DESIGN AND EVALUATION OF OPHTHALMIC FORMULATIONS IN AN UNLICENSED MEDICINES MANUFACTURING UNIT

by
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Copyright Statement

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Abstract

Bespoke unlicensed medicines manufactured as 'specials' often have quality attributes that are minimally qualified with little formal assessment of the benefits and risks associated with their clinical use. Mitomycin C is a cytotoxic antibiotic used as an unlicensed ophthalmic medicine in ocular surgery and the treatment of ocular malignancies. Amphotericin B is an antimycotic agent used in ophthalmology to treat eye fungal infections. Both compounds have limited aqueous stability which restricts its clinical usage. For this reason, the development of innovative ophthalmic formulations with superior stability was pursued.

As part of the design, drug stability of the formulations was determined using a validated HPLC method with samples tested periodically. Also, characterisation of the pharmaceutical products was carried out as part of the evaluation.

The novel mitomycin C formulation represents a substantial improvement in shelf life of at least 16 months at 4°C compared to 6 weeks shelf life of the existing clinical formulation. A toxicological evaluation of the novel mitomycin C eye drops showed that this formulation had a comparable profile to the aqueous mitomycin C solution.

On the other hand amphotericin B products were also developed as eye drops, in situ and ready to use gel formulation. The instability and the possible drug-excipient interaction of the in-situ gel formulation make this option unfit for purpose. In terms of the other 2 products, either the eye

drops or the ready to use gel stored at 4 °C have superior stability than the formulation currently manufactured at the Pharmacy Production Unit within NHS in GG&C. Although the characterization of the eye drops showed better results and the manufacturing process is easier, the ophthalmic gel formulation could imply a better acceptance by patients.

These findings will reduce manufacturing costs and extend patient access improving the treatment of ocular pathologies. *In vivo* research to evaluate the ocular bioavailability of the novel ophthalmic formulations would be desirable.

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List of abbreviations

5-FU	5-Fluorouracil
ACN	Acetonitrile
AmB	Amphotericin B
AmB-PLP	Amphotericin B-Pyridoxal phosphate complex
AME	Amphotericin methyl esther
API	Active Pharmaceutical Ingredient
AUC	Area under the curve
BP	British Pharmacopoeia
CQA	Critical Quality Attributes
DCR	Dacryocystorhinostomy
DMEM	Dulbeco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DSC	Differential Scanning Calorimeter
EMA	European Medicines Agency
EPI-LASIK	Epithelial laser in situ keratomileusis
FDA	Food and Drug Administration
GG&C	Greater Glasgow and Clyde
GDP	Good Distribution Practice
GMP	Good Manufacturing Practice
HPLC	High Performance Liquid Chromatography
IC_{50}	Inhibitory concentration
ICH	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IV	Intravenous
IVIS	*In vivo* imaging system
LASEK	Laser epithelial keratomileusis
LASIK	Laser-assisted in situ keratomileusis
MA	Marketing authorisation
MHRA	Medicines and Healthcare products Regulatory Agency
MMC	Mitomycin C
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduce form of NAPD
NHS	National Health care System
OSSN	Ocular surface squamous neoplasia
PAM	Primary acquired melanosis
PBS	Phosphate Buffered Saline
PG	Propylene glycol
PEG	Polyethylene glycol

PKR	Photorefractive keratectomy
PPU	Pharmacy Production Unit
PVC	Polyvinyl chloride plastic
QbD	Quality by Design
QTPP	Quality Target Product Profile
RNA	Ribonucleic acid
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RQAS	Regional Quality Assurance Service
RSD	Relative Standard Deviation
t_{90}	Time to reach 90% of the content of the active ingredient
RT	Retention time
TSA	Trypticase soy agar
USP	United States Pharmacopoeia
UV	UltraViolet
WHO	World Health Organisation

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Chapter 1. Unlicensed medicines: An overview.

1.1. Introduction

Licensed pharmaceutical products are suitable for the majority of the population. However, there are groups of patients such us children or elderly people that cannot be administered the commercialised product. This is sometimes because of the dose or in other cases due to the pharmaceutical form in which a particular formulation is presented. For example, some patients are not able to swallow solid oral dosage forms and need a liquid form of medicine as an alternative presentation. In other circumstances, allergies to formulation components may also lead to a prescriber's request for unlicensed formulations to avoid any adverse reactions in the patient. Finally, there are patients with rare illnesses that cannot be treated with marketed licensed products. In these special cases, patient-specific formulations would be developed for them. For all of the aforementioned patients treatment with an unlicensed medicine is the solution (East of England Collaborative Procurement Hub Specials Sourcing Group, 2010).

Unlicensed medicines are those medicines manufactured under the requirements of a special license or prepared as extemporaneous products under the supervision of a pharmacist. This type of medicine will be requested by an authorised healthcare professional to address patient medical requirements unmet by licensed products. Therefore, unlicensed medicines do not have marketing authorisation (MA) in its country of use (NHS GG&C Unlicensed Medicines Working Group, 2013). Within the definition of unlicensed medicines are not only included novel

pharmaceutical products but also extemporaneous products prepared from a licence drug and licensed products imported from another country (East of England Collaborative Procurement Hub Specials Sourcing Group, 2010).

Sometimes when the quality of an imported medicine cannot be guaranteed, manufacturing units holding a specials license may develop formulations with identical or similar ingredients to the required quality (Parkinson *et al.*, 2004).

Unlicensed medicines should be distinguished from the off label use of licensed products. Every licensed product is approved by the specific agency in each country to be used under the terms of the MA. Those licensed products are assessed for quality, efficacy and safety before being granted MA. However, each individual granted MA is specific for the treatment of certain clinical conditions and appropriate patient information and labelling specifications are therefore a condition of the authorisation. Off label use is when a licensed medicine is used for a clinical indication that is not within the approved MA (NHS GG&C Unlicensed Medicines Working Group, 2013).

Figure 1.1 shows the different stages to be followed in the event that a licensed product does not meet the needs of a patient, is unavailable or is out of stock, and where a special formulation is indicated to treat the patients.

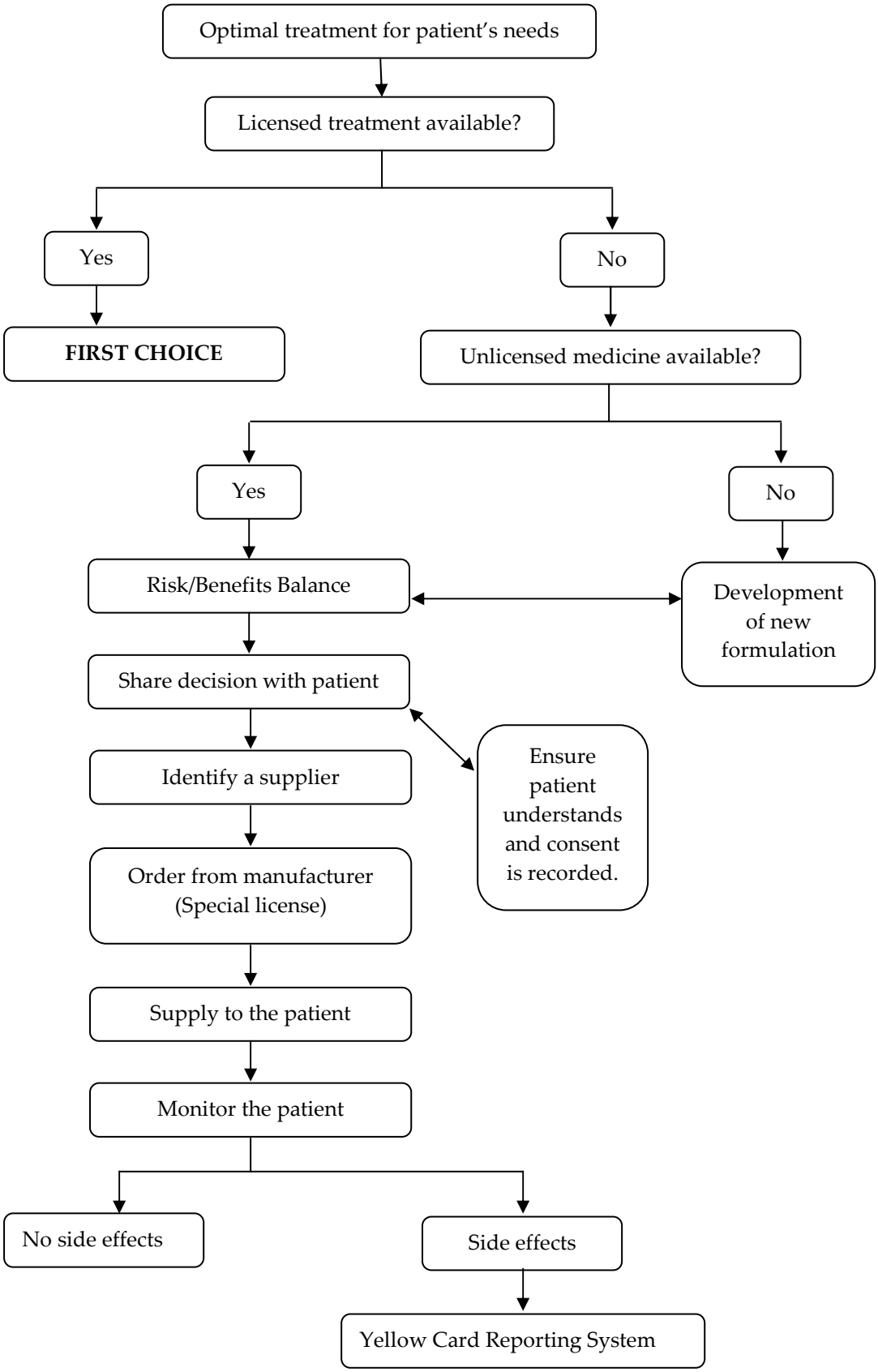

Figure 1.1 - Process map to follow when prescribing an unlicensed medicine.

Before prescribing an unlicensed medicine for a particular patient or a group of patients, the healthcare professionals need to ensure that there are no licensed products in the marketplace that meets the requirements of those patients. This decision should be based only on clinical grounds. The costs of the product, convenience or operational need should never be factors in the assessment. Doctors and dentists, supplementary prescribers and nurse or pharmacist independent prescribers registered in the United Kingdom (UK) are entitled to prescribe unlicensed medicines. Pharmacists in hospitals, healthcare centres or registered pharmacies can supply specials. Wholesale dealers with a specials license can manufacture unlicensed medicines and supply them directly to any of the professionals mentioned above. Some manufacturers are also licensed to import unlicensed medicines from other countries (MHRA, 2009).

Making the decision to prescribe an unlicensed medicine, the prescriber should assess the risks and the benefits of the treatment to the patient or patient group. Apart from the fact that there is no suitable licensed product available and a positive benefit-risk assessment, other factors to be considered includes existing local policies, the quality of the product prescribed and continuity treatment should be assured (MHRA, 2009). Appropriate ongoing clinical monitoring should occur and any adverse reactions detected should be reported through the Medicines and Healthcare products Regulatory Agency (MHRA) yellow card (MHRA, 2015).

The patient as well as healthcare professionals should be actively involved in the decision making process to use an unlicensed medicines as well. The patient should be informed about the treatment, its unlicensed status,

including any known risks associated with the unlicensed medicines. It is very important that the patient or representative understands all the information surrounding the product due to the fact that they have to sign a written consent to this effect prior to the initiation of treatment. Every person involved in the chain of supply of the unlicensed medicines is responsible for ensuring that the process is optimally followed (NHS GG&C Unlicensed medicines short life working group, 2013).

The Association of Pharmaceutical Specials Manufacturers reported that in the United Kingdom every year approximately 1% of all prescriptions are Specials. Although the number of prescriptions has risen, the costs per item are decreasing. The total spent in primary care for specials in 2015 was £82.2m. Normally the specials are manufactured for individuals, but in selected cases products are manufactured in small batches for groups of patients with similar clinical needs (APSM, 2015).

In Europe, there is extensive use of unlicensed medicines and off label use of licensed pharmaceutical products in paediatric populations, particularly during surgical procedures and in intensive care units. This represents a high risk for this patient population. However, this reflects the paucity of licensed medicines available for children. It would be therefore impractical and unethical to restrict the use of unlicensed medicines in children. Oncology as a speciality also has a high rate of unlicensed or off label medicines prescribed (Conroy et al., 2000).

1.2. Development of unlicensed medicines

MA for medicines was introduced in the UK via the 1968 Medicines Act legislation to ensure that a pharmaceutical product meets the principles of safety, efficacy and quality. In the United Kingdom the MA for a given medicine can be issued either by the MHRA, or by the European Medicines Agency (EMA) (NHS, 2016).

Although unlicensed products are formulated and manufactured under a Manufacturer's Specials Licence granted by the MHRA and meet internationally accepted standards, these medicines do not possess MA (NHS GG&C Unlicensed medicines working group, 2013). This supposes that these medicines are only superficially qualified, meaning that they often have short shelf-lives attributed to them, creating supply issues and interfering with the continuity of a patient's treatment (MHRA, 2014). There are also the possibility of variations in manufacturing and compounding between different special providers. That is the reason why quality risk management during the development and the manufacturing process are so important. A systemic risk monitoring system to measure, control and review any risks that can affect the quality during the lifecycle of the medicines should be in place for every product. This means that when a risk is identified, it should be analysed and evaluated, and appropriate control measures should be designed and introduced to reduce the risk to acceptable levels. To complete the quality risk management cycle, any risks identified should be regularly reviewed (ICH, 2005b).

The Scottish NHS has two manufacturing units for specials. These are the Pharmacy Production Unit (PPU) in Glasgow and Tayside Pharmaceuticals in Dundee. Both are approved manufacturing sites with the licence issued by the Medicines and Healthcare products Regulatory Agency to manufacture and distribute unlicensed medicines. Once a production facility is granted a specials licence, the MHRA will regularly undertake inspections to ensure they comply with Good Manufacturing Practice (GMP) and Good Distribution Practice (GDP).

By law, a manufacturing unit for specials or an importer may advertise the services that they provide but they must not publicize any specific specials that they are supplying. However, a list of prices or a catalogue can be sent to authorised healthcare professionals in response to an enquiry for information about the range of products that they are able to supply (MHRA, 2014).

Eudralex guidelines defines GMP as a collective of practices to ensure that a pharmaceutical product is manufactured under controlled circumstances to maintain quality standards suitable and fit for purpose and as required by MA, clinical trials or product specification. In the same way, GDP is defined as a group of rules to ensure that a pharmaceutical product is stored, transported and handle under suitable conditions to preserved the quality of the product when it reaches the costumer (Eudralex, 2013).

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) introduced the Quality by Design (QbD) concept in 2009 under the quality guidelines ICH Q8 (Pharmaceutical development), ICH Q9 (Quality Risk Management), ICH Q10

(Pharmaceutical Quality System) and ICH Q11 (Development and manufacture of drug substance). These documents deliver a number of directions about the development process of pharmaceuticals, defining QbD as it applies to the pharmaceutical industry and also providing guidance for marketing applications.

Within the pharmaceutical industry QbD is an approach for the development of medicines with high quality that meet patient's requirement in a safe way (EMA, 2014). As it is shown in Figure 1.2, the first step of this approach will be to identify the Quality Target Product Profile (QTPP), determining critical quality attributes (CQA's) of the product taking into consideration the intended use, the route of administration, stability, dosage form, and strength. The CQA's are chemical, physical, biological and microbiological attributes that can be defined, measured, and continually monitored to ensure final product outputs remain within acceptable quality limits. The CQA's that can have an impact on the quality of the product will be controlled in the development stage and also will influence in the selection of excipients of the formulation. The manufacturing process will be selected based on the QTPP, describing also the control strategy and all the risks that can have an effect on the quality of the final product. It is also important to establish the relationship between the attributes of the components of the formulation and the process parameters, and understand how those factors can affect the formulation. This analysis will be the guideline to monitor, control and optimise the manufacturing process and ensure the quality of the final pharmaceutical product. (ICH, 2009).

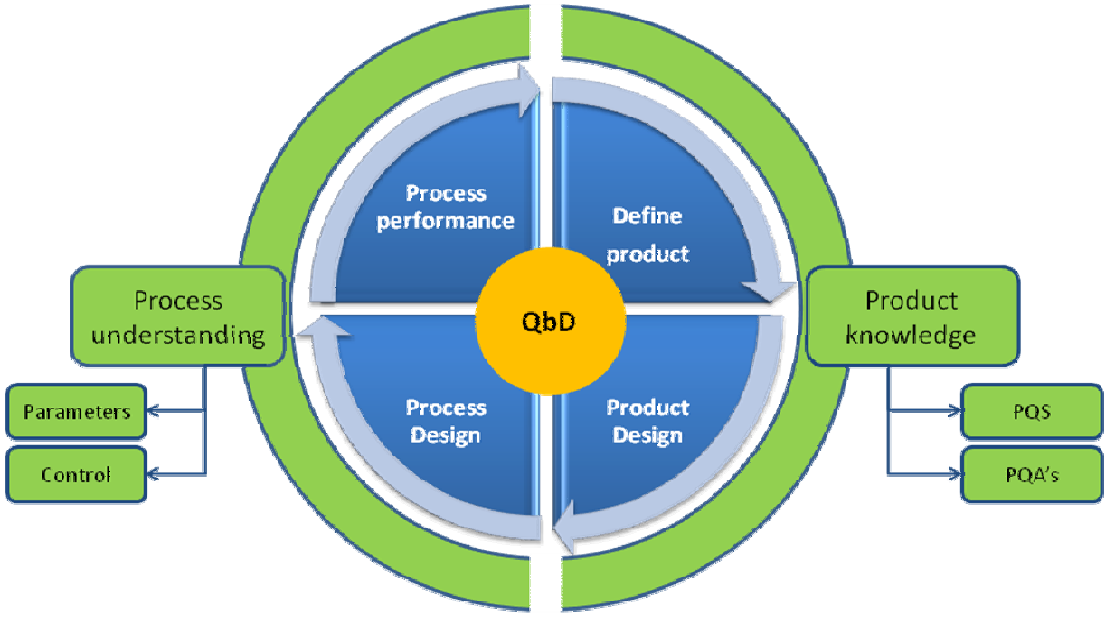

Figure 1.2 - Quality by design (QbD) chart (Based on QbD concept presented by U.S. Food and Drug Administration)

The manufacturing process and the use of pharmaceutical products always represent a degree of risk. Therefore quality risk management is also important in QbD philosophy. The quality risk should be based in 2 fundamental principles; the quality should be based on scientific knowledge and focus on the protection of the patient. The level of effort, formality and documentation of the quality risk management process should be proportional to the level of risk (ICH, 2009).

1.3. Study objectives

The aim of this research is to design innovative unlicensed medicines for use in areas where there are existing and urgent unmet needs. These areas were identified and prioritised by a situational awareness at the PPU within Greater Glasgow and Clyde National Healthcare Service (NHS GG&C). Due to the importance of the manufacturing of mitomycin C (MMC) and

amphotericin B (AmB) eye drops at the PPU, those products were chosen for further development as part of this research project.

At the start of the project batches of 0.15% w/v AmB eye drops were manufactured solely by the PPU. The original formulation was assigned just one week of shelf life, which meant that stability was limiting formulation availability. Since patients with ocular infections require immediate treatment, development of a more stable formulation could reduce the waiting times for patients and enhance clinical outcomes. Although MMC eye drops had better stability than AmB eye drops, a 6 week shelf-life remains very limited for a pharmaceutical product.

The focus of this research was the improvement in unlicensed medicines quality and safety. In order to do so the following steps were taken:

- Evaluating formulations that are currently available as specials but which are possible to manufacture as larger batches with definitive formal stability studies performed to confirm 'fit for purpose' and appropriate shelf-life.

- Developing formulations with superior stability in comparison with the pharmaceutical products currently manufactured in the NHS GG&C. Creating additional capacity within the supply chain to permit a greater range of medicines to be manufactured, widening availability and consolidating patient access.

- Carrying out stability studies of the novel ophthalmic formulations developed to enhance the quality of the medicines. Investigating the

influence of different storage conditions and different components of the formulations in the stability of the pharmaceutical product.

- Performing *in vitro* studies to compare the activity profile of the novel formulations and the formulation currently manufactured in the PPU. In relation to MMC formulations, it is important to assess whether or not there is a comparable acute toxicity profile, and investigate if the excipient used in the novel formulation affects the toxicity of the novel formulation. With regard to AmB formulation antifungal activity was studied.

- Characterisation of the innovative formulations to investigate the pharmaceutical product. Some aspects studied included the compatibility of the excipients with the active ingredient, pH and homogeneity of the final product.

This research project has the potential to transform the quality of life of many thousands of patients within the UK and will help to establish an evidence base on the design, manufacture and use of these medicines. This will also enhance clinical practice and maximise patient safety and wellbeing.

Chapter 2. Mitomycin C Ophthalmic formulation

2.1. General aspects of Mitomycin C

2.1.1 Properties and chemical structure of Mitomycin C

Mitomycins are natural products discovered by the Japanese researchers Hata *et al.* (1955), and isolated by Wakaki *et al.* (1958). Since then, several studies have been published around the world about synthesis, stability aspects of the compound, clinical applications and complications of treatment with mitomycins. Nearly six decades after the discovery, mitomycins are continued to be researched. It is a large family of active compounds, however MMC is the most widely known and pharmacologically active mitomycin of the family (Andrez, 2009). It took 20 years of development from its discovery until MMC (Figure 2.1) was used as a licensed medicine. Clinically approval in the U.S.A. by the Food and Drug Administration (FDA) was granted in 1974 (Bradner, 2001).

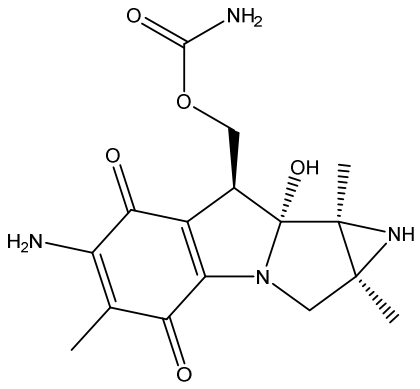

Figure 2.1 - Structure of Mitomycin C

MMC has antitumor-antibiotic activity (Abraham, *et al.,* 2006). However, it is not used as an antibiotic due to its high toxicity, making its principal use as an anticancer cytotoxic product.

MMC is obtained from two different bacteria belonging to the *Streptomyces* genus: *Streptomyces lavendulae and Streptomyces caespitosus* (Danshiitsoodol *et al.,* 2006). The method of producing MMC requires the fermentation of *Streptomyces caespitosus* in suitable media. After the fermentation cycle, MMC is extracted from the broth. However, during this process, impurities are also removed (Gourevitch A. *et al.,* 1962). Amongst those related compounds, it could be found mitomycin A, mitomycin B and albomitomycin C. The British Pharmacopoeia (BP) includes a monograph for MMC powder where the limits for those compounds are not more than 0.5% for each impurity or a total of 2%.

2.1.2 Mechanism of action

The mechanism of action of MMC consists in the inhibition of the synthesis of nucleic acids (DNA & RNA) due to the formation of linkages between the two nucleobases, adenine and guanine damaging DNA (Teus *et al,* 2009). MMC does not act in a specific stage of the cell-cycle, but studies have shown that is more likely to be active during the late G1 (Growth) and early S (DNA synthesis) phases of the cycle (Verweij J. *et al.*, 1990). The MMC is a prodrug with a pyrrolo-indole ring system incorporating an aziridine ring. It remains inactive until an enzymatic transformation of MMC produces an alkylating agent which can bind and damage DNA (Danshiitsoodol *et al*., 2006).

There are two different mechanisms to activate MMC. A two electron reduction (Figure 2.2) through quinone oxidoreductase I, quinone oxidoreductase II and xanthine dehydrogenase to obtain a MMC hydroquinone (Paz *et al.*, 2012). And also a reduction of MMC by one electron at a time, in this case, the inactive compound is reduced to the semiquinone first and then a further reduction by a subsequent electron to the MMC hydroquinone. Enzymes such as cytochrome P450 reductase, xanthine oxidase, cytochrome b5 reductase and NADPH ferredoxin reductase have been shown to reduce MMC via a one-electron path (Paz *et al.*, 2012).

Once the MMC is activated it acts differently depending if it is under aerobic or anaerobic conditions. Under anaerobic conditions, reduced MMC intermediates bind double-stranded DNA, however the fate of MMC after enzymatic reduction in aerobic conditions is different. In aerobic conditions, as it is the case of ophthalmic use of MMC, the semiquinone or the hydroquinone form of MMC reacts with molecular oxygen to generate free radicals highly cytotoxic. This cytotoxicity is cause via peroxidation and DNA and protein damage (Abraham, *et al.*, 2006).

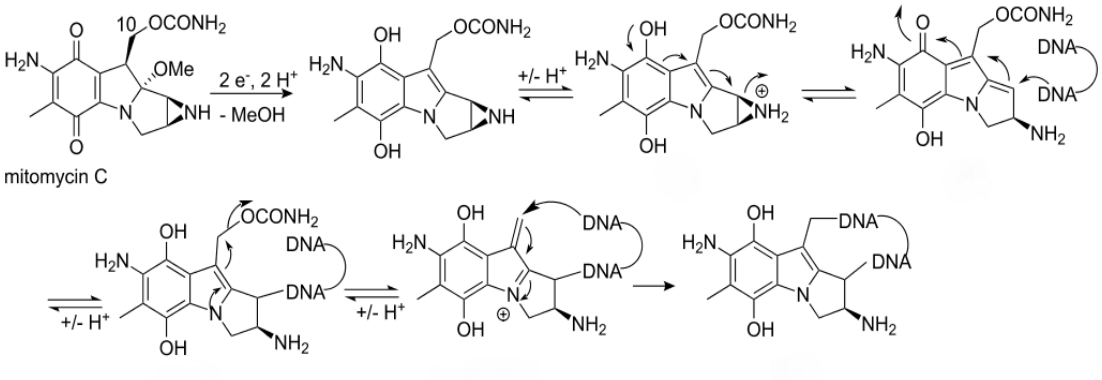

Figure 2.2 - Mechanism of action: 2-electron reductive activation and DNA alkylation of MMC (Andrez, 2009).

2.1.3 Clinical uses

Cancer can be defined as a group of pathologies in which the cells of some parts of the body experience an uncontrolled growth. The World Health Organisation (WHO) reported that there are 14 million of new cases of cancer, 8.2 million of people died in 2012 over the world, and the 30% of cancers could be prevented with a healthy life style and avoiding the risk factors of each type of cancer.

The clinical applications of MMC can be divided in two categories (Figure 2.3), its licensed use, and its use as an unlicensed topical medicine in ophthalmic practice. In its licensed use it is administered intravenously as a mono-therapy in sterile solution or in combination with other cytostatic agents against a range of tumours such as breast, cervix, pancreas, lung, liver and oesophageal cancer. It has also been used against bowel, head and neck, skin and prostate cancers (eMC, 2017). Although MMC's clinical prominence has declined with the advent of new anticancer compounds with superior specificity, efficacy or reduced side effect profiles, it is commonly used as mono-therapy to treat superficial bladder tumours after resection of the tumour (Ferakis et al., 2010). In ophthalmic practice is used as an unlicensed medicine. MMC is topically applied to treat ocular malignancies such as ocular surface squamous neoplasia, primary acquired melanosis with atypia and in conjunctival melanoma. Also, due to its inhibitory effects on fibroblasts, it is used as an anti-scarring agent in a variety of ocular surgeries such as glaucoma, pterygium, lacrimal surgery or refractive surgery (Abraham *et al*., 2006).

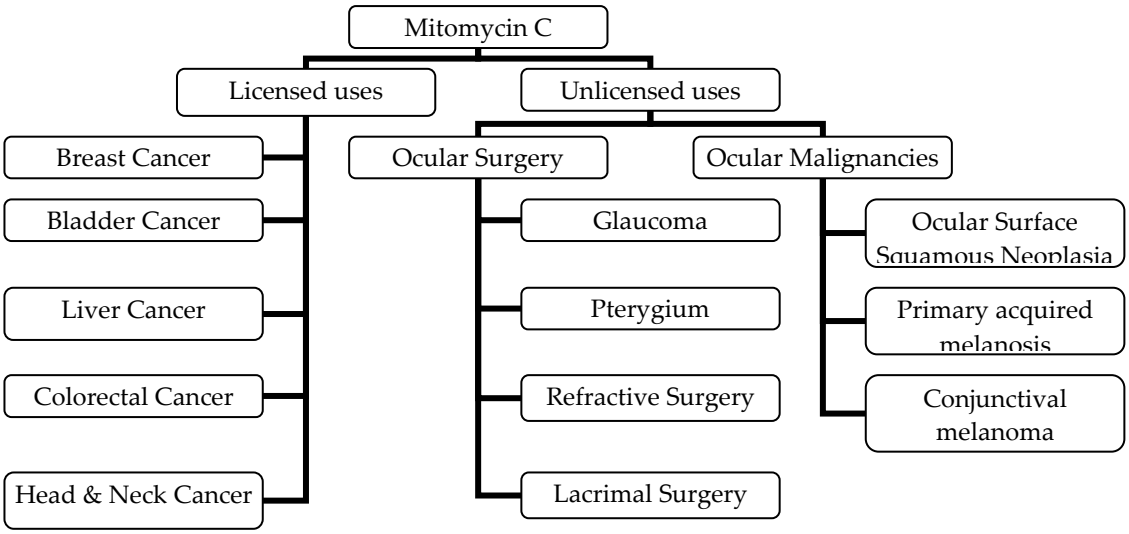

Figure 2.3 - Licensed and unlicensed uses of MMC

The NHS reported in 2014 that breast cancer is the most common cancer of females in the UK, and although it is more prevalent among women, men can also have breast cancer (Cancer Research, 2014). There are different types of breast cancer and different ways to treat them. The most common treatments are surgery, chemotherapy or radiotherapy. As chemotherapy licensed drug to treat breast cancer, MMC is used more often in combinations with other antineoplastic agents than as a single therapy. The vinca alkaloids are the most common drugs chosen as a treatment, however, other drugs such as MMC, cyclophosphamide, methotrexate, 5-fluorouracil (5-FU), are commonly used to treat it in multi therapy (Fukuda et al., 2015).

Over 10,000 new cases of bladder cancer are diagnosed each year in UK and more often we can find this cancer in men than women. Among the risks factors for this type of cancer we can find the influence of the tobacco, family history, the diet and hormonal factors (NHS, 2015). In 2013, the percentage of incidence of bladder cancer related with tobacco and life style were 44% and 37% respectively. Bladder cancer can be divided into non-muscle invasive

(superficial), invasive and advance cancer, the treatment can vary depending on the stage and the type of cancer, but the most usual treatments used are surgery to remove the affected area with adjuvant chemotherapy treatment to reduce the rate of recurrence, radiotherapy or radiotherapy combined with chemotherapy (Cancer Research UK, 2015). To treat non-muscle invasive bladder tumours MMC has an important role and is used as an intravesical instillation of 20 to 40 mg of MMC once or three times a week. The number of cycles to be applied to the patient of MMC depends on the stage of the cancer (Bolenz et al., 2006).

The treatment in colorectal cancer depends on the stage of the cancer and also on the type of tumour. Usually the most often procedure is excision of the tumour by laparoscopy technique and adjuvant chemotherapy treatment. Sometimes is also used radiotherapy to reduce the risk of recurrence. The first-line treatment recommended in a stage III of colon cancer is capecitabine as a monotherapy or oxaliplatin in combination with 5-FU and folinic acid (NICE guidelines, 2006). MMC is often used as a single therapy or combined with other drugs such as 5-FU. However, there is not much clinical support on this use and a lack of evidence of effectiveness (Ferrarotto *et al.*, 2012).

In patients with head and neck cancers surgical excision of the tumour is the best option, however sometimes the tumour is too large to be operable. In those instances, radio-chemotherapy is required. Budach W. *et al., (*2006) published a research article with regards the application of chemotherapy and radiotherapy concurrently and use of radiotherapy alone. The conclusion of this article was that a prolongation of survival of 24 months, and 16.8 months was seen in patients treated with 5-FU and cisplatin

respectively in combination with radiotherapy. This supposed a largest benefit in terms of survival of patients with head and neck tumours. Although treatments with carboplatin and MMC were less effective, it was reported an increment of survival of 6.7 and 4 months respectively. Even though MMC is not the preferable treatment in neck and head cancer due to less efficacy and high toxicity, some recent findings showed that MMC entrapped in nanoparticulate carriers has selective binding to cancer cells which could potentially reduce the side effect in patients with head and neck cancer treated with MMC (Bachar et al., 2011).

In regard to the unlicensed uses, the first clinical application of MMC for eye disorders was in 1960's as an adjuvant treatment of pterygium. The popularity of MMC grew from that date onwards amongst ophthalmologists (Gaston O. Lacayo, 2011). In February 2012, Mitosol® was approved by FDA in U.S.A. This is a kit manufactured by Mobius Therapeutics for ophthalmic use, contains a 0.2 mg vial of MMC, a 1 ml syringe of sterile water for injection, sponges for administration and several devices to avoid contamination. It was approved to be used in glaucoma surgery (Formulary journal, 2012). Apart from the cytotoxic effects of MMC, the anti-fibrotic effects of MMC are also a clinically important feature. It can reduce the growth of scar tissue and conjunctival wound healing when is applied as adjunctive treatment in ocular surgeries such us pterygium surgery, primary trabeculectomy in glaucoma or lacrimal surgery. MMC and 5-FU are anti-fibrotic drugs widely used in ophthalmologic practice as adjuvant treatment in surgery (Abraham LM et al., 2006).

Pterygium is in a non - cancerous degenerative lesion of the eye resulting in excessive growth of the conjunctiva. Clinical causes are not fully understood but sunlight exposure and other factors such as genetics or dust may be contributory (Bekibele *et al.*, 2012). It has been reported that the use of MMC as an adjunctive anti-fibrotic agent decreases the rate of pterygium recurrence (Kareem *et al.*, 2012).

In glaucoma filtering surgery, there is evidence that after treatment of a patient's eye with MMC there is an induction of apoptosis of Tenon's capsule fibroblasts in the subconjunctiva which improves the success rate of this surgery (Crowston *et al*., 1998). MMC is not only used in glaucoma but also in other ocular surgeries such as primary trabeculectomy, combined cataract and glaucoma surgeries, laser eye surgery or dacryocystorhinostomy. There is no consensus as to the optimal dose or exposure time to MMC. The range dose used is typically between 0.02 – 0.05%, with 0.02% more commonly adopted due to the fact that lower concentrations reduces the potential risk of MMC-related side effects. MMC treatment is usually intraoperative or postoperative with an exposure time between 2 – 5 minutes and is typically applied to the eye on a sponge soaked with MMC solution (Abraham *et al.*, 2006). Different studies regarding the use of MMC in glaucoma surgery have been performed in paediatric population (Badeeb O., 2008). In Saudi Arabia there is a higher incidence of congenital glaucoma than in other parts of the world, so in 1998 Ali Al-Hazmi (1998) studied the effectiveness and complications of the use of MMC in children as an adjuvant treatment in paediatric glaucoma surgery. The results showed that the success of that surgery depends on the age of the child, but in general suggests that

outcomes after surgical procedures are similar to adults, improving with postoperative application of MMC.

To perform refractive surgery there are different techniques such as excimer laser refractive keratomy, photorefractive keratectomy (PKR), laser-assisted in situ keratomileusis (LASIK), laser epithelial keratomileusis (LASEK) or epithelial laser in situ keratomileusis (Epi-LASIK). All of these techniques have been used historically or presently to treat eye conditions such as myopia, hyperopia or astigmatism (Mcalinden, 2012). However, currently LASIK and PKR are more commonly used, with LASIK being most preferred since post-surgical recovery is faster and the risk of corneal haze and postoperative pain is the least of all techniques. To prevent corneal haze, MMC is applied to regulate corneal wound healing (Abraham *et al.,* 2006).

Dacryocystorhinostomy (DCR) is a procedure to unblock the tear drainage system by re-opening the connection between the tear sac and the inner aspect of the nose to where it drains. Silicone tubes are implanted during the operation, and are normally removed three months post-surgery. The main complications of DCR are obstruction of the canaliculus and the closure of the osteotomy site. Several studies report reduced failure rates when MMC is topically applied at the time of surgery (Abraham *et al.*, 2006).

Apart from the use of MMC in non-cancerous ocular surgery, MMC is also used to treat ocular surface tumours. The principal therapy for ocular surface squamous neoplasia is surgical excision followed by topical application of MMC. Since 1994, MMC has been adopted as a chemotherapeutic agent either peri- or post-operatively. The clinical effectiveness of MMC treatment

in this indication has been confirmed after years of use. Chen *et al.* (2004) compared the adjuvant use of MMC following surgical excision of lesions in primary ocular surface squamous neoplasia (OSSN). The main problem of using the primary excision technique by itself is the high rate of recurrence (15-52%). However they reported that there was no evidence of clinical recurrence after 25 months in patients with OSSN where all lesions were surgical excised and treated with MMC after complete epithelial healing.

Primary acquired melanosis (PAM) with atypia is a precancerous lesion on the surface of the eye characterised by an excessive proliferation of melanocytes, which can become malignant resulting in a conjunctival melanoma (Pe'er *et al.*, 2005). Several studies have shown a positive clinical response to topical ocular application of MMC in patients with this condition. Finger P. *et al.*, 1998 investigated the ophthalmic application of 0.04% w/v MMC in 10 patients as an adjuvant treatment. The results were satisfactory either in PAM or conjunctival melanoma. Pe'er *et al.* (2005) concluded that topical ocular application of MMC is an acceptable alternative treatment to surgical excision and cryotherapy in treating conjunctival PAM with atypia.

2.1.4 Clinical risks assessment of the use of mitomycin C eye drops

Although MMC use in the eye is not licensed, in many decades of use the side effect profile of MMC appears to be acceptable with few reports of major clinical side effects. In Table 2-1 is presented a summary of major and minor side effects related with the uses of MMC as unlicensed medicine in ophthalmologic practice.

