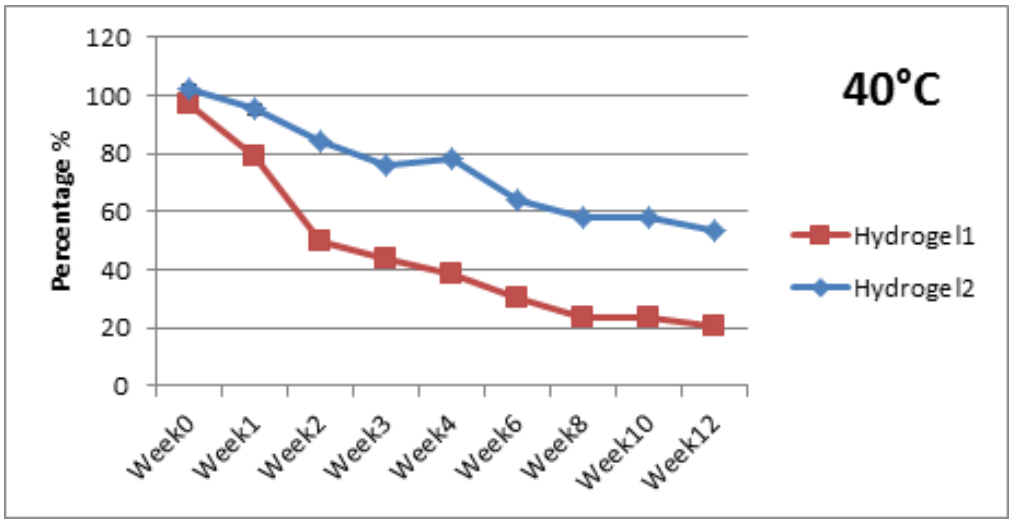


Diagram 18 *Graph comparing the stabilities of Hydrogel 1 and Hydrogel 2 at 40 °C*

The hydrogel 2 containing antioxidants and chelating agents is more stable than hydrogel1 (**Diagram 18**)

7. Comparisons of stability of Hydrogel 2 vs Hydrogel 3 at 4 °C.

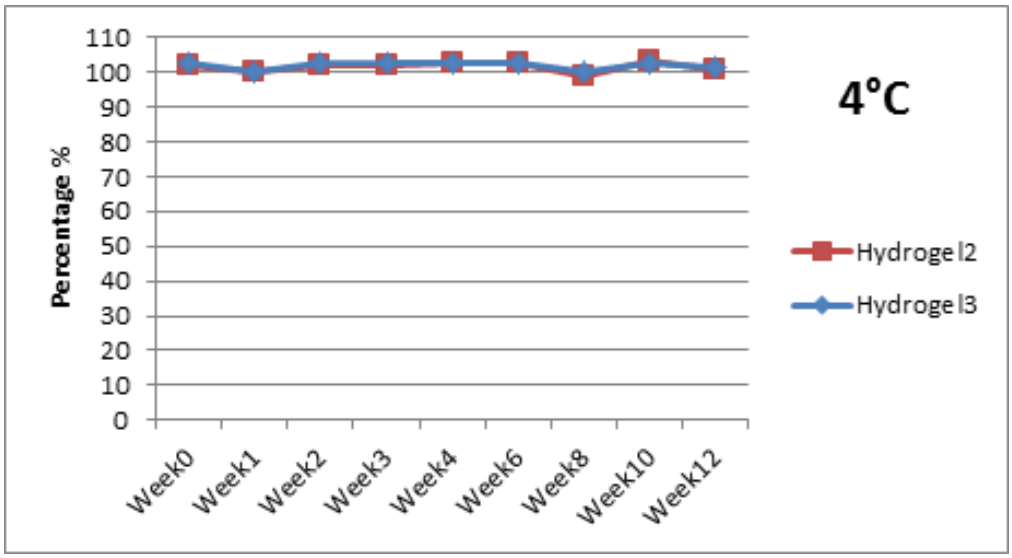


Diagram 19 *Graph comparing the stabilities of Hydrogel 2 & and Hydrogel 3 at 4 °C*

Hydrogel 2 was comprised of 2 antioxidants and 1 chelating agent, whereas hydrogel 3 was comprised of 2 antioxidants and 2 chelating agents. Synergetic effect was created between the 2 chelating agents. At 4°C, both the hydrogels are stable (**Diagram 19**).

8. Comparisons of stability of Hydrogel 2 vs Hydrogel 3 at 25°C.

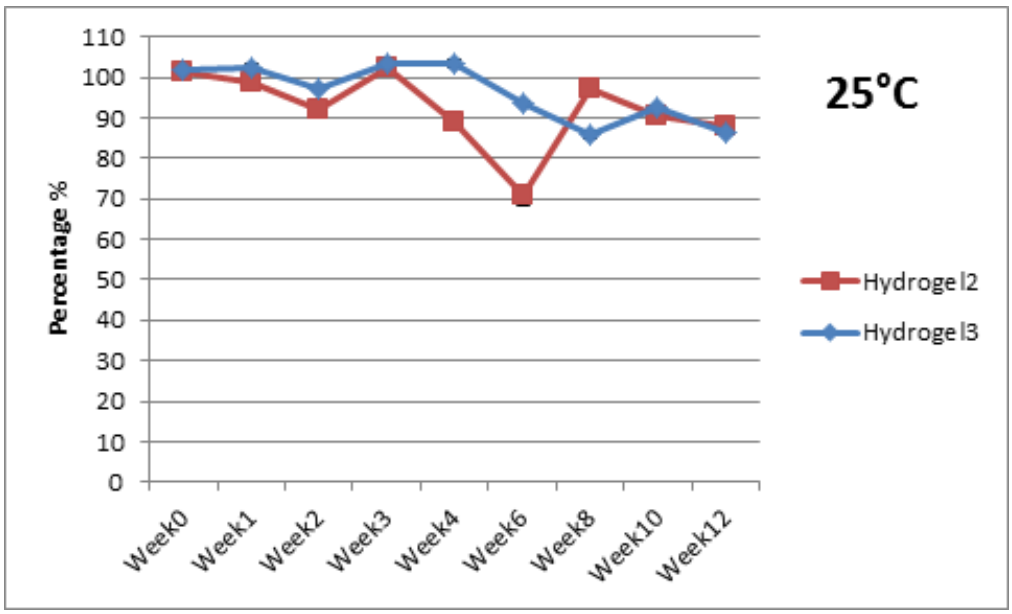


Diagram 20 Graph comparing the stabilities of Hydrogel 2 & Hydrogel 3 at 25 °C

Hydrogel 2 was comprised of 2 antioxidants and 1 chelating agent, whereas hydrogel 3 was comprised of 2 antioxidants and 2 chelating agents. Synergetic effect was created between the 2 chelating agents. From the **Diagram 20** it is evident that the hydrogel containing synergetic effect of chelating agents is more stable.

9. Comparisons of stability of Hydrogel 2 vs Hydrogel 3 at 40 °C.

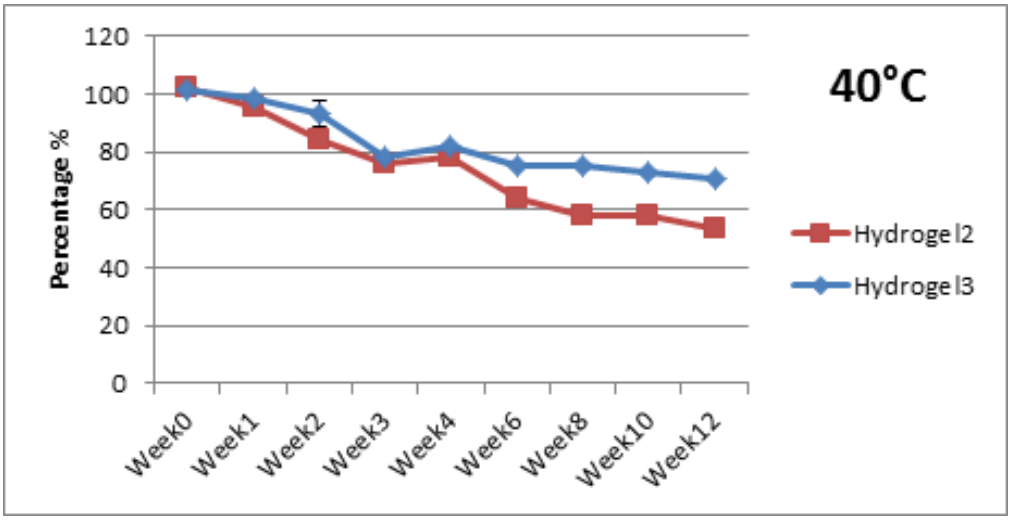


Diagram 21 *Graph comparing the stabilities of Hydrogel 2 & Hydrogel 3 at 40°C*

Hydrogel 2 was comprised of 2 antioxidants and 1 chelating agent, whereas hydrogel 3 was comprised of 2 antioxidants and 2 chelating agents. Synergetic effect was created between the 2 chelating agents. From the **Diagram 21** it is evident that the hydrogel containing synergetic effect of chelating agents is more stable than the hydrogel with single chelating agent. This might be also due to increase in concentration of chelating agent, as 2 chelating agents were present in the formulation.

10. Comparisons of stability of Hydrogel3 vs Hydrogel 9 at 4 °C.

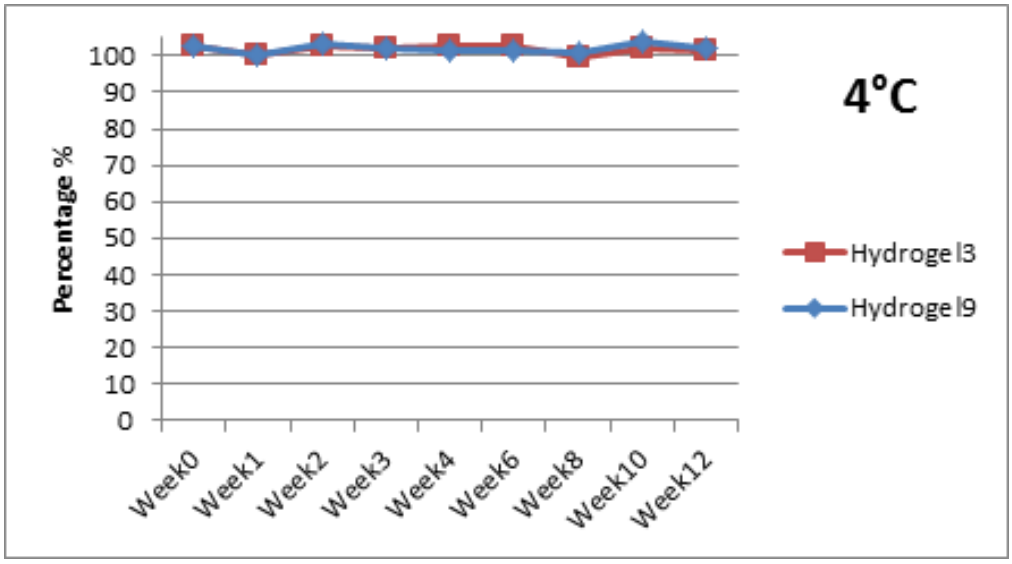


Diagram 22 *Graph comparing the stabilities of Hydrogel 3 & Hydrogel 9 at 4 °C*

Both hydrogels consisted of the same 2 antioxidants and 2 chelating agents, but the difference was that hydrogel 9 had lower concentrations of antioxidants and it contained only one mucoadhesive polymer, HPMC. The antioxidants concentration in hydrogel 3 was 0.32 %w/w of sodium thiosulfate and 0.32% of sodium

metabisulfate, whereas hydrogel 9 consisted of 0.2 %w/w of sodium thiosulfate and sodium metabisulfate each. Stabilities at 4 °C are similar (**Diagram 22**) and the hydrogel with lesser antioxidants concentration is slightly more stable.

11. Comparisons of the stability of Hydrogel 3 vs Hydrogel 9 at 25 °C.

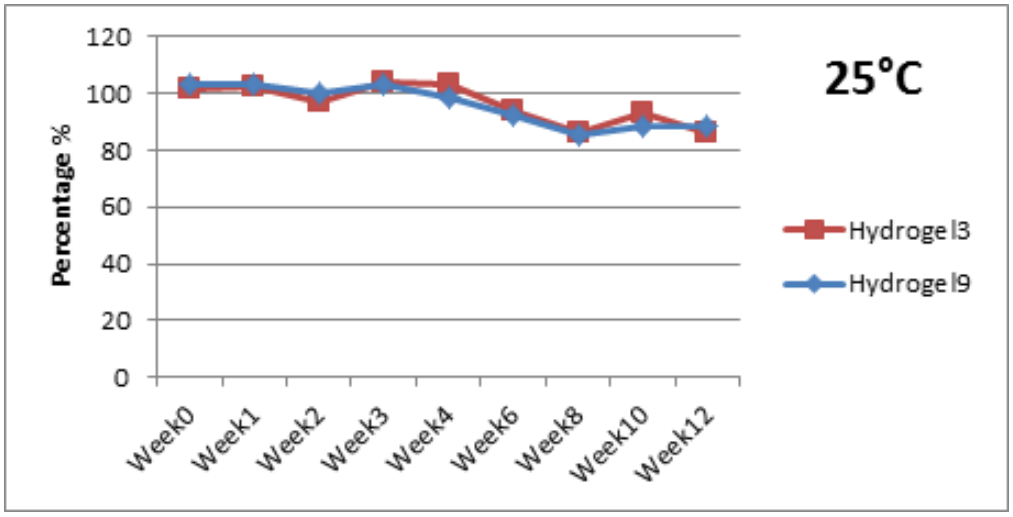


Diagram 23 *Graph comparing the stabilities of Hydrogel 3 & Hydrogel 9 at 25 °C*

Hydrogel 3 was comprised of 2 antioxidants 0.31 %w/w each and 2 chelating agents, whereas hydrogel 9 was also comprised of 2 antioxidants but in lower concentrations i.e. 0.2 %w/w each and 2 chelating agents. Both the hydrogels showed almost similar stabilities at 25 °C (**Diagram 23**).

12. Comparisons of stability of Hydrogel 3 vs Hydrogel 9 at 40 °C.

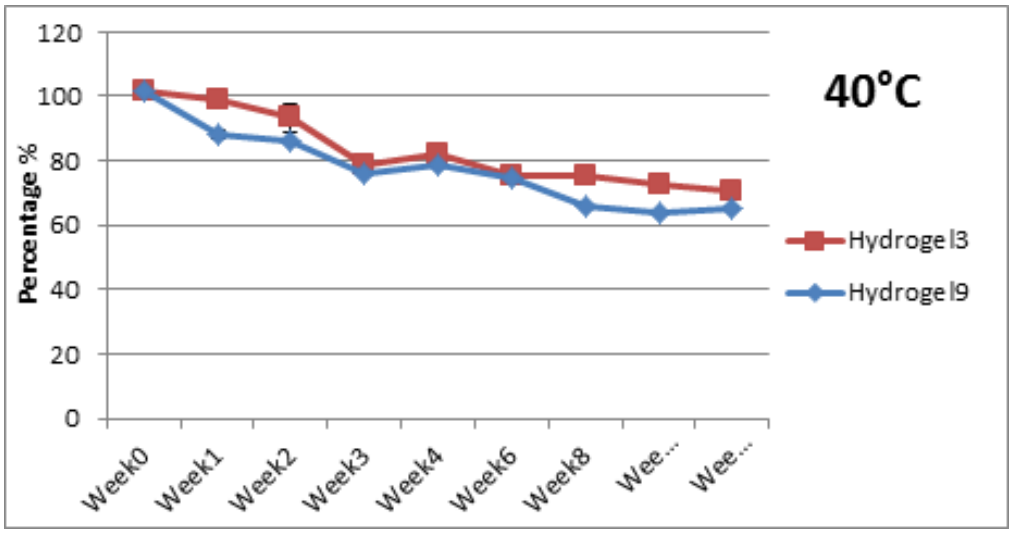


Diagram 24 *Graph comparing the stabilities of Hydrogel 3 and Hydrogel 9 at 40 °C*

Hydrogel 3 was comprised of 2 antioxidants 0.32 % w/w each and 2 chelating agents, whereas hydrogel 9 was also comprised of 2 antioxidants but in lower concentrations i.e. 0.2 % w/w each and 2 chelating agents. At 40 °C it is evident that higher concentrations of antioxidants gave more stability (**Diagram 24**).

13. Comparisons of stability of Hydrogel 4 vs Hydrogel 5 at 4 °C.

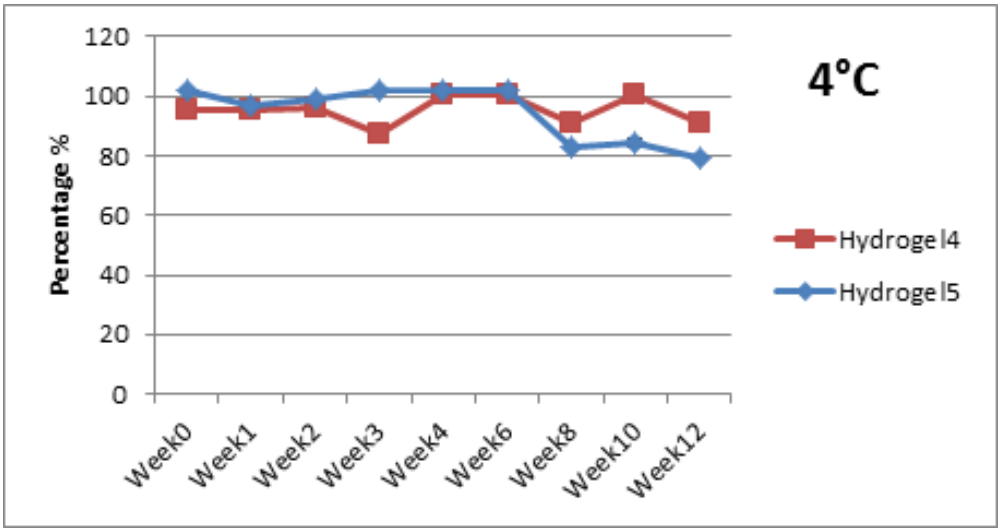


Diagram 25 *Graph comparing the stabilities of Hydrogel 4 and Hydrogel 5 at 4 °C*

Hydrogel 4 was comprised of doxycycline-HPβCD complex whereas hydrogel 5 comprised of complex and 2 antioxidants. Hydrogel 5 was the only formulation that has shown significant degradation at 4°C (**Diagram 25**). Indicating that the cyclodextrins might be acting as barrier between the antioxidants and the doxycycline and also cyclodextrins might have increased the hydrophilicity of drug and exposed it more readily to water molecules within the surfactant. Hydrogel 1 did not contain any antioxidants or chelating agents and it was almost 100 % stable. There are three possibilities either only antioxidants could not impart 100 % stability to the doxycycline, or cyclodextrins were acting as barriers between the drug and antioxidants or pH of the hydrogel was 6.55 which might be the most favourable (pH) region to impart the stability. At 4 °C the degradation was because of exposure to water molecules with in the formulation, and presence of HPβCD increased the hydrophilicity of drug, which caused more exposure to water molecules with in the surfactant.

14. Comparisons of stability of Hydrogel 4 vs Hydrogel 5 at 25 °C.

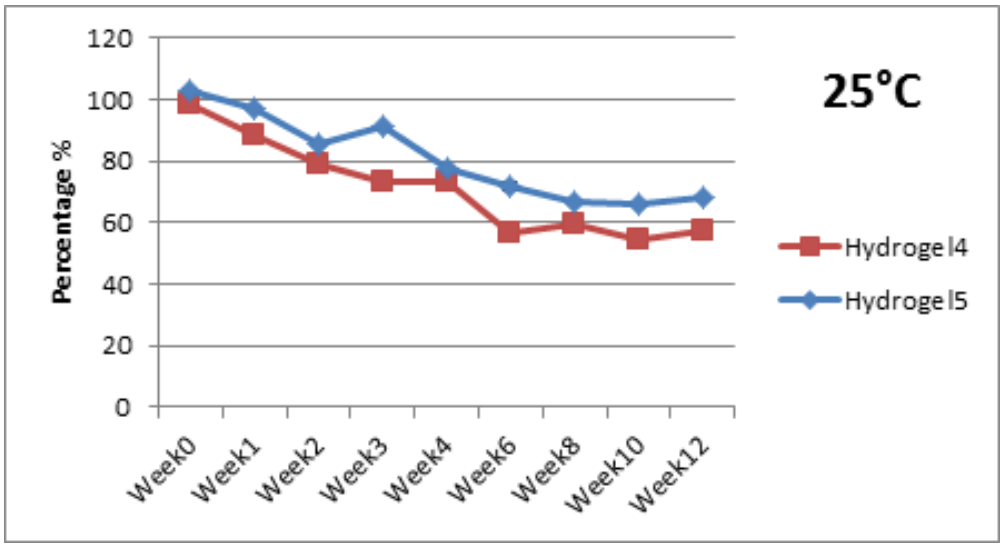


Diagram 26 *Graph comparing the stabilities of Hydrogel 4 & Hydrogel 5 at 25 °C*

Hydrogel 4 was comprised of doxycycline-HPβCD complex whereas hydrogel 5 comprised of complex and 2 antioxidants. At 25°C the degradation was rapid in both the formulations, but significantly the presence of antioxidants halted the degradation in hydrogel5 (**Diagram 26**). For hydrogel 5 at 25 °C the degradation was because of 2 reasons, 1. Exposure to water molecules with in the surfactant, 2. Because of increase in temperature. Antioxidants significantly reduced the degradation by halting the degradation due to oxidation that was in turn due to increased temperature and increased penetration of water molecules with in the surfactant as it is known that, as temperature increases, water becomes more lipophilic. But unlike at 4°C the hydrogel 5 was more stable than hydrogel 4 because the predominant effect was increased temperature and antioxidants significantly halted oxidation due to increased temperature.

15. Comparisons of stability of Hydrogel 4 vs Hydrogel 5 at 40 °C.

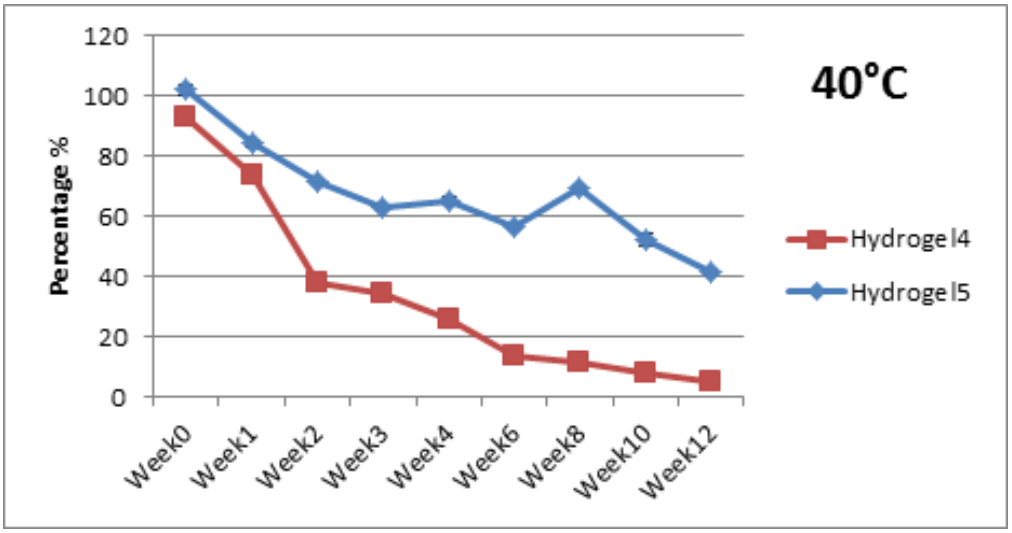


Diagram 27 *Graph comparing the stabilities of Hydrogel 4 & Hydrogel 5 at 40 °C*

Hydrogel 4 was comprised of doxycycline-HPβCD complex whereas hydrogel 5 comprised of complex and 2 antioxidants. At 40°C the degradation was rapid in both the formulations, but the presence of antioxidants halted the degradation to a high extent in hydrogel 5 (**Diagram 27**). At 40 °C the degradation was predominantly due to high temperature and antioxidants have significalntly reduced this effect in hydrogel 5. In hydrogel 5 another unfavourable factor was change in pH when antioxidants were added and at this changed pH (from 6.55 to around5.91) doxycycline degradation might be rapid.

16. Comparisons of stability of Hydrogel 5 vs Hydrogel 6 at 4 °C.

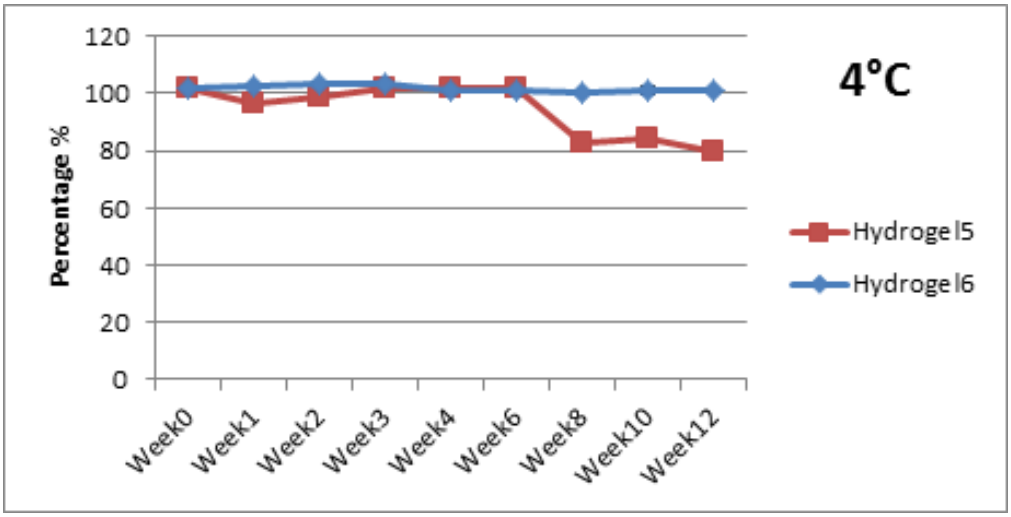


Diagram 28 *Graph comparing the stabilities of Hydrogel 5 & Hydrogel 6 at 4 °C*

Hydrogel 5 was comprised of HPβCD and 2 antioxidants, whereas hydrogel 6 was comprised of HPβCD, 2 antioxidants and 1 chelating agent. It is evident from the **Diagram 28** that antioxidants alone cannot impart stability to doxycycline and presence of chelating agent is a must.

17. Comparisons of stability of Hydrogel 5 vs Hydrogel 6 at 25 °C.

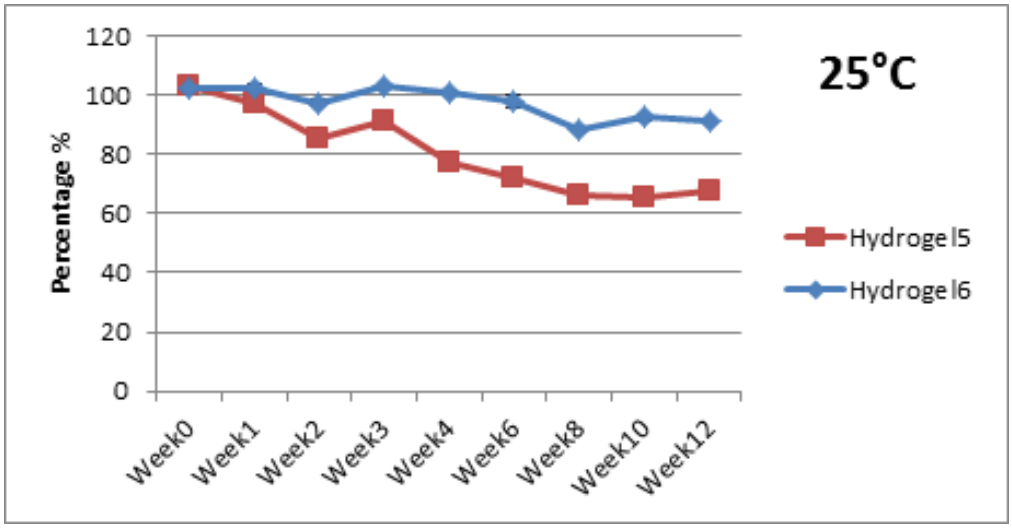


Diagram 29 *Graph comparing the stabilities of Hydrogel 5 & Hydrogel 6 at 25 °C*

Hydrogel 5 was comprised of HPβCD and 2 antioxidants, whereas hydrogel 6 was comprised of HPβCD, 2 antioxidants and 1 chelating agent. Hydrogel 6 was more stable when compared with hydrogel 5 (**Diagram 29**).

18. Comparisons of stability of Hydrogel 5 vs Hydrogel 6 at 40 °C.

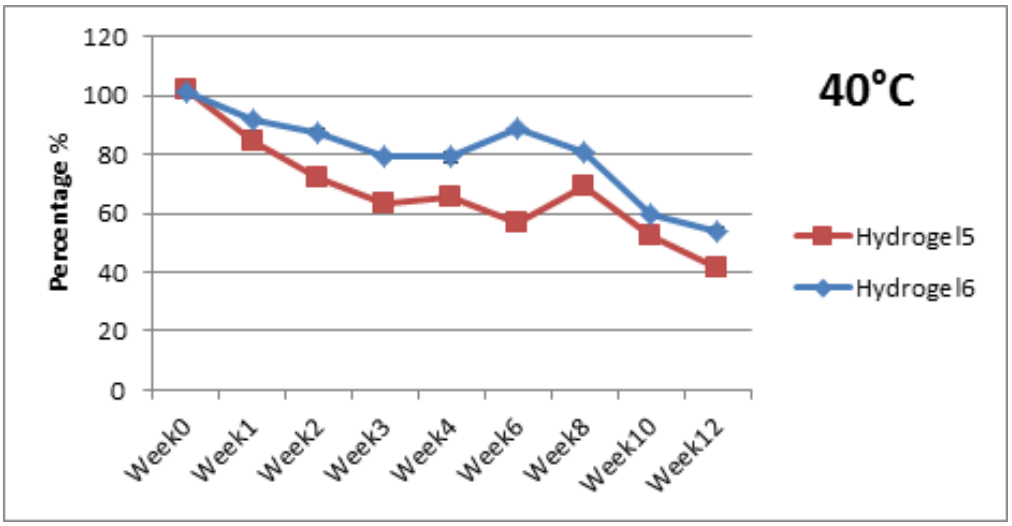


Diagram 30 *Graph comparing the stabilities of Hydrogel5 & Hydrogel6 at 40°C*

Hydrogel 5 was comprised of HPβCD and 2 antioxidants, whereas hydrogel 6 was comprised of HPβCD, 2 antioxidants and 1 chelating agent. Hydrogel 6 was more stable (**Diagram 30**) when compared with hydrogel 5 at 40 °C, because of presence of chelating agent.

19. Comparisons of stability of Hydrogel 6 vs Hydrogel 7 at 4 °C.

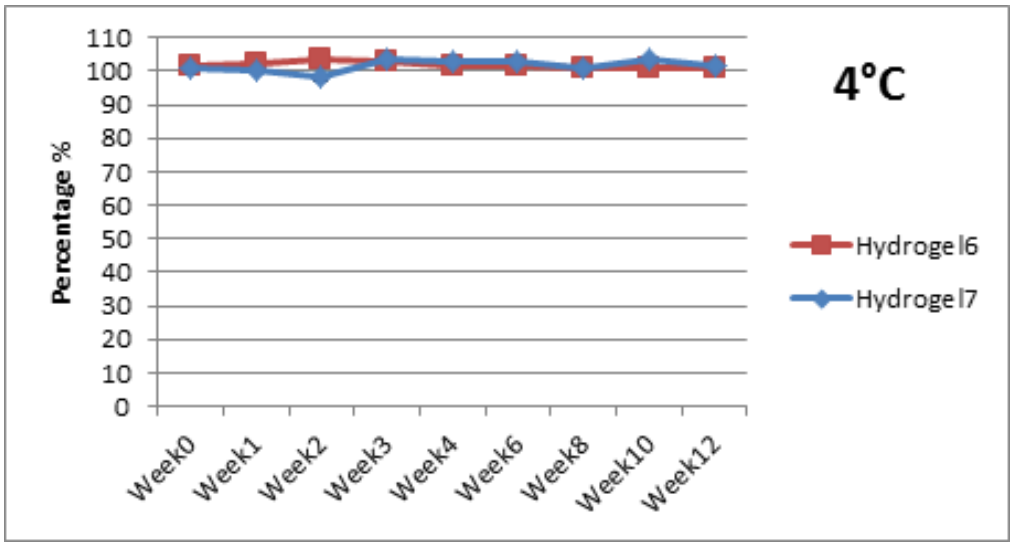


Diagram 31 *Graph comparing the stabilities of Hydrogel 6 & Hydrogel 7 at 4 °C*

Hydrogel 6 was comprised of HPβCD'S, 2 antioxidants and a chelating agent, whereas hydrogel 7 was comprised of HPβCD'S, 2 antioxidants and 2 chelating agents. The stabilities at 4 °C are almost similar (**Diagram 31**).

