A BIOADHESIVE FORMULATION FOR THE DELIVERY OF ANTI-FUNGAL AGENTS TO THE OESOPHAGUS

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Degree of Master of Philosophy

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June 2005

ABSTRACT

Oesophageal candidiasis is an increasing clinical condition associated mainly with immunocompromised patients; effective topical therapy minimises the amount of drug used and reduces the many unwanted side effects associated with systemic therapy. This study will investigate the *in vitro* potential of a drug delivery device designed to target the oesophagus for the delivery of antifungal agents in the treatment of oesophageal candidiasis.

Xanthan gum and chitosan were selected respectively as a bioadhesive hydrogels that can coat the infected area and to deliver locally acting drugs. Metronidazole and miconazole nitrate were used as model drugs incorperated into the bioadhesive system to examine the factors that control the extent and duration of adhesion of formulation as well as the controlled release of drug from such systems. PEG and glycerol were used as excipients aid to the solubility of the model drugs.

The diffusion of the model drug from the formulation to the site of action was assessed using Franz cell apparatus. The retention of the formulation on the biological substrate was evaluated using an in vitro model (Batchelor et al., 2002). The efficacy of the model drug was measured according to a microdilution method with the minimal inhibitory concentration (MIC) values (National Committee for Clinical Laboratory Standards, 1997). The hydrogels containing anti-fungal agents showed good release ability over 30 minutes and were retained on the oesophageal mucosa up to 30 minutes after washing.

Keywords: Oesophageal candidiasis, bioadhesive, chitosan, xanthan gum, metronidazole, miconazole nitrate, antifungal efficacy.

To my parents and my sister

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Hannah Batchelor for her guidance, advice and especially her great encouragement over my past two years. Thanks to Dr. Yvonne Perrie for her valuable advice in my first year report.

Thanks to Mr Chris Bache for his technical assistance and patience in the lab. Thanks to Mr Jiteen Kansara for his friendly help and happiness taken to the lab.

Thanks to all the research students in the lab for all their help.

Finally, I would like to thank to my parents and my sister for their continuous encouragement and financial support over all the past years.

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CHAPTER 1 INTRODUCTION

1.1 THE OESOPHAGUS

1.1.1 Anatomy and physiology of the oesophagus

The oesophagus is a muscular tube, located behind the trachea and heart, in front of the spinal column. It passes through the chest diaphragm and enters the stomach. The average length of oesophagus is 23 cm with a range from 17-30 cm (Li et al., 1994) and 1.5-2 cm diameter (Mercer et al., 1988). The anatomical structure of the oesophagus consists of the upper oesophageal sphincter, lower oesophageal sphincter and the oesophageal muscular body.

The upper oesophageal sphincter is located at the meeting of pharynx and oesophagus. The sphincter is striated muscle forming a ring that acts to open and close during swallowing. It is in a state of tonic contraction with relaxation mediated from vagal stimulation. This relaxation and contraction of the upper sphincter is caused by the stimulation of food swallowing initiating primary peristalsis to propel food boluses down the oesophageal tube (Lang et al., 1997).

The lower oesophageal sphincter (LOS) represents the transition between the oesophagus and stomach and is about 3-4 cm in length (Sloan et al, 1992). It is at the junction of squamous and columnar epithelium and at this point the oesophagus meets the stomach.

The oesophageal body, between the upper and lower oesophageal sphincters, serves to transit swallowed boluses to the stomach. The resting pressure of the oesophagus changes due to breathing. During inspiration the pressure is between -5 to -10 mmHg and during expiration it is 0 to 5 mmHg (Washington et al, 2001).

There are four tissue layers within the oesophagus shown in Figure 1; a fibrous external layer, a muscular layer, a submucous layer and an internal mucous-type layer (Washington et al., 2001). The external fibrous layer consists of elastic fibres. The muscular layer is composed of circular muscle surrounded by longitudinal muscle. The lower third of the oesophagus is smooth muscle, with striated muscle in the upper part and both types are found in the middle section (Washington et al., 2001). The submucosal layer contains larger blood vessels, nerves and mucous glands that loosely connect the mucous and muscular layers. The internal mucosal is covered with a layer of stratified squamous epithelium lining extending from the buccal cavity continuing through the pharynx and down to the oesophagus and ends at the LOS. It provides a tough impermeable lining resisting the abrasive nature of food boluses.

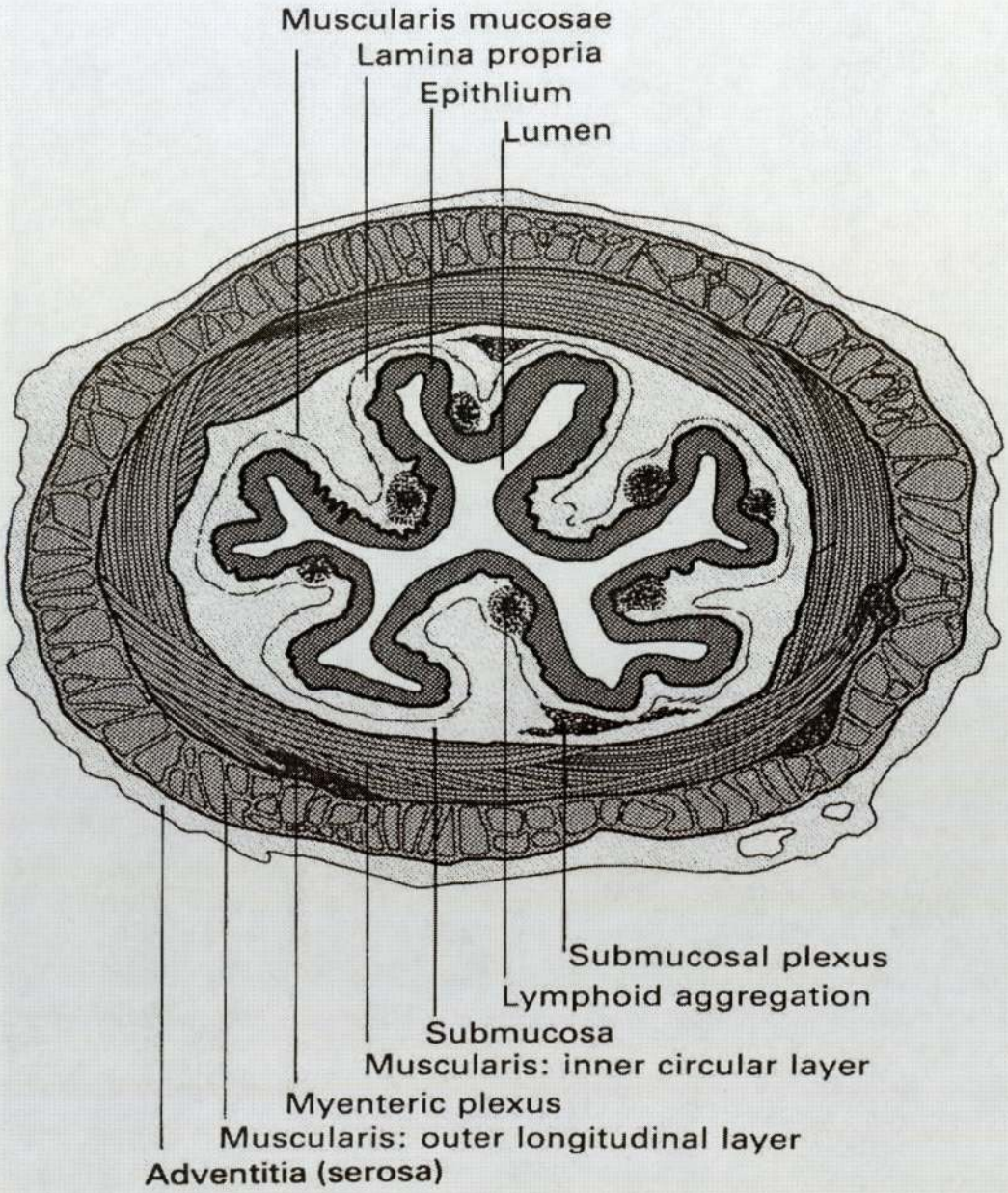


Figure 1.1: Cross-section schematic diagram of the human oesophagus (from Leeson et al., 1988).

The oesophageal glands are located in submucosa and distributed throughout the length of the oesophagus, they are small racemose glands of mucus type and each with a long duct opening in the lumen (Washington et al, 2001). About 600 – 700 glands in total are present in the oesophagus making the oesophagus moist rather than wet (Namiot et al, 1994a). The main function of the secretions is to lubricate the tube and protect the lower part of the oesophagus from gastric reflux via the pre-epithelial defences within the oesophagus.

1.1.2 Functions of oesophagus

The main function of the oesophagus is transport of food from the oral cavity to the stomach. Transport within the oesophagus requires cooperation of three major structures: the upper oesophageal sphincter, the lower oesophageal sphincter and the oesophageal body as mentioned in the previous part. These three parts act in synchrony to permit and promote smooth progression of bolus from mouth to stomach. The swallow is associated with a transient decrease in oesophageal pressure followed by a primary peristaltic wave of high pressure which travels towards the stomach at a speed of 2 to 6 cm/s in the proximal oesophagus and gradually becoming faster to the distal portion (Washington et al, 2001). The average speed of this peristaltic wave is 3.0 – 3.5 cm/s (Humphries et al, 1977) and each wave lasts about 7-10 seconds. Oesophageal luminal acidification and bolus distension initiate secondary peristalsis for further clearance of the residue left in the oesophageal lumen (Humphries et al, 1977).

A secondary function lies in the control of physiological reflux. Gastric reflux is a physiological event that occurs in all individuals that is reported to occur more frequently after meals (Ippoliti, 1994). Two important features associated with oesophageal defence are the lower oesophageal sphincter and the inherent epithelial resistance. The resting pressure at the lower oesophageal sphincter is maintained at a sufficiently high level, compared to the pressure within the stomach, to prevent the reflux of the gastric contents into the oesophageal cavity. In instances where the lower oesophageal sphincter has failed to prevent gastric reflux the oesophageal mucosa is exposed to the refluxed material. The mucosal layer has inherent systems in place that aid in protecting the tissue from damage that may be caused by the refluxed material.

1.1.3 Oesophageal Candidiasis

Oesophageal candidiasis is a fungal infection frequently associated with severe immunological impairment. Up to 50% of HIV patients suffer from this condition (Vandercam et al., 1998). The spread of infection is believed to be via oral secretions and the high prevalence in AIDS patients indicates the role of cell-mediated immunity in protecting the oesophagus from infection (Powderly, 1994). Key reports have suggested that oropharyneal candidiasis is a predisposing factor for oesophageal infection (Chiou et al., 2000; Samonis et al., 1998). Typical manifestations of oesophageal candidiasis include dysphagia, odynophagia and retrosternal and epigastral pain.

However, the oesophagus has a comparatively poor blood supply in relation to the oral

cavity and therefore the drug delivered to this site after systemic administration will be found at lower levels that may not be clinically effective. Systemic administration of antifungal agents also leads to many drug interactions as well as unwanted side effects (Klastersky, 2004). In targeting an oesophageal infection high doses of systemic antifungal agents are required which is not only costly but may contribute to resistance to antifungal agents observed with immunocompromised patients.

1.2 ANTIFUNGAL AGENTS

The drugs considered for the topical treatment of fungal infections may be partitioned into three groups: azoles, polyenes and novel peptide agents. Polyenes are broad-spectrum antifungal agents that are virtually insoluble in water; they are not absorbed from the GI tract or from mucous membranes. Two polyenes drugs used in the treatment of *Candida*: nystatin and amphotericin. These drugs are generally associated with systemic therapy and they are injected. Both nystatin and amphoterin are available as lozenges and as oral suspensions in the treatment of candidiasis within the gastro-intestinal tract, primarily the oral cavity. However, systemic administration of polyenes leads to many unwanted side effects including nephrotoxicity.

Antimicrobial peptides are a new class of antifungal therapy are currently in the development stage that work published has shown great promise from such agents (Ruissen et al., 2002). Antimicrobial peptides are often cationic and amphipathic; these properties allow interaction with membranes leading to antifungal efficacy. Much work

has been performed on histatin and lactoferrin-derived peptides (Lupetti et al., 2002; Ruissen et al., 2002; Kuipers et al., 2002).

Azoles are the most widely used drugs in the treatment of oropharyngeal candidiasis. Fast dissolving formulations are beneficial to patients with oropharyngeal infections as saliva concentrations of the drug are increased (Vandergam et al., 1998). In this study, miconazole nitrate and metronidazole were selected as model drugs of antifungal agents. Metronidazole and miconazole nitrate have been established as first line treatment of candidiasis. Miconazole nitrate is a broad-spectrum antifungal agent of the imidazole group. It acts by means of a combination of two mechanisms: ergosterol biosynthesis inhibition, which causes lysis of fungal cell membrane because of the change in both membrane integrity and fluidity, and direct membrane damage of the fungal cells. It has been extensively applied for the management of dermal (Minghetti et al., 1999), buccal (Bouckaert et al., 1993), and vaginal (Mandal, 2000) candidiasis.

Metronidazole is a nitroimidazole compound with a broad spectrum of activity against protozoa and anaerobic bacteria. Metronidazole exerts an antimicrobial effect in an anaerobic environment by the following possible mechanism: Once metronidazole enters the organism, it readily permeates bacterial cell membranes to achieve a steady-state, intracellular concentration. Because of this alteration to the metronidazole molecule, a concentration gradient is maintained which promotes the drug's intracellular transport. The antimicrobial effects of the drug depend upon its selective reactivity in the unstable and reduced form, which is achieved through the actions of electron transport proteins of

susceptible bacteria. Once in the cell, metronidazole binds to the DNA and disrupts the helical structure of molecules. The breakage of DNA strands occur, which ultimately leads to cell death. This process results in very rapid killing of anaerobic microorganisms (Seymour and Heasman, 1995). Currently, metronidazole is indicated in the treatment of bone and joint, brain, CNS, endocardial, intra-abdominal, vagina, lower respiratory tract, pelvic, septicemic, and skin and soft tissue infections caused by *Bacteroides* species.

1.3 BIOADHESION

1.3.1 Bioadhesion and mucoadhesion

Bioadhesion can be described as the attachment of a synthetic or natural macromolecule to a biological tissue. The adhesive bond may form with either the epithelial surface, the continuous mucus or a combination of the two. The term *mucoadhesion* is used specifically to describe the phenomenon when the bond involves mucous coating, e.g. a mucosal surface and an adhesive polymeric device. Early pioneering work on bioadhesive-based drug delivery system has identified that bioadhesive polymers can significantly improve the performance of many drugs. Bioadhesive polymers can target drugs to a specific body site to improve the drug bioavailability and control the release rate to enhance patient compliance.

1.3.2 Bioadhesive polymers

When any bioadhesive material and a tissue link together, there are many types of interaction forces; not only between chemical groups of these two substrates, but also between the chemical bonds within the bioadhesive polymer. There are three types of forces that can be used to group the bioadhesion; covalent, hydrogen bond and electrostatic interactions.

Natural polymers, synthetic polymers and semi-natural polymers are three types of bioadhesive. Natural bioadhesive polymers can be found easily in life, such as chitin found in crustaceans such as shells of crabs, crawfish and lobsters; gelatin which is a product of the structural and chemical degradation of collagen and cellulose found in plants, etc. Semi-natural bioadhesive polymers include chitosan and various gums such as xanthan, alginate and gellan. Most synthetic bioadhesive polymers that have been investigated are either polyacrylic acids such as carbopol, polycarbophil and polyacrylic acid, or cellulose derivatives including carboxymethyl cellulose, methyl cellulose and hydroxyethyl cellulose. Compared with natural polymers, the biocompatibility and biodegradability of synthetic polymers are much more limited than those of natural polymers such as cellulose, chitin, chitosan and their derivatives. However, these naturally abundant materials also exhibit a limitation in their reactivity and processability (Mass et al., 1998; Illum et al., 1998).

Bioadhesive polymers can be classified into water-soluble and water-insoluble. Most

water-soluble polymers have linear or random chain structures. The polymers entrap the drug within their loose network. The duration of residence time on the tissue and the drug release rate depend on the dissolution rate of the polymer. For the water-insoluble polymers, typically cross-linked structure with strong chemical bond can form a swellable network which gives the polymer a lack of solubility in common solvents. Therefore, the drug release rate from cross-linked polymer is based on its swelling property. The residence time on the target site is based on the rate of mucus/tissue turnover.

