MASTER THESIS FOR THE DEGREE MASTER OF PHARMACY

DEVELOPMENT OF MUDS FOR IMPROVED VAGINAL THERAPY

BY
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Abstract

Bacterial vaginosis is a common cause of vaginitis, condition often treated with oral metronidazole. However, due to unpleasant side effects, patient compliance is often reduced. Vaginal tablet with bioadhesive properties could enable a prolonged residence time and a sustained release of a drug at the site of infection. Vagina as a drug administration site is attractive due to high surface area, rich blood supply and avoidance of first pass metabolism. The aim of the study was development and characterisation of bioadhesive multiparticulate delivery system (MUDS) for vaginal delivery of metronidazole. Metronidazole was mixed with chitosan of different degrees of deacetylation (DD) and/or pectin of different degrees of methoxylation (DM). The drug content was kept constant at 10 % (w/w) throughout the experiments. A 2^2 – factorial design with centre point for was used to screen for the combination of 50:50 ratios of chitosan type (DD 77%, 82% and 92%) and pectin type (DM 10%, 25% and 40%) with high bioadhesion to cow vaginal tissue. The max detachment force (Fmax) and work of adhesion (AUC) were determined using 11 mm flat faced tablets and vaginal tissue by Texture Analyser (Stable Microsystem, UK). Chitosan DD 92 % was selected as the chitosan type for further optimization studies since this was found to have the largest influence on the bioadhesion of 11 mm tablets to vaginal tissue. For further optimization studies, different ratios of pectin (DM 10% and 40%) and chitosan (DD 92%) were mixed with the drug (10%, w/w), and concave mini-tablets (2 mm) were produced by direct compression (compression force 241.49 ± 10.22 MPa) using 15–tip multiple tooling. Tensile strength, friability and simulated wetting time of the mini-tablets were tested. Dissolution rate of mini–tablets was determined using the paddle method (100 rpm and 37 ℃) in vaginal fluid stimulant (VFS) pH 4.5 and 6.8. A modified version of rotating cylinder method was applied for bioadhesive studies of mini–tablets using cow rectal tissue to determine the percentage remaining mini-tablets on the tissue after rotating at 250 rpm for 10 minutes. The combination of 70 % chitosan DD 92 % and pectin DM 40% showed the strongest bioadhesive properties as well as the slowest release of metronidazole from the mini–tablets in VFS. The highest degree of cross–linking was seen in media of high pH (pH 6.8) and for higher amount of esterification in the molecule. All formulations showed low friability and tensile strength between 12-25 N/mm^2, except those containing only pectin. To conclude, it is possible to obtain desired mucoadhesive strength and sustained release of metronidazole in mini–tablets by combining chitosan of high deacetylating degree with pectin type of low methoxylation degree. The tablets show sufficient tensile strength and friability.

Abbreviations

AUC:	Work of adhesion
C-77:	Chitosan deactylation degree 77%
C-82:	Chitosan deacetylation degree 82%
C-92:	Chitosan deacetylation degree 92%
DD:	Degree of deacetylation
DM:	Degree of methoxylation
Fmax:	Maximum detachment force
HM:	High methoxyl pectin
HPC:	Hydroxypropyl cellulose
MUDS:	Multiparticulate delivery system
P-10:	Pectin degree of methoxylation 10 %
P-40:	Pectin degree of methoxylation 40 &
SWT:	Simulated wetting test
VFS:	Vaginal fluid stimulant

1. Introduction

1.1 Background

Bacterial vaginosis is a common cause of vaginitis and arises from an overgrowth mainly of anaerobic bacteria. Metronidazole is the drug of choice, but due to unpleasant side effects like gastric intolerance and metallic taste it can result in failure of completing the therapy. Vagina as a drug administration site possess advantages such as less systemic side effects, lower dosing frequency and lower amount of drug administrations per dose. Thus resulting in higher patient compliance and achieving better therapeutic outcome, which reduces the risk of recurrence or even worse, the development of antibiotic resistance. Moreover, the vagina possesses characteristics that are favouring drug absorption like high surface area of the vaginal wall, rich blood supply and avoidance of hepatic first pass metabolism.

Traditional formulations like gels, creams and tablets exhibit low residence time at the vaginal site due to gravity and self-cleansing effect of the vaginal tract. To achieve desirable therapeutic effect, vaginal delivery systems should reside at the sites of infection for prolonged period of time and enable sustained release of the incorporated drug. This can be achieved by using bioadhesive vaginal tablets. Multiparticulate delivery system (MUDS) is a promising system for vaginal drug delivery of drugs due to their small size, which contributes to increased surface area and spread throughout the vaginal cavity. Hormonal activity, pH, vaginal fluid and patient's age must be taken into consideration in development of a new vaginal formulation.

Chitosan is a natural biopolymer derived from chitin by chemical processing. Chitosan is considered as non–toxic, biocompatible and biodegradable with mucoadhesive properties. Cross-linking with pectin is a possibility for achieving prolonged and controlled release of the drug.