Table 2-1 - Complications and side effects of MMC application in ocular surgery and ocular malignancies (Abraham L.M. *et al.*, 2006)

Application	Type of application	Complications
Glaucoma filtering surgery	Ocular surgery	Corneal epithelial toxicity Bleb leaks Blebitis Hypotonic maculopathy Choroidal effusion Cataract formation Shallow anterior Chamber Suprachoroidal haemorrhage Endophthalmitis Hyphaemia Upper eyelid retraction Retina haemorrhage
Pterygium surgery	Ocular surgery	Photophobia Sclera thinning Irritation Ocular pain Infection Sclera ulceration Cataracts Superficial scleral melting Corneal perforation Secondary Glaucoma
Refractive surgery	Ocular surgery	Vascular endothelial injury Tissue necrosis Cytotoxicity Ocular pain
Dacryocystorhinostomy	Ocular surgery	Foreign body sensation Photophobia Corneal toxicity Ocular pain
OSSN	Ocular malignancy	Allergy reactions Punctal stenosis Epiphora Limbal stem cell deficiency Toxicity
PAM	Ocular malignancy	Ocular irritation Tearing Intumescent Corneal oedema Keratitis
Conjunctival melanoma	Ocular malignancy	Cataract Ocular pain

Amongst the side effects derived from the uses of MMC in ophthalmology practice is important to highlight the cytological toxicity, hypotony or allergy and epiphora, as the complications that are more likely to appear when MMC is applied into the eye. It is known that the antifibrotic effect that leads MMC to have the clinical properties is the same mechanism that provokes the cytological toxicity of this compound (Lama *et al.*, 2003). Mietz *et al.*, (2005) evaluated the complications of the postoperative MMC use in primary trabeculectomy to treat glaucoma. Their study consisted in a prospective randomized trial, where all the 52 patients were performed the primary trabeculectomy but only in 26 eyes were applied MMC after the surgery. The follow up was performed for at least 24 months after the surgery, and the most common side effect associated with the use of MMC was found to be hypotony (Intraocular pressure < 6mmHg). This complication is mainly caused because MMC provokes a thinning effect in the bleb that result in micro bleb leaks (Al Habash *et al.*, 2015) developing in some cases endophthalmitis, also known as intraocular infection (Abraham *et al.*, 2006). Within this study 50% of the group of patients treated with MMC after surgery and 35% of controls suffered this side effect. However no other major complications were developed. Regarding minor side effects, this study did not found significant differences between the control and the postoperative MMC group.

The allergy reactions are common side effects in patients treated with MMC, itchiness or dermatitis are some of the symptoms of this complication. It is more likely to develop this adverse reaction after the application of the second cycle of the drug than with the first contact; however there have been

of a few cases in which there was an allergy reaction during the first course of treatment (Khong *et al.*, 2006).

Although MMC is used intraoperatively in DCR to increase the success of this surgery avoiding the obstruction of the canaliculus (Liao *et al.*, 2000), this drug can cause as a complication epiphora or watering eye. It is not known the cause of this complication or whether or not the number of applications of MMC can influence. It was believed that it could be caused by a toxoallergic reaction, developing an inflammatory response that could provoke punctual stenosis or stenosis of the canaliculus. However it cannot be probed a relation between allergy reaction and epiphora with the data recovery until the moment (Kopp, 2004).

Cytologic changes and cytotoxicity can be observed when MMC is applied in the eye. Salomao *et al.* (1999) described changes observed in the conjunctiva after the application of MMC in eyes affected with PAM with atypia. It is important to highlight that the changes provoked by MMC in the conjunctiva can be mistaken by conjunctival squamous neoplasia which could cause difficulties in the differentiation of the effects of this two conditions in patients treated with MMC.

2.1.5 Stability profile of MMC

Stability is the capacity of a compound to maintain purity, quality and identity over a defined period of time within established limits. This period of time will be considered as the shelf life of the final pharmaceutical product. All drugs are susceptible to degradation because of different factors

including inappropriate storage or incorrect use. Conditions such as temperature, pH or light could affect the stability of a drug. Humidity, some types of additives or excipients, oxygen, and also the material of the container could also have an important impact on the potency and quality of the final product compromising the stability of the formulation and therefore risking the safety of the patient (ICH, 2003).

MMC is presented as a crystalline blue-violet powder soluble in water. Although it has good chemical stability in its solid form at room temperature, several studies have reported that MMC is unstable in aqueous solution. Chemical degradation of MMC in aqueous solution is pH-dependent, with greatest stability at pH 7 - 8 (Beijnen *et al.*, 1986). The kinetics of MMC degradation in aqueous solution and the influence of pH or temperature are well recognised. In the 1980s Beijnen *et al.* studied the chemical stability of MMC in different pH solutions. MMC shows ultraviolet (UV) absorbance at 365nm. However above pH 13, different compounds absorbing at 313 nm instead of 365 nm were detected by UV which indicates a dramatic change in the structure of MMC at this pH (Beijnen, *et al.* 1985). The eye drop formulation are developed with a pH close to physiological, therefore those degradations products formed at pH 13 should not be detected during the stability studies. Under acidic conditions aqueous degradation pathways are much more complicated. For example, at acidic pH, the C9a function of MMC is cleaved, forming a double bond, leading to opening of the aziridine ring to yield isomeric mitosene compounds (Beijnen, *et al.*, 1985). Apart from the relation between pH and degradation process of MMC, Beijnen *et al.* (1986) also studied the influence of the buffer components and temperature on the degradation process of MMC in basic

solution. It was understood that in alkaline conditions the type of buffer have an impact on the degradation of MMC at 25 °C or lower temperatures, being more unstable in borate and phosphate buffer. Acetate, nitrate and chloride ions do not influence the stability of this compound in alkaline conditions. Although Beijnen *et al* research about the stability of MMC in different conditions was extensive, changes of the structure of the molecule were reported previous to them (Garrett, 1963). The commercial manufacturer of MMC, Kiowa, has also published stability data in different non-buffered solvents and conditions for clinical practice. MMC stored at room temperature for 2 days suffers 10%, 13% and 15% degradation when is reconstituted with water for injection, normal saline and 5% w/v glucose respectively. When the storage temperature is lowered to 5°C there is little difference on degradation rates compared to room temperature. MMC showed a limited stability reaching 90% of the starting content after 3 days under refrigeration in all 3 reconstitution solutions mentioned above (Kyowa Hakko kirin Co., 2013). When 5% w/v dextrose is used as a vehicle, stability is highly dependent on pH (Dorr R.T., 1995). Those findings reaffirm the theory that MMC stability depends on pH. Benvenuto *et al.* (1981) studied the influence of glass and plastic storage containers on the stability of MMC in different solvents over 24 hours stored at room temperature, and concluded that although MMC dissolved in NaCl 0.9% w/v was stable in polyvinyl chloride plastic (PVC), MMC in 5% w/v glucose was not stable in either glass or plastic containers.

All of the above studies reinforce the point that is essential to control the pH of the solvent vehicle in order to enhance MMC chemical stability in the finished product. In addition, Quebbeman *et al* (1985) published a study on

the stability of MMC admixtures, and reported that MMC dilution in unbuffered admixtures of either 0.9% w/v NaCl or 5% w/v glucose exhibited low stability within 24h. In contrast, buffered MMC solutions exhibited no significant changes in concentration over 120 days when stored at 5°C.

2.2 Formulation and stability studies of mitomycin C eye drops

2.2.1 Introduction

Presently, at the PPU (NHS GG&C), MMC eye drops are aseptically prepared dissolving the commercial intravenous (IV) MMC powder in sterile Phosphate Buffered Saline (PBS) obtaining the desired final MMC concentration of 0.02% - 0.04%. The shelf life assigned to this unlicensed formulation by the PPU is 6 weeks under refrigeration. The strategy started confirming the shelf life of the MMC eye drops manufactured at the PPU within NHS GG&C, followed by developing a novel ophthalmic product containing MMC with an extended shelf life.

When it comes to reformulation, it has to be taken into account that water-based solvent vehicles are commonly chosen for the manufacture of a range of pharmaceutical products due to their high biological compatibility and low cost. However occasionally there may be compounds that have poor aqueous solubility or are unstable in aqueous solutions. In those cases a water-miscible co-solvent or non-aqueous solvent can be used as an alternative, although, the biological toxicity of such systems can limit their use to specific routes. The instability of MMC powder in aqueous solution limits its usage as eye drops. Therefore, a formulation with reduced water content to provide long-term stability and then, adding the aqueous solution

at point of reconstitution for patient use would be desirable. The stability of a ready to use formulation would also be investigated to limit the health and safety risks during the reconstitution stage.

Bristol Myers Squibb Company published a patent in 1991 about the stability of MMC in non-aqueous systems such as glycerine, PG, PEG, dimethylacetamide, N-methylpyrrolidinone or 2-pyrrolidinone. The aim of this invention was to have a MMC ready to use intravenous formulation and therefore avoid or reduce the health hazard that reconstitution presented. Some of those solvents are not recommendable for ophthalmic applications but others such as PG or PEG can be considered safe. The results published shown a superior stability of MMC in PG/Water co-solvent compared with the other solvents in a concentration range between 0.5 mg/ml and 12 mg/ml. In addition to the stability profile of the drug in PG, MMC has also a good solubility in PG, approximately of 10.4 mg/ml at a pH of 7.4 (Mehdi Paborji *et al.*, 1991).

Based on the patent, a novel MMC eye drops formulation was designed using a non-aqueous excipient to improve stability of MMC. It was envisaged that the final concentration of MMC in this formulation would be 0.2 mg/ml. The pharmacologically inactive ingredient chosen was PG. It is considered to have very low human toxicity, and is widely used as a pharmaceutical excipient in ophthalmic preparations. The FDA (2015) stated that the maximum potency of PG as an inactive ingredient in ophthalmic solution is 10%, therefore this was used as the upper limit during the development stage. PG is also used in topical pharmaceutical formulations due to its penetration enhancing properties, which may increase skin and

mucosa permeability improving the transport of drugs (Lane, 2013). Per contra, this characteristic of PG could have a negative impact in the context of MMC application to the eye. This is because it may cause an increased ocular bioavailability of the active ingredient, which could raise the amount of localised ocular side effects.

Stability studies of pharmaceutical products ensure the quality, safety and efficacy of the product throughout the shelf life period. For that reason, testing of a pharmaceutical product over a period of time is a pre-requisite before regulatory approval and it is also mandatory for unlicensed medicines. During the design of those studies, all the environmental aspects that could have an effect upon the stability of the product need to be evaluated (WHO, 2009).

When carrying out stability studies the first stage is developing and validating a stability indicating method to test the finished product. Reverse Phase High Performance Liquid Chromatography (RP-HPLC) is an analytical technique widely used in the assessment of stability of pharmaceutical compounds. Chromatography is a technique for the separation of a mixture by passing through a medium in which the components move at different rates. RP-HPLC consists of a relatively non-polar stationary phase column and a polar mobile phase typically aqueous-based solution. The basic principle of this technique is that the individual analytes will elute from the column at different velocities depending on their polarity and relative affinity for the reverse and polar phases. In RP-HPLC the most polar compounds elute first and non-polar molecules later (Lindsay, 1992).

The HPLC system includes a pump to deliver the mobile phase with a defined composition and at a defined flow rate. The mobile phase is filtered and degassed before use to eliminate any particles and air bubbles that could influence the pressure of the system and its subsequent performance. The sample is placed in the auto sampler module and it is introduced automatically into the system at the desire volume through the injector device. Once the sample is injected chromatographic separation of analytes occurs due to different partitioning activities between mobile phase and column. The temperature of the column should be constant for the achievement of accurate retention times (RT). An increment on the temperature of the column could increase the speed of the separation process. Therefore it is recommended to regulate the temperature with an oven. Finally, the separated sample components go through a detector. All data obtained from the analysis is captured and analysed using the system software chosen (WHO, 2015).

During the development stage the type of instrumentation including the detector needs to be defined, and also a suitable column for RP-HPLC to optimise separation. Once this has been decided, the experimental conditions to run a reference standard will be determined. Initial settings depend upon the physicochemical characteristics of the analytes. A run with the initial conditions will be performed to establish the suitability of the method to separate a mixture of compounds, and iterative changes to improve separation made. After those changes have taken place, experimental conditions will be established for the final method and the validation tests can be performed to ensure the method is adequate for purpose. When the

methodology has been developed and validated, the stability study can be designed (Lindsay, 1992).

As it can be seen in Table 2-2 different HPLC methods have been described over the past years to study the stability of MMC. Quebbeman *et al.* (1985) described an HPLC-UV method used to study the stability of MMC. A similar analytical method was used by Gupta *et al* in 1997. Also Beijnen *et al.* (1985a, b; 1986) published 3 papers relating to degradation paths of MMC in alkaline and acidic solution and the stability of this active ingredient in solutions for intravesical instillation respectively.

Table 2-2 - Published methods to quantify MMC.

Author	Year	Stationary phase	Mobile phase	Gradient	System	Flow rate	UV detection	Cal curve range
Quebbeman	1985	Alltech C_{18} (250 x 4.6mm) 10um particle size	50 mM monobasic sodium phosphate buffer and methanol (70/30 vol/vol) pH 5.5	Isocratic	Waters - 6000A pump, 440 UV detector, WISP 710b autosampler, 730 data module	2ml/min	365 nm	0-400 ug/ml
Beijnen *et al.*	1985	Lichrosorb 10 RP-18 (300 x 3.9 mm)	Methanol/ Water (40/60) 0.5% (v/v) Acetic acid was added. pH adjusted to 4.3 with NaOH	Isocratic	Waters - 6000A pump, U6K injection device,440 UV detector, SP4000 integrator	1 ml/min	254 nm	$3*10^{-6}$ to $2*^{10-6}$ M
Beijnen *et al.*	1985	Lichrosorb 10 RP-18 (300 x 3.9 mm)	Methanol/0.5M Phosphate buffer(pH 7.0)	Isocratic	Waters - 6000A pump, U6K injection device,440 UV detector, SP4290 integrator	1 ml/min	365/313nm	$3*10^{-5}$ to $9*10^{-7}$ M
Beijnen *et al.*	1986	Lichrosorb 10 RP-8 (125x4mm) 5um	ACN/Water (15/85) glacial acetic acid pH3.1	Isocratic	Waters - M-45 solvent delivery system,440 UV detector, SP4270 integrator	1 ml/min	254/313nm	$1.5*10^{-4}$ to $2*10^{-6}$ M
Beijnen *et al.*	1986	Radial-Pak C_{18} (100x8mm) 10um	Methanol/1mM Phosphate buffer (pH7.0)	Gradient	Waters - M-45 solvent delivery system,440 UV detector, SP4270 integrator	3ml/min	254/313nm	$1.5*10^{-4}$ to $2*10^{-6}$ M
Yamazoe *et al.*	1996	Cosmosil 10 C_{18} (250 x 4.6mm) 10um particle size	0.01 M Phosphate buffer (pH 3.0) / ACN	Gradient	Waters - 600E system controller, 490 UV detector, WISP 712 sample processor	2ml/min	220nm	0.3-8 ug/ml
Carlucci *et al.*	1999	Nova-Pak (150x3.9mm) 4um	Methanol/0.01M Phosphate buffer (pH6.5) (30/70)	Isocratic	Waters - 510 Pump, 484 UV detector, 740 data module integrator, 7125 sample injector system	1 ml/min	360	10-2000 ng/ml
Velpandian *et al.*	2008	C8 kromosil (250x4.6 mm) 5um	phosphate buffer (pH 6.5):ACN (80/20 vol/vol)	Isocratic	Thermo surveyor HPLC system	1 ml/min	365nm	Not specified

Once a suitable method for stability testing is developed and validated the next stage is to design the stability study. All potential factors that could have an impact on the stability of the final product should be studied. The stability characteristics of the compound to be studied will help to decide parameters such as storage conditions for the final drug products, sampling methodology or testing frequency (WHO, 2009). The storage conditions are not only based on the degradation paths of the molecule but also on the climatic conditions of the territories where the product will be used and the intended conditions in which the product will be kept when is commercialized (WHO, 2009).

A sampling plan has to be set up as part of the stability testing design. This includes not only the set-up of the testing time points but also the number of samples that need to be evaluated at each time point. In the case where multiple product sizes and different strengths of the formulation are to be manufactured, it is advisable to include matrixing and bracketing design to facilitate batch selection and simplify the testing process (ICH, 2002). The ICH guidelines define bracketing as the design of a stability study where only samples on the extremes of certain design factors are tested at all time points. Under this circumstance it is assumed that the stability of any intermediate levels is represented by the stability of the extremes tested. And matrixing is defined as the design of a stability schedule where a selected subset of the total number of possible samples for all factor combinations would be tested at a specified time point. Therefore, only at time zero and at the end of the study all the batches on stability needs to be tested (ICH, 2002).

2.2.2 Materials

To manufacture MMC eye drop formulations, sterile freeze dried vials containing 10 mg MMC and 95 mg sodium chloride were used. These vials (license product) were kindly supplied by the PPU within NHS GG&C and were obtained from Kyowa Hakko Kirin UK Ltd., (Berkshire, United Kingdom). PG was purchased from Fagron UK Ltd., (Newcastle upon Tyne, United Kingdom) and the sterile phosphate buffer saline (pH 7.4) was obtained from Tayside Pharmaceuticals (Dundee, United Kingdom). The PPU also donated the batches of MMC eye drops they currently manufacture (Glasgow, United Kingdom).

To develop and validate the HPLC method, potassium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Sigma Aldrich Inc. (Dorset, United Kingdom), the methanol and acetonitrile (ACN) was obtained from Fisher Scientific (Leicestershire, United Kingdom). All chemicals were HPLC grade and in-house 18MOhm deionised water was used.

2.2.3 Methods

2.2.3.1 Formulation of novel MMC eye drops

To prepare the novel MMC eye drops formulation, 5 mL of an 80:20 (v/v) PG/PBS was added to a 10 mg vial of freeze dried MMC and the contents were gently agitated until the powder had dissolved. Aliquots containing 1mg of MMC were transferred to 10 ml diagnostic bottle made of type 1 amber glass with screw. The dimensions of the eye drop bottle were (OD x

H) 29.4 mm x 56.7 mm and they were capped with a glass bulb dropper. Material was stored at 4 °C until required. At that time the sample was diluted with a further 4 ml of PBS to provide a final concentration of 0.02% w/v and 8% v/v of MMC and PG respectively.

2.2.3.2 Reverse phase high performance liquid chromatography (RP-HPLC) method for MMC quantification

2.2.3.2.1 Development of the method

To carry out the testing of MMC samples, a Thermo Finnigan HPLC system was chosen. This instrument is equipped with a Surveyor LC pump, Surveyor auto sampler and finally a Surveyor photo diode-array detector (PDA). Chromeleon software was used for the integration of the chromatograms obtained during the testing.

The physical and chemical characteristics of the sample impact on what type of column should be used. Because of that, it is important to study the structure of all the components of the given sample, and to identify its physicochemical characteristics such as pKa and solubility, pH of the solution to be tested or concentration range of those compounds (Lindsay, 1992). The only compound to be studied was MMC and Table 2-3 shows the basic properties of the compound.

Table 2-3 - Physicochemical characteristics of MMC

Mitomycin C	
Solubility in water @ 25 °C[1]	0.8 mg/mL
Solubility in water @ 15 °C[1]	0.5 mg/mL
Solubility in water @ 5 °C[1]	0.3 mg/mL
pKa*[2]	2.7 – 3
pKa**[2]	5.1
Molecular Weight[3]	334.33

* pKa value of oxidize form of MMC **pKa value of reduce form of MMC

There are several types of columns available in the market with different dimensions, size particles or range of pH stabilities. The selection of the column can directly impact on the final results of the separation such as peak asymmetry, RT, pressure of the system and resolution. However, there is always the possibility to manipulate the conditions of the system to achieve optimal separation (Lindsay, 1992). In this particular method development a bonded-phase silica-based HPLC column (C18) was used. LiChrospher® 5 µm RP-18 100 Å, 125 x 4 mm was obtained from Phenomenex® (Macclesfield, UK)

The effect of different mobile phases in the RT and the shape of the peak of MMC were studied. ACN was initially evaluated as the organic component of the mobile phase. The aqueous buffer chosen to mix with the organic

[1] Source from Kyowa Hakko Kirin Co.

[2] Source from Boruah, R. C., & Skibo, E. B. (1996). Determination of the pKa Values for the Mitomycin C Redox Couple by Titration, pH Rate Profiles, and Nernst-Clark Fits. Studies of Methanol Elimination, Carbocation Formation, and the Carbocation Quinone Methide Equilibrium, (7), 2232–2243.

[3] Source from sigma-aldrich.com

solvent in different proportions was phosphate buffer pH 5.5 described by Quebbeman *et al.* (1985).

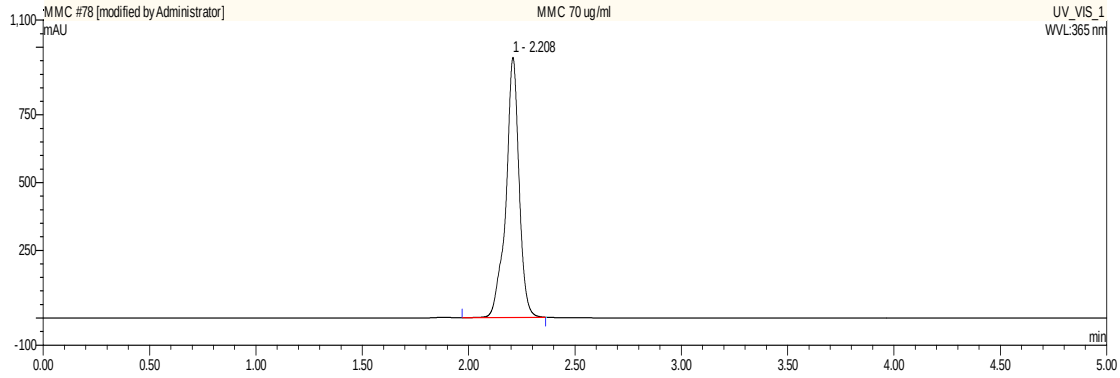

Figure 2.4 - Option A of mobile phase (ACN:Phosphate buffer pH 5.5 – 30:70 v/v)

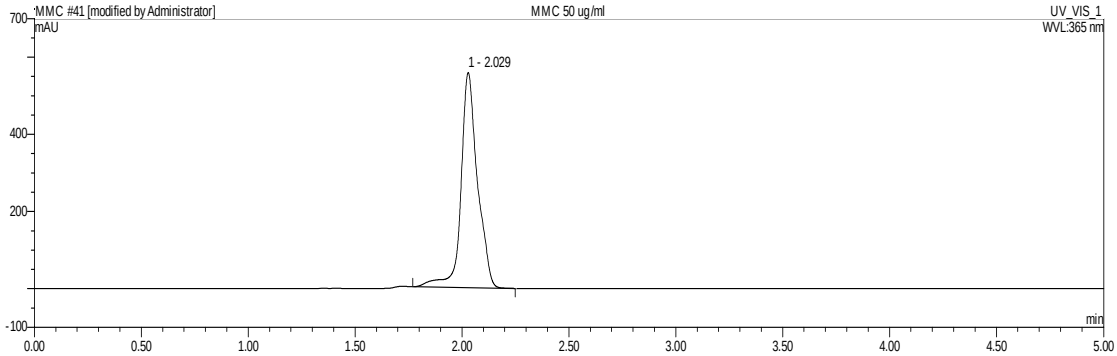

Figure 2.5 - Option B of mobile phase (ACN:Phosphate buffer 5.5 - 35:65 v/v)

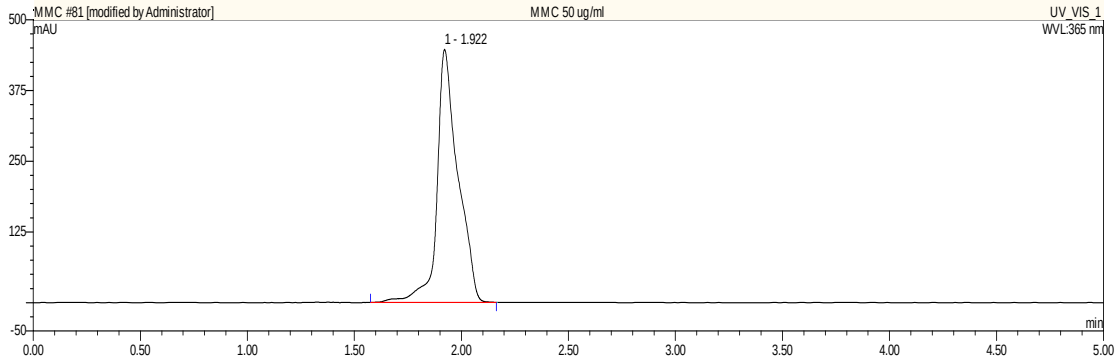

Figure 2.6 - Option C of mobile phase (ACN:Phosphate buffer pH5.5 – 40:60 v/v)

Initial experiment using ACN solvent as part of the mobile phase showed that the optimal composition was ACN:Phosphate buffer pH:5.5 (30:70 v/v).

However, all three chromatograms (Figure 2.4, Figure 2.5 & Figure 2.6) showed that the main peak was not clearly defined suggesting a minor shoulder peak present. It looks like there is a co-elution of MMC and a different compound suspected to be a fermentation product.

It was decided to substitute the solvent used for methanol, the new mobile phase was then composed by Methanol:Phosphate Buffer pH:5.5 (30:70 v/v), and the comparison highlighted a better resolution when methanol was used as part of the mobile phase (Figure 2.7). In contrast with the 30:70 (v/v) ACN mobile phase, MMC eluted at 2.6 min and had a peak symmetry of 1.02. Using methanol as a solvent not only the symmetry of the main peak has improved but also the resolution between the main peak and the possible fermentation product.

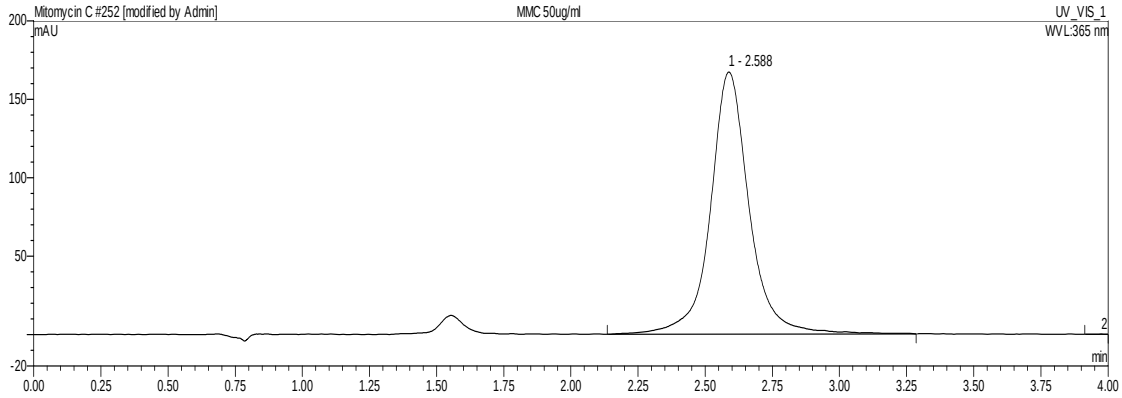

Figure 2.7 - Option D of mobile phase (Methanol:Phosphate buffer pH5.5 – 30:70 v/v)

Variations in the flow rate have an effect on the elution time of the peak in the chromatogram, on the band broadening and on the resolution (The Theory of HPLC Band Broadening, 2012). Figure 2.8 – 2.10 show the chromatograms resulting from injecting a 50 µg/ml MMC sample onto a LiChrospher® 5 µm RP-18 100 Å, 125 x 4 mm at different flow rates 0.8

ml/min, 1 ml/min and 1.5 ml/min respectively. The mobile phase used was Methanol:Phosphate buffer pH:5.5 (30:70 v/v).

The RT of MMC is 5 min, 4 min and 2.6 min at a flow rate of 0.8 min/ml, 1 ml/min and 1.5ml/min respectively. Although the peak at 1.5 ml/min appears soon in the chromatogram, the shape of the peak is the best of all of them. The peaks in Figure 2.8 and Figure 2.9 are broad and are tailing comparing with the peak at a flow rate of 1.5 ml/min. Based on these experiments the flow rate of 1.5 ml/min was selected as being the preferred mobile flow rate.

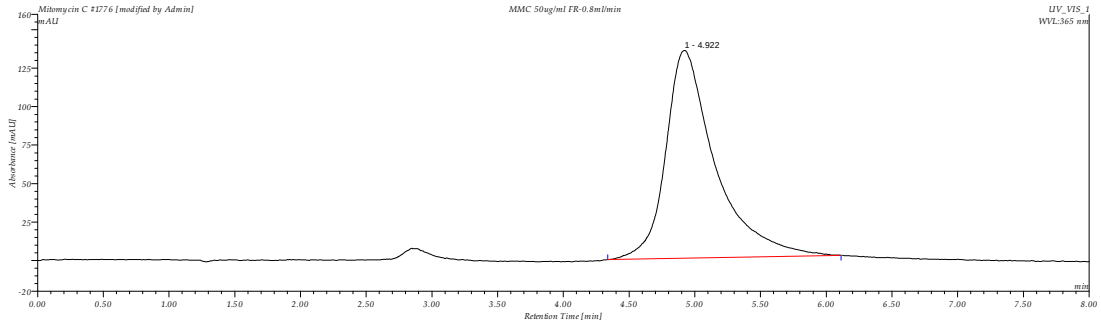

Figure 2.8 - MMC peak at a flow rate of 0.8 ml/min

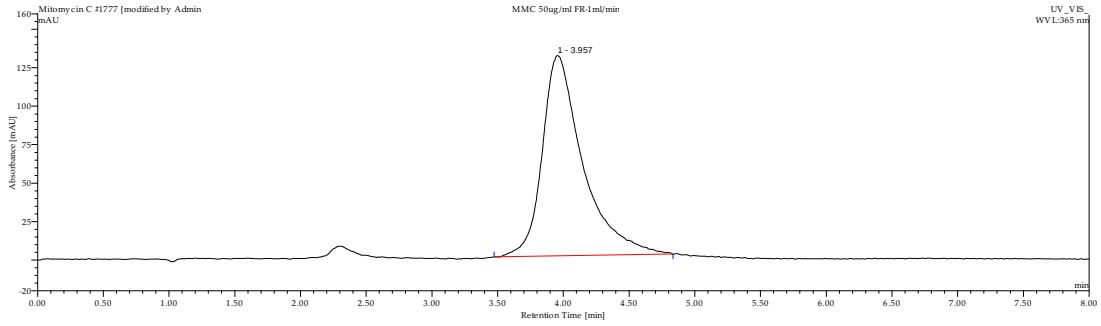

Figure 2.9 - MMC peak at a flow rate of 1 ml/min

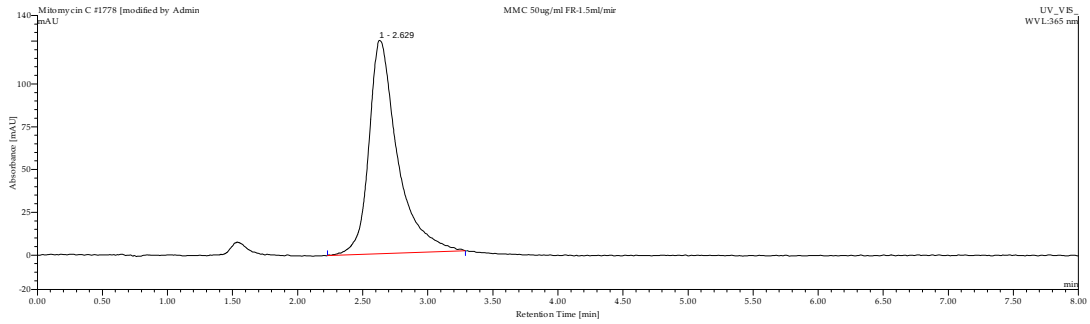

Figure 2.10 - MMC peak at a flow rate of 1.5 ml/min

Following from the preliminary method development, the final chromatographic conditions of the RP-HPLC method are summarized in Table 2-4.

Table 2-4 - Chromatographic conditions of the HPLC method develop to assay

Chromatographic conditions	
Mobile phase	Methanol:Phosphate buffer pH 5.5 (70:30 v/v)
Flow rate	1.5ml/min
Detection Wavelength	365nm
Temperature	21°C

2.2.3.2.2 Validation of the method

Validating the analytical method is essential to demonstrate that is suitable to perform stability testing of MMC eye drops (ICH, 1996). This section describes the results obtained from the validation of the analytical procedure developed. The validation procedure was carried out according to the ICH guidelines and conforming to the rules governing medicinal products in the European Union, Eudralex guidelines. The document ICH Q2 (R1) 'Validation of analytical procedures: Text and methodology' describes the characteristics and requirements needed to validate different types of

analytical procedures such as identification, test for impurities or assay (Table 2-5).

Table 2-5 - Test attributes and their validation parameters

	Identification	Assay
Accuracy	-	+
Repeatability	-	+
Intermediate precision	-	+
Specificity	+	+
Detection limit	-	-
Quantification limit	-	-
Linearity	-	+
Range	-	+

Source: ICH Guidelines

All attributes were validated in this research, including those related to the identification of MMC. And also the attributes related to the quantification of the active ingredient (assay).

- **Identification**

The purpose of the identification test is to detect and differentiate the compounds in a sample. Only specificity needs to be studied (ICH, 2005). The ICH Q2 R(1) defines specificity as the capability to distinguish different compounds with similar structures. To assess the specificity a forced degradation study was carried out. The objective of this assessment was to prove that the analytical method developed was able to separate the API, MMC, from degradation products potentially formed during the study.

MMC is not stable at alkaline pH (Beijnen *et al.* 1985), so degradation studies were carried out to stress a MMC sample and observe whether degradation products and their resultant peaks could be suitably characterised. A sample of 50 μg/ml was degraded in 0.05 M NaOH.

Figure 2.11 demonstrates the impact of incubation in alkali solution upon MMC stability. It can be observed that the area under the curve (AUC) of the MMC peak (RT 2.909 mins) is dramatically reduced with the simultaneous appearance of two degradation products with RT of 0.9 & 1.1 mins respectively. The peak eluting at 1.7 minutes was also detected before any forced degradation of the sample, therefore this peak is likely to be a fermentation product or a contaminant from the manufacturing process.

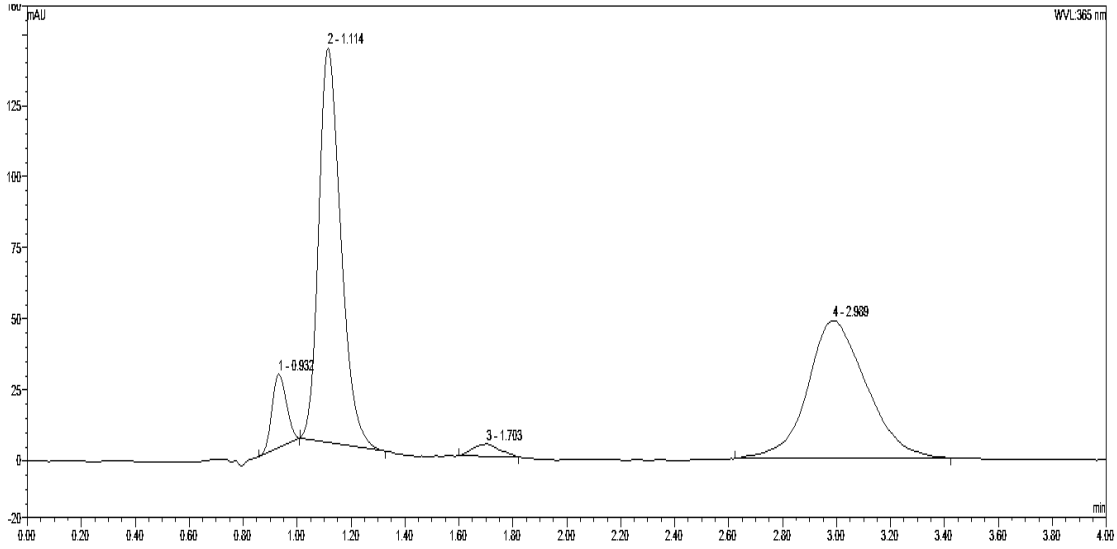

Figure 2.11 - Degradation of a MMC sample prepared in 0.05M of NaOH.

- **Assay of mitomycin C**

The main objective of the assay procedures is to quantify the active ingredients in a sample. The following parameters have been studied in the

validation process of the HPLC method: specificity, linearity, accuracy, range, intermediate precision and repeatability (ICH, 2005).

Specificity was already treated under identification study. The linearity demonstrates the relationship between the concentration of the active ingredient of a sample and the AUC obtained with the HPLC apparatus (ICH, 2005). In this case, the linearity was assessed in a range from 30μg/ml to 70μg/ml (60% - 140%). The test was carried out at 5 different concentrations corresponding to 5 calibration levels, each point of the calibration curve was injected and measured three times, the mean was calculated and the resultant value was used for linearity analysis.

Table 2-6 shows the mean values of the data collected from HPLC runs over 5 different concentration levels and the Figure 2.12 demonstrates a coefficient of determination >0.9988.

Table 2-6 - Summarised all the data collected to assess the linearity of the procedure (n=3).