1.3.3 Theories for the mechanism of bioadhesion

A complete and clear mechanism and theory of how and why the bioadhesive and a mucosal tissue surface attach together is not available at the moment. But there are three steps or processes that describe how the dosage form is attached: at first, the bioadhesive needs to initiate intimate contact with mucosal surface by wetting and swelling; secondly, the chains of the polymer interpenetrate and entangle into the mucus substrate with mucin chains to create a greater contact; finally attraction and repulsion in entangled chains and the weak chemical bonds formed. However, adhesion between mucin and mucoadhesive polymers is usually analyzed based on five classic theories: electronic theory, adsorption theory, wetting theory, diffusion theory and fracture theory (Lee et al., 2000).

The *electronic theory* comments that attractive electrostatic forces between the glycoprotein mucin network and the bioadhesive material maintain the contact. When

these two substrates contact with each other, electron transfer occurs between the two forming a double layer of electric charge at the interface. The *adsorption theory* assumes that the adherence between adhesion and mucosal substrate depends on the surface forces resulting in chemical bonding. These forces include strong primary, covalent bonds and weak secondary forces such as ionic bonds, hydrogen bonds and van der Waal's forces. The *wetting theory* states that the intimate contact between mucus membranes and bioadhesion depends on the spreading ability of the polymers. This spreading ability is predicted by interfacial tension and measured by a spreading coefficient. Thus the wetted surface is controlled by structural similarity, degree of cross-linking of the adhesive polymer, and use of a surfactant. The *fracture theory* is developed to analyse the maximum tensile stress during detachment of the bioadhesive-dosage form from the mucosal surface. The *diffusion theory* is the most widely accepted physical theory. The essences of this theory are the physical entanglement of mucin strands and the flexible polymer chains and the interpenetration of mucin strands into the porous structure of the polymer substrate. Sufficient depth of interpenetration and entanglement can create a semi-permanent adhesive bond. The penetration rate is based on the diffusion coefficient of the polymer which is associated with the molecular weight and cross-linking density and also is influenced by some important parameters such as segment mobility, flexibility of bioadhesive polymer, mucus glycoprotein and expanded nature of both networks. The mechanism of bioadhesion has been reviewed extensively by Lee et al., 2000.

1.3.4 Pharmaceutical applications of bioadhesion

Because of the ability to maintain a delivery system at a particular location for both local disease treatment as well as systemic drug bioavailability, bioadhesives can target many sites with mucosal membrane such as oral cavity, eye, nasal cavity, vagina, and GI tract.

There is a small total surface of ~50cm^2 in oral cavity with a highly permeable mucosa and rich blood supply. It is a considered popular site for drug delivery for a variety of reasons, such as avoiding first-pass metabolism, greater permeability than skin, a smooth and relatively immobile surface and accessibility. Moreover, the oral cavity is a good route for drugs with a short biological half-life, requiring a sustained-release effect, or poor solubility (Gandhi et al., 1994). The nasal cavity with a surface area of 150cm^2 is considered attractive for drug delivery. The nasal mucosa provides a large, highly dense vascular network for efficient absorption. Also, the nasal cavity has a important feature that the blood is drained directly from nose into systemic circulation, thereby avoiding the first-pass effect and reducing metabolism (Dondeti et al., 1996). The vaginal route has been frequently used for drug delivery of therapeutic and contraceptive agents to exert a local effect, such as antifungal and spermicidal agents, as well as for the systemic delivery of drugs (Robinson et al., 1994). The drugs delivered by this route can also avoid gastrointestinal degradation or hepatic metabolism following peroral delivery. The GI (gastrointestinal) route is the most popular and probably most complex route of drug delivery. A lot of disadvantages, such as high turnover rate of mucus, variable range of pH, gastrointestinal degradation, first-pass effect, longer time to achieve therapeutic

blood levels and rapid luminal enzymatic degradation need to be considered. A bioadhesive is developed to retain and localize the drug delivery system in selected regions of the GI. Also, a bioadhesive formulation can provide a specific window for the absorption of drug by an active, saturable absorption process (Deshpande et al., 1996).

1.3.5 Evaluation of bioadhesive-based dosage forms

The best approach to evaluate bioadhesive-based dosage form is to evaluate the effectiveness of a mucoadhesive polymer to prolong the residence time of drug at the site of absorption, thereby increasing absorption and bioavailability of the drug.

1.4 CHITOSAN

1.4.1 Origins of chitosan

Chitosan is a semi-natural bioadhesive obtained by the hydrolysis of chitin, a natural bioadhesive polymer that can be found easily in crustaceans including the shells of crabs. During the past 20 years, a substantial amount of work has been published on this polymer and its potential use in various applications. Recently, chitosan has been considered for pharmaceutical formulation and drug delivery systems in which attention has been focused on its absorption-enhancing, controlled release and bioadhesive properties. Synthesized from a naturally occurring source, this polymer has been shown to be both biocompatible and biodegradable (Hirano et al., 1990).

1.4.2 Properties of chitosan

Structural properties

The structures of cellulose, chitin and chitosan are shown in Figure 2, which clearly indicates the differences among these three bioadhesive polymers. Chitin is well known to consist of 2-acetamido-2-deoxy-β-D-glucose through a β-(1→4) linkage, which also may be regarded as cellulose with hydroxyl at position C-2 replaced by an acetamido group.

Chitosan is a linear copolymer of β-(1→4) linked 2-acetamido-2-deoxy-β-D-glycopyranose (GlcNAc) and 2-amino-2-deoxy-β-D-glycopyranose which is the N-deacetylated derivative of chitin. Because a sharp nomenclature with respect to the degree of N-deacetylation has not been defined between chitin and chitosan (Muzzarelli et. al., 1973; Zikakis et. al., 1984), chitosan is not easily defined in term of its exact chemical composition. It usually refers to a family of polymers that are characterized by the number of sugar units per polymer molecule, which defines the molecular weight, and the degree of deacetylation that affects the solubility of chitosan in aqueous solutions.

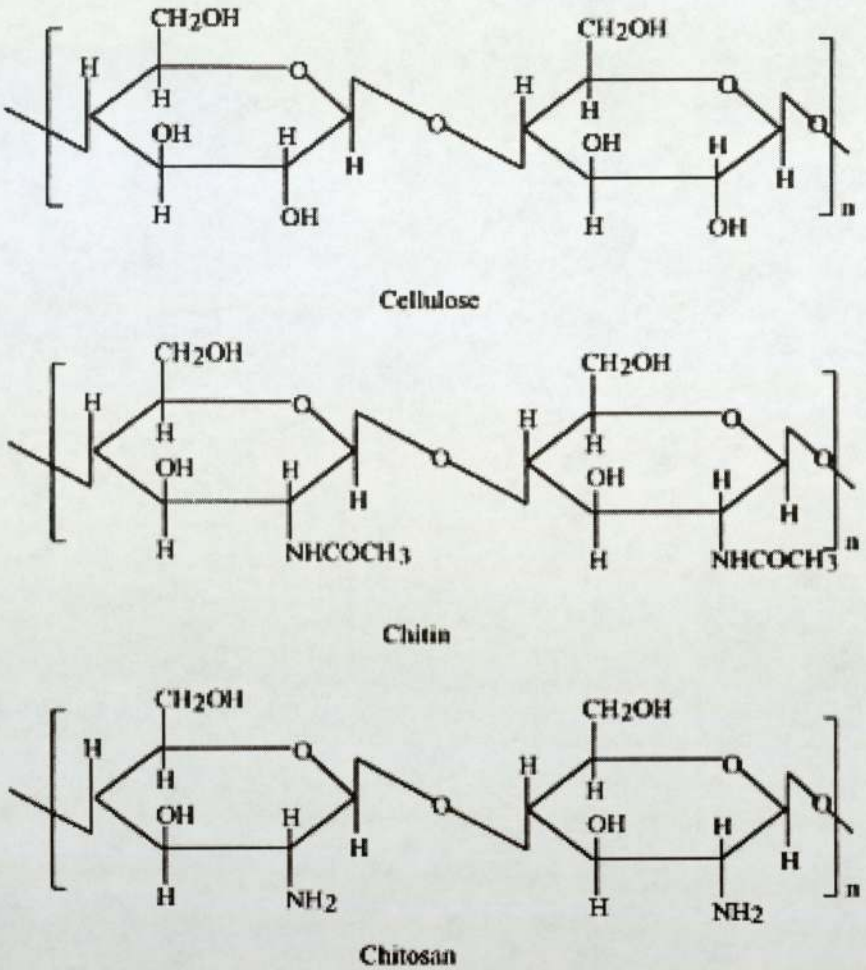


Figure 1.2: Structures of cellulose, chitin and chitosan (Majeti et al., 2000).

Chemical and physical properties

Since chitosan is obtained by partial deacetylation of chitin, it comprises a series of polymers varying in their degree of deacetylation, molecular weight, viscosity, etc. (Muzzarelli R.A.A. et. al., 1997). Owing to the presence of a number of amino groups, chitosan reacts with anionic systems leading to modification of the physicochemical characteristics of its combinations with these systems. For the gelled system of chitosan,

hydrogels with the desired characteristics can be obtained by varying the ration of chitosan or using chitosan with different molecular weights to the anionic system used.

Both the number of GlcNAc units (degree of acetylation) and the molecular weight (MW) of chitosan have been shown to influence the physical and biological properties (Alimuniar & Zainuddin et al., 1992; Muzzarelli et al., 1977; No & Mayers et al., 1997). Even though the degree of N-deacetylation affects the solubility of chitosan in aqueous solutions, chitosan is soluble in weak acidic solutions such as acetic acid, formic acid, and slightly soluble in weak alkaline solutions (Filar et al., 1977). Among chitosans of various ranges of molecular-weight, better mucoadhesion was observed for higher-molecular weight (approximately 1400 kDa) compared to lower-molecular weight chitosans (500 to 800 kDa) (Chae et al., 2004).

1.4.3 Functions of chitosan

Chitosan has valuable properties as a biomaterial because it is considered to be biocompatible, biodegradable and non-toxic (Singla et al., 2001). The cationic character and the potential functional groups make it an attractive biopolymer for many biomedical and pharmaceutical applications. The unique properties of this highly basic polysaccharide include polyoxysalt formation, ability to form films, chelate metal ions and optical structural characteristics (Hench Larry et al., 1998).

From the biopharmaceutical viewpoint, chitosan has a novel characteristic of

mucoadhesion and enhancement of paracellular drug transport via transient opening of tight junction between epithelial cells (Hench Larry et al., 1998). The amino groups of chitosan may allow the establishment of different types of interactions with both non-ionic and ionic drugs, and also provide pH-sensitive systems, which swell in certain gastric conditions allowing a site-specific release. In mucosal drug delivery such as oral, ocular, nasal, and gastrointestinal drug delivery systems, chitosan has been considered one of the most safe and excellent candidate for drug adsorption enhancer.

As a pharmaceutical excipient, chitosan has been used in various formulations, including powders, tablets, emulsions, and gels. In addition to its unmodified form, the chemically or physically modified chitosan such as trimethyl chitosan chloride (TMC), monocarboxymethylated chitosan (MCC), and chitosan nanoparticals (ionic complex micelle of chitosan with tripolyphosphate or heparin) have also been widely utilized for drug delivery system.

1.5 XANTHAN GUM

1.5.1 Origins of xanthan gum

Xanthan gum is a high molecular weight microbial desication-resistant polymer which is produced by a pure culture aerobic submerged fermentation of a carbohydrate with the microorganism *xanthamonas campestris.* It is naturally produced to stick the bacteria to the leaves of cabbage-like plants. It has been widely used in oral and topical formulations,

cosmetics and food (Wade and Weller, 1994).

1.5.2 Properties of xanthan gum

Chemical structure

Xanthan gum is a hydrophilic polymer with a long chain polysacharide composed of the sugars glucose, mannose, and glucuronic acid. The backbone is similar to cellulose, with added sidechains of trisacharides (three sugars in a chain). The structural unit of xanthan in figure shows that xanthan gum is an anionic polyelectrolyte with a β-(1→4)-D-glucopyranose glucan (as cellulose) backbone with side chains of -(3→1)-α-linked D-mannopyranose-(2→1)-β-D- glucuronic acid-(4→1)- β-D- mannopyranose on alternating residues. Slightly less than half (~40%) of the terminal mannose residues are 4, 6-pyruvated and the inner mannose is mostly 6-acetylated (i.e. the side chains are mainly β-D-mannopyranosyl-(1→4)-(α-D-glucuronopyranosyl)-(1→2)-β-D-mannopyranoside-6-acetate-(1→3)-. Some side chains may be missing (Chaplin Martin, 2005).

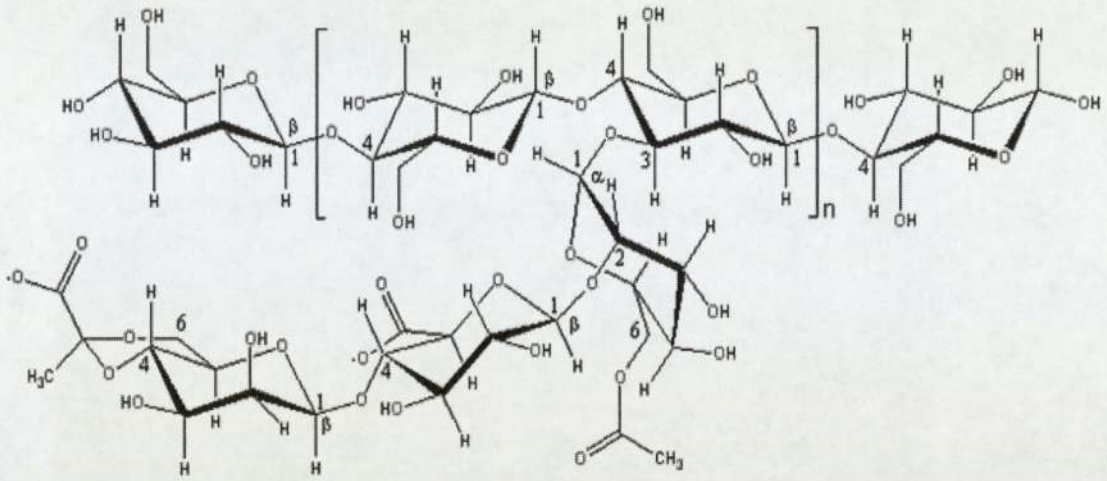


Figure 1.3: The structural unit of xanthan gum (adapted from Chaplin Martin, 2005)

Molecular structure

Xanthan gum has a relatively reproducible specification as it is produced by fermentation. Each molecule consists of about 7000 pentamers and the gum is less polydisperse than most hydrocolloids. Its natural state has been proposed to be bimolecular antiparallel double helices. It may form a very stiff intramolecular (single molecule hairpin) double stranded helical conformation by the annealing of the less stiff 'natural' denatured elongated single stranded chains. The glucan backbone is protected by the side chains which lie alongside, making it relatively stable to acids, alkalis and enzymes. Use of different strains or fermentation conditions may give rise to differing degrees of actelylation and pyruvylation, which moderates the functionality.

Chemical and physical properties

The conversion between the ordered double helical conformation and the single more-flexible extended chain may take place over hours of annealing (equilibrating) at between 40°C - 80°C. The weakly bound network formed is highly pseudoplastic, with viscosity reducing considerably a shear increase and returning in full immediately on release. High viscosity solutions (~1% M/V) appear gel-like but still shear-thin. The rationale for this strange behaviour is the hydrogen-bonded and entangled association between the side chains of the highly extended molecules, which resists dissociation. Shear thinning with greater strain is mainly due to the conformation of the side chains flattening against the backbone under shear, reducing the intermolecular interactions.

1.5.3 Functions of xanthan gum

Xanthan gum is mainly considered to be non-gelling and used for the control of viscosity due to the weak associations demonstrated by weak-gel shear-thinning properties. It hydrates rapidly in cold water without lumping to give a reliable viscosity, encouraging its use as thickener, stabilizer, emulsifier and foaming agent. The consistent water holding ability may be used for the control of syneresis and to retard ice recrystallization (ice crystal growth) in freeze-thaw situations; xanthan gel strength being improved on freeze-thaw.