20. Comparisons of stability of Hydrogel 6 vs Hydrogel 7 at 25 °C

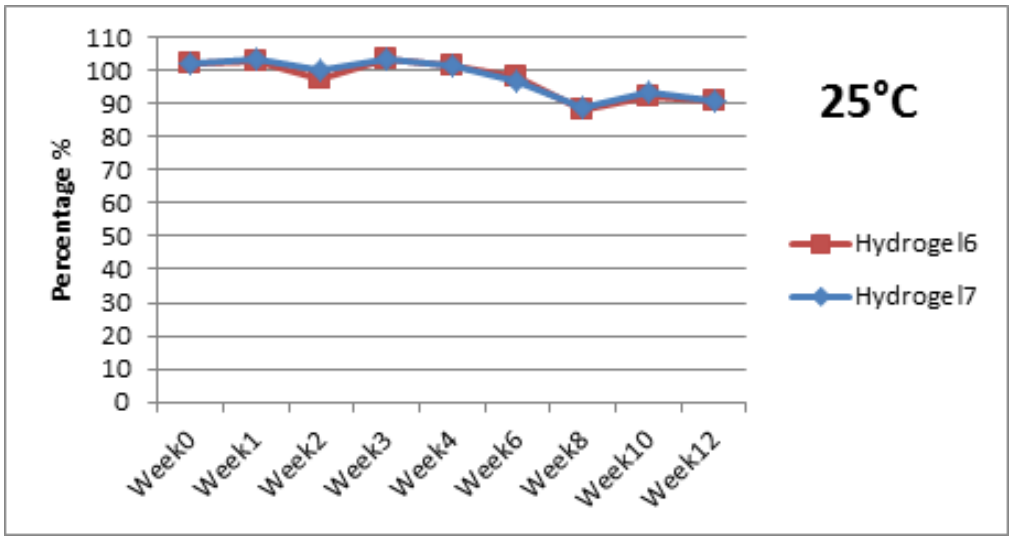


Diagram 32 *Graph comparing the stabilities of Hydrogel 6 & Hydrogel 7 at 25 °C*

Hydrogel 6 was comprised of HPβCD'S, 2 antioxidants and a chelating agent, whereas hydrogel 7 was comprised of HPβCD'S, 2 antioxidants and 2 chelating agents. The results are evident from the **Diagram 32** and both the hydrogels have shown similar stabilities.

21. Comparisons of stability of Hydrogel 6 vs Hydrogel 7 at 40 °C.

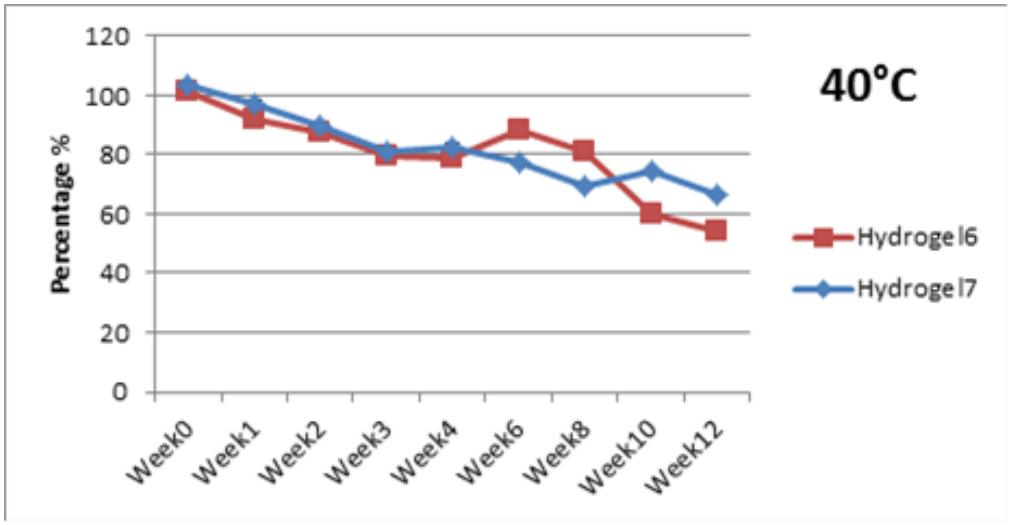


Diagram 33 *Graph comparing the stabilities of Hydrogel 6 & Hydrogel 7 at 40 °C*

Hydrogel 6 was comprised of HPβCD'S, 2 antioxidants and a chelating agent, whereas hydrogel 7 was comprised of HPβCD'S, 2 antioxidants and 2 chelating agents. The stability of hydrogel 7 is slightly greater than hydrogel 6 (**Diagram 33**) at 40 °C.

22. Comparisons of stability of Hydrogel 7 vs Hydrogel 8 at 4 °C.

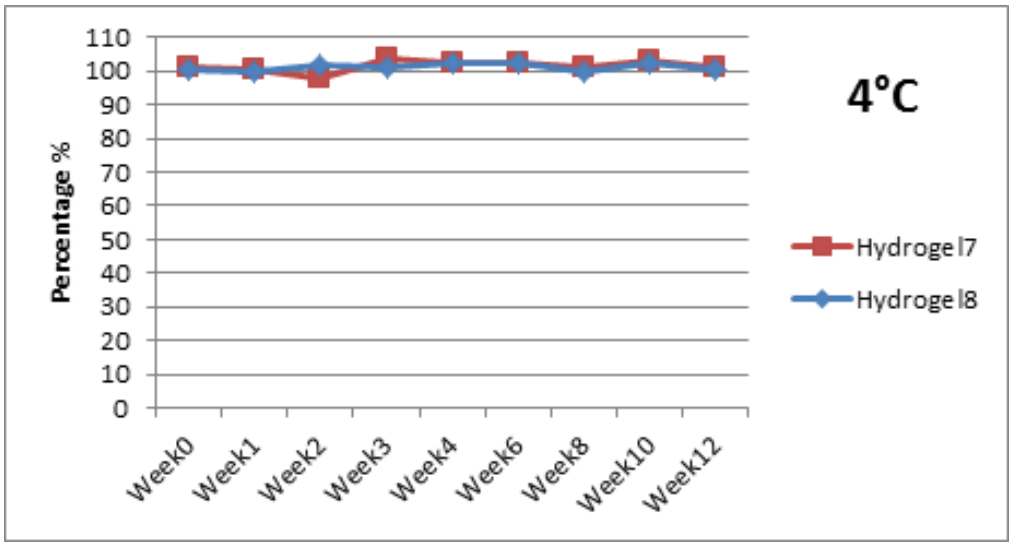


Diagram 34 *Graph comparing the stabilities of Hydrogel 7 & Hydrogel 8 at 4 °C*

Hydrogel 7 was comprised of HPβCD's, 2 antioxidants and 2 chelating agents, whereas hydrogel 8 was comprised of HPβCD's, 4 antioxidants and 2 chelating agents. As it was known that, different antioxidants gave different stabilities to doxycycline, so 2 new antioxidants were added to the existing antioxidants. From the **Diagram 34**, the results show that the stability actually decreased when 2 extra antioxidants were added to the hydrogel 8.

23. Comparisons of stability of Hydrogel 7 vs Hydrogel 8 at 25 °C.

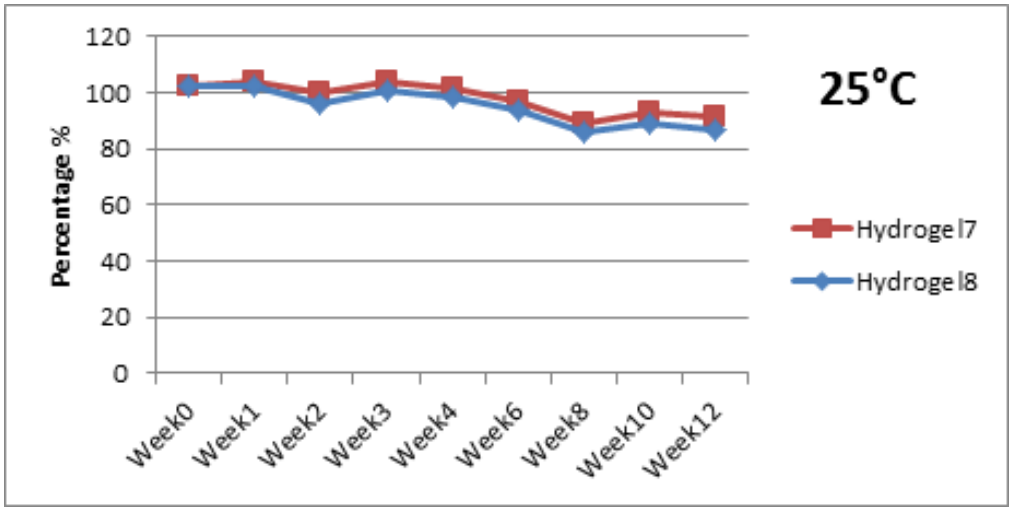


Diagram 35 *Graph comparing the stabilities of Hydrogel 7 & Hydrogel 8 at 25 °C*

Hydrogel 7 was comprised of HPβCD's, 2 antioxidants and 2 chelating agents, whereas hydrogel 8 was comprised of HPβcd's, 4 antioxidants and 2 chelating agents. As it was known that, different antioxidants gave different stabilities to doxycycline, so 2 new antioxidants were added to the existing antioxidants. From the **Diagram 35** the results show that the stability actually decreased when 2 extra antioxidants were added to the hydrogel 8.

24. Comparisons of stability of Hydrogel 7 vs Hydrogel 8 at 40 °C.

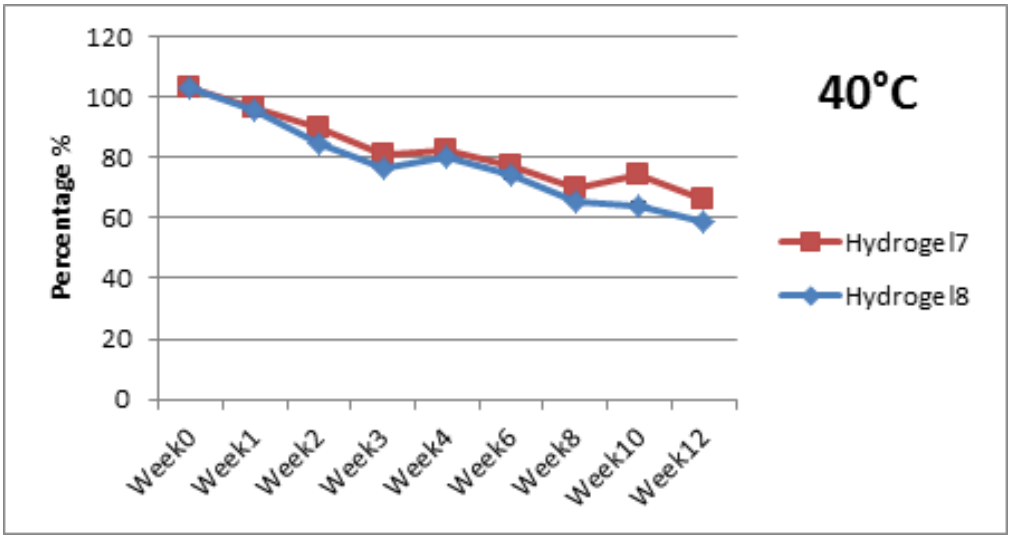


Diagram 36 *Graph comparing the stabilities of Hydrogel 7 & Hydrogel 8 at 40 °C*

Hydrogel 7 was comprised of HPβCD's, 2 antioxidants and 2 chelating agents, whereas hydrogel 8 was comprised of HPβCD's, 4 antioxidants and 2 chelating agents. As it was known that, different antioxidants gave different stabilities to doxycycline, so 2 new antioxidants were added to the existing antioxidants. From the **Diagram 36** the results show that the stability actually decreased when 2 extra antioxidants were added to the hydrogel 8.

25. Comparisons of stability of Hydrogel 1 vs Hydrogel 3 at 4 °C.

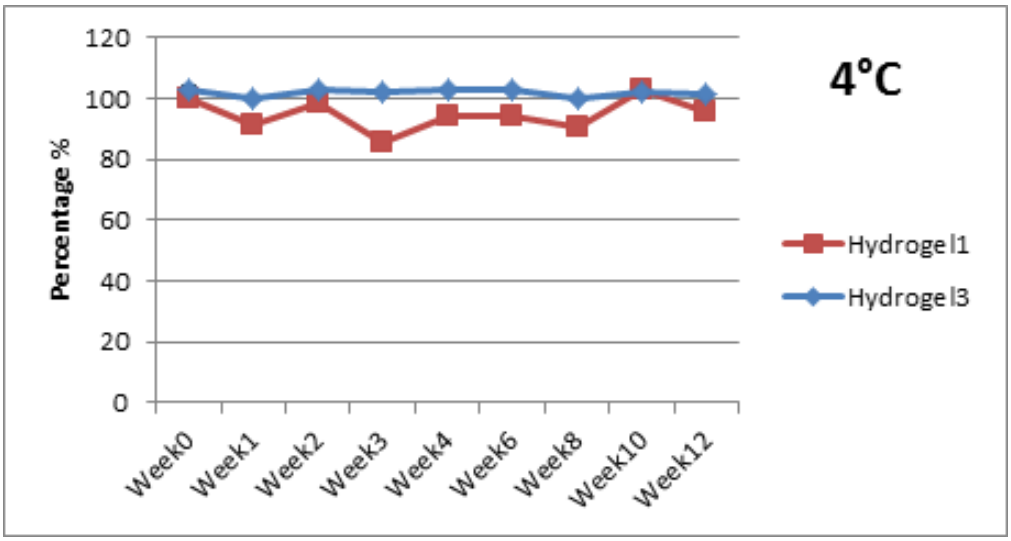


Diagram 37 *Graph comparing the stabilities of Hydrogel 1 & Hydrogel 3 at 4 °C*

Hydrogel 1 did not contain any antioxidants or chelating agents, whereas hydrogel 3 consisted of 2 antioxidants, and 2 chelating agents. Hydrogel 1 is stable, but the values were not consistent (**Diagram 37**), it might be due to, the hydrogel itself might be stable but when diluted with 0.01 M HCl, it might be getting oxidised, but there were no degradation peaks seen. Hydrogel 3 when observed from the graph, there is not much fluctuations in the stability values, it

is due to presence of antioxidants and chelating agents. In the entire experiment at 4 °C the fluctuation occurred only in hydrogel no's 1 and 4, and there were no antioxidants in both of this hydrogels.

26. Comparisons of stability of Hydrogel 1 vs Hydrogel 3 at 25 °C.

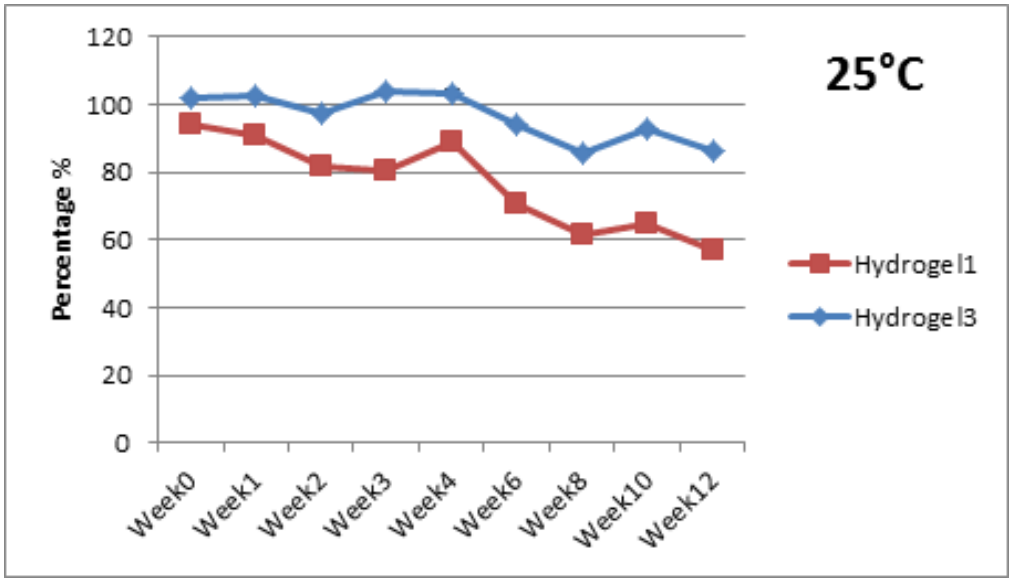


Diagram 38 *Graph comparing the stabilities of Hydrogel 1 & Hydrogel 3 at 25 °C*

Hydrogel 1 did not contain any antioxidants or chelating agents, whereas hydrogel 3 consisted of 2 antioxidants, and 2 chelating agents. Hydrogel 3 was the most stable hydrogel at 25 °C. There is almost 30 % difference in stabilities between hydrogel 3 and hydrogel1 (**Diagram 38**).

27. Comparisons of stability of Hydrogel 1 vs Hydrogel 3 at 40 °C.

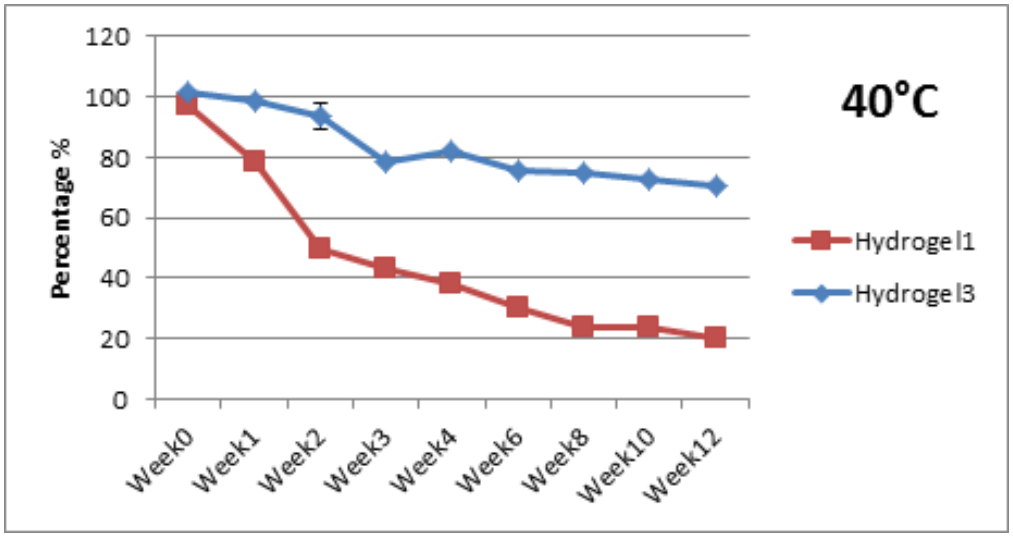


Diagram 39 *Graph comparing the stabilities of Hydrogel 1 & Hydrogel 3 at 40 °C*

Hydrogel 1 did not contain any antioxidants or chelating agents, whereas hydrogel 3 consisted of 2 antioxidants, and 2 chelating agents. Hydrogel 3, was the most stable at 40 °C (**Diagram 39**). Temperature increased the degradation

due to oxidation, while the presence of antioxidants and chelating agents halted the degradation due to oxidation, which was induced due to high temperature.

28. Comparisons of stability of Hydrogel 4 vs Hydrogel 7 at 4 °C.

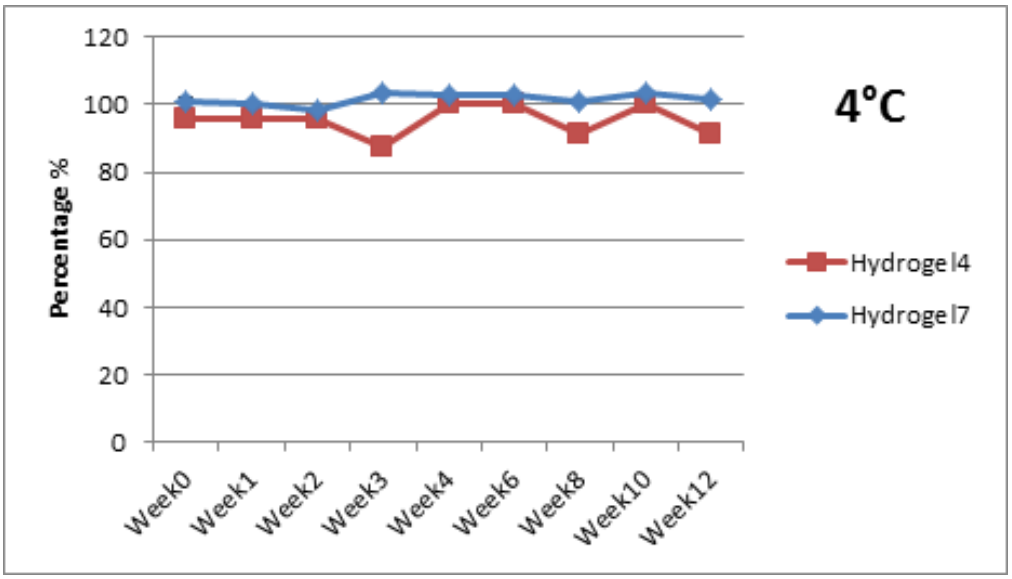


Diagram 40 *Graph comparing the stabilities of Hydrogel 4 & Hydrogel 7 at 4 °C*

Hydrogel 4 consisted of HPβCD, while hydrogel 7 consisted of HPβCD, 2 antioxidants and 2 chelating agents. Hydrogel 7 was consistently stable whereas hydrogel 4 was stable but values were fluctuating (**Diagram 40**).

29. Comparisons of stability of Hydrogel 4 vs Hydrogel 7 at 25 °C.

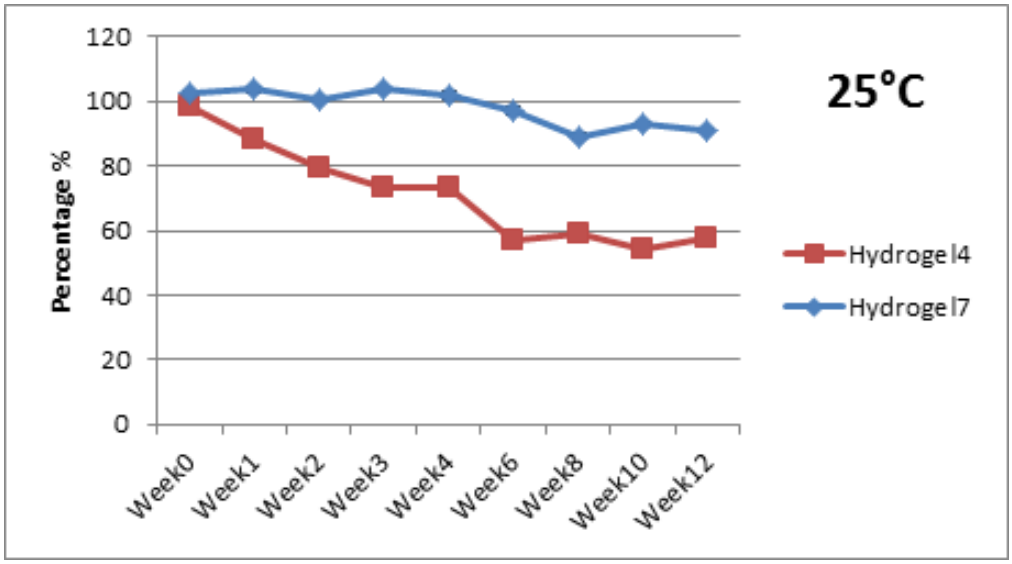


Diagram 41 *Graph comparing the stabilities of Hydrogel 4 & Hydrogel 7 at 25 °C*

Hydrogel 4 consisted of HPβCD, while hydrogel 7 consisted of HPβCD, 2 antioxidants and 2 chelating agents. The presence of antioxidants and chelating agents in the hydrogel 7, have halted the degradation (**Diagram 41**) due to oxidation, which in-turn was induced due to high temperature. HPβCD itself did

not seem to impart any thermal stability to doxycycline. HPβCD's in the formulations have infact reduced the stability of doxycycline by increasing the hydrophilicity of doxycycline, and exposing it to water molecules with in the surfactant and there by caused degradation due to oxidation.

30. Comparisons of stability of Hydrogel 4 vs Hydrogel 7 at 40 °C.

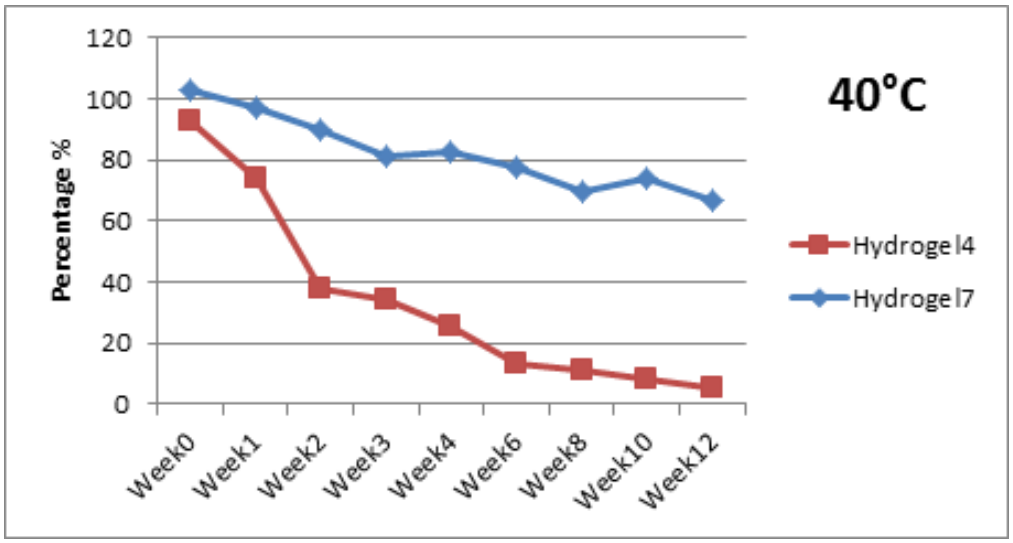


Diagram 42 *Graph comparing the stabilities of Hydrogel 4 & Hydrogel 7 at 40 °C*

Hydrogel 4 consisted of HPβCD, while hydrogel 7 consisted of HPβCD, 2 antioxidants and 2 chelating agents. It is clearly evident from the graph that the presence of antioxidants and chelating agents in the hydrogel 7, have halted the degradation due to oxidation (**Diagram 42**), which in-turn was induced due to high temperature. Hydrogel 4 was the most unstable formulation among hydrogels stored at 40 °C, by the end of 3 months period. Even hydrogel 1, which did not have any HPβCD's, antioxidants and chelating agents was significantly more stable than hydrogel 4. This indicates that the stability in the absence of antioxidants and chelating agents was due to poloxamers itself. Poloxamers are the surfactants having lipophilic and hydrophilic components within itself. Doxycycline is hydrophilic and lipophilic but predominantly more lipophilic. So when doxycycline was added to poloxamers it has occupied the lipophilic part of the surfactant and was protected from water molecules with in the surfactant. When Cyclodextrins were added to the formulation, they might have increased the hydrophilicity of the doxycycline, and caused them to more readily expose to the water molecule with in the surfactant and undergo oxidation. Temperature was one more factor that acted as catalyst to undergo oxidation when in aqueous solvent.

1. Comparisons of stabilities of 9 hydrogels at 25°C, from week 1 to week 12.

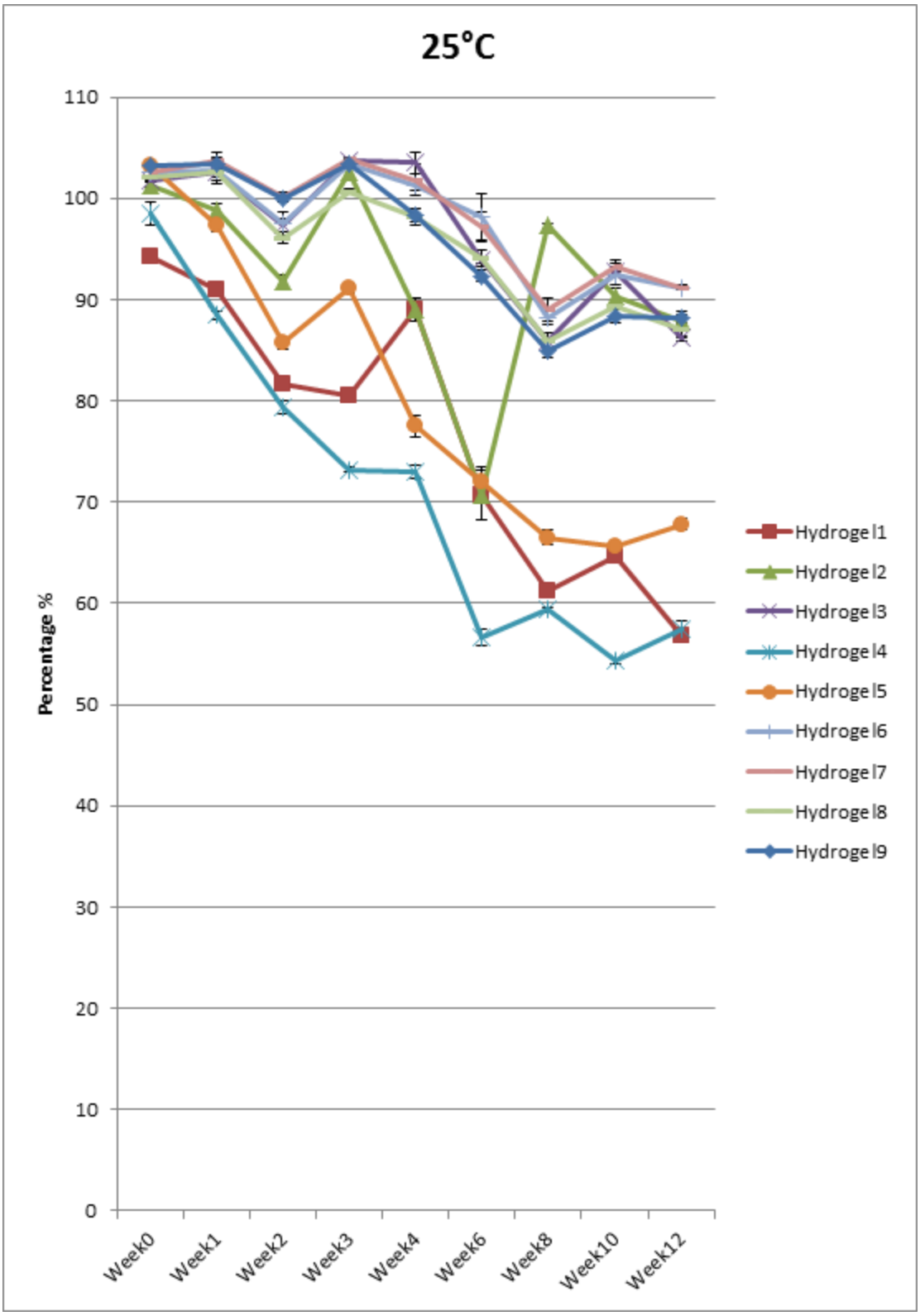


Diagram 43 *Comparison of stabilities of 9 hydrogels 25°C, from week 0-week 12*

2. Comparisons of stabilities of 9 hydrogels at 40 °C, from week 1 to week 12.

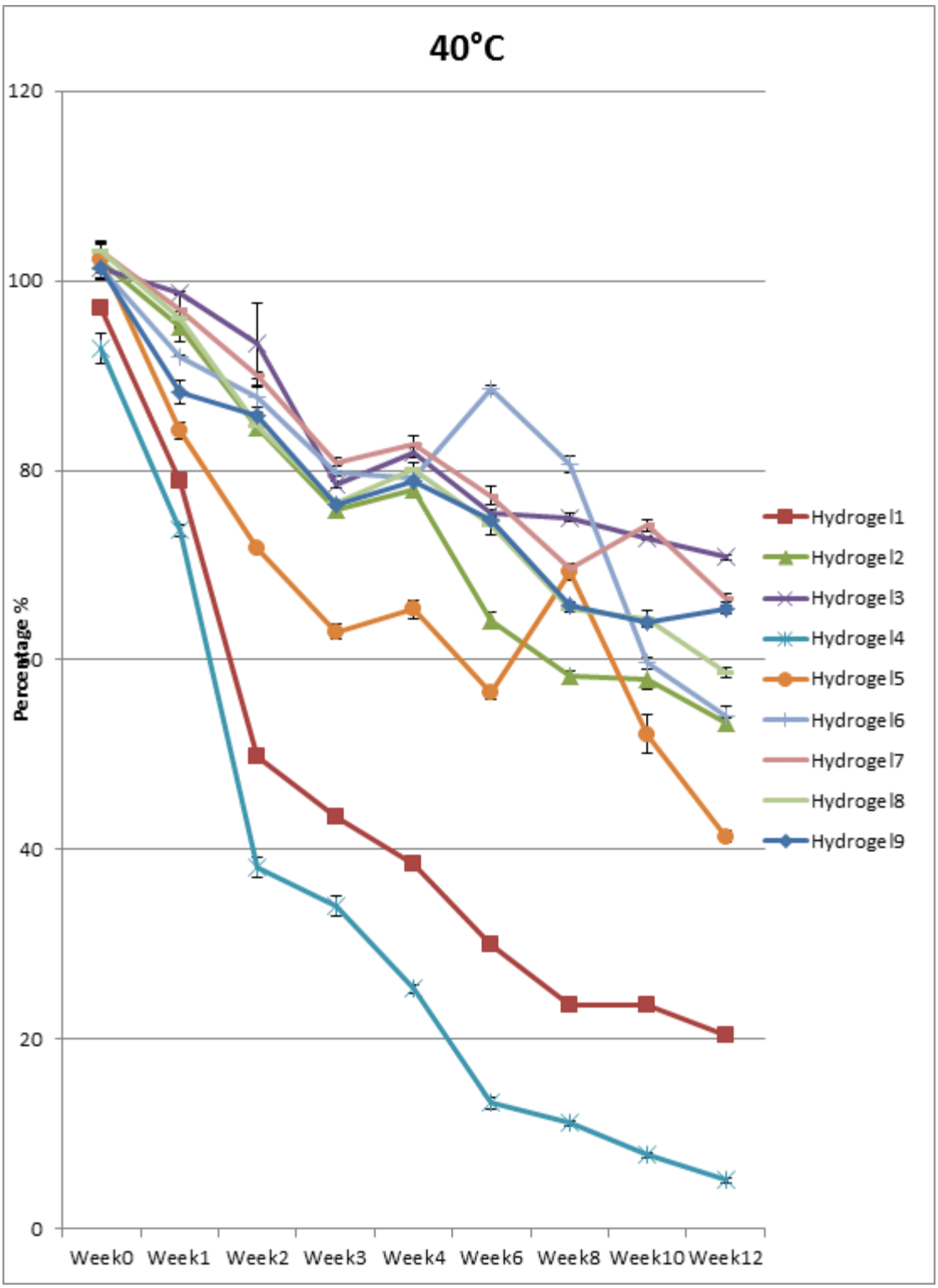


Diagram 44 *Comparison of stabilities of 9 hydrogels at40°C, from week0-week12*

4.6.1 Longterm stability studies (23 months)

Some of the hydrogels tested were more than a year old. Their initial stabilities could not be recorded as there was a problem with the HPLC method at that time, which was replaced by a new method later.