Level of calibration curve	Concentration (μg/ml)	Mean AUC±SD
1	30	18.55±0.26
2	40	24.70±0.29
3	50	30.70±1.03
4	60	35.50±0.38
5	70	42.79±0.86

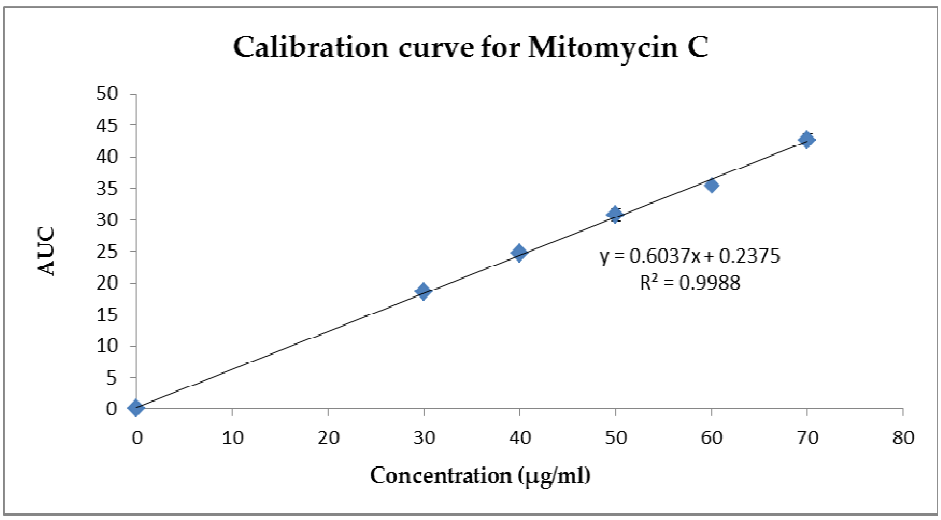

Figure 2.12 - A typical calibration curve obtained to quantify MMC (n=3)

To assess the precision of the system two different factors need to be evaluated, the repeatability or intra-assay precision, and the intermediate precision (ICH, 2005).

Two types of test were carried out to assess repeatability. The first one consisted of 3 replicates of 3 different concentrations levels to determine the experimental amount of MMC, calculating the average of the values and the relative standard deviation (RSD). The level of concentration chosen had to cover the assumed range for the analytical method to demonstrate the precision over those concentrations (30µg/ml - 70µg/ml).

Table 2-7 summarizes the information obtained once the samples were injected onto the HPLC. As the results show, only the concentration of 50 µg/ml had a RSD (2.59%) above the permitted limit of 2%. The RSD of the AUC in the rest of the levels was below 2% (RSD < 2%).

Table 2-7 - Repeatability data obtained from HPLC (n=3)

Sample	Theoretical amount (μg/ml)	Experimental amount (%)	MEAN	%RSD
1		100.13	99.56	1.45
2	30	100.63		
3		97.91		
1		97.88	101.14	2.59
2	50	101.10		
3		104.80		
1		102.10	100.74	1.90
2	70	102.41		
3		98.66		

Another approach to assess repeatability is to study a sample at 100% of the test concentration. In this case, 6 preparations of MMC with a concentration of 50 μg/ml were injected into the system. The RSD of the experimental amount obtained with the HPLC was also calculated. The main reason for choosing 50 μg/ml as the calibration level was that this represents the concentration of the test sample and also confirms the results obtained in the repeatability test where the RSD was above 2%. In this new experiment was confirmed that the RSD value is less than 2% suggesting that human error or random variation may have been contributory to the result.

Table 2-8 - Results of repeatability of the system

Sample	Theoretical amount (μg/ml)	Experimental amount (μg/ml)
1	50	49.63
2	50	49.47
3	50	49.08
4	50	49.17
5	50	50.36
6	50	50.40
	Mean	49.69
	%RSD	1.16

The methodology to assess intermediate precision depends upon the conditions that the procedure is intended to be used (ICH, 2005). The variation factor studied was the precision of the system on different days. The experiment consisted in preparing and running a calibration curve on 3 different days to determine the content and calculate the RSD and therefore the precision intra-day of the method. Table 2-9 shows that the RSD was not more than 2% in all cases except for 30 μg/ml where the RSD was 2.28%.

Table 2-9 - Results of the inter-day precision study (n=3)

Theoretical amount (μg/ml)	Day	Experimental amount (%)	MEAN	SD	%RSD
30	1	30.21	29.76	0.68	2.28
	2	28.98			
	3	30.09			
40	1	40.59	40.41	0.50	1.24
	2	40.8			
	3	39.85			
50	1	50.97	50.87	0.42	0.82
	2	51.23			
	3	50.41			
60	1	58.85	59.05	0.17	0.30
	2	59.18			
	3	59.11			
70	1	71.08	70.44	0.66	0.93
	2	69.77			
	3	70.48			

Accuracy should be performed after linearity, specificity and precision have been established. To confirm the accuracy of the method the recovery of 9 determinations of 3 different concentration levels was assess in the range of 70% to 130% (ICH, 2005). The mean recovery results (Table 2-10) of 35 μg/ml, 50 μg/ml and 65 μg/ml test samples was 97.73%, 99.04% and 97.74% respectively.

Table 2-10 - Results of accuracy test

Theoretical amount (μg/ml)	Experimental amount (μg/ml)	Experimental amount (%)	MEAN	%RSD
35	33.71	96.32	97.73	1.48
	34.18	97.66		
	34.72	99.21		
50	50.45	100.9	99.04	2.62
	48.03	96.07		
	50.07	100.14		
65	62.11	95.56	97.74	2.66
	65.4	100.61		
	63.08	97.05		

The range for this procedure was established from linearity, precision and accuracy studies. The analytical method needs to be linear, accurate and precise within or at the extremes of the specified range. In this validation, the test concentration is 50 μg/ml and the range was validated for 60% to 140% of the MMC test concentration (30 μg/ml to 70 μg/ml), so we can confirm that the chosen range complies with the ICH guidelines requirements (80% to 120%) (ICH, 2005).

2.2.3.2.3 Summary of validation results

Table 2-11 summarises all the results obtained from the validation of the method summarized.

Table 2-11 - Summery of validation results

Procedure	Attributes	Results
Identification	Specificity	Demonstrated the separation of the active ingredient from the degradation products.
Assay for MMC	Specificity	Demonstrated the separation of the active ingredient from the degradation products.
	Linearity	60%-140%
	Range	60%- 140%
	Repeatability	RSD< 2.6%
	Repeatability of the system	RSD< 1.15%
	Intermediate precision	RSD< 2.3%
	Accuracy (Recovery)	35 μg/ml - 97.73% 50 μg/ml – 99.04% 65 μg/ml – 97.74%

2.2.3.3 Stability study design

A pre-stability study was initially performed on the formulation manufactured at the PPU within NHS GG&C and also on the novel formulation pre-reconstitution. A further long-term study of the pre-reconstituted MMC in PG was carried out. In parallel, a stability study of the novel formulation already reconstituted was also performed, to assess the stability of a ready to use form of the eye drops.

During the initial study, three batches of 15 bottles of 0.02% w/v MMC eye drops were aseptically manufactured and donated by the PPU (NHS GG&C). Also 9 bottles per batch of the novel ophthalmic formulation at a concentration of 0.02% w/v were manufactured in uncontrolled (non-sterile) conditions in the University of Strathclyde. One batch of each formulation were stored at 4 °C, 25 °C and 37 °C and tested frequently for stability over 6 months.

With the data obtained in this pre-study, kinetics of chemical decomposition was determined. The decomposition of a drug can follow different types of reactions. When the decomposition occurs in a constant rate and it does not depend on the concentration of the active ingredient then this is considered a zero-order reaction kinetics. However, if the degradation is determined by the concentration of the drug, the reaction then follows first order kinetics. There are also second and third order decomposition reactions where 2 or 3 reacting species may be involved (Florence, 2011). The proposed models were tested by fitting the experimental data into the respective kinetic equations, and the model with the best coefficient of determination was used for calculations. Once the reaction order was determined the Arrhenius equation was used to predict the shelf-life of the formulations, and to predict the effect of the temperature on drug decomposition (Florence, 2011).

The decision of storing the novel formulation only at 4 °C in long-term studies was made after analysing the data obtained from the pre-study. The data demonstrated better stability of MMC at lower temperatures, fact that is in agreement with other researchers (Georgopoulos, 2002). The testing frequency complied with ICH guidelines. Therefore, the novel formulation

was tested approximately every 3 months for at least one year. Due to the fact that the novel product was kept in the refrigerator or cold room at 4°C during stability testing the humidity does not have to be under control during the long term study.

In the long-term stability study, 3 batches (10 bottles each) of the novel ophthalmic formulation at a concentration of 0.02% w/v were prepared under uncontrolled conditions. Before reconstitution the samples were stored at 4 °C and tested over a 14 months period. The same storage conditions were used to study the stability of the ready to use form of the eye drop.

2.2.3.4 Sample preparation for HPLC test

2.2.3.4.1 Preparation of calibration standard solutions

A stock solution was prepared by reconstituting a MMC vial with a content label of 10 mg of MMC with 10 mL of water. This solution was transferred to a 50 mL volumetric flask and the volume was made up with deionised water. The calibration standards of 30, 40, 50, 60 and 70 μg/ml were prepared by diluting the 200 μg/ml stock solution and used as calibration standards.

2.2.3.4.2 Preparation mitomycin C 0.02% NHS eye drops formulation for HPLC testing

Bottles of MMC 0.02% w/v formulation were allowed to equilibrate to room temperature before use and the content of the eye drops was gently mixed to ensure uniformity of composition. A 250 μl quantity of the eye drops was

mixed with 750 µl of PBS, obtaining a solution of MMC with a concentration of 50 µg/ml for analysis by HPLC.

2.2.3.4.3 Preparation of mitomycin C 0.02 % w/v novel ophthalmic formulation for short and long-term stability studies

A unit of MMC eye drops were brought to room temperature and reconstituted with 4.5 ml of PBS using gentle agitation to ensure a homogenous solution was obtained. To obtain a final concentration of MMC 50 µg/ml, 250 µl of the reconstituted product was further diluted by addition of 750 µl of PBS and mixed again prior to HPLC analysis.

2.2.3.4.4 Preparation of mitomycin C 0.02 % w/v ready to use formulation for stability studies

The samples kept at 4 °C where brought to room temperature. The content of the eye drops was gently agitated before 250 µl of the eye drops was mixed with 750 µl of PBS to provide a final MMC concentration of 50µg/ml. This was then analysed by HPLC.

2.2.4 Results

2.2.4.1 Pre-stability data of the two mitomycin C eye drop formulations

Stability of the two different MMC eye drops formulations was performed on samples stored at 4 °C, 25 °C or 37 °C. Typical degradation profiles of MMC in both formulations are presented in Figure 2.13 & Figure 2.14. Figure 2.13 shows that the concentration of MMC in the current NHS ophthalmic

formulation maintained over 90% labelled content for 7 weeks when stored at 4 °C. However, less than 90% of MMC remained after one week when stored at 25 °C, and over 30% degraded after one week when stored at 37 °C.

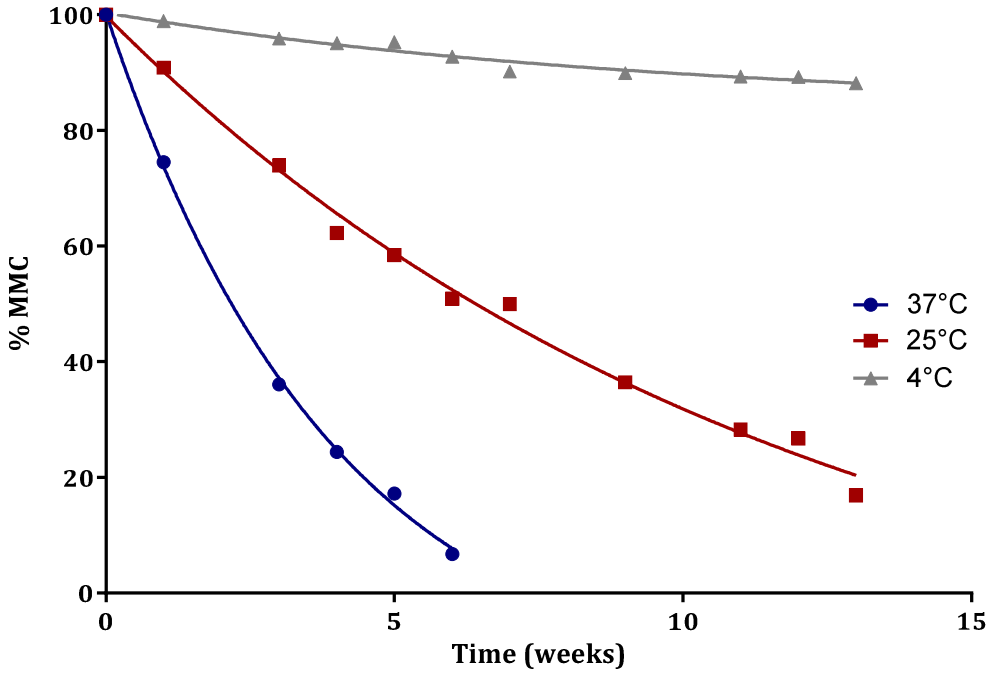

Figure 2.13 - Stability data for MMC eye drops manufactured in the NHS PPSU following storage at 4 °C, 25 °C or 37°C (n=1). Testing period was 13 weeks for 4 °C and 25 °C batches, and 6 weeks for 37 °C batch.

In contrast, for the novel formulation of MMC concentration remained stable for 6 months with approximately only 1% of the content lost when the stored at 4 °C (Figure 2.14). However, batches stored at 25°C or 37°C lost 10% and 30% of the starting concentration in less than 3 weeks respectively.

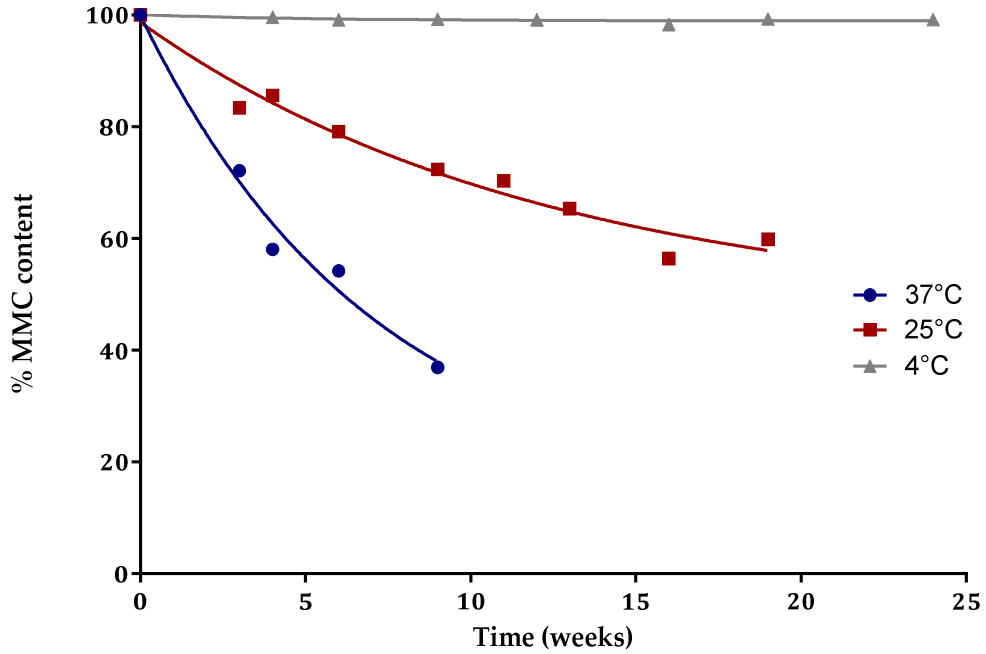

Figure 2.14 - Stability data for the novel MMC eye drops non-aseptically manufactured in the University of Strathclyde at 4 °C, 25 °C or 37°C (n=1). Testing period was 24 weeks for 4 °C batch, 19 weeks for 25 °C batch and 9 weeks for 37 °C batch.

Kinetics of MMC were analysed to predict the shelf-life of the products. The order of reaction in both formulations could be described by a first order rate process. The data obtained from the pre-stability study was plotted as time against the logarithm to base 10 of MMC concentration remaining in the formulation. In the kinetic studies both of the formulations were stored at different temperatures and a good correlation was obtained from the data fitted to a first order reaction model.

Table 2-12 - Linearity obtained from the stability data fitted to the first order rate kinetics model.

Formulation	Storage temp (°C)	Coefficient of determination (r^2)
NHS formulation	4	0.9233
	25	0.9712
	37	0.9621
Novel formulation	4	0.2676
	25	0.9411
	37	0.9327

Table 2-12 highlights the fact that the plots corresponded to first order reaction kinetics. However, the novel formulation stored at 4 °C shows a coefficient of determination of 0.2676, this is due to the fact that there is very little chemical degradation over the period of time during it was tested. Only 1% of MMC was lost in this formulation after 6 months. And therefore it can't be assumed first order reaction kinetics in this case, to stablish the kinetics reaction prolonged time interactions are required.

Once the kinetic order of reactions was determined, the constant rate was calculated from the gradient of the plot (Equation 1). The observed shelf-life was compared with the theoretical shelf-life calculated from Kinetics (Equation 2) (Florence, 2011).

Equation 1

$$SLOPE = -\frac{k_1}{2.303}$$

Equation 2

$$t_{90} = \frac{0.105}{k_1}$$

Table 2-13 - Observed degradation rate constant of MMC and observed and predicted shelf-life of both formulations storage at 4 °C, 25°C and 37°C.

Formulation	Storage temp(°C)	k_1 obs	Shelf-life observed(weeks)	Shelf-life predicted(weeks)
NHS formulation	4	0.0097	7	11
	25	0.1237	1	1.21
	37	0.4187	0.58	0.25
Novel formulation	4	-	-	-
	25	0.0281	2.17	3.74
	37	0.1543	1.12	0.68

The kinetics of the novel formulation stored at 4 °C cannot be calculated with the same equations that were used in the other cases. However the activation energy could be determined for both formulations with the data already obtained. From there, using Arrhenius equation, the rate constant of the new MMC eye drops stored 4 °C was calculated, and with this data the shelf-life or t_{90} was estimated.

The Arrhenius equation is,

Equation 3

$$\log k = \log \mathrm{A} - \frac{E_a}{2.303RT}$$

So the activation energy could be calculated with the constant rate of the same formulation at different temperatures. By plotting the logarithm of the rates of reaction against reciprocal temperature, the slope of the graphic will be $-E_a/2.303R$, where R is the gas constant (8.314 J mol^{-1} K^{-1}).

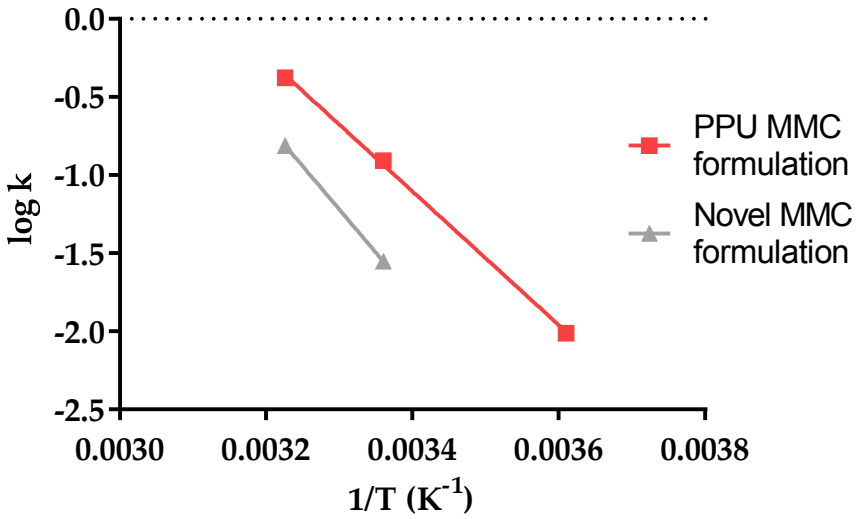

Figure 2.15 - This plot represents the logarithm of the elimination constant rate of MMC formulation manufactured at PPU against the reciprocal temperature (red), and the novel MMC formulation (grey).

Figure 2.15 the slope of the formulation manufactured at the PPU (in red) was -4279, therefore, the activation energy was 81.9 kJ/mol for the MMC in buffer solution. On the other hand the slope of the MMC novel formulation (in grey) is -5531. The activated energy calculated in this case is 105.9 kJ/mol.

Equation 4(Florence, 2011),

$$\log\left[\frac{k_2}{k_1}\right] = -\frac{(T_2 - T_1)Ea}{2.303RT_2T_1}$$

Substituting all the values in Equation 4, the constant rate of the decomposition of the MMC formulation stored at 4 °C was calculated, and a value of 1.09×10^{-3} $week^{-1}$ was obtained. Finally, the shelf-life or t_{90} was estimated with the Equation 2 and the nominal expiry date was 95 weeks,

approximate 22 months. However, long-term stability studies at 4 °C were developed to confirm this theoretical calculation.

2.2.4.2 Long-term stability data of novel formulation

Demonstrating long-term stability is essential to establish quality of a marketed pharmaceutical product, but also it is a crucial stage when unlicensed medicines are developed.

MMC content was assayed from 3 batches of the new ophthalmic product before being reconstituted and stored at 4 °C. Although analytical variations are expected, the pharmaceutical products should be formulated to deliver 100% of the labelled amount of the drug substance. At time zero the MMC content in all 3 batches was 103.5% ± 3.5%. Although this differs from the 100% label content for stability purposes at time zero it will be considered the content assayed in each batch as 100%.

The test period lasted 14 months and the stability data obtained showed slow degradation of MMC over the study duration with a loss of 7% observed. At 11 months, one batch of MMC eye drops was found to have a concentration of 84.2% which would have meant that the batch was out of specification. However, upon retesting at 14 months, the MMC concentration obtained was 91.71% suggesting an anomaly in analysis at 11 months (Figure 2.16).

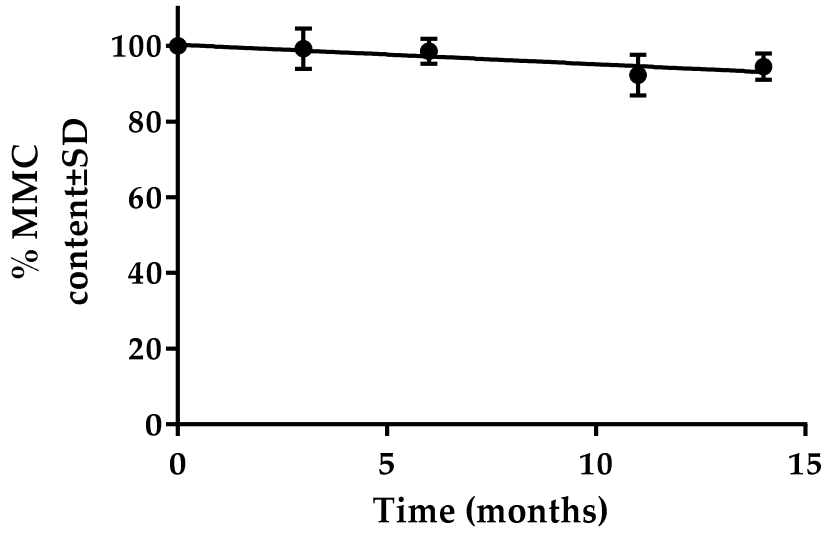

Figure 2.16 - Stability data of the novel MMC eye drops before reconstitution over 14 months (n=3) stored at 4 °C.

Using the data gained from this long-term stability study for MMC formulated in PG and stored at 4°C, the first order constant rate can be calculated in a more reliable manner than previously as 3 points can be used in the new analysis. The logarithm of the % of MMC remaining in the formulation was plotted over time, and from the gradient of the regression line the rate was predicted ($k_1 = 6.2x10^{-3}$). The predicted data showed that the potency of MMC would reach 90% 16 months after being manufactured. No degradation products were detected over the study duration.

2.2.4.3 Stability study of the new formulation after reconstitution

It is desirable to have a ready to use MMC eye drops formulation so, in addition stability testing post-reconstitution was performed. An acceptable shelf-life of the ready to use formulation will avoid manipulation of the product by other clinical professionals after manufacturing, which reduces the risk of potential exposure to cytotoxic compound.

Therefore, 3 batches of MMC eye drops already reconstituted were kept at 4 °C over a period of time for stability purposes. At time zero the MMC content in all 3 batches was 104% ± 6%, although it exist a variance at time zero between the 100% label content and the assay, for stability purposes at time zero it will be considered the content assayed in each batch as 100%.

One batch was analysed every week over an 8 week period and a loss of 1%was found (Figure 2.17). The remaining 2 batches were analysed over a longer time period and after 24 weeks the % of the MMC concentration remaining in the formulation was 93%. However, by one year nearly 10% of the starting content of MMC was lost (Figure 2.18).

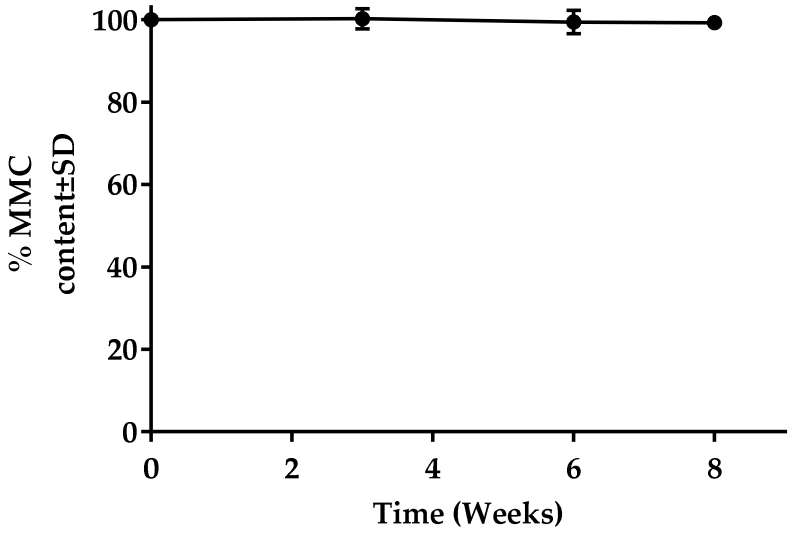

Figure 2.17 - Stability data of the novel MMC eye drops already reconstituted over 8 weeks (n=3) stored at 4 °C.

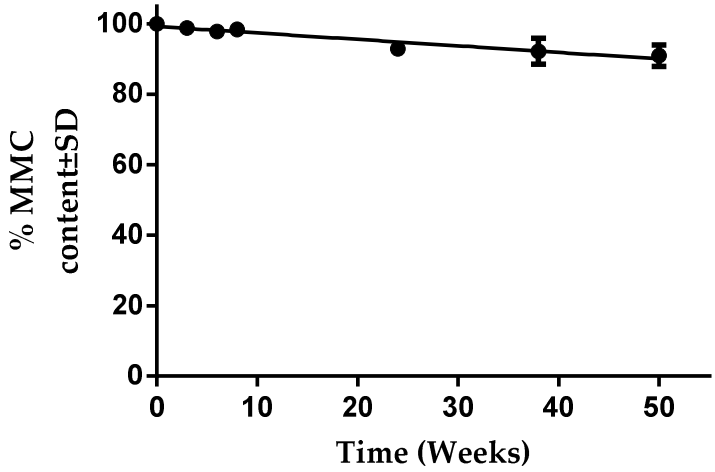

Figure 2.18 - Stability data of the eye drops already reconstituted over 50 weeks (n=2) stored at 4°C.

Although the MMC novel formulation after reconstitution reaches t90 after 42.1 weeks, after week 24 degradation products have been detected in both batches of the ready to use formulation.

Table 2 - 14 shows the analysis of the degradation products in both batches. Two degradation products were distinguished, one with a RT of 1 min and other at 1.2 min. These degradation products were measured as a percentage, comparing their AUC with the AUC of the MMC 50 µg/ml calibration level. The degradation products were not identified.

Table 2-14 - Degradation products results of ready to use eye drops

Batch	Time (weeks)	Degradation product	%Degradation product	%Total
RTU02	24	1	0.19	0.19
	38	1	0.03	0.1
		2	0.07	-
	50	1	0.19	0.36
		2	0.17	-
RTU03	24	1	0.23	0.23
	38	1	0.04	0.1
		2	0.06	-
	50	-	-	-

2.2.5 Discussion

An analytical method was necessary to carry out a meaningful comparison of the stability of a novel and existing MMC ophthalmic formulation. It is well known that HPLC is a widely used technique to test the stability of pharmaceutical products to ensure the quality and safety of final products (WHO, 2015). Authors such as Beijnen *et al.* (1985), Gupta (1997) or Quebbeman *et al.* (1985) have published different assay methods for MMC. From these methods, the initial conditions for the identification of MMC and its degradation products and the assay of the MMC were established and tested.

Methanol and ACN were tried in conjunction with phosphate buffer (pH 5.5) as a mobile phase. The proportions of polar and non-polar mobile phase components was varied during the process to determined which solvent and proportions optimised the shape of the eluting peak and gave best separation

of formulation components. Although ACN as a solvent is more commonly used in liquid chromatography due to its separation strengths, this study showed that ACN in combination with buffer resulted in peak fronting of MMC. After replacing ACN by methanol in the mobile phase, two peaks were observed in the chromatogram. The main peak corresponding to MMC presented a symmetry value of 1.02 and RT of 2.6 minutes at a flow rate of 1.5 ml/min, and a secondary peak eluted at 1.7 minutes. At the development stage it was uncertain if this peak corresponded to an impurity or degradation product. However, taking into account that a licensed MMC powder for injection was used to prepare the calibration levels, the more likely explanation is that the peak corresponds to a closely related compound of MMC.

Once the mobile phase composition was optimised, the effect of flow rate was evaluated. Three different flow rates of 0.8 ml/min, 1 ml/min or 1.5 ml/min were tested. The best MMC peak shape was obtained with a flow rate of 1.5 ml/min, providing a RT of 2.6 min. The column oven was maintained at 21° C to provide consistency of column temperature and elution time, also to reduce viscosity of solvent system that reduces pump pressures. The wavelength was 365 nm and the injection volume was 20 µl due to good instrument sensitivity.

Several studies supported the fact that MMC degradation is pH dependant (Beijnen *et al*., 1985 & 1986). Due to the prohibitive cost of the standard reference samples for MMC, it was decided to force the degradation of the marketed powder for injection of MMC in a basic pH solution to demonstrate that these breakdown products do not appear at the same RT as

the main peak of MMC. The results confirmed that the MMC peak decreased upon alkali incubation and simultaneously two degradation products appeared with RTs of 0.9 min and 1.1 min respectively. Therefore, stability studies of MMC formulations could be performed, with the specificity of the method confirmed.

In addition, during the degradation studies, the peak appearing at 1.703 min during the development phase does not experience any modification in the AUC suggesting that it is not a degradation product. It could possibly be a fermentation product or a contaminant closely related to the MMC, present in the commercial vial of MMC-Kyowa.

It was confirmed through the work performed that the HPLC method used to quantify MMC was successfully developed and validated. A linear relationship was observed between the AUC of the MMC peaks versus the concentration in a range from 0 to 70 μg/ml, and the coefficient of determination is 0.999. The total time for analysis of an individual run was about 7 min. An internal standard was not used due to the fact that there were no extraction procedures to be carried out during studies, consequently there were no need to compare the active ingredient with any other substance with similar structure during the test for corrections (FDA, 1994).

In terms of validation, the actual method is suitable for the identification of MMC. Furthermore, the method is appropriate for the assay of MMC from different ophthalmic formulations. Although some degradation products have been successfully detected with this method, they have not been identified. The degradation products levels have been measured by

comparison to the response of the active ingredient. As a result of those findings, the method was used to study the stability of ophthalmic MMC formulations.

Three different stability studies were performed. An initial pre-study to check the stability of both MMC eye drop formulations stored at 4°C, 25°C and 37°C. Once this pre-study was completed and results analysed, the storage conditions for the long-term stability studies were designed. Due to the fact that MMC is a cytotoxic compound, a ready to use formulation would be the best option to avoid further exposure or microbial contamination due to manipulation by healthcare professionals.

For the pre-stability study, 3 batches of the unlicensed formulation of MMC manufactured at the PPU were stored at 4°C, 25°C or 37 °C, one batch at each temperature to confirm the shelf life. One of the limitations of this study was that the novel MMC ophthalmic formulation was prepared in uncontrolled conditions (non-sterile), however in real practice this product will be prepared under aseptic conditions. One batch of the new formulation of MMC was manufactured in the University of Strathclyde. Bottles were divided and stored at one of the three temperatures. During the manufacturing process of the new formulation, it was observed that it was difficult to accurate aliquot 0.5 ml of MMC dissolved in PG into different bottles for the stability study due to the viscosity of the medium. This fact was reflected in the variance of the results of the stability study of the new formulation.

The visual inspection at time zero does not show any signs of precipitation in any of the formulations. And the colour of the aqueous and non-aqueous solutions were both purple. As soon as they were manufactured, all batches were transferred to the intended storage conditions. Only temperature was monitored, humidity was not measured as the bottles were vapour impermeable.

The unlicensed medicine currently in use within the NHS lost 10% of active after 7 weeks when stored at 4 °C. These findings mapped onto the 6 weeks shelf-life currently given by PPU within NHS GG&C for this formulation. During this 6 weeks the formulation was not only stored at 4 °C but also at 25 °C and 37 °C retaining 50% and less than 10% of the started content respectively. Excellent stability was observed with the novel MMC formulation stored at 4 °C. Approximately 99% of the starting MMC concentration remained at 6 months. Although stability at 25 °C and 37 °C was poorer than 4 °C, the stability was superior in comparison to the NHS formulation currently manufactured.

A previous study on the stability of MMC in buffered admixtures (pH 7.8) propose no substantial decrease in the starting concentration over 120 days at 5 °C and a reduction of 10% after 15 days when stored at 25 °C (Quebbeman *et al.* 1985). Also the patent US5216011 suggests that MMC in PG lost 15% and 2% of the starting amount after 6 months stored a 25 °C and 4°C respectively. The commercial supplier of clinical grade MMC powder, Kyowa Japan, states that the stability of their product after reconstitution with water (0.4 mg/ml) is 2 days and 3 days when stored at room temperature and at 5 °C respectively (Kyowa Hakko kirin Co., 2013). The

result of this pre-study coincide with the authors mention above suggesting that the most appropriate storage temperature of the novel MMC formulations is 4°C. At this temperature, the ophthalmic formulation remains physically and chemically stable over 25 weeks.

The pre-stability assessment allowed kinetics of MMC degradation from the different formulations to be performed. Several studies have been previously published about this relationship. Edwards *et al.* (1979) investigated the decomposition of MMC in saline solution at different temperatures and pH. Edwards *et al.* (1979) described the degradation of MMC as a first order reaction and the activation energy for the decomposition of MMC to be 81.1 kJ/mol. In this research, it was found that either the decomposition of MMC buffered at pH 7.5 or MMC in non-aqueous solvent also followed a first order reaction with a coefficient of determination at all temperatures above 0.92. There was an exception to this, the novel formulation of MMC eye drops had a coefficient of determination at 4° C of 0.2676. This formulation stored at 4° C could not be fully characterised as less than 1% of content was lost during 6 months. With the data obtained from the degradation of novel MMC product at 25°C and 37°C it was possible to calculate the activation energy of the decomposition of the MMC in non-aqueous solvent and from there estimating the theoretical shelf-life of the product at 4° C.

The activation energy calculated for the formulation prepared by the PPU in aqueous buffer (81.9 kJ/mol) did not differ from that reported by Edwards *et al* in 1979 (81.1 kJ/mol). And the activation energy calculated from the degradation kinetics of the novel formulation at two different temperatures

was 105.9 kJ/mol. Using this data and the degradation constant rates a t_{90} of 2 years was predicted. However due to the limitations of theoretical estimation of shelf life, this data should be verify with long term stability studies.

Therefore, the next step was to develop a long-term stability study of the novel formulation at a storage temperature of 4° C. In this new study, 3 batches of MMC novel product were tested to observe for consistency in results. There were no signs of precipitation or change in colour during the 14 months that the study lasted. Stability data obtained with MMC in PG showed an acceptable stability over 14 months reaching 93.2% of the original MMC content. As it was mention before, during the manufacturing was noticed that the viscosity of MMC in PG was high, this means that when trying to aliquot the formulation into individual bottles was not as accurate as anticipated. This fact impacted on the variance in the assay of MMC within batches at different time points, reaching in some cases a RSD of 7%.

The theoretical t_{90} calculated for the novel formulation from the Arrhenius equation was 22 months. However after 14 months, a loss of 7% was observed. With the new data obtained over a period of time of 14 months, the Arrhenius equation was used again to estimate a more accurate shelf life when the new product is stored at 4°C. At this time it was established a predicted t_{90} of 16 months after manufacturing which differs from the initial calculation by a period of 6 months.

The accelerated stability studies are commonly used to predict shelf life based on the relationship between the pharmaceutical product and the thermal degradation using the Arrhenius equation. Although this is a

common practice, the drug regulatory authorities insist that adequate real-time stability testing should be conducted alongside the accelerated studies to provide evidence of the performance of a drug so the recommended storage conditions and shelf life can be assigned.

The stability of the formulation reconstituted was also studied. The same variance in the assay results intra batch caused by the viscosity of the system as previously seen with the long-term study formulation was also experienced. One batch of the novel MMC formulation was reconstituted to be tested for a few weeks, and when favourable results were obtained, 2 more batches were prepared and tested to see if results were consistent. The novel formulation in the ready to use form reached t_{90} after 10 months. Two degradation products were observed at 1 and 1.2 minutes during the HPLC chromatographic analysis at weeks 24, 38 and 50. Both degradation products were inconsistent and were observed at different time points in each batch studied.

2.3 *In vitro* cytotoxicity assessment of a novel eye drop formulation of mitomycin C.

2.3.1 Introduction

In terms of toxicity the objectives are to evaluate if there is a comparable acute toxicity profile between the novel eye drops formulation and the formulation currently manufactured within the NHS; and also, to assess if there is any impact in the toxicity of the MMC formulation due to the penetration enhancement properties of the excipient propylene glycol (PG). Also, cytotoxic studies is a legal requirement prior to approval of any

pharmaceutical product, consequently, a comparison of the toxicity of both formulations is essential.

The *in vitro* cytotoxicity studies have some limitations, one of them is the difficulty of imitate the *in vivo* pharmacokinetics for absorption, distribution, metabolism and elimination. The stability of the drugs in a toxicity *in vitro* study depends only on the metabolism of the cells used for the study. However, the stability *in vivo* may depend upon many factors such as the influence of many types of tissue, body fluids or enzymatic reactions amongst others.