Xanthan most important property being its very high low-shear viscosity coupled with its

strongly shear-thinning character. The relatively low viscosity at high shear means that it is easy to mix, pour and swallow but its high viscosity at low shear gives good suspension and coating properties and lends stability to colloidal suspensions (Chaplin Martin, 2005).

1.6 AIMES AND OBJECTIVES

The aim of this study is to investigate the in vitro potential of a drug delivery device designed to target the oesophagus for the delivery of antifungal agents in the treatment of oesophageal candidiasis. A bioadhesive system will be developed according to the physicochemical properties of model drugs.

The objectives are:

1 To measure the bioadhesive potential of these formulations

2 To measure the rate of drug release from the formulations

3 To measure the overall efficacy of the drug within these formulations

CHAPTER 2 MATERIALS AND METHODS

2.1 INTRODUCTION

In this study, chitosan and xanthan gum were selected respectively as adhesive formulations to be used within bioadhesive drug delivery systems. An *in vitro* release study and an *ex vivo* retention experiment investigated the possibility and the capability of these two bioadhesive excipients in a formulation target to the oesophagus. The MIC (minimal inhibitory concentration) was defined as the minimum concentration of miconazole nitrate salt as a model drug to show full inhibition of *Candida* growth.

2.2 MATERIALS

2.2.1 Porcine oesophageal tissue for *ex vivo* experiments

Porcine oesophagi from freshly slaughleed pigs were obtained from a local abattoir. The white inner epithelial tubes were dissected out by cutting the muscular layers and peeling off these outer muscular layers. The epithelium was then stored at -70°C. The frozen oesophageal tube was thawed at room temperature in saline solution before use.

2.2.2 Bioadhesives investigated in the formulation selection

2.2.2.1 Chitosan

Chitasan, medium molecular weight, (Lot 17813LU) (Aldrich Chemical Company, Inc. USA), was used as one of the bioadhesives in the drug delivery systems.

A 2% w/v concentration was chosen in the experiments, Acetate acid buffer (pH=4) was used to solubilise chitosan. The solution was prepared using a Heidolph rotary mixer with a 20 mm diameter, four-blade propeller. Acetate buffer was stirred at a speed of approximately 1800 rpm and a set mass of chitosan powder was added slowly. Increasing the rotary speed to 2000 rpm , the chitosan was dissolved fully over approximately 20 minutes.

2.2.2.2 Xanthan

Gum Xanthan, (Lot 59H0718) (Sigma Chemical CO. St. Louis, MO), was used at 2% w/v as an alternative biadhesive in the experiments. With the acetate buffer at pH 4, the preparation of xanthan gel was the same as chitosan.

2.2.3 Drugs used as anti-fungal agents

2.2.3.1 Metronidazole

Metronidazole (Lot 061K1384) (Sigma-Aldrich, Inc., St. Louis, MO), a synthetic 5-nitroimidazole, has an antibiotic action that is based on the modification of the genetic substance of microorganisms. Metronidazole base is sparingly soluble in water and in alcohol and is stable in air but darkens following prolonged exposure to light (AHFS Drug Information, 2000).

2.2.3.2 Metronazole nitrate

(±)-Miconazole nitrate salt (Lot 043K1249) (Sigma-Aldrich, Inc., St. Louis, MO), a synthetic 1-phenethylimidazole derivative exhibits *in-vitro* activity against species of the genus *Candida*. *In-vitro* fungicidal activity has been demonstrated particularly against *Candida albicans*. It is absorbed after oral administration from the GI tract. Miconazole nitrate shows poor water solubility.

2.2.4 Excipients in the formulations

PEG (Polyethylene glycol) (MW 200), (Lot 38H0600) (Sigma Chemical CO. St. Louis, MO),

Glycerol, (Lot 61K0019) (Sigma Chemical CO. St. Louis, MO)

These two solutions both were selected as solubility aids to enhance dissolution of the drug.

2.2.5 Acetate buffer

Acetate buffer was used at pH 4 to solubilise the chitosan and xanthan.

Acetate Buffer	A	0.2N acetic acid
	B	0.2N sodium acetate
pH	4	
500ml	205ml A+45ml B →500ml water	
1000ml	410ml A+90ml B →1000ml water	

Table 2.1: The preparation of acetate buffer

2.2.6 Materials used in release study

2.2.6.1 Fluorescein disodium salt

Fluorescein disodium salt was used as a model drug to mimic release and also as a labeling agent to quantify retention of the bioadhesives.

2.2.6.2 Hydrated dialysis membrane

Hydrated dialysis membrane was used as an alternative biological substrate to porcine oesophageal tissue. A previous study has compared the retention on both hydrated dialysis membrane and showed that these surfaces are comparable (Tang et al., 2000). When wet, the surface of dialysis membrane is viscous, which may contribute to increase the surface adhesion.

2.2.6.3 Glass microfibre (GF/C) filter paper

Glass microfibre (GF/C) filter paper, (Waterman), was used as a membrane in the Franz cell experiments for separating the formulation from the receptor phase.

2.2.6.4 Parafilm®

Parafilm® is used to create a seal to prevent leakages during the Franz cell experiments. It is impermeable to water thus retention on such a substrate should be minimal, thus it was a negative control to validate the methodology for the retention model.

2.2.6.5 Methanol

Methanol (UN 1230) was used in Franz cell experiment for drug release study. Methanol was required so that the drug would be soluble in the receptor phase. Although this

receptor phase is not physiologically similar to that found within the oesophagus the experiments were a useful way to compare the rate of release from the various formulations tested.

2.2.7 Radiochemical materials for retention study

For the opaque formulations, a radioactive label was used to quantify the retention. Element 43, Technetium, in the periodic table, has twenty-two isotopes with masses ranging from 90 to 111 that are all radioactive. Also, Technetium as a fission product from the fission of uranium in nuclear reactors has several long-life radioactive isotopes, ^{97}Tc (Half-life (T1/2) = $2.6x10^6$ days), ^{98}Tc (Half-life (T1/2) = $4.2x10^6$ days), ^{99}Tc (Half-life (T1/2) = $2.1x10^5$ days), ^{95}Tc (Half-life (T1/2) = 61 days). In this retention study, $^{99}Tc_m$ was selected because of its suitable half-life (T 1/2 = 6.01 hours); This short half-life and the gamma energy emitted of technetium have been used in previous studies that measure oesophageal adhesion *in vivo* (Potts et al. 1997).

2.2.8 Materials used in the MIC procedure

The oral *Candida* isolates (630G) were used in this study. The organisms were grown and maintained aerobically at 35°C on an agar plate which was prepared with Sabouraud dextrose agar, (Lot 092k1520) (Sigma-Aldrich CO. St. Louis, MO). The *Candida* cells for use in the experiments were suspended in 0.85% w/v sterile saline.

Barium chloride and H_2SO_4 were used to prepare the standard solution at 530nm on the plate reader. To prepare the standard: 100ml 1.175g of barium chloride was prepared in water, then 0.5ml of this solution was added to 99.5ml of 0.18mol/L solution of H_2SO_4.

RPMI 1640; was used as broth medium by addition of the powder to 1 litre of sterile water; according to the manufactures instructions and this solution was stored in the fridge once prepared.

2.3 METHODS

2.3.1 Viscosity study

2.3.1.1 Introduction

Viscosity is a measure of the flow properties of a liquid; a high viscosity indicates a slow flowing liquid. A frequent reason for the measurement of rheological properties is to characterise and classify fluids and semi-solids. Flow behaviour is responsive to properties such as molecular weight and molecular weight distribution. Viscosity of semi-solids and biological materials produces useful correlations with bioavailability and function (Aulton, 1988).

2.3.1.2 Viscosity apparatus

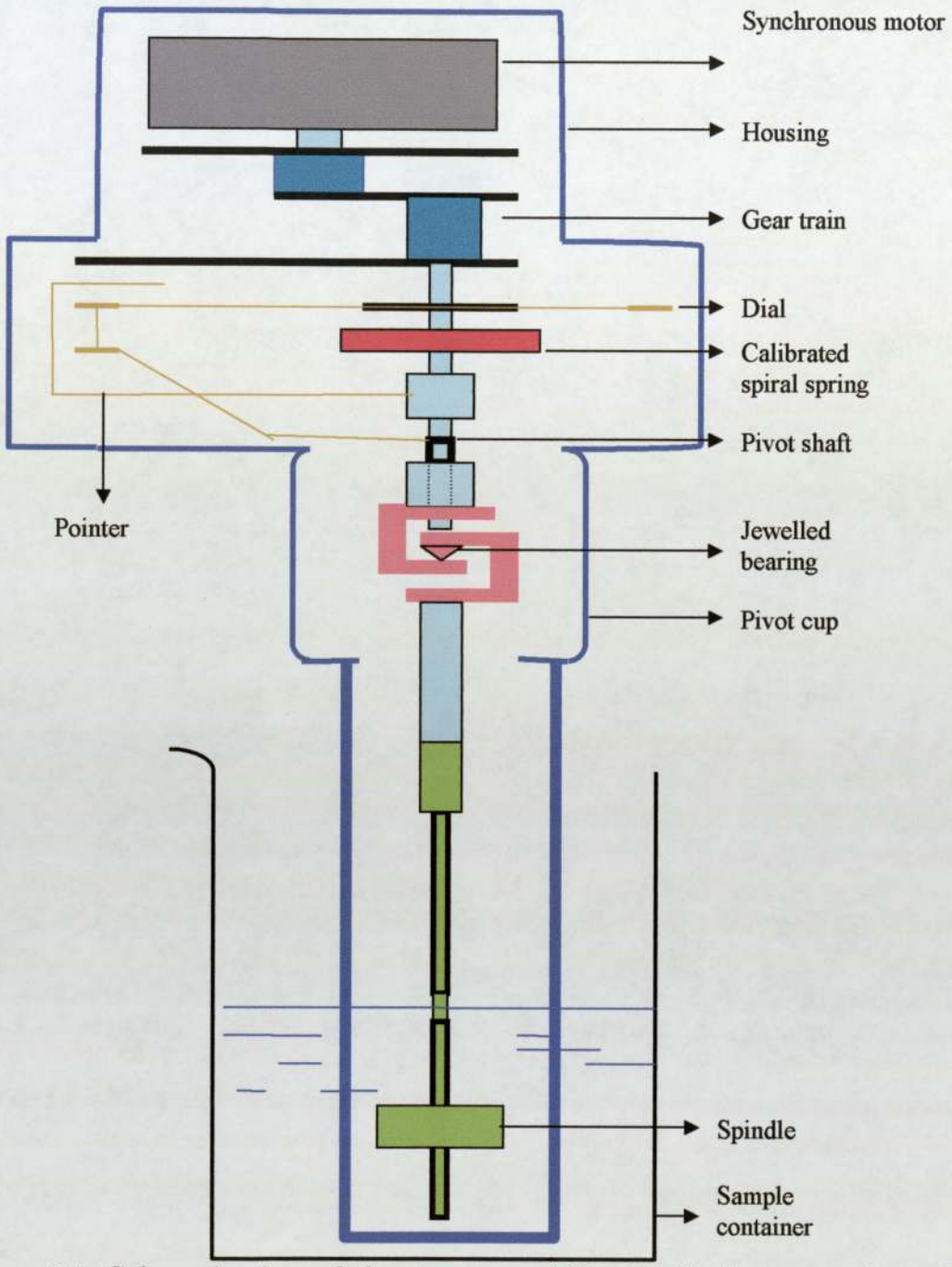


Figure 2.1: Schematic view of the structure of Brookfield viscometer (from Tang, 2004).

A Brookfield DV-I was used in this study with the guard leg. The working principal of the Brookfield viscometer is to measure the torque required to rotate an immersed element (the spindle) in a fluid. The spindle is driven by a synchronous motor through a calibrated spring. For a given viscosity, the viscous drag, or resistance to flow is proportional to the spindle's rotation speed and is related to the spindle's size and shape. For a given spindle geometry and speed, an increase in viscosity will be indicated by an increase in the deflection of the spring.

The viscometer comprised of several mechanical sub-assemblies that are schematically presented in Figure 2.1. A synchronous drive motor and multiple-speed transmission are located at the top of the instrument inside the housing. The main case of the viscometer is a calibrated beryllium-copper spring, one end of which is attached to the pivot shaft; the other end of which is connected to the dial. The dial was driven by the transmission and, in turn, drives the pivot shaft through the spring. The viscosity measured was displayed on a digital screen that was read directly to gain a measurement.

2.3.1.3 Viscosity procedure

A Heidolph rotary mixer was used to make the adhesive solutions. The caculated mass of powder was gently poured into the beaker with 100ml accurately measured acetate buffer (pH=4), whilst stirring at 200 rpm. After stirring for 2 hours there were no visible clumps in the beaker, the beaker of viscous liquid was removed from the mixer and left to equilibrate for 5 minutes.

The guard leg was present with the Brookfield viscometer and spindle 4 was attached to the viscometer. The viscosity of adhesion was read at set period of time. Because of the high viscosity of adhesion, a longer time was needed to wait for the liquid recover back to the original form after the guard leg and the spindle were inserted. The shear rate was set at 5 sec^{-1}.

2.3.2 Release model

2.3.2.1 Introduction

This study investigated the possibility and capability of drug release using bioadhesive drug delivery systems. Fluorescently labelled solution was introduced to mimic drug particles to study the release properties of chitosan and xanthan. Metronidazole and miconazole nitrate salt were used as model drugs incorporated with PEG or glycerol as a solubility aid to dissolve the drugs. A relationship between drug release rate and the viscosity of different matrixes was found.

2.3.2.2 Frans Cell apparatus

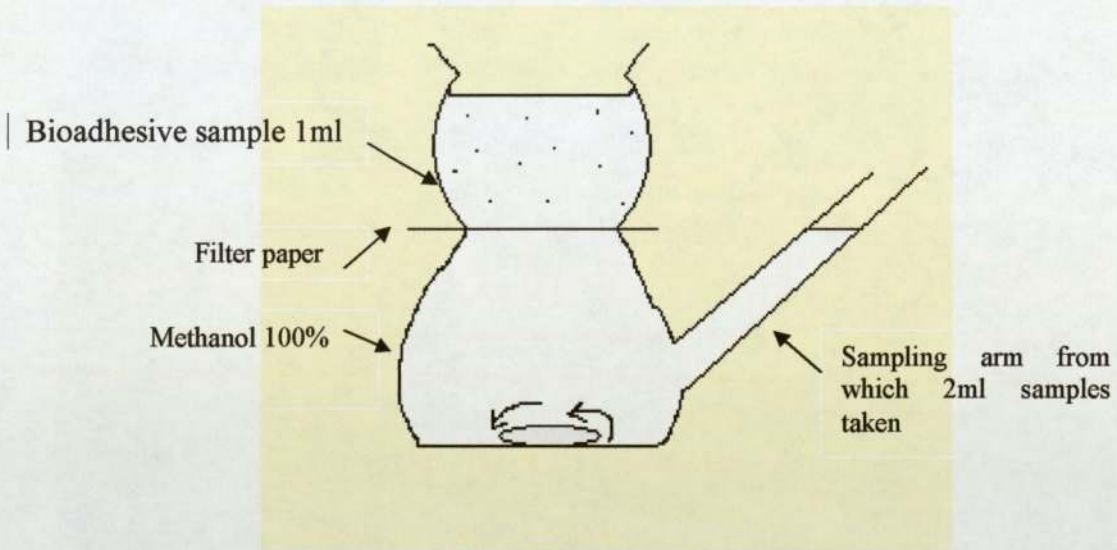


Figure 2.2: The release model apparatus

The Franz diffusion cells had a 2.52 cm^2 diffusion area (diameter = 1.7 cm) and 30ml receptor volume, filter paper was used as the membrane that separated the formulation from the receptor phase. Uniform mixing of the receptor medium was provided by magnetic stirring.

2.3.2.3 Release procedure

The filter paper was cut to size and mounted on the diffusion area and the edges were sealed with parafilm. A fixed dose (1ml) of sample was dispensed onto the surface of the

filter paper which was inside the diffusion cell. Samples of 2ml were taken from the medium at certain time intervals and replaced with the same amount of medium. Acetate buffer (pH=4) was used in receptor as a medium in the release study of fluorescent labelled adhesion and the fluorescence spectrophotometer was used to measure the sample. To investigate the release from the formulations with model drugs, methanol was used as the medium and the results were analysed via UV Unicam Helios β spectrophotometer. The receptor temperature was controlled at physiological temperature between 36.5 and 37.4°C via circulating water.