Table 33 Data for preparation of hydrogels

15 grams of 0.1% doxycycline *in-situ* forming hydrogel (pH adjusted to 6.6)									
s.no	**DOXYCYCLINE**	**HP-β-CD**	**Poloxamer- 407 (21%w/w)**	**Poloxamer-188 (10%w/w)**	**HPMC (0.5-2%)0.5% w/w**	**Na2S2O3 (0.1-0.5%) 0.2%w/w**	**Na2S2O5 (0.1-0.5%) 0.2%w/w**	**EDTA 0.2%w/w**	**MgCl2.6H2O 1:4 Molar ratio with doxycycline**
Hydrogel1	15mg	--	3.15gm	1.5gm	75mg				
Hydrogel2	15mg		3.15gm	1.5gm	75mg	30mg	30mg	30mg	24mg

Date of manufacture of hydrogels: 15-06-2012

Date of analysis of hydrogels: 20-05-2013 (at 11.5months), 4-10-2013 (at 15months), 27-01-2014 (at 20 months)

Table 34 Stabilities of hydrogels upto 20 months

Stabilities of hydrogels with pH 6.6					
	At 4°C stabilities after			**At 22°C stabilities after 11.5months**	**At 40°C stabilities after 11.5months**
S.no	**11.5months**	**15months**	**20 months**		
Hydrogel 1 (only Doxycycline)	100.3 %	99.5 %	99.5 %	47 %	No peaks
Hydrogel 2 (doxycycline + antioxidants + chelating agent)	99.5 %	100.2 %	98.5 %	78 %	No peaks

Hydrogel 1 (Table 34) which consisted of only doxycycline without any antioxidants and chelating agents (Table 33) (manually adjusted to pH 6.6 with 1 M NaOH and 1 M HCl) was 100 % stable by the end of 11.5 months, 99.5 % stable by the end of 15 months and 99 % stable (Table 34) after 20 months at 4 °C. Surprisingly hydrogel 1 without any antioxidants and chelating agents was 100 % stable after 15 months. This might be because of pH 6.6 might be the most favourable pH region to protect doxycycline from degrading. It was evident from previous studies that doxycycline degraded rapidly when pH was 7.4 and moderately degraded when pH was below 6.0. The hydrogel 2 with antioxidants and chelating agent was 99.5 % stable at the end of 11.5 months and 100.2 % stable by the end of 15 months. At 22°C the hydrogel 1 was 47 % stable whereas hydrogel 2 was 78 % stable. At 40 °C there were no peaks seen, even the degradation compounds have degraded due to high temperature.

Table 35 Data for preparation of hydrogels

15 grams of 0.1% doxycycline *in-situ* forming hydrogel (in 7.4 pH buffer solution)									
S.no	DOXYCYCLINE	HP-β-CD	Poloxamer-407 (21%w/v)	Poloxamer-188 (10 % w/v)	HPMC (0.5-2%) 0.5% w/v	Na2S2O3 (0.1-0.5%) 0.2% w/v	Na2S2O5 (0.1-0.5%) 0.2% w/v	EDTA 0.2%w/v	Mgcl2.6H2O 1:4 Molar ratio with doxycycline
Hydrogel 1	15mg		3.15gm	1.5gm	75mg				
Hydrogel 2	15mg		3.15gm	1.5gm	75mg	30mg	30mg	30mg	24mg
Hydrogel 3	15mg	360mg	3.15gm	1.5gm	75mg				

Date of manufacture of hydrogels: 7-3-2012

Date of analysis of hydrogels: 20-05-2013 (after 15 months)

Date of analysis of hydrogels: 04-10-2013 (after 19 months)

Date of analysis of hydrogels: 27-01-2014 (after 23 months)

Table 36 Stabilities of hydrogels stored at 4°C, after 15months having a pH 7.4

Stabilities of Hydrogels with pH 7.4			
S.no	**At 4°C stabilities after 15 months**	**At 4°C Stabilities after 19 months**	**At 4 °C after 23 months**
Hydrogel1	44 %	35 %	27.5 %
Hydrogel2	93.5 %	85 %	83 %
Hydrogel3	71 %	------	

At pH 7.4 the hydrogel 1 which contained only doxycycline without any antioxidants and chelating agents (Table 35) was 44 % stable by the end of 15 months, 35 % stable by the end of 19 months and 27.5 % stable at the end of 23 months at 4 °C (Table 36). The effect of pH of hydrogels on stability of doxycycline is clearly evident from the above data. The same hydrogel at pH 6.6 was 100 % stable after 15 months and 99 % stable after 20 months, whereas at pH 7.4 the doxycycline rapidly degraded to 44 % by the end of 15 months. Hydrogel 2 at pH 7.4, which contained antioxidants and chelating agent was 93.5 % stable at 4 °C by the end of 15months, 85 % stable by the end of 19 months and 83 % stable at the end of 23 months. Even at this unfavourable pH region (7.4), the presence of antioxidants and chelating agents in the hydrogel were successful in protecting the doxycycline from degrading but not by 100 %. Whereas at pH 6.6 in the presence of antioxidants and chelating agents, the hydrogels were 100 % stable after 19 months. Hydrogel 3, which contained

doxycycline-HPβCD complex was 71 % stable, cyclodextrins have halted the degradation significantly at pH 7.4, whereas when cyclodextrins were added to pH 6.6 hydrogels they actually decreased the stability to below 100 %, and the hydrogels without cyclodextrins were 100 % stable even after 20 months (Table 34). At pH 7.4 in the absence of antioxidants and chelating agents there were 2 degradation compounds seen, one is 4-epidoxycycline and the other is 6-epidoxycycline, whereas in the presence of antioxidants and chelating agents only 4-epidoxycycline was seen.

4.7 Viscosity measurements

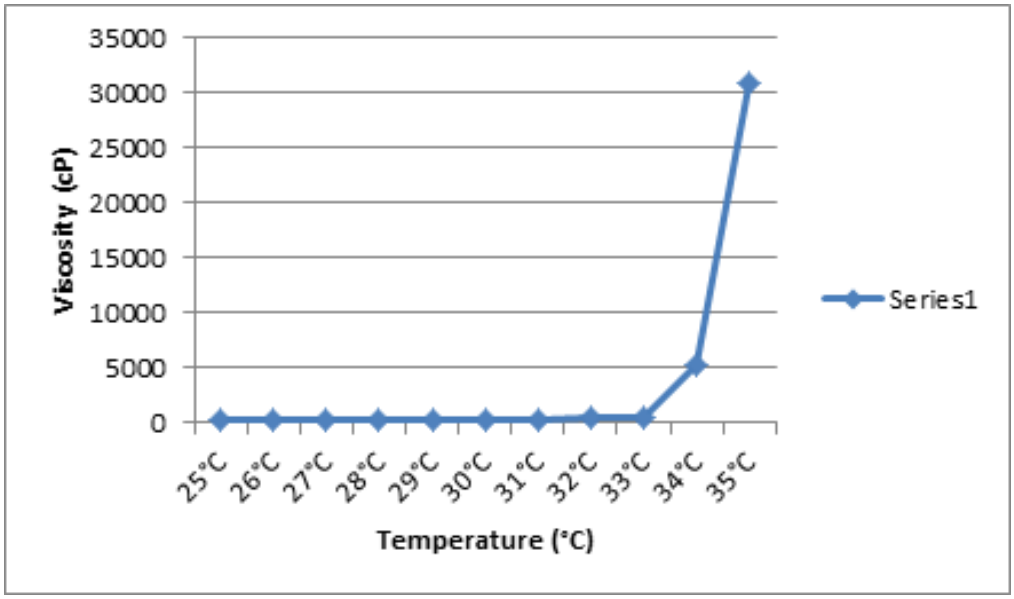


Diagram 45 *Graph showing the effect of increase in temperature on viscosity of poloxamer hydrogel*

The viscosity values were recorded to see the effect of temperature on viscosity values. The viscosity values were recorded starting from room temperature (25°C). The values significantly increased till 33°C and near to the gelation temperature the values increased exponentially (**Diagram 45**). The values could only be recorded till 35°C as the viscometer's upper limit for testing viscosity was reached (max 31000 cP). When fully gelled the hydrogel was estimated to possess a viscosity (cP) range of about 1,50,000-2,00,000 cP.

Table 37 Viscosities of hydrogels tested under mucoadhesion screening tests

s.no	Polymer concentration	Temperature °C	ViscositycP			Rpm	Torque %	Spindle no
			Reading1	Reading2	Reading3			
1	Only poloxamers	25	320	325	317	3	11.9	Cpe 52
2	0.5% HPMC	25	420	430	411	3	13.8	Cpe 52
3	1% HPMC	25	539	535	530	3	17.4	Cpe 52
4	1.5% HPMC	25	967	940	965	1.5	15.6	Cpe 52
5	2% HPMC	25	1184	1180	1183	1.5	19.1	Cpe 52
6	0.5% CMC	25	579	572	578	3	18.6	Cpe 52
7	1% Chitosan	---	---	---	---	---	---	---
8	0.5% PEG6000	25	496	492	495	3	16	Cpe 52
9	0.5% Carbopol 974P	25	514	534	530	3	16.7	Cpe 52
10	0.25% HPMC + 0.25% povidone	25	440	438	441	3	14.2	Cpe 52
11	0.5 % povidone	25	477	475	477	3	15.4	Cpe 52
12	0.5% polyvinyl alcohol	---	---	---	---	---	---	---
13	0.2% HPMC + 0.2% povidone + 0.1% polyvinyl alcohol	---	---	---	---	---	---	---
14	0.25% povidone + 0.25 % polyvinyl alcohol	---	---	---	---	---	---	---
15	0.25% HPMC + 0.25% carbopol 974P	25	483	483	485	3	15.6	Cpe 52

4.8 Mucoadhesion Measurements

↓Viscosities of formulations with different mucoadhesive polymers↓

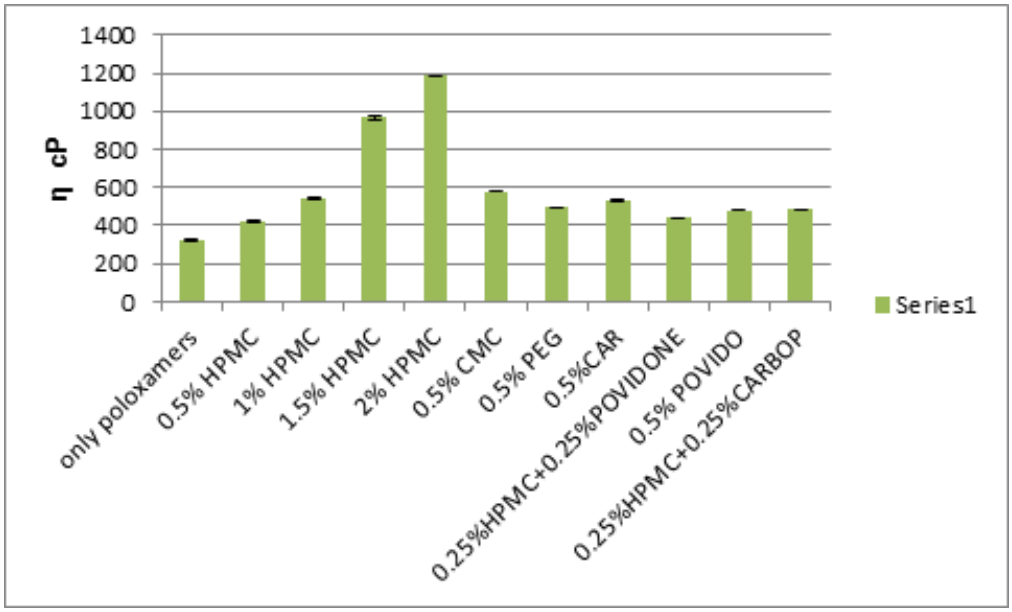


Diagram 46 *Graph comparing the viscosities of hydrogels under mucoadhesion screening tests at room temperature (25 °C)*

The aim of the test was to select a mucoadhesive polymer which does not make the formulation too viscous, mainly to maintain the free flowability nature of the *in situ* hydrogels. The formulation containing only poloxamers without mucoadhesive polymers showed the lowest viscosity value. As the concentration of the HPMC was increased, the viscosity values were also increased. Viscosity of 0.5% CMC was higher than hydrogel containing 1 % HPMC. Carbopol974P was not uniformly dispersed into the formulation, so the viscosity values varied depending upon presence of carbopol in different regions within the hydrogel. 0.5 % HPMC, 0.5 % povidone, 0.5 % PEG and 0.25 % HPMC and 0.25 % povidone containing hydrogels showed lower viscosity values among different combinations of mucoadhesive (**Table 37**) polymers tested in the *in situ* forming hydrogels. So the hydrogels containing 0.5 % HPMC, 0.5 % povidone, 0.5 % PEG and 0.25 % HPMC and 0.25 % povidone can be selected as the viscosity values are minimum (**Diagram 46**). Further tests were carried out by Texture analyser to select the most ideal mucoadheve polymer.

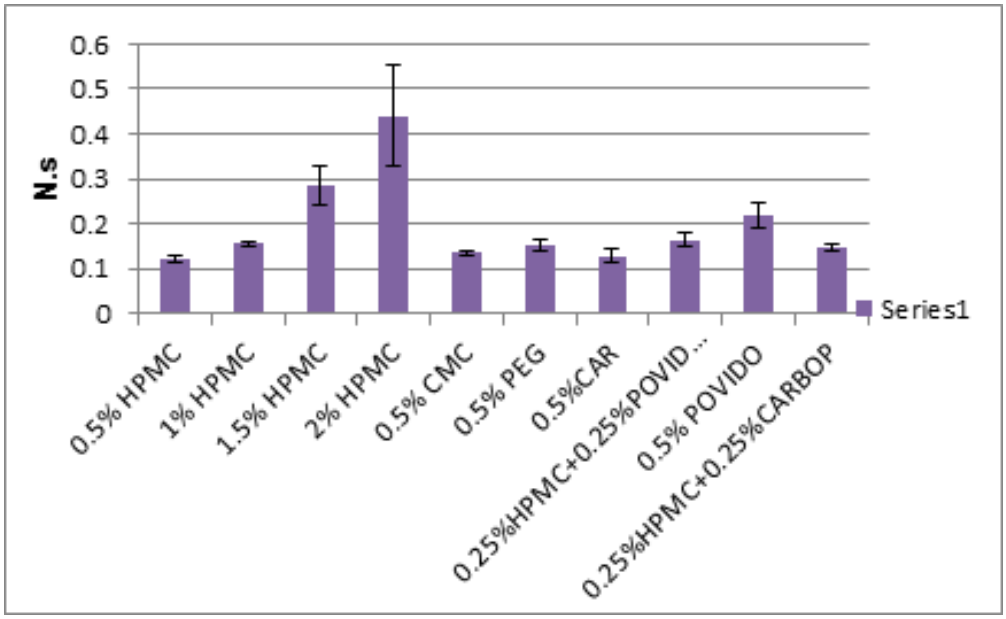


Diagram 47 *Graph comparing the AUC's under force-time plot of hydrogels under mucoadhesion screening tests*

The area under the curve values was calculated from the force versus time plots. The higher the value, the greater is the mucoadhesion. Formulation containing 2% HPMC showed the highest AUC value, but the hydrogel gelled at room temperature. 0.5% povidone containing hydrogel showed good mucoadhesion capacity (**Diagram 47**) than hydrogels containing 0.5% HPMC, 1% HPMC, 0.5% CMC, 0.5% PEG, and 0.5% carbopol. From the AUC values 3 hydrogels containing 1% HPMC, 0.5% povidone and 0.25% HPMC and 0.25% povidone hydrogels might be selected as the mucoadhesive polymers.

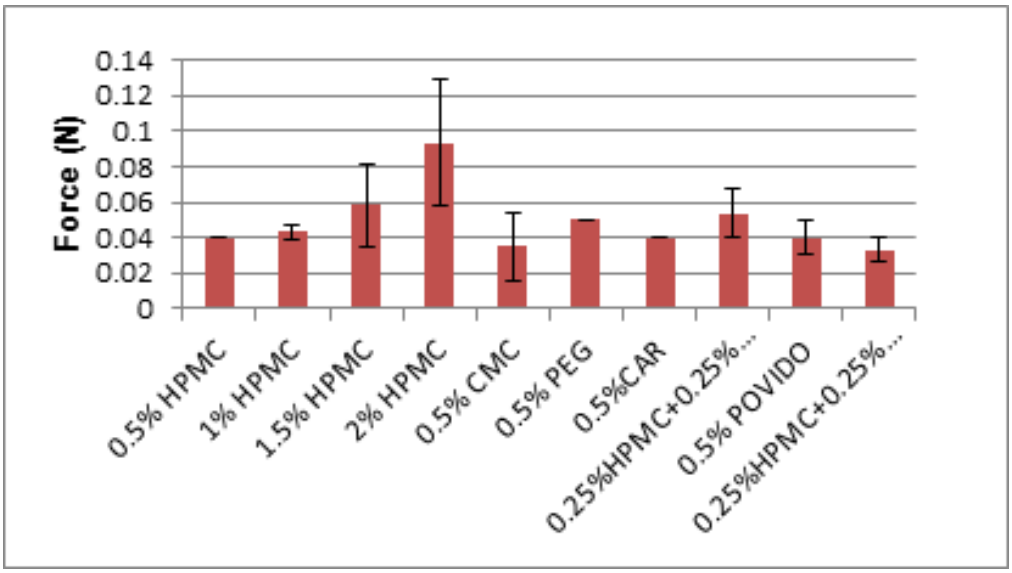


Diagram 48 *Graph comparing the Peak detachment forces of hydrogels under mucoadhesion screening tests*

The peak detachment force measures the peak force required to detach the hydrogel from the mucus membrane. The greater the peak detachment force

values, the greater will be the mucoadhesion. Peak detachment force was highest for the hydrogel containing 2 % HPMC, but the formulation has gelled below the room temperature. Even the hydrogel containing 1.5% HPMC has gelled below the room temperature. Hydrogels containing 0.5 % CMC and 0.25 % HPMC and 0.25 % carbopol showed the lowest peak detachment force values. Hydrogels containing 0.5% PEG, and 0.25 % HPMC and 0.25 % povidone showed good (**Diagram 48**) peak detachment values.

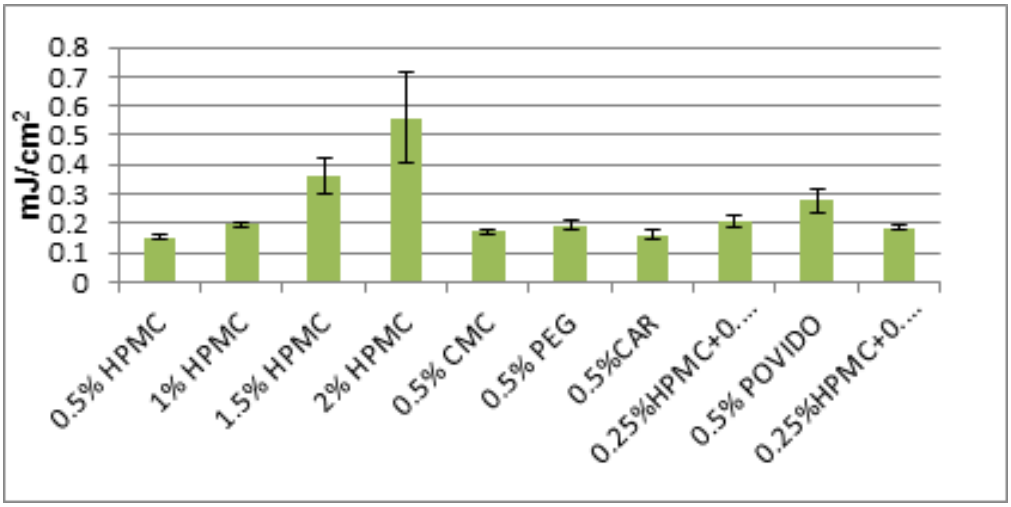


Diagram 49 *Graph comparing work done by mucoadhesion of different hydrogels under mucoadhesion screening tests*

The work of mucoadhesion measures the work done to detach the hydrogel from the mucus membrane. The greater the values of work of adhesion, the greater will be the mucoadhesion. Hydrogels containing 2 % HPMC and 1.5 % HPMC showed the highest work of adhesion values (**Diagram 49**) but the hydrogels have gelled below the room temperature. 0.5 % HPMC and 0.5 % carbopol containing hydrogels showed the lowest work of adhesion values. 0.5 % povidone containing hydrogels showed the highest work of adhesion values but the gel strength was not good when compared to HPMC containing formulations. The formulations containing 1 % HPMC, 0.5 % CMC, 0.5 % PEG, 0.25 % HPMC and 0.25 % povidone, and 0.25 % HPMC and 0.25 % carbopol, mucoadhesive polymers can be incorporated into the hydrogels.

In the mucoadhesion analysis multiple measurements (6-10) were recorded for each hydrogel. In some instances the values were out of range, in such cases the values which repeated most number of times was considered.

1. Hydrogel with 0.5 % HPMC: This hydrogel has showed optimum mucoadhesion capacity and was less viscous at room temperature. At this concentration HPMC did not affect the gelation temperature of poloxamers
2. Hydrogel with 1 % HPMC: At this concentration the mucoadhesion capacity did not improve much when it was compared with 0.5% HPMC containing hydrogel. And the solution was 100 cP more viscous than the 0.5 % HPMC hydrogel.

3. Hydrogel with 1.5 % HPMC: The mucoadhesion capacity was good but the formulation no longer behaved as *in situ* hydrogel, instead they gelled below room temperature

4. Hydrogel with 2 % HPMC: The mucoadhesion capacity was excellent at this concentration but the solutions gelled below room temperature. The mucoadhesive capacity values increased with increase in concentrations of HPMC. This also proves that the *in vitro* method for analysing mucoadhesion is appropriate.

5. Hydrogel with 0.5 % CMC: The mucoadhesive capacity was similar to that of 0.5 % HPMC but the viscosity of solution was 150 cP more than that of 0.5 % HPMC

6. Hydrogel with 1 % chitosan: Chitosan did not dissolve in hydrogel. It should be dissolved in dilute acetic acid and then added to the hydrogel. The stability of active compound might have been affected if acetic acid was used to dissolve chitosan, so chitosan was not considered for further studies.

7. Hydrogel with 0.5 % PEG 6000: PeG 6000 is FDA approved. Polyethylene glycol is mainly used as a laxative, it was rarely used as a mucoadhesive polymer. It gave good mucoadhesion to the hydrogels but the only drawback was that, the gels were easily getting liquefied, i.e. they might be having very low gel strength. Unlike HPMC or CMC the PEG completely mixed into the poloxamers, and formed clear liquids.

8. Hydrogels with 0.5 % Carbopol 974P: The mucoadhesion was slightly greater than HPMC, and equal to that of 0.5 %CMC. The main drawback with carbopol was that it formed thick spheroidal masses, which settled at the bottom of hydrogel. Even after shaking the spheroidal masses did not get dispersed into the hydrogel. So some parts of hydrogel might have high mucoadhesion while some parts have less.

9. Hydrogels with 0.25 % HPMC + 0.25 % Povidone: This combination was ideal among all the polymers tested. It had good mucoadhsion which was greater than 0.5 % HPMC, CMC and carbopol. It had less viscosity at room temperature compared to other polymers tested. It had good gel strength because of presence of HPMC.

10. Hydrogel with 0.5 % Povidone: This non-ionic polymer gave clear hydrogels, they mixed completely with the poloxamers, unlike HPMC, CMC and carbopol, which can be seen as tiny swollen grains (water imbibed) of polymer. It had very good mucoadhesion but low gel strength. Repeated measurements were taken to crosscheck the values. But the literature review showed that povidone has very little mucoadhesion capacity. At the molecular level chemical bonds might be the necessary for mucoadhesion but the general stickiness of the formulations (like " glue") can also improve the mucoadhesion, povidone when mixed with poloxamers, the stickiness of the formulation might have been increased. This effect might be subjective.

11. Hydrogel with 0.5 % polyvinyl alcohol: Polyvinyl alcohol was considered as it is a non-ionic mucoadhesive polymer. It dissolved well but when the

hydrogels were stored at 4 °C, the polymer formed hard crystal stones which settled at the bottom of the hydrogel. Polvinyl alcohol might be insoluble at low temperatures. Some of the non-ionic polymers are soluble at room temperature but insoluble at high temperatures[351] e.g. methyl cellulose, poly(ethylene oxide).

12. Hydrogel with 0.2 % HPMC + 0.2 % povidone + 0.1% polyvinyl alcohol: Even in low concentrations polyvinyl alcohol formed immiscible solid crystal stones at 4 °C.

13. Hydrogel with 0.25 % povidone + 0.25 % polyvinyl alcohol: Polyvinyl alcohol formed thick crystal stones when stored at 4 °C.

14. Hydrogel with 0.25 % HPMC + 0.25 % carbopol 974P: This hydrogel has shown mucoadhesion similar to 0.5 % CMC, and sometimes even less (in repeated measurements) . Viscosity was less than 0.5 % CMC and greater than 0.5 % HPMC. It appeared to have good gel strengths. Over all the combination of CMC and HPMC did not improve the mucoadhesion.

❖ The final mucoadhesive polymer was selected after considering 4 factors:

1. **viscosity:** The formulation should have less viscosity at room temperature as they are *in situ* forming hydrogels. A total of 4 formulations i.e. 0.5 % HPMC, 0.5 % povidone, 0.5 % PEG and 0.25 % HPMC and 0.25 % povidone qualified for having low viscosity values at room temperature

2. **Area under force-time curve:** The greater the AUC values the greater will be the mucoadhesion capacity of the formulation. So the formulations containing higher AUC values were preferred. 3 hydrogels containing 1 % HPMC, 0.5 % povidone and 0.25 % HPMC and 0.25 % povidone, qualified the test.

3. **Peak detachment force:** The greater the peak detachment force values the greater will be the mucoadhesion capacity of the formulation. 2 hydrogels containing 0.5 % PEG, and 0.25 % HPMC and 0.25 % povidone, qualified the test.

4. **Work of adhesion (mJ/cm^2):** The greater the work done to detach the hydrogel from mucus membrane the greater is the mucoadhesion. So the formulations with high Work of adhesion values were preferred. 5 hydrogels containing 1 % HPMC, 0.5 % CMC, 0.5 % PEG, 0.25 % HPMC and 0.25 % povidone, and 0.25 % HPMC and 0.25 %, qualified the test

<u>1. The formulation containing HPMC 0.25 % + povidone 0.25 % was selected because of the following reasons:</u>

→Both polymers are non-ionic polymer, which was the most important parameter, considering the stability of doxycycline.

→They did not increase the viscosities of formulation much.

→ The formulation possessed good AUC values, peak detachment force values and work of adhesion values.

→At this concentrations, the gelation temperature and gel strength of poloxamers was not affected.
→HPMC was recommended for oral formulations.
→Polyvinyl pyrrolidone was previously used in many mucoadhesive buccal tablets[5].
→There are many commercially available buccal formulations which used the combination of two or more mucoadhesive polymers[5].
→Presence of small amounts of HPMC improved the gel strength.

4.9 *In vitro* release studies

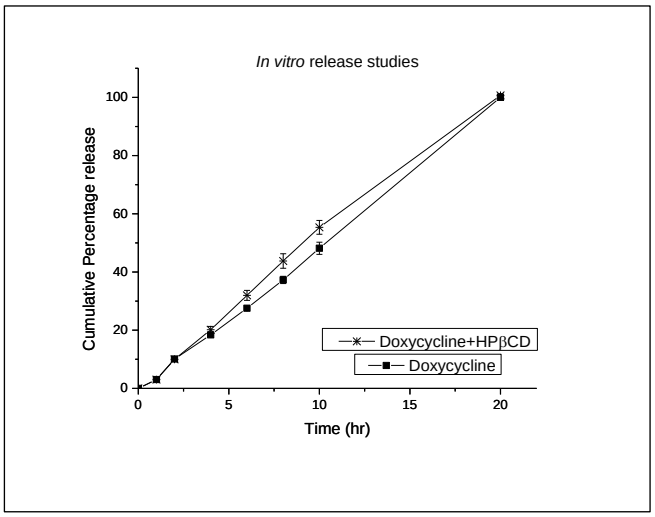


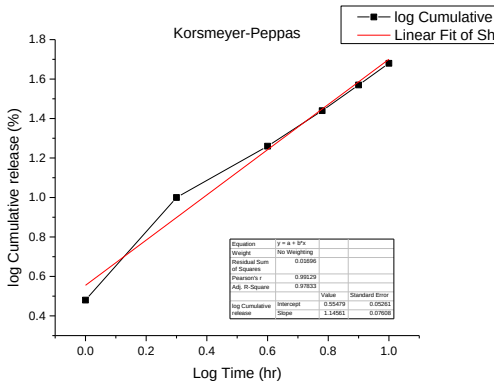
Diagram 50 *Graph showing the release of doxycycline from polymer matrices over a period of 20 hours*

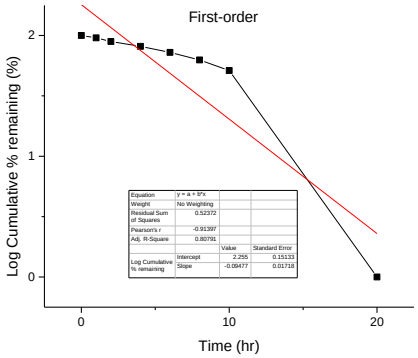
In vitro release studies were first attempted with Franz diffusion cells, but had to be replaced by the membrane-less model. While using the Franz diffusion cells the receptor phase migrated into the donor phase. It was thought that the phenomenon was due to ionic imbalance between the donor phase and receptor phase, but when ionic equilibrium was provided by adding same number of ions in receptor phase, even then the receptor phase was migrating into the donor phase. The problem occurred due to osmolality, as high concentrations of poloxamers were present in the hydrogel, they were drawing in the receptor phase. Especially for poloxamers membrane-less model is widely used method to evaluate the *in vitro* release behaviour. The drug was released slowly over a period of 20 hours. From the **Diagram 50** it is evident that the release of drug is slightly greater in the presence of HPβCD, but the overall release time was same for both the hydrogels. The plot was non-linear for Higuchi model indicating that the release of drug from the polymer matrices did not occur by diffusion. The non-linear curve obtained from the Hixson-Crowell[352] model indicated that the drug release was not through dissolution or erosion of the hydrogel. The drug release was not concentration dependent which was evident from the non-linear curve obtained from first order model. From the plot (**Diagram51**, **Diagram52**) obtained by zero order, which was a linear curve, it was evident that the drug release was not concentration and time dependent and was constant. To find out the exact release mechanism of drug from the polymer matrices the data was fitted in Korsmeyer-Peppas model. The release exponent “n” for both the

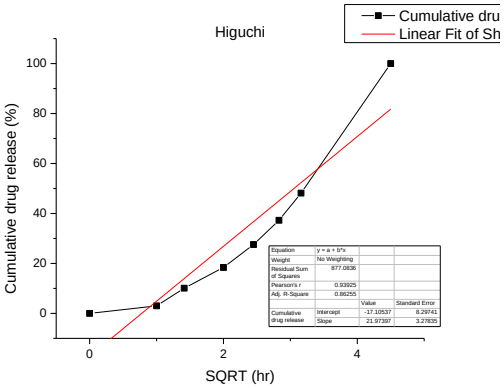
hydrogel was “>1”, indicating that the drug release mechanism occurred through Super case II transport. From literature review, some of the sustained release, mucoadhesive, buccal formulations, followed a similar drug release characteristics with combination of zero order and super case II transport[353-355]. R^2 values obtained from different models are shown in Table 38

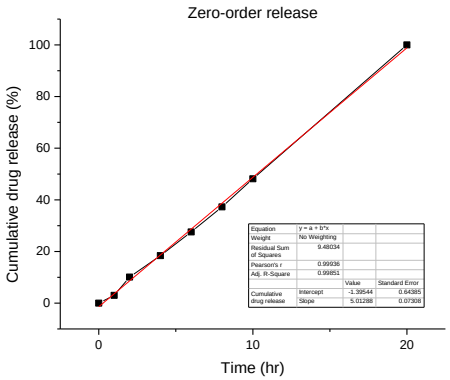
Table 38: R^2 values for different Kinetic models

	Zero order R^2	**First order R^2**	**Higuchi R^2**	**Hixson-Crowell R^2**	**Korsmeyer-Peppas R^2**
Hydrogel containing only doxycycline	0.99	0.80	0.88	0.85	0.98 (release exponent n=1.14)
Hydrogel containing doxycycline + HPβCD	0.99	0.83	0.89	0.88	0.99 (release exponent n=1.09)