There are also advantages in performing *in vitro* studies prior to *in vivo* research. The number of animals used during *in vivo* testing can be decreased significantly reducing the cost of the study. Reduces the number of test replicates in comparison with *in vivo* testing and therefore simplifies the analysis of data. It is easy to perform and therefore it has a lower cost in comparison with the toxicological test performed with animals. Also the concentration of the cytotoxic product and the time of exposure can be controlled more accurately than *in vivo* research.

To carry out the *in vitro* evaluation a bioluminescent assay was chosen, and the proliferation of B16-F0 luciferase cells line was monitored with an imaging device, *in vivo* imaging system (IVIS) to localize the bioluminescence. Bioluminescence is defined as the act of releasing light due to a chemical reaction of an enzyme called luciferase with the substrate D-luciferin [(S)-2-(6′-hydroxy-2′-benzothiazolyl)thiazoline-4-carboxylic acid] which is a light emitting pigment. This enzyme and substrate is used by

fireflies to emit light (Meroni *et al.*, 2009). The luciferase can be inserted into cancerous cells, so the D-luciferin penetrates into the cell through the membrane and under the effects of luciferase and ATP, a bluish-green light is produced. That reaction helps to determine the presence of life in the cells. The light emitted can be captured by a high sensitive camera and analysed by the appropriate computer software. This imaging system used to study the toxicological effect of pharmaceutical products *in vitro* or *in vivo* in small animals is an easy and highly sensitive method (Zinn et al., 2008).

To study the cytotoxicity of MMC, a B16 melanoma cell line was chosen. This cell line comes from mouse melanoma which produces melanin. Isaiah Fidler (1977) developed the first B16 melanoma cell line. Different subcultures were created with different metastatic levels and B16 cell lines have been used in an extensive range of studies related to cancer research (Fidler *et al.*, 1977).

2.3.2 Materials

The luciferase expressing B16-F0 melanoma cell line was purchased from American Type Culture Collection (ATCC) in Middlesex, UK. Formulations were manufactured at the PPU within NHS GG&C and in Strathclyde University as described in Section 2.2.3.

The following reagents and chemicals were obtained to carry out the *in vitro* study: Dulbeco's modified Eagle's medium (DMEM) and TrypLE Express were purchased from Invitrogen (Paisley, UK). L-Glutamine was purchased from Lonza Wokingham Ltd. (Berkshire, UK). Penicillin, Streptomycin and Trypan Blue were obtained from Sigma-Aldrich (Dorset, UK). Foetal calf serum was purchased from Biosera Ltd (East Sussex, UK).

2.3.3 Methods

A mouse epithelial–like melanoma cell line, B16-F0, was grown in a high glucose medium, Dulbecco's modified Eagle's medium, supplemented with 8% fetal calf serum , antibiotics (penicillin and streptomycin) and 2 mM L-glutamine. Cells were incubated at 37°C in a humidified atmosphere and 5% CO_2/95% air conditions.

A cryotube of luciferase expressing B16-F0 cell line stored in nitrogen liquid was defrosted and added to 10 mL of complete DMEM. The cell suspension was centrifuge at 1250 rpm at 4°C for 10 minutes. The supernatant was discarded and 10mL of fresh medium was added to the flask to re-suspend the cell pellet obtained by centrifugation. The resultant suspension was incubated in a 40 mL sterile flask at 37 °C in an atmosphere of 5% CO_2/95% air conditions. After 24 h, cells were harvested and passaged. Medium was discarded and 5 mL of triple express added to the 40 mL flask for 5mins to detach cells. After 5mins 15 mL of complete DMEM was added to the flask to neutralise the tryple express and the cell suspension was transferred to a 200 mL sterile flask to incubate again for 2-3 days. Normally 2 to 4 passages were carried out before using the cells in for toxicity evaluation. When cell cultures showed 70% of confluency, the cells were harvested for *in vitro* cytotoxic experiments.

Proliferation studies were previously carried out by other in house researchers to determine optimal number of cells to be experimentally used (Al-Gawhari, 2012). Prior to toxicity experiments, cells were detached as described and re-suspended in 20ml complete DMEM. Cells were

enumerated under a microscope by mixing 100 µl of the cell suspension with 100 µl of trypan blue and transferring 10 µl of the resultant mixture on to a haemocytometer.

The 96 well plates were set up as shown in Figure 2.19. The cell density used in the individual wells was 1 x 10^6. All wells situated in the edges (wells are shown in red in the diagram) contained 200 µl of complete DMEM. Wells situated in positions B2-G2 were the negative control (C-), containing only DMEM. And B11-G11 were positive control (C+), containing DMEM, luciferin and cells. Position B3 contained the PPU formulation, the luciferase cells and the luciferin (OF). Serial dilutions were performed from B3 to B10 with a MMC concentration range from 200 µg/ml to 1.56 µg/ml. This was performed in triplicate (C3-C10 & D3-D10). The same set up was performed using the novel formulation in positions E3-E10, F3-F10 & G3-G10 of the 96 well plate (NF).

Once the 96 wells plate was set up it was incubated and imaged using the IVIS system at different times (20 min, 40 min, 1 hour, 2 hours, 4 hours and 6 hours and finally 24 hours) to assess the effects of exposure time on outcomes. The *in vitro* experiment was repeated 3 times and the setup of the plate is shown in Figure 2.19.

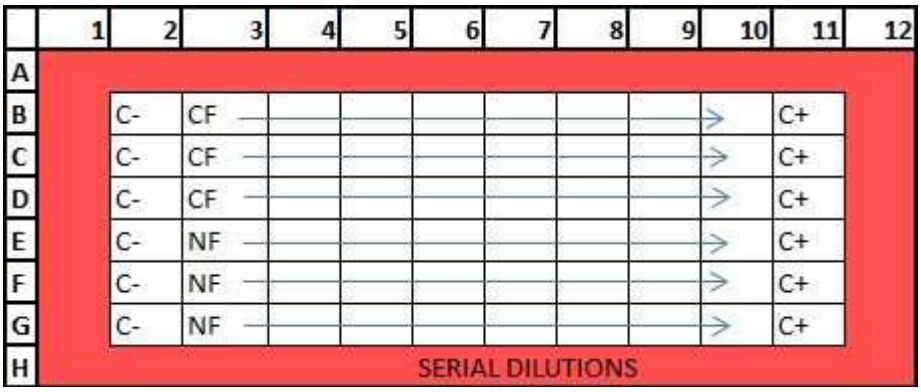

Figure 2.19 - Setting of the 96 wells plate with both formulations. Rows B, C and D correspond to the current formulation manufactured in the Pharmacy Production Unit GG&C and the rows E, F and G correspond to the novel MMC eye drops. The dilutions of both products started at a concentration of 100 μg/ml (column 3) to a concentration of 0.78 μg/ml (column 10).

2.3.4 Results

The proliferation of B16 F0 against MMC eye drops was investigated. There was a direct correlation between formulation exposure time, concentration and cytotoxic effect. Figure 2.20 shows the percentage of cells killed by both MMC eye formulations at a fixed concentration of 0.02% each at different times over 24 hours. The data obtained was analysed for normality by D'Agostino test using GraphPad Prism 6 software and the results showed $p>0.05$ which means that this dataset follows a normal distribution model. Student's 2 t-test was used to statistically analyse the cytotoxicity of both treatments and no significant differences in cytotoxicity were found between both eye formulations ($p>0.05$).

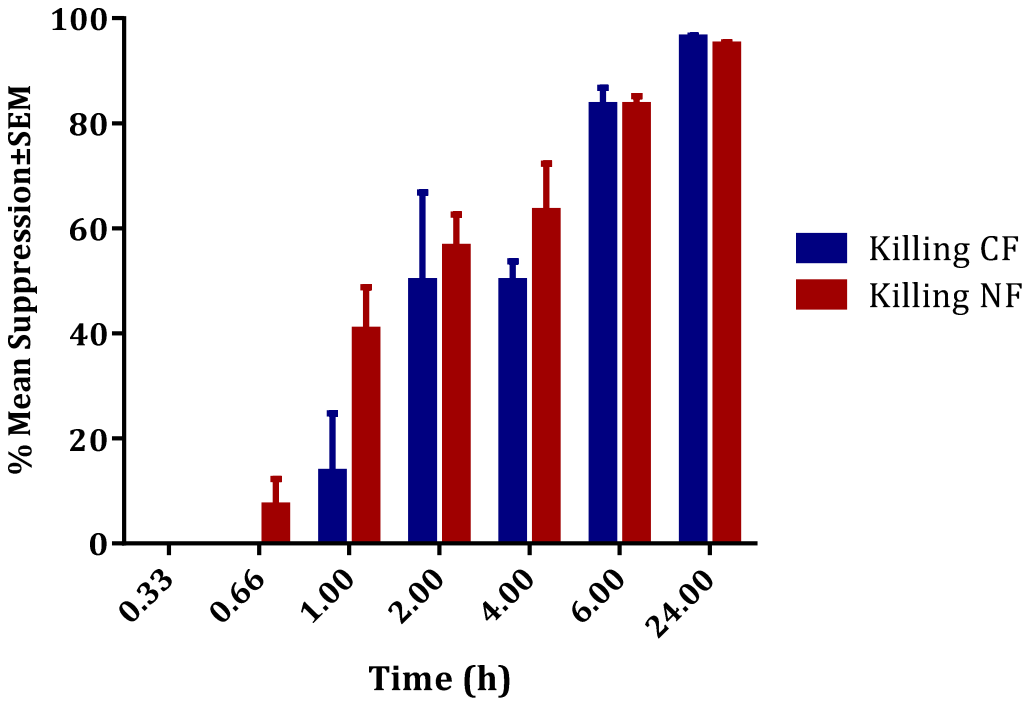

Figure 2.20 - The effect of the MMC formulation currently in use and the novel MMC eye drops with a concentration of 0.02% on the growth of the B16-F0 cells over time (n=3).

In Figure 2.21 – 2.23, the *in vitro* emission of fluorescent light after 2h, 6h and 24h of exposure to eye drops MMC formulations is shown.

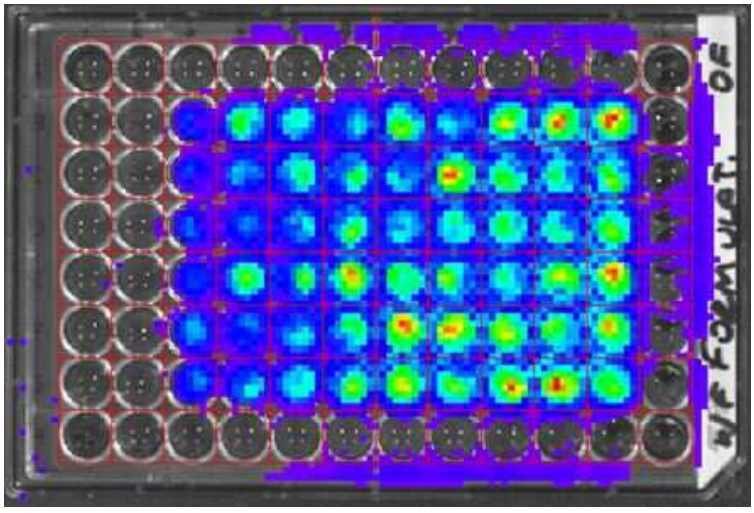

Figure 2.21 - The 96 well plate with B16-F0 and MMC novel formulation in rows 2 to 4, and existing MMC formulation in rows 5 to 7 after 2h of contact time.

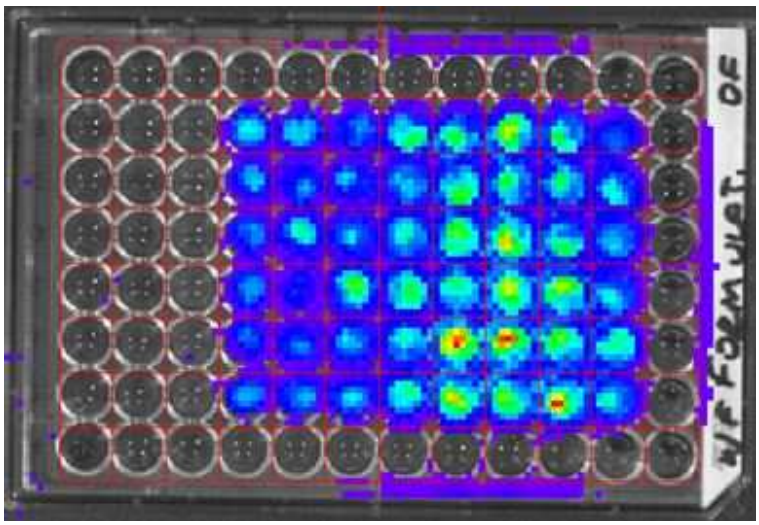

Figure 2.22 - The 96 well plate with B16-F0 and MMC novel formulation in rows 2 to 4, and existing MMC formulation in rows 5 to 7 after 6h of contact time.

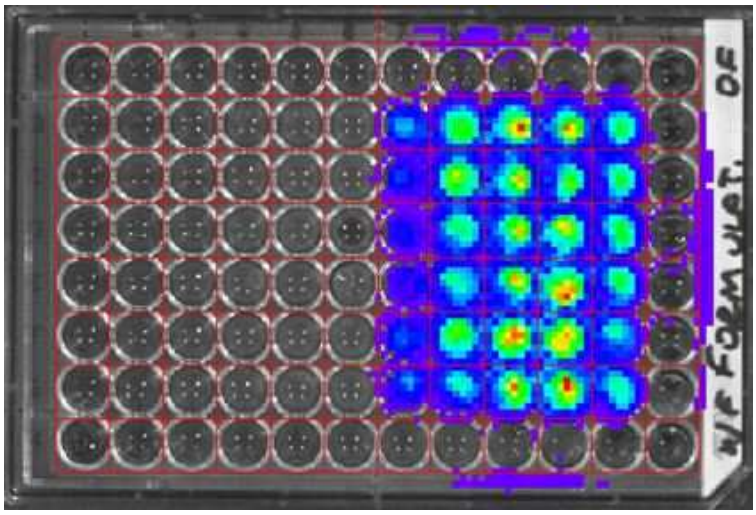

Figure 2.23 - The 96 well plate with B16-F0 and MMC novel formulation in rows 2 to 4, and existing MMC formulation in rows 5 to 7 after 24h of contact time.

The inhibitory concentration (IC_{50}) values of both formulations at different times were also calculated. The data obtained was analysed for normality by D'Agostino test using GraphPad Prism 6 software and the results showed $p>0.05$ which means that this dataset follows a normal distribution model. Student's 2 t-test was used to statistically analysed the cytotoxicity of both treatments and no significant difference in the overall cytotoxicity was found

between both eye formulations ($p>0.05$). Each formulation was also compared at each time point and the analysis showed significant difference in the cytotoxicity effect of both formulations after 4 hours of exposure ($p<0.05$).

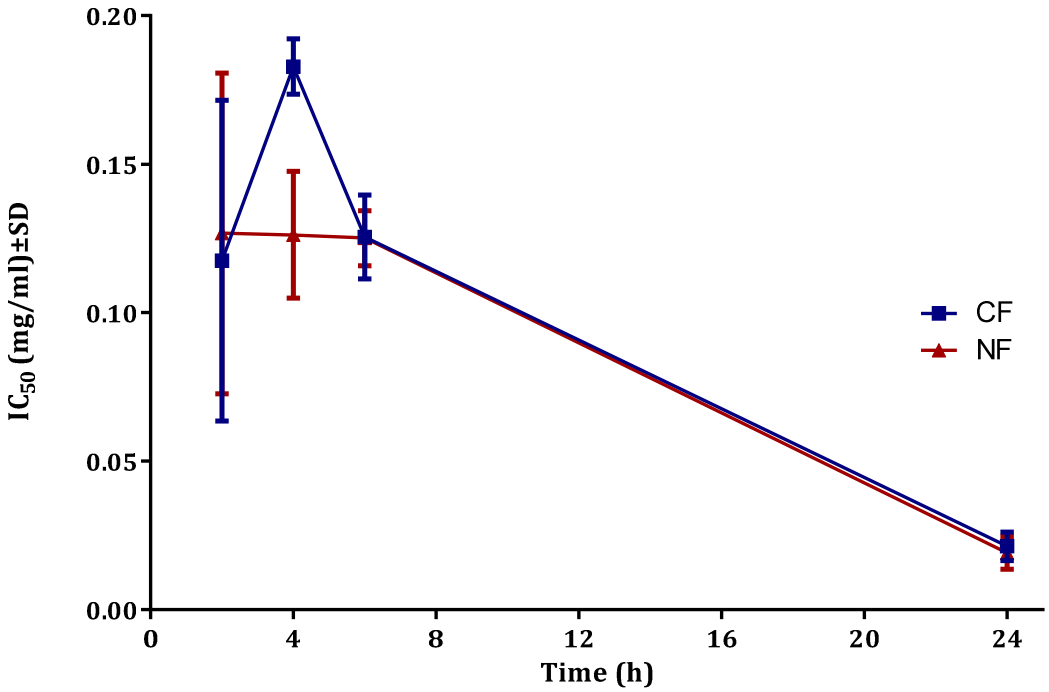

Figure 2.24 - A comparison of the cytotoxicity between the MMC formulations on the survival rate of B16-F0 melanoma cell line over time. IC_{50}±SD of the new formulation (NF) and the old formulation (CF) at concentration of 0.02% have been measured in different time points during 24h (n=3 for each concentration level).

The results obtained showed a relationship between the concentration of MMC used and the inhibition of cell growth. At lower concentration of MMC, the number of cells dying was less than with higher concentration of MMC. The data obtained after 24h of exposition was fitted in a sigmoidal dose-response curve (Figure 2.25) using GraphPad Prism 6 software and the IC_{50} values for the eye drops currently in used and the novel ophthalmic formulation were 0.049 and 0.040 mM respectively.

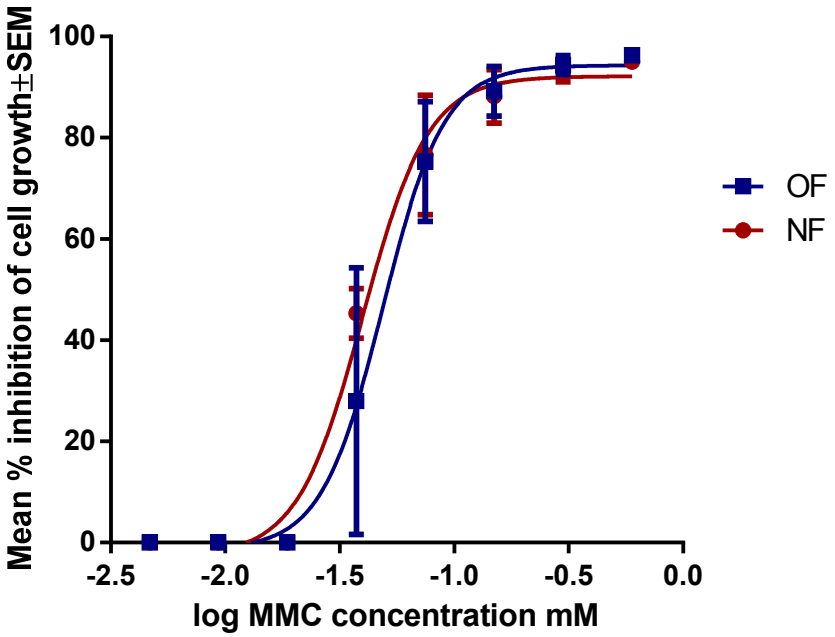

Figure 2.25 - Dose response curve of the activity of both treatments against B16 F0 melanoma cell line after 24h, serial dilutions starting at 0.6 mM (n=3).

2.3.5 Discussion

In the 16th century Paracelsus wrote that "*The right dose differentiates a poison from a remedy*" which is the basic principle of toxicology. This theory states that there is a relationship between the drug amounts received by a human being and a toxic reaction, even oxygen in the wrong concentration can be harmful for an individual's body. The time of exposure is also an important factor to take into account when the toxicity of a drug is evaluated, as well as the mechanism of action of the compound.

MMC is a well-known compound used as an anticancer drug. It has demonstrated to be a potent therapeutic agent for ophthalmology use including melanoma. In this research the objective was to demonstrate that

the novel MMC formulation had no more cytotoxicity than the existing manufactured product.

A viability assay was used to compare the survival rate of cytotoxic cells when they are exposed to both MMC products. Bioluminescence assay was used to measure the proliferation of B16 melanoma cells.

In previous ocular research, B16 cell lines have been inoculated into murine eyes to study the growth of melanoma cancer in the eye and the metastatic effect of some of those cells in the lung. el Filali *et al.* (2012) published a paper detailing the use of murine B16 F10 cell line to growth uveal melanoma inside the eye and also for *in vitro* experiments to determine the effect of bevacizumab.

In the present research B16 F0 cells have been used. The main difference between the cell lines B16 F0 and B16 F10 is the availability to form metastases in *in vivo* systems. B16 F0 is characterised because of its low metastatic effect. In contrast, the variant of the cell line B16 F10 has an aggressive metastatic effect from subcutaneous site to lungs.

Three different aspects were compared to assess the toxicity of the novel formulation, the mean suppression of both formulation at a concentration of 0.02% over time, the IC_{50} of the MMC eye drops over time and the dose-response curve after 24 hours of treatment.

The results showed that both treatments at a concentration of 0.02% have a comparable cytotoxic profile against B16 F0 when analysing the suppression

effect over time. When comparing the dose-response curve after 24h of exposition it showed no significant difference.

However the comparison of the IC_{50} of both products over a period of time indicated that at 4h of exposure, the IC_{50} of the new formulation was significantly lower in comparison with the other treatment. This can be due to the enhanced penetration effect of PG. This excipient is commonly used in dermatology to increase the penetration of some drugs through the skin. Although the killing rate is increased after 4 hours of exposure to the novel treatment in the subsequent time points the suppression rate is stabilised and there is no significant difference between both formulations. Those results suggested that the novel MMC ocular formulation could have been transported into the cells quicker than the eye drops currently in use but after 24h of exposition the concentration needed to kill the 50% of the cells is the same for both products. Although this could imply an improvement in drug efficacy, it could also mean a higher rate of side effects. *In vivo* studies will need to be performed to know the real impact of this finding.

The standard deviation of the formulations in the early stages of the experiment is high. This could be explained due to the fact that the drug takes time to distribute in the 96 wells plate, the diffusion rate of the drug into the cells depends on its cycle stage and also in the case of the novel formulation the high viscosity of the PG.

In ocular drug administration there are different anatomical systems playing an important role. First of all there are several layers acting as cellular barriers affecting the penetration of the formulation. Those layers include the

sclera, cornea and the conjunctiva. The cornea will control the influx of the eye drops in topical routes and the lacrimal drainage will wash away most of the volume of the topical MMC eye drop apply into the eye (Patel *et al.*, 2013)

Although *in vitro* studies suppose a lot of benefits in ocular drug development, the existing ocular barriers make the delivery of the drugs into the eye a challenge. Therefore it is recommendable that *in vivo* assessment is carried out as future work to confirm *in vitro* findings.

Chapter 3 Amphotericin B ophthalmic formulation

3.1 General aspects of Amphotericin B

3.1.1 Chemical structure & mechanism of action of amphotericin B

AmB is an antifungal agent isolated from a strain of *Streptomyces nodosus* in 1953. It was first isolated from a sample of soil obtained on the Orinoco River in South America at Squibb laboratories. However it was in 1959 when it was first reported the antifungal activity of this drug (Dutcher *et al.*, 1968).

Immediately after the discovery, there were some attempts to characterise the structure of this compound, however it was not until 1970 when the full structure of this antibiotic was elucidated by X-ray single crystal analysis (Ganis *et al.*, 1970). Figure 3.1 shows the structure of AmB. It is an asymmetrical macrocyclic with a mycosamine sugar head group and a free carboxylic acid. This confers amphoteric properties to this drug. AmB has a hydrophobic side characterised by a conjugated heptadiene backbone and also a hydrophilic side characterised by the presence of seven hydroxyl groups (Torrado et al., 2008).

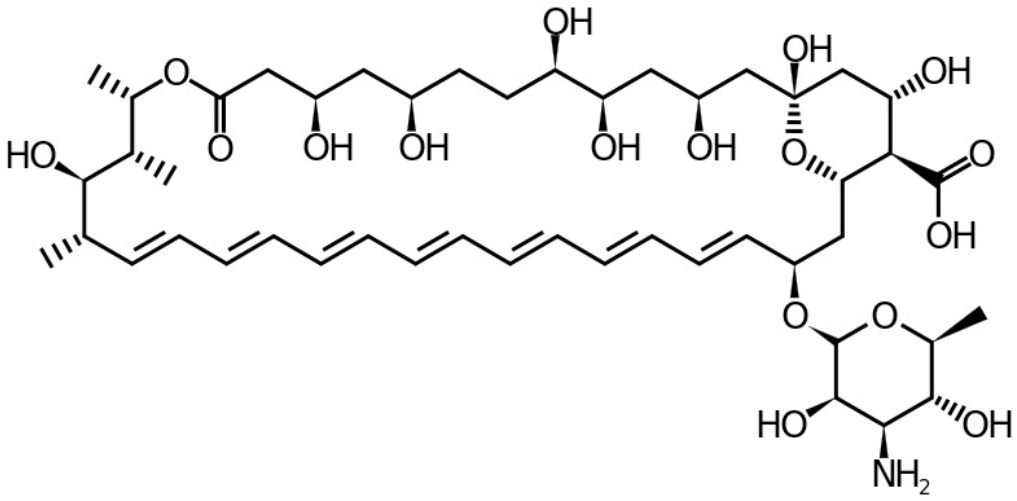

Figure 3.1 - Structure of amphotericin B

AmB belongs to the polyene antimycotic agents, within this group of pharmaceuticals are also found compounds such as Amphotericin A, Nystatin and Natamycin. In 1962 Lampen *et al.* investigated the relationship between Nystatin and the membranes of yeast, however it was not until 1991 when a full investigation about the complexity of the mechanism of action of AmB was published by the Hartsel group. They suggested that the molecular mechanism of action for AmB is more complex than previously evaluated. Differences between AmB-induced ions cations and large molecule permeability have been observed among vesicles containing ergosterol, cholesterol, or no sterol, which could point to a mixture of mechanisms of action (Hartsel *et al.*, 1991).

In Figure 3.2 is represented the channel hypothesis as the mechanism of action of AmB against fungal cells. AmB binds to ergosterol, which is an essential sterol component of the fungal cell membrane. As a result of this binding, ion transporting channels across the membrane will be opened, which alters the permeability of the cell. This leads to the leakage of

important intracellular components, and ultimately causes cellular death (Brajtburg & Bolard, 1996)

The same early 1950s investigations that identified antimycotic activity of AmB also highlighted that AmB was not active against bacteria. It was only later discovered that the absence of ergosterol in bacterial cell membranes was an explanation for this (Baginski, *et al*, 1997).

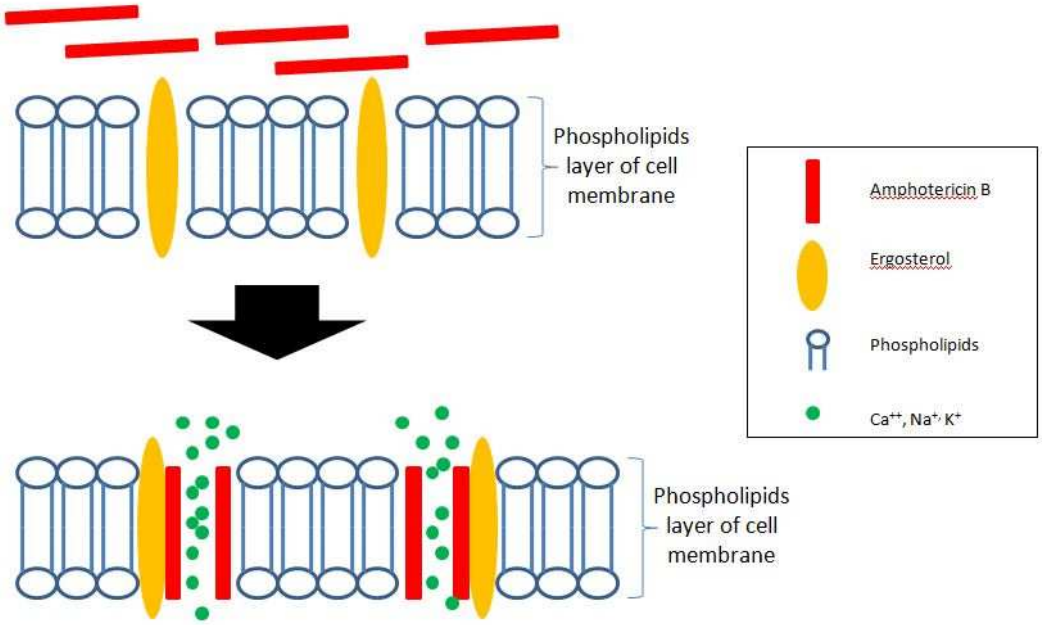

Figure 3.2 - Mechanism of action of amphotericin B as an antifungal agent.

Ergosterol is not only found in the membrane of fungi. It is also the main sterol component in the cell membrane of parasites belonging to the *Leishmania* genus and has important clinical applications against this organism (Saha *et al*., 1986).

In parallel, it was also found that AmB binds to cholesterol, the principal sterol in mammalian cells. Although investigations have revealed that the

affinity of AmB and cholesterol is much lower, the relatively poor selectivity of AmB to these sterols explains the clinical toxicities observed with AmB in humans (Paquet *et al.*, 2002).

3.1.2 Properties

AmB is a bright yellow, odourless powder. The solubility of AmB (Table 3-1) is very poor in solvents such as water, ether or benzene. However, it is slightly soluble in methanol and soluble in dimethylformamide or dimethylsulfoxide. The solubility of AmB in water, methanol and dimethylformamide can be increased at extreme low pH. But not only acidic ionization of the amino groups of AmB enhances its solubility, the conjugates formed between AmB with surfactants such as sodium lauryl sulphate or sodium deoxycholate (Fungizone®) improves also the solubility of this compound by formation of a mixed micelle structure (Florey, 1977). Good solubility of drugs in water is essential to achieve good bioavailability, independently of the route of administration. For this reason, the poor solubility of AmB in aqueous and non-aqueous media supposes a real challenge in pharmaceutical development (Khadka et al., 2014).

Table 3-1 - Amphotericin B solubility in certain solvents (Florey,1977)

Solvents	Solubility of AmB(mg/ml)
DMSO	30-40
Dimethylformamide	2-4
PG	1-2
Methanol	1.60
Water (pH 2 or pH 11)	0.1
Ethanol	0.5
Benzene	0.06
Ether	0.01
Toluene	0.00

Liposomal drug delivery systems are an alternative use to improve the solubility and bioavailability of drugs such as AmB. Although liposomes were discovered in the 1965, it was not until the early 1990s when liposomes were used as drug delivery systems for low solubility drugs (Torrado et al., 2008). In the case of AmB the liposome-based formulation was first approved by the FDA in 1997 under the trade name of AmBisome® (FDA, 1998). The side effects of this formulation are reduced in comparison with Fungizone®, and there are also potentially better clinical outcomes because potency gains due to targeting to site of infection. However the high cost of the liposomal form of Amphotericin B constrains its use (Van de Ven *et al.*, 2012).

More recent studies have used different pharmaceutical techniques to increase the solubility of AmB. Nanosuspension technology has demonstrated the ability to improve the solubility of water insoluble drugs, increasing also the bioavailability in most cases. This is obtained through the formation of biphasic systems consisting of API dispersed in an aqueous vehicle and stabilized by surfactants. The fact that this technology involves

simple production methods means that the balance cost-effectiveness will be encouraging (Al-Quadeib *et al.*, 2015).

The aggregation of AmB plays an important role in the toxicity caused by this molecule. Its physical state is of a great interest due to the fact that it is not only linked with the effectiveness of this drug but also with the side effects causes by this molecule (Barwicz *et al.*, 1992).

Due to its amphipathic nature AmB forms self-associates and aggregates. The physical state of this molecule has been studied for years because it is understood that it is directly related to the toxic effects that it exerts. Although the monomeric form has been linked with the lowest toxicity because it preferentially associates only with sterols in fungal membranes, AmB in its aggregation state can also form pores in membranes containing cholesterol causing host toxicity (Barwicz *et al.*, 1992).

Several factors can influence in its physical state such as solution pH, final concentration (Torrado et al., 2008), the organic solvent used (Legrand *et al.*, 1992) or the excipients included in the formulation. These factors still however remain poorly understood (Tancrède *et al.*, 1990).

The UV-visible spectrum of AmB is very sensitive to the changes in association state provoked by the processing factors mention above. Therefore, the spectral modifications can be monitored and used as a proxy for aggregation. This is typically done by assessing the ratio of absorbance at two different peaks $A_{325/348}/A_{407}$. In the monomeric form of AmB this ratio is

~0.25, however when AmB is in an aggregated form this ratio increases to ~ 2 (Barwicz *et al*, 1992).

3.1.3 Clinical uses

AmB is an antimycotic agent with a broad range of activity against yeasts and moulds, but not only acts as an antifungal, it also has activity against *Leishmania* sp. (Mullen *et al.*, 1997). This active ingredient can be administered as a topical formulation (mucocutaneous and ocular infections), as an intravenous product (systemic infections), via oral administration (oropharyngeal or intestinal infections) or inhaled (pulmonary infections) depending upon the source of infection and pathogen.

AmB is used topically in fungal infections located in the eye that can rapidly lead to ulceration in the cornea if inadequately treated. However, this antifungal agent is not only used to combat keratomycosis but also as a treatment of life-threating systemic fungal infections administered as an intravenous infusion (Riddell *et al*, 2011).

Due to its low solubility in water and the poor gastro intestinal permeability it is classified as a class IV drug in the Biopharmaceutics classification system. This system was developed by Amidon *et al.* in 1995 with the main objective to classify two key properties of orally administered drugs that impact upon bioavailability: solubility and permeability. The hydrophobicity of the polyene structure reduces aqueous solubility whilst the polar hydroxyl moiety reduces gastrointestinal permeability meaning it has very poor oral bioavailability. However, AmB is used orally for selective

digestive tract decontamination (SDD) in intensive care unit patient. Such treatment reduces the possibility of having a ventilator-associated infection and reduces mortality (Bonten *et al.*, 2000).

AmB is also used intravenously either as a prophylactic or active therapy against systemic fungal infections. This is important particularly in immunocompromised patients e.g. cancer, transplant patients or those with HIV. In those cases, the objective of the therapy with AmB is to prevent or treat an invasive fungal infection, first cause of morbidity and mortality among this population (Miceli *et al.*, 2012). Different studies have focused on the use of AmB to prevent those systemic infections cause by *Candida albicans* or *Aspergillus sp* in immunocompromised patients. In 2001, a prospective study about the use of liposomal AmB to prevent invasive fungal infections in paediatric population with chemotherapy related neutropenia was published by Uhlenbrock *et al*. (2001). The findings showed that there were no differences in the incidence of invasive fungal infections between the group of patients receiving prophylactic systemic antifungal therapy and the control group. Also, Schwarz *et al* (2006) reported that liposomal AmB alone or combined with flucytosine was successful in a murine model of cryptococcal meningoencephalitis which is a common infection in immunocompromised patients.

AmB can also be administered by nebulisation or intravenously to patients with pulmonary infections such as invasive pulmonary Aspergillosis. Patients with neutropenia fever and candidaemia are also treated with AmB as an alternative to echinocandin drugs (Carol A Kauffman, 2017).

As it has been previously mentioned, AmB is also very effective as an anti-parasitic agent in the treatment of leishmaniasis (Matlashewski *et al.*, 2011). AmB is used to treat systemic forms such as visceral leishmaniasis. It may be also useful in the treatment of American mucocutaneous leishmaniasis, but it is not the drug of choice in primary therapy (Palumbo, 2010).

Returning to the ocular administration, there is a real challenge in ophthalmology practice to treat fungal eye infections. Keratitis is one of the main causes of blindness worldwide and although it is preventable, it can be irreversible once it occurs. Keratitis consists of corneal inflammation. Many types of fungi can provoke ocular infections, therefore the first clinical priority is to identify the causative pathogen and treat it properly (Ansari *et al.*, 2013).

Fungal infections of the cornea are rare but can occur after a traumatic injury, especially in humid climates. Age, debility, or immunosuppression can encourage fungal proliferation. Cases of systemic invasion secondary to initial ocular infection have been reported, therefore an early diagnosis and treatment is essential (Thomas, 2003).

3.1.4 Amphotericin B formulations

AmB powder for injection is a licensed product widely used as an antifungal agent due to the broad spectrum and the low resistance to the drug. However this drug has to be used as a complex due to the poor solubility in water. The conventional AmB formulation is the complex formed between AmB and deoxycholate (Fungizone®), but its use is limited due to the toxicity

exhibited. Because of this reason other formulations of AmB have been developed. Although these alternatives have shown less toxicity, they are more expensive options. Those non-conventional formulations incorporated into clinical practice are lipid based preparations such as AmB lipid complex, liposomal AmB and colloidal dispersion (Hamill, 2013).

Polyene macrolides were the first choice to treat fungal infections, but more recently newer treatments have been developed. For fungal infections in adults or in immunocompromised patients the therapy for decades has been limited to the use AmB, flucytosine or azoles such as fluconazole (Johnson *et al.*, 2004). However new options have become available to treat this condition. New potent azole drugs such as voriconazole and posiconazole with a broad antifungal activity have been included in the guidelines to treat fungal infections, the problem derived with the use of these compounds are the many azole-related drug interactions in patients with HIV or transplant related medication. But they are not the only antifungal compounds, the newer class of drugs called echinocandins have also been used to treat those infections (Trofe-Clark *et al*, 2013).

In ophthalmologic practice, AmB is used to treat fungal keratitis. It is administered to the patient's eye as an unlicensed medicine manufactured from Fungizone®. However, due to the fact that Fungizone® contains deoxycholate, this product produces discomfort in the patient eye increasing the probabilities of treatment failure. This is not the only disadvantage of this unlicensed eye drops. The chemical instability of Fungizone® in aqueous solution is also an issue to consider. Currently, this product is manufactured in small batches due to its instability resulting in a very short shelf life once

prepared. This restricts patient access to the medicine and frequently treatment initiation is delayed with the resultant clinical impacts.