1.2. Vagina

1.2.1 Anatomy and histology

Vagina is a fibromuscular tube that extends 6-12 cm from cervix of the uterus (Hussain and Ahsan, 2005, Valenta, 2005). The surface of the vagina is composed of numerous folds, often called rugae. These folds keep distensability, support and provide an increased surface area of the vaginal wall (Hussain and Ahsan, 2005). The vaginal wall is compromised of three layers: the epithelial layer, tunica adventia and the muscular coat. The epithelial layer creates the superficial layer, which is about 200 μm thick (Valenta, 2005). The smooth muscular fibres of the muscular coat are running along in both circular and longitudinal directions. This gives the vagina an excellent elastic character. Further, the connective tissue of the tunica adventia also increases the flexibility of the vagina. The vaginal wall compromises of a dense network of blood vessels and extends from internal iliac artery, uterine, middle rectal and internal pudental arteries (Washington et al., 2001). The blood enters systemic circulation via rich venous plexus which empties primarily into internal iliac vein. The vagina lacks the direct release of mucus because it does not contain any goblet cells. But still it discharges a large amount of fluid. The fluid has its origin from transudates through the epithelium, cervical mucus, exfoliating cells, leukocytes, endometrial and tubual fluids (Owen and Katz, 1999, Washington et al., 2001). The vaginas nerve supply comes from two sources. The peripheral, which primarily supplies the lower quarter of the vagina, makes it a highly sensitive area: the autonomic primarily supplies upper three quarters. Autonomic fibres respond to stretch and are not very sensitive to pain or temperature. The upper vagina is a very insensitive area because of few sensory fibres. This is why women rarely feel localized sensations or any discomfort when using vaginal rings, and are often unaware of the presence of such items in vagina (Alexander et al., 2004).

1.2.2. Physiological changes and the influence on drug absorption

Women in post-menopausal stage experiences many physiologically alterations. These changes manifests as reduction in production of estrogen through pre-and postmenopause (Owen and Katz, 1999). This leads to reduced glycogen content and elevated pH to 6.0-7.5 due to less conversion of lactic acid from glycogen by *lactobacillus.* The increased vaginal pH could often result in frequent vaginal infections. Furthermore, reduced vaginal discharges and thinning of epithelial layer occurs. The reduction is estimated to 50 % compared to that produced by women of reproductive age (Alexander et al., 2004).

Environmental changes in fertile women are associated with hormonal events in menstruation cycle. The epithelial layer changes in thickness approximately 200-300 μm during menstruation cycle (Valenta, 2005, Hussain and Ahsan, 2005). The pH is kept around 3.8 -4.2 and tends to be lowest when estrogen is highest (ovulation) (Hussain and Ahsan, 2005). In this period, glycogen and epithelial desquamation is at its maximum which causes an increased viscosity.

The physiological changes in vagina would affect the absorption of drugs and must be taken into consideration in the development of formulations. The thickness of epithelial layer could affect the permeability, where thinner epithelium causes increased absorption. Amount of fluid is important because drugs must be in solution before absorption (Richardson and Illum, 1992). For bioadhesive systems, wetting of the tablet is crucial for bioadhesion and increasing the residence time (Karasulu et al., 2002). Viscosity may present as a barrier for drug absorption and continuous secretion can result in removal of the dosage form (Valenta, 2005). The influence of pH is important for absorption, where the unionized form is more readily absorbed (Richardson and Illum, 1992).

1.2.3 Vaginal infections

Vaginitis is the most frequent gynaecological diseases and is caused by bacterias, candidas and *Trichemonas Vaginalis*. Infections caused by *Trichemonas vaginalis* is quite rare, whereas most frequent is candidas and bacteria (Kukner et al., 1996). Bacterial vaginosis is caused by an overgrowth mainly by anaerobic bacteria, including *Gardernalla Vaginalis*, *Prevotella spp*., *Peptostreptococci* and *Mobiluncus spp*. (Donders et al., 2000a). This is leading to a replacement of lactobacilli and an increased vaginal pH >4.5. No inflammation responses, like edema and redness, arises (Donders, 2010). The overgrowth of anaerobic bacteria can be caused by the disappearance of lactobacilli due to environmental changes, such as vaginal douching or increased pH after sexual intercourse. Another reason for diminished lactobacilli is attacks of specific virus (bacteriophages). The symptoms are production of a thin and grey vaginal discharge which has a fishy odour (Kukner et al., 1996). It occurs most often in women of childbearing age, but may also occur in menopausal women. Children are rarely infected. Normally, bacterial infections are just annoying due to the odour and voluminous discharge. However, it is associated with some risk factors including increased risk of getting sexual transmitting disease, especially genital herpes and HIV, a

higher risk of spontaneous miscarriage ranging from 13-24 gestational week and preterm birth (Donders et al., 2000b).

1.2.3.1 Metronidazole as a model drug

Metronidazole is a nitroimidazole derivate which has a bactericide effect against anaerobic bacteria and protozoic effect (e.g *trichemonas vaginalis*). The drug is activated inside the microorganism by reduction of the nitro group and binds covalently to DNA. This results in DNA damage in the form of loss of helical structure, impaired template function and strand breakage with subsequent death of the microbe. The activated drug can also interact with essential cellular components (Gardner and Hill, 2001). Metronidazole is the drug of choice in the treatment of bacterial vaginosis and