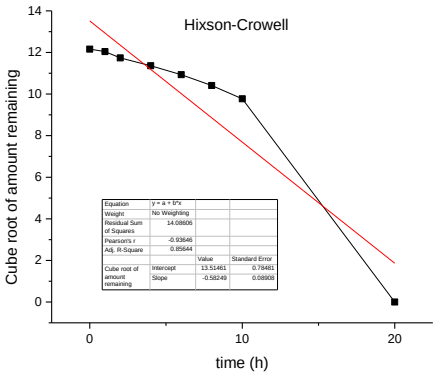


Diagram 51 *Figures showing release Kinetics, model fitting of release data for Hydrogel containing only doxycycline.*

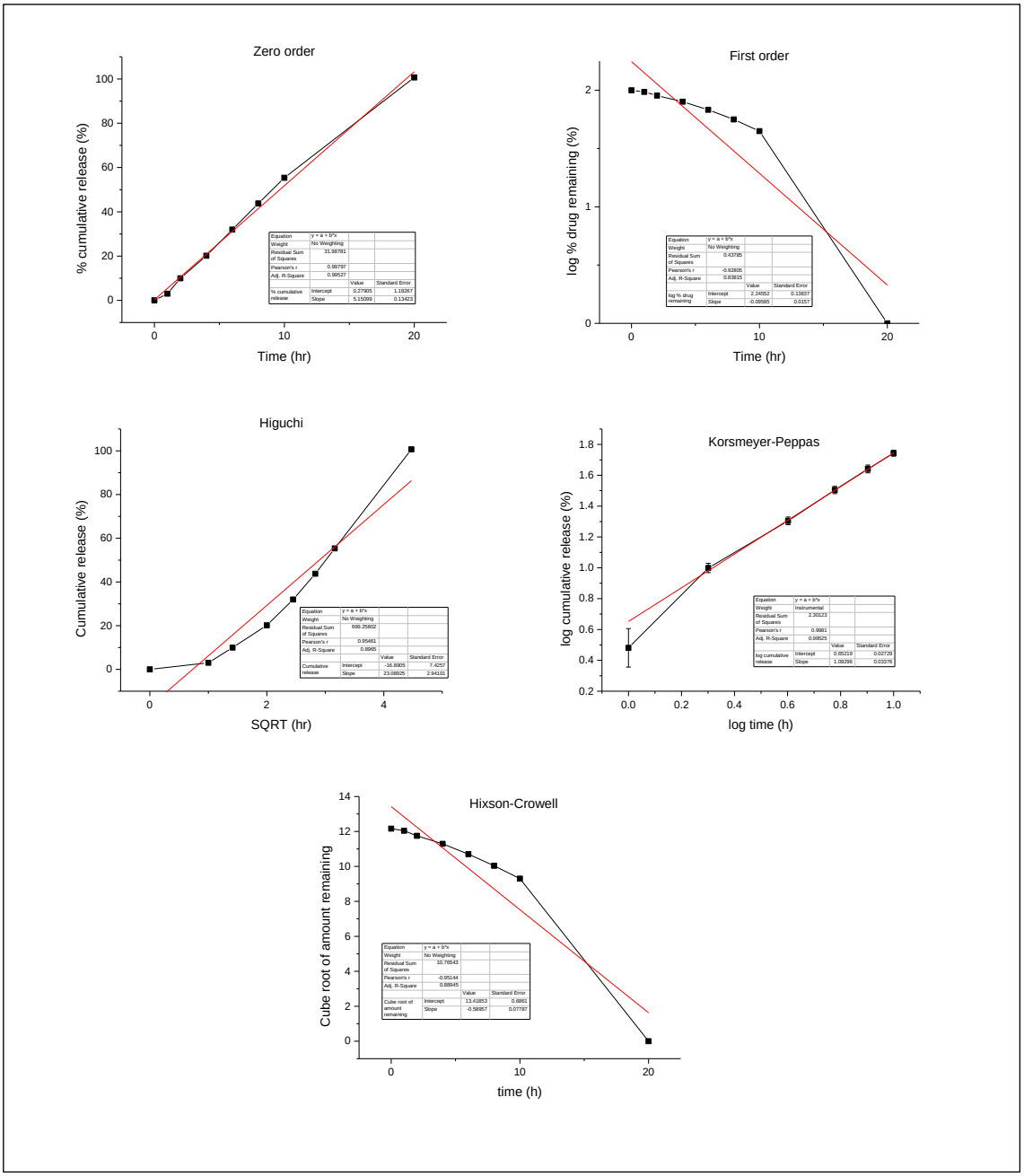


Diagram 52 *Figures showing release Kinetics, model fitting of release data for Hydrogel containing doxycycyline and HPβCD*

5 Discussion

5.1 HPLC method

Skuli's HPLC method was sensitive to changes in the pH of mobile phase. The excipients in the hydrogel especially antioxidants were increasing the pH of the dilution medium for HPLC analysis. The pH of the dilution medium was actually 2.5, when the hydrogel containing antioxidants were added the pH increased to 2.6. At pH 2.6, the peak areas were increasing and the HPLC results were showing 1.5 times higher values of what it contained originally (it is known that, change in pH greatly effects the UV absorbance[321], especially for acidic and basic compounds). The reason for high values in the presence of antioxidants was a change in pH. When only a doxycycline containing hydrogel was analysed or even when the doxycycline was directly dissolved in water, the results were never 100 %. But when a small quantity of antioxidant was added to the dilution medium the results were 100 %. It means that the perchloric acid in the mobile phase, which was actually added for maintaining buffer capacity, was oxidising the doxycycline and interestingly no degradation peaks were seen. Perchloric acid is a strong oxidising agent. Because of the presence of oxygen atom in its anionic ring its oxidising capacity even got intensified. Because the buffer capacity of the mobile phase was not sufficiently good, even the small amounts of antioxidants in hydrogel were affecting the pH of the mobile phase. So to improve the buffer capacity it was considered to add buffer salts. But it was advised by DIONEX executives not to add any buffer salts to acetonitrile containing mobile phases. There were some methods which existed with having acetonitrile and buffer salts together. Also attempts were done to readjust the pH to 2.5, and go on for analysis, but the results were not consistent. So considering all the above drawbacks it was finally decided to change the HPLC method to European Pharmacopoeia method. After changing the method the results were always consistent and accurate. The only factor which affected the peaks in the European pharmacopoeia method was dilution medium. The dilution medium was 0.01M HCl, if it was prepared on the same day of analysis the peaks were sharp and base line was stable and if the same solution was used after a few days then the peaks were distorted and the baseline was very unstable.

- When the dilutions were carried out in distilled water or 0.01M HCl and analysed by HPLC the results were not 100 % in 8 out of ten times. When a small concentration of antioxidants was added to distilled water then results were 100 %. When the antioxidants were added to 0.01M HCl the antioxidants precipitated causing a turbid solution.
- Effect of excipients on HPLC peaks in Skuli's method: It was clearly demonstrated that the change in the pH of mobile in the presence of antioxidants and chelating agents was causing high values. But on the other side, in the absence of antioxidants and chelating agents, the reason why the values were most times less than 100% or some times greater than 100% is unexplainable. When the hydrogels containing only doxycycline was analysed the results were always less than 100 % and in the presence of antioxidants and

chelating agents the results were 1.5 time higher (130 %), this effect was not a onetime incident, even when the entire batch of hydrogels were freshly prepared and analysed same type of error always existed. For this reasons the method was replaced by European Pharmacopoeia method. In the European Pharmacopoeia method also in the beginning the values of hydrogels were below 100 %, but never above 100 %. This was due to fact that doxycycline was not equally dispersed with in the hydrogel. Manual stirring with glass rod and magnetic stirring, could not effectively distribute the doxycycline with in the hydrogel. Later the hydrogels were mixed by placing on a VORTEX shaker, which was very effective in distributing the doxycycline uniformly with in the entire hydrogel. After this there were no fluctuations in stabilities atleast not above 100% as like in the case of Skuli's method. After the results were stabilized the hydrogels stability was crosschecked with Skuli's method and it showed 100% stability same as European Pharmacopoeia method. But in the beginning why the values were 1.5 times higher is unexplainable to most extent though effect of excipients is confirmed. It might look like vortexing the hydrogels might have caused equal distribution of doxycycline and this might be the reason why Skuli's method is showing accurate results lately. But when the dilutions were done in pure distilled water same kinds of error existed. Overall it can be concluded that the Skuli's method showed lot of unexplainable fluctuations in values (ranging from 60% - 150%) compared to European Pharmacopoeia method (95% -100%). The buffer capacity of European Pharmacopoeia method was good whereas in the Skuli's method perchloric acid was used to create the buffer capacity. Also Perchloric acid itself is a very strong oxidising agent and might be causing some spontaneous degradation to doxycycline. This might be the cause of low stabilities in the absence of antioxidants and chelating agents.

5.2 Stability studies

Degradation compounds mentioned in European Pharmacopoeia and British Pharmacopoeia's were, metacycline and 6-epidoxycycline. There were no traces of metacycline or 6-epidoxycycline in any of the hydrogels with pH 6.55-6.6 tested at 4°C, 25 °C and 40 °C up to 3 months and in some cases up to 20 months. The only degradation compound seen was 4-epidoxycycline at all 3 temperatures. This degradation compound seems to be appearing at a constant rate depending upon the temperature which the formulation was stored. 4-epidoxycycline was not available commercially to quantify. At 4°C all the formulations were stable at pH 6.55-6.6. But when the pH was in basic region the degradation was rapid. It indicates that pH itself might induce degradation and catalyse the reaction. The formulation containing only doxycycline and pH adjusted to 6.55 was 100 % stable after 1 year at 4°C. Whereas, the same formulation at 4 °C containing only doxycycline, when pH was adjusted to 7.4, by adding a buffer, its stability was only 44 % after 15 months. So at 4°C pH 6.55-6.6 is the most favourable region for doxycycline stability if there are no antioxidants and chelating agents in the formulation. Even pH 7.4 formulations, were stabilized by adding antioxidants and chelating agents but not by 100 %. At 25°C the highest stability achieved at the end of 3 months was 91 %. And at 40°C the highest stability at the end of 3 months was 71 %. After 6 months there

were no peaks found in formulations stored at 40 °C, which means that even impurities degraded at high temperatures.

Doxycycline is both hydrophilic and lipophilic but predominantly more lipophilic. When surfactants like poloxamers were added to the formulation doxycycline occupied the lipophilic regions (micelles) of surfactant and was protected from exposure to water molecules within the surfactant. Poloxamers induced good stability to doxycycline.

- Addition of HPβCD increased the hydrophilicity of doxycycline, and thereby it was more readily exposed to water molecules with in the surfactant and hence more degradation was observed.
- At 4°C majority of formulations were 100 % stable over a tested period of 3 months and in some cases they were stable over a tested period of 20 months.
- At 4°C there were fluctuations in stabilities of hydrogels which did not contain any antioxidants and chelating agents. The stabilities of hydrogels fluctuated between 85-100 %. This phenomenon was observed in hydrogels 1 and 4. These both hydrogels did not contain any antioxidants or chelating agents. In other hydrogels containing combination of antioxidants and chelating agents, the fluctuations in stabilities were not observed. These stability fluctuations might be due to following reasons-

1. Doxycycline might be getting oxidised when exposed to strong oxidising agents within the mobile phase for HPLC analysis or even distilled water (to support this theory when dilutions were done in either pure water or 0.01N HCl or 2.5 pH mobile phase the values were never 100 % and there were no visible degradation peaks. But when small amounts of antioxidants were added to the water then the values were 100 %. When the antioxidants were added to 0.01N HCl, the resulting solution turned into turbid).

2. Doxycycline reversibly converts into epimers i.e. in unfavourable conditions like, increase in temperature, exposure to strong oxidising agents etc. and when the conditions favour it might be converted back into doxycycline. To support this theory NMR and IR structure analysis studies are needed.

3. While preparing the sample the time gap (from waiting for the hydrogel to be weighed into the volumetric flask until preparation of vials and till its turn comes in HPLC injection) and the temperature of the dilution medium itself, might affect the stability of hydrogels which are devoid of antioxidants and chelating agents.

4. After adding the doxycycline into the hydrogel, say for some initial weeks doxycycline completely might not be able to reach the expected lipophilic compartment with in the surfactant, and the left over doxycycline might be getting exposed to water molecules and might be causing the fluctuations in stability values. Previously also when doxycycline was added to distilled water and assayed the results were not always 100 % even on the same day of analysis and the stabilities were fluctuating in the similar manner. Doxycycline when exposed to water molecules in the absence of antioxidants might be temporarily/reversible in the process of converting into its epimer (degradation product) and during this time if the hydrogel stability was assayed, it might be the cause for fluctuating values and there were no degradation peaks seen. After some time (after 3 months) the stabilities were completely stabilised (100 % from

3 - 20 months) and there were no fluctuations. This might be because the doxycycline might be completely encapsulated within the lipophilic component of the surfactant (poloxamers). This also suggests that during mixing of hydrogels by vortexing, extra time might be needed to anchor the more lipophilic doxycycline into the lipophilic compartment with in the surfactant.

- At 4 °C in the hydrogel containing only antioxidants (hydrogel 5) without chelating agents, strange phenomenon was observed. The hydrogel was stable upto 4-6 weeks and there was a sudden dip in the stability. This phenomenon might be due to following reason
  - Antioxidants might be precipitating the doxycycline out from the micelles, and exposing it to aqueous atmosphere, and only antioxidants in the aqueous atmosphere were not sufficient to protect doxycycline from oxidising beyond 4 weeks.
- At 25 °C, one of the formulation was 100 % stable up to 1 month and 91 % stable after 3 months.
- At 40 °C, one of the formulation contained 71 % of doxycycline over a period of 3 months, which is the highest stability achieved till date.
- From the experiment it was evident that presence of different types of mucoadhesive polymers in non-ionic surfactants, did not affect the stability of doxycycline.
- Effect of pH on stability of doxycycline was clearly evident from the experiment results. At pH 6.6, the formulation was 99 % stable after 20 months, whereas the same formulation when prepared in 7.4 pH buffer solution, only 44 % of doxycycline remained at the end of 15 months. Both the formulations contained no antioxidants or chelating agents or any stabilizing agents. Just the poloxamers, pH 6.6 and storage temperature 4 °C imparted 99 % stability to doxycycline.
- The effect of pH of hydrogels on stability of doxycycline even in the presence of antioxidants and chelating agents was evident. The hydrogel 2 at pH 7.4 in the presence of antioxidants and chelating agents was only 83 % stable by the end of 23 months whereas same hydrogel at 6.6 pH was 99 % stable even after 20 months.
- pH 6.55-6.6 might be the most favourable regions to prevent doxycycline from degradation. At pH 7.4 rapid degradation occurred and from the previous studies it was evident that at pH below 6.0 doxycycline degradation was significant.
- At pH 6.55 the only degradation compound seen in all hydrogels at all 3 temperatures was 4-epidoxycycline, whereas at pH 7.4 the hydrogels showed 2 degradation products i.e. 4-epidoxycycline and metacycline, in the absence of antioxidants and chelating agents. In the presence of antioxidants and chelating agents the stabilities at pH 6.55 were 100 % after 15 months whereas At 7.4 pH, the stabilities after 15 months was 93.5 %, after 19 months the stability was 89.5 %, and after 23 months the stability was 83 %.
- 4-epidoxycycline was the only degradation product evident in all the formulations stored at 4 °C, 25 °C and 40 °C. At 4 °C the formation of 4-epidoxycycline was very slow and it did not affect the stability of doxycycyline. 4-epidoxycycline and doxycycline (reversible) inter-conversion depends on their kinetic equilibrium. At 4 °C, after 20 months the 4-epidoxycycline was less than 0.25 % of the area due to 1.6 mg of 6-epidoxycycline in 100 mL

solution (HPLC analysis) according to European Pharmacopoeia. The amount of 4-epidoxycycline was minimum in formulations that contained only doxycycline and its amount significantly increased with increasing amounts of excipients like antioxidants and chelating agents. At 4 °C, and at pH 6.55 - 6.6, the formulations with and without antioxidants and chelating agents preserved the negligible amounts of 4-epidoxycycline until 20 months, as it was evident from observing their HPLC UV absorbance areas, which were either constant or were increasing at a very slow (negligible) pace. At 4 °C, in pH 7.4 hydrogels, the 4-epidoxycycline was decreasing with time and for hydrogels stored at 25 °C and 40 °C the 4-epidoxycycline was slowly disappearing with time. So the amount of 4-epidoxycycline present at pH 7.4 hydrogels at 4 °C or for hydrogels stored at 25 °C and 40 °C, at that particular time does not actually represent the 4-epidoxycycline formed from the initial day of preparation of formulation and the European Pharmacopoeia set limit for 4-epidoxycycline cannot be considered.

5.3 Colour of the formulations

The colour of the formulations was clear/colourless when the formulations pH was around 6.6. When the pH was 7.4 the colour of the formulation was dark yellow. So pH played important role in the colour of the formulation. If there was any degradation the formulation turned into brown colour. The intensity of brown colour depended on the level of degradation, especially at 40 °C the formulations were dark brown. In the presence of antioxidants the formulation did not turn into brown colour even though there was slight degradation at 25 °C. So the colour of formulation should not be taken as a parameter in estimating the stability of formulations.

5.4 Complexation of doxycycline-HPβCD

Doxycycline and cyclodextrins were pre-complexed before adding to the formulations. Cyclodextrins have the ability to bind to the drug molecule when dissolved together in water directly. But in the formulation pre-complexation was necessary because of following reasons.

- The cyclodextrins can bind to the drug molecule when added to pure solution, but when they are added individually into a 350 cP viscous solution, the free movement of cyclodextrins and drug will be a question. They might be just suspended in viscous hydrogels and complexation might not take place. And the free cyclodextrins instead of binding to doxycycline might bind to excipients in the formulation.
- The other solution was to add cyclodextrins and drug to pure water before adding any poloxamers. But later in the process of addition of poloxamers, stirring and manual agitation will be required and bubbles will be formed in the formulations as the poloxamers are surfactants and by doing so, the doxycycline might be oxidised because of excessive bubbles in formulation. Some of the formulation will stick to walls of the beaker because of foam and gets dried, there could be some loss of drug in the process.

5.5 Viscosity measurements

The viscosities were measured after the formulations were uniformly redispersed with the mucoadhesive polymers. The formulations were redispersed by placing on a vortex shaker. On leaving the formulation idle, the mucoadhesive polymers settled in the lower zones of the beaker. The viscosities were measured when the hydrogels were idle, by collecting samples from lower regions where the mucoadhesive polymers settled, it showed high viscosity values whereas when the sample was collected from the superficial hydrogel regions the viscosities were lower because of the absence of the mucoadhesive polymers. When the mucoadhesive polymers were equally dispersed the viscosity values were different.

5.6 Mucoadhesion analysis

The mucoadhesive values were calculated from AUC, peak detachment forces and work of mucoadhesion. A total of 14 hydrogel were screened and finally a hydrogel containing a combination of 0.25 % HPMC and 0.25 % Povidone polymers, showed optimum values in all the screening tests. Both the polymers were non-ionic polymers, which was also an important parameter for selection as it was thought that charge on polymers could affect the stability of doxycycline.

5.7 *In vitro* release studies

In vitro release studies were first attempted with Franz diffusion cells but due to a high concentration of poloxamers, the receptor phase was migrating into the donor chamber. This method was replaced with polymer non membrane method, which is the most widely used method for poloxamer hydrogels. The drug release was very slow and constant from the polymer matrices, which followed the zero order kinetics, and the mechanism of drug release was not through either diffusion or dissolution but occurred through super case II transport, indicating a more complex release mechanism. The slow drug release was observed for up to 20 hours.

6 Conclusion

The main aim of this project of formulating a stable hydrogel, that is stable for over 2 years is almost achieved at 4 °C, and the stabilities achieved at 25 °C and 40 °C are the highest among all the previous studies. These stabilities were achieved as a combinatorial effect of all the excipients present in the hydrogel at 25 °C and 40 °C, whereas at 4 °C, the 99 % stability after 20 months was achieved only from the surfactant polymer (poloxamer) and the right pH region 6.55-6.6. Overall 7 out of 9 hydrogels were stable over a tested period of 3 months at 4 °C. 2 of the 7 hydrogels at 4 °C were 98.5 % and 99 % stable after 20 months, and they are expected to be stable for another 7-10 months.

In situ formation of hydrogels will improve the patient compliance and ease of administration. The hydrogels have the ability to attach at the site of administration and release drug constantly over a period of 20 h. But due to constant salivary flow, and chewing of the food, the residence time of hydrogel in real life conditions, will be less when compared to *in vitro* simulation models.

The current treatment of aphthous ulcers, which is based upon inhibiting MMPs with a topical formulation containing sub-antimicrobial concentration of doxycycline, looks as a promising alternative to the existing treatment regimens, as the healing time and pain reduction was significantly rapid when compared to other treatments and is also safe for long-term management.

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9 APPENDIX

Data of HPLC runs with high %Yields

Total of 3 runs were carried out i.e. initial day, after 9 days, after 5 weeks.

Table: A1 HPLC data for batch 1 (starting day). (15-06-2012)

Sample No.	Sample Name	Ret.Time min Doxycycline UV_VIS_1	Area mAU*min Doxycycline UV_VIS_1	Height mAU Doxycycline UV_VIS_1	Amount Doxycycline UV_VIS_1	Type Doxycycline UV_VIS_1	Plates (EP) Doxycycline UV_VIS_1
1	STANDARD 1	27.687	0.2576	0.2611	0.0048	M *^	6187
2	STANDARD 2	27.473	0.4254	0.4743	0.0053	MB*^	9907
3	STANDARD 3	27.513	2.7003	3.0788	0.0115	MB*^	9618
4	STANDARD 4	27.513	6.6402	7.2569	0.0223	MB*^	8331
5	STANDARD 5	27.58	13.2066	12.7147	0.0403	MB*^	5738
6	1A1	27.513	3.3349	3.9673	0.0132	BMB*^	8741
7	1A2	27.507	2.1122	2.6488	0.0099	BMB*^	8955
8	1A3	27.52	3.7421	4.271	0.0144	bMB*^	8418
9	1B1	27.52	2.6938	3.2117	0.0115	BMB*^	8311
10	1B2	27.467	3.3712	3.8912	0.0133	BMB*^	8260
11	1B3	27.5	3.1792	3.753	0.0128	BMB*^	8382
12	STANDARD 1	27.58	0.3098	0.2796	0.0049	M *^	5660
13	STANDARD 2	27.553	0.4627	0.4944	0.0054	bMB*^	8316
14	STANDARD 3	27.473	3.6901	3.642	0.0142	MB*^	7133
15	STANDARD 4	27.507	7.0187	6.7143	0.0234	MB*^	6442
16	STANDARD 5	27.573	13.6445	11.4205	0.0415	MB*^	4356
17	1C1	27.487	4.0386	4.5478	0.0152	BMB*^	7524
18	1C2	27.473	2.7411	3.0956	0.0116	BMB*^	7639
19	1C3	27.48	3.5022	3.7911	0.0137	bMB*^	7741
20	2A1	27.473	6.4386	7.7838	0.0218	BMB*^	8176
21	2A2	27.447	6.807	8.1636	0.0228	BMB*^	8256
22	2A3	27.467	5.7075	7.0923	0.0198	BMB*^	8393
23	STANDARD 1	27.667	0.2818	0.2511	0.0049	MB*^	6294
24	STANDARD 2	27.58	0.5366	0.475	0.0056	M *^	4932
25	STANDARD 3	27.52	3.2509	3.3759	0.013	MB*^	6977
26	STANDARD 4	27.547	6.5519	6.2997	0.0221	MB*^	6144
27	STANDARD 5	27.62	13.8204	10.97	0.042	MB*^	4045
28	2B1	27.52	7.5713	8.5118	0.0249	BMB*^	7459
29	2B2	27.533	6.5755	7.7622	0.0221	BMB*^	7806
30	2B3	27.513	6.631	7.6742	0.0223	BMB*^	7593
31	2C1	27.507	6.9124	8.1533	0.0231	BMB*^	8078
32	2C2	27.507	6.4025	7.4824	0.0217	BMB*^	8030
33	2C3	27.487	6.6999	7.8984	0.0225	BMB*^	7956
Average:		27.524	4.8866	5.1942	0.0175		7448
Rel.Std.Dev:		0.20%	74.60%	65.80%	57.18%		18.74%

2. **Table A2:** HPLC data for batch1 after 9 days (24-06-2012)

Sample No.	Sample Name	Ret.Time min Doxycycline UV_VIS_1	Area mAU*min Doxycycline UV_VIS_1	Height mAU Doxycycline UV_VIS_1	Amount Doxycycline UV_VIS_1	Type Doxycycline UV_VIS_1	Plates (EP) Doxycycline UV_VIS_1
1	STANDARD 1	25.447	0.559	1.0009	0.0044	BMB*^	13369
2	STANDARD 2	25.34	0.9674	1.6892	0.0055	MB*^	13491
3	STANDARD 3	25.327	3.4101	6.2698	0.0119	BMB*^	15370
4	STANDARD 4	25.28	7.1669	13.3199	0.0219	BMB*^	15550
5	STANDARD 5	25.227	14.1264	26.3558	0.0404	BMB*^	15270
6	1A1	25.26	5.842	10.5952	0.0184	BMB*^	15450
7	1A2	25.233	5.7932	10.5371	0.0183	BMB*^	15579
8	1A3	25.213	5.7111	10.4753	0.0181	BMB*^	15686
9	1B1	25.18	5.5453	10.5615	0.0176	BMB*^	16083
10	1B2	25.167	5.657	10.6395	0.0179	BMB*^	15873
11	1B3	25.153	5.6552	10.6248	0.0179	BMB*^	15811
12	STANDARD 1	25.2	0.6789	1.1948	0.0047	MB*^	15112
13	STANDARD 2	25.213	1.0508	1.9083	0.0057	BMB*^	14688
14	STANDARD 3	25.147	3.8631	7.1377	0.0131	BMB*^	15408
15	STANDARD 4	25.127	7.8032	14.7358	0.0236	BMB*^	15789
16	STANDARD 5	25.107	14.5879	27.9449	0.0416	BMB^	15642
17	1C1	25.133	4.5173	8.6267	0.0149	BMB*^	15820
18	1C2	25.167	4.4771	8.5932	0.0148	BMB*^	15997
19	1C3	25.133	4.5353	8.6014	0.0149	BMB*^	15764
20	2A1	25.1	8.6609	17.0652	0.0259	BMB*^	16603
21	2A2	25.093	8.8351	17.2516	0.0263	BMB*^	16570
22	2A3	25.087	9.3063	17.5212	0.0276	BMB*^	16206
23	STANDARD 1	25.153	0.6203	1.2073	0.0045	BMB*^	16316
24	STANDARD 2	25.133	1.0216	1.9337	0.0056	BMB*^	15932
25	STANDARD 3	25.113	3.8626	7.1945	0.0131	BMB*^	15795
26	STANDARD 4	25.107	7.9695	14.9408	0.024	BMB*^	16024
27	STANDARD 5	25.093	14.3405	28.0127	0.041	BMB*^	15995
28	2B1	25.12	8.6132	17.0124	0.0258	BMB*^	16812
29	2B2	25.107	8.4929	17.0064	0.0254	BMB*^	17017
30	2B3	25.107	8.5498	17.1076	0.0256	BMB*^	16980
31	2C1	25.12	7.025	14.1275	0.0215	BMB*^	17061
32	2C2	25.113	7.0112	14.1806	0.0215	BMB*^	17178
33	2C3	25.127	7.0245	14.2082	0.0215	BMB*^	17158
Average:		25.171	6.16	11.8055	0.0192		15861
Rel.Std.Dev:		0.33%	60.90%	61.70%	51.75%		5.56%

3. **Table: A3** HPLC data for batch1 after 5 weeks (13-07-2012)

Sample No.	Sample Name	Ret.Time min Doxycycline UV_VIS_1	Area mAU*min Doxycycline UV_VIS_1	Height mAU Doxycycline UV_VIS_1	Amount Doxycycline UV_VIS_1	Type Doxycycline UV_VIS_1	Plates (EP) Doxycycline UV_VIS_1
1	STANDARD 1	25.927	1.1151	1.7998	0.0033	BMB*^	12355
2	STANDARD 2	25.82	2.1601	3.6622	0.006	BMB*^	13417
3	STANDARD 3	25.76	5.3532	9.5007	0.0142	BMB*^	14171
4	STANDARD 4	25.72	8.8024	15.9491	0.023	BMB*^	14669
5	STANDARD 5	25.68	15.7197	28.8836	0.0408	BMB*^	14840
6	1A1	25.707	7.9238	13.3788	0.0208	BMB*^	13957
7	1A2	25.693	7.8298	13.198	0.0205	BMB*^	13780
8	1A3	25.68	7.6508	12.9793	0.0201	BMB*^	13802
9	1B1	25.667	6.8811	11.6323	0.0181	BMB*^	13654
10	1B2	25.667	6.8108	11.6177	0.0179	BMB*^	13959
11	1B3	25.647	6.8092	11.5881	0.0179	BMB*^	13695
12	STANDARD 1	25.68	1.1446	1.8485	0.0033	BMB*^	11911
13	STANDARD 2	25.667	2.1862	3.7543	0.006	BMB*^	13752
14	STANDARD 3	25.633	5.321	9.5096	0.0141	BMB*^	14245
15	STANDARD 4	25.607	8.6639	15.7659	0.0227	BMB*^	14677
16	STANDARD 5	25.573	15.7484	28.5634	0.0409	BMB*^	14512
17	1C1	25.64	2.6655	4.4419	0.0073	BMB*^	12868
18	1C2	25.633	2.6131	4.3453	0.0071	BMB*^	12967
19	1C3	25.627	2.5529	4.2862	0.007	BMB*^	13158
20	2A1	25.567	8.8087	16.2929	0.023	BMB*^	15237
21	2A2	25.573	8.916	16.6776	0.0233	BMB*^	15541
22	2A3	25.56	9.0073	16.8358	0.0235	BMB*^	15579
23	STANDARD 1	25.58	1.1449	2.0679	0.0033	BMB*^	14735
24	STANDARD 2	25.613	2.1708	3.9271	0.006	BMB*^	14606
25	STANDARD 3	25.587	5.3926	9.7962	0.0143	BMB*^	14634
26	STANDARD 4	25.573	8.7753	15.9397	0.023	BMB*^	14688
27	STANDARD 5	25.56	15.5733	28.5416	0.0404	BMB*^	14692
28	2B1	25.567	8.1834	15.367	0.0214	BMB*^	15533
29	2B2	25.567	8.1651	15.5584	0.0214	BMB*^	15860
30	2B3	25.567	8.2648	15.6759	0.0216	BMB*^	15761
31	2C1	25.58	6.4263	12.447	0.0169	BMB*^	16260
32	2C2	25.58	6.4713	12.5381	0.017	BMB*^	16203
33	2C3	25.587	6.399	12.5119	0.0168	BMB*^	16384
Average:		25.639	6.7167	12.1479	0.0177		14427
Rel.Std.Dev:		0.32%	58.26%	59.74%	56.93%		7.75%

Table:B1 Data for HPLC runs in stability studies (previously prepared hydrogels, after 1 year) (20-05-2013)

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	16.287	1.7005	0.9737	0.0039	BMB*^	547
2	standard2	16.213	3.7319	2.1193	0.0086	BMB*^	523
3	standard3	16.067	8.9911	5.0432	0.0206	BMB*^	510
4	standard4	15.993	16.5939	9.4649	0.038	BMB*^	516
5	standard5	15.967	25.7235	14.4631	0.0589	BMB*^	507
6	1	15.92	8.7231	4.8839	0.02	BMB*^	498
7	2	15.84	8.7349	4.9046	0.02	BMB*^	499
8	3	15.847	8.8089	4.9304	0.0202	BMB*^	500
9	4	15.787	8.3181	4.706	0.0191	BMB*^	504
10	5	15.747	8.2942	4.7376	0.019	BMB*^	503
11	6	15.68	8.3368	4.8046	0.0191	BMB*^	509
12	7	15.707	4.0066	2.3518	0.0092	BMB*^	515
13	8	15.613	4.1213	2.4006	0.0094	BMB*^	517
14	9	15.587	4.1258	2.4002	0.0095	BMB*^	519
15	standard1	15.62	1.6464	0.9903	0.0038	BMB*^	550
16	standard2	15.52	3.7581	2.2323	0.0086	BMB*^	525
17	standard3	15.527	9.0771	5.2563	0.0208	BMB*^	506
18	standard4	15.507	16.5908	9.8262	0.038	BMB*^	534
19	standard5	15.453	25.7365	15.0192	0.059	BMB*^	511
20	10	15.387	6.8397	4.0031	0.0157	BMB*^	508
21	11	15.407	6.8169	4.0393	0.0156	BMB*^	512
22	12	15.373	6.8542	4.0365	0.0157	BMB*^	519
23	13	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
24	14	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
25	15	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
26	16	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
27	17	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
28	18	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
29	standard1	15.247	1.6355	1.0527	0.0037	BMB*^	584
30	standard2	15.187	3.7268	2.2794	0.0085	BMB*^	538
31	standard3	15.207	9.1309	5.433	0.0209	BMB*^	511
32	standard4	15.1	16.3213	10.0743	0.0374	BMB*^	546
33	standard5	15.12	25.828	15.4583	0.0592	BMB*^	515
34	19	15.06	3.9002	2.3398	0.0089	BMB*^	526
35	20	15.067	3.8463	2.3474	0.0088	BMB*^	527
36	21	15.087	3.8133	2.3282	0.0087	BMB*^	530
37	22	15.053	8.1996	4.9904	0.0188	BMB*^	525
38	23	14.967	8.2362	5.0069	0.0189	BMB*^	524
39	24	14.96	8.047	4.9927	0.0184	BMB*^	528
40	25	14.953	6.1653	3.7221	0.0141	BMB*^	513
41	26	14.96	6.2519	3.7669	0.0143	BMB*^	524
42	27	14.827	6.1467	3.7987	0.0141	BMB*^	524
Average:		15.468	8.5772	5.0327	0.0197		521
Rel.Std.Dev:		2.56%	74.87%	74.28%	74.87%		3.28%

Data for HPLC runs in “how excipients were interfering the HPLC results”.