3.1.5 Side effects derived from the clinical use of amphotericin B.

The use of AmB in different types of fungal infections is limited because it is associated with severe and sometimes life-threatening toxicity. Most of these side effects are associated with intravenous administration, being nephrotoxicity the adverse reaction most commonly observed with Fungizone®. AmB administered in a colloidal drug delivery system (Fungizone®) can cause chronic renal impairment in patients with cumulative dosage. Fever, chills, pain and thrombophlebitis at the injection site due to chemical irritation of the dosage form on the venous endothelium and haematologic effects are commonly observed (Laniado-Laborín *et al,* 2009). It is also known that these adverse reactions are caused by water soluble aggregates rather than water-insoluble aggregates (Espada et al., 2008).

Renal toxicity occurs in almost all patients, but it is more severe in patients given cumulative intravenous doses. Although renal toxicity can improve with the cessation of therapy, the risks of permanent harm are considerable. Nephrotoxicity is more frequently observed with Fungizone® compared to lipid formulations of AmB (Deray, 2002).

The febrile reaction associated with the AmB treatment could be prevented or ameliorated by the administration of analgesic and antipyretic drugs before AmB therapy. Other reactions such as nausea, vomiting, anorexia,

headache, myalgias and arthralgias have been reported to be associated to the administration of AmB parenterally (Goodwin *et al.*, 1995).

Cases of anaphylaxis reactions produced after the administration of liposomal AmB have been described more frequently when compared with conventional formulations. Although these reactions are very rare, it is recommended to carefully monitor the patient being treated with AmB first time (Cesaro *et al.*, 1999).

3.1.6 Stability profile of amphotericin B

AmB powder is stable for long periods of time if stored in an air tight container protected from light at 4 °C. However, in aqueous solution the stability of this compound is reduced dramatically. AmB is not only unstable in aqueous solution it is also sensitive to several chemical and physical attacks. This compound shows poor stability when it is exposed to ambient parameters such as heat and light or chemical parameters such as extreme pH (Hung *et al,* 1988).

Hung *et al.* (1988) studied the stability of AmB in aqueous solution. The study revealed that maximum aqueous stability of AmB in solution is achieved when kept in darkness, in the absence of oxygen and at low temperatures. Solution pH or the use of surfactants can be used to improve solubility. Polysorbate-20 was observed to have similar solubilizing effects to sodium deoxycholate acid. High surfactant concentration at an acidic pH was optimal for prolonging stability.

Analogues of AmB have been also studied in terms of stability. Bonner *et al.* (1975) compared the stability of AmB and its analogue amphotericin methyl ester (AME). The results obtained showed that AME had increased aqueous solubility but was more sensitive to heat and acidic pH than AmB.

3.2 Formulation and stability studies of Amphotericin B ophthalmic formulation

3.2.1 Introduction

Presently, at the PPU (NHS GG&C), AmB eye drops are prepared dissolving the commercial intravenous (IV) AmB deoxicholate powder in water for injection obtaining the desired final concentration of 0.15%. The shelf life assigned to this unlicensed formulation by the PPU is 28 days under refrigeration. The strategy started confirming the shelf life of the product manufactured at the PPU, followed by the development of novel ophthalmic products with an extended shelf life. The instability of AmB in aqueous solution and also the poor solubility of the API in water limit its usage as eye drops. Therefore, a re-formulation strategy improving solubility of the compound and also stability is mandatory to develop new ophthalmic products containing AmB.

Day et al. studied in 2011 the synthesis of a complex formed by AmB and Pyridoxal Phosphate. Pyridoxal phosphate is the active form of Vitamin B_6, and it is safely used in the eye as an anti-glycating agent to prevent the cataract formation located in the lens of the eye. The cataracts are formed due to a phenomenon called glycation, which is when simple sugars react with

proteins to form proteins cross-links. This process ends with the production of advance glycation end (AGEs) products (Lehman *et al*, 2001).

Pyridoxal phosphate forms stable imine bonds with primary amines at physiological pH in aqueous conditions. They applied that model to insoluble drugs such as AmB or Nystatin to form pro-drugs (Day et al., 2011). This model is based in the chemical reaction known as a Schiff base reaction (Figure 3.3) where the amine group of AmB reacts with the aldehyde group of the pyridoxal-5′-phosphate to form the Schiff base (imine) (Dhar *et al.*, 1982).

Figure 3.3 - Schiff base reaction

Although the formation of the Schiff base is the main reaction, what confers the stability to that chemical union is the hydrogen bonds form. H-bonds occur when 2 electronegative atoms interact with the same hydrogen. In this case, it is the imine nitrogen and the 5-OH group of PLP what are involved in the formation of the H-bond (Day et al., 2011).

In Figure 3.4 is described in detailed the reaction between PLP and AMB. It consists in protonating the ring nitrogen of the pyridine at physiological pH. The primary amine group of AmB will attack the aldehyde function of the pyridoxal phosphate to form a Schiff base. The phenolic alcohol acts as an acid/base catalyst by protonating the imine and forming an intramolecular

hydrogen bond that will make the complex AmB-pyridoxal phosphate more stable (Day *et al*., 2011).

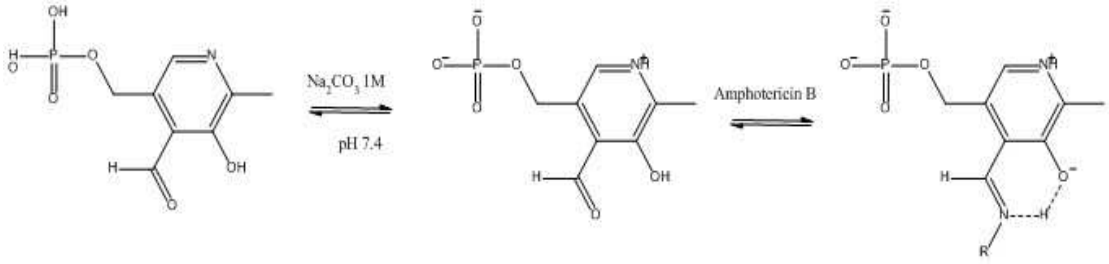

Figure 3.4 - Complex formed by amphotericin B and Pyridoxal phosphate (R = AmB).

Based on Day *et al.* research, the re-formulation strategy has been to develop two conventional ocular drug delivery systems containing the AmB-PLP complex, eye drops and gel formulations. Eye drop solutions are the most common used delivery systems for topical treatments in ocular disease. However, those formulations have poor bioavailability due to the rapid elimination of the active ingredient through high tear fluid turnover and ocular biological barriers, such as the corneal epithelium. These factors limit ocular absorption of drug (Patel *et al*, 2013). On the other hand, the development of gel formulations in ophthalmic practice as drug delivery systems means extension of drug contact time with the eye surface. This helps to increase the ophthalmic bioavailability of the drug and also reduces dosing frequency improving patient compliance (Gaudana *et al*, 2010).

For these reasons mention above, apart from a novel eye drops product, two ophthalmic gels were developed to improve the bioavailability in the administration of drugs to the eye. Two different types of gel were targeted during the developing of the formulations, ready to use gel and in situ gel-forming.

Once the formulations were developed stability studies of the novel ophthalmic products were performed to assign an expiry date to the medicines ensuring the quality of the product.

3.2.2 Materials

3.2.2.1 Synthesis of amphotericin B-pyridoxal phosphate complex

To synthetize amphotericin B–pyridoxal phosphate complex (AmB-PLP), AmB powder was kindly supplied by the PPU within NHS GG&C. Pyridoxal Phosphate and Na_2CO_3 were purchased from Sigma Aldrich (Irvine, UK). The methanol was obtained from Fisher Scientific (Leicestershire, United Kingdom).

3.2.2.2 Ophthalmic formulations

The eye drops currently used in the NHS were kindly manufactured and supplied by the PPU within NHS GG&C.

Carbopol 974P was donated by Surfachem (Leeds, UK), Methocel K4M Premium (HPMC) was obtained from Colorcon Ltd (Dartford, UK). Pluronic F128 and sodium hydroxide pellets were purchased from Sigma Aldrich (Irvine, UK). Phosphate Saline Buffer tablets were purchased in Thermo Fischer Scientific (Loughborough, UK)

3.2.2.3 Stability Studies

To validate the analytical method developed by Italia *et al.* (2009), sodium acetate trihydrate and ACN HPLC grade were purchased from Fisher Scientific (Leicestershire, UK). These reagents were used to prepare the chromatographic buffer. All chemicals and solvents were HPLC grade and the water used was 18MOhm deionised water.

3.2.3 Methods

3.2.3.1 Synthesis of amphotericin B-pyridoxal phosphate complex

The method developed by Day *et al.* was used to enhance the solubility of AmB. However dimethyl formaldehyde was substitute by methanol due to a desire to improve downstream processing capabilities. The production of the AmB-PLP involved 2 steps, the reaction process between AmB and PLP to synthetize the conjugate, and the freeze drying process to isolate the complex as a powder. The synthesis of the conjugate was reached by dissolving the pyridoxal-5'-phosphate in water (pH adjust to 7.4 with Na_2CO_3) and the AmB in methanol. The solution of AmB was added to the PLP solution drop by drop while stirring. The mixture was left stirring over 24h in the dark at room temperature. During that time the majority of the methanol evaporated and the volume left corresponding to the water used to dissolve the PLP was filtered and made up to 100 ml with water. The filtered solution was frozen and then freeze dried to eliminate the water and any residual traces of methanol from the product and to turn the solution into a dry solid powder.

The freeze drying process, also known as lyophilisation, is the best approach to process heat-sensitive drugs. With this technique the complex can be dried at low temperatures without compromised the activity of the AmB. The freeze dryer used was a Christ-EPSILON 2-4 LSC. The solution was loaded into the freeze dryer and the freezing stage started. The freezing stage lasted for 4 hours and 30 minutes at a temperature of -80 °C. Once the solution was in a frozen state, the primary drying was commenced. This step went on for about 24 hours and the temperature was increased up to -10 °C, and then the final drying where the temperature increased to 20 °C for 10 hours. The pressure will drop from atmosphere pressure to 0.011 mbar gradually during the main and final drying.

In order to optimize the synthesis process different factors were tested. The efficiency of the reaction using different molar ratio of AmB/PLP was the first aspect investigated. The AmB yield obtained by synthetizing the complex using three different molar ratios between AmB and PLP were compared (1:1, 1:2 and 1:4) to determine whether or not there was any difference in the final product. The AmB yield of the chemical reaction is the relation between the theoretical amount of AmB that could be produced during the reaction (theoretical yield) and the actual AmB amount of product obtained after the reaction and calculated by HPLC (actual yield). Other factor studied during the development of the formulation was if the complex filtration had any impact on the yield obtained. In this case only 2 molar ratios AmB:PLP were used (1:1 & 1:2).

Solubility is the physical propensity of a solid, gas or liquid to dissolve in a solvent. Therefore the solubility of the AmB-PLP complex depends upon the

solvent used. The British Pharmacopoeia (2016) classifies solubility ranging from very soluble (less than 1 part of solvent required per part of solute) to practically insoluble (more than 10,000 part of solvent required per part of solute). In pharmaceutical practice it is very important to investigate the solubility of compounds as part of the new product development. More than 40% of the new chemicals discovered have poor solubility (Kavitha *et al.*, 2015). This is not only a challenge during the formulation stage but also for the subsequent bioavailability after administration. AmB is insoluble in water, however Day *et al.* (2011) reported that the solubility of the AmB and PLP complex in water was improved significantly.

The solubility of the AmB and PLP complex was determined by the saturation shake flask method. An excess amount of freeze dried powder was added to distilled water until visual saturation was achieved, as judged by the appearance of a suspension. The suspension was shaken for 24h protected from light at room temperature. After 24h, visual inspection was performed to confirm undissolved material was still present. The sample was then centrifuged at 3000 rpm and visually inspected to observe that a solid pellet had formed in the tube. The clear supernatant was isolated for quantification by HPLC-UV.

3.2.3.2 Novel Ophthalmic formulations

Using the AmB-PLP powder as a starting material, novel ophthalmic formulations were manufactured and assessed for stability. Three batches of all AmB-PLP formulations variants were manufactured in the University of Strathclyde under uncontrolled conditions. Each batch contained 30 units and was sub-divided into lots stored at different temperatures.

3.2.3.2.1 Amphotericin B eye drops

The freeze dried powder of AmB-PLP complex was diluted in Phosphate Saline Buffer (PBS) to provide an aqueous eye drop containing 0.15% w/v of active.

3.2.3.2.2 Amphotericin B in situ ophthalmic gel

The selection of the excipients to be used in the formulation of the in situ gel is a key factor during the development stage. A formulation of composition detailed below was produced using the cold manufacturing method described by Soga et al. (2005).

Table 3-2 - Composition of Pluronic F127 gel containing AmB

Formulation component	% w/v
Pluronic F127	20
AmB-PLP complex	0.15 (as AmB base)
Phosphate buffered saline	q.s

Pluronic F127 powder was added to PBS buffer solution and stirred at 4 °C until completely dissolved. At this point an appropriate quantity of the AmB-PLP complex powder was added and allowed to dissolve in the solution before being made up to the desired final volume.

3.2.3.2.3 Amphotericin B ready to use gel: Carbopol 974/HPMC K4M

A formulation of composition detailed below was manufactured.

Table 3-3 - Composition of Carbopol 974/HPMC K4M gel containing AmB

Formulation component	% w/v
Hydroxypropyl methylcellulose K4M	1
Carbopol 974	0.4
AmB-PLP complex	0.15 (as AmB base)
Phosphate buffered saline	q.s

Hydroxypropyl methylcellulose K4M (HPMC) and Carbopol 974 were added to the buffer solution and allowed to hydrate with stirring. Once the excipients were completely wet, the required quantity of the AmB-PLP complex was dissolved in a small quantity of purified water and then transferred into the wetted HPMC/carbopol solution. The pH of the solution was adjusted to 7.5 using 0.1 M NaOH and the volume made up with additional buffer solution.

3.2.3.3 Validation of the reverse phase high performance liquid chromatography (RP-HPLC) method for AmB quantification

The method was developed by Italia *et al.* (2009). The characteristics of the method are described in Table 3-4.

Table 3-4 - Chromatographic conditions of the HPLC method develop to assay

Chromatographic conditions			
Method type	Gradient		
Column	Gemini 5 μm C18		
Mobile phase	Acetate buffer pH 4.0 / ACN		
	Time (min)	%Buffer	%ACN
	0	60	40
	4	20	80
	8	20	80
	9	60	40
	12	60	40
Flow rate	1 ml/min		
Volume of injection	20 μl		
Wavelength	407 nm		

To carry out the stability study of the AmB-PLP powder and ophthalmic formulations two different HPLC systems were used. The validation of the method and the stability study of the formulation currently manufactured at the PPU within NHS GG&C were performed in the equipment based in the regional quality assurance service (RQAS) in Stobhill Hospital. This HPLC consisted in a pump SpectraSYSTEM P4000 with an X-ACT 4 channel degassing unit, the autosampler model was SpectraSYSTEM AS3000 and also a UV SpectraSYSTEM detector model UV6000LP.

To perform the stability studies and characterization of the AmB complex powder and the new ophthalmic formulations, the method was transferred to different equipment based at the University of Strathclyde. It was the same

equipment used to develop and validate the method used to carry out the stability studies of MMC.

The method was validated under the same standards described for the validation of the HPLC procedure to study the stability of MMC. The same parameters described previously were tested to validate this method. Specificity, accuracy, range, linearity, repeatability and intermediate precision were assessed to ensure the appropriate method was chosen for stability purposes (ICH, 2005).

- **Identification**

AmB is unstable at high temperatures dissolved in DMSO, therefore to assess the specificity a sample of AmB in 60% DMSO with a concentration of 5 μg/ml was prepared and heated in a water bath at 50 °C for 30 min not protected from light to stress the active ingredient and to check the degradation products after the process.

Figure 3.5 shows the chromatogram obtained from the sample actively degraded. The main peak of AmB reduces in height whilst new the degradation products start to appear in the chromatogram.

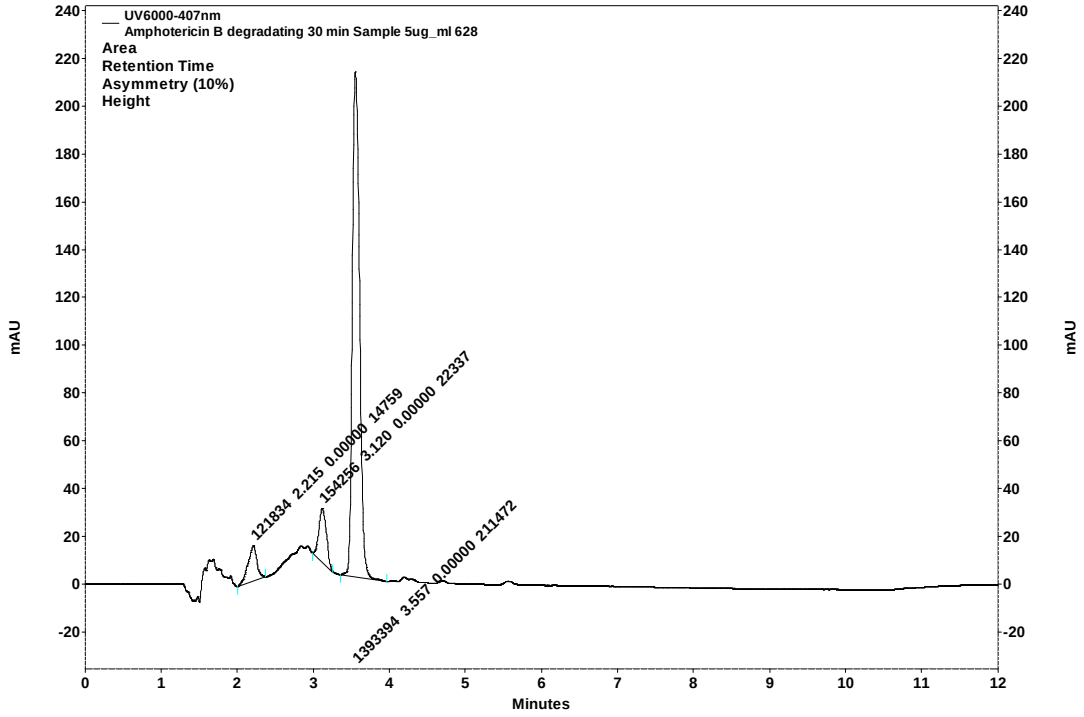

Figure 3.5 - Degradation of a sample of amphotericin B. The highest peak corresponds to the sample of amphotericin B 5 μg/ml in 60% DMSO.

- **Assay of amphotericin B**

Linearity, accuracy, range, intermediate precision and repeatability have been assessed to validate the method (ICH, 2005).

Specificity was already treated under identification study. The linearity was assessed measuring the AUC of the active ingredient peaks at concentrations of AmB in a range between 1.5 μg/ml to 10 μg/ml. Each level was injected and measured three times and the mean was calculated, the resultant value was used for linearity testing. Table 3-5 shows all the data collected from the linearity assay and the Figure 3.6 demonstrates a coefficient of determination of 0.9997.

Table 3-5 - Summary of the data collected to assess the linearity of the procedure.

Level of calibration curve	Concentration (µg/ml)	Mean AUC±SD
1	10	4484581±17579
2	7.5	3434260±8651
3	5	2250236±12648
4	2.5	1107042±2795
5	1.5	702326±2219

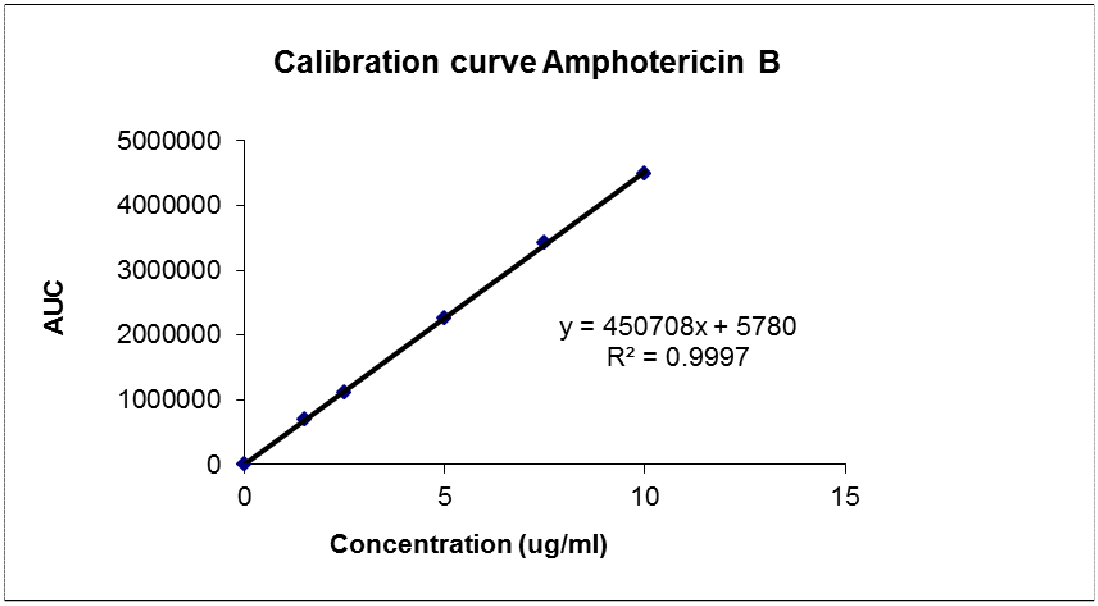

Figure 3.6 - Calibration curve of amphotericin B and equation.

Repeatability and intermediate precision were tested to assess precision. The repeatability test consisted in 7 determinations of the test sample to establish the RSD of the mean. In Table 3-6 & Table 3-7 is summarized the information obtained. As it is shown, the RSD of the AUC is 0.86% and for the RT is 0.58%, both under the 2% value recommended by ICH guidelines.

Table 3-6 - Repeatability of the AUC and RT of the peak corresponding to amphotericin B.

Sample	RT (min)	AUC
1	3.518	2283100
2	3.55	2264814
3	3.58	2252541
4	3.492	2255794
5	3.53	2245438
6	3.548	2254677
MEAN	3.54	2259394
%RSD	0.86	0.58

Intermediate precision was also tested as part of the precision study. There are different ways to assess intermediate precision, the test chosen depend on the circumstances under the procedure is intended to be used (ICH, 2005). The parameter studied in this case is the precision in different days. The experiment consisted in running a test sample of 5 μg/ml over 3 days to determine the RSD of the sample tested for precision intraday.

Table 3-7 - Data collected from intermediate precision in a sample of 5 ug/ml.

Day	AUC	MEAN	%RSD
1	2256013	2315237.667	2.64604
2	2311348		
3	2378352		

Table 3-7 shows that the RSD of the AUC values of different concentrations of AmB in different days exceeded the 2% limit imposed by the ICH guidelines.

Accuracy test should be carried out after linearity, specificity and precision have been established. To confirm the accuracy of the method the recovery of 6 determinations of 3 different concentration levels was assessed between a range of 60% to 120%. The recovery results of 4 μg/ml, 5 μg/ml and 6 μg/ml test samples was 97.18%, 98.46% and 99.77% respectively, verifying the accuracy of the reverse-phase HPLC method (Table 3-8).

Table 3-8 - Results of accuracy test

Theoretical amount (μg/ml)	Experimental amount (μg/ml)	Experimental amount (%)	MEAN	%RSD
6	5.99	99.89	99.77	0.18
	5.98	99.64		
5	4.96	99.11	98.46	0.91
	4.89	97.84		
4	3.91	97.82	97.18	0.93
	3.86	96.53		

For AmB HPLC procedure the test concentration is 5 μg/ml and the range was validated for linearity, accuracy and precision between 30% and 200% of the analyte concentration. As per ICH guidelines requirement, the range has to be validated at least between 80% and 120% of the active ingredient test concentration. Therefore, the range of this method was confirmed.

- **Summary of validation results**

All the results obtained from the validation of the method summarized are shown in table 3-9. It was verified that there was a linear relationship between the AUC and the concentration of the active ingredient over the chosen range. The method has also proven to be accurate and precise.

Table 3-9 - Summary of validation results

Procedure	Attributes	Results
Identification	Specificity	Demonstrated the separation of the active ingredient from the degradation products.
Assay for amphotericin B	Specificity	Demonstrated the separation of the active ingredient from the degradation products.
	Linearity	30%-200%
	Range	30%- 200%
	Repeatability AUC	RSD< 0.60%
	Repeatability RT	RSD< 0.90%
	Intermediate precision	RSD< 2.65%
	Accuracy (Recovery)	6 μg/ml – 99.77% 5 μg/ml – 98.46% 4 μg/ml – 97.18%

3.2.3.4 Design of the stability study

On the same manner that MMC stability studies were planned, the AmB project followed the same stages. While the method was validated, the strategy to study the stability of different formulations was planned (Figure 3.7). First of all a stability study of the product manufactured at the PPU within NHS GG&C was carried out to confirm the shelf life assigned by the manufacturer. If the stability of this pharmaceutical product was confirmed to be less than 3 months then a re-formulation process would be the next step including new stability studies of the novel formulation. If on the other hand, the AmB eye drops already manufactured had a shelf life greater than 3 months, no reformulation work would be needed.

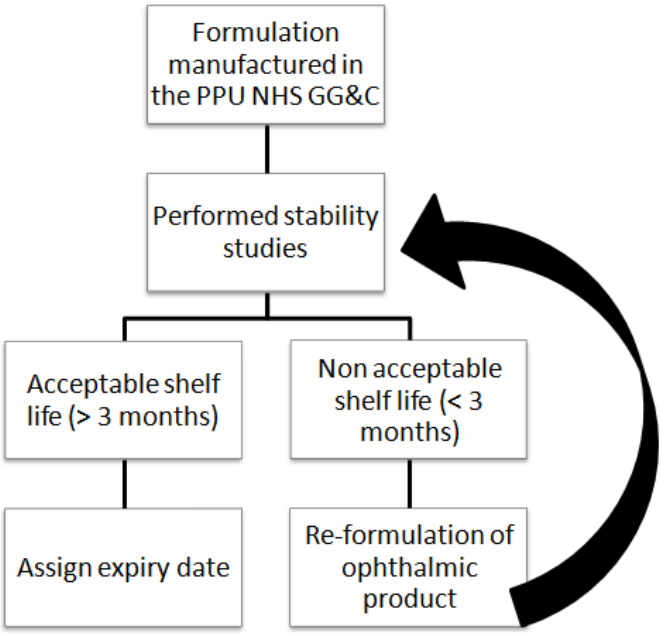

Figure 3.7 - Development strategy of the stability studies of ophthalmic formulation manufactured in the Pharmacy Production Unit.

Three batches of the AmB eye drops at a concentration of 0.15% w/v were manufactured at the PPU within NHS GG&C and stored at 4 °C. Each batch was tested every week. The stability testing of this formulation was performed before any development and characterisation work of the novel formulations.

The decision of storing the ophthalmic final product at refrigerated conditions during the stability studies was made because is well known than AmB is quickly degraded at higher temperatures or when exposed to light. Due to the well-known instability of AmB in aqueous solution, the testing frequency was established as once per week. Although humidity is a parameter that should normally be controlled during the stability study, because the product is an aqueous solution the humidity will not have an impact in the stability of the product (ICH, 2003). Therefore, the humidity was not controlled in any of the ophthalmic formulations studied.

On the other hand, the storage conditions during the stability assessment of the novel products were different. In terms of the AmB-PLP complex powder, it was stored at 4°C, 25°C and 37°C as it is known that some compounds are usually more stable in powder state than in aqueous solution. And the novel formulations were stored at either 4°C or 25°C as these would be the typical storage conditions for an ophthalmic product.

3.2.3.5 Sample preparation

3.2.3.5.1 Preparation of calibration standard solutions

A stock solution of 100 μg/ml was prepared by reconstituting a 50 mg vial of Fungizone® with 10 ml of water for injection. Mixing thoroughly to dissolve the entire contents and transferring the solution to a vial. Then, 100 μl of this solution was placed into a 5 ml volumetric flask and made up to volume with the same solvent. Afterwards, appropriate aliquots of the standard stock solution were prepared diluting with purified water, with resulting concentrations of 10 μg/ml, 7.5 μg/ml, 5 μg/ml, 2.5 μg/ml and 1.5 μg/ml. AmB powder was also used to make the standard solutions instead of Fungizone® and the solvent used to prepared them was a mixture of DMSO/water (60/40 v/v).

3.2.3.5.2 Preparation AmB 0.15% w/v PPU ophthalmic formulation for HPLC testing

The eye drop bottles were brought to room temperature and the content of the eye drops was gently agitated before being diluted with distilled water to a nominal concentration of 50 μg/ml and analysed by HPLC. This solution was further diluted to 5 μg/ml and mixed before being transferred into an HPLC vial.

3.2.3.5.3 Preparation of AmB-PLP complex powder for stability studies

The vials containing the freeze dry powder were brought to room temperature and 3 ml of HPLC grade water added to each vial to dissolve the AmB-PLP complex. The solution was then transferred to a volumetric flask and the volume was made up to 10 ml and then further diluted to 7.5 μg/ml with HPLC grade water. Once the sample was prepared it was transferred to an HPLC vial for analysis.

3.2.3.5.4 Preparation of AmB-PLP complex eye drops 0.15 % for stability studies

The vials were brought to room temperature and the content of the eye drops gently agitated before being diluted with distilled water to a nominal concentration of 50 μg/ml and analysed by HPLC. This solution was further diluted to 7.5 μg/ml and mixed before being transferred into an HPLC vial.

3.2.3.5.5 Preparation of amphotericin B - pyridoxal phosphate complex 0.15 % ophthalmic gel preparation for stability studies

Each vial of gel formulation was brought to room temperature. Then, samples at this point were further diluted to a nominal AmB concentration of 50 μg/ml. This solution was further diluted to 7.5 μg/ml and mixed before being transferred into an HPLC vial.

3.2.4 Results

3.2.4.1 Synthesis of amphotericin B-pyridoxal phosphate complex

Figure 3.8 shows the percentage of AmB yield obtained in each reaction, the results were analysed and showed no significant difference in the quantity of AmB in the final product at different molar ratios of AMB:PLP ($p \geq 0.05$).

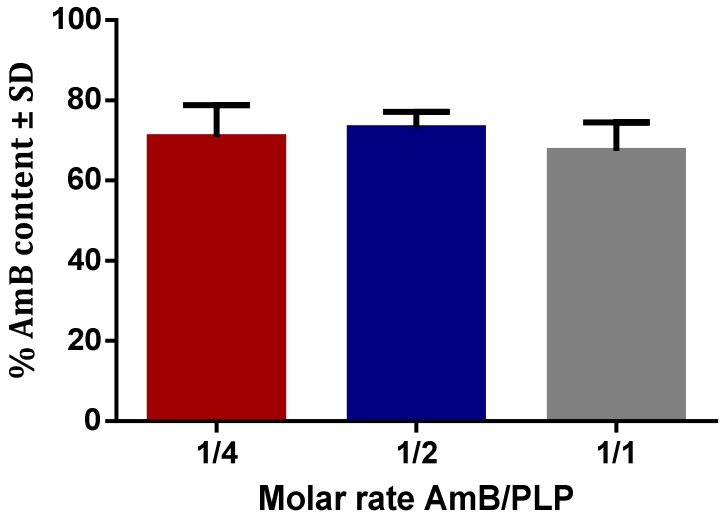

Figure 3.8 - % yield of amphotericin B±SD in the final product at 3 different molar ratio (n=3).

In Figure 3.9 is shown the final AmB yield in the complex calculated when the filtration stage is carried out before freeze drying and also after freeze drying the product. The analysis of the results showed that there is no significant difference between carrying out the filtration of the complex before or after the freeze drying process ($p \geq 0.05$) in any of the molar ratios (1:1 & 1:2).

The yield obtained from the reaction of AmB:PLP at a molar ratio of 1:1 & 1:2 when the filtration was carried out before the freeze drying step was also compared. And finally the yield obtained from the reaction of AmB:PLP at a molar ratio of 1:1 & 1:2 when the filtration was carried out after the freeze drying step was also compared. In none of those cases was there significant differences in the quantity of AmB in the final complex between molar ratio 1:1 and 1:2 either if the filtration is made before or after the freeze drying process ($p \geq 0.05$).

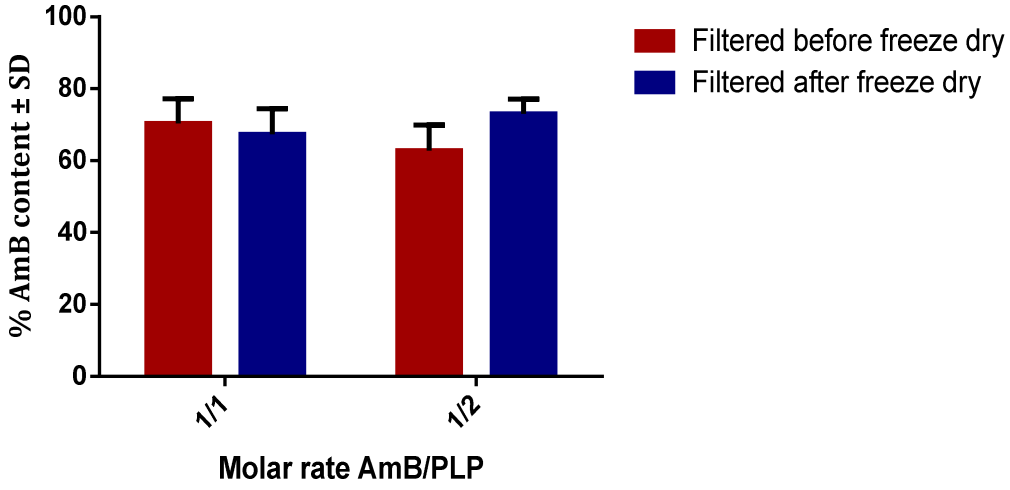

Figure 3.9 - % of amphotericin B±SD in two final complex at a molar rate of 1:2 and 1:1 before and after the freeze dry process (n=3).

The experimental results showed that the solubility of AmB-PLP complex in water was 90 mg/ml.

3.2.4.2 Stability Studies

3.2.4.2.1 Stability data of amphotericin B eye drops manufactured in the NHS

The stability of 3 batches of eye drops manufactured from Fungizone® in the Pharmacy Production Unit was tested and the results are shown in Figure 3.10. The rates of degradation of this formulation tested every week and stored at 4 °C reached 10% after 6 weeks.

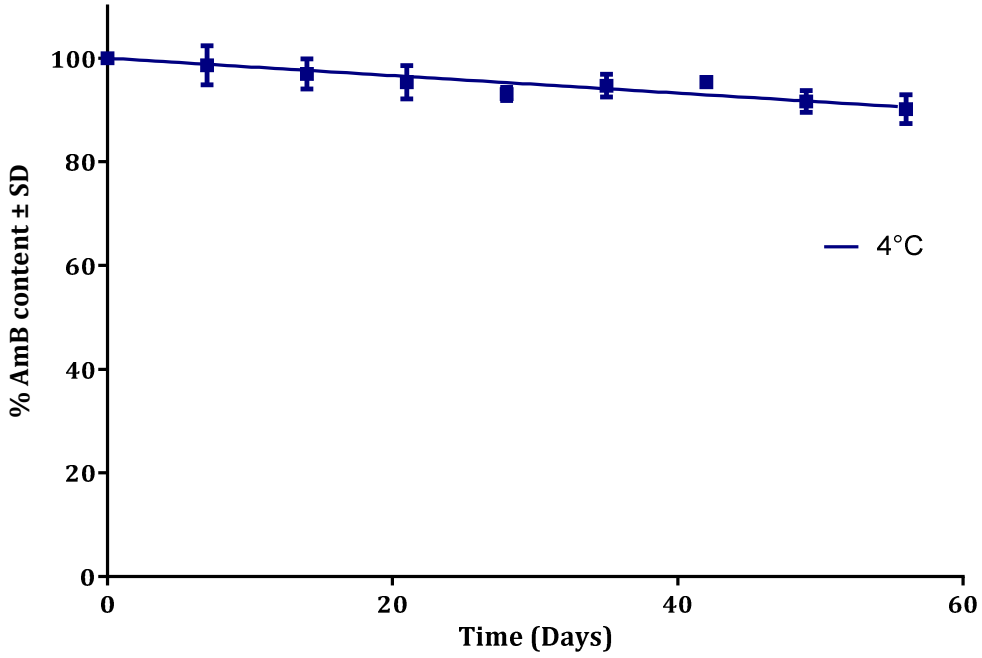

Figure 3.10 - Stability of amphotericin B eye drops manufactured in the NHS over 2 months (n=3) using Fungizone® as a starting material.

3.2.4.2.2 Stability data of AmB-PLP powder

The freeze dry powder of AmB-PLP was stored at 4 °C, 25 °C and 37 °C and tested for stability to check the rate of degradation of the active ingredient over a period of 98 days.

The powder shows a good stability when kept at 4 °C and 25 °C, with reduced stability when stored at 37 °C. The content of AmB did not reach the t_{90} during the 98 days that the study lasted when stored at 4 °C. When the formulation was stored at 25 °C the AmB content decreased 5% in 84 days, and finally at 37 °C reached 88% after 98 days.