2.3.2.4 Calibration curve

2.3.2.4.1 Determination of the absorbance peak of model drugs with UV spectrophotometer

As UV Unicam Helios β spectrophotometer was used to analyse the samples with model drug in the release study, thus a wavelength needed to be determined by analysis. In order to find the absorbance peak of the drug, the model drugs were dissolved in methanol at concentration of 0.25mg/ml which was measured with the blank solution (pure methanol solution) zeroing the spectrophotometer. Both metronidazole and miconazole nitrate salt had an obvious absorbance peak at 224nm so that two calibrations were set up at this wavelength to quantify the release of the model drugs from the formulations.

2.3.2.4.2 Calibration curve of fluorescent labelled adhesion

0.25g disodium fluorescein was dissolved in 100ml distilled water from which 1ml solution was removed and taken out to dissolve in 100ml buffer at pH 4. 1ml solution was taken from the 100ml flask and diluted with buffer in 10ml and 100ml flasks respectively. The fluorescence intensity of the eight solutions (2.5E-06g/ml, 2.5E-07g/ml, 2.5E-08g/ml, 2.5E-09g/ml, 2.5E-10g/ml, 2.5E-11g/ml, 2.5E-12g/ml, 2.5E-13g/ml) was determined using a spectrophotometer. A calibration line was drawn from it. Figure 2.3 Shows an example calibration line.

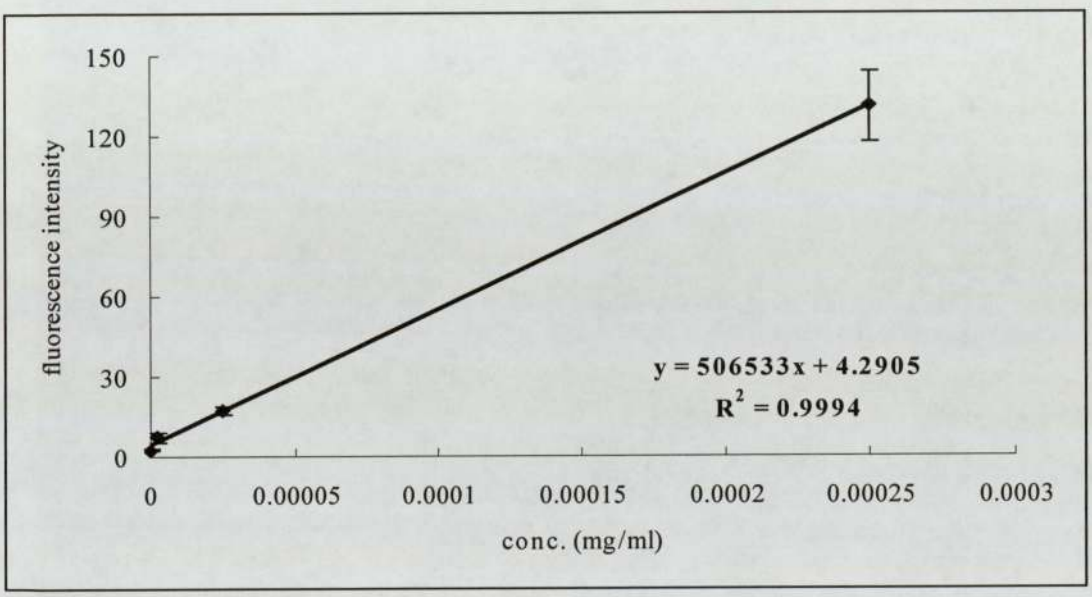


Figure 2.3: Calibration curve of fluorescent labelled adhesion (data shows mean±s.d. dev. n≥4).

2.3.2.4.3 Calibration curve of metronidazole

The 0.125g metronidazole was dissolved in 100ml methanol from which 1ml solution was removed and taken out to dilute to 1.25E-4g/ml. The solution was serially diluted twofold to 1:2, 1:4, 1:8, etc. The solutions were assayed at 224 nm using a UV Unicam Helios β spectrophotometer. A calibration line was drawn from it. Figure 2.4 Shows an example calibration line.

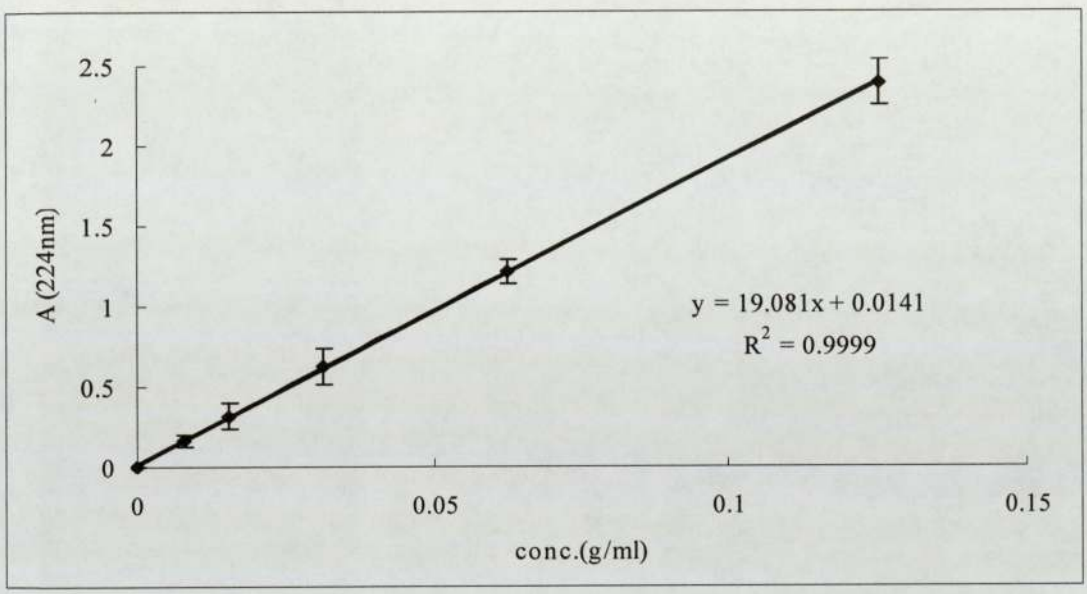


Figure 2.4: Calibration curve of metronidazole in release experiment (data shows mean±s.d. dev. n≥4).

2.3.2.4.4 Calibration Curve of Miconazole Nitrate Salt

The 0.05g miconazole nitrate salt was dissolved in 100ml methanol from which 1ml solution was removed and taken out to dilute to 5E-4g/ml. The solution was serially

diluted twofold to 1:2, 1:4, 1:8, etc. The solutions were assayed at 224 nm using a spectrophotometer. A calibration line was drawn from it. Figure 2.5 Shows an example calibration line.

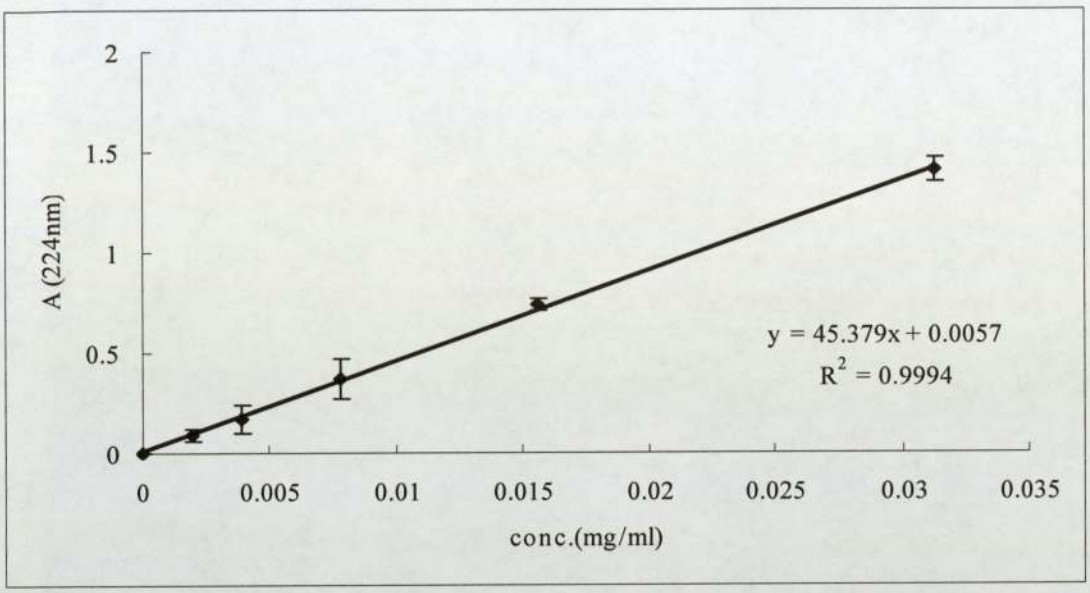


Figure 2.5: Calibration of miconazole nitrate in release experiment (data shows mean±s.d. dev. n≥4).

2.3.2.5 Preparation of Fluorescent Labelled Adhesion

0.25 g disodium fluorescein was dissolved in 100 ml distilled water from which 1ml solution was removed and taken out to dissolve in 100ml buffer. Chitosan and xanthan were prepared separately within the second solution to make 2% w/v solution as the gel. The rate of fluorescein release from each bioadhesive was measured via a previously constructed calibration curve.

2.3.3 Retention procedure

2.3.3.1 Introduction

This study investigated the adhesion of polymer to oesophageal mucosa. Porcine oesophagi were used in this *ex vivo* experiment. Radioactive label – technetium 99m was used as an analytical tool to quantity retention. This method measured not only the percentage of removed formulation, but also the retained amount on the surface.

2.3.3.2 Retention Model

The adhesion of the formulation on a biological substrate was evaluated using an *in vitro* model [Batchelor et al. 2002]. The *in vitro* apparatus comprised a biological substrate maintained at 37°C and high humidity (>90%RH); a 1 ml dose of the formulation was dispensed onto the biological substrate. The substrate was washed to mimic saliva flow and the eluent was collected at designated time points. Analysis of the eluent allowed the fraction of the formulation retained on the substrate to be calculated over time.

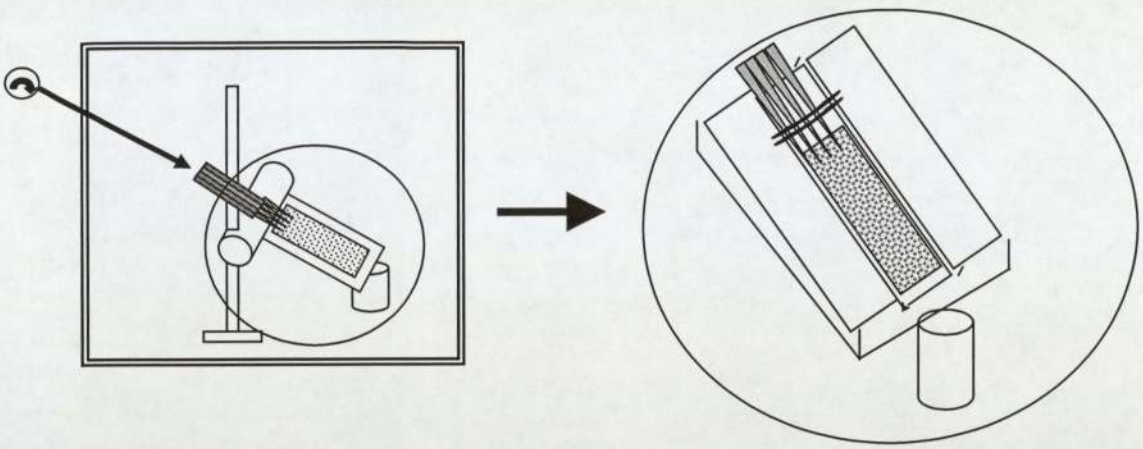


Figure 2.6: Retention model apparatus

Two similar techniques have been described to evaluate semisolid/liquid retention on mucosal surfaces (Young and Smart, 1998; Batchelor, 2002). These involved monitoring the retention of a maker molecule incorporated into a liquid or semisolid formulation when applied to a model mucosal surface and challenged with a flow of simulated intestinal liquid. An issue is the possible loss of the marker molecule as it diffuses out of the dispersion into the tissue or surrounding medium. The incorporation of the label as part of the polymer backbone means that the distribution of the polymer can be determined with confidence without having to significantly alter its physicochemical properties (Riley et al., 2002).

2.3.3.3 Preparation of Radio Chemical ($^{99}Tc_m$) Labelled adhesion and Experimental Procedure

A strip of porcine oesophageal tissue was cut to suitable size and mounted onto the platform so that an enough tissue area was exposed to the test adhesive polymer. Then a dose of labelled polymer was added to the oesophageal surface for an appropriate time with washing media running over the surface. The eluate was collected at designated time points for analysis. A special procedure was introduced as follows:.

A preparation of about 10-20 MBq of technetium tin colloid within 5 ml was supplied from City Hospital Birmingham. This material was kept within a lead pot for the duration of the experiment and was only opened for short periods for access to the material.

0.1 ml of this solution was withdrawn from the vial using a syringe. This volume contained 0.2-0.4 MBq of radioactivity. The radiolabel was added to a screw-top plastic vial containing 1.5 ml solution of adhesion that had been prepared previously. The syringe used to dispense the radiolabel was discarded into a solution of 5% Decon. The radiolabel was blended with the adhesive solution using a vortex mixer for 30 seconds.

Approximately 1 ml of the blended mix was drawn into a clean syringe; this syringe was then weighed using a weighing boat on a 4 decimal place balance. The volume was then dispensed onto the tissue surface and the syringe was reweighed to calculate the exact mass that had been added. When handling the syringe it was placed within a test-tube to

avoid contamination of surfaces with radiolabel.

A further volume (0.5 ml) of the original blend was drawn into a syringe and the syringe was weighed. This volume was then dispensed into a scintillation tube and the syringe was reweighed. This scintillation tube acted as a control to measure the counts per unit mass of the blended formulation.

Once the volume of material had been dispensed onto the tissue surface the eluate was collected directly into scintillation tubes. Once material had been collected the scintillation tubes were capped to prevent spillage. All apparatus was placed on a tray that could collect radiochemical material should there be a spillage.

Retention was measured for all formulations tested. The presence of drug was examined as well as the presence of additional excipients including those that aided the solubility of the drug.

2.3.4 MIC procedure

2.3.4.1 Introduction

This study was to establish whether a topical adhesive delivery device of the antifungal agents had a significant action against *Candida albicans*. Antifungal activities of the formulations were determined by the macro dilution method. A score assessment criteria

was also set up.

2.3.4.2 Efficacy procedure of adhesion

The oral *Candida isolates* (630G) were grown on an agar plate for 24 hr at 35°C in air. Then 5 colonies of *Candida* were selected and suspended in 0.85% w/v sterile saline. The suspension was vortexed for 15 seconds and the cell density adjusted so that it read the same as the standard at 530nm. When the cell suspension gave the same reading as the standard this meant that the suspension of *Candida* was approximately $1x10^6$ cells/ml.

The formulation was diluted to 1:2; 1:4; 1:8; 1:16; 1:32 etc so that they went across all 8 wells respectively. 100µl of each diluted solution was added into a sterile 96 well plate.

$1x10^6$ cells/ml suspension was made a 1:100 dilution so that there were $1x10^4$ cells/ml in sterile saline. Using RPMI 1640 broth medium, $1x10^4$ cells/ml of suspension was made a 1:20 dilution so that there were $5x10^2$ cells/ml.

A volume of 100 µl of final diluted suspension was added to each well already containing 100 µl of adhesive samples in the dilution series and a positive control well containing only *Candida* cells shown in Table 2.2.

Samples	control	1	2	3	4	5
Formulation dilutions	N/A	1:2	1:4	1:8	1:16	1:32
Cells concentration (cells/ml)	$5x10^2$	$5x10^2$	$5x10^2$	$5x10^2$	$5x10^2$	$5x10^2$

Table 2.2: The mixture samples in the plate for adhesive formulation efficacy study.

The plate was incubated at 35°C for 24 hours. After incubation, each well was scored according to a score assessment criteria by naked eyes to note whether there was any fungal growth and confirmed by a plate reader at 550nm.

A score assessment criteria was also set up as followed: 0=optically clear (no growth); 1=slight growth; 2= approximately one third of the well exhibits growth; 3= approximately half the area shows growth; 4=same as control (almost total surface covered with growth).