1. Effect of antioxidants (28-06-12)

Table: C1

Sample No.	Sample Name	Ret.Time min Doxycycline UV_VIS_1	Area mAU*min Doxycycline UV_VIS_1	Height mAU Doxycycline UV_VIS_1	Amount Doxycycline UV_VIS_1	Type Doxycycline UV_VIS_1	Plates (EP) Doxycycline UV_VIS_1
1	STANDARD 1	26.067	0.8799	1.3679	0.0035	bMB*^	14174
2	STANDARD 2	25.947	1.702	2.9912	0.0054	BMB*^	14154
3	STANDARD 3	25.893	4.5724	8.2719	0.0121	BMB*^	15303
4	STANDARD 4	25.853	8.9467	16.7039	0.0223	BMB*^	15774
5	STANDARD 5	25.793	16.1016	30.2056	0.0389	BMB*^	15723
6	1a	25.8	6.2278	11.473	0.016	BMB*^	15591
7	1b	25.8	6.4002	11.9234	0.0164	BMB*^	15796
8	1c	25.793	6.5958	12.0318	0.0168	BMB*^	15488
9	2a	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
10	STANDARD 1	25.807	0.901	1.5316	0.0036	BMB*^	14391
11	STANDARD 2	25.787	2.0272	3.6645	0.0062	BMB*^	15229
12	STANDARD 3	25.747	5.4182	10.0224	0.0141	BMB*^	15474
13	STANDARD 4	25.733	9.9456	18.6058	0.0246	BMB*^	15736
14	STANDARD 5	25.707	17.149	32.2848	0.0413	BMB*^	15726
15	2b	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
16	2c	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
17	3a	25.727	8.1728	15.1696	0.0205	BMB*^	15750
18	3b	25.72	8.0438	15.0903	0.0202	BMB*^	15895
19	STANDARD 1	25.747	0.8837	1.487	0.0035	BMB*^	14908
20	STANDARD 2	25.74	1.9452	3.5435	0.006	BMB*^	14643
21	STANDARD 3	25.733	5.4453	9.9548	0.0141	BMB*^	15511
22	STANDARD 4	25.713	9.7761	18.3282	0.0242	BMB*^	15755
23	STANDARD 5	25.693	17.0486	32.0511	0.0411	BMB*^	15666
24	3c	25.733	8.0825	15.1078	0.0203	BMB*^	15846
25	4a	25.74	8.8854	16.6581	0.0221	BMB*^	15876
26	4b	25.74	8.9313	16.7233	0.0222	BMB*^	15975
27	4c	25.747	9.187	16.8332	0.0228	BMB*^	15645
Average:		25.782	7.2195	13.4177	0.0183		15418
Rel.Std.Dev:		0.33%	66.11%	67.32%	60.72%		3.57%

2. Effect of chelating agents (3-07-2012)

Table: C2

Sample No.	Sample Name	Ret.Time min Doxycycline UV_VIS_1	Area mAU*min Doxycycline UV_VIS_1	Height mAU Doxycycline UV_VIS_1	Amount Doxycycline UV_VIS_1	Type Doxycycline UV_VIS_1	Plates (EP) Doxycycline UV_VIS_1
1	STANDARD 1	25.413	1.1648	2.1672	0.003	BMB*^	15562
2	STANDARD 2	25.313	2.0814	3.7572	0.0051	BMB*^	14507
3	STANDARD 3	25.293	5.5941	10.4599	0.0132	BMB*^	15808
4	STANDARD 4	25.253	9.6127	18.4287	0.0225	BMB*^	16062
5	STANDARD 5	25.207	16.8761	33.1953	0.0392	BMB*^	16256
6	1A	25.213	10.5211	20.489	0.0246	BMB*^	16080
7	1B	25.2	10.6081	20.7558	0.0248	BMB*^	16259
8	1C	25.187	10.644	20.8234	0.0249	BMB*^	16207
9	2A	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
10	2B	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
11	2C	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
12	STANDARD 1	25.187	1.2747	2.4274	0.0033	BMB*^	15587
13	STANDARD 2	25.167	2.3925	4.6629	0.0058	BMB*^	16274
14	STANDARD 3	25.147	6.3749	12.5548	0.015	BMB*^	16377
15	STANDARD 4	25.12	10.3663	20.3966	0.0242	BMB*^	16296
16	STANDARD 5	25.1	17.5877	34.8484	0.0409	BMB*^	16305
17	3A	25.127	12.6176	24.8008	0.0294	BMB*^	16482
18	3B	25.12	12.5346	24.7806	0.0292	BMB*^	16641
19	3C	25.107	12.6159	24.813	0.0294	BMB*^	16420
20	4A	25.113	12.251	23.7474	0.0286	BMB*^	16322
21	4B	25.113	12.2216	23.7576	0.0285	BMB*^	16311
22	4C	25.12	12.2828	23.7698	0.0286	BMB*^	16249
23	STANDARD 1	25.167	1.28	2.4327	0.0033	BMB*^	15020
24	STANDARD 2	25.147	2.4163	4.7145	0.0059	BMB*^	15915
25	STANDARD 3	25.127	6.3771	12.5985	0.015	BMB*^	16542
26	STANDARD 4	25.107	10.2523	20.3048	0.024	BMB*^	16384
27	STANDARD 5	25.093	17.488	34.7424	0.0406	BMB*^	16355
28	5A	25.113	8.602	16.578	0.0202	BMB*^	16147
29	5B	25.127	8.5978	16.5752	0.0201	BMB*^	16118
30	5C	25.127	8.6016	16.523	0.0202	BMB*^	16095
31	6A	25.12	8.8582	17.1216	0.0207	BMB*^	16319
32	6B	25.113	8.8499	17.13	0.0207	BMB*^	16078
33	6C	25.133	8.9665	17.2446	0.021	BMB*^	16161
Average:		25.162	8.9971	17.5534	0.0211		16105
Rel.Std.Dev:		0.29%	51.85%	52.65%	51.05%		2.75%

3. Effect of chelating agents (same vials, injected the next day) (5-07-2012)

Table: C3

Sample No.	Sample Name	Ret.Time min Doxycycline UV_VIS_1	Area mAU*min Doxycycline UV_VIS_1	Height mAU Doxycycline UV_VIS_1	Amount Doxycycline UV_VIS_1	Type Doxycycline UV_VIS_1	Plates (EP) Doxycycline UV_VIS_1
1	STANDARD 1	26.653	1.2781	2.2366	0.0031	BMB*^	15241
2	STANDARD 2	26.627	2.4193	4.4082	0.0057	BMB*^	16084
3	STANDARD 3	26.58	6.1708	11.4245	0.0142	BMB*	16402
4	STANDARD 4	26.553	10.5138	19.5005	0.0241	BMB*	16181
5	STANDARD 5	26.513	17.6389	32.7369	0.0404	BMB*	16023
6	1A	26.533	11.2598	20.6929	0.0258	BMB*	16069
7	1B	26.533	11.357	20.7469	0.0261	BMB*	16004
8	1C	26.513	11.283	20.7866	0.0259	BMB*	16132
9	2A	26.507	11.1589	20.4879	0.0256	BMB*	15897
10	2B	26.5	11.1929	20.5147	0.0257	BMB*	16018
11	2C	26.487	11.1157	20.4533	0.0255	BMB*	16002
12	STANDARD 1	26.48	1.2859	2.2801	0.0031	BMB*	15727
13	STANDARD 2	26.513	2.4341	4.4439	0.0057	BMB*	15947
14	STANDARD 3	26.493	6.1378	11.4738	0.0142	BMB*	16384
15	STANDARD 4	26.46	10.4581	19.5239	0.024	BMB*	16232
16	STANDARD 5	26.44	17.4403	32.6042	0.04	BMB*	16163
17	3A	26.46	12.4636	23.0569	0.0286	BMB*	16221
18	3B	26.467	12.4006	23.0605	0.0285	BMB*	16340
19	3C	26.453	12.3917	23.0798	0.0284	BMB*	16279
20	4A	26.46	11.7354	21.7759	0.0269	BMB*	16321
21	4B	26.473	11.7548	21.7166	0.027	BMB*	16237
22	4C	26.473	11.8697	21.7977	0.0272	BMB*	16105
23	STANDARD 1	26.513	1.268	2.3609	0.003	BMB*	16353
24	STANDARD 2	26.513	2.4794	4.4758	0.0058	BMB*	15703
25	STANDARD 3	26.493	6.1637	11.4975	0.0142	BMB*	16463
26	STANDARD 4	26.473	10.3948	19.51	0.0239	BMB*	16304
27	STANDARD 5	26.46	17.3665	32.3896	0.0398	BMB*	16133
28	5A	26.493	8.142	14.879	0.0187	BMB*	16097
29	5B	26.487	8.1124	14.848	0.0187	BMB*	16110
30	5C	26.487	8.0227	14.7663	0.0185	BMB*	16154
31	6A	26.48	8.423	15.3659	0.0194	BMB*	16113
32	6B	26.487	8.2787	15.2409	0.019	BMB*	16045
33	6C	26.493	8.3521	15.3308	0.0192	BMB*	16217
Average:		26.502	9.1746	16.9536	0.0211		16112
Rel.Std.Dev:		0.18%	48.95%	49.35%	48.63%		1.46%

4. Column precision new column (11-07-2012)

Table: C4

Sample No.	Sample Name	Ret.Time min Doxycycline UV_VIS_1	Area mAU*min Doxycycline UV_VIS_1	Height mAU Doxycycline UV_VIS_1	Amount Doxycycline UV_VIS_1	Type Doxycycline UV_VIS_1	Plates (EP) Doxycycline UV_VIS_1
1	STANDARD 1	28.7	1.1541	1.8971	0.003	BMB*^	15344
2	STANDARD 2	28.68	2.441	4.1019	0.0057	BMB*^	16172
3	STANDARD 3	28.633	6.4292	11.0134	0.0142	BMB*^	16241
4	STANDARD 4	28.593	11.1123	19.0887	0.0242	BMB*^	16104
5	STANDARD 5	28.547	18.5346	31.7834	0.04	BMB*^	16042
6	1	28.587	7.4013	12.4047	0.0163	BMB*^	16006
7	2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Average:		28.623	7.8454	13.3815	0.0172		15985
Rel.Std.Dev:		0.21%	80.84%	81.57%	78.38%		2.04%

5. Column precision old column (12-07-2012)

Table: C5

Sample No.	Sample Name	Ret.Time min Doxycycline UV_VIS_1	Area mAU*min Doxycycline UV_VIS_1	Height mAU Doxycycline UV_VIS_1	Amount Doxycycline UV_VIS_1	Type Doxycycline UV_VIS_1	Plates (EP) Doxycycline UV_VIS_1
1	STANDARD 1	25.607	1.1923	2.0817	0.0029	BMB*^	13794
2	STANDARD 2	25.527	2.5442	4.637	0.0058	BMB*^	14863
3	STANDARD 3	25.467	6.6151	12.7519	0.0144	BMB*^	16082
4	STANDARD 4	25.44	11.1833	22.1031	0.024	BMB*^	16678
5	STANDARD 5	25.4	18.7618	37.2176	0.04	BMB*^	16506
6	1	25.447	7.781	14.6697	0.0168	BMB*^	15967
7	2	25.44	8.9286	16.9967	0.0192	BMB*^	16060
Average:		25.475	8.1437	15.7797	0.0176		15707
Rel.Std.Dev:		0.27%	71.73%	74.23%	70.15%		6.52%

6. Different brand doxycycline (12-07-2012)

Table: C6

Sample No.	Sample Name	Ret.Time min Doxycycline UV_VIS_1	Area mAU*min Doxycycline UV_VIS_1	Height mAU Doxycycline UV_VIS_1	Amount Doxycycline UV_VIS_1	Type Doxycycline UV_VIS_1	Plates (EP) Doxycycline UV_VIS_1
1	STANDARD 1	25.78	1.1875	2.0921	0.0027	BMB*^	13793
2	STANDARD 2	25.727	2.5616	4.6471	0.0056	BMB*^	15097
3	STANDARD 3	25.673	6.7323	12.8171	0.0144	BMB*^	15959
4	STANDARD 4	25.647	11.3428	22.005	0.0242	BMB*^	16299
5	STANDARD 5	25.607	18.7841	36.8376	0.04	BMB*^	16363
6	1A	25.653	7.1962	13.2648	0.0154	BMB*^	15436
7	1B	25.647	7.0883	13.0933	0.0152	BMB*^	15375
8	2A	25.633	9.2928	17.2018	0.0199	BMB*^	15518
9	2B	25.62	9.353	17.1836	0.02	BMB*^	15353
10	3A	25.62	8.5955	15.6536	0.0184	BMB*^	15249
11	3B	25.613	8.5583	15.5625	0.0183	BMB*^	15168
12	4A	25.627	8.3422	15.1125	0.0179	BMB*^	15163
13	4B	25.613	8.2845	15.0544	0.0177	BMB*^	15241
14	5A	25.62	7.1798	12.9804	0.0154	BMB*^	15145
15	5B	25.62	7.2067	12.9474	0.0154	BMB*^	14921
16	STANDARD 1	25.647	1.3157	2.3741	0.003	BMB*^	14450
17	STANDARD 2	25.647	2.6096	4.9304	0.0057	BMB*^	15588
18	STANDARD 3	25.6	6.8277	13.2103	0.0146	BMB*^	16240
19	STANDARD 4	25.587	11.2482	22.2326	0.024	BMB*^	16666
20	STANDARD 5	25.567	18.6994	36.8881	0.0398	BMB*^	16440
21	6A	25.613	8.3919	15.673	0.018	BMB*^	15677
22	6B	25.62	8.3507	15.3928	0.0179	BMB*^	15470
23	7A	25.62	7.1735	13.472	0.0154	BMB*^	15772
24	7B	25.627	7.2993	13.6663	0.0156	BMB*^	15857
25	8A	25.62	7.3894	13.473	0.0158	BMB*^	15364
26	8B	25.64	7.4174	13.3342	0.0159	BMB*^	15036
27	9A	25.633	7.9247	14.8614	0.017	BMB*^	15668
28	9B	25.627	7.9885	15.0034	0.0171	BMB*^	15725
29	10A	25.627	9.0494	17.0969	0.0194	BMB*^	15813
30	10B	25.633	9.151	17.1633	0.0196	BMB*^	15712
Average:		25.634	8.0847	15.1742	0.0173		15519
Rel.Std.Dev:		0.15%	46.89%	49.61%	46.42%		3.80%

7. Doxycycline analysis from Hovione container (14-08-2012)

Table: C7

Sample No.	Sample Name	Ret.Time min Doxycycline UV_VIS_1	Area mAU*min Doxycycline UV_VIS_1	Height mAU Doxycycline UV_VIS_1	Amount Doxycycline UV_VIS_1	Type Doxycycline UV_VIS_1	Plates (EP) Doxycycline UV_VIS_1
1	STANDARD 1	33.727	1.2181	1.1751	0.0029	BMB*^	9395
2	STANDARD 2	33.607	2.3403	2.5543	0.0068	BMB*^	10467
3	STANDARD 3	33.58	3.8203	4.2621	0.0118	BMB*^	10974
4	STANDARD 4	33.473	9.0116	11.0653	0.0295	BMB*^	12448
5	STANDARD 5	33.433	12.3327	14.9159	0.0408	BMB*^	12204
6	1	33.467	7.3525	8.85	0.0238	BMB*^	11958
7	2	33.427	7.3289	8.742	0.0238	BMB*^	11648
8	3	33.46	7.2348	8.704	0.0234	BMB*^	12015
9	STANDARD 1	33.5	1.1848	1.2543	0.0028	BMB*^	10055
10	STANDARD 2	33.46	2.2734	2.5415	0.0065	BMB*^	10448
11	STANDARD 3	33.473	3.6331	4.0251	0.0112	BMB*^	10742
12	STANDARD 4	33.38	8.8774	10.7015	0.029	BMB*^	11792
13	STANDARD 5	33.38	11.8574	13.9789	0.0392	BMB*^	11546
14	4	33.38	11.7482	13.9512	0.0388	BMB*^	11525
15	5	33.367	11.8507	13.8848	0.0391	BMB*^	11376
16	6	33.38	11.8537	13.7976	0.0392	BMB*^	11272
17	STANDARD 1	33.553	1.1725	1.204	0.0028	BMB*^	9250
18	STANDARD 2	33.42	2.2719	2.4492	0.0065	BMB*^	10136
19	STANDARD 3	33.453	3.3818	3.6067	0.0103	BMB*^	9683
20	STANDARD 4	33.4	8.7296	9.9906	0.0285	BMB*^	10987
21	STANDARD 5	33.413	11.2065	12.7889	0.037	BMB*^	10770
22	7	33.473	5.4567	6.032	0.0174	BMB*^	10420
23	8	33.433	5.463	6.0459	0.0174	BMB*^	10485
24	9	33.48	5.5087	6.0058	0.0176	BMB*^	10120
Average:		33.463	6.5462	7.6053	0.0211		10905
Rel.Std.Dev:		0.25%	59.96%	62.41%	63.37%		8.16%

8. Doxycycline analysys Hovione container (2nd time) (16-08-2012)

Table: C8

Sample No.	Sample Name	Ret.Time min Doxycycline UV_VIS_1	Area mAU*min Doxycycline UV_VIS_1	Height mAU Doxycycline UV_VIS_1	Amount Doxycycline UV_VIS_1	Type Doxycycline UV_VIS_1	Plates (EP) Doxycycline UV_VIS_1
1	STANDARD 1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	STANDARD 2	25.733	2.0616	3.2428	0.0055	BMB*^	12849
3	STANDARD 3	25.667	5.9608	10.4848	0.0146	BMB*^	14726
4	STANDARD 4	25.633	9.4985	17.0181	0.0228	BMB*^	15120
5	STANDARD 5	25.6	17.3604	32.181	0.041	BMB*^	15693
6	1	25.62	9.142	17.7128	0.0219	BMB*^	16710
7	2	25.633	9.2693	18.006	0.0222	BMB*^	16787
8	3	25.627	9.277	18.1508	0.0223	BMB*^	16972
9	4	25.613	10.2571	20.0466	0.0245	BMB*^	16606
10	5	25.607	10.3986	20.0007	0.0249	BMB*^	16410
11	STANDARD 1	25.64	1.2857	2.3708	0.0037	BMB*^	15388
12	STANDARD 2	25.647	2.0306	3.7739	0.0055	BMB*^	15459
13	STANDARD 3	25.607	6.1709	11.7231	0.0151	BMB*^	16248
14	STANDARD 4	25.593	9.5169	18.1519	0.0228	BMB*^	16288
15	STANDARD 5	25.573	17.3037	33.3072	0.0409	BMB*^	16344
16	6	25.593	10.1771	19.5074	0.0243	BMB*^	16323
17	7	25.6	8.7867	16.6182	0.0211	BMB*^	16171
18	8	25.6	8.649	16.5605	0.0208	BMB*^	16320
19	9	25.6	8.7056	16.5946	0.0209	BMB*^	16262
20	10	25.593	9.0846	17.2868	0.0218	BMB*^	16265
21	STANDARD 1	25.653	1.2416	2.2475	0.0036	BMB*^	15288
22	STANDARD 2	25.627	2.014	3.7646	0.0054	BMB*^	15813
23	STANDARD 3	25.613	6.1217	11.6476	0.0149	BMB*^	16245
24	STANDARD 4	25.6	9.29	17.8688	0.0223	BMB*^	16343
25	STANDARD 5	25.573	16.9997	33.1126	0.0402	BMB*^	16483
26	11	25.607	9.0063	17.2911	0.0216	BMB*^	16410
27	12	25.613	8.9442	17.2773	0.0215	BMB*^	16594
28	13	25.613	8.5076	16.3428	0.0205	BMB*^	16406
29	14	25.613	8.5526	16.3651	0.0206	BMB*^	16418
30	15	25.633	8.5407	16.3948	0.0205	BMB*^	16479
Average:		25.618	8.4191	16.0362	0.0203		16049
Rel.Std.Dev:		0.12%	49.71%	50.42%	47.84%		5.04%

9. Effect of pH on HPLC peaks (23-08-2012)

Table: C9

Sample No.	Sample Name	Ret.Time min Doxycycline UV_VIS_1	Area mAU*min Doxycycline UV_VIS_1	Height mAU Doxycycline UV_VIS_1	Amount Doxycycline UV_VIS_1	Type Doxycycline UV_VIS_1	Plates (EP) Doxycycline UV_VIS_1
1	STANDARD 1	25.367	0.3991	0.4974	0.0023	BMB*^	9575
2	STANDARD 2	25.327	2.1852	3.7691	0.0063	BMB*^	14129
3	STANDARD 3	25.267	5.8632	10.6959	0.0147	BMB*^	15005
4	STANDARD 4	25.24	10.2652	19.2099	0.0247	BMB*^	15555
5	STANDARD 5	25.193	17.0402	32.216	0.0401	BMB*^	15683
6	1	25.22	7.0621	12.6878	0.0174	BMB*^	14990
7	2	25.213	6.9519	12.5645	0.0171	BMB*^	14993
8	3	25.193	9.4292	17.5558	0.0228	BMB*^	15595
9	4	25.187	9.3877	17.5797	0.0227	BMB*^	15719
10	5	25.18	8.9232	16.6882	0.0216	BMB*^	15601
11	6	25.173	8.8951	16.6526	0.0216	BMB*^	15615
12	7	25.18	8.4387	15.8609	0.0205	BMB*^	15623
13	8	25.18	8.3029	15.7533	0.0202	BMB*^	15733
14	STANDARD 1	25.227	0.3262	0.432	0.0021	BMB*^	11613
15	STANDARD 2	25.207	2.1535	3.8993	0.0062	BMB*^	14721
16	STANDARD 3	25.173	5.9236	11.139	0.0148	BMB*^	15582
17	STANDARD 4	25.147	10.1203	19.3464	0.0243	BMB*^	15769
18	STANDARD 5	25.133	16.8371	32.1752	0.0396	BMB*^	15865
19	9	25.16	5.8838	10.7408	0.0147	BMB*^	15242
20	10	25.16	5.8569	10.6897	0.0146	BMB*^	15137
21	11	25.153	8.568	16.0528	0.0208	BMB*^	15513
22	12	25.167	8.4963	15.9712	0.0206	BMB*^	15552
23	13	25.173	8.7932	16.7201	0.0213	BMB*^	15803
24	14	25.167	8.8467	16.6359	0.0214	BMB*^	15694
25	15	25.167	8.7744	16.7252	0.0213	BMB*^	15873
26	16	25.173	8.7391	16.6929	0.0212	BMB*^	15781
27	STANDARD 1	25.333	0.3032	0.4128	0.002	BMB*^	13242
28	STANDARD 2	25.22	2.0621	3.7899	0.006	BMB*^	14726
29	STANDARD 3	25.2	5.8047	10.9472	0.0145	BMB*^	15670
30	STANDARD 4	25.18	10.0775	19.2066	0.0242	BMB*^	15689
31	STANDARD 5	25.18	16.7072	31.9345	0.0393	BMB*^	15867
32	17	25.24	4.2484	7.7277	0.011	BMB*^	14699
33	18	25.247	4.2339	7.6026	0.011	BMB*^	14737
34	19	25.22	9.3328	18.3439	0.0225	BMB*^	16617
35	20	25.227	9.2355	18.4166	0.0223	BMB*^	16795
36	21	25.24	5.5878	10.7023	0.014	BMB*^	16137
37	22	25.253	5.5953	10.5282	0.0141	BMB*^	15725
Average:		25.207	7.45	14.0152	0.0183		15186
Rel.Std.Dev:		0.21%	54.54%	55.87%	50.53%		8.59%

10. Effect of manual pH readjustment to 2.5 (29-12-2012)

Table: C10

Sample No.	Sample Name	Ret.Time min Doxycycline UV_VIS_1	Area mAU*min Doxycycline UV_VIS_1	Height mAU Doxycycline UV_VIS_1	Amount Doxycycline UV_VIS_1	Type Doxycycline UV_VIS_1	Plates (EP) Doxycycline UV_VIS_1
1	standard1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	standard2	25.4	1.8041	3.152	0.0051	BMB*^	15195
3	standard3	25.347	6.2453	12.066	0.0154	BMB*^	16273
4	standard4	25.273	10.7589	21.1729	0.0257	BMB*^	16639
5	standard5	25.253	16.4122	31.9995	0.0387	BMB*^	16422
6	1	25.247	8.9892	17.7458	0.0217	BMB*^	16628
7	2	25.24	9.0031	17.854	0.0217	BMB*^	16643
8	3	25.227	9.0574	18.0162	0.0218	BMB*^	16771
9	4	25.227	7.9753	15.5928	0.0193	BMB*^	16482
10	5	25.22	7.9999	15.517	0.0194	BMB*^	16414
11	6	25.22	7.8527	15.3946	0.0191	BMB*^	16497
12	7	25.213	9.1086	18.0353	0.0219	BMB*^	16536
13	standard1	25.247	0.4114	0.6852	0.0019	BMB*^	15369
14	standard2	25.233	1.6496	3.0393	0.0048	BMB*^	15374
15	standard3	25.213	6.2548	12.3886	0.0154	BMB*^	16741
16	standard4	25.187	11.0299	21.8271	0.0264	BMB*^	16501
17	standard5	25.187	16.4836	32.6695	0.0389	BMB*^	16597
18	8	25.207	9.0275	17.9093	0.0218	BMB*^	16696
19	9	25.207	9.0591	17.9152	0.0218	BMB*^	16659
20	10	25.213	8.9494	17.6802	0.0216	BMB*^	16560
21	11	25.22	8.8234	17.528	0.0213	BMB*^	16569
22	12	25.22	8.9251	17.5869	0.0215	BMB*^	16414
23	13	25.24	8.2374	16.1839	0.0199	BMB*^	16511
24	14	25.253	8.1516	16.0727	0.0197	BMB*^	16600
25	standard1	25.34	0.3619	0.5317	0.0018	BMB*^	14607
26	standard2	25.28	1.5319	2.8338	0.0045	BMB*^	15780
27	standard3	25.267	6.1969	12.0745	0.0152	BMB*^	16439
28	standard4	25.273	10.9325	21.6781	0.0261	BMB*^	16639
29	standard5	25.267	16.174	31.9998	0.0382	BMB*^	16558
30	15	25.313	8.0764	15.9143	0.0196	BMB*^	16631
31	16	25.313	7.4108	14.5803	0.018	BMB*^	16679
32	17	25.333	7.4898	14.6061	0.0182	BMB*^	16479
33	18	25.347	7.3901	14.4656	0.018	BMB*^	16699
34	19	25.367	8.9149	17.407	0.0215	BMB*^	16498
35	20	25.387	8.7048	17.2826	0.021	BMB*^	16728
36	21	25.407	8.6814	17.2366	0.021	BMB*^	16730
Average:		25.268	8.1164	15.9612	0.0197		16387
Rel. Std. Dev:		0.25%	46.88%	47.43%	44.50%		3.05%

Data of HPLC runs in stability studies (Main experiment)

1. Batch 1 week 0: (27-03-2013)

Table: D1

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	19.627	1.8611	1.1557	0.0045	BMB*^	897
2	standard2	19.567	3.6055	2.2397	0.0087	BMB*^	921
3	standard3	19.587	9.6564	5.6441	0.0232	BMB*^	841
4	standard4	19.447	15.773	9.3454	0.0379	BMB*^	855
5	standard5	19.453	27.3147	16.206	0.0656	BMB*^	863
6	1	19.427	8.4172	5.0816	0.0202	BMB*^	878
7	2	19.387	8.3652	4.9928	0.0201	BMB*^	859
8	3	19.387	8.2741	4.9692	0.0199	BMB*^	861
9	4	19.367	8.4993	5.3283	0.0204	BMB*^	899
10	5	19.293	8.4183	5.3033	0.0202	BMB*^	910
11	6	19.273	8.5799	5.3296	0.0206	BMB*^	884
12	7	19.26	8.7125	5.3775	0.0209	BMB*^	885
13	8	19.287	8.5867	5.3085	0.0206	BMB*^	896
14	9	19.273	8.383	5.2753	0.0201	BMB*^	891
15	standard1	19.32	1.7377	1.1302	0.0042	BMB*^	1004
16	standard2	19.313	3.6486	2.2717	0.0088	BMB*^	905
17	standard3	19.247	9.1183	5.4275	0.0219	BMB*^	848
18	standard4	19.207	15.8129	9.3401	0.038	BMB*^	844
19	standard5	19.207	26.9257	16.3697	0.0647	BMB*^	878
20	10	19.187	7.9409	4.793	0.0191	BMB*^	863
21	11	19.267	7.8684	4.7394	0.0189	BMB*^	869
22	12	19.26	8.0669	4.7945	0.0194	BMB*^	866
23	13	19.207	8.4177	5.4357	0.0202	BMB*^	934
24	14	19.193	8.4698	5.4318	0.0203	BMB*^	924
25	15	19.2	8.561	5.472	0.0206	BMB*^	925
26	16	19.147	8.4335	5.3839	0.0203	BMB*^	907
27	17	19.12	8.4001	5.3701	0.0202	BMB*^	914
28	18	19.113	8.5446	5.43	0.0205	BMB*^	907
29	standard1	19.04	1.6893	1.0578	0.0041	BMB*^	886
30	standard2	19.153	3.6434	2.2956	0.0088	BMB*^	908
31	standard3	19.127	9.0584	5.4955	0.0218	BMB*^	859
32	standard4	19.107	15.7678	9.5555	0.0379	BMB*^	862
33	standard5	19.08	26.5313	16.4159	0.0637	BMB*^	886
34	19	19.087	8.4887	5.3641	0.0204	BMB*^	901
35	20	19.047	8.1958	5.2947	0.0197	BMB*^	918
36	21	19.067	8.4806	5.3754	0.0204	BMB*^	904
37	22	19.067	8.5434	5.4345	0.0205	BMB*^	906
38	23	19.08	8.3024	5.3846	0.0199	BMB*^	929
39	24	19.047	8.2513	5.3422	0.0198	BMB*^	926
40	25	19.007	8.66	5.3641	0.0208	BMB*^	884
41	26	19.06	8.6342	5.3633	0.0207	BMB*^	891
42	27	18.993	8.3115	5.3131	0.02	BMB*^	897
Average:		19.228	9.4988	5.8572	0.0228		893
Rel.Std.Dev:		0.84%	60.33%	58.68%	60.33%		3.43%

2. Batch 1 week 1 : (03-04-2013)

Table: D2

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	19.507	1.7055	0.9907	0.0039	BMB*^	812
2	standard2	19.527	3.0316	1.8844	0.0069	BMB*^	880
3	standard3	19.553	9.3686	5.4701	0.0214	BMB*^	831
4	standard4	19.473	15.7235	9.171	0.0358	BMB*^	827
5	standard5	19.473	26.3774	15.3589	0.0601	BMB*^	831
6	1	19.487	8.0374	4.7169	0.0183	BMB*^	834
7	2	19.48	8.131	4.7372	0.0185	BMB*^	830
8	3	19.44	7.946	4.7132	0.0181	BMB*^	838
9	4	19.387	8.784	5.3129	0.02	BMB*^	855
10	5	19.333	8.7957	5.3102	0.0201	BMB*^	857
11	6	19.333	8.8087	5.3358	0.0201	BMB*^	855
12	7	19.333	8.832	5.269	0.0201	BMB*^	843
13	8	19.34	8.7739	5.2818	0.02	BMB*^	851
14	9	19.387	8.822	5.2969	0.0201	BMB*^	852
15	standard1	19.327	1.4578	0.915	0.0033	BMB*^	927
16	standard2	19.313	3.2589	1.9461	0.0074	BMB*^	849
17	standard3	19.32	9.1731	5.469	0.0209	BMB*^	845
18	standard4	19.327	15.5961	9.2705	0.0356	BMB*^	840
19	standard5	19.287	26.3144	15.5026	0.06	BMB*^	835
20	10	19.347	8.2467	4.9073	0.0188	BMB*^	842
21	11	19.287	8.4583	4.9676	0.0193	BMB*^	823
22	12	19.32	8.4387	4.9578	0.0192	BMB*^	826
23	13	19.313	8.5376	5.0392	0.0195	BMB*^	831
24	14	19.32	8.4775	5.0253	0.0193	BMB*^	845
25	15	19.253	8.4145	5.0241	0.0192	BMB*^	836
26	16	19.22	8.9704	5.4315	0.0205	BMB*^	851
27	17	19.26	8.973	5.415	0.0205	BMB*^	855
28	18	19.227	8.969	5.4331	0.0204	BMB*^	854
29	standard1	19.253	1.3905	0.9428	0.0032	BMB*^	962
30	standard2	19.193	3.285	1.9895	0.0075	BMB*^	833
31	standard3	19.287	9.494	5.5807	0.0216	BMB*^	835
32	standard4	19.22	15.8884	9.3635	0.0362	BMB*^	829
33	standard5	19.213	26.5504	15.6276	0.0605	BMB*^	834
34	19	19.227	8.7979	5.379	0.0201	BMB*^	865
35	20	19.253	8.8027	5.3814	0.0201	BMB*^	858
36	21	19.2	8.8159	5.3873	0.0201	BMB*^	855
37	22	19.193	8.8007	5.3454	0.0201	BMB*^	859
38	23	19.227	8.7085	5.3427	0.0199	BMB*^	865
39	24	19.2	8.7267	5.3668	0.0199	BMB*^	869
40	25	19.167	8.7222	5.387	0.0199	BMB*^	868
41	26	19.167	8.8313	5.4225	0.0201	BMB*^	858
42	27	19.193	8.7708	5.3988	0.02	BMB*^	875
Average:		19.313	9.5716	5.7159	0.0218		850
Rel.Std.Dev:		0.55%	59.00%	57.60%	59.00%		3.10%