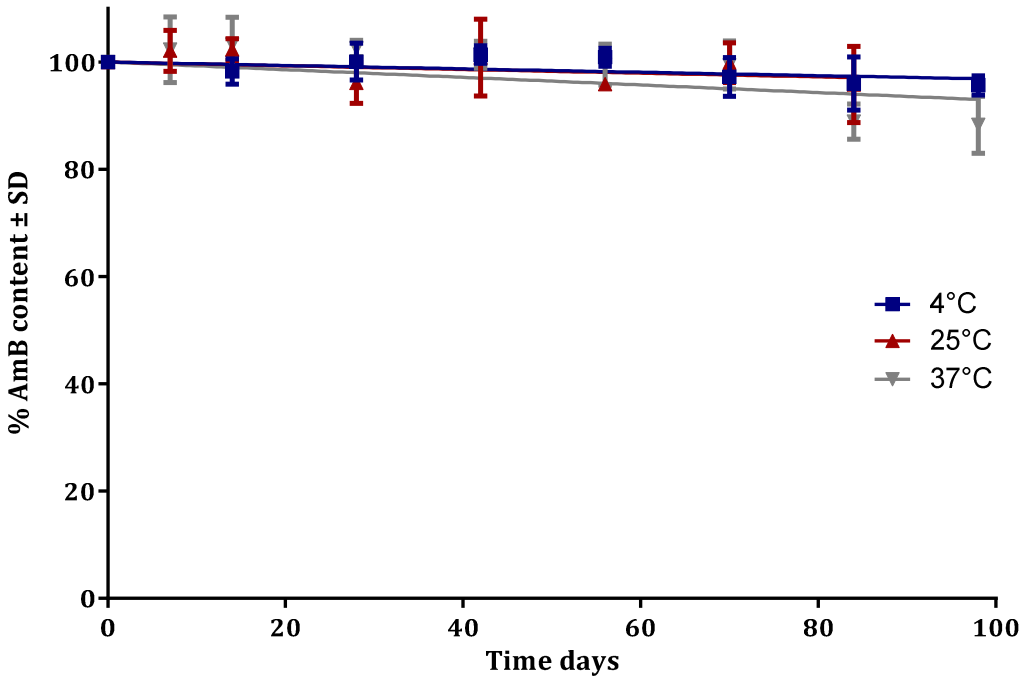

Figure 3.11 - Stability of amphotericin B-PLP freeze dried powder. The product was kept in vials, protected from light at 4 °C, 25 °C and 37 °C (n=3). The testing was performed over 3 months.

3.2.4.2.3 Stability study of the novel eye drops formulation

Three batches of the AmB-PLP complex eye drops were manufactured and kept at 4 °C and 25 °C over a period of time for stability purposes, no samples were stored at 37 °C. One bottle of eye drops from each batch was tested every 2 weeks until the end of the study.

In terms of content of AmB over time the formulations stored at 4 °C and 25 °C stayed stable over 122 days (Figure 3.12).

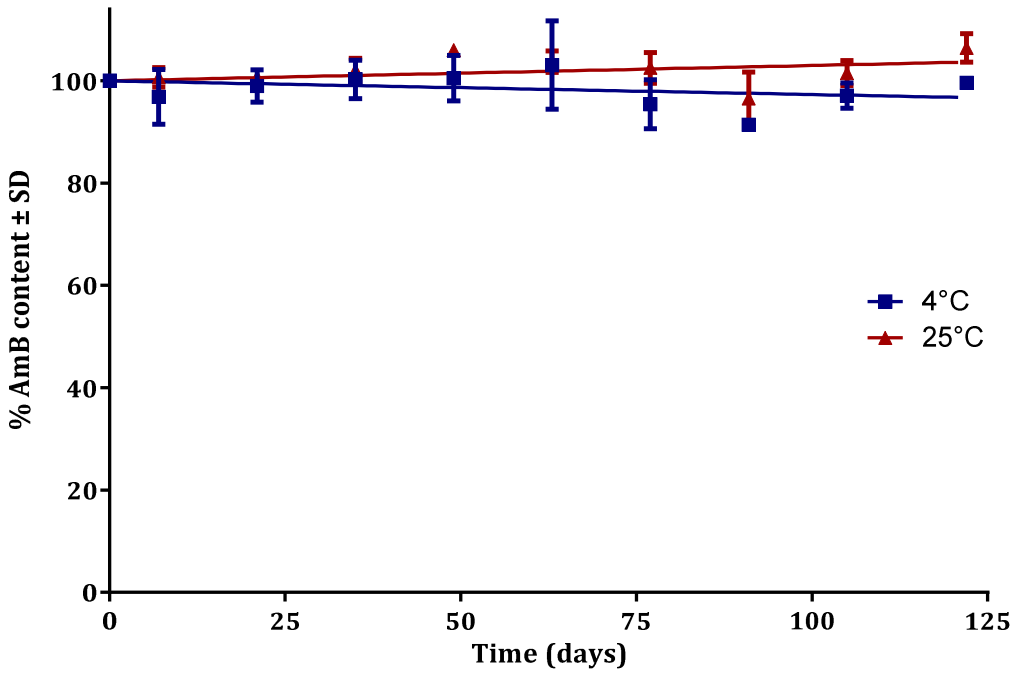

Figure 3.12 - Stability of novel eye drop formulation manufactured at the University of Strathclyde. The product was kept protected from light at 4 °C and 25 °C (n=3). The testing was performed every 2 weeks over 122 days.

3.2.4.2.4 Stability study of the new ophthalmic gel formulations

Two different gel systems were developed and assessed for stability. The in situ gel (Pluronic) formulation was assessed for stability at 4 °C and 25 °C

and was tested every 2 weeks. After 122 days stored at 4 °C the content of AmB dropped to 91%. However, the same formulation kept at 25 °C experiments experienced a fall to 67% of starting AmB content.

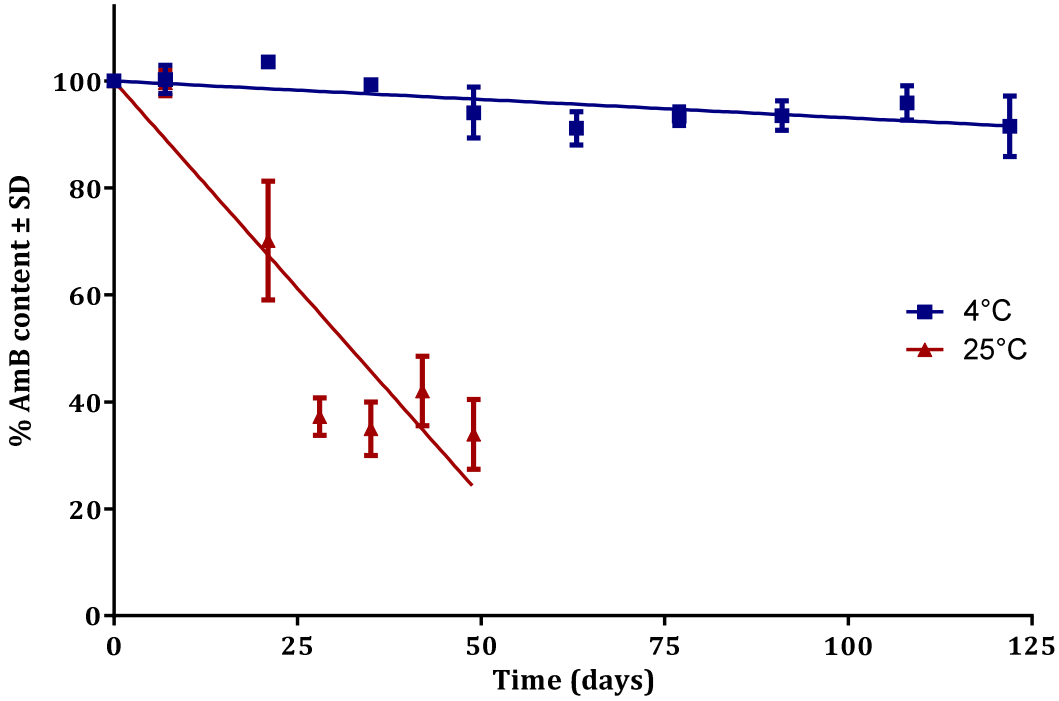

Figure 3.13 - Stability of novel pluronic gel formulation manufactured in Strathclyde University. The product was kept protected from light at 4 °C and 25 °C (n=3). The testing was performed every 2 weeks over 4 months and 1.5 months respectively.

The other product developed was the ready to use carbopol gel formulation. When stored at 4 °C it was found that at 122 days post manufacturing the AmB content was slightly lower than when stored at 25 °C (97% and 99% respectively). Visual inspection indicated a change in the clarity of the product stored at 25 °C.

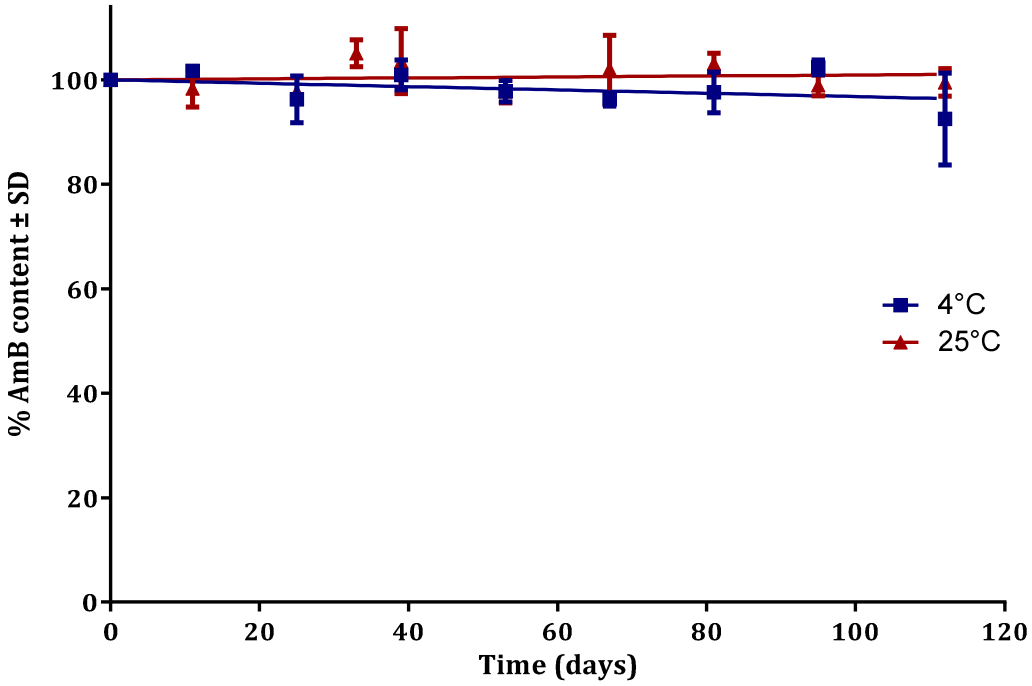

Figure 3.14 - Stability of novel Carbopol gel formulation manufactured in Strathclyde University. The product was kept protected from light at 4 °C and 25 °C (n=3). The testing was performed every 2 weeks over nearly 4 months.

3.2.5 Discussion

The main objective of this research was to develop new ophthalmic pharmaceutical products containing AmB with a superior stability. To achieve this, it was mandatory to improve the solubility of the active ingredient in aqueous solution. It has to be mention that the variation of the process in comparison with the method described by Day et al. (2011) to synthetize the complex AmB-PLP did not make any impact. The results obtained were very similar, with a dramatic improvement in the solubility of the complex (90mg/ml) in comparison to that of AmB (insoluble) in water at pH 6-7 (Lemke *et al.*, 2005).

In terms of the synthesis of the complex, the molar ratio 1:2 of AmB:PLP exhibited a higher yield of AmB and that is more feasible to filter the solution before the freeze drying process. Due t the fact that only a 65% of AmB was recovered further development of the API manufacturing method should be carried out and extra purification of the complex is advisable.

There are several methods published to quantify this compound, however to carry out the stability studies a method developed by Italia *et al.* (2009) was chosen to quantify the AmB in solution. However Gemini 5 μm C18 column was used.

The validation tests were performed in the equipment based in Regional Quality Assurance Service, the degradation study was the first parameter to be investigated. This experiment was especially important due to the fact that AmB is a natural compound and even when a high purity powder is used, traces of amphotericin A could be detected. The structure of this impurity only differs from AmB in a double bond between C28-C29, therefore it is very common to see the peaks of both components eluting at similar RT (Aszalos *et al.*, 1985). To study the specificity of the method, AmB was stressed with heat. This active ingredient is very sensitive to high temperatures therefore this was the approach chosen to stress the sample. Although extreme pH or light also affect the stability of AmB, these parameters were not used during the degradation experiment due to the fact that the finished product will be formulated in a buffer and stored in an amber container. The chromatogram resultant from this experiment showed that the main peak of the active ingredient reduced its AUC, at the same time the secondary peaks increase the AUC and new peaks corresponding to

possible degradation products appeared in the chromatogram. Therefore it was proven that the method is thus stability indicating and degradation products could be observed while the AmB is degrading. Although the peaks of the impurities were resolved from the AmB peak and no interferences were observed in the region of the AmB, this analytical method was not able to discriminate the degradation products resulting from heat conditions. Therefore any degradation products were not identified or quantified.

For the analytical method, the correlation coefficient (r^2) was 0.9997, so the method was considered linear over the range of concentration chosen (1.5 μg/ml to 10 μg/ml). Moreover, the repeatability showed an RSD below 2%. The precision intraday was also measured by calculating the RSD of a 5 μg/ml sample tested in 3 different days. The results showed that the RSD was 2.6% exceeding slightly the limits, therefore the precision intraday was repeated and the same result was obtained. In order to finish the validation this result was accepted due to the fact that the deviation from the limit state in the ICH guidelines is minimal and variances in the temperature of the room could have a huge impact in the precision intraday. Furthermore, accuracy was measured obtaining a recovery range within 97% - 100%. With these results the method was considered accurate, repeatable, linear and with an acceptable intermediate precision.

The first stage to study the stability properties of AmB in different ophthalmic formulations was to carry out the validation work of this analytical method, and prove that the method was suitable for its intended use. The next stage was to design the stability studies of each formulation;

parameters such as testing frequency, storage conditions and number of batches were planned before any formal stability work was performed.

The AmB eye drops manufactured at the PPU within the NHS GG&C was the first formulation studied to ensure that the shelf life of 2 weeks assigned by the manufacturer was appropriate. Three batches were prepared aseptically and tested in the HPLC once per week, the length of the study resulted in 2 months due to the fact that during that time the formulation was proven to be unstable. The eye drop bottles were kept at 5±3 °C and the containers were protected from light at all the times not to induce the degradation of AmB. Once the results were obtained and they were analysed, the product shelf life was assigned. Although the t_{90} was reached at about 6 weeks after manufacturing, the shelf life assigned was only 28 days. This was due to the fact that the assay in all 3 batches at time zero only reached the 96% of the label content. The AmB powder used to manufactured the eye drops was Fungizone, although is a marketed product the state label content may not be exact which could have an influence in the low content of the eye drops. Also, the multiple manipulations during processing the samples could have an impact on the content of the final formulation. For all these reasons a reduce shelf life was assigned to compensate the low assay results obtained at time zero. Nevertheless, it is an improvement taking into account the 2 weeks shelf life assigned by the manufactured, a longer shelf life would be ideal for this product.

After those findings the development of novel ophthalmic formulations was carried out, and stability studies were performed on the formulations

containing a complex formed by AmB and PLP to ensure an improvement in the stability of the product.

Additionally, the stability of the freeze dried power complex was also tested. Each batch had samples stored at 4 °C, 25 °C and 37 °C and were tested every 2 weeks. There were no signs of changes in the physical appearance over time. Between t_0 and t_8, day 0 and day 84, the powder experimented a fall of less than 5% when stored at 4 °C and 25 °C (Figure 3.11). However when the lyophilised powder was stored at 37 °C the concentration of AmB dropped to 88% after 84 days post manufacturing. Those findings agree with the fact that even though AmB powder is more stable than AmB dissolved in aqueous solution, one of the degradation paths of this molecule is to be exposed to high temperature (The pharmaceutical codex, 1994).

Using the same stability indicating method the novel ophthalmic preparations were also studied to demonstrate that a superior stability in comparison with the formulation manufactured at the PPU within NHS GG&C. The same number of batches of each formulation was manufactured and tested at the same frequency as the powder, however only two different storage conditions were applied, with 3 batches of each formulation stored at 4 °C and 25 °C.

The AmB content in the eye drops stored at 4 °C remained considerably stable over 122 days only decreasing its concentration by 1%, however when the eye drops were stored at 25 °C it was observed that AmB content remained stable until day 105 post manufacturing and increase the content of AmB in 5% from previous time point, t_8. A review of the results obtained

with the HPLC was undertaken and no difference in the AUC obtained with the calibration samples compared to other time points of the study. However, an increment in the AUC of the samples stored at 25 °C that were tested at the end of the procedure was noticed. A comparison between each time point of the formulation stored at 4 °C and 25 °C was performed confirming a significant difference at time point corresponding to 122 days after manufacturing. Due to the fact that the difference was in only one time point it was considered that it was due to the method preparation or the equipment. Small changes in the pressure or the temperature of the HPLC could have contributed to experimental error. Other possible explanation could be a loss of volume due to solvent evaporation during the study in the later samples, although this was considered unlikely.

Apart from the eye drops formulated with the complex there were also two different ophthalmic gel products tested for stability. As it was done with the other formulations, the gel formed by pluronic F127 was also stored at 4 °C and 25 °C. However the test frequency changed for the samples stored a 25 °C due to evident instability of AmB in this preparation in the early stages of the study as indicated by a change in clarity. As a result, the samples stored at 4 °C were tested every 2 weeks for 122 days and the samples stored at 25 °C ever week for only 50 days. The content of AmB lost about 9% of the starting content over 122 days at 4°C, and only 33% of AmB remained after 50 days when stored at 25 °C. It was noticed that the concentration obtained after testing the samples stored at 25 °C was highly variable. That was due to the sampling procedure, at 25 °C the pluronic formulation was in a gel state, due to the semi-solid consistency it was more difficult to accurately sample the formulation to perform the test.

AmB is much more stable when stored at 4 °C than at higher temperatures. This coincides with need for this ophthalmic gel being stored at lower temperatures to maintain the liquid state of the product prior to application on the patient's eye where it gels at 34 °C. However, it cannot be ignored that the formulation rapidly decomposes when stored at 25 °C.

Finally, the gel formed by Carbopol and HPMC was analysed and showed similar stability at 4 °C and 25 °C. Therefore, either the eye drops or the ready to use gel containing AmB-PLP were considered chemically stable over time when they are stored at different temperatures.

3.3 Characterisation of new ophthalmic formulations

3.3.1 Introduction

Once a pharmaceutical formulation is developed the characterization of the new product is an essential stage to ensure that the physico-chemical properties of the drug and of the formulation do not change. By evaluating these characteristics such as appearance, pH or gelation properties in case of an ophthalmic gel, the formulations profile can be optimized achieving better products at the end (Shashank *et al.*, 2012). Other factors such as aggregation state or drug-excipient compatibility can also be studied.

It is important to study the physical appearance of the ophthalmic formulations over time. A clear product is one of the objectives when developing ophthalmic formulations. Any visible particles or any sign of bacterial growth could damage the patient's eye when the formulation is administered. Also, any change in the colour of the ophthalmic formulation

from bright yellow to dark-yellow could be a sign of degradation of the active ingredient (Baranowski et al., 2014). In order to study the physical appearance of all products stored at 4 °C and 25 °C, a visual inspection was carried out alongside the stability studies of the products. The goal of this inspection was to detect any changes in colour, loss in clarity of the product or any sign of precipitation.

The adjustment of the pH is an essential factor during the development of ophthalmic formulation. A formulation with an optimal pH will reduce the discomfort and intolerability produced in patient's eye, making the product more suitable for ocular administration. The physiological pH of tears is 7.4 and possesses some buffer capacity, therefore an ophthalmic formulation with this pH will minimise the risk of lacrimation and therefore will reduce the dilution of applied drug in tear fluid. Although a pH of 7.4 would be desirable, the range of pH in ophthalmic products can vary from 4 to 8 (Baranowski et al., 2014). In terms of selecting the appropriate buffer is also important to take into account that the isotonicity of the buffer selected. Finally, the chemical stability of the active ingredient at that pH is also important during the development stage. There are some cases where the optimal pH for the eye is not the best in regards to the stability of the molecule (WHO, 2016).

Determining the degree of aggregation of the new complex and also investigating how different factors could influence the physical state of AmB was imperative as it is known how the aggregation state of AmB could impact on the side effects caused by this compound.

Drug-excipient compatibility represents an important stage during pharmaceutical development. Any interactions between the active ingredient and the excipients in the final product can influence the safety, efficacy and stability of the product. There are several methods to predict incompatibilities in the early stages of the formulation phase. Thermal analytical techniques such as Differential Scanning Calorimetry (DSC), Isothermal Microcalorimetry and Hot Stage Microscopy (HSM) or non-thermal techniques for instance Vibrational Spectroscopy, Powder X-ray Diffraction (PXRD) and Solid State Nuclear Magnetic Resonance Spectroscopy (ss NMR) are some examples of analytical methods used to investigate drug-excipient compatibility. In this research Differential Scanning Calorimetry was chosen to investigate the thermotropic behaviour of the components of the ophthalmic formulations.

Gelation capacity is the ability of some solids to form a semisolid product in contact with a liquid medium. When they become gels, they have the ability of staying at the administration site for a longer period of time than liquid formulations increasing the absorption of the active ingredient. Some products, like carbopol, have the quality of bioadhesion. This is a process by which the polymer will adhere to a biological substrate. When this biological structure is a mucosa then it is known as mucoadhesion (Palacio *et al.*, 2012).
The carbopol and HPMC eye formulation are already in a gel-state when applied into the eye. The mechanism of action of carbopol is to slightly hydrate after being added to water, followed by molecular uncoiling producing minimal swelling. However neutralization of the carboxylic groups of the molecule to a salt fully uncoils the carbomer causing a thickening effect and the formation of the gel system (Braun *et al.*, 2000).

HPMC is used as a viscosity enhancing agent and it is also considered as a mucoadhesive material like Carbopol (Rowe *et al.*, 2009).

On the other hand, Pluronic F127 also known as poloxamer 407 is a polymer used in pharmaceutical formulation as a gelling agent with thermoreversible properties. Figure 3.15 shows the mechanism of action of pluronic F127 transforming from a liquid solution into a gel (Rowe *et al.*, 2009).

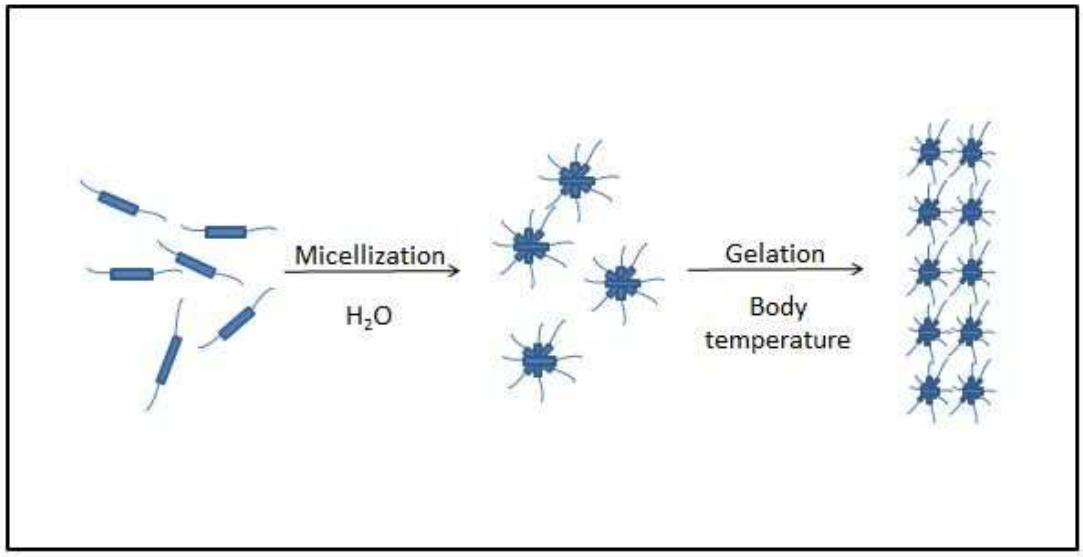

Figure 3.15 - Gelation process of pluronic F127 (Devi, Sandhya, & Hari, 2013).

This polymer provides a liquid system the consistency of a gel and its behaviour depends on the temperature. It is in liquid form at 4 °C and becomes a gel at body temperature. That property is known as an in situ gelling. Those formulations are relatively new delivery drug systems for pharmaceutical formulations (Devi et al., 2013).

The drug content and the uniform distribution of the active ingredient are important parameters to ensure the homogeneity of the formulations. During the manufacturing process of pharmaceutical products it is very common to

assess uniformity of content of the active ingredient within a batch. In the case of semisolid drug products such as ophthalmic gels, due to the physical separation that can occur during the manufacturing process it is essential to ensure homogeneity of the final product (US Pharmacopoeia, 2013).

Batch size, mixing process or the vehicle used in the formulation are factors that can affect the homogeneity of a pharmaceutical product. Using an appropriate sampling protocol and carrying out the test with a validated analytical method is imperative to appropriately qualified homogeneity. The most extended and reliable technique to be used is chromatography, therefore in this study HPLC-UV was chosen to carry out the homogeneity study (Whitmire *et al.*, 2010).

3.3.2 Materials

The freeze dried powder of AmB-PLP complex and the novel ophthalmic formulations were manufactured at the University of Strathclyde. The AmB powder and the formulation currently manufactured in the NHS was donated by the PPU within NHS GG&C. Sodium chloride (NaCl) was obtained from BDH Laboratory Supplies (Poole, UK), Sodium bicarbonate ($NaHCO_3$) was purchased from Melford Laboratories Ltd. (Ipswich, UK), calcium chloride dihydrate ($Ca_2Cl \bullet 2H_2O$) and potassium chloride (KCl) were obtained from Sigma-Aldrich Co. (Dorset, UK).

3.3.3 Methods

3.3.3.1 pH measurement

The pH of the novel ophthalmic formulations was measured over a period of 93 days. For the measurements the only materials used were the three formulations manufactured in Strathclyde University and a Methrom pH meter to carry out the readings.

3.3.3.2 Aggregation state of Amphotericin B

The simulated tear fluid was prepared weighing 0.68 g of Na_2Cl, 0.22 g of $NaHCO_3$, 0.008g Ca_2Cl and 0.15g of KCl and dissolving those salts in 100 ml of distilled water (pH 7.4) (Liu et al., 2010).

In order to study the aggregation state of the new complex of AmB and PLP, an ultraviolet spectroscopy technique was used. The spectra of AmB in DMSO and the novel complex dissolved in water were compared. The new complex of AmB-PLP in the final gel formulations was also studied to confirm whether or not the excipients would have an impact on the physical state of the complex. Absorbance spectroscopy was performed using a Shimazu UV-1700 Series. The wavelength range used was between 200 and 500 nm with a sampling interval of 0.2.

The ratio of absorbance at different wavelengths is considered to be a "fingerprint" for measuring the aggregation states of AmB. When the ratio of absorbances A_{325}/A_{409} is about 2 is an indication of the AmB being in

aggregated state, if it is lower, approximately 0.25 is it an indicative of the monomeric state of this molecule (Barwicz *et al.*, 1992).

The UV spectrum of AmB-PLP complex in simulated tear fluid was also obtained over a period of time to simulate real conditions when the formulation is administered in the eye.

3.3.3.3 Drug compatibility

All the samples were scanned with a DSC822e differential scanning calorimeter (DSC) at a heating rate of 10 °C/min in the range from 20 °C to 250 °C to determine thermal properties. DSC scans were run for the active ingredient, excipients and also the physical mixtures used in the preparation of the ophthalmic formulation. The samples were run under a 20 ml/min stream of nitrogen gas. Approximately 3 mg of each sample was weighed and placed into standard aluminium pans, those pans were hermetically sealed before being run in the DSC.

3.3.3.4 *In vitro* gelation

The simulated tear fluid were prepared weighting 0.68 g of Na_2Cl, 0.22 g of $NaHCO_3$, 0.008g Ca_2Cl and 0.15g of KCl and dissolving those salts in 100 ml of distilled water (pH 7.4) (Liu et al., 2010).

To perform the experiment a container filled with water was placed in a hot plate-stirrer at 50 °C, the temperature of the water was equilibrated at 34 °C ± 2 °C which is the temperature of the eye surface (Liu et al., 2010). When the water reached the desired temperature, a holder with 3 test tubes was placed

in the water bath, and then, STF was added to each test tube. The temperature of the STF was measured to be sure that it also reaches 34 °C ± 2 °C.

The gelling capacity of the in situ gel and the ready to use gel was evaluated. In the case of Pluronic gel the test was carried out adding a drop of 15 μl of the Pluronic solution to the STF and the time to form the gel was taken as well as the time that the gel took to dissolved. The experiment was performed with 3 different batches of the product and 3 repeats for each batch.

The ready to use formulation went through the same process, however the formulation was added as a gel not as a liquid, and only the time that takes the gel to dissolved was noted (Adam *et al.*, 2010). The way to perform the experiment is the same as per the in situ gel.

3.3.3.5 Drug content and homogeneity

The same RP-HPLC method used during the stability studies was also used to quantify AmB during this assessment. Three batches were manufactured with a size batch of 30 g for each gel formulation, in situ gel and ready to use gel. Also, 3 batches of 30 ml of the eye drop product were prepared. Sampling a pharmaceutical batch for homogeneity test is a critical step in the process. Depending on where you get the sample the results can change significantly. To obtain accurate results 3 samples should be taken, one from the top of the container, one from the middle part and one from the bottom of the jar (US Pharmacopoeia, 2013).

Figure 3.16 shows a container with the gel with a vertical line separating both sides of the jar. All samples must be taken from the left or the right of the middle line with a syringe. It was essential that this syringe was long enough to reach the bottom of the container. The first sample to be taken was from the top part of the container, afterwards the middle sample was taken and finally the bottom of the container was sampled.

Each sample weight was approximately 0.5 g and was placed in a 10 ml volumetric flask previously tared, and the exact weight was noted. The volume of the flask was made to volume with water, and properly mixed. Previously to inject the sample into the HPLC the solution was diluted 1 in 10.

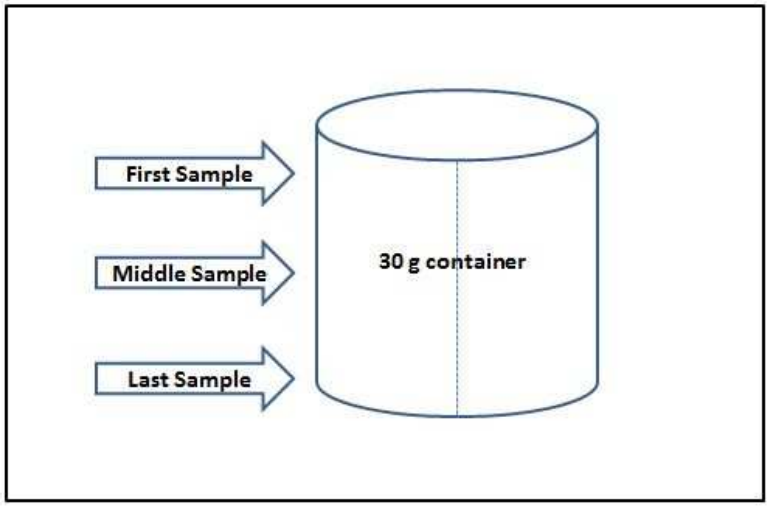

Figure 3.16 - Sampling from a 30 g container of AmB-PLP gel form homogeneity study.

3.3.4 Results

3.3.4.1 Physical appearance

No change was observed in any of the formulations kept at 4 °C (Table 3-10). Table 3-11 shows that after thirty days from manufacturing all formulations stored at 25 °C became turbid. That could be due to the fact that the batches were not aseptically manufactured. For this reason, microbiological tests were performed in those samples to check if there was any type of bacteriological growth in the formulations. RQAS department within the NHS GG&C identified the contamination through physical and chemical testing and also with the Biolog's Microbial Identification Systems software (OmniLog® Data Collection) used to identify the microorganisms from its phenotypic pattern in the GEN III MicroPlate. The results showed growth in all formulations which was identified as *Burkholderia cepacia.* This Gram-negative rod is a common contaminant of water and its presence may indicate inadequate environmental control during manufacturing. Therefore, a repeat stability study with product manufactured under aseptic conditions is warranted.

On the other hand, a change in the colour of one of the formulations was also observed. A more intense yellow-brownish colour in the ready to use ophthalmic gel was detected when stored at 25 °C after 30 days of storage. The intensification of the colour could be an indication of the degradation of the AmB.

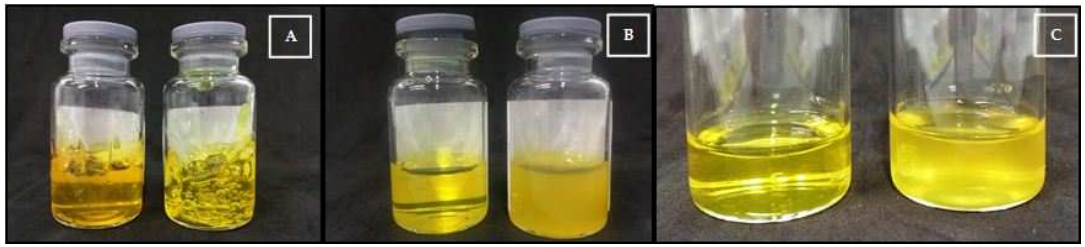

Figure 3.17 - Physical appearance of amphotericin B formulations. Picture A) Carbopol/HPMC gel at 4 °C bottle in the left and 25 °C bottle placed in the right. Picture B) Pluronic F127 gel storage at 4 °C bottle placed in the right and 25 °C bottle in the left. Picture C) eye drops storage at 4 °C bottle placed in the right and 25 °C bottle in the left.

Table 3-10 - Physical appearance of various ophthalmic formulations with amphotericin B freshly prepared and after 30, 60 and 90 days storage at 4 °C.

Formulations	**Freshly prepared**		**After 30 days**		**After 60 days**		**After 90 days**		**After 120 days**	
	Turbidity	Colour change	Turbidity	Colour change	Turbidity	Colour change	Turbidity	Colour change	Turbidity	Colour change
Eye drops	No	No	No	No	No	No	No	No	No	No
Pluronic gel	No	No	No	No	No	No	No	No	No	No
Carbopol/HPMC	No	No	No	No	No	No	No	No	No	No

Table 3-11 - Physical appearance of various ophthalmic formulations with amphotericin B freshly prepared and after 30, 60 and 90 days storage at 25 °C.

Formulations	**Freshly prepared**		**After 30 days**		**After 60 days**		**After 90 days**		**After 120 days**	
	Turbidity	Colour change	Turbidity	Colour change	Turbidity	Colour change	Turbidity	Colour change	Turbidity	Colour change
Eye drops	No	No	Yes	No	Yes	No	Yes	No	Yes	No
Pluronic gel*	No	No	Yes	No						
Carbopol/HPMC	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

*Physical appearance was only monitor for 30 days.

3.3.4.2 pH measurement

In order to perform statistical analysis, the results were examined for each individual formulation to confirm that the pH of the final products stays stable over time. A multiple comparison analysis using ANOVA was performed to compare each time point of every product.

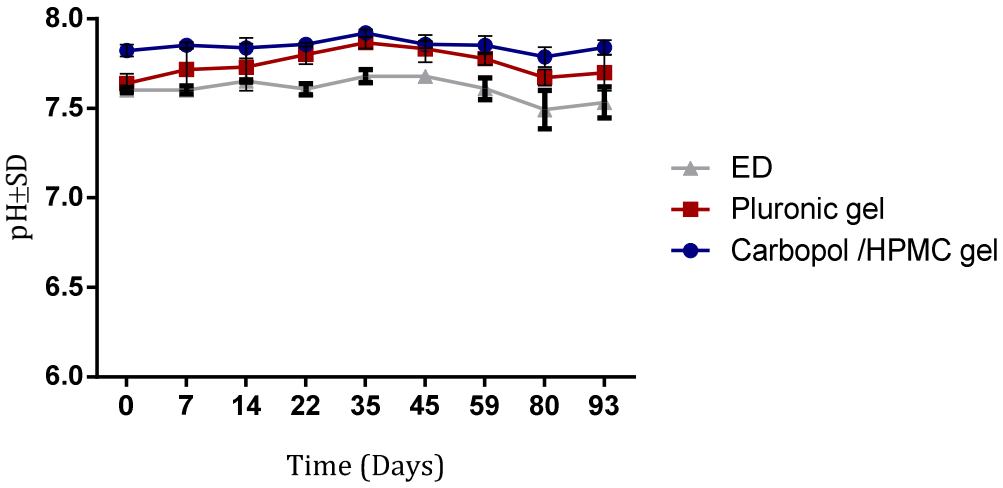

Figure 3.18 - pH measurements of the novel formulations over 93 days (n=3).

Statistical analysis showed no significant difference in the pH results between time point zero and the end of the study, in any of the 3 ophthalmic formulations tested (Figure 3.18; $p > 0.05$).

The pH of the ocular formulations varied between 7.6 and 7.8, which is considered to be stable within the tolerated pH range for this type of pharmaceutical products.

3.3.4.3 Aggregation state of Amphotericin B

The UV spectrum of molecular AmB in DMSO in Figure 3.19 (blue line) was obtained and analysed, showing the lowest peak at around 353 nm and the highest peak at 416 nm. The ratio that measures aggregation was calculated for AmB in DMSO, and it was found to be 0.25 which corresponds to the monomeric (molecular) form of AmB. This result was obtained using the absorbance values at wavelengths of 352.8 nm and 416 nm.

In Figure 3.19 the UV-spectrum of the complex AmB-PLP powder dissolved in water is also shown (purple line). In this case the maximum peaks are shown at 408.0 nm, 384.4 nm, 365.0 nm and 325.0 nm, being the highest peak at 325 nm. This can be considered as the first sign of aggregation. The ratio was also calculated as A_{325}/A_{408} and the result was found to be 1.17 which indicates a higher aggregation state.