2.3.4.3 Determination of minimal inhibitory concentration of drug via growth on agar plates

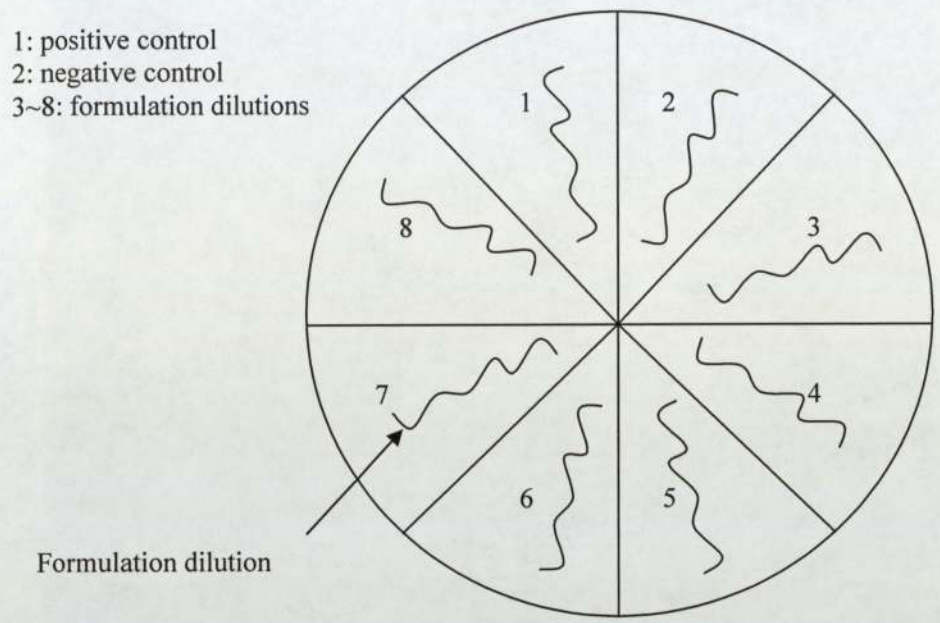


Figure 2.7: The distribution of samples on agar plate in MIC of formulation study.

The intensity of light at this wavelength is inversely proportional to the extent of fungal growth. Therefore, formulations used in this part were optically opaque could not be measured by reading at 550nm to calculate the percentage inhibition of light passage.

A suspension of *Candida cells* was added to each well of a sterile 96 well plate containing the samples of the dilution series of the formulation and a positive control containing *Candida* alone and a negative control containing formulation alone. The procedure was as same as described at 2.3.4.2.

The Agar plates were prepared and divided into approximate 8 sectors from the centre small volume from each well was swabbed onto an agar plate prior to incubation for 24 hours at 35°C.

The presence of *Candida* growth on the agar plates was then determined and the minimum concentration of formulation that inhibited growth of *Candida* was recorded. The same score assessment criteria was also set up to note whether there was any fungal growth.

2.3.5 Incorporation of drugs

Drugs were incorporated at a concentration of 25mg/ml in each formulation. The drug was first dissolved in PEG or glycerol in combination with the buffer prior to addition of chitosan or xanthan at a concentration of 2 % m/v. There were eight formulations with metronidazole and miconazole nitrate salt respectively used in the experiment.

2.3.6 Statistical significance test

Statistical significance test report the distinction between statistical significance and practical importance. It is possible for a difference of little practical importance to achieve a high degree of statistical significance. It is also possible for clinically important differences to be missed because an experiment lacks the power to detect them.

A P value was described as the observed significance level in experiments using statistical program (two-way ANOVA) in Excel. A null hypothesis was proposed at the 0.05 lever that was know as the critical region. Data collected from experiments were said to be significant at the 0.05 level or less.

	Adhesion		excipient			Drug
	Chitosan (w/v)	Xanthan (w/v)	Glycerol (v/v)	PEG (v/v)	Acetate buffer (pH=4)	concentration (mg/ml)
1	2%		10%		90%	25
2	2%		25%		75%	25
3	2%			10%	90%	25
4	2%			25%	75%	25
5		2%	10%		90%	25
6		2%	25%		75%	25
7		2%		10%	90%	25
8		2%		25%	75%	25

Table 2.3: The compositions in bioadhesive formulation study.

CHAPTER 3 RESULTS AND DISCUSSION

3.1 VISCOSITY STUDY

3.1.1 Effect of solution viscosity on the interaction of chitosan and PEG/glycerol

100 ml chitosan solution was prepared at a concentration of 2% w/v in acetate buffer (pH=4) with PEG/glycerol at concentrations of either 10% v/v or 25% v/v. A solution containing only chitosan (2% w/v) in acetate buffer was used as a control. The influence of the type and the concentration of the added solubility enhancers is shown in Figure 3.1. It is clear to see that glycerol enhanced the viscosity of the chitosan solution to a greater extent than PEG at the shear rate of 5 sec^{-1}. The viscosity of the chitosan alone was 7110cps and in the presence of 10% v/v glycerol this rose to 7900cps and 4940cps with 25 % v/v glycerol; at 10 % v/v PEG the viscosity was 5450cps and 2750cps at 25 % v/v. The data showed that the viscosity of the chitosan solution reduced as the concentration of added polymers was increased.

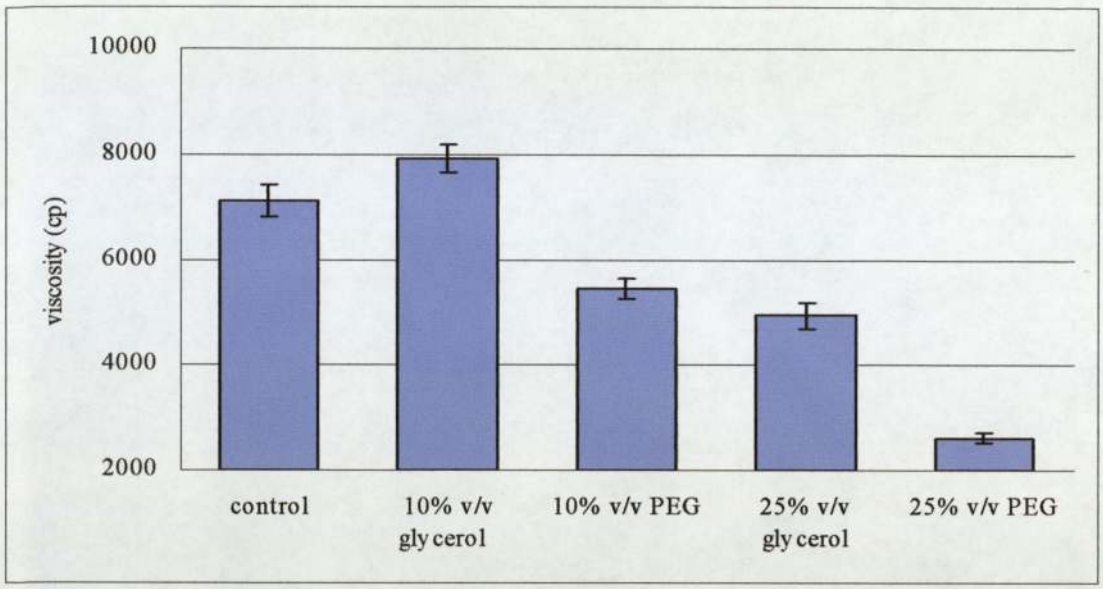


Figure 3.1: The viscosities of chitosan solutions with added polymers (data shows mean±s.d. dev. n≥4; D=5 sec^{-1}).

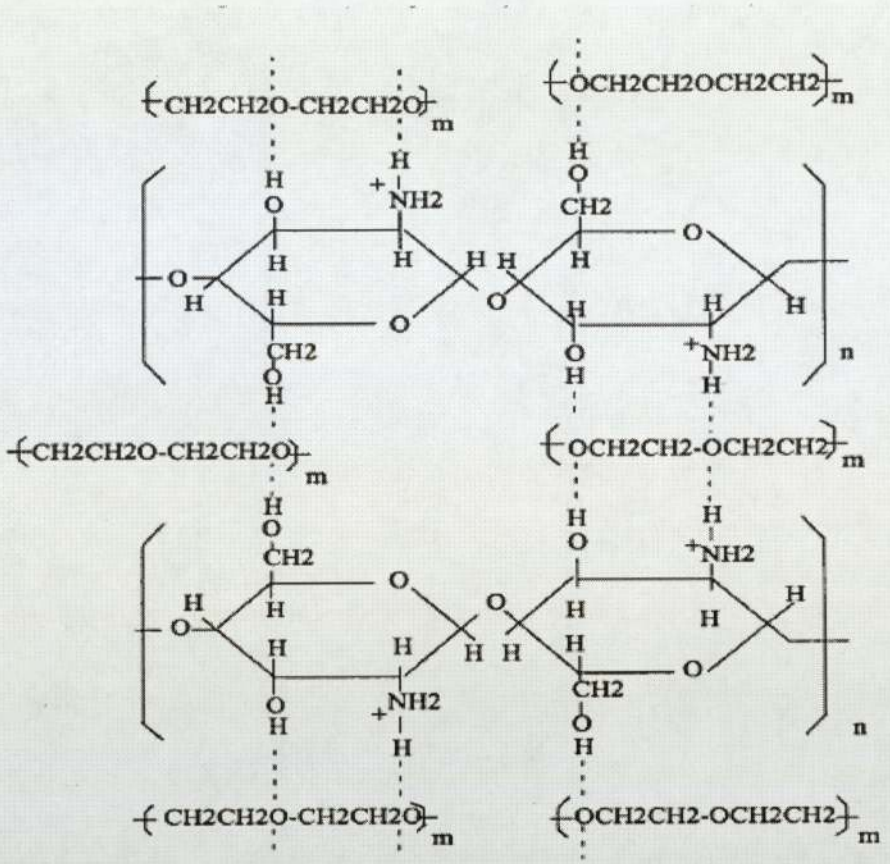
Chitosan within acetate buffer at pH 4 displays cationic polyelectrolyte behaviour due to the amino groups within the chitosan molecule dissociating. PEG bonds to water via H-Bonds. When PEG and chitosan are mutually dissolved in water, the following bonds can occur: PEG-PEG, PEG-water, PEG-chitosan, Chitosan-chitosan, Chitosan-water. Viscosity provides a measure of the relative amounts of these bonds that occur.

The presence of PEG in combination with chitosan leads to a reduction in the overall viscosity. Chitosan-chitosan bonds are responsible for the greatest viscosity observed as they interact to form a 3-D gel mesh associated with high viscosity. As PEG was introduced the number of PEG-chitosan bonds increased which, in turn lead to a reduction in the number of H-bonding sites available for chitosan to self-associate and form a gel net work.

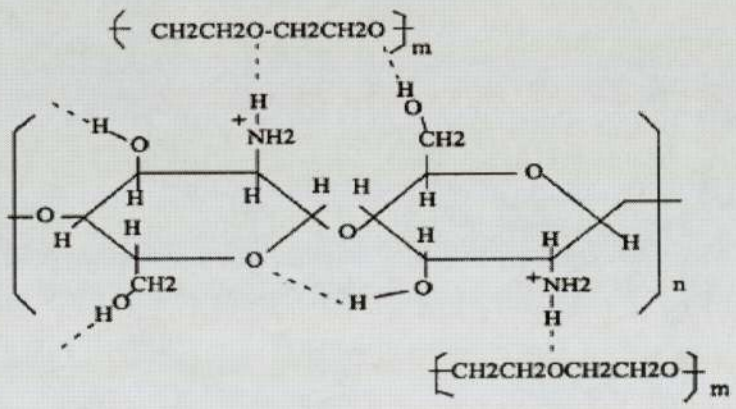
form a gel net work.

This has been fully described by Jiang et al (1999), who investigated the interactions between chitosan and PEG, and showed the structures of the polyblend at lower and higher PEG concentrations respectively (Figure 3.2). When the PEG concentration is low, each oxygen atom of the PEG molecules forms two hydrogen bonds with chitosan molecules, however, only one hydrogen bond was formed at a higher PEG concentration. The decrease of the hydrogen bonds between the oxygen atom of the part of the PEG molecules and the chitosan molecules results in an increase of the intramolecular hydrogen bonds in chitosan molecule and gives the chitosan solution an increasing viscosity.

In this study, when the concentrations of added polymers, PEG and glycerol, were increased, more hydrogen bonds between added polymers and chitosan were formed and resulted in an decrease of the intramolecular hydrogen bonds formed in the chitosan molecule. The viscosity of the chitosan solution was decreased as the intramolecular hydrogen bonds decreased.



(a) at lower PEG concentration



(b) at higher PEG concentration

Figure 3.2: The structure of chitosan-PEG complex: intramolecular bonds are formed in the chitosan molecular (from Jiang et al., 1999)

3.1.2 Effect of added polymers in xanthan solution

Xanthan solutions were prepared at 2% w/v with PEG and glycerol at 10% v/v and 25% v/v respectively as copolymers in this experiment. The results of the viscosity at the shear rate of 5 sec^{-1} shown in Figure 3.3 indicated that the added polymers increased the viscosity of the xanthan solution. When the concentration of the added polymers was 10% v/v, the viscosities of xanthan solutions were both increased around 20400 cps compared to the control solution with 19130cps. The viscosity of xanthan at the glycerol concentration of 25% v/v was greatly increased to 21400cps. Whereas PEG as an added polymer in xanthan solution gave a lower viscosity at 21150cps at 25% v/v concentration which was still much higher than the control (xanthan alone).

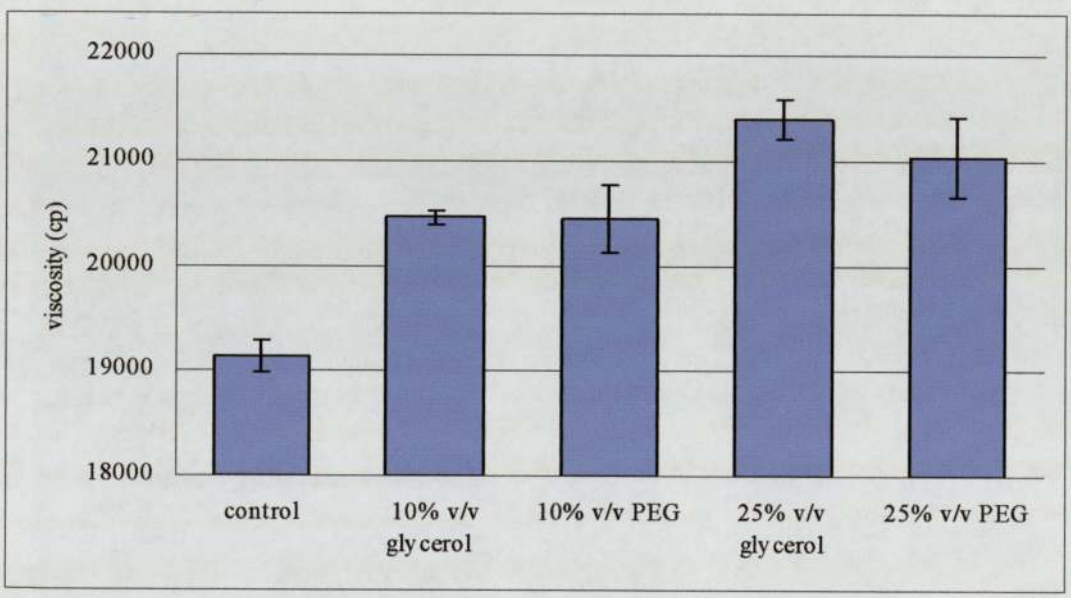


Figure 3.3: The viscosities of xanthan solutions with added polymers (data shows mean±s.d. dev. n≥4; D=5 sec^{-1}).

Xanthan is an anionic polymer. Langevin et al (2001) studied the changes of the surface tension by putting the excipients in the anionic polymer solutions. The origin of viscosity increase when xanthan is added to PEG/glycerol, is probably due to the fact that the added polymer does not bind to the xanthan chains. Their investigation also indicates that when the concentration of the added polymer is increased, the surface tension of the solution is increased. Therefore, because the interaction between xanthan and added polymer has no significant effect on the solution, the viscosity of the polyblend was higher than xanthan control and improved with a rising percent of added polymer. The results indicated that added polymers and xanthan do not form associations but exclude one another. Therefore, each polymer demonstrates an effective increase in concentration as its effective volume is decreased. As concentration is linked to viscosity, the observed viscosity is increased.