3. Batch 1 week2: (10-04-2013)

Table: D3

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	18.4	1.5073	0.9193	0.0034	BMB*^	777
2	standard2	18.373	3.8389	2.2692	0.0088	BMB*^	736
3	standard3	18.333	9.884	5.6693	0.0226	BMB*^	699
4	standard4	18.3	16.7547	9.5793	0.0383	BMB*^	695
5	standard5	18.24	28.6224	16.1822	0.0654	BMB*^	690
6	1	18.213	8.4142	4.9103	0.0192	BMB*^	715
7	2	18.213	8.7376	4.9577	0.02	BMB*^	685
8	3	18.233	8.7134	4.9677	0.0199	BMB*^	693
9	4	18.147	9.1472	5.2509	0.0209	BMB*^	695
10	5	18.193	9.1406	5.2618	0.0209	BMB*^	698
11	6	18.14	9.1594	5.2811	0.0209	BMB*^	697
12	7	18.407	8.8917	5.2086	0.0203	BMB*^	716
13	8	18.393	9.0724	5.2572	0.0207	BMB*^	712
14	9	18.373	9.0034	5.2704	0.0206	BMB*^	716
15	standard1	18.393	1.566	0.9364	0.0036	BMB*^	747
16	standard2	18.32	3.5683	2.1034	0.0082	BMB*^	711
17	standard3	18.287	10.0491	5.7024	0.023	BMB*^	693
18	standard4	18.307	16.6907	9.5279	0.0382	BMB*^	704
19	standard5	18.233	28.0874	16.0383	0.0642	BMB*^	704
20	10	18.32	8.4739	4.7405	0.0194	BMB*^	697
21	11	18.22	8.414	4.7517	0.0192	BMB*^	693
22	12	18.227	8.3075	4.7164	0.019	BMB*^	700
23	13	18.24	8.6762	4.9585	0.0198	BMB*^	698
24	14	18.26	8.7074	4.9704	0.0199	BMB*^	699
25	15	18.287	8.612	4.9545	0.0197	BMB*^	709
26	16	18.213	9.155	5.3531	0.0209	BMB*^	711
27	17	18.247	9.1483	5.3603	0.0209	BMB*^	721
28	18	18.213	9.0709	5.3497	0.0207	BMB*^	716
29	standard1	18.267	1.541	0.9346	0.0035	BMB*^	759
30	standard2	18.233	3.5086	2.0853	0.008	BMB*^	731
31	standard3	18.247	9.8128	5.6816	0.0224	BMB*^	711
32	standard4	18.213	16.4699	9.5056	0.0377	BMB*^	700
33	standard5	18.207	28.0015	16.0265	0.064	BMB*^	703
34	19	18.233	8.6639	4.9786	0.0198	BMB*^	708
35	20	18.207	8.5004	4.9415	0.0194	BMB*^	710
36	21	18.18	8.6215	4.9472	0.0197	BMB*^	696
37	22	18.227	8.9342	5.0733	0.0204	BMB*^	705
38	23	18.193	8.9535	5.0883	0.0205	BMB*^	697
39	24	18.193	8.8435	5.0597	0.0202	BMB*^	707
40	25	18.187	9.0918	5.1367	0.0208	BMB*^	693
41	26	18.18	9.0136	5.1026	0.0206	BMB*^	700
42	27	18.187	9.0188	5.1133	0.0206	BMB*^	707
Average:		18.254	9.9616	5.7172	0.0228		708
Rel.Std.Dev:		0.39%	60.82%	60.17%	60.82%		2.60%

4. Batch 1 week 3: (17-04-2013)

Table: D4

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	18.687	1.6765	0.9528	0.0042	BMB*^	668
2	standard2	18.647	3.3559	1.9082	0.0083	BMB*^	690
3	standard3	18.613	8.698	4.8054	0.0216	BMB*^	656
4	standard4	18.54	15.0236	8.0191	0.0372	BMB*^	621
5	standard5	18.5	25.6472	13.9788	0.0636	BMB*^	630
6	1	18.52	6.851	3.7913	0.017	BMB*^	658
7	2	18.46	6.7964	3.7904	0.0168	BMB*^	641
8	3	18.427	7.0655	3.9074	0.0175	BMB*^	643
9	4	18.413	8.4054	4.619	0.0208	BMB*^	644
10	5	18.347	8.3886	4.4489	0.0208	BMB*^	617
11	6	18.367	8.2241	4.6282	0.0204	BMB*^	629
12	7	18.387	8.3043	4.5923	0.0206	BMB*^	647
13	8	18.307	8.225	4.5563	0.0204	BMB*^	642
14	9	18.267	8.2095	4.5868	0.0204	BMB*^	653
15	standard1	18.427	1.4694	0.8457	0.0036	BMB*^	674
16	standard2	18.467	3.1822	1.7418	0.0079	BMB*^	643
17	standard3	18.3	9.057	4.9702	0.0225	BMB*^	634
18	standard4	18.327	14.8689	8.108	0.0369	BMB*^	634
19	standard5	18.26	25.0568	14.0039	0.0621	BMB*^	651
20	10	18.307	6.9638	3.7545	0.0173	BMB*^	627
21	11	18.3	7.1281	3.7906	0.0177	BMB*^	610
22	12	18.327	6.9971	3.7738	0.0173	BMB*^	623
23	13	18.26	8.2674	4.5059	0.0205	BMB*^	637
24	14	18.26	8.1882	4.4835	0.0203	BMB*^	630
25	15	18.293	8.2583	4.5292	0.0205	BMB*^	645
26	16	18.273	8.2536	4.7295	0.0205	BMB*^	669
27	17	18.193	8.2726	4.7549	0.0205	BMB*^	668
28	18	18.173	8.4269	4.7924	0.0209	BMB*^	665
29	standard1	18.307	1.2872	0.7529	0.0032	BMB*^	652
30	standard2	18.28	3.0861	1.7659	0.0077	BMB*^	665
31	standard3	18.28	8.9754	4.9597	0.0223	BMB*^	647
32	standard4	18.233	15.1191	8.2747	0.0375	BMB*^	640
33	standard5	18.213	25.7707	14.1959	0.0639	BMB*^	645
34	19	18.673	8.4455	4.5126	0.0209	BMB*^	623
35	20	18.593	8.3742	4.547	0.0208	BMB*^	622
36	21	18.56	8.2539	4.6927	0.0205	BMB*^	646
37	22	18.573	8.274	4.5023	0.0205	BMB*^	618
38	23	18.5	8.0836	4.5209	0.02	BMB*^	650
39	24	18.513	8.1999	4.5231	0.0203	BMB*^	633
40	25	18.56	8.2659	4.5627	0.0205	BMB*^	644
41	26	18.487	8.3088	4.5808	0.0206	BMB*^	638
42	27	18.427	8.4705	4.6366	0.021	BMB*^	634
Average:		18.401	9.0042	4.9618	0.0223		643
Rel.Std.Dev:		0.77%	60.85%	60.58%	60.85%		2.68%

5. Batch 1 week 4: (24-04-2013)

Table: D5

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	19.067	1.4431	0.7127	0.0037	BMB*^	485
2	standard2	19.207	2.653	1.1355	0.0068	BMB*^	391
3	standard3	19.213	8.0822	3.3061	0.0206	BMB*^	367
4	standard4	19.08	13.4451	5.4628	0.0343	BMB*^	357
5	standard5	18.987	24.1501	9.9167	0.0616	BMB*^	361
6	1	18.933	7.46	3.1105	0.019	BMB*^	365
7	2	19.08	7.4906	3.0684	0.0191	BMB*^	357
8	3	18.947	7.3078	3.0591	0.0186	BMB*^	363
9	4	18.887	8.0904	3.4806	0.0206	BMB*^	377
10	5	18.827	8.0666	3.4814	0.0206	BMB*^	380
11	6	18.787	8.0651	3.4882	0.0206	BMB*^	378
12	7	18.86	8.1207	3.4908	0.0207	BMB*^	378
13	8	18.733	8.075	3.501	0.0206	BMB*^	377
14	9	18.867	7.9412	3.432	0.0203	BMB*^	376
15	standard1	18.94	1.2893	0.6096	0.0033	BMB*^	441
16	standard2	18.933	2.7608	1.1591	0.007	BMB*^	360
17	standard3	18.733	7.9848	3.3343	0.0204	BMB*^	358
18	standard4	18.733	13.3151	5.4962	0.034	BMB*^	356
19	standard5	18.707	24.2964	10.0388	0.062	BMB*^	357
20	10	18.893	7.7847	3.2246	0.0199	BMB*^	358
21	11	18.68	7.9033	3.2487	0.0202	BMB*^	348
22	12	18.693	7.883	3.2409	0.0201	BMB*^	351
23	13	18.7	8.1336	3.3862	0.0207	BMB*^	356
24	14	18.647	8.0431	3.3634	0.0205	BMB*^	357
25	15	18.6	7.8458	3.3235	0.02	BMB*^	363
26	16	18.633	8.0197	3.5056	0.0205	BMB*^	375
27	17	18.687	7.9359	3.4944	0.0202	BMB*^	384
28	18	18.62	7.9167	3.4961	0.0202	BMB*^	378
29	standard1	18.587	1.7833	0.7749	0.0045	BMB*^	344
30	standard2	18.607	2.8856	1.2084	0.0074	BMB*^	347
31	standard3	18.673	7.9527	3.3371	0.0203	BMB*^	360
32	standard4	18.587	13.2467	5.4973	0.0338	BMB*^	353
33	standard5	18.6	24.0865	10.1088	0.0614	BMB*^	359
34	19	18.54	8.5821	3.7855	0.0219	BMB*^	373
35	20	18.573	8.4969	3.7844	0.0217	BMB*^	381
36	21	18.44	8.6912	3.8096	0.0222	BMB*^	365
37	22	18.6	8.0394	3.5065	0.0205	BMB*^	377
38	23	18.533	8.1314	3.5126	0.0207	BMB*^	368
39	24	18.56	7.9012	3.4826	0.0202	BMB*^	373
40	25	18.567	7.8315	3.5058	0.02	BMB*^	386
41	26	18.44	7.9408	3.527	0.0203	BMB*^	376
42	27	18.467	8.0648	3.5431	0.0206	BMB*^	372
Average:		18.749	8.6937	3.6893	0.0222		371
Rel.Std.Dev:		1.08%	58.53%	56.63%	58.53%		6.52%

6. Batch 1 week 6: (08-05-2013)

Table: D6

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	19.067	1.4431	0.7127	0.0037	BMB*^	485
2	standard2	19.207	2.653	1.1355	0.0068	BMB*^	391
3	standard3	19.213	8.0822	3.3061	0.0206	BMB*^	367
4	standard4	19.08	13.4451	5.4628	0.0343	BMB*^	357
5	standard5	18.987	24.1501	9.9167	0.0616	BMB*^	361
6	1	18.933	7.46	3.1105	0.019	BMB*^	365
7	2	19.08	7.4906	3.0684	0.0191	BMB*^	357
8	3	18.947	7.3078	3.0591	0.0186	BMB*^	363
9	4	18.887	8.0904	3.4806	0.0206	BMB*^	377
10	5	18.827	8.0666	3.4814	0.0206	BMB*^	380
11	6	18.787	8.0651	3.4882	0.0206	BMB*^	378
12	7	18.86	8.1207	3.4908	0.0207	BMB*^	378
13	8	18.733	8.075	3.501	0.0206	BMB*^	377
14	9	18.867	7.9412	3.432	0.0203	BMB*^	376
15	standard1	18.94	1.2893	0.6096	0.0033	BMB*^	441
16	standard2	18.933	2.7608	1.1591	0.007	BMB*^	360
17	standard3	18.733	7.9848	3.3343	0.0204	BMB*^	358
18	standard4	18.733	13.3151	5.4962	0.034	BMB*^	356
19	standard5	18.707	24.2964	10.0388	0.062	BMB*^	357
20	10	18.893	7.7847	3.2246	0.0199	BMB*^	358
21	11	18.68	7.9033	3.2487	0.0202	BMB*^	348
22	12	18.693	7.883	3.2409	0.0201	BMB*^	351
23	13	18.7	8.1336	3.3862	0.0207	BMB*^	356
24	14	18.647	8.0431	3.3634	0.0205	BMB*^	357
25	15	18.6	7.8458	3.3235	0.02	BMB*^	363
26	16	18.633	8.0197	3.5056	0.0205	BMB*^	375
27	17	18.687	7.9359	3.4944	0.0202	BMB*^	384
28	18	18.62	7.9167	3.4961	0.0202	BMB*^	378
29	standard1	18.587	1.7833	0.7749	0.0045	BMB*^	344
30	standard2	18.607	2.8856	1.2084	0.0074	BMB*^	347
31	standard3	18.673	7.9527	3.3371	0.0203	BMB*^	360
32	standard4	18.587	13.2467	5.4973	0.0338	BMB*^	353
33	standard5	18.6	24.0865	10.1088	0.0614	BMB*^	359
34	19	18.54	8.5821	3.7855	0.0219	BMB*^	373
35	20	18.573	8.4969	3.7844	0.0217	BMB*^	381
36	21	18.44	8.6912	3.8096	0.0222	BMB*^	365
37	22	18.6	8.0394	3.5065	0.0205	BMB*^	377
38	23	18.533	8.1314	3.5126	0.0207	BMB*^	368
39	24	18.56	7.9012	3.4826	0.0202	BMB*^	373
40	25	18.567	7.8315	3.5058	0.02	BMB*^	386
41	26	18.44	7.9408	3.527	0.0203	BMB*^	376
42	27	18.467	8.0648	3.5431	0.0206	BMB*^	372
Average:		18.749	8.6937	3.6893	0.0222		371
Rel.Std.Dev:		1.08%	58.53%	56.63%	58.53%		6.52%

7. Batch 1 week 8: (22-05-2013)

Table: D7

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	16.16	2.115	1.2457	0.0047	BMB*^	549
2	standard2	16.153	4.2529	2.4854	0.0095	BMB*^	565
3	standard3	16.133	10.3999	6.03	0.0231	BMB*^	558
4	standard4	16.04	17.1957	10.0947	0.0382	BMB*^	560
5	standard5	15.98	28.7928	16.8372	0.064	BMB*^	554
6	1	16.033	8.203	4.8081	0.0182	BMB*^	564
7	2	15.953	8.1861	4.8294	0.0182	BMB*^	561
8	3	15.847	8.1434	4.8328	0.0181	BMB*^	558
9	4	15.84	8.8478	5.2428	0.0197	BMB*^	558
10	5	15.813	8.8339	5.2514	0.0196	BMB*^	565
11	6	15.707	8.8723	5.2905	0.0197	BMB*^	559
12	7	15.72	8.9586	5.2809	0.0199	BMB*^	562
13	8	15.753	8.902	5.286	0.0198	BMB*^	565
14	9	15.727	8.8515	5.2692	0.0197	BMB*^	561
15	standard1	15.693	2.1478	1.3148	0.0048	BMB*^	593
16	standard2	15.673	4.1422	2.5606	0.0092	BMB*^	581
17	standard3	15.593	10.3052	6.2553	0.0229	BMB*^	564
18	standard4	15.627	17.4081	10.4266	0.0387	BMB*^	562
19	standard5	15.607	28.6768	17.3585	0.0637	BMB*^	566
20	10	15.56	8.2113	4.9784	0.0182	BMB*^	563
21	11	15.533	8.1403	4.9497	0.0181	BMB*^	559
22	12	15.58	8.2089	4.9783	0.0182	BMB*^	563
23	13	15.547	7.5177	4.6023	0.0167	BMB*^	568
24	14	15.493	7.4598	4.5834	0.0166	BMB*^	573
25	15	15.48	7.4786	4.6031	0.0166	BMB*^	568
26	16	15.467	9.0916	5.545	0.0202	BMB*^	568
27	17	15.46	9.0623	5.5578	0.0201	BMB*^	567
28	18	15.42	9.0615	5.5712	0.0201	BMB*^	565
29	standard1	15.447	2.0589	1.3104	0.0046	BMB*^	608
30	standard2	15.387	4.2848	2.6504	0.0095	BMB*^	566
31	standard3	15.36	10.409	6.4143	0.0231	BMB*^	570
32	standard4	15.333	17.3294	10.6605	0.0385	BMB*^	570
33	standard5	15.293	28.8055	17.81	0.064	BMB*^	567
34	19	15.307	9.1258	5.665	0.0203	BMB*^	571
35	20	15.3	9.0555	5.6542	0.0201	BMB*^	576
36	21	15.3	9.1707	5.6889	0.0204	BMB*^	576
37	22	15.24	8.9819	5.5635	0.02	BMB*^	567
38	23	15.247	8.992	5.5864	0.02	BMB*^	573
39	24	15.2	9.0088	5.6105	0.02	BMB*^	565
40	25	15.193	9.1257	5.7288	0.0203	BMB*^	577
41	26	15.173	9.1496	5.7132	0.0203	BMB*^	571
42	27	15.107	9.0783	5.7368	0.0202	BMB*^	576
Average:		15.583	10.0486	6.092	0.0223		567
Rel.Std.Dev:		1.86%	61.38%	60.88%	61.38%		1.77%

8. Batch 1 week 10: (05-06-2013)

Table: D8

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	16.473	2.0089	1.247	0.0043	BMB*^	654
2	standard2	16.333	4.0262	2.4518	0.0087	BMB*^	643
3	standard3	16.28	9.7654	5.8499	0.0211	BMB*^	620
4	standard4	16.26	16.6884	10.1041	0.0361	BMB*^	630
5	standard5	16.193	27.7702	16.8056	0.06	BMB*^	626
6	1	16.187	9.3361	5.7227	0.0202	BMB*^	633
7	2	16.14	9.5662	5.8025	0.0207	BMB*^	620
8	3	16.1	9.575	5.8181	0.0207	BMB*^	621
9	4	16.033	9.595	5.8734	0.0207	BMB*^	622
10	5	16.013	9.6115	5.871	0.0208	BMB*^	626
11	6	15.953	9.4978	5.8678	0.0205	BMB*^	623
12	7	15.967	9.4114	5.888	0.0203	BMB*^	635
13	8	15.933	9.5289	5.9315	0.0206	BMB*^	635
14	9	15.88	9.4735	5.9227	0.0205	BMB*^	631
15	standard1	15.88	2.0645	1.3099	0.0045	BMB*^	672
16	standard2	15.833	3.9704	2.5043	0.0086	BMB*^	632
17	standard3	15.853	9.5702	5.9312	0.0207	BMB*^	630
18	standard4	15.827	16.7473	10.3679	0.0362	BMB*^	628
19	standard5	15.813	27.6282	17.1451	0.0597	BMB*^	625
20	10	15.827	9.3241	5.7492	0.0202	BMB*^	623
21	11	15.747	9.2771	5.7507	0.0201	BMB*^	620
22	12	15.753	9.2014	5.729	0.0199	BMB*^	627
23	13	15.727	7.7189	4.8949	0.0167	BMB*^	636
24	14	15.693	7.6559	4.8836	0.0166	BMB*^	632
25	15	15.673	7.9956	4.9606	0.0173	BMB*^	624
26	16	15.713	9.4009	5.8655	0.0203	BMB*^	635
27	17	15.627	9.1729	5.8509	0.0198	BMB*^	634
28	18	15.66	9.5101	5.909	0.0206	BMB*^	619
29	standard1	15.66	2.114	1.3403	0.0046	BMB*^	686
30	standard2	15.647	4.0551	2.5732	0.0088	BMB*^	641
31	standard3	15.58	9.5321	5.9799	0.0206	BMB*^	618
32	standard4	15.553	16.7527	10.5251	0.0362	BMB*^	622
33	standard5	15.56	27.7883	17.448	0.0601	BMB*^	624
34	19	15.513	9.559	6.0299	0.0207	BMB*^	628
35	20	15.527	9.5274	6.039	0.0206	BMB*^	628
36	21	15.527	9.573	6.0529	0.0207	BMB*^	627
37	22	15.527	9.4775	6.1124	0.0205	BMB*^	645
38	23	15.487	9.545	6.1265	0.0206	BMB*^	633
39	24	15.46	9.4301	6.1059	0.0204	BMB*^	635
40	25	15.487	9.5882	6.2588	0.0207	BMB*^	647
41	26	15.44	9.6256	6.2716	0.0208	BMB*^	638
42	27	15.373	9.6741	6.3171	0.0209	BMB*^	635
Average:		15.803	10.2699	6.4093	0.0222		632
Rel.Std.Dev:		1.74%	56.78%	56.02%	56.78%		2.11%

9. Batch 1 week 12: (19-06-2013)

Table: D9

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	16.82	1.9075	1.2094	0.004	BMB*^	708
2	standard2	16.847	3.6625	2.2627	0.0077	BMB*^	689
3	standard3	16.72	9.5104	5.8008	0.0199	BMB*^	676
4	standard4	16.747	16.2193	9.8522	0.034	BMB*^	679
5	standard5	16.653	26.8816	16.3115	0.0563	BMB*^	672
6	1	16.647	9.2089	5.6002	0.0193	BMB*^	666
7	2	16.62	9.122	5.5955	0.0191	BMB*^	684
8	3	16.573	9.1178	5.6267	0.0191	BMB*^	680
9	4	16.493	9.5949	5.9746	0.0201	BMB*^	688
10	5	16.46	9.6763	6.0198	0.0203	BMB*^	672
11	6	16.473	9.589	5.9978	0.0201	BMB*^	693
12	7	16.427	9.655	5.8801	0.0202	BMB*^	675
13	8	16.387	9.6588	5.89	0.0202	BMB*^	676
14	9	16.407	9.6515	5.876	0.0202	BMB*^	667
15	standard1	16.4	1.9167	1.24	0.004	BMB*^	707
16	standard2	16.367	3.7418	2.3474	0.0078	BMB*^	680
17	standard3	16.34	9.6466	5.9722	0.0202	BMB*^	664
18	standard4	16.38	16.2102	10.0642	0.034	BMB*^	683
19	standard5	16.333	26.8408	16.6264	0.0563	BMB*^	672
20	10	16.373	8.6524	5.3291	0.0181	BMB*^	667
21	11	16.36	8.7446	5.3558	0.0183	BMB*^	667
22	12	16.347	8.7404	5.362	0.0183	BMB*^	674
23	13	16.313	7.5377	4.7185	0.0158	BMB*^	676
24	14	16.327	7.609	4.7432	0.0159	BMB*^	671
25	15	16.24	7.613	4.7401	0.016	BMB*^	669
26	16	16.307	9.5844	5.9905	0.0201	BMB*^	680
27	17	16.28	9.6553	6.0066	0.0202	BMB*^	672
28	18	16.28	9.6311	6.0091	0.0202	BMB*^	669
29	standard1	16.253	1.9751	1.2677	0.0041	BMB*^	698
30	standard2	16.307	3.7739	2.3497	0.0079	BMB*^	675
31	standard3	16.227	9.6417	6.0107	0.0202	BMB*^	680
32	standard4	16.227	16.0338	10.0986	0.0336	BMB*^	682
33	standard5	16.187	26.6833	16.6723	0.0559	BMB*^	676
34	19	16.18	9.6523	6.0585	0.0202	BMB*^	675
35	20	16.24	9.7388	6.0791	0.0204	BMB*^	677
36	21	16.18	9.6547	6.0488	0.0202	BMB*^	670
37	22	16.2	9.6194	5.8639	0.0202	BMB*^	661
38	23	16.16	9.5859	5.8779	0.0201	BMB*^	665
39	24	16.207	9.5245	5.8776	0.02	BMB*^	672
40	25	16.173	9.7478	6.1382	0.0204	BMB*^	677
41	26	16.127	9.7939	6.1548	0.0205	BMB*^	678
42	27	16.12	9.7824	6.1297	0.0205	BMB*^	666
Average:		16.374	10.114	6.2626	0.0212		677
Rel.Std.Dev:		1.16%	55.69%	55.41%	55.69%		1.54%

10. Batch 2 week 0: (24-03-2013)

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11. Batch 2 week 1: (31-03-2013)

Table: D11

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	19.22	1.6286	0.999	0.0038	BMB*^	818
2	standard2	19.12	2.7328	1.6917	0.0063	BMB*^	846
3	standard3	19.127	9.0593	5.2146	0.0209	BMB*^	775
4	standard4	19.08	15.1956	8.7094	0.0351	BMB*^	774
5	standard5	19.027	26.3305	15.2376	0.0608	BMB*^	783
6	1	19.053	7.8548	4.5238	0.0181	BMB*^	778
7	2	19.02	7.9258	4.5465	0.0183	BMB*^	777
8	3	19.047	7.888	4.5478	0.0182	BMB*^	783
9	4	19.013	8.4959	5.0087	0.0196	BMB*^	793
10	5	18.953	8.6201	5.043	0.0199	BMB*^	787
11	6	18.98	8.5886	5.061	0.0198	BMB*^	793
12	7	18.967	8.9295	5.2547	0.0206	BMB*^	794
13	8	18.947	8.7988	5.2608	0.0203	BMB*^	799
14	9	18.913	8.939	5.2793	0.0206	BMB*^	797
15	standard1	18.967	1.3923	0.8454	0.0032	BMB*^	847
16	standard2	18.92	3.1354	1.8617	0.0072	BMB*^	799
17	standard3	18.94	9.0285	5.2073	0.0208	BMB*^	768
18	standard4	18.927	15.6232	8.9692	0.0361	BMB*^	776
19	standard5	18.88	26.2136	15.3721	0.0605	BMB*^	793
20	10	18.913	7.6069	4.534	0.0176	BMB*^	798
21	11	18.86	7.7165	4.5148	0.0178	BMB*^	785
22	12	18.867	7.6704	4.5281	0.0177	BMB*^	791
23	13	18.813	8.3478	4.9635	0.0193	BMB*^	787
24	14	18.853	8.4355	4.9565	0.0195	BMB*^	782
25	15	18.8	8.4903	4.9842	0.0196	BMB*^	788
26	16	18.84	9.0577	5.3447	0.0209	BMB*^	796
27	17	18.787	8.8513	5.2969	0.0204	BMB*^	803
28	18	18.773	8.8159	5.297	0.0203	BMB*^	798
29	standard1	18.78	1.5276	0.9346	0.0035	BMB*^	788
30	standard2	18.78	3.1392	1.9158	0.0072	BMB*^	833
31	standard3	18.773	9.028	5.2623	0.0208	BMB*^	773
32	standard4	18.773	15.2853	9.04	0.0353	BMB*^	788
33	standard5	18.76	26.3206	15.5358	0.0607	BMB*^	793
34	19	18.74	9.2933	5.4898	0.0214	BMB*^	786
35	20	18.747	8.9408	5.4064	0.0206	BMB*^	809
36	21	18.74	9.1333	5.418	0.0211	BMB*^	789
37	22	18.707	8.9095	5.3428	0.0206	BMB*^	797
38	23	18.753	8.876	5.3363	0.0205	BMB*^	804
39	24	18.707	8.8393	5.3205	0.0204	BMB*^	801
40	25	18.707	9.0197	5.4277	0.0208	BMB*^	803
41	26	18.68	8.9393	5.4024	0.0206	BMB*^	810
42	27	18.68	8.9048	5.411	0.0206	BMB*^	810
Average:		18.879	9.465	5.5785	0.0218		795
Rel.Std.Dev:		0.73%	59.41%	58.64%	59.41%		2.17%

12. Batch 2 week 2: (7-04-2013)

Table: D12

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	18.66	1.5676	1.0961	0.0035	BMB*^	1002
2	standard2	18.58	4.0694	2.433	0.0091	BMB*^	790
3	standard3	18.613	9.6695	5.8207	0.0215	BMB*^	806
4	standard4	18.547	16.0512	9.611	0.0358	BMB*^	796
5	standard5	18.533	27.0584	16.3424	0.0603	BMB*^	807
6	1	18.547	7.3016	4.4583	0.0163	BMB*^	809
7	2	18.433	7.2917	4.4951	0.0162	BMB*^	809
8	3	18.473	7.3844	4.4983	0.0165	BMB*^	795
9	4	18.433	8.2452	5.1046	0.0184	BMB*^	819
10	5	18.407	8.3186	5.1269	0.0185	BMB*^	809
11	6	18.46	8.1497	5.0869	0.0182	BMB*^	823
12	7	18.413	8.7768	5.3194	0.0196	BMB*^	791
13	8	18.36	8.732	5.3225	0.0195	BMB*^	806
14	9	18.347	8.6796	5.3395	0.0193	BMB*^	814
15	standard1	18.427	1.6408	1.1046	0.0037	BMB*^	926
16	standard2	18.413	3.7016	2.2957	0.0082	BMB*^	804
17	standard3	18.42	9.0923	5.5866	0.0203	BMB*^	801
18	standard4	18.373	15.7401	9.4206	0.0351	BMB*^	789
19	standard5	18.333	27.3753	16.541	0.061	BMB*^	799
20	10	18.34	7.2361	4.4446	0.0161	BMB*^	805
21	11	18.4	7.06	4.4119	0.0157	BMB*^	817
22	12	18.307	7.1284	4.4214	0.0159	BMB*^	813
23	13	18.333	7.616	4.7161	0.017	BMB*^	806
24	14	18.32	7.6573	4.7066	0.0171	BMB*^	804
25	15	18.313	7.7788	4.7681	0.0173	BMB*^	806
26	16	18.327	8.8649	5.3672	0.0198	BMB*^	799
27	17	18.327	8.7558	5.3679	0.0195	BMB*^	812
28	18	18.253	8.6364	5.3281	0.0192	BMB*^	812
29	standard1	18.293	1.7465	1.1288	0.0039	BMB*^	874
30	standard2	18.213	3.7049	2.3021	0.0083	BMB*^	812
31	standard3	18.28	8.8333	5.507	0.0197	BMB*^	815
32	standard4	18.26	15.7863	9.5192	0.0352	BMB*^	788
33	standard5	18.273	27.1996	16.6145	0.0606	BMB*^	805
34	19	18.227	8.9455	5.524	0.0199	BMB*^	800
35	20	18.24	9.0309	5.5426	0.0201	BMB*^	797
36	21	18.253	9.0113	5.5552	0.0201	BMB*^	808
37	22	18.26	8.5721	5.334	0.0191	BMB*^	806
38	23	18.253	8.6451	5.3646	0.0193	BMB*^	824
39	24	18.24	8.618	5.3148	0.0192	BMB*^	799
40	25	18.293	8.9841	5.4912	0.02	BMB*^	798
41	26	18.253	8.9274	5.4887	0.0199	BMB*^	792
42	27	18.247	9.0116	5.5107	0.0201	BMB*^	797
Average:		18.364	9.4428	5.7794	0.021		814
Rel.Std.Dev:		0.62%	61.83%	60.73%	61.83%		4.62%

13. Batch 2 week 3: (14-04-2013)

Table: D13

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	18.067	1.3338	0.8572	0.0034	BMB*^	783
2	standard2	17.98	3.4623	2.034	0.0088	BMB*^	709
3	standard3	17.96	8.0579	4.7722	0.0205	BMB*^	714
4	standard4	17.9	13.7329	8.1599	0.0349	BMB*^	721
5	standard5	17.86	23.765	14.1683	0.0604	BMB*^	719
6	1	17.86	6.3443	3.6508	0.0161	BMB*^	691
7	2	17.807	6.3573	3.6686	0.0162	BMB*^	687
8	3	17.827	6.2842	3.6477	0.016	BMB*^	697
9	4	17.7	8.1143	4.8653	0.0206	BMB*^	721
10	5	17.713	8.0968	4.8759	0.0206	BMB*^	728
11	6	17.7	7.9832	4.8528	0.0203	BMB*^	730
12	7	17.633	8.1959	4.9737	0.0208	BMB*^	720
13	8	17.64	8.132	4.9632	0.0207	BMB*^	734
14	9	17.587	8.1313	4.9832	0.0207	BMB*^	724
15	standard1	17.72	1.3129	0.7995	0.0033	BMB*^	726
16	standard2	17.58	3.2629	1.9226	0.0083	BMB*^	696
17	standard3	17.613	7.7894	4.6631	0.0198	BMB*^	712
18	standard4	17.553	13.8349	8.3543	0.0352	BMB*^	712
19	standard5	17.547	23.8933	14.4394	0.0608	BMB*^	718
20	10	17.513	5.7297	3.3887	0.0146	BMB*^	694
21	11	17.507	5.7323	3.3797	0.0146	BMB*^	688
22	12	17.5	5.7775	3.4032	0.0147	BMB*^	692
23	13	17.453	7.1903	4.3888	0.0183	BMB*^	728
24	14	17.46	7.1918	4.3566	0.0183	BMB*^	715
25	15	17.46	7.1372	4.3605	0.0181	BMB*^	721
26	16	17.473	8.13	5.018	0.0207	BMB*^	737
27	17	17.413	8.1485	5.012	0.0207	BMB*^	719
28	18	17.393	8.116	5.0197	0.0206	BMB*^	732
29	standard1	17.453	1.2622	0.7926	0.0032	BMB*^	744
30	standard2	17.413	3.142	1.9337	0.008	BMB*^	721
31	standard3	17.413	7.8239	4.7698	0.0199	BMB*^	726
32	standard4	17.4	13.9665	8.5056	0.0355	BMB*^	721
33	standard5	17.36	24.2064	14.627	0.0616	BMB*^	717
34	19	17.32	8.1954	5.1587	0.0208	BMB*^	739
35	20	17.327	8.1987	5.1877	0.0208	BMB*^	745
36	21	17.32	8.1753	5.1408	0.0208	BMB*^	731
37	22	17.333	7.9192	4.9289	0.0201	BMB*^	730
38	23	17.32	7.936	4.8931	0.0202	BMB*^	732
39	24	17.287	7.9425	4.9036	0.0202	BMB*^	725
40	25	17.273	8.1687	5.1068	0.0208	BMB*^	731
41	26	17.24	8.1777	5.128	0.0208	BMB*^	736
42	27	17.213	8.2019	5.1246	0.0209	BMB*^	725
Average:		17.55	8.4418	5.1233	0.0215		721
Rel.Std.Dev:		1.26%	60.95%	60.49%	60.95%		2.47%