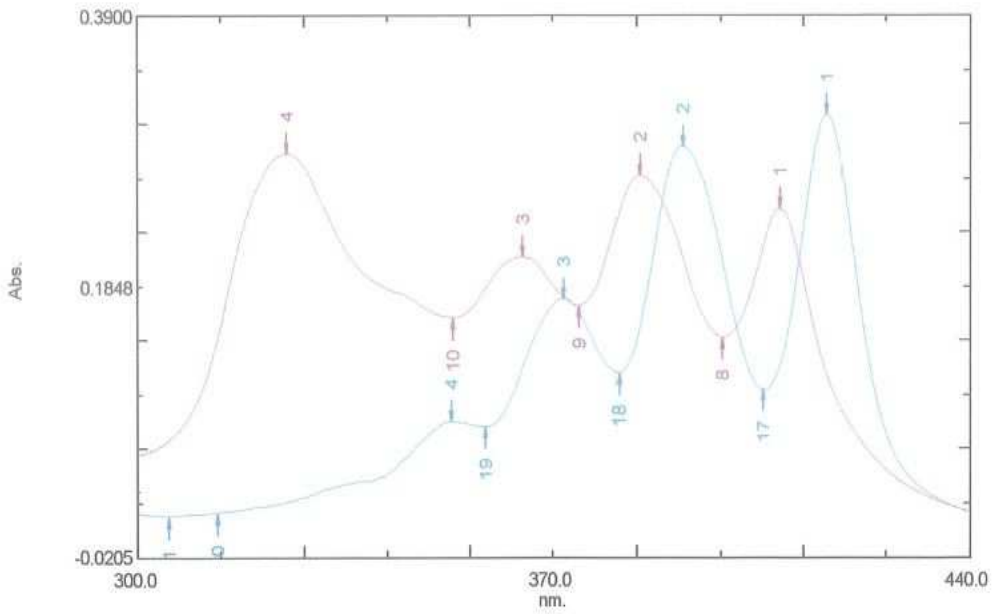

Figure 3.19 - Absorption spectrum of AmB in DMSO (blue line) & the freeze dried powder of AmB-PLP in water UV-spectrum (purple line)

An aliquot of AmB-PLP in pluronic ophthalmic gel was diluted in water and tested in the UV. The absorption spectrum is shown in Figure 3.20. This figure revealed a similar UV spectrum to AmB-PLP powder in water with peaks at 408.0 nm, 384.4 nm, 364.2 nm and 322.2 nm. It can be noticed a small shift in the highest peak at 322.2 which can be an indicative of aggregation of AmB. The ratio of absorbencies was also calculated and the result was 2.29.

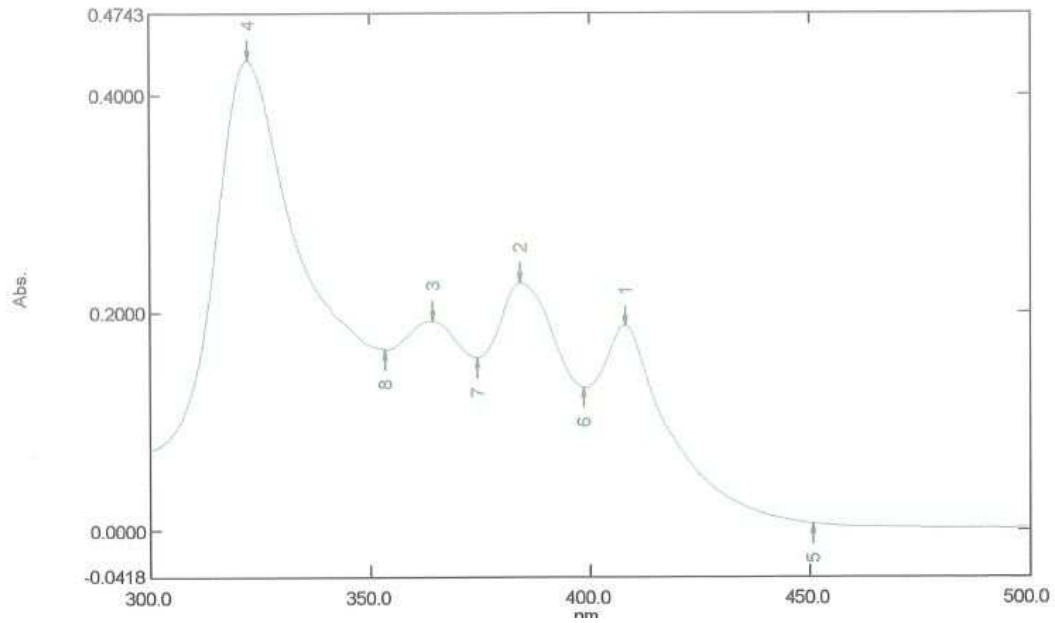

Figure 3.20 - Absorption spectra of AmB-PLP in Pluronic ophthalmic gel.

The spectrum of AmB-PLP in Carbopol/HPMC ophthalmic gel diluted in water is shown in Figure 3.21. The peaks are shown at 407.8 nm, 385.0 nm, 364.2 nm and 325.8 nm and the ratio of absorbencies was 2.05.

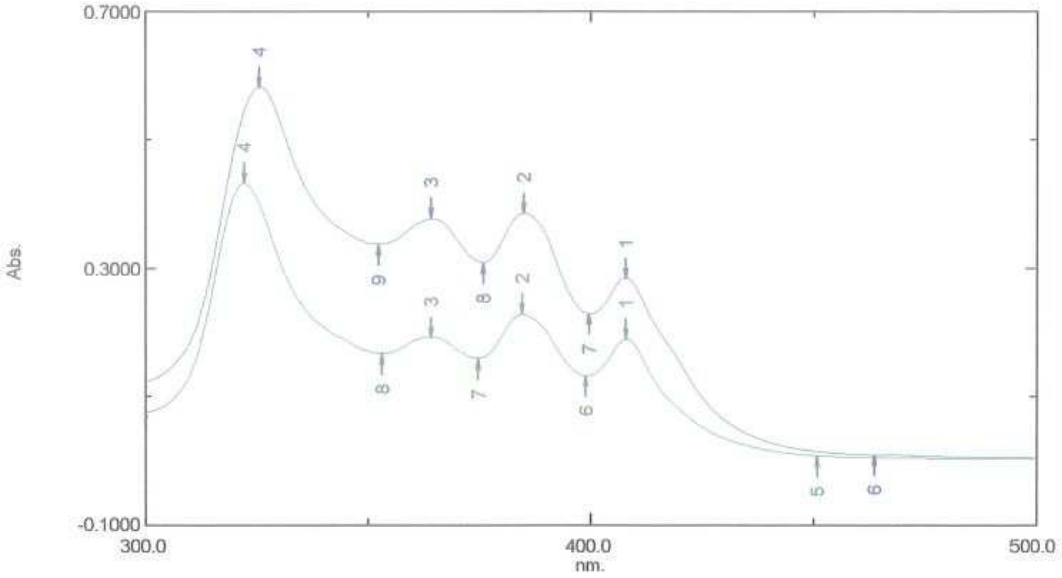

Figure 3.21 - Absorption spectra of AmB-PLP in Carbopol/HPMC gel (upper line) & AmB-PLP in Pluronic gel (bottom line)

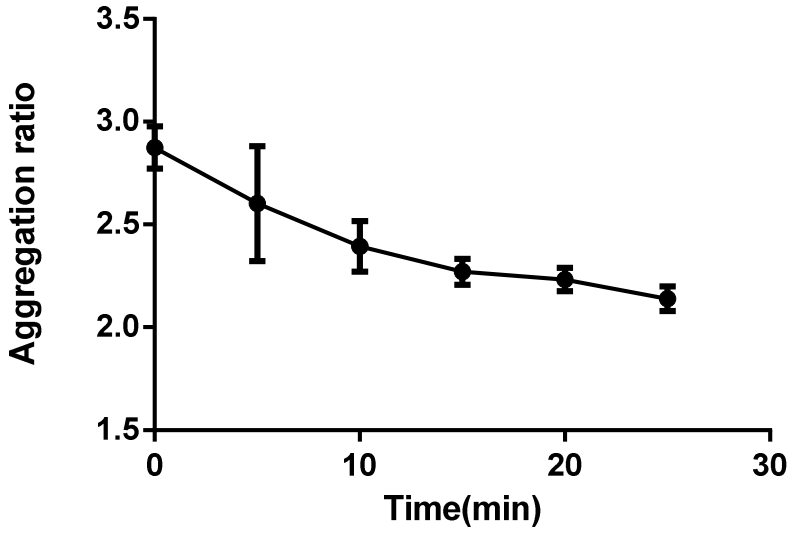

Figure 3.22 - The mean±SD of the aggregation ratio of AmB-PLP complex in simulated tear fluid at 34°C. Measurements were taken every 5 minutes over a period of time of 25 minutes (n=3). The measurement of one of the test samples was missed at 5 minutes time point.

The changes of the aggregation state of AmB-PLP complex over time when it is in contact with simulated tear fluid were also studied. In Figure 3.22 is shown how the aggregation rate is reduced over time when it is in contact to

the simulated tear fluid at 34°C. The ratio of aggregation at each time point was compared with the ratio obtained at time zero. The results were analysed with one way ANOVA multiple comparisons and the results showed a significant difference between the aggregation state at the different time points and the control group at time zero ($p<0.05$).

3.3.4.4 Drug compatibility

The thermograms of the component of both ophthalmic gel products are shown in Figure 3.23 & Figure 3.28.

Figure 3.23 corresponds to the thermogram of the different components of the Carbopol/HPMC gel (A, C, D) and also the physical mixture (B). The AmB-PLP complex thermogram (C) showed a broad endothermic peak starting from 30 °C to 100 °C. AmB is a hygroscopic molecule and therefore this peak could be due to the loss of absorbed water. There is also an endothermic peak observed at 188 °C that has shifted in comparison to the normal endothermic peak reported by some authors at about 168 °C, this is likely to correspond to the melting point of the drug.

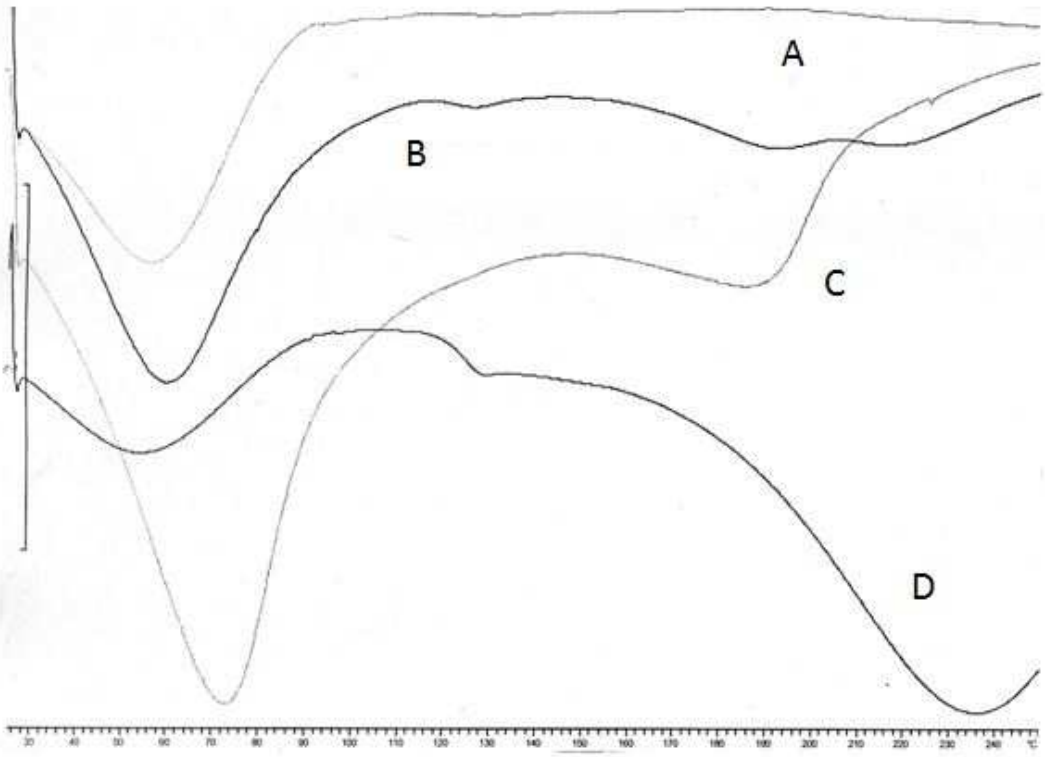

Figure 3.23 - Differential scanning calorimetry (DSC) thermograms of pure HPMC-K4M (A), AmB-PLP/HPMC-K4M/Carbopol mixture (B), pure AmB-PLP (C) and pure Carbopol (D)

The pan containing AmB was opened to visually inspect the physical appearance of the powder after the test was carried out. In Figure 3.24 it is shown the powder corresponding to the AmB-PLP complex that was turned from yellow to brown-black indicating the typical degradation process that AmB powder suffers at high temperatures.

Figure 3.24 - Amphotericin B-PLP powder after the test was carried out

Hydroxypropyl methyl cellulose, also known as Hypromellose, has the same property as AmB, it absorbs water from the atmosphere. Its thermogram (A) shows an enthalpy event between 30 °C and 90 °C due to the removal of humidity. This compound does not change its physical state before start degradation process, which means that it will degrade rather than melt. This compound will start becoming brown at 200 °C - 220 °C which coincides with a slight shoulder seen in the temperature region from 200 °C - 220 °C. As well as AmB, HPMC pan was also opened to check the physical stage of the compound. The powder has started to darken as seen in Figure 3.25.

Figure 3.25 - HPMC powder after the test was carried out.

Carbopol thermogram (D) shows 3 endothermic peaks. The thermic event between 30 °C and 80 °C corresponds to the evaporation of moisturise. The thermogram shows a different endothermic event that occurs at about 130 °C, this peak corresponds to the glass transition temperature for Carbopol (Kanis et al., 2000). And finally there is a broad peak starting at about 180 °C that could be related to the residual solvents used for the synthesis of Carbopol that evaporates easily when the temperature increase (Gómez-Carracedo *et al.*, 2004).

Figure 3.26 - Carbopol powder after the test was carried out.

The thermogram (B) of the physical mixture showed a broad peak between 30 °C and 100 °C as it was shown in all the individual components of the mixture corresponding to the moisturize water evaporation. Then, there is an endothermic peak with the onset at 115 °C and the endset at 134.7 °C matching with the glass transition temperature of Carbopol. The endothermic event corresponding to the melting point seen in the thermogram of AmB-PLP powder at 188 °C was also visible in the physical mixture. The last peak seen at 204 °C can be due to the degradation of the HPMC and the evaporation of solvents used to synthetize the Carbopol. Finally, the same increase in the AmB-PLP thermogram is seen in the physical mixture.

Figure 3.27 - Physical mixture powder of the components of HPMC/Carbopol gel formulation after the test was carried out.

In Figure 3.28 is shown the thermogram of the individual components that form the Pluronic ophthalmic gel (A & C) as well as the physical mixture (B). As it was described before the AmB-PLP complex thermogram showed a broad endothermic peak starting from 30 °C to 100 °C and also an endothermic peak observed at 188 °C. The DSC curve of Pluronic F128 showed an endothermic peak at 57.18 °C, this is associated with its melting point at 56 °C (BASF Corporation). The physical mixture thermogram (B) only differs from the Pluronic curve in the initial peak, being this peak broader in the physical mixture. However, no signs of the thermo events related to the AmB-PLP have been identified in the mixture.

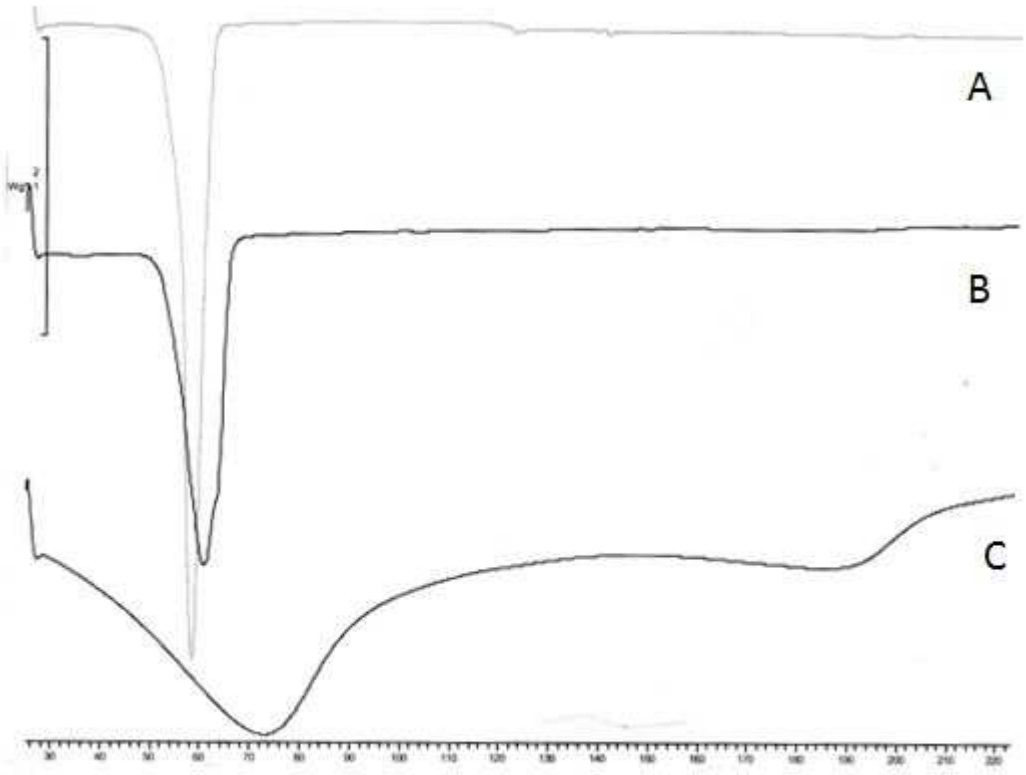

Figure 3.28 - Differential scanning calorimetry (DSC) thermograms of pure PLU F-127 (A), the AmB-PLP/PLU F-127 mixture (B) and pure AmB-PLP (C)

3.3.4.5 *In vitro* gelation

The gelling capacity was evaluated in both of the gel products. Three batches of Carbopol/HPMC ophthalmic gel were prepared for evaluation, and the results obtained showed that this formulation added to STF remains as a gel during 4.5 hours. It was completely dissolved in the media after that period of time. All the batches took the same amount of time to disappear in the STF.

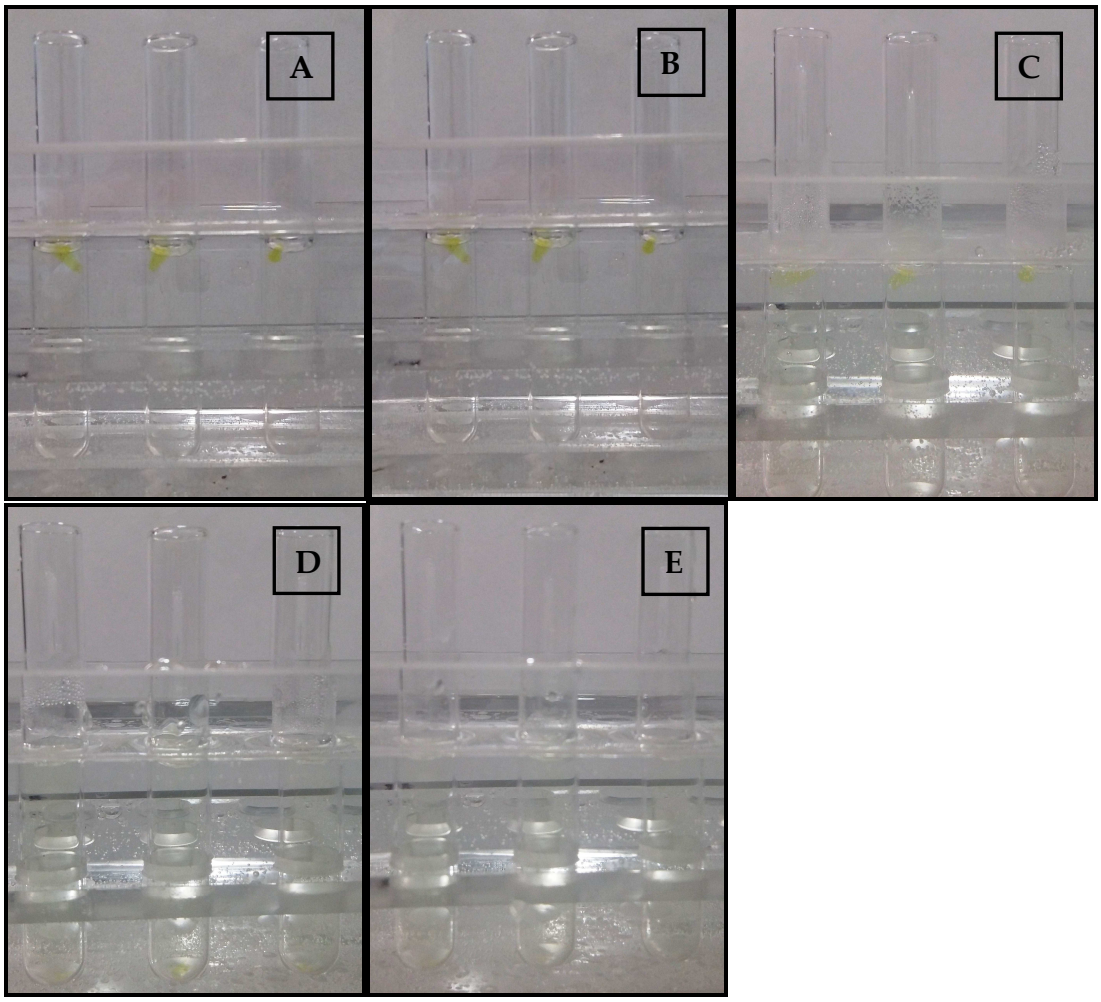

Figure 3.29 - Photographs taken of the test tubes with STF at 34 °C at different times over a 4.5 hours period with Carbopol-HPMC formulation. A) represents the formulation at t_0 when the formulation is added to the STF in gel form. B) represents the formulation after 1 hour in the STF. Photograph C) represents the process after 3 hours; D) represents the formulation after 4 hours and finally E) it is the formulation in STF after 4.5 hours.

Figure 3.29 shows the different stages of the gel in STF at different time points. After 3 hours (C) it is appreciated how the gel starts to dissolve, after 4 hours (D) the gel remains in the bottom of the test tube and finally half an hour later there is no residue in the bottom of the tube (E).

On the other hand the in situ formulation was added to the tear system as a liquid. Figure 3.30 shows the phases of the process, when the drop of the liquid formulation is added to the system forming the gel and when the drop of AmB-PLP pluronic gel is dissolved. One 50 µl drop of AmB-PLP in pluronic gel takes one second to change from liquid form to gel when it is added to the STF equilibrated at 34 °C. There is no significant difference in the time to form the gel of the 3 batches ($p<0.05$).

All batches tested were completely dissolved after 12 minutes since the formulation was added to the STF. The mean of the time to get all batches dissolved was 11.5±0.4 minutes and there were no significant difference between the 3 batches ($p<0.05$).

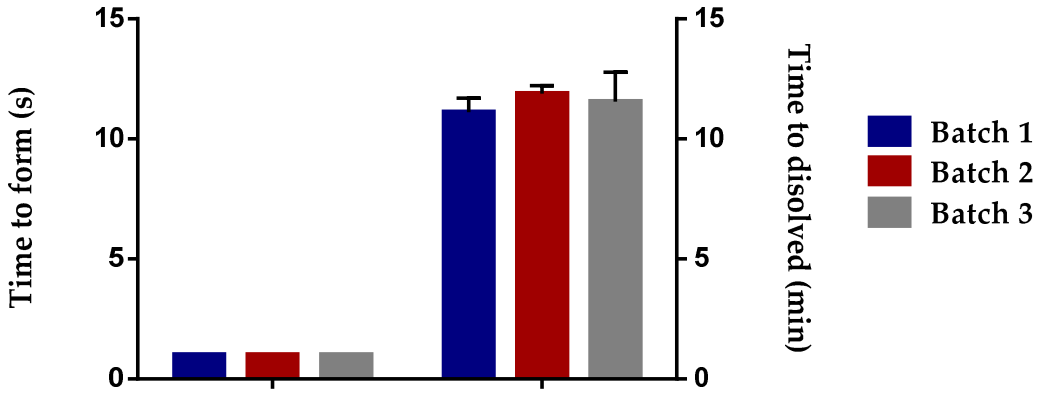

Figure 3.30 - Gelling capacity of amphotericin B pluronic gel. The data represents the mean ± SD of each batch tested; each point was measured in triplicate.

Figure 3.31 shows the different stages of the process, the formulation is added to the system in liquid form and it is transformed in gel immediately staying in the surface of the STF (A). When the gel starts to dissolve in the fluid the drop of gel moves to the bottom of the test tube staying there until it is completely dissolved (B & C).

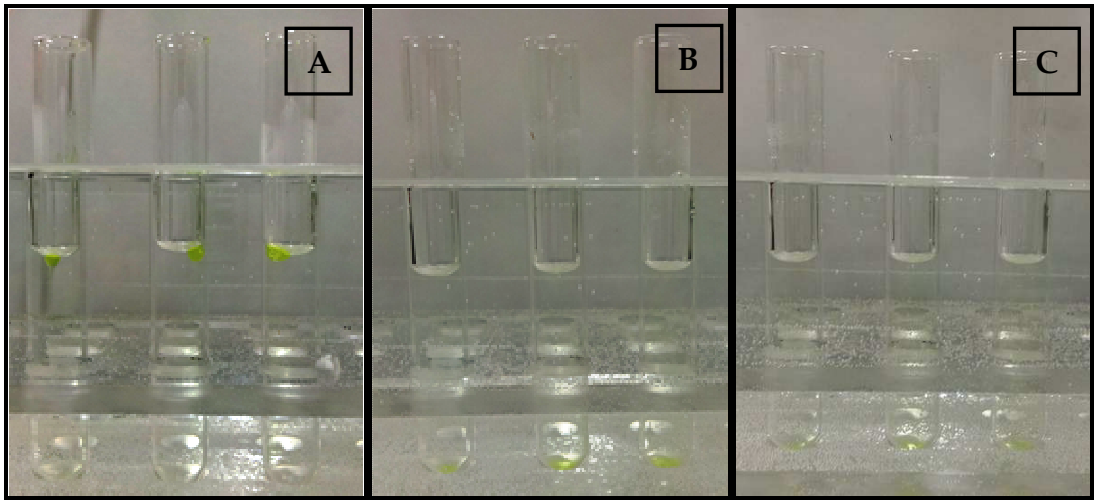

Figure 3.31 - Photographs taken of the test tubes with STF at 34 °C at different times over a 12 minutes period with pluronic formulation. A) represents the formulation at t0 when the formulation is added to the STF. B) represents the formulation after 5 minutes being in the STF. And photograph C) represents the process after 10 minutes.

3.3.4.6 Drug content and homogeneity

The assay of AmB was carried out to find out the drug content in all samples. In situ gel was the formulation with better assay rate, 98.69% of the label content. The ready to use formulation had the lowest rate of AmB, 91.33% followed by the 92.11% of active ingredient in eye drops formulation.

Table 3-12 - Concentration±SD (%) of AmB of pluronic gel, Carbopol/HPMC gel and eye drops freshly prepared (n=3).

Formulation	Mean±SD (mg/ml)	Assay (%)
Pluronic gel	1.48±0.15	98.67
Carbopol/HPMC	1.37±0.08	91.33
eye drops	1.38±0.10	92.11

All formulations were also tested for homogeneity of the final product. Different batches of each formulation were sampled (n=3) for start point, middle point and final point as describe in previous section.

The data obtained from testing each formulation was analysed individually using ANOVA. The results of the concentration of AmB were compared to investigate any difference between the batches tested from the same formulation.

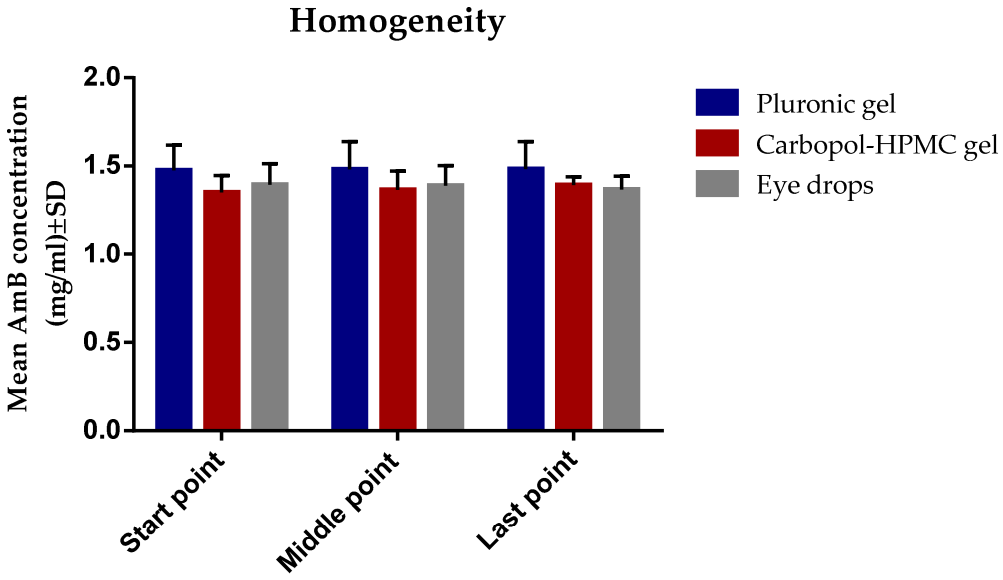

Figure 3.32 - The concentration of AmB (mg/ml) in pluronic gel, Carbopol-HPMC and eye drops formulation (n=3) freshly made determined in 3 samples taken from different locations in the final batch.

The homogeneity results (Figure 3.32) showed that there is no significant difference in the concentration of AmB of the samples taken from different locations from the final container ($p > 0.05$) in all of the formulations.

3.3.5 Discussion

Aqueous solubility is an important characteristic when developing ophthalmic products. Although the development of pharmaceutical products containing AmB could be challenging due to its low solubility in water, the complex formed by AmB and Pyridoxal phosphate has a better solubility, making possible the development of different ocular formulations. The type of product developed can influence the contact time and effectiveness of the treatment. Consequently, a conventional ophthalmic solution easy to instil and two gel-based products that increased contact time are the products to be studied.

The conventional eye drop formulation was developed and investigated. The physical appearance of this product did not suffer any change over time when it was stored at 4 °C. However, the samples stored at 25 °C became turbid after 30 days of storage. It was investigated to determine if it was due to microbiological contamination or precipitation. The conclusions were that there was bacterial growth in the samples stored at 25 °C, this was due to the fact that the initial manufacturing of the ophthalmic products were not under aseptic conditions. The microorganism was identified as *Burkholderia cepacia* also known as *Pseudomas cepacia.* This is a Gram negative organism that can be typically found in water and soil and can survive for long periods of time in moist environments. It is understood it came from the water used in the formulation or during the washing process of any of the instruments used in

the preparation of the ophthalmic products. Despite the inability to manufacture the product as a sterile, any ophthalmologic preparation will be manufactured under aseptic conditions or will be terminally sterilized reducing significantly the risk of microbiological contamination. Quality control checks would be routinely performed to ensure that the pharmaceutical product complies with the BP specifications if it is carried into clinical usage.

The pH was also tested over a period of time of 3 months and no changes were detected. Keeping the pH within the physiological range can improve the tolerability of the formulation by the patient.

The aggregation state of the AmB complex was also studied to determine whether or not the presence of the excipients would impact upon aggregation state. In the case of the eye drops, the molecular state of the complex in aqueous solutions was studied and compared to AmB dissolved in DMSO. The results obtained showed that the spectrum of the AmB dissolved in DMSO corresponds to the monomeric state of the antifungal compound, this has been reported previously by different authors (Legrand *et al.*, 1992; Nishi et al., 2007). However, the water soluble complex formed by AmB and PLP seems to be in a dimeric form based on its spectrum (Espada et al., 2008). The absorbance ratio of 1.17 suggests that the aggregation state was higher in comparison to the ratio of AmB dissolved in DMSO (monomeric). Different authors reported the relationship between the toxicity of AmB and its aggregation state administered in mice, confirming through their research that during systemic application of AmB the dimeric state of AmB is linked with a higher toxicity than the monomeric state

(Barwicz *et al.*, 1992). Despite all these findings about AmB toxicity during systemic application, following topical eye application, systemic absorption is minimal. While there is an increasing incidence of fungal eye infections, there is also limited information available about eye epithelial toxicity. This is a consequence of the reduce number of topical agents commercially available, most of the treatments are prepared in hospitals or manufacturing units as unlicensed medicines. Kimakura *et al.* (2014) compared the toxicity profile of different antifungal agents including AmB with an *in vitro* model using human corneal epithelial cells. Their findings showed that AmB reduces cellular viability, compromises the membrane integrity, and also reduces the healing capacity after 30 minutes of exposure.

The aggregation ratio of AmB – PLP complex was also studied in physiological conditions over time. The eye drops formulation was added to a simulated tear fluid kept at 34 °C and the aggregation ratio was calculated over a period of time of 25 minutes. In this case the aggregation state decreases over time, this could be due to the fact that the formulation is kept at 34 °C. Similar findings were already reported by other authors. Gaboriau *et al.* (1997) suggested the possibility of minimising the toxicity of AmB deoxycholate to mammalian cells with a pre-heating treatment of Fungizone®. Their findings showed that AmB deoxycholate solution leads to a progressive dissociation of the aggregates into the monomeric form at room temperature. He also published that if the AmB deoxycholate solution has been pre-heated at 70 °C for 20 minutes then the super aggregates dissociates into monomers. However, due to the instability of AmB exposed to high temperatures this pre-heating treatment could also lead to a loss in

the antifungal activity. In 2006 Hargreaves *et al.* studied also the influence of the temperature in the AmB physical state obtaining similar results.

The eye drops were also tested for drug content, and the assay showed a content of AmB of 92.11%. Although the results are low, the BP limits for topical products is ±10% of the content state in the label.

Although homogeneity tests are not a critical parameter for a solution, tests to ensure content uniformity within batches were also performed. The concentration of three samples was determined and the RSD calculated. In the case of the eye drops the RSD was less than 1% which confirms a good homogeneity of the batch samples. Apart from the eye drop formulation two gels containing the complex formed by AmB and PLP were also developed. The in situ gelling systems have been developed with the advantage that it could be presented in two different dosage forms. The finished product is presented as an eye drop formulation, however it changes to a gel system when there is a change in the environment, this transition could be triggered by different factors such as a change in temperature or a change in the pH of the medium. In the present work temperature-induced in situ gelling system of AmB-PLP was formulated. This means that the product will be kept as a solution at low temperatures and will be easily administered in the patient's eye, as soon as the drop is in contact with the eye.

The same parameters as for the eye drops were tested, but also characteristics such as gelling capacity and compatibility of excipients were studied. Therefore, the in situ gelling system was visually inspected and the results obtained were the same as for the eye drops. The gel formulated with

Pluronic as excipient did not show any changes when it was stored at 4 °C, however at 25 °C bacterial growth was also detected. After the identification test was carried out in the microbiological department in RQAS within NHS GG&C, the same microorganism, *P. cepacia*, was identified. No changes in the pH were detected over time in this formulation.

Aggregation studies were also performed to see whether or not the excipient used to formulate the gel had an influence in the physical state of the active ingredient. It was found that when the AmB-PLP complex was formulated in the Pluronic gel the aggregation ratio was indicative of a higher level of aggregation in comparison with the eye drop product. It was also notice that the wavelength of the maximum peak in the AmB-PLP in Pluronic gel in relation to the complex in the aqueous solution was displaced from 325 nm to 322.2 nm. This is also indicative of AmB in its aggregation form.

For the gel formulations a compatibility study of the excipients was also performed. A run of the ingredients themselves was performed and then a mixture of excipients was also studied and compared with DSC. Pluronic showed an initial endothermic peak at 57 °C that corresponds with the melting point of the compound. The complex formed by AmB and PLP also showed a broad endothermic event due to dehydration of the AmB due to the hygroscopic nature. Although the curves for the individual components of this ophthalmic formulation showed clear endothermic peaks, in the thermogram equivalent to the physical mixture (AmB-PLP:Pluronic F127) the thermal event corresponding to the Pluronic melting point is the only identifiable event. The peak corresponding to the Pluronic-127 has shifted slightly, and was broader which could be due to the moisture loss of the

AmB-PLP. The fact that the peak corresponding to the AmB-PLP melting point does not appear in the mixture could be an indicator of interaction between the excipient and the active ingredient. Therefore, it would be advisable to perform further testing of the physical mixture at different proportions and also further study with FT-IR to better establish the nature, if any, of the interactions.

The gelling capacity was also a parameter studied. Both in situ and ready to use gel products were assessed for *in vitro* gelation using simulated tear fluid at 34 °C. Patil *et al,* (2015) described the gelling capacity as high when a gel forms within 60 seconds and remains for 6 hours, medium when gel forms within 60 seconds and remains stable for 3 hours and low gelling capacity when gel forms in 60 seconds and dissolves rapidly. The data obtained showed that the gel formed with the Pluronic F127 as excipient shows an instantaneous gelation when the drop is in contact with the tear fluid at 34 °C, the capacity to stay as a gel in the simulated tear fluid is low, which will reduce the contact time between the ophthalmic formulation and the ocular surface. This could be a disadvantage due to the fact that one of the objectives of this research was to increase the contact time between the formulation and the ocular surface but may be enhanced in future work with an increase in the concentration of Pluronic or with the addition of another gelling agent.

Content assay was performed in 3 batches of the in situ gel, the results showed a mean drug content of 98.7% which is within the BP limits. The homogeneity test gave satisfactory results for drug content uniformity as

there was no significant difference observed in the % of drug in different locations and the RSD calculated was below 2%.

The last formulation analysed was the gel containing the AmB-PLP complex, the gelling agent Carbopol 974 and the viscosity enhancer HPMC. The ophthalmic formulation was preliminary evaluated for visual appearance looking for any changes in colour or clarity and also any changes in pH. There was no changes in appearance in the product when was stored at 4 °C and the formulation remained clear, however the colour of this formulation turned from a bright yellow to a dark yellow when it was stored at 25 °C. Although this could suggest chemical degradation of AmB, the stability studies did not showed any noticeable chemical degradation. The same change in clarity that was observed in the other two formulations was also detected in the ready to use gel. It was also performed microbiological test on the finished product and the same microorganism, *Burkholderia cepacia,* was identified. The pH was also monitored for 3 months and no changes were detected.