3.1.3 Comparison of the viscosity behaviour between chitosan and xanthan

To better understand the difference of the viscosities between xanthan and chitosan solutions, Figure 3.4 was presented to show the viscosities of both xanthan and chitosan solutions at the same situation. It is clear to see that xanthan solution had a significantly higher viscosity than chitosan in every sample no matter PEG or glycerol used as an added polymer ($p<0.05$; two-way ANOVA). Compared to the viscosities of the xanthan solutions around 19000~22000cps, chitosan solutions at the same situation could only give around 3000~8000cps at the shear rate of 5 sec^{-1}.

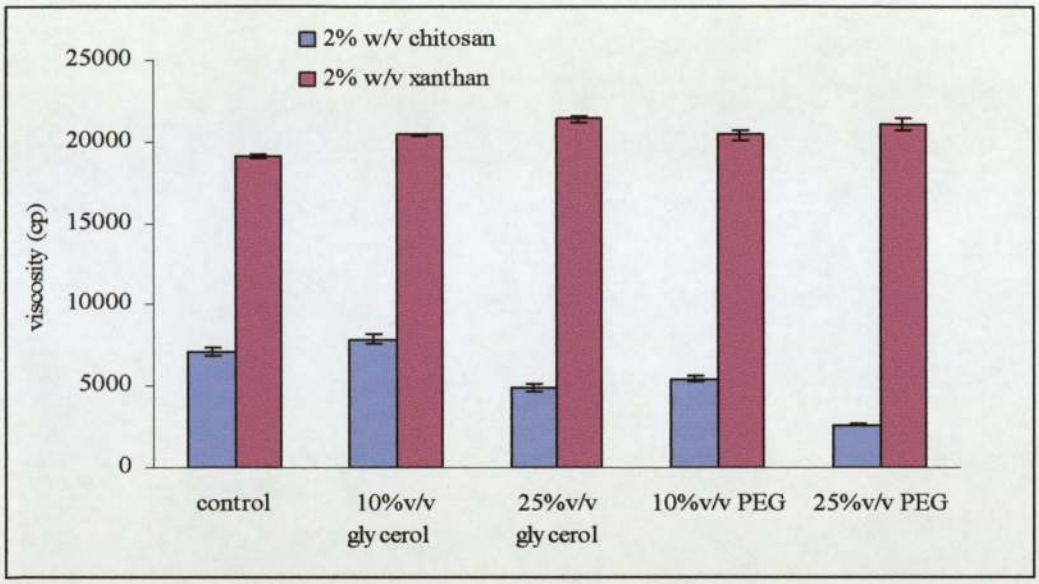


Figure 3.4: The viscosities of chitosan and xanthan solutions with added polymers ($D=5\ sec^{-1}$; $P<0.05$; two-way ANOVA)

This phenomenon can be explained by essential difference of the characters between chitosan and xanthan. Although they are both polyelectrolyte polymers, their molecular structures are not similar. Chitosan is a linear copolymer presenting rod-like and rarely entangled properties. When chitosan was supplied as a polyelectrolyte solution at a very small concentration at 2% w/v, the viscosity of the solution remained small (Langevin et al., 2001). Compared with chitosan, xanthan has a relatively complicated structure with reproducible bimolecular antiparallel double helices. It may form a very stiff intramolecular double stranded helical conformation by the annealing of the less stiff 'natural' denatured elongated single stranded chains. Therefore at a low concentration, the viscosity of xanthan is much higher than chitosan.

3.2 Release study

3.2 Release study

3.2.1 Theory of dissolution

The dissolution of a solid in a liquid may be regarded as being composed of two consecutive stages. At first, solute molecules are liberated from the solid phase by an interfacial reaction. The second stage is a transport of these solute molecules away from the interface into the bulk of liquid phase under the influence of diffusion or convection. In the absence of a chemical reaction between solute and solvent, the diffusion of dissolved solute across the static boundary layer of liquid that exists at a solid=liquid interface is slowest stage. The rate of dissolution of a solid in a liquid may be determined by the rate of diffusion of the slowest stage and described quantitatively by the Noyes-Whitney equation (Eqn 1):

$$\frac{dm}{dt} = kA(Cs - C) \qquad \textbf{(Eqn 1)}$$

where m is the mass of solute that has passed into solution in time t, i.e. *dm/dt* represents the rate of dissolution, A is the surface area of the undissolved solid in contact with the solvent, *Cs* is the concentration of solute required to saturate the solvent at the experimental temperature, *C* is the solute concentration at time *t* and *k* is the intrinsic dissolution rate constant or simply the dissolution rate constant. This constant has the dimensions of length^{-2} time^{-1} and it can be shown that:

$$k = \frac{D}{Vh} \qquad \textbf{(Eqn 2)}$$

where D is the diffusion coefficient of the solute in the dissolution medium (or solvent), V is the volume of the dissolution medium and h is the thickness of the boundary layer.

As an *in vitro* release experiment, when the dissolution medium and the thickness of the boundary layer are given at the same conditions, diffusion coefficient D is an important role that influences the release ability of a formulation. Some studies have been done to determine the diffusion coefficient of solute in hydrate matrix, especially in gel-matrix (Kuu et al., 1992; Westrin et al., 1994; Mohammad et al., 1997). The viscosity of the dissolution medium and the size of the diffusion molecules are considered as main effects of the diffusion coefficient.

3.2.2 Diffusion study based on the Noyes-Whiteney

Drug release from liquid vehicles may be compared to solid dissolution from a liquid medium as described by the Noyes-Whiteney equation (Eqn 1). In these drug release experiments we can assume that Cs-C is approximately equal to Cs. The Noyes-Whiteney equation may be simplified to

$$\frac{dm}{dt} = kACs \qquad \textbf{(Eqn 3)}$$

In a graph of *dm/dt*, the gradients is therefore equal to *kACs* as in Eqn 3. *Cs* is the concentration in the formulation and *A* is the area of the Franz cell ($2.52cm^2$) in this study. Therefore, *k* can be calculated and then *D* can also be calculated as we know *V* and *h* from Eqn 2.

Figure 3.5 showed the example for *k* and *D* data analysis. *K* was calculated from the release vs time curve. The gradient of the linear portion of the line (at least 6 data points were used) was 1.9576, taken as shown in the Figure 3.5. The area of Franz cell, *A* was $2.52cm^2$; the concentration of metronidazole, *Cs* was 25 mg/ml.

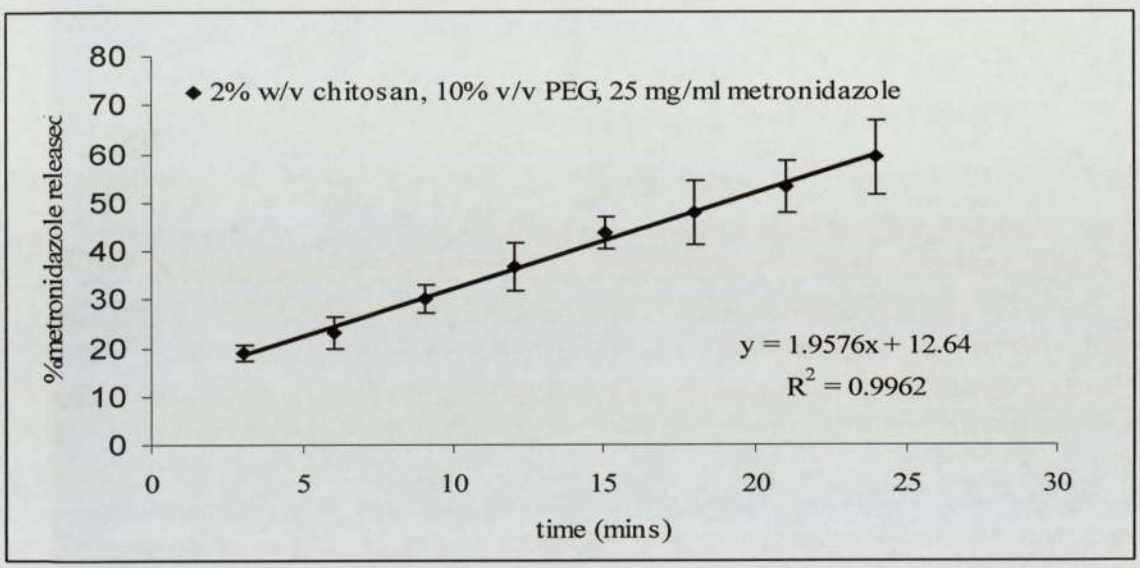


Figure 3.5: The linear of release study related to the k and D of the formulation

This data analysis is not providing a true value of *k* and *D* as it is not measuring dissolution of a solid, as described by the Noyes-Whiteney equation (Eqn 1). However, it does provide useful comparative data on the release rates from these formulations. Table

shows the *k* and *D* of formulations.

	K	D
Formulations containing 25mg/ml metronidazole		
2% w/v chitosan as control	0.053	0.021
2% w/v chitosan, 10% v/v PEG	0.031	0.012
4% w/v chitosan, 10% v/v PEG	0.029	0.011
2% w/v xanthan, 10% v/v PEG	0.012	0.005
Formulations containing 12.5mg/ml metronidazole		
2% w/v chitosan, 10% v/v PEG	0.050	0.020
2% w/v chitosan, 25% v/v PEG	0.030	0.012
Formulations containing 25mg/ml miconazole nitrate		
2% w/v chitosan as control	0.019	0.007
2% w/v chitosan, 10% v/v PEG	0.028	0.011
2% w/v chitosan, 25% v/v PEG	0.023	0.009
2% w/v chitosan, 10% v/v glycerol	0.028	0.011
2% w/v chitosan, 25% v/v glycerol	0.016	0.006

Table 3.1: The k and D of formulations based on the Noyes-Whiteney equation.

From the table, when the hydrogel concentration was increased, the *k* and *D* were reduced which meant that the release rate was slower. When xanthan and chitosan were used at same concentration at 2% w/v, xanthan solution had lower value of *k* and *D* than chitosan. *K*, the diffusion rate constant is affected by viscosity of diffusion medium. In the

viscosity study, xanthan solution was much more viscous than chitosan solution resulting in a lower diffusion coefficient of solute in the diffusion medium. However, increasing the concentration of hydrogel can also increase the viscosity of solution that may decrease the diffusion coefficient (Veyries et al., 1999; Ricci et al, 2004; Senel et al 1999). The data analysis in miconazole nitrate formulation showed that excipients can increase the value of k and D compared to the control formulation. This might because that the solubility of drug was improved by the excipients.

3.2.3 Release of fluorescent labelled adhesion

To investigate the release properties from the adhesives, 2%w/v chitosan and 2%w/v xanthan which had been labeled with fluorescence were applied to the release Franz cell shown in 2.3.2.2. The preparation of the labeled gels has been described in 2.3.2.5. The medium in the receptor was the acetate buffer (PH=4) which was shown in 2.2.5.

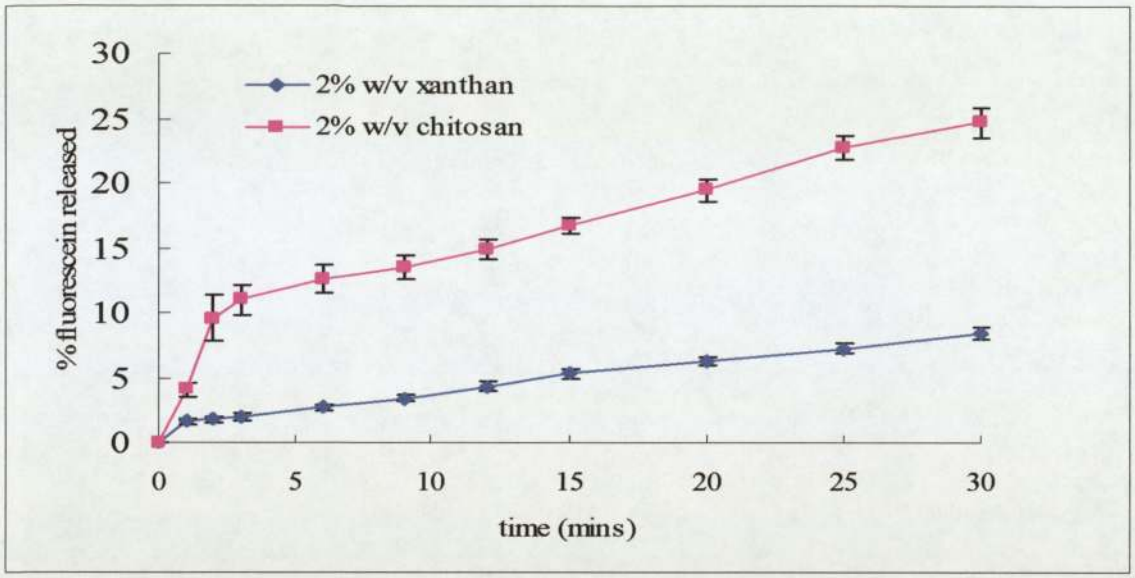


Figure 3.6: Comparison of release from labelled adhesions (data shows mean±s.d. dev. $n \geq 4$; $p < 0.05$; two-way ANOVA).

From the results shown in Figure 3.6, chitosan showed much better release property than xanthan. In the first 3 minutes, the percentage of the fluorescein released from chitosan was significantly greater at 11±1% compared to xanthan at 2% ($p<0.05$; two-way ANOVA). Over 30 minutes, chitosan showed greater release than xanthan with 25±1% and 8% of fluorescein released respectively. This can be explained by the different viscosities of these two formulations. Anderson et al 2001 indicated that drug release is affected by the viscosity of the gel. In the previous viscosity part 3.1.3, the viscosity of 2% w/v pure chitosan solution is much lower than pure xanthan solution at the same concentration. The effect of the diffusion coefficient on the *in vitro* release study demonstrated that more viscous gels had higher diffusion coefficients.

3.2.4 Release of model drugs in formulations

3.2.4.1 Release study in model drug I: metronidazole

3.2.4.1.1 Effect of bioadhesive concentration on drug release

When metronidazole as a model drug was incorporated in different concentrations of chitosan solutions, the release of the drug showed dependence on the gel-matrix concentration (Figure 3.7). The 4% w/v chitosan solution released metronidazole slower than 2% w/v chitosan solution at the beginning of the experiment, however, both of the formulations released drug around 60% over 30 minutes.

Several studies on the effect of polymer concentration in drug release have been reported recently (Veyries et al., 1999; Ricci et al, 2004; Senel et al 1999). Increased polymer concentration increases gel viscosity that reduces the drug diffusion coefficient. This could alter the dissolution and prolong the process of drug release from gel.

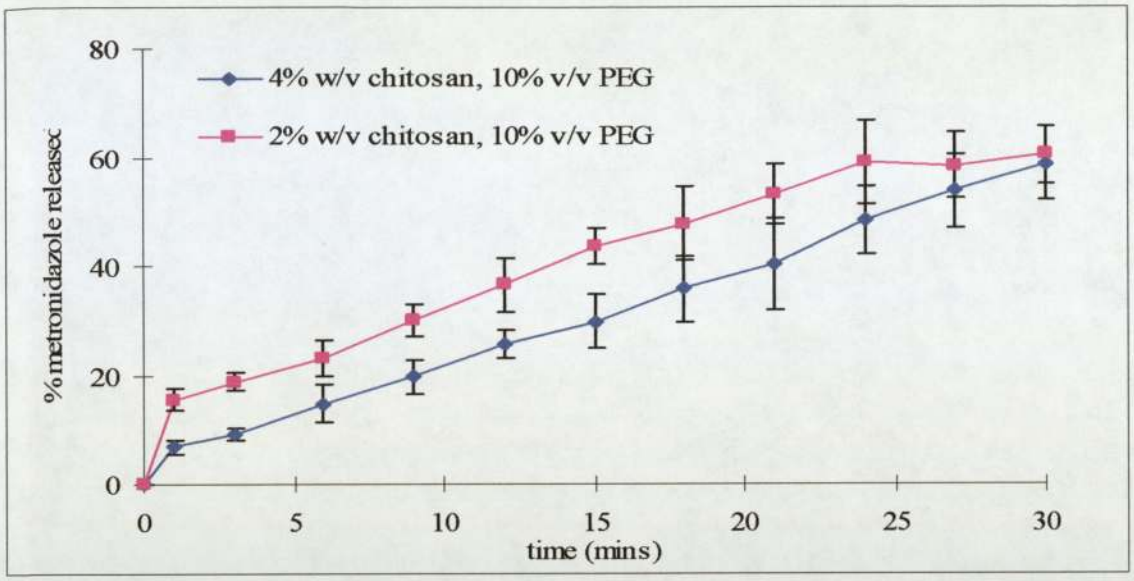


Figure 3.7: Effect of chitosan concentration on metronidazole release (data shows mean±s.d. dev. n≥4).