14. Batch 2 week 4: (21-04-2013)

Table: D14

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	23.34	1.8117	0.7756	0.0046	BMB*^	644
2	standard2	22.907	3.6586	1.2283	0.0093	BMB*^	345
3	standard3	22.98	8.9482	3.337	0.0227	BMB*^	446
4	standard4	22.927	14.3686	5.3336	0.0365	BMB*^	441
5	standard5	22.827	23.4352	8.6571	0.0596	BMB*^	439
6	1	22.94	7.0655	2.412	0.018	BMB*^	390
7	2	22.893	6.8716	2.3732	0.0175	BMB*^	401
8	3	22.687	7.0396	2.3999	0.0179	BMB*^	383
9	4	22.673	7.4313	2.8811	0.0189	BMB*^	457
10	5	22.673	7.4749	2.8854	0.019	BMB*^	460
11	6	22.74	7.7185	2.9605	0.0196	BMB*^	443
12	7	22.567	8.2363	2.9684	0.0209	BMB*^	416
13	8	22.613	8.19	2.9918	0.0208	BMB*^	411
14	9	22.607	8.0206	2.942	0.0204	BMB*^	435
15	standard1	22.553	1.4806	0.5745	0.0038	BMB*^	465
16	standard2	22.567	3.4018	1.1218	0.0086	BMB*^	326
17	standard3	22.567	8.5772	3.2642	0.0218	BMB*^	439
18	standard4	22.5	14.5762	5.4491	0.037	BMB*^	437
19	standard5	22.593	23.1202	8.7053	0.0588	BMB*^	441
20	10	22.593	5.7195	2.1587	0.0145	BMB*^	443
21	11	22.46	5.7246	2.1646	0.0145	BMB*^	437
22	12	22.567	5.8258	2.1961	0.0148	BMB*^	450
23	13	22.547	5.9736	2.3202	0.0152	BMB*^	457
24	14	22.413	6.1288	2.3283	0.0156	BMB*^	430
25	15	22.473	6.1886	2.4064	0.0157	BMB*^	450
26	16	22.447	7.8928	3.0719	0.0201	BMB*^	459
27	17	22.407	7.9721	3.013	0.0203	BMB*^	437
28	18	22.373	8.0163	3.0149	0.0204	BMB*^	434
29	standard1	22.473	1.3704	0.5607	0.0035	BMB*^	515
30	standard2	22.3	3.2765	1.1265	0.0083	BMB*^	346
31	standard3	22.493	8.6256	3.2581	0.0219	BMB*^	446
32	standard4	22.407	14.3331	5.4143	0.0364	BMB*^	435
33	standard5	22.467	23.3338	8.7495	0.0593	BMB*^	434
34	19	22.347	7.9608	3.0078	0.0202	BMB*^	434
35	20	22.473	8.2053	3.0894	0.0209	BMB*^	433
36	21	22.32	7.8939	2.9904	0.0201	BMB*^	438
37	22	22.407	7.8052	2.9783	0.0198	BMB*^	446
38	23	22.307	7.6478	2.9505	0.0194	BMB*^	446
39	24	22.367	7.7695	2.9757	0.0197	BMB*^	442
40	25	22.347	7.7773	2.9444	0.0198	BMB*^	436
41	26	22.293	7.7515	2.9493	0.0197	BMB*^	430
42	27	22.313	7.6704	2.9346	0.0195	BMB*^	438
Average:		22.565	8.3879	3.1396	0.0213		437
Rel.Std.Dev:		1.00%	60.00%	60.05%	60.00%		10.68%

15. Batch 2 week 6: (05-05-2013)

Table: D15

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	19.02	1.4651	0.8386	0.0037	BMB*^	668
2	standard2	18.813	2.5481	1.3217	0.0064	BMB*^	559
3	standard3	18.753	8.4624	4.068	0.0211	BMB*^	530
4	standard4	18.707	13.8119	6.7027	0.0345	BMB*^	528
5	standard5	18.62	24.8293	12.0968	0.062	BMB*^	527
6	1	18.627	5.9132	2.8966	0.0148	BMB*^	536
7	2	18.6	5.6022	2.796	0.014	BMB*^	540
8	3	18.56	5.4485	2.7438	0.0136	BMB*^	539
9	4	18.507	7.4676	3.6717	0.0186	BMB*^	515
10	5	18.42	7.5307	3.7375	0.0188	BMB*^	537
11	6	18.42	7.3936	3.7276	0.0185	BMB*^	546
12	7	18.373	7.5287	3.7935	0.0188	BMB*^	539
13	8	18.38	7.473	3.7862	0.0187	BMB*^	541
14	9	18.447	7.5358	3.7972	0.0188	BMB*^	543
15	standard1	18.327	1.3524	0.7666	0.0034	BMB*^	639
16	standard2	18.447	3.032	1.5103	0.0076	BMB*^	528
17	standard3	18.413	8.3772	4.1307	0.0209	BMB*^	532
18	standard4	18.38	13.5918	6.7913	0.0339	BMB*^	531
19	standard5	18.32	24.3165	12.241	0.0607	BMB*^	539
20	10	18.453	4.4623	2.2986	0.0111	BMB*^	564
21	11	18.393	4.614	2.3325	0.0115	BMB*^	543
22	12	18.32	4.5557	2.3252	0.0114	BMB*^	539
23	13	18.287	5.6852	2.9767	0.0142	BMB*^	545
24	14	18.36	5.6769	2.9601	0.0142	BMB*^	560
25	15	18.227	5.9395	3.0187	0.0148	BMB*^	535
26	16	18.3	7.6413	3.9042	0.0191	BMB*^	546
27	17	18.193	7.8328	3.9954	0.0196	BMB*^	546
28	18	18.16	8.0898	3.974	0.0202	BMB*^	506
29	standard1	18.12	1.3669	0.7572	0.0034	BMB*^	607
30	standard2	18.227	2.743	1.4158	0.0068	BMB*^	538
31	standard3	18.193	8.0754	4.1624	0.0202	BMB*^	550
32	standard4	18.213	13.7827	6.9403	0.0344	BMB*^	539
33	standard5	18.147	24.6547	12.5123	0.0616	BMB*^	543
34	19	18.14	7.6481	4.0068	0.0191	BMB*^	568
35	20	18.2	7.927	4.0568	0.0198	BMB*^	545
36	21	18.127	7.7793	4.0454	0.0194	BMB*^	561
37	22	18.173	7.4501	3.8993	0.0186	BMB*^	568
38	23	18.107	7.5405	3.9162	0.0188	BMB*^	553
39	24	18.127	7.6096	3.9799	0.019	BMB*^	564
40	25	18.1	7.3427	3.832	0.0183	BMB*^	556
41	26	18.107	7.4603	3.8686	0.0186	BMB*^	556
42	27	18.087	7.4106	3.8651	0.0185	BMB*^	561
Average:		18.355	8.0231	4.0586	0.02		550
Rel.Std.Dev:		1.19%	67.70%	66.30%	67.70%		5.25%

16. Batch 2 week 8: (19-05-2013)

Table: D16

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	16.527	1.641	0.9237	0.0038	BMB*^	518
2	standard2	16.3	3.5141	1.9956	0.008	BMB*^	521
3	standard3	16.34	8.9783	4.8565	0.0205	BMB*^	475
4	standard4	16.213	16.7255	9.0814	0.0382	BMB*^	479
5	standard5	16.173	25.7907	13.8932	0.059	BMB*^	471
6	1	16.1	5.3841	2.8991	0.0123	BMB*^	468
7	2	16.127	5.2961	2.8824	0.0121	BMB*^	464
8	3	16.067	5.3842	2.9095	0.0123	BMB*^	471
9	4	15.887	8.5113	4.7783	0.0195	BMB*^	492
10	5	15.893	8.4746	4.7976	0.0194	BMB*^	498
11	6	15.907	8.5098	4.8114	0.0195	BMB*^	498
12	7	15.913	7.5438	4.1644	0.0173	BMB*^	478
13	8	15.847	7.5473	4.1829	0.0173	BMB*^	479
14	9	15.813	7.4044	4.1611	0.0169	BMB*^	482
15	standard1	15.827	1.6885	0.9549	0.0039	BMB*^	489
16	standard2	15.76	3.7682	2.1144	0.0086	BMB*^	465
17	standard3	15.687	9.0655	5.0381	0.0207	BMB*^	467
18	standard4	15.693	16.62	9.4534	0.038	BMB*^	493
19	standard5	15.687	25.7641	14.4137	0.0589	BMB*^	478
20	10	15.667	5.18	2.8739	0.0118	BMB*^	473
21	11	15.58	5.1828	2.8803	0.0119	BMB*^	468
22	12	15.54	5.2113	2.8824	0.0119	BMB*^	464
23	13	15.6	5.7487	3.2938	0.0131	BMB*^	484
24	14	15.6	5.8407	3.289	0.0134	BMB*^	470
25	15	15.533	5.8442	3.3071	0.0134	BMB*^	476
26	16	15.453	7.6425	4.4134	0.0175	BMB*^	485
27	17	15.507	7.6995	4.4348	0.0176	BMB*^	488
28	18	15.453	7.7647	4.4742	0.0178	BMB*^	479
29	standard1	15.407	1.7836	1.0327	0.0041	BMB*^	495
30	standard2	15.42	3.7602	2.1712	0.0086	BMB*^	488
31	standard3	15.367	9.0746	5.2157	0.0208	BMB*^	489
32	standard4	15.327	16.5607	9.8054	0.0379	BMB*^	513
33	standard5	15.307	25.7802	14.9418	0.059	BMB*^	486
34	19	15.3	7.6691	4.5255	0.0175	BMB*^	496
35	20	15.26	7.8333	4.5611	0.0179	BMB*^	482
36	21	15.267	7.8606	4.5686	0.018	BMB*^	488
37	22	15.2	7.3887	4.4133	0.0169	BMB*^	495
38	23	15.193	7.568	4.4769	0.0173	BMB*^	494
39	24	15.113	7.5522	4.4873	0.0173	BMB*^	491
40	25	15.173	7.4952	4.3801	0.0171	BMB*^	493
41	26	15.067	7.365	4.3506	0.0168	BMB*^	485
42	27	15.093	7.4986	4.4099	0.0171	BMB*^	494
Average:		15.647	8.5456	4.8453	0.0195		485
Rel.Std.Dev:		2.40%	68.39%	67.65%	68.39%		2.81%

17. Batch 2 week 10: (02-06-2013)

Table: D17

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	16.1	2.0826	1.2033	0.0046	BMB*^	518
2	standard2	16.02	4.3737	2.4661	0.0097	BMB*^	510
3	standard3	15.907	10.6207	5.9622	0.0236	BMB*^	500
4	standard4	15.887	16.8153	9.3727	0.0373	BMB*^	497
5	standard5	15.853	29.3707	16.4322	0.0652	BMB*^	497
6	+	15.8	5.8014	3.2219	0.0129	BMB*^	493
7	2	15.733	5.8212	3.2423	0.0129	BMB*^	492
8	3	15.713	5.864	3.2685	0.013	BMB*^	491
9	4	15.627	8.0613	4.6316	0.0179	BMB*^	506
10	5	15.627	8.2381	4.666	0.0183	BMB*^	493
11	6	15.593	8.1076	4.6522	0.018	BMB*^	499
12	7	15.6	8.3273	4.7297	0.0185	BMB*^	501
13	8	15.513	8.4298	4.773	0.0187	BMB*^	496
14	9	15.52	8.3377	4.7527	0.0185	BMB*^	503
15	standard1	15.4	2.0613	1.2208	0.0046	BMB*^	501
16	standard2	15.46	4.3187	2.5242	0.0096	BMB*^	499
17	standard3	15.407	10.5402	6.0814	0.0234	BMB*^	495
18	standard4	15.407	16.5153	9.5104	0.0366	BMB*^	490
19	standard5	15.393	29.119	16.8212	0.0646	BMB*^	496
20	10	15.36	4.9115	2.7118	0.0109	BMB*^	479
21	11	15.44	4.9186	2.7319	0.0109	BMB*^	485
22	12	15.3	4.9347	2.7232	0.0109	BMB*^	487
23	13	15.347	5.9461	3.3999	0.0132	BMB*^	484
24	14	15.293	5.888	3.3925	0.0131	BMB*^	487
25	15	15.333	5.8863	3.4049	0.0131	BMB*^	490
26	16	15.26	8.2039	4.8412	0.0182	BMB*^	500
27	17	15.24	8.3631	4.8648	0.0186	BMB*^	491
28	18	15.213	8.4299	4.8946	0.0187	BMB*^	489
29	standard1	15.253	2.0632	1.2407	0.0046	BMB*^	529
30	standard2	15.16	4.3745	2.5496	0.0097	BMB*^	491
31	standard3	15.16	10.5251	6.1762	0.0233	BMB*^	494
32	standard4	15.18	16.3914	9.5915	0.0364	BMB*^	498
33	standard5	15.107	29.1118	17.1604	0.0646	BMB*^	497
34	19	15.06	8.3473	4.9113	0.0185	BMB*^	487
35	20	15.093	8.4596	4.95	0.0188	BMB*^	498
36	21	15.06	8.434	4.9611	0.0187	BMB*^	494
37	22	14.987	8.012	4.7523	0.0178	BMB*^	496
38	23	15.04	8.0904	4.7709	0.0179	BMB*^	498
39	24	14.96	8.0708	4.7852	0.0179	BMB*^	496
40	25	15	7.9893	4.6939	0.0177	BMB*^	486
41	26	14.967	8.0183	4.661	0.0178	BMB*^	484
42	27	14.987	7.903	4.6806	0.0175	BMB*^	497
Average:		15.39	9.1923	5.2948	0.0204		496
Rel.Std.Dev:		2.00%	70.70%	70.65%	70.70%		1.81%

18. Batch 2 week 12: (16-06-2013)

Table: D18

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	16.78	1.7661	1.0253	0.0039	BMB*^	609
2	standard2	16.787	3.3962	1.9545	0.0076	BMB*^	591
3	standard3	16.673	8.8597	4.9224	0.0197	BMB*^	559
4	standard4	16.653	14.3123	8.0724	0.0318	BMB*^	567
5	standard5	16.587	23.8266	13.3756	0.053	BMB*^	561
6	1	16.54	5.1642	2.8824	0.0115	BMB*^	545
7	2	16.58	5.0405	2.8354	0.0112	BMB*^	548
8	3	16.473	5.1403	2.8736	0.0114	BMB*^	561
9	4	16.453	7.9114	4.522	0.0176	BMB*^	567
10	5	16.36	7.9367	4.5334	0.0177	BMB*^	554
11	6	16.433	7.8223	4.5008	0.0174	BMB*^	560
12	7	16.353	7.7332	4.521	0.0172	BMB*^	574
13	8	16.333	7.7262	4.5335	0.0172	BMB*^	573
14	9	16.333	7.7941	4.5273	0.0173	BMB*^	570
15	standard1	16.433	1.7426	1.025	0.0039	BMB*^	581
16	standard2	16.287	3.3668	1.9967	0.0075	BMB*^	575
17	standard3	16.273	8.6329	4.9456	0.0192	BMB*^	558
18	standard4	16.293	14.4447	8.2199	0.0321	BMB*^	552
19	standard5	16.273	23.7525	13.5223	0.0528	BMB*^	550
20	10	16.267	5.0982	2.791	0.0113	BMB*^	536
21	11	16.227	5.1718	2.8005	0.0115	BMB*^	525
22	12	16.267	5.238	2.8542	0.0117	BMB*^	535
23	13	16.2	6.0203	3.4469	0.0134	BMB*^	538
24	14	16.213	6.1254	3.5084	0.0136	BMB*^	546
25	15	16.227	6.1566	3.4929	0.0137	BMB*^	537
26	16	16.2	8.1761	4.692	0.0182	BMB*^	547
27	17	16.14	8.1994	4.69	0.0182	BMB*^	546
28	18	16.16	8.2179	4.7084	0.0183	BMB*^	559
29	standard1	16.153	1.5676	0.9857	0.0035	BMB*^	597
30	standard2	16.107	3.3562	1.985	0.0075	BMB*^	553
31	standard3	16.133	8.6205	4.9737	0.0192	BMB*^	554
32	standard4	16.073	14.3284	8.2991	0.0319	BMB*^	551
33	standard5	16.04	23.6622	13.6181	0.0526	BMB*^	546
34	19	16.047	8.2019	4.7768	0.0182	BMB*^	551
35	20	16.033	8.1732	4.7912	0.0182	BMB*^	558
36	21	16.067	8.2227	4.8073	0.0183	BMB*^	559
37	22	16.08	7.8823	4.5622	0.0175	BMB*^	551
38	23	15.987	7.8844	4.5642	0.0175	BMB*^	544
39	24	16.047	7.7259	4.5601	0.0172	BMB*^	570
40	25	16.033	7.9187	4.6918	0.0176	BMB*^	569
41	26	15.92	7.9904	4.7155	0.0178	BMB*^	561
42	27	15.933	8.1403	4.7366	0.0181	BMB*^	555
Average:		16.273	8.2964	4.7581	0.0185		558
Rel.Std.Dev:		1.38%	62.64%	62.10%	62.64%		2.99%

19. Batch 3 week 0: (21-03-2013)

Table: D19

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	19.767	1.9894	1.3153	0.0047	BMB*^	1013
2	standard2	19.827	3.3876	2.2246	0.008	BMB*^	1043
3	standard3	19.74	9.1677	5.4924	0.0217	BMB*^	907
4	standard4	19.713	15.3018	9.246	0.0362	BMB*^	917
5	standard5	19.667	25.5893	15.5881	0.0605	BMB*^	915
6	1	19.613	8.3078	4.836	0.0196	BMB*^	881
7	2	19.553	8.1682	4.7745	0.0193	BMB*^	874
8	3	19.54	8.2136	4.8773	0.0194	BMB*^	901
9	4	19.527	8.4419	5.394	0.02	BMB*^	964
10	5	19.493	8.832	5.5473	0.0209	BMB*^	950
11	6	19.447	8.5813	5.5104	0.0203	BMB*^	958
12	7	19.373	8.6625	5.4122	0.0205	BMB*^	923
13	8	19.427	8.6008	5.3955	0.0203	BMB*^	944
14	9	19.373	8.4684	5.3811	0.02	BMB*^	942
15	standard1	19.253	1.7524	1.1934	0.0041	BMB*^	1122
16	standard2	19.34	3.6143	2.2544	0.0085	BMB*^	807
17	standard3	19.333	9.0876	5.6025	0.0215	BMB*^	895
18	standard4	19.26	14.9395	9.4119	0.0353	BMB*^	932
19	standard5	19.24	25.6946	16.0088	0.0607	BMB*^	918
20	10	19.307	7.6576	4.6884	0.0181	BMB*^	934
21	11	19.267	7.9445	4.8041	0.0188	BMB*^	882
22	12	19.287	7.9519	4.8422	0.0188	BMB*^	915
23	13	19.147	8.4376	5.4873	0.0199	BMB*^	952
24	14	19.187	8.8109	5.5108	0.0208	BMB*^	923
25	15	19.1	8.7308	5.5537	0.0206	BMB*^	900
26	16	19.06	8.5541	5.7211	0.0202	BMB*^	967
27	17	19.093	8.4556	5.5917	0.02	BMB*^	977
28	18	19.127	8.6991	5.6909	0.0206	BMB*^	967
29	standard1	19.033	1.7419	1.1533	0.0041	BMB*^	984
30	standard2	19.06	3.0971	2.0625	0.0073	BMB*^	927
31	standard3	19.067	9.0515	5.7049	0.0214	BMB*^	939
32	standard4	19.033	14.9626	9.5654	0.0354	BMB*^	943
33	standard5	19.027	25.0576	16.3367	0.0592	BMB*^	953
34	19	18.98	8.6124	5.7962	0.0204	BMB*^	987
35	20	18.947	8.7771	5.8464	0.0207	BMB*^	964
36	21	19.007	8.8222	5.8734	0.0209	BMB*^	979
37	22	18.993	8.6276	5.8149	0.0204	BMB*^	991
38	23	18.913	8.7716	5.8977	0.0207	BMB*^	982
39	24	18.973	8.7937	5.9659	0.0208	BMB*^	1012
40	25	18.96	8.5319	5.5763	0.0202	BMB*^	952
41	26	18.94	8.6469	5.5755	0.0204	BMB*^	942
42	27	18.88	8.5644	5.5779	0.0202	BMB*^	949
Average:		19.259	9.3834	5.9548	0.0222		946
Rel.Std.Dev:		1.38%	57.05%	56.18%	57.05%		5.38%

20. Batch 3 week 1: (28-03-2013)

Table: D20

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	19.653	1.5064	0.9523	0.0037	BMB*^	909
2	standard2	19.6	3.6049	2.1316	0.0087	BMB*^	824
3	standard3	19.58	8.8537	5.0549	0.0215	BMB*^	802
4	standard4	19.5	15.0967	8.7384	0.0366	BMB*^	806
5	standard5	19.487	26.5371	15.409	0.0644	BMB*^	815
6	1	19.52	6.5066	3.8055	0.0158	BMB*^	822
7	2	19.487	6.4795	3.8368	0.0157	BMB*^	825
8	3	19.44	6.4949	3.8314	0.0158	BMB*^	825
9	4	19.36	7.6674	4.5786	0.0186	BMB*^	840
10	5	19.42	7.8552	4.6185	0.0191	BMB*^	833
11	6	19.38	8.0086	4.6702	0.0194	BMB*^	821
12	7	19.367	8.1476	4.8529	0.0198	BMB*^	835
13	8	19.313	8.1293	4.8396	0.0197	BMB*^	815
14	9	19.327	8.1405	4.8502	0.0197	BMB*^	828
15	standard1	19.433	1.725	1.0773	0.0042	BMB*^	937
16	standard2	19.34	3.7366	2.2226	0.0091	BMB*^	839
17	standard3	19.32	8.8698	5.1555	0.0215	BMB*^	807
18	standard4	19.3	15.6541	8.9953	0.038	BMB*^	799
19	standard5	19.293	26.8319	15.681	0.0651	BMB*^	815
20	10	19.293	6.1445	3.6663	0.0149	BMB*^	838
21	11	19.28	6.0601	3.6106	0.0147	BMB*^	827
22	12	19.267	6.0318	3.5868	0.0146	BMB*^	820
23	13	19.287	6.8889	4.0491	0.0167	BMB*^	818
24	14	19.26	7.043	4.0735	0.0171	BMB*^	795
25	15	19.307	6.902	4.0359	0.0167	BMB*^	815
26	16	19.24	7.5805	4.5514	0.0184	BMB*^	830
27	17	19.24	7.5959	4.5509	0.0184	BMB*^	837
28	18	19.273	7.5766	4.568	0.0184	BMB*^	848
29	standard1	19.18	1.7531	1.1293	0.0043	BMB*^	939
30	standard2	19.22	3.7271	2.2686	0.009	BMB*^	871
31	standard3	19.267	9.4188	5.3424	0.0228	BMB*^	778
32	standard4	19.187	15.4143	9.0567	0.0374	BMB*^	822
33	standard5	19.187	27.1783	15.9566	0.0659	BMB*^	825
34	19	19.233	7.7831	4.7836	0.0189	BMB*^	864
35	20	19.173	8.0998	4.8558	0.0196	BMB*^	829
36	21	19.16	8.0977	4.8827	0.0196	BMB*^	847
37	22	19.22	7.8147	4.7285	0.019	BMB*^	852
38	23	19.147	7.876	4.7646	0.0191	BMB*^	842
39	24	19.14	8.0406	4.8288	0.0195	BMB*^	838
40	25	19.2	7.1359	4.3317	0.0173	BMB*^	841
41	26	19.193	7.3157	4.411	0.0177	BMB*^	842
42	27	19.087	7.368	4.4291	0.0179	BMB*^	833
Average:		19.313	8.7784	5.1849	0.0213		834
Rel.Std.Dev:		0.69%	66.76%	65.49%	66.76%		3.82%

21. Batch 3 week 2: (04-04-2013)

Table: D21

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	18.713	1.3215	0.9	0.003	BMB*^	946
2	standard2	18.607	3.0837	1.9571	0.0071	BMB*^	841
3	standard3	18.573	9.0794	5.6394	0.0209	BMB*^	840
4	standard4	18.547	15.5839	9.6309	0.0359	BMB*^	838
5	standard5	18.547	26.089	16.0629	0.0601	BMB*^	841
6	1	18.613	4.343	2.6977	0.01	BMB*^	850
7	2	18.513	4.3079	2.674	0.0099	BMB*^	830
8	3	18.487	4.3585	2.7272	0.01	BMB*^	847
9	4	18.387	7.3795	4.6972	0.017	BMB*^	864
10	5	18.38	7.3978	4.6882	0.017	BMB*^	845
11	6	18.38	7.2349	4.6495	0.0167	BMB*^	859
12	7	18.333	7.7588	4.9285	0.0179	BMB*^	858
13	8	18.32	7.7948	4.9108	0.018	BMB*^	844
14	9	18.287	7.8597	4.9287	0.0181	BMB*^	843
15	standard1	18.28	1.4613	0.9832	0.0034	BMB*^	902
16	standard2	18.327	3.2431	2.087	0.0075	BMB*^	845
17	standard3	18.3	9.3318	5.8492	0.0215	BMB*^	841
18	standard4	18.287	15.7814	9.8905	0.0364	BMB*^	845
19	standard5	18.253	26.1418	16.404	0.0602	BMB*^	847
20	10	18.353	3.4145	2.1381	0.0079	BMB*^	848
21	11	18.26	3.2179	2.096	0.0074	BMB*^	850
22	12	18.247	3.3172	2.1618	0.0076	BMB*^	886
23	13	18.213	6.2098	3.9371	0.0143	BMB*^	841
24	14	18.2	6.2483	3.9282	0.0144	BMB*^	846
25	15	18.167	6.2522	3.9553	0.0144	BMB*^	839
26	16	18.193	7.5099	4.8624	0.0173	BMB*^	875
27	17	18.133	7.5876	4.8731	0.0175	BMB*^	857
28	18	18.153	7.747	4.9238	0.0178	BMB*^	851
29	standard1	18.12	1.5206	1.0039	0.0035	BMB*^	870
30	standard2	18.167	3.2739	2.0943	0.0075	BMB*^	858
31	standard3	18.167	9.4477	5.9196	0.0218	BMB*^	840
32	standard4	18.127	15.8069	10.0168	0.0364	BMB*^	851
33	standard5	18.1	25.8806	16.4742	0.0596	BMB*^	847
34	19	18.12	7.7623	4.969	0.0179	BMB*^	855
35	20	18.087	7.837	4.975	0.0181	BMB*^	854
36	21	18.12	7.8099	4.9695	0.018	BMB*^	853
37	22	18.047	7.3824	4.7711	0.017	BMB*^	866
38	23	18.047	7.3349	4.8008	0.0169	BMB*^	871
39	24	18.033	7.3473	4.7764	0.0169	BMB*^	869
40	25	18.06	7.4469	4.8018	0.0172	BMB*^	873
41	26	18.053	7.4324	4.7712	0.0171	BMB*^	855
42	27	18.013	7.4544	4.7968	0.0172	BMB*^	850
Average:		18.269	8.2094	5.1981	0.0189		856
Rel.Std.Dev:		1.00%	73.10%	72.02%	73.10%		2.34%

22. Batch 3 week 3: (11-04-2013)

Table: D22

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	17.853	1.5704	0.983	0.0036	BMB*^	770
2	standard2	17.853	3.711	2.1885	0.0085	BMB*^	697
3	standard3	17.827	9.8822	5.9311	0.0226	BMB*^	728
4	standard4	17.773	16.7218	9.9859	0.0382	BMB*^	731
5	standard5	17.7	28.2691	16.8559	0.0646	BMB*^	724
6	1	17.72	3.814	2.3024	0.0087	BMB*^	721
7	2	17.653	3.7732	2.2975	0.0086	BMB*^	741
8	3	17.613	3.8437	2.3153	0.0088	BMB*^	721
9	4	17.6	6.704	4.113	0.0153	BMB*^	749
10	5	17.507	6.6265	4.0999	0.0151	BMB*^	745
11	6	17.493	6.6269	4.1149	0.0151	BMB*^	747
12	7	17.467	6.879	4.2732	0.0157	BMB*^	752
13	8	17.447	6.8294	4.2746	0.0156	BMB*^	747
14	9	17.453	6.921	4.2965	0.0158	BMB*^	743
15	standard1	17.473	1.6612	1.0463	0.0038	BMB*^	785
16	standard2	17.433	3.7187	2.2693	0.0085	BMB*^	721
17	standard3	17.4	9.9399	6.1136	0.0227	BMB*^	734
18	standard4	17.38	16.755	10.2577	0.0383	BMB*^	732
19	standard5	17.36	28.0813	17.2036	0.0641	BMB*^	730
20	10	17.327	2.9228	1.8748	0.0067	BMB*^	754
21	11	17.4	2.9069	1.8837	0.0066	BMB*^	775
22	12	17.38	3.1271	1.9323	0.0071	BMB*^	734
23	13	17.353	5.4979	3.395	0.0126	BMB*^	728
24	14	17.273	5.4408	3.3675	0.0124	BMB*^	725
25	15	17.313	5.6024	3.3923	0.0128	BMB*^	709
26	16	17.24	6.9769	4.3981	0.0159	BMB*^	743
27	17	17.26	6.9521	4.428	0.0159	BMB*^	764
28	18	17.207	7.0093	4.4192	0.016	BMB*^	742
29	standard1	17.24	1.6083	1.0602	0.0037	BMB*^	778
30	standard2	17.153	3.6317	2.3006	0.0083	BMB*^	735
31	standard3	17.2	10.0703	6.2416	0.023	BMB*^	734
32	standard4	17.16	16.9096	10.4389	0.0386	BMB*^	728
33	standard5	17.16	28.1205	17.4738	0.0642	BMB*^	736
34	19	17.1	7.047	4.4994	0.0161	BMB*^	754
35	20	17.093	7.1367	4.5116	0.0163	BMB*^	736
36	21	17.107	7.0706	4.4886	0.0161	BMB*^	753
37	22	17.16	6.6871	4.2232	0.0153	BMB*^	746
38	23	17.087	6.6721	4.2353	0.0152	BMB*^	745
39	24	17.04	6.7432	4.2529	0.0154	BMB*^	741
40	25	17.053	6.6774	4.2525	0.0153	BMB*^	747
41	26	17.053	6.6663	4.2561	0.0152	BMB*^	751
42	27	17.06	6.708	4.2537	0.0153	BMB*^	736
Average:		17.367	8.1075	5.0119	0.0185		741
Rel.Std.Dev:		1.37%	82.00%	80.56%	82.00%		2.36%

23. Batch 3 week 4: (18-04-2013)

Table: D23

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	18.813	1.3293	0.7154	0.0033	BMB*^	622
2	standard2	18.693	3.0016	1.6039	0.0076	BMB*^	602
3	standard3	18.707	8.6322	4.4505	0.0218	BMB*^	587
4	standard4	18.653	14.5216	7.3619	0.0366	BMB*^	566
5	standard5	18.607	24.9948	12.8218	0.063	BMB*^	571
6	1	18.627	3.0949	1.6008	0.0078	BMB*^	556
7	2	18.707	3.0122	1.561	0.0076	BMB*^	585
8	3	18.553	3.074	1.5929	0.0077	BMB*^	580
9	4	18.48	6.2844	3.2948	0.0158	BMB*^	586
10	5	18.493	6.1612	3.2575	0.0155	BMB*^	588
11	6	18.467	6.1393	3.2455	0.0155	BMB*^	588
12	7	18.493	6.5628	3.4405	0.0165	BMB*^	588
13	8	18.433	6.4815	3.4287	0.0163	BMB*^	591
14	9	18.453	6.4875	3.4139	0.0163	BMB*^	589
15	standard1	18.44	1.1417	0.6932	0.0029	BMB*^	715
16	standard2	18.427	2.9355	1.5223	0.0074	BMB*^	575
17	standard3	18.467	8.6998	4.5407	0.0219	BMB*^	580
18	standard4	18.387	14.6755	7.4966	0.037	BMB*^	563
19	standard5	18.38	25.3479	13.093	0.0639	BMB*^	570
20	10	18.447	2.0479	1.106	0.0052	BMB*^	613
21	11	18.373	2.0005	1.0915	0.005	BMB*^	614
22	12	18.427	1.9861	1.0959	0.005	BMB*^	591
23	13	18.42	5.2484	2.6789	0.0132	BMB*^	569
24	14	18.353	5.2403	2.7097	0.0132	BMB*^	574
25	15	18.427	5.0632	2.6572	0.0128	BMB*^	582
26	16	18.373	6.4652	3.3755	0.0163	BMB*^	586
27	17	18.333	6.2397	3.3432	0.0157	BMB*^	597
28	18	18.387	6.1449	3.3893	0.0155	BMB*^	649
29	standard1	18.293	1.5941	0.8282	0.004	BMB*^	566
30	standard2	18.46	2.9899	1.5916	0.0075	BMB*^	588
31	standard3	18.347	8.8445	4.5883	0.0223	BMB*^	578
32	standard4	18.373	14.88	7.5574	0.0375	BMB*^	557
33	standard5	18.333	25.1605	13.0899	0.0634	BMB*^	576
34	19	18.307	6.6758	3.5196	0.0168	BMB*^	590
35	20	18.353	6.5561	3.5177	0.0165	BMB*^	613
36	21	18.313	6.4573	3.4108	0.0163	BMB*^	600
37	22	18.253	6.3678	3.3964	0.016	BMB*^	583
38	23	18.3	6.3225	3.3753	0.0159	BMB*^	583
39	24	18.273	6.5292	3.4448	0.0165	BMB*^	583
40	25	18.233	5.9851	3.2414	0.0151	BMB*^	593
41	26	18.293	5.9009	3.229	0.0149	BMB*^	598
42	27	18.287	6.0767	3.2538	0.0153	BMB*^	583
Average:		18.434	7.2227	3.7768	0.0182		590
Rel.Std.Dev:		0.74%	82.60%	80.92%	82.60%		4.50%