The effects of the excipients in the physical state of AmB complex were evaluated. As mentioned before, when the ratio between absorbance at 325/348nm and 409nm increases from 0.25 to 2 is an indicative of AmB being in its aggregation state (Barwicz *et al*, 1992). In the case of the complex AmB-PLP in the ready to use gel, the A325/409 ratio was calculated as 2.05. This result suggests the presence of AmB in its aggregated state.

DSC curves for the physical mixture of Carbopol, HPMC and AmB-PLP and for the individual components were recorded to determine interactions

between the components of the formulation. A totally different result was obtained with this formulation in comparison with the in situ gel product. In this case, it was observed that the qualitative thermal characteristics of the mixture coincided with the sum of the thermal events of the individual components. Therefore, it is suggestive that there is no evidence of drug-excipient interactions.

The gelling capacity of this product was better than the gelling capacity of the in situ gel formulation. It was considered that the gelling capacity of Carbopol/HPMC is medium which means that the gel remains for a few hours in the simulated tear fluid. This will improve the contact time of the active ingredient with antifungal activity and the ocular surface which could potentially reduce the application frequency of the product in the patient's eyes.

Although the distribution of the active ingredient through the batch was good, the drug content was found to be low at 91.33%. It is considered to be within the ±10% limits for topical products stated in the BP however the RSD value between different batches was calculated and was found to be high, which could be an indicative that the manufacturing method was not robust. This drug content variability batch to batch has been observed in all three formulations. Therefore, it is likely that this is due to an inconsistency during the synthesis of the AmB-PLP powder more than during the formulation of the finished product. This is an indicative that further work should be conducted to improve the synthesis and purification of the active ingredient.
Characterisation studies are an important part during the development of ophthalmic pharmaceutical products. These studies will predict any further

problems that could arise when the formulation are tested *in vivo.* After performing the characterisation of all 3 formulations it is understood that only the eye drops and the ready to use gel are fit for purpose. Both products have advantages and disadvantages. The aggregation state of the eye drops is indicative that the side effects could be reduced in comparison with the other formulations. However, the improvement on the contact time regarding the gel formulations is an advantage for patient compliance with the treatment. *In vitro* studies are also important to compare possible effects of the formulations on the antifungal activity. For this reason *in vitro* studies and also stability of the final products will be taken into account in the decision of which formulation is more suitable for patient's application.

3.4 *In vitro* antifungal activity

3.4.1 Introduction

Historically, Pasteur already described laboratory methodologies supporting the concept of *in vitro* antibiosis well before the discovery of the antibiotics (Sirot *et al.*, 1996). But it was Alexander Fleming in 1928 who discovered by chance the inhibitory effect of a mould against bacteria, the methodology he described is nowadays known as agar diffusion test (Tan *et al.*, 2015). Unlike antimicrobial susceptibility tests, antifungal susceptibility test was first used in the 1950s when AmB was introduced as an antifungal therapy (Vandeputte *et al.*, 1950). The antifungal susceptibility test is based on an *in vitro* response of an organism to an antifungal agent. Currently, different methods have been developed to detect resistance mechanisms to antifungal therapies, and also to help in the discovery and development of new drugs

or active ingredients used against fungus. Some of the techniques used in this field are broth-based methodology, agar-based method, flow cytometry and commercially available test such as ETEST®. Techniques that directly measures the alterations in ergosterol synthesis have also been performed in the case of azole antifungal agents (Balouiri *et al.*, 2016).

To compare the *in vitro* antifungal activity between the new formulations and the formulation manufactured at the PPU within NHS GG&C, two different tests were designed. A broth-based method was used as a qualitative experiment to detect growth of *Candida albicans* when it is exposed to the ophthalmic formulations, and a quantitative experiment to compare the inhibitory effect of the Fungizone® and the complex formed by AmB and PLP.

3.4.2 Materials

The novel formulations containing AmB complex were prepared at the University of Strathclyde. Fungizone® eye drops were manufactured at the PPU within NHS GG&C. Quality control *Candida albicans* ATCC 10231 in 2 different concentrations were selected to perform the experiment and was obtained from Microbiologic EZ-Accu shot bought in Thermo Fisher Scientific, Paisley (UK). The medium used was RPMI-1640 with L-glutamine and without glucose from Whittaker and was obtained from VWR, Leighton (UK). Trypticase soy agar (TSA) used to grow the *Candida albicans* to carry out the experiment was bought in Thermo Fisher Scientific, Paisley (UK). Sabouraud dextrose agar prefilled cassettes were acquired at Milipore SA, in Ireland. Peptone water was bought in Biotrading, Netherlands. The modified

lecithin broth was obtained from Biomerieux, France. The 100 ml 0.45 μm sterilised filtration funnels were acquired from Milliflex, France. An Oxoid Turbidimeter was used to calibrate the solutions to 0.5 McFarlane.

3.4.3 Methods

For the qualitative experiment, the broth dilution method was used. This is a technique in which sterile test tubes holding identical volumes of broth with antifungal drug solution in different concentrations are inoculated with a known number of fungi.

To start the experiment, *Candida albicans* was plated in TSA and incubated for 24 hours. After that period of growth, 0.5 McFarlane solutions of *Candida albicans* were prepared with a turbidimeter and reserved. The test tubes used were cleaned and autoclaved, they were labelled from 1 to 10 for each formulation, 4 formulations were tested, Fungizone® eye drops, complex eye drops, complex Carbopol gel and complex pluronic gel. As Figure 3.33 shows, serial dilutions of all the formulations were carried out in the test tubes, from a concentration of 75 μg/ml to 0.07 μg/ml. When the dilutions were performed, RPMI-1640 was added as well as 0.5 ml of the Macfarlane solution to all the test tubes. Two drug free controls were also prepared, a positive control with the medium and the inoculum suspension and the negative control with only RPMI-1640 medium. The solutions were incubated at 36 °C for 48 hours and then visually inspected to detect any growth in the different test tubes. Each formulation in each concentration as well as the controls was repeated in triplicate.

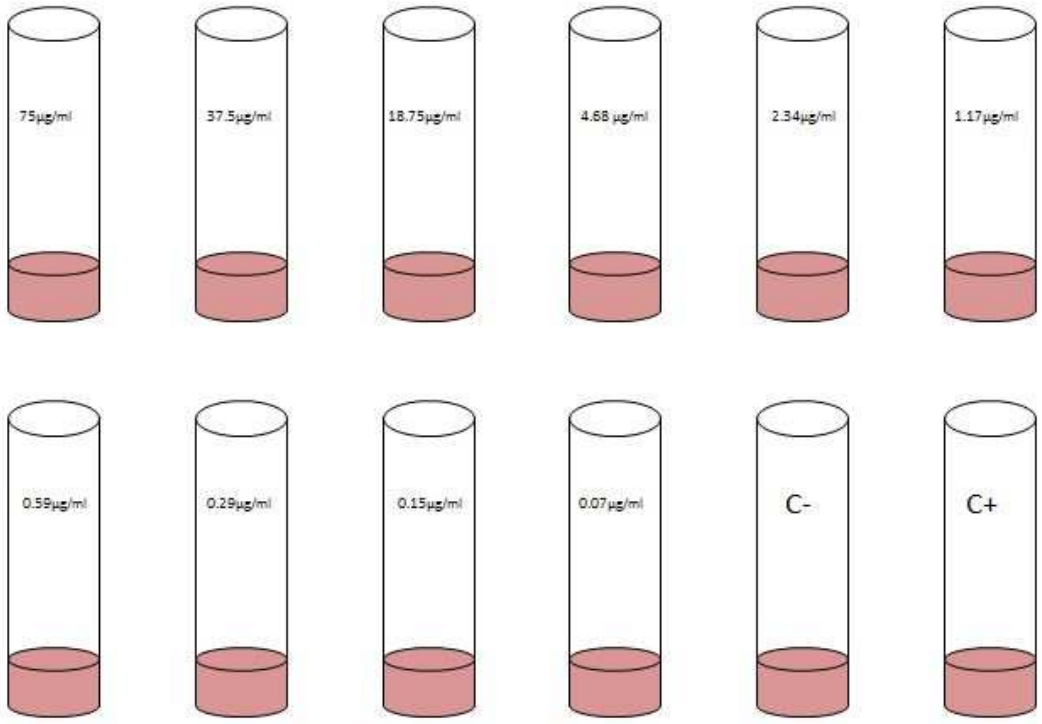

Figure 3.33 - Test tubes for broth dilution method

The quantitative experiment consisted in total viable count using membrane filtration method was only performed to compare the activity of the Fungizone® powder and the AmB-PLP complex. This method is designed to permit the passage of a large quantity of fluids through a filter membrane retaining some microorganisms on its surface. It is an effective test planned to isolate colonies of *Candida albicans* added to the eye drop formulations.

Dilutions of Fungizone® and AmB-PLP complex eye drops in RPMI-1640 were prepared in sterile bottles. Final concentrations from 37.5 µg/ml to 1.5 µg/ml were used in this experiment to compare the activity of both products. Each concentration of the active ingredients was challenged with 1-6 x10^2 CFU/ml of *Candida albicans* for 1h. The positive control contained *Candida albicans* but no antifungal agent, and the negative control which contained only media were also prepared. The final concentration of CFU

was also plated in TSA to be compared with the positive control and study the performance of the filter. After 1h of contact time, each formulation was gently mixed and 1 ml of each preparation was added to letheen broth. Then this was further added to a solution containing peptone water. This preparation was vacuum filtered through a sterile membrane filtered cup, and those filters laid into a Sabouraud dextrose agar plate. Those plates are sealed and incubated for 3-5 days at 35 °C and then, any colony growing in the plates was counted by eye inspection and compared with the colonies found in the positive control.

3.4.4 Results

All test tubes from the broth dilution susceptibility test were visually inspected after an incubation period of 24h. Each tube was compared against the positive and the negative control to identify growth.

Table 3-13 - Turbidity detected during the broth dilution susceptibility test by visual inspection

Test tube	Concentration (μg/ml)	Fungizone eye drops	AmB-PLP eye drops	Ready to use gel	In situ gel
1	75	-	-	-	-
2	37.5	-	-	-	-
3	18.75	-	-	-	-
4	4.6875	-	-	-	-
5	2.3437	-	+	-	-
6	1.1718	-	-	-	-
7	0.5859	-	-	-	-
8	0.2929	-	-	-	-
9	0.1465	+	+	-	-
10	0.0732	+	+	-	+
Control +		+			
Control -		-			

***Visual turbidity suspected to be growth**

As the Table 3-13 shows, turbidity was found in test tube 5, 9 and 10, however not in all formulations. In the Fungizone® eye drops as well as in the eye drops containing the complex, the test tube with concentrations of 0.1465 and 0.0732 μg/ml were turbid. The novel eye drops formulation also showed turbidity in test tube 5. No growth was detected in any test tube corresponding to the ready to use gel formulation; they were all clear when visually inspected. Test tube number 10 corresponding to the in situ gel was also visually turbid. All test tubes that were found turbid were plated in TSA and incubated to confirm if there was any type of contamination or if *Candida albicans* was growing on them.

In the case of the eye drops containing Fungizone or AmB-PLP complex it was discovered that plates 9 and 10 had yeast growth. However, the colonies corresponding to plates 5 for the complex eye drops and plate 10 for the Pluronic the growth was found to be a contamination of a Gram negative rod. This microorganism was not fully identified, however it was likely contamination during the preparation of the experiment in that particular dilution test tube.

Figure 3.34 - Figure 3.40 show the photographs taken from the test tubes with suspected growth and controls.

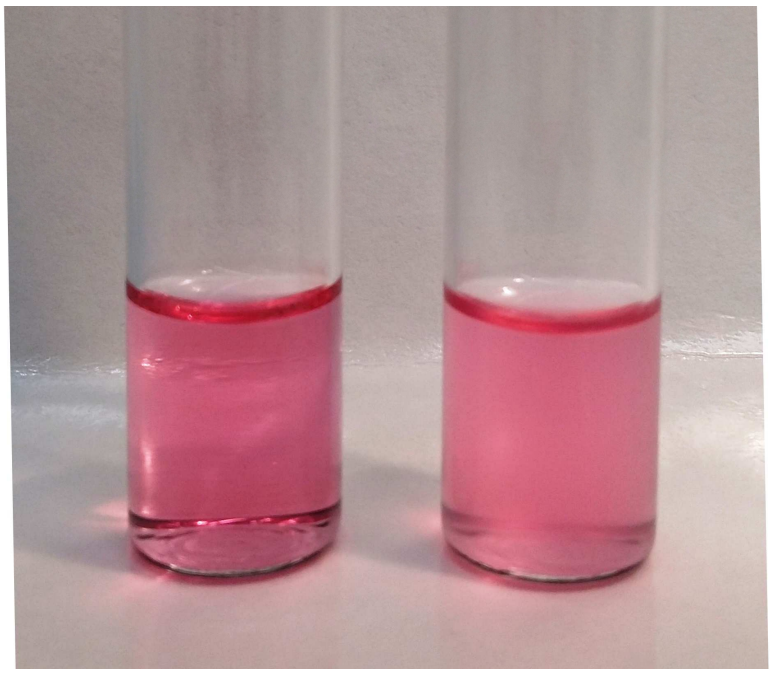

Figure 3.34 - Test tubes corresponding to the negative control (left) & positive control (right) after 48h of incubation.

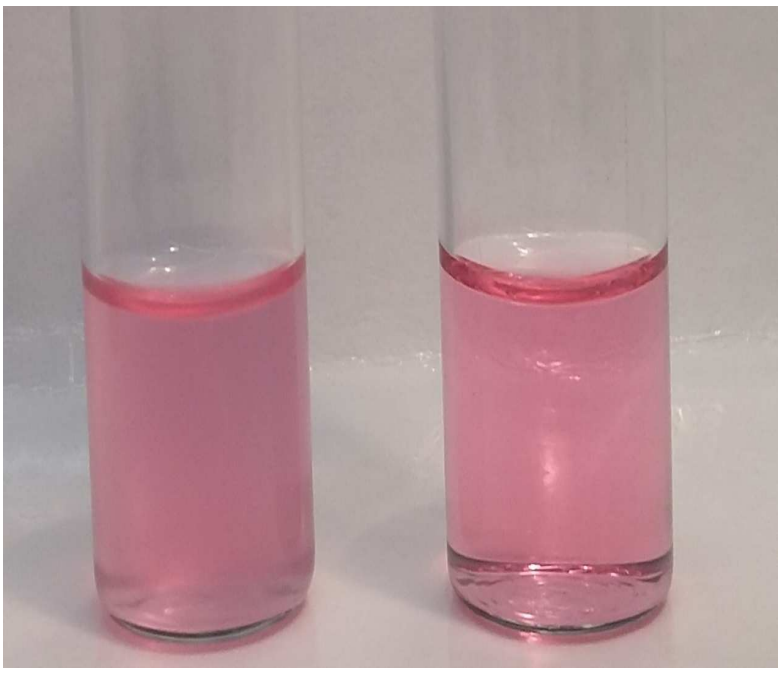

Figure 3.35 - Fungizone® tube 10 (left) and negative control (right) after 48h of incubation.

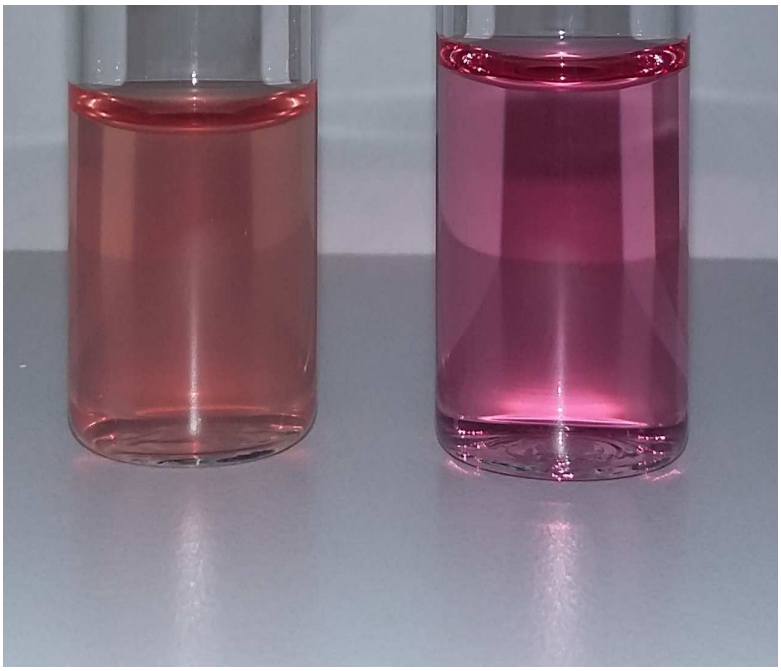

Figure 3.36 - Fungizone® 9 (left) & negative control (right) after 48h of incubation

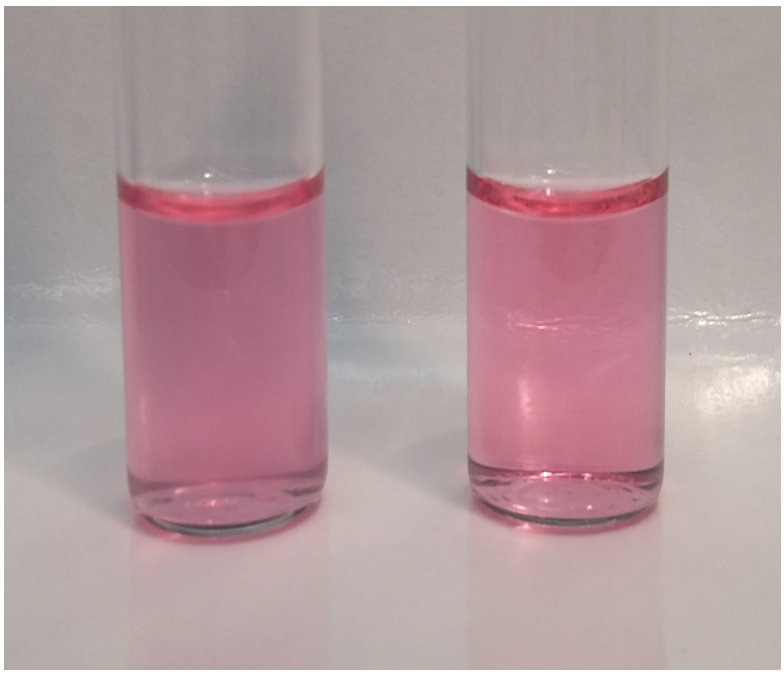

Figure 3.37 - Pluronic 10 (left) & negative control (right) after 48h of incubation.

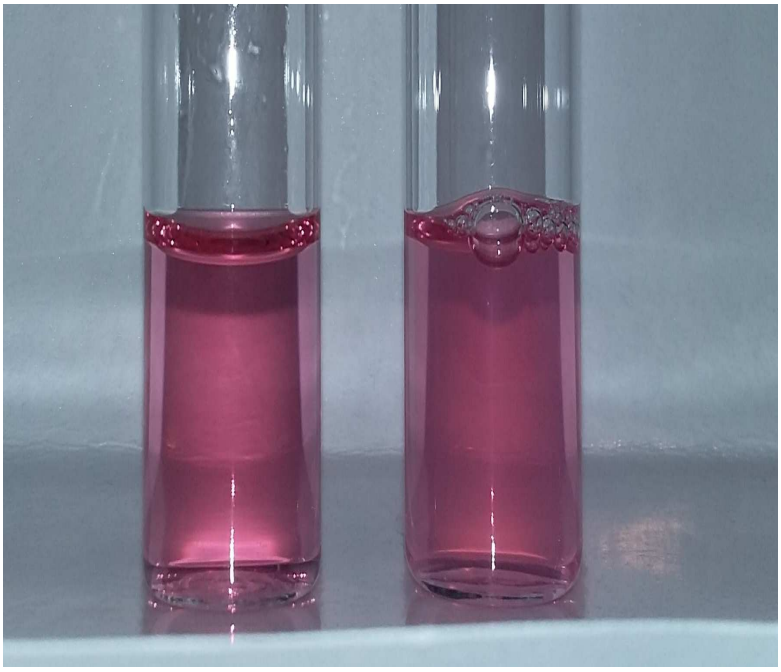

Figure 3.38 - Negative control (left) and AmB-PLP eye drops 5 (right) after 48h of incubation

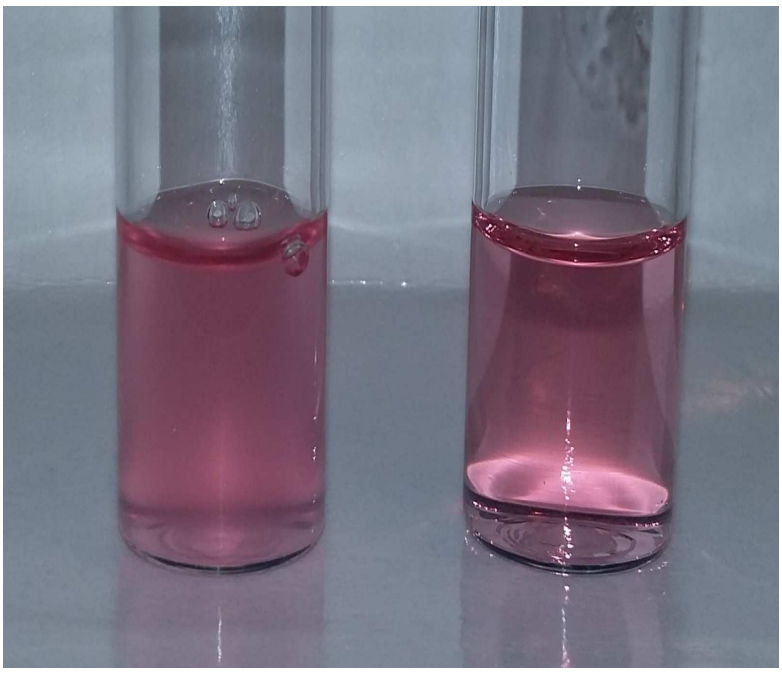

Figure 3.39 - AmB-PLP eye drops 9 (left) & negative control (right) after 48h of incubation

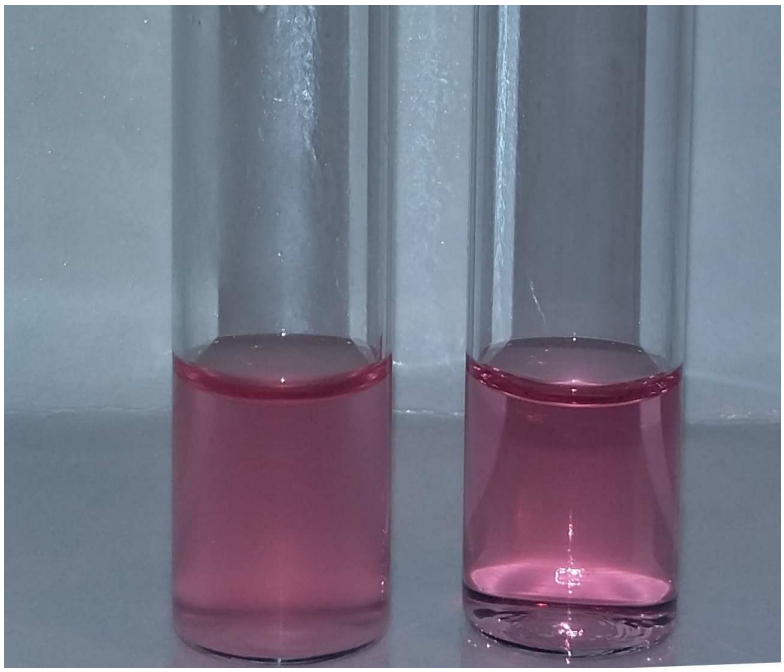

Figure 3.40 - AmB-PLP eye drops 10 (left) & negative control (right) after 48h of incubation.

The *in vitro* susceptibility test to Fungizone® and AmB-PLP complex at different concentrations was carried using *Candida albicans*. Both active

ingredients were in contact with the quality control microorganism for one hour, the different solutions were filtered and Figure 3.42 shows the *Candida albicans* colonies that were grown in the Sabouraud Dextrose Agar cassettes after incubation.

Figure 3.41 - Total viable count using membrane filtration method. The picture shows the growth of *Candida Albicans* in Sabouraud Dextrose Agar cassettes after incubation at 37 °C for 48 h. These cassettes correspond to the formulations of Fungizone® (left) & AmB-PLP (right) at a concentration of 3.75 μg/ml.

Figure 3.42 - Total viable count using membrane filtration method. The picture shows the growth of *Candida Albicans* in Sabouraud Dextrose Agar cassettes after incubation at 37 °C for 48 h. These agar cassettes correspond to Fungizone® (left) and AmB-PLP (right) at a concentration of 1.5 μg/ml.

The activity of Fungizone® powder and AmB-PLP powder was compared and is shown in Figure 3.43. The Fungizone® inhibited the growth of *Candida albicans* at concentrations from 15.0 µg/ml to 37.5 µg/ml and the novel complex form by AmB and Pyridoxal Phosphate inhibited the fungi at concentration from 7.5 µg/ml to 37.5 µg/ml.

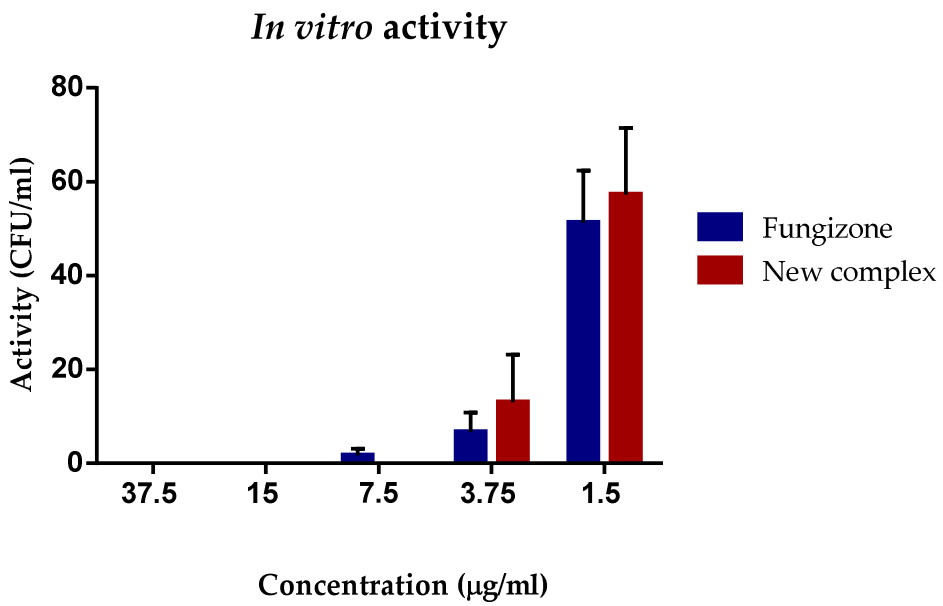

Figure 3.43 - Shows the fungal activity of Fungizone and AmB-PLP complex at an endpoint of 1h against *Candida Albicans* (n=3).

The potency to inhibit *Candida albicans* by both drugs at different concentrations was analysed, and the data obtained revealed that there was no significant difference in the *in vitro* activity of both drugs in all concentration points ($p>0.05$). Which means that the novel complex formed between AmB-PLP is as potent as Fungizone®.

3.4.5 Discussion

The activity of AmB deoxycholate against *C. albicans* has been demonstrated in several studies over the years. However the main problem resides in extrapolating the *in vitro* activity results to an *in vivo* scenario. The reason for that is the host toxicity of AmB which is related to the dose and also to the aggregation state of the molecule.

In the present study, a broth dilution method (qualitatively) and total viable count (quantitatively) using membrane filtration method were used to compare the *in vitro* activity. An initial experiment was carried out to quantitatively compare the antifungal activity of two agents, the known AmB deoxycholate and the novel complex formed by AmB and Pyridoxal phosphate. Real conditions were simulated, which means that the active ingredients were in contact with the microorganisms only over 1 hr. As expected, it was found that *Candida albicans* growth was inhibited by different concentrations of AmB deoxycholate. The antifungal activity of the complex formed by AmB and PLP was comparable to the antifungal activity AmB deoxycholate.

After the comparison of both powders, a qualitative experiment to visually assess the activity of all the formulations was performed. In this case the same concentration of *C albicans* was exposed to different concentrations of each formulation over a period of time of 24h. The minimum concentration used was 0.0732 μg/ml and although growth was found at different concentrations, only both eye drop formulations at a concentration of 0.1465 μg/ml and 0.0732 μg/ml were found to have positive growth for yeast. This

suggests that all ophthalmic formulations have comparable antifungal activity.

The results presented suggest that the antifungal activity of both drugs tested against *Candida albicans* is comparable. However, it will be necessary more investigations to obtain more clinical data to confirm if this good *in vitro* efficacy is predictive for clinical outcome. *In vivo* studies would be recommended to confirm the *in vitro* investigations.

Chapter 4 General conclusions and further work

The Pharmacy Production Unit within NHS Grater Glasgow and Clyde is license by the MHRA to prepare many different unlicensed medicines also known as specials. One of the disadvantages of the use of specials is that they are often superficially qualified and lack of the necessary stability in comparison to licensed products. Therefore, the aim of the present project was to study new ophthalmic formulations more qualified and with superior stability.

Two ophthalmic products were selected to be the focus of this research, MMC and AmB eye drops. The development of innovative ophthalmic formulations with superior stability will improve patient care, creating additional manufacturing capacity in the unlicensed medicines supply chain, whilst widening availability and improving patient access to MMC and AmB products.

MMC 0.02% eye drops formulation has a shelf life of 6 weeks after manufacturing. Therefore, improving the formulation to obtain a product with higher stability is advisable to improve patients care. The strategy was to develop a formulation where MMC powder was dissolved in a non-aqueous co-solvent to decrease the degradation rate of the active ingredient. Once this was achieved using propylene glycol as a co-solvent, stability studies of the novel product were developed.

HPLC is a well-established technique to perform stability analysis. The method designed and validated for use in the study permitted stability assessment of the different MMC formulations. From the subsequent stability studies performed it can be concluded that the novel formulation has an acceptable shelf life in both its un-reconstituted and ready to use form when stored at 4 °C. There are no signs of degradation products or precipitation of the active ingredient over the proposed shelf-life in the product that is reconstituted before administration. Regarding the ready to use form signs of degradation products are seen from week 24 after manufacturing. Further studies and toxicology profile of those degradation products is required

The stability of the novel MMC formulation before reconstitution had a slightly longer shelf-life under refrigerated storage conditions than the ready to use form - 16 and 10 months respectively. However, the hazards of manipulating a cytotoxic compound by health professionals in an uncontrolled environment would favour the use of the reconstituted, ready to use product. It would be necessary in future work to identify and quantify the degradation products appeared during the stability study of the ready to use formulation. Otherwise, the shelf life could be established before the appearance of the degradation products.

Given that the existing formulation manufactured at the PPU within NHS GG&C has a current shelf-life of 6 weeks, this represents a significant improvement in formulation stability.

Undertaking an *in vitro* cytotoxicity test is an essential aspect in drug discovery and formulation development. The characterization of the toxicity

profile of new chemicals or pharmaceutical products before animal tenting is appropriate and cost effective to predict the toxic potential of new products for humans (Chapman *et al*., 2013).

MMC is a known anti-cancer drug with a well stablished toxicity profile, however, i*n vitro* studies to compare the toxicity of the novel formulation with the formulation currently in use have provided more information about the toxicity and activity profile of the new product. From the results of this study the novel treatment and the formulation currently manufactured in the NHS have a comparable profile in terms of toxicity. The new product is able to develop its anti-cancerous effect over B16 F0 melanoma cells. However, the penetration of the drug to the site of action will depend on the physiological barriers, in conjunction with the physicochemical properties of the formulation. Consequently, even though both formulations showed a comparable toxicity profile, *in vivo* studies are recommendable to demonstrate those findings.

The Pharmacy Production Unit in NHS Greater Glasgow and Clyde also manufacture a 0.15% AmB eye drops formulation using Fungizone® as a starting material. The stability of this formulation is very limited so three new ophthalmic formulations were developed and characterised with the objective of improving the stability and solubility issues of the current product.

The potential of the derivatives of AmB to provide stable ophthalmic formulations was investigated. The poor aqueous solubility of the antifungal agent makes difficult the formulation of pharmaceutical products containing

this active ingredient. However, it is known that AmB improves its solubility when form complex with surfactants such as sodium deoxycholate marketed as Fungizone® or when liposomes are used as delivery system which is the case of Ambisome®. Although these currently exist in the marketplace, the cost of Ambisome® is prohibitive, and the side effect profile of Fungizone® could be improved. Therefore newer formulations containing AmB which can be given less frequently or new alternatives such as echinocandins and next generation azoles group are now more commonly being used.

This project outlines the possibility of using the complex formed between AmB and Pyridoxal Phosphate in ophthalmic formulations. Day *et al* carried out the synthesis of the complex using dimethylformamide, however in the present research dimethylformamide was not used during the manufacturing of the active ingredient. Substitution of this solvent with methanol was used for health & safety reasons and to facilitate processing. Although both organic solvents belongs to the group class 2 of residual solvents used in pharmaceutical manufacturing the permitted limits of methanol are higher than the limits for dimethylformamide (USP). An evaluation of the powder characteristics such as yield of reaction, solubility and stability was performed. While the study showed an improvement in the aqueous solubility of the complex and it was also found to be stable for 3 months with no physical changes, further stability studies are advisable to confirm long-term stability. The major challenge during the synthesis of this compound was the final reaction yield. The batch to batch variability in the content of AmB influenced in the drug content of the ophthalmic products developed to treat fungal infections. Therefore, additional studies in optimising the synthesis of AmB-PLP powder will need to be undertaken. *In*

vitro quantitative studies were also performed, and it was found that the new complex AmB-PLP and AmB deoxycholate have a comparable activity profile.

Three different types of ophthalmic formulations were developed, eye drops, ready to use gel and in situ gel. Those novel formulations developed were definitely superior in terms of stability when they were compared to the formulation currently manufactured in the Pharmacy Production Unit. Although the thermoreversible gel would be the preferable option due to the fact that possess the advantages of the eye drop and the ready to use gel formulations, the high degradation rate of this product at 4 °C and 25 °C in comparison with the rest of the novel products, makes it the least desirable option. Preliminary characterisation studies of the in-situ gel indicated the possibility of existence of interactions between the excipient Pluronic 127 and AmB-PLP. These findings match with the results obtained from the stability study. This drug-excipient incompatibility could explain the lack of formulation stability. Apart from those difficulties, it was also discovered that it has a low gelation capacity, and also a high rate of aggregation of the AmB when it is added to this formulation. If all those factors are brought together, it is conclusive that this formulation is not fit for purpose. Nevertheless it would be interesting to carry out supplementary studies to confirm the drug-excipient interactions and also to determine their cause.

While the in situ gel formulation was rejected as an option to treat ocular fungal infections, the eye drops and the ready to use gel formulation manufactured with the complex AmB-PLP were still good ophthalmic treatment options. In terms of stability, both products are viable with a

potency loss of 1% and 3% the eye drops and the ready to use gel respectively over a period of 122 days. Even though both formulations are stable, the gel has the advantage of a longer time contact between the active ingredient and the eye due to the higher viscosity of the product. In eye infections the frequency of application of the eye drops is approximately every 2 hours. In consequence, a longer contact time per application is an important improvement in this type of treatments. Long-term stability studies should be performed as future work. Further evaluation of the characteristics of both products was carried out. In the case of the eye drops no changes were found in the formulation pH or during the visual inspection of samples kept at 4 °C. Although microbiological growth was detected in the samples kept at 25 °C, this will be avoided in real scale batches due the fact that any ophthalmic preparation would be manufactured either under aseptic conditions or terminal sterilization. The optimal storage conditions would be under refrigeration due to the fact that the formulation is preservative free. This also means that once the container is opened you could only retain for a week before having to discard in case there is excessive microbial growth.

The absorption spectrum of AmB-PLP eye drops seems to coincide with that of the marketed product of Fungizone® what has been associated to side effects. Although the spectrum suggests AmB is in an aggregated state, it has been proven that the aggregation ratio reduces over time when the compound is exposed to physiological conditions such as simulated tear fluid at 34 °C. This could potentially reduce the side effects derived from the physical state of AmB, additional *in vivo*/*ex vivo* studies would be advisable. Homogeneity within batch was found to be good, which was expected due to

the fact that it is an API dissolved in an aqueous solution, but the drug content was found to be low, which could potentially be due to the challenges faced over the synthesis development stage of the complex.

The characterization of the ready to use gel proved no change either in physical appearance or pH over time in the formulation kept under refrigeration. Although the stability study did not show a significance degradation in the gel when is kept at 25 °C, a change in colour was observed which normally is indicative of chemical degradation of the active ingredient. From comparing the physical state of AmB in the eye drops with the UV-spectrum and the absorbance ratio of the AmB in this formulation it was notice that the excipients used to formulate the gel had an impact on the aggregation state of AmB, and showed an aggregation level higher than the one calculated for the eye drops. Even if the aggregation state of the active ingredient is associated with side effects, *in vivo*/*ex vivo* studies will be recommended to compare the toxicity profile of the novel formulation against the ophthalmic formulation currently used in the NHS.

Even though the compatibility study showed a possible interaction drug-excipient in the in situ gel, the same study was performed in the ready to use gel formulation and no interactions were detected.

In a theoretical view, if all three formulations selected to be developed were compared, the in-situ gelling system would be the best choice due to the fact that it shares the advantages of the other two products. It is easy to use as conventional eye drops and the contact time with the ocular surface is increased like in ready to use gel systems. However, once all the data was

analysed, it came clear the fact that the in situ formulation was not the preferable product choice due to the stability and the possible drug-excipient interaction. In terms of the other 2 products, either the eye drops or the ready to use gel stored at 4 °C have superior stability than the formulation currently manufactured at the PPU within NHS GG&C. It is advisable to do further studies to determine why there was a change in the appearance in the ready to use gel stored at 25 °C but not significant decrease on the stability results. Although the characterization of the eye drops showed better results and the manufacturing process is easier, the ophthalmic gel formulation could imply a better acceptance by patients.

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