3.2.4.1.2 Effect of drug concentration on drug release

When metronidazole was incoporated into the formulation at different concentration at 12.5mg/ml and 25mg/ml respectively, the formulation with more drug showed faster release rate. From the release profile in Figure 3.8, when the samples were applied on the boundary layer in the Franz diffusion cells shown in 2.3.2.2, similar amount of the dissolved drug passed into medium phase from both formulations immediately. According to the Noyes-Whitney equation, the term *(Cs-C)* for the formulation with 25mg/ml metronidazole is higher; therefore the diffusion rate should be faster, than the formulation with metronidazole at 12.5mg/ml. Over 30 minutes of release, 62±4% of metronidazole was released from the formulation with higher drug concentration, however only 35±7%

from the lower one, as expected.

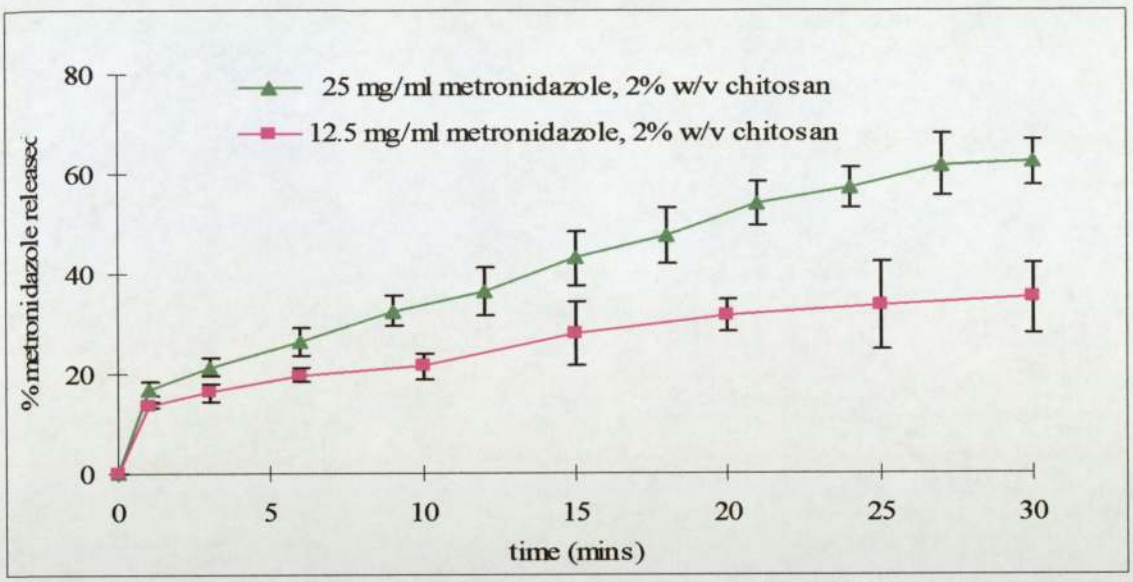


Figure 3.8: Effect of metronidazole concentration in release profile (data shows mean±s.d. dev. n≥4).

3.2.4.2 Release study in model drug II: miconazole nitrate

3.2.4.2.1 Effect of excipients concentration on drug release

3.2.4.2.1.1 Glycerol as excipient

For the further release study, miconazole nitrate salt was introduced as another model drug in the same way as metronidazole. Not only PEG, but also glycerol was used as the excipients aid to the solubility of the model drug to note the release properties. From the release profile shown in Figure 3.9, miconazole nitrate from both formulations containing

glycerol exhibits no measurable lag period before release, which means that drug in these formulations can be available immediately. Compared to the control formulation, the role of glycerol was to aid the solubility of miconazole nitrate. A dose dependent difference was shown in the release profile.

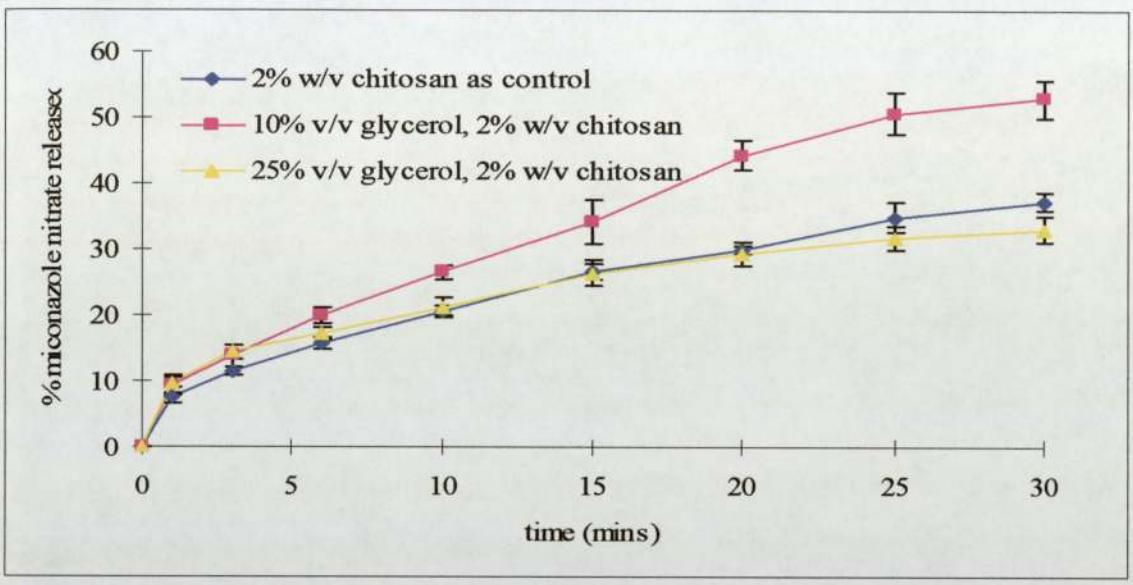


Figure 3.9: Effect of glycerol concentration on miconazole nitrate release (data shows mean±s.d. dev. n≥4).

The formulation with 10% v/v glycerol showed the greatest release with 53±2% of miconazole nitrate released after 30 minutes. Compared to the control formulation without glycerol, the diffusion rate of miconazole nitrate from the formulation with 10% v/v glycerol was faster during the 30 minutes release. However, when the concentration of glycerol was increased to 25% v/v, the drug diffusion rate was decreased, with only 33±1% of drug was released in total over 30 minutes.

Glycerol is an FDA approved excipient for topical ocular and parenteral administration capable of forming hydrogen bonds thus slowing the release rate of formulations. Furthermore, glycerol has been studied to control the rate of release as an excipient in a formulation especially for mucosal delivery based on a chitosan carrier by Brown et al (2000). When glycerol was added at lower concentration, it showed the main function that can improve the solubility of miconazole nitrate resulting in a faster diffusion rate over 30 minutes. Increased glycerol concentration reduced the amount of miconazole nitrate released from chitosan solution. This result may be related to the solubility coefficient of the drug between the donor and receptor mediums. If the drug is much more soluble in the donor, it will partition into this phase rather than the receptor phase where its solubility is reduced.

3.2.4.2.1.2 PEG as excipient

When PEG was incorporated into the formulation to improve miconazole nitrate solubility at 10% v/v and 25% v/v, both formulations with PEG showed better release compared to the control (Figure 3.10). At the first 10 minutes release, there was no significant difference among the formulations and about 20% of miconazole nitrate was released from each formulation. The formulation containing 10% v/v PEG showed the fastest release rate in the following 20 minutes that over 30% of drug was released and around 50% of miconazole nitrate was released totally over 30 minutes.

Chitosan with PEG has shown lower viscosity compared to the pure chitosan solution in

the viscosity part. In general, a lower viscous formulation has a higher release property; therefore, the formulations containing PEG showed better release than chitosan control. However, the profile showed that the formulation containing 10% v/v PEG at a lower viscosity had a better release compared to the formulation with 25% v/v PEG which is more viscous. Again, this is probably due to the solubility in each phase affecting the partition of the drug.

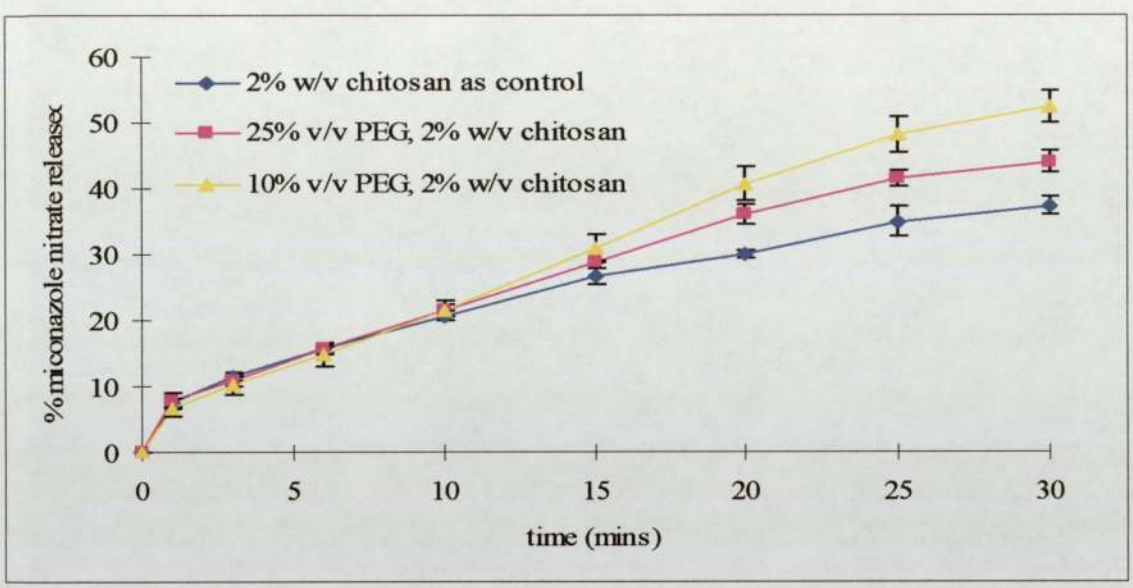


Figure 3.10: Effect of PEG concentration on miconazole nitrate release (data shows mean±s.d. dev. n≥4).

3.2.4.2.2 Comparison of drug release using different excipients

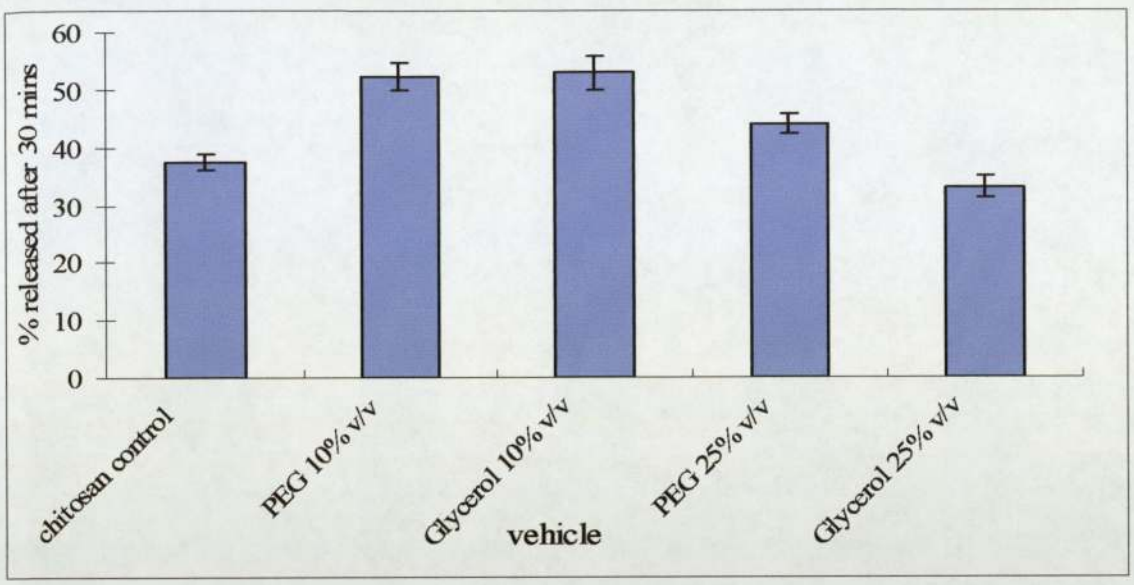


Figure 3.11: Comparison of drug release using different excipients in chitosan-based hydrogel (data shows mean±s.d. dev. n≥4).

Figure 3.11 shows the influence of the vehicle on the miconazole nitrate release. The incorporation of PEG of both concentration lead to an overall increase in the percent released over 30 minutes. The glycerol at 25% v/v was not statistically different to the control. Both PEG and glycerol at 10% v/v as excipients gave formulations with the best release with over 50% of drug released after 30 minutes. When the concentration of PEG was increased to 25% v/v, the release of miconazole nitrate was reduced less over 30 minutes; however, it was still more than chitosan control with 37%.

The influence of PEG and glycerol on the drug release in chitosan can be summarized in three ways: the influence of viscosity of formulations, the influence of solubility of drug

and the influence of the hydrogen bonding between chitosan and excipients. In the previous experiment, the viscosity of formulation was decreased when the excipients were added. As an effect of the diffusion coefficient in vitro release study, less viscous formulation has lower diffusion coefficient, therefore the release rate of drug from formulation is faster. It has been reported that both PEG and glycerol have been reported that both of them can improve the solubilities of hydrophobic drugs (Abuchowski et al ., 1977a,b). In general, when the solubility of drug is increased, the release of the drug from formulation is improved. Hydrogen bonds are considered to be barrier to release of drug. Not only PEG but also glycerol has been reported to form hydrogen bond in gel formulations that aid diffusional resistance (Brown et al., 2000). Although PEG at 25% v/v gave the formulation the lowest viscosity, the release from this formulation is lower. When polymers were added into chitosan hydrogel at high concentration, the formation of hydrogen bond shows more influence than the influence of viscosity in a decrease release of drug. Moreover, 25% v/v glycerol added into the formulation resulted in the lowest release of miconazole nitrate even less than chitosan control.

3.3 Retention Study

3.3.1 Effect of vehicle on mucosal retention of chitosan hydrogel formulation

Figure 3.12 shows the influence of vehicle on the mucosal retention of chitosan hydrogel formulations on oesophageal tissue in the *in vitro* experiment. In all cases with chitosan hydrogel, the retention profiles were biphasic, with rapid detachment of chitosan in first

minutes of washing (Stage I) followed by a more controlled loss detachment of chitosan unable to establish a retentive interaction with the mucosa, whereas Stage II probably represents the loss of chitosan that had adhered to the mucosa. This result is in agreement with Riley et al (2002) and Smart et al (2003) that have studied retention of polymers on mucosa and observed a two-stage retention profile dependent on the ability of the polymer to interact with the mucosa.