24. Batch 3 week 6: (02-05-2013)

Table: D24

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	18.9	1.4151	0.8343	0.0035	BMB*^	699
2	standard2	18.86	3.4687	1.9733	0.0086	BMB*^	727
3	standard3	18.787	8.5483	4.6362	0.0212	BMB*^	654
4	standard4	18.787	13.3537	7.1414	0.0331	BMB*^	645
5	standard5	18.713	25.0669	13.3969	0.0621	BMB*^	644
6	1	18.707	2.3984	1.3754	0.0059	BMB*^	708
7	2	18.673	2.4706	1.3881	0.0061	BMB*^	660
8	3	18.613	2.4165	1.3739	0.006	BMB*^	676
9	4	18.567	5.2756	2.9309	0.0131	BMB*^	670
10	5	18.527	5.1127	2.8736	0.0127	BMB*^	665
11	6	18.493	5.1427	2.9087	0.0127	BMB*^	679
12	7	18.533	6.1019	3.4148	0.0151	BMB*^	656
13	8	18.447	6.1339	3.4452	0.0152	BMB*^	666
14	9	18.513	6.0605	3.4037	0.015	BMB*^	656
15	standard1	18.587	1.4895	0.8555	0.0037	BMB*^	690
16	standard2	18.453	2.6613	1.5008	0.0066	BMB*^	649
17	standard3	18.5	8.4565	4.5617	0.021	BMB*^	631
18	standard4	18.467	13.3487	7.2574	0.0331	BMB*^	644
19	standard5	18.393	24.8304	13.5135	0.0615	BMB*^	643
20	10	18.34	1.1392	0.6688	0.0028	BMB*^	689
21	11	18.467	1.0869	0.659	0.0027	BMB*^	744
22	12	18.473	1.0192	0.6282	0.0025	BMB*^	787
23	13	18.393	4.6138	2.5283	0.0114	BMB*^	638
24	14	18.307	4.4863	2.4896	0.0111	BMB*^	638
25	15	18.347	4.6048	2.5329	0.0114	BMB*^	649
26	16	18.153	7.1521	3.7219	0.0177	BMB*^	532
27	17	18.107	7.194	3.7575	0.0178	BMB*^	524
28	18	18.227	7.1036	3.7242	0.0176	BMB*^	539
29	standard1	18.333	1.4767	0.8827	0.0037	BMB*^	728
30	standard2	18.267	2.8065	1.5793	0.007	BMB*^	650
31	standard3	18.293	8.4274	4.6383	0.0209	BMB*^	646
32	standard4	18.267	13.7412	7.5211	0.034	BMB*^	649
33	standard5	18.227	24.9165	13.782	0.0617	BMB*^	654
34	19	18.233	6.2675	3.5794	0.0155	BMB*^	677
35	20	18.213	6.1476	3.5559	0.0152	BMB*^	686
36	21	18.213	6.3197	3.6096	0.0157	BMB*^	678
37	22	18.167	6.0801	3.4695	0.0151	BMB*^	661
38	23	18.193	5.9121	3.4115	0.0146	BMB*^	674
39	24	18.173	5.9702	3.424	0.0148	BMB*^	679
40	25	18.167	6.0394	3.4715	0.015	BMB*^	665
41	26	18.147	6.0597	3.454	0.015	BMB*^	664
42	27	18.08	6.0154	3.4486	0.0149	BMB*^	674
Average:		18.412	6.865	3.7934	0.017		662
Rel.Std.Dev:		1.19%	86.44%	84.43%	86.44%		7.23%

25. Batch 3 week 8: (16-05-2013)

Table: D25

Sample No.	Sample Name	Ret.Time min doxtcycline UV_VIS_1	Area mAU*min doxtcycline UV_VIS_1	Height mAU doxtcycline UV_VIS_1	Amount doxtcycline UV_VIS_1	Type doxtcycline UV_VIS_1	Plates (EP) doxtcycline UV_VIS_1
1	standard1	16.433	2.0544	1.1677	0.0047	BMB*^	508
2	standard2	16.34	3.3543	1.845	0.0076	BMB*^	487
3	standard3	16.253	7.8751	4.2706	0.0178	BMB*^	475
4	standard4	16.16	16.3297	8.9313	0.037	BMB*^	481
5	standard5	16.153	27.201	14.7617	0.0616	BMB*^	476
6	1	16.193	2.1209	1.1639	0.0048	BMB*^	494
7	2	16.1	2.0983	1.1608	0.0048	BMB*^	490
8	3	16.013	2.0497	1.1704	0.0046	BMB*^	517
9	4	15.96	5.1875	2.9161	0.0118	BMB*^	489
10	5	15.88	5.2213	2.9382	0.0118	BMB*^	483
11	6	15.847	5.1232	2.9288	0.0116	BMB*^	497
12	7	15.753	6.5992	3.7582	0.015	BMB*^	490
13	8	15.807	6.677	3.7919	0.0151	BMB*^	491
14	9	15.693	6.5866	3.7863	0.0149	BMB*^	496
15	standard1	15.807	1.9888	1.1866	0.0045	BMB*^	540
16	standard2	15.687	3.2496	1.8793	0.0074	BMB*^	486
17	standard3	15.64	7.8362	4.3451	0.0178	BMB*^	464
18	standard4	15.633	16.2355	9.2327	0.0368	BMB*^	485
19	standard5	15.567	26.5867	15.0485	0.0602	BMB*^	477
20	10	15.493	0.983	0.5753	0.0022	BMB*^	507
21	11	15.533	0.9843	0.5998	0.0022	BMB*^	531
22	12	15.493	1.0136	0.5903	0.0023	BMB*^	520
23	13	15.48	6.2072	3.4784	0.0141	BMB*^	503
24	14	15.447	6.0749	3.4828	0.0138	BMB*^	514
25	15	15.453	6.0481	3.488	0.0137	BMB*^	514
26	16	15.44	7.1695	4.3413	0.0162	BMB*^	545
27	17	15.38	7.2114	4.3571	0.0163	BMB*^	536
28	18	15.34	6.998	4.3125	0.0159	BMB*^	551
29	standard1	15.247	1.9582	1.2104	0.0044	BMB*^	548
30	standard2	15.28	3.3692	1.9961	0.0076	BMB*^	499
31	standard3	15.2	7.9983	4.5847	0.0181	BMB*^	471
32	standard4	15.16	16.2683	9.5991	0.0369	BMB*^	495
33	standard5	15.153	26.5603	15.5485	0.0602	BMB*^	488
34	19	15.047	6.1532	3.6155	0.0139	BMB*^	475
35	20	15.14	6.1529	3.6335	0.0139	BMB*^	489
36	21	15.033	6.1931	3.6516	0.014	BMB*^	476
37	22	15.027	5.7672	3.3988	0.0131	BMB*^	477
38	23	14.993	5.7597	3.4021	0.0131	BMB*^	482
39	24	14.98	5.7197	3.413	0.013	BMB*^	485
40	25	14.907	5.8394	3.4797	0.0132	BMB*^	493
41	26	14.907	5.7927	3.4847	0.0131	BMB*^	493
42	27	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Average:		15.562	7.3316	4.208	0.0166		498
Rel.Std.Dev:		2.74%	90.19%	88.58%	90.19%		4.50%

26. Batch 3 week 10: (30-05-2013)

Table: D26

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	16.113	1.9683	1.2325	0.0044	BMB*^	621
2	standard2	16.013	4.1067	2.5507	0.0091	BMB*^	626
3	standard3	16.007	10.0815	6.2279	0.0223	BMB*^	619
4	standard4	15.887	16.9679	10.4688	0.0375	BMB*^	616
5	standard5	15.833	28.6107	17.6173	0.0633	BMB*^	614
6	1	15.833	2.0707	1.3656	0.0046	BMB*^	656
7	2	15.747	2.1392	1.393	0.0047	BMB*^	657
8	3	15.74	2.2237	1.3975	0.0049	BMB*^	626
9	4	15.653	5.3801	3.3301	0.0119	BMB*^	628
10	5	15.573	5.1878	3.3096	0.0115	BMB*^	637
11	6	15.5	5.1626	3.2968	0.0114	BMB*^	638
12	7	15.573	6.597	4.2011	0.0146	BMB*^	636
13	8	15.513	6.57	4.2071	0.0145	BMB*^	633
14	9	15.453	6.5975	4.2394	0.0146	BMB*^	626
15	standard1	15.427	2.1073	1.3479	0.0047	BMB*^	623
16	standard2	15.453	4.2068	2.6938	0.0093	BMB*^	626
17	standard3	15.347	10.1995	6.5605	0.0226	BMB*^	621
18	standard4	15.347	17.0815	10.9644	0.0378	BMB*^	624
19	standard5	15.327	28.5815	18.4187	0.0632	BMB*^	626
20	10	15.253	0.7151	0.5429	0.0016	BMB*^	781
21	11	15.147	0.7146	0.5552	0.0016	BMB*^	784
22	12	15.147	0.7359	0.5666	0.0016	BMB*^	773
23	13	15.253	4.9699	3.0311	0.011	BMB*^	611
24	14	15.22	4.5723	2.9561	0.0101	BMB*^	643
25	15	15.18	4.6292	2.9535	0.0102	BMB*^	622
26	16	15.167	5.3496	3.5642	0.0118	BMB*^	647
27	17	15.127	5.3994	3.6146	0.0119	BMB*^	644
28	18	15.067	5.4622	3.6211	0.0121	BMB*^	631
29	standard1	15.153	2.0386	1.3848	0.0045	BMB*^	673
30	standard2	15.06	4.0141	2.7318	0.0089	BMB*^	659
31	standard3	15.013	10.2675	6.7825	0.0227	BMB*^	637
32	standard4	14.967	17.0003	11.3636	0.0376	BMB*^	644
33	standard5	14.967	28.4946	19.0949	0.063	BMB*^	643
34	19	14.92	6.7715	4.5224	0.015	BMB*^	638
35	20	14.88	6.6782	4.5193	0.0148	BMB*^	644
36	21	14.92	6.66	4.5535	0.0147	BMB*^	656
37	22	14.9	5.7967	4.0034	0.0128	BMB*^	663
38	23	14.847	5.7382	3.9944	0.0127	BMB*^	670
39	24	14.8	5.9303	4.0537	0.0131	BMB*^	648
40	25	14.76	5.7592	4.0223	0.0127	BMB*^	669
41	26	14.747	5.838	4.0564	0.0129	BMB*^	668
42	27	14.72	5.8091	4.0422	0.0128	BMB*^	663
Average:		15.299	7.5044	4.8894	0.0166		649
Rel.Std.Dev:		2.50%	94.12%	92.62%	94.12%		6.19%

27. Batch 3 week 12: (13-06-2013)

Table: D27

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	16.553	1.9492	1.1127	0.0043	BMB*^	556
2	standard2	16.48	3.9066	2.1893	0.0086	BMB*^	537
3	standard3	16.327	9.353	5.2986	0.0206	BMB*^	549
4	standard4	16.3	15.661	8.8934	0.0345	BMB*^	544
5	standard5	16.24	25.9732	14.7946	0.0572	BMB*^	546
6	1	16.167	1.9	1.0634	0.0042	BMB*^	511
7	2	16.24	1.7935	1.0559	0.0039	BMB*^	545
8	3	16.107	1.8869	1.0934	0.0042	BMB*^	535
9	4	16.047	4.8953	2.8004	0.0108	BMB*^	539
10	5	15.993	4.8262	2.7932	0.0106	BMB*^	548
11	6	15.987	4.7934	2.8158	0.0106	BMB*^	555
12	7	15.987	6.4547	3.8192	0.0142	BMB*^	556
13	8	15.987	6.4538	3.7973	0.0142	BMB*^	555
14	9	15.927	6.415	3.816	0.0141	BMB*^	558
15	standard1	15.947	1.8779	1.1263	0.0041	BMB*^	550
16	standard2	15.94	3.7612	2.2198	0.0083	BMB*^	549
17	standard3	15.873	9.3037	5.4511	0.0205	BMB*^	548
18	standard4	15.9	15.7132	9.1963	0.0346	BMB*^	547
19	standard5	15.88	26.2558	15.2369	0.0578	BMB*^	547
20	10	15.673	0.4341	0.335	0.001	BMB*^	753
21	11	15.667	0.4758	0.3456	0.001	BMB*^	809
22	12	15.573	0.4808	0.3497	0.0011	BMB*^	695
23	13	15.793	3.8282	2.3022	0.0084	BMB*^	559
24	14	15.753	3.6831	2.237	0.0081	BMB*^	569
25	15	15.753	3.7748	2.2621	0.0083	BMB*^	571
26	16	15.753	4.7755	2.9011	0.0105	BMB*^	557
27	17	15.72	4.9518	2.9504	0.0109	BMB*^	560
28	18	15.76	5.0108	2.9783	0.011	BMB*^	546
29	standard1	15.693	1.8781	1.1493	0.0041	BMB*^	580
30	standard2	15.66	3.753	2.2416	0.0083	BMB*^	568
31	standard3	15.64	9.1796	5.5054	0.0202	BMB*^	550
32	standard4	15.633	15.5933	9.3061	0.0343	BMB*^	552
33	standard5	15.64	25.9742	15.4773	0.0572	BMB*^	552
34	19	15.613	5.9937	3.5891	0.0132	BMB*^	550
35	20	15.593	6.0879	3.6473	0.0134	BMB*^	555
36	21	15.607	6.0437	3.62	0.0133	BMB*^	552
37	22	15.607	5.2742	3.2809	0.0116	BMB*^	572
38	23	15.607	5.3755	3.2917	0.0118	BMB*^	565
39	24	15.613	5.3358	3.2902	0.0118	BMB*^	569
40	25	15.527	5.9072	3.6363	0.013	BMB*^	561
41	26	15.567	5.9961	3.6738	0.0132	BMB*^	567
42	27	15.46	5.9032	3.6491	0.013	BMB*^	573
Average:		15.852	6.8782	4.0617	0.0151		568
Rel.Std.Dev:		1.71%	94.18%	92.50%	94.18%		9.61%

Stability after 15 months: 4-10-2013

Table: D28

Sample No.	Sample Name	Ret.Time min Doxycycline	Area mAU*min Doxycycline	Height mAU Doxycycline	Amount Doxycycline	Type Doxycycline	Plates (EP) Doxycycline
		UV_VIS_1	UV_VIS_1	UV_VIS_1	UV_VIS_1	UV_VIS_1	UV_VIS_1
1	STANDARD1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	STANDARD2	13.833	3.9408	2.5716	0.0085	BMB*^	536
3	STANDARD3	13.82	10.1031	6.6836	0.0219	BMB*^	552
4	STANDARD4	13.787	16.5797	10.9451	0.0359	BMB*^	549
5	STANDARD5	13.76	27.7557	18.5497	0.0602	BMB*^	553
6	1	13.74	9.0799	6.0394	0.0197	BMB*^	544
7	2	13.72	9.0414	6.052	0.0196	BMB*^	551
8	STANDARD1	13.72	1.9401	1.3624	0.0042	BMB*^	582
9	STANDARD2	13.68	3.8643	2.6169	0.0084	BMB*^	555
10	STANDARD3	13.64	10.1691	6.798	0.022	BMB*^	549
11	STANDARD4	13.567	16.5003	11.0964	0.0358	BMB*^	548
12	STANDARD5	13.567	27.5645	18.765	0.0598	BMB*^	555
13	3	13.56	9.0727	6.1843	0.0197	BMB*^	549
14	4	13.553	9.2314	6.2602	0.02	BMB*^	553
15	5a	14.78	3.1832	2.1448	0.0069	BMB*^	627
16	5b	14.74	3.2888	2.1981	0.0071	BMB*^	626
17	6a	14.633	8.3284	5.5643	0.0181	BMB*^	631
18	6b	14.747	8.1941	5.514	0.0178	BMB*^	634
19	6c new mobile ph	15.253	8.1568	5.1132	0.0177	BMB*^	616
20	invitro concn test	0.033	0.0065	0.1033	0	BMB	1
Average:		13.27	9.7895	6.5559	0.0212		543
Rel.Std.Dev:		24.48%	78.09%	78.46%	78.09%		25.00%

Stability after 20 months: 27-01-2014

Table: D29

Sample No.	Sample Name	Ret.Time min Doxycycline	Area mAU*min Doxycycline	Height mAU Doxycycline	Amount Doxycycline	Type Doxycycline	Plates (EP) Doxycycline
		UV_VIS_1	UV_VIS_1	UV_VIS_1	UV_VIS_1	UV_VIS_1	UV_VIS_1
1	STANDARD1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	STANDARD2	15.28	2.6119	1.8944	0.0056	BMB*^	765
3	STANDARD3	15.247	6.6328	4.6548	0.0143	BMB*^	741
4	STANDARD4	15.173	10.9866	7.6978	0.0237	BMB*^	722
5	STANDARD5	15.173	18.5261	13.0552	0.04	BMB*^	740
6	1	15.147	9.4943	6.4327	0.0205	BMB*^	691
7	2	15.107	8.9642	6.3633	0.0193	BMB*^	749
8	3	15.12	8.7953	6.225	0.019	BMB*^	744
9	4	15.08	9.107	6.2564	0.0197	BMB*^	715
10	5	15.087	9.1081	6.3904	0.0197	BMB*^	733
11	6	15.093	9.1054	6.3942	0.0197	BMB*^	741
12	STANDARD1	15.133	1.3192	0.9801	0.0028	BMB*^	808
13	STANDARD2	15.153	2.5175	1.8577	0.0054	BMB*^	775
14	STANDARD3	15.087	6.3341	4.5789	0.0137	BMB*^	754
15	STANDARD4	15.047	11.2918	7.9248	0.0244	BMB*^	726
16	STANDARD5	15.053	18.6825	13.2026	0.0403	BMB*^	740
17	7	15.067	9.5387	6.9689	0.0206	BMB*^	758
18	8	15.047	9.3995	6.9233	0.0203	BMB*^	763
19	9	15.013	2.5522	1.7863	0.0055	BMB*^	722
20	10	15.013	2.5329	1.8064	0.0055	BMB*^	716
21	11	15.073	7.5974	5.5575	0.0164	BMB*^	757
22	12	15.04	7.7946	5.6077	0.0168	BMB*^	744
Average:		15.106	8.2329	5.8361	0.0178		743
Rel.Std.Dev:		0.47%	55.89%	55.38%	55.89%		3.33%

In vitro Release studies:

Table: E1 Data for in vitro release experiment (11-06-2013)

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	16.467	14.2741	8.2682	0.0301	BMB*^	588
2	standard2	16.4	30.0772	17.5707	0.0634	BMB*^	590
3	standard3	16.347	75.1031	44.0029	0.1584	BMB*^	592
4	standard4	16.287	125.7728	73.4267	0.2653	BMB*^	589
5	standard5	16.227	208.3702	122.1864	0.4395	BMB*^	592
6	1	16.093	0.0958	0.1033	0.0002	BMB*^	3120
7	2	16.107	0.0968	0.0992	0.0002	BMB*^	1866
8	3	16.147	0.1386	0.1272	0.0003	BMB*^	1646
9	4	16.18	0.259	0.1809	0.0005	BMB*^	629
10	5	16.307	0.2779	0.219	0.0006	BMB*^	844
11	6	16.14	0.2111	0.1739	0.0004	BMB*^	1034
12	7	15.967	0.2771	0.216	0.0006	BMB*^	927
13	8	16.033	0.2155	0.1577	0.0005	BMB*^	934
14	9	16.08	0.1775	0.1461	0.0004	BMB*^	842
15	standard1	16.033	13.8832	8.0689	0.0293	BMB*^	568
16	standard2	15.987	30.5203	18.1712	0.0644	BMB*^	591
17	standard3	15.98	75.8701	45.272	0.16	BMB*^	593
18	standard4	15.96	125.9285	75.1859	0.2656	BMB*^	593
19	standard5	15.92	207.7588	124.8162	0.4382	BMB*^	596
20	10	15.873	0.2673	0.2064	0.0006	BMB*^	846
21	11	16.153	0.2226	0.1665	0.0005	BMB*^	686
22	12	16.013	0.1963	0.1587	0.0004	BMB*^	746
23	13	15.82	0.416	0.258	0.0009	BMB*^	610
24	14	15.86	0.1949	0.164	0.0004	BMB*^	1187
25	15	15.867	0.3579	0.2523	0.0008	BMB*^	778
26	16	15.633	0.238	0.1867	0.0005	BMB*^	727
27	17	15.787	0.2218	0.1642	0.0005	BMB*^	744
28	18	15.913	0.2819	0.2005	0.0006	BMB*^	713
Average:		16.056	32.5609	19.2911	0.0687		885
Rel.Std.Dev:		1.23%	188.95%	189.00%	188.95%		60.84%

Table E2: Data for in vitro release experiment (12-06-2013)

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	standard1	16.787	13.1074	7.4283	0.0279	BMB*^	590
2	standard2	16.72	30.2466	17.4459	0.0643	BMB*^	606
3	standard3	16.613	75.1273	43.7399	0.1597	BMB*^	608
4	standard4	16.56	125.1861	72.838	0.2661	BMB*^	607
5	standard5	16.507	206.2356	120.8889	0.4384	BMB*^	611
6	1	16.52	0.8603	0.5536	0.0018	BMB*^	686
7	2	16.493	0.7643	0.4832	0.0016	BMB*^	681
8	3	16.56	0.5892	0.3778	0.0013	BMB*^	707
9	4	16.447	0.464	0.3379	0.001	BMB*^	774
Average:		16.579	50.2868	29.3437	0.1069		652
Rel.Std.Dev:		0.67%	144.39%	144.80%	144.39%		9.62%

In vitro release repeat: (6-10-2013)

Table: E3

Sample No.	Sample Name	Ret.Time min doxycycline UV_VIS_1	Area mAU*min doxycycline UV_VIS_1	Height mAU doxycycline UV_VIS_1	Amount doxycycline UV_VIS_1	Type doxycycline UV_VIS_1	Plates (EP) doxycycline UV_VIS_1
1	STANDARD1	15.32	1.3644	0.856	0.0029	BMB*^	588
2	STANDARD2	15.167	2.7087	1.6661	0.0058	BMB*^	588
3	STANDARD3	15.227	6.9193	4.1343	0.0149	BMB*^	561
4	STANDARD4	15.18	11.0223	6.6881	0.0237	BMB*^	561
5	STANDARD5	15.133	18.6085	11.3729	0.04	BMB*^	569
6	1	15.22	0.2206	0.1457	0.0005	BMB*^	479
7	2	15.213	0.1828	0.1475	0.0004	BMB*^	996
8	3	15.04	0.1717	0.1562	0.0004	BMB*^	1208
9	4	15.107	0.4282	0.311	0.0009	BMB*^	811
10	5	15.12	0.4951	0.3162	0.0011	BMB*^	679
11	6	15.167	0.4041	0.293	0.0009	BMB*^	749
12	7	15.147	0.5928	0.3832	0.0013	BMB*^	603
13	8	15.067	0.389	0.2763	0.0008	BMB*^	663
14	9	15.053	0.4733	0.316	0.001	BMB*^	669
15	10	15.087	0.6294	0.4376	0.0014	BMB*^	693
16	11	15	0.4964	0.3541	0.0011	BMB*^	683
17	12	15.16	0.6016	0.3767	0.0013	BMB*^	604
18	13	15.033	0.6783	0.4136	0.0015	BMB*^	527
19	14	15.153	0.5808	0.3909	0.0012	BMB*^	665
20	15	15	0.5322	0.3525	0.0011	BMB*^	653
21	16	15.1	0.8087	0.5029	0.0017	BMB*^	542
22	17	14.973	0.6501	0.422	0.0014	BMB*^	566
23	18	15.007	0.7248	0.4594	0.0016	BMB*^	590
24	19	15.107	0.1368	0.1333	0.0003	BMB*^	1301
25	20	15.227	0.2737	0.1701	0.0006	BMB*^	552
26	21	14.86	0.2068	0.1524	0.0004	BMB*^	902
27	22	14.993	0.4526	0.3355	0.001	BMB*^	687
28	STANDARD1	15.107	1.5006	0.8763	0.0032	BMB*^	558
29	STANDARD2	15.047	2.6679	1.6491	0.0057	BMB*^	571
30	STANDARD3	15.013	6.7893	4.1542	0.0146	BMB*^	559
31	STANDARD4	15.007	10.9224	6.7576	0.0234	BMB*^	566
32	STANDARD5	15.033	18.781	11.5201	0.0403	BMB*^	568
33	23	15.18	0.4366	0.3013	0.0009	BMB*^	689
34	24	15.08	0.534	0.3568	0.0011	BMB*^	614
35	25	15.053	0.6491	0.4357	0.0014	BMB*^	603
36	26	15	0.722	0.4425	0.0016	BMB*^	562
37	27	15.047	0.6626	0.4475	0.0014	BMB*^	634
38	28	14.913	0.76	0.5004	0.0016	BMB*^	571
39	29	14.913	0.7197	0.4862	0.0015	BMB*^	602
40	30	14.913	0.9119	0.5771	0.002	BMB*^	561
41	31	14.973	0.7163	0.4976	0.0015	BMB*^	653
42	32	14.973	0.8655	0.5551	0.0019	BMB*^	591
43	33	15.02	0.7932	0.5248	0.0017	BMB*^	609
44	34	14.993	0.7884	0.5161	0.0017	BMB*^	627
45	35	14.993	0.7352	0.4964	0.0016	BMB*^	643
46	36	15.113	0.7889	0.507	0.0017	BMB*^	616
47	37	15.067	2.738	1.7269	0.0059	BMB*^	575
48	38	14.98	2.6185	1.6236	0.0056	BMB*^	561
49	39	15	2.7127	1.6809	0.0058	BMB*^	567
50	40	14.973	2.6842	1.6755	0.0058	BMB*^	585
51	41	14.947	3.0134	1.8087	0.0065	BMB*^	538
52	42	14.98	2.8929	1.7893	0.0062	BMB*^	587
Average:		15.061	2.2723	1.4129	0.0049		644
Rel.Std.Dev:		0.64%	178.94%	175.44%	178.94%		23.65%

Data for mucoadhesion analysis tests:

Table: F1

1. ↓Mucoadhesion of hydrogel composed of 0.5% hydroxyl propyl methyl cellulose. ↓

s.no	Peak detachment force (N)	Area under Force-Time curve (AUC) (N.s)	Work of adhesion $mJ/cm^2 = AUC/\pi r^2$	Ref code from texture analyser software
1	0.04	0.11	0.14	Venu/test 053
2	0.04	0.13	0.165	Venu/test 054
3	0.04	0.12	0.152	Venu/test 055
4	0.04	0.13	0.165	Venu/test 056
5	0.035	0.11	0.14	Venu/test 057
6	0.04	0.13	0.165	Venu/test 051
Average	0.04	0.12	0.1545	
Standard Deviation	0	0.00830949	0.011236	

2. ↓Mucoadhesion of hydrogel composed of 1% hydroxyl propyl methyl cellulose. ↓

Table: F2

s.no	Peak detachment force (N)	Area under Force-Time curve (AUC) (N.s)	Work of adhesion $mJ/cm^2 = AUC/\pi r^2$	Ref code from texture analyser software
1	0.04	0.17	0.216	Venu/test 026
2	0.04	0.16	0.204	Venu/test 027
3	0.04	0.15	0.19	Venu/test 028
4	0.05	0.15	0.19	Venu/test 030
5	0.04	0.15	0.19	Venu/test 031
6	0.05	0.15	0.19	Venu/test 032
Average	0.043	0.155	0.196	
Standard deviation	0.004364	0.007071068	0.10044	

3. ↓Mucoadhesion of hydrogel composed of 1.5% hydroxyl propyl methyl cellulose. ↓

Table: F3

s.no	Peak detachment force (N)	Area under Force-Time curve (AUC) (N.s)	Work of adhesion $mJ/cm^2 = AUC/\pi r^2$	Ref code from texture analyser software
1	0.1	0.32	0.407	Venu/test 035
2	0.04	0.24	0.305	Venu/test 036
3	0.06	0.35	0.44	Venu/test 037
4	0.06	0.23	0.29	Venu/test 039
5	0.07	0.24	0.305	Venu/test 040
6	0.2	0.33	0.42	Venu/test 041
Average	0.058333	0.285	0.361	
Standard deviation	0.022939	0.045591	0.621	

4. ↓Mucoadhesion of hydrogel composed of 2% hydroxyl propyl methyl cellulose. ↓

Table: F4

s.no	Peak detachment force (N)	Area under Force-Time curve (AUC) (N.s)	Work of adhesion $mJ/cm^2 = AUC/\pi r^2$	Ref code from texture analyser software
1	0.05	0.61	0.776	Venu/test 042
2	0.05	0.33	0.42	Venu/test 043
3	0.16	0.60	0.763	Venu/test 044
4	0.08	0.38	0.483	Venu/test 045
5	0.11	0.31	0.394	Venu/test 047
6	0.11	0.41	0.522	Venu/test 048
Average	0.0933	0.44	0.56	
Standard deviation	0.035724	0.112122	0.15	

5. ↓Mucoadhesion of hydrogel composed of 0.5% carboxy methyl cellulose. ↓

Table: F5

s.no	Peak detachment force (N)	Area under Force-Time curve (AUC) (N.s)	Work of adhesion $mJ/cm^2 = AUC/\pi r^2$	Ref code from texture analyser software
1	0.03	0.14	0.178	Venu/test 017
2	0.03	0.12	0.152	Venu/test 018
3	0.02	0.13	0.165	Venu/test 020
4	0.02	0.14	0.178	Venu/test 019
5	0.08	0.14	0.178	Venu/test 023
6	0.08	0.14	0.178	Venu/test 024
Average	0.035	0.135	0.1715	
Standard deviation	0.019086	0.007071	0.0099	

6. ↓Mucoadhesion of hydrogel composed of 0.5% polyethylene glycol 6000. ↓

Table: F6

s.no	Peak detachment force (N)	Area under Force-Time curve (AUC) (N.s)	Work of adhesion $mJ/cm^2 = AUC/\pi r^2$	Ref code from texture analyser software
1	0.05	0.18	0.229	Venu/test 064
2	0.05	0.15	0.19	Venu/test 066
3	0.05	0.15	0.19	Venu/test 067
4	0.05	0.13	0.165	Venu/test 071
5	0.05	0.16	0.203	Venu/test 072
6	0.05	0.15	0.19	Venu/test 073
Average	0.05	0.15333	0.1945	
Standard deviation	0	0.013801	0.01912	

7. ↓Mucoadhesion of hydrogel composed of 0.5% carbopol 974P. ↓

Table: F7

s.no	Peak detachment force (N)	Area under Force-Time curve (AUC) (N.s)	Work of adhesion $mJ/cm^2 = AUC/\pi r^2$	Ref code from texture analyser software
1	0.04	0.12	0.152	Venu/test 077
2	0.04	0.15	0.19	Venu/test 078
3	0.04	0.15	0.19	Venu/test 080
4	0.04	0.12	0.152	Venu/test 081
5	0.04	0.11	0.14	Venu/test 082
6	0.04	0.12	0.152	Venu/test 083
Average	0.004	0.128333	0.16266	
Standard deviation	0	0.14557	0.019788	

8. ↓ Mucoadhesion of hydrogel composed of 0.25 % HPMC + 0.25 % Povidone. ↓

Table: F8

s.no	Peak detachment force (N)	Area under Force-Time curve (AUC) (N.s)	Work of adhesion $mJ/cm^2 = AUC/\pi r^2$	Ref code from texture analyser software
1	0.03	0.17	0.216	Venu/test 084
2	0.05	0.14	0.178	Venu/test 085
3	0.08	0.18	0.229	Venu/test 086
4	0.05	0.17	0.216	Venu/test 088
5	0.06	0.14	0.178	Venu/test 089
6	0.05	0.18	0.229	Venu/test 092
Average	0.0533	0.1633	0.2076	
Standard deviation	0.013801	0.015736	0.0216	

9. ↓ Mucoadhesion of hydrogel composed of povidone 0.5%. ↓

Table: F9

s.no	Peak detachment force (N)	Area under Force-Time curve (AUC) (N.s)	Work of adhesion $mJ/cm^2 = AUC/\pi r^2$	Ref code from texture analyser software
1	0.03	0.20	0.255	Venu/test 093
2	0.04	0.19	0.242	Venu/test 094
3	0.06	0.24	0.305	Venu/test 095
4	0.04	0.20	0.255	Venu/test 096
5	0.03	0.20	0.255	Venu/test 098
6	0.04	0.28	0.356	Venu/test 099
Average	0.04	0.218333	0.278	
Standard deviation	0.009258	0.02948	0.0402	

10.↓Mucoadhesion of hydrogel composed of 0.5% HPMC + 0.5% carbopol 974P.↓

Table: F10

s.no	Peak detachment force (N)	Area under Force-Time curve (AUC) (N.s)	Work of adhesion $mJ/cm^2 = AUC/\pi r^2$	Ref code from texture analyser software
1	0.03	0.15	0.19	Venu/test 100
2	0.03	0.13	0.165	Venu/test 101
3	0.05	0.15	0.19	Venu/test 102
4	0.03	0.15	0.19	Venu/test 103
5	0.03	0.15	0.19	Venu/test 104
6	0.03	0.15	0.19	Venu/test 105
Average	0.033	0.1466	0.1858	
Standard deviation	0.006901	0.006901	0.009317	