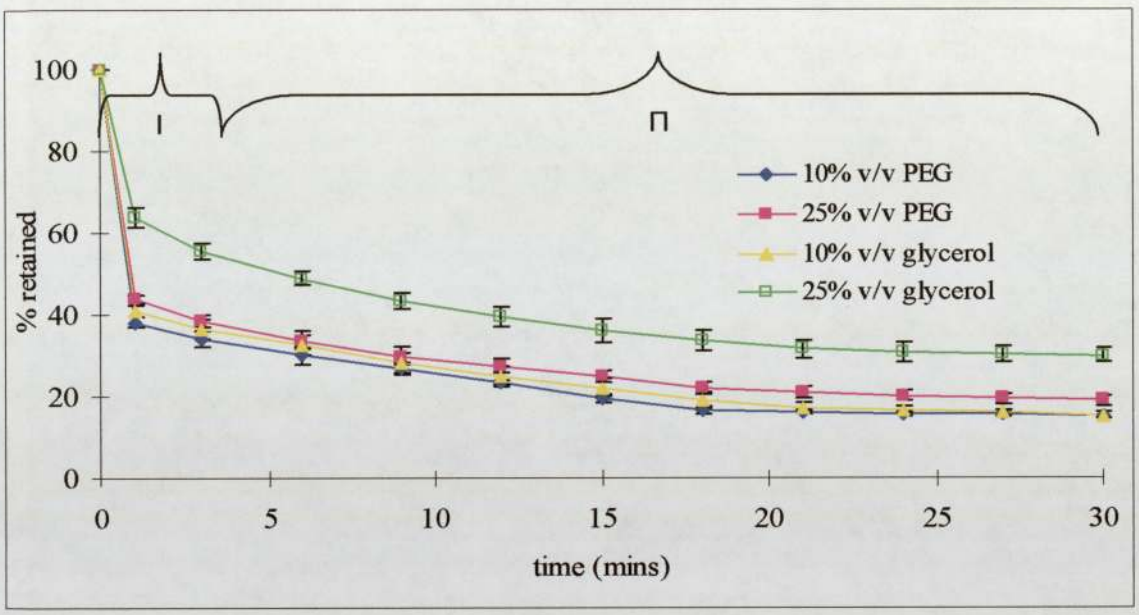


Figure 3.12: the retention profile of chitosan-based formulation in different vehicles (data shows mean±s.d. dev. n≥4).

In Figure 3.13 it is apparent that the chitosan hydrogel formulations containing miconazole nitrate had better retention than without drug. It has been reported that water-insoluble drugs can improve the viscosity of hydrogel formulation, resulting in a longer time retention on mucosal tissue. Glycerol as an excipient at 25% v/v showed significantly better retention compared to the other three samples ($p<0.05$; two-way

ANOVA). The formulation with 25% v/v glycerol retained 30% and 25% of the applied dose (1ml) over 30 minutes with and without drug respectively; however, there was no significant difference on the retention of other formulations which were lower from 10% to 15% after minutes washing.

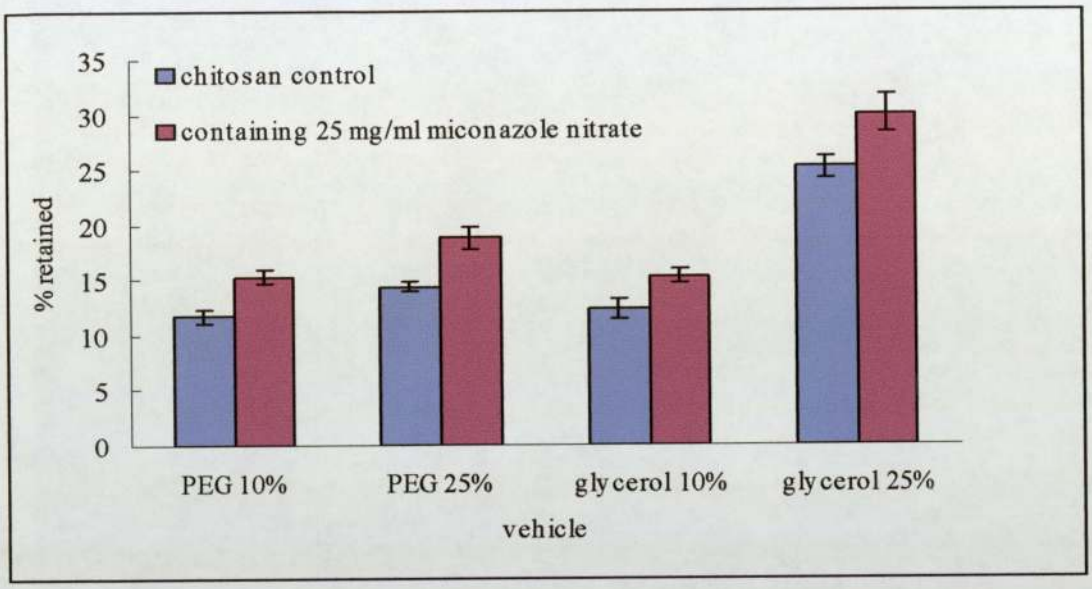


Figure 3.13: Effect of vehicle on mucosal retention of chitosan-based formulations (data shows mean±s.d. dev. n≥4).

Recently, Richardson et al (2004a) and Richardson et al (2004b) investigated the bioadhesive liquid dosage form using glycerol and PEG as vehicles to specifically adhere to the oesophageal mucosa and indicated that glycerol can rapidly hydrate and swell to form a viscous adherent layer on the mucosa that can resist elution. The degree of hydration and viscosity of vehicle are considered to be more influential compared to the effects of washing. When the particle hydration of vehicle is more extensive, swelling and forming an adhesive layer on mucosa, the formulation can be better retained during

washing. Also, Needleman et al (1998) have shown how the viscosity of a retained polymer layer can influence its resistance to displacement from a tissue surface during washing.

3.3.2 Effect of vehicle on mucosal retention of xanthan hydrogel formulation

As the previous discussion, the retention of xanthan-based hydrogrel formulations was increased when miconazole nitrate was added shown in Figure 3.14. Compared to the vehicle of glycerol, the influence of drug in PEG as vehicle was significant. When PEG was added at 10% v/v, the percent of formulation retained was improved from 95% to 97% as miconazole nitrate was added. Increasing the percent of PEG to 25% v/v, there were 96% and 97% formulation retained with and without drug respectively. However, glycerol as an excipient gave xanthan-based formulation a better retention than PEG that around 98% of each formulation with glycerol retained over 30 minutes. There was no significant difference in retention between the formulations prepared with and without miconazole nitrate. The difference of retention of formulations added glycerol at 10% v/v and 25% v/v was also not significant. This results also was indicated that glycerol can improve the resistance of elution supported by Richardson et al (2004a) and Richardson et al (2004b).

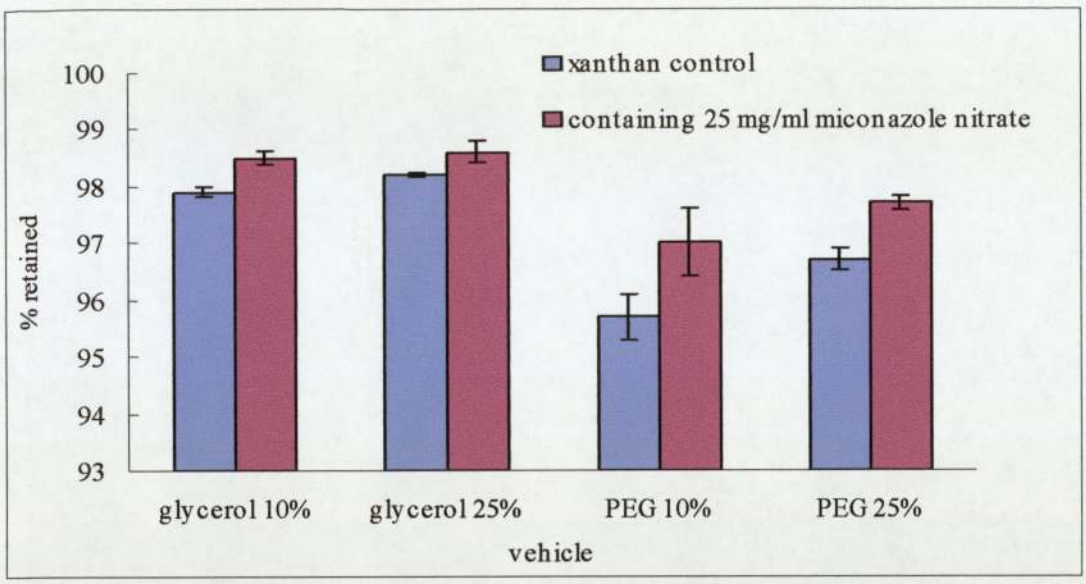


Figure 3.14: Effect of vehicle on mucosal retention of xanthan-based formulations (data shows mean±s.d. dev. n≥4).

From the retention profile in Figure 3.15 below, xanthan-based formulation showed good retention. Also, the profiles showed smooth displacement from the tissue surface during washing. In all cases, there was over 95% of formulation retained after 30 minutes. The viscosity study in part 3.1 gives the highly retention property of xanthan-based hydrogel formulation the mainly support with the highly viscous property. Because of the lower viscosity of xanthan-based hydrogel formulation added with PEG, the formulations prepared with PEG were more easily to be washed off. When increasing the percent of PEG from 10% v/v to 25% v/v, the viscosity of the formulation was increased resulting in a significant increase of resistance ($p<0.05$). Compared to the big influence of the amount of PEG in the formulation, the retention profiles were similar for the xanthan-based formulation cooperated with glycerol at 10% v/v and 25% v/v.

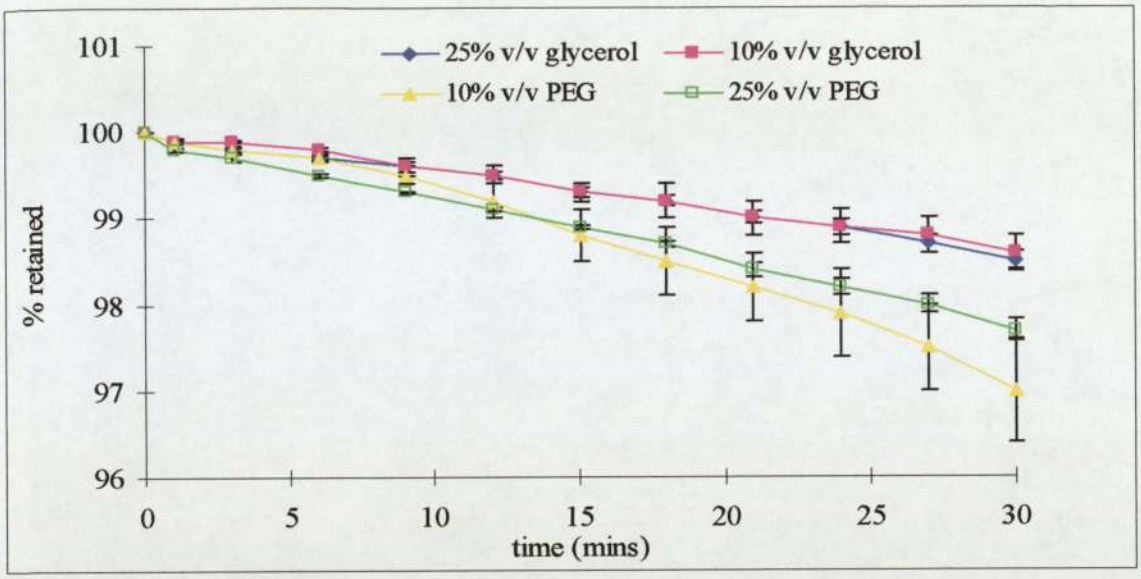


Figure 3.15: The retention profile of xanthan-based formulation in different vehicles (data shows mean±s.d. dev. n≥4).

3.4 Microbiology

3.4.1 Effect of formulation on *Candida* growth

The efficacy of the formulation along without model drug was studied in this section. The dilution series of 2% w/v chitosan and 2% w/v xanthan hydrogel were investigated with a sample containing *Candida cells* in acetate buffer as a positive control. The scores of visible fungal growth are shown on Table 3.2. From the data of results, chitosan hydrogel as a formulation exerted an antifungal activity alone compared to xanthan gum. The MIC of 2% w/v chitosan hydrogel on *C. albicans* was 10 mg/cm^3. Senel et al (1999) has also studied the antifungal activity of chitosan and reported a MIC value of 10 mg/cm^3 for *C. albicans*.

albicans.

	$20mg/cm^3$	$10mg/cm^3$	$5mg/cm^3$	$2.5mg/cm^3$
chitosan-based hydrogel	0	0	1	3
xanthan-based hydrogel	1	1	2	3
positive control (*Candida* alone)	4	4	4	4

Table 3.2: The scores of fungal growth at the conditions outlined in the table (0 indicated no growth; 4 is positive control)

The antimicrobial activity of chitosan has been reported by other studies (Ikinci et al., 2001; Statroniewicz et al., 1994). Although the mechanism by which chitosan affects Candidal species is not clearly understood, Seo et al 1994 has suggested that an ionic interaction between the cations due to the amino groups of chitosan and antonic parts of bacterial cell wall such as phospholipids and carboxylic acids as the mechanism for the antimicrobial activity of chitosan.

3.4.2 Comparison of MIC of model drug in chitosan-based and xanthan-based hydrogel formulations

When the drug was incorporated, the anti-fungal activitiy of the formulation is clearly comparable with the result obtained with chitosan and xanthan gum respectively (Table 3.3). In chitosan, the MIC values were significantly higher compared with xanthan. This effect was obviously due to the anti-fungal activity of chitosan itself. It has been reported

that chitosan, due to its cationic nature is capable of opening tight junctions in a cell membrane. This property has led to investigate the use of chitosan as a permeation enhancer for hydrophilic drugs that may otherwise have poor oral bioavailability, such as miconazole. The absorption enhancement is caused by interactions between the cell membrane and positive charges on the polymer.

	Concentration of drug (mg/ cm^3)					
	25	12.5	6.25	3.125	1.563	0.781
10% v/v glycerol	0	0	0	2	2	3
25% v/v glycerol	0	0	0	2	3	3
10% v/v PEG	0	0	0	1	2	3
25% v/v PEG	0	0	0	2	2	3
positive control (*Candida* alone)	4	4	4	4	4	4

Table 3.3: The efficacy of miconazole nitrate in chitosan hydrogel formulations (0 indicated no growth; 4 is positive control)

	Concentration of drug (mg/ cm^3)					
	25	12.5	6.25	3.125	1.563	0.781
10% v/v glycerol	0	0	0	2	3	3
25% v/v glycerol	0	0	1	3	3	3
10% v/v PEG	0	0	0	2	3	3
25% v/v PEG	0	0	1	3	3	3
positive control (*Candida* alone)	4	4	4	4	4	4

Table 3.4: The efficacy of miconazole nitrate in xanthan hydrogel formulations (0 indicated no growth; 4 is positive control)

With miconazole nitrate salt, the MIC values of 2% w/v chitosan gel shows 6mg/cm^3, no matter the concentration of PEG or glycerol contained in the formulation. On the other hand, increasing the concentration of PEG or glycerol resulted an increase on the MIC values of the formulations containing 2% w/v xanthan gum. The results showed on the Table 3.4 indicated that a promising application of miconazole nitrate at a low concentration can be incorporated in 2% w/v chitosan gel with excipients or in 2% w/v xanthan gum with lower concentration of excipients.

CHAPTER 4 CONCLUSIONS

The investigation has developed and demonstrated the applicability of a bioadhesive drug delivery system for anti-fungal agent targeted to the oesophagus. The MIC study showed that the concentration required to be effective against *Candida* is 6.25mg/cm^3 of miconazole nitrate. With higher concentrations of PEG and glycerol, the efficacy of the formulation was reduced with some growth with xanthan at a drug concentration of 6.25mg/cm^3. Because of anti-fungal activity of chitosan, the efficacy of the formulation with chitosan was 6.25mg/cm^3 at 10% v/v and 25% v/v added excipients.

Drug release studies agreed with the MIC data. When the excipients were added aid to the solubility of miconazole nitrate, more excipients improved the solubility of the drug resulting in a reduced release of the drug. The percent of drug released over 30 minutes was 10% for xanthan and approximately 50% for chitosan with excipients. Both bioadhesive formulations with xanthan and chitosan showed no lag period release which is suitable for *in vivo* therapy.

The bioadhesive formulations were proven with well retained ability. Xanthan was retained a far greater extent compared to chitosan. Over 90% of formulation with xanthan was retained after applied on the oesophageal tissue for 30 minutes, compared to chitosan with 30% formulation retained.

As 1cm^3 formulation containing 25mg/cm^3 was applied on the 6cm^2 area of oesophageal

tissue (1 cm width, 6 cm length) in retention study, there was 4.17mg of drug per unit area present. With xanthan where over 90% was retained, there was approximately 3.75mg of drug present per unit area. Increasing drug concentration lead to a faster release has been studied in Section 3.2.4.1.2. The MIC was found to be 6.25mg/cm^3, therefore a greater concentration of drug within the formulation may be required. On the other hand, the MIC challenge may not be realistic for the therapeutic situation. This formulation could be taken at regular intervals over a prolonged period of time.

This preliminary study suggests that a bioadhesive formulation containing antifungal agents may be beneficial in the treatment of oesophageal *Candida.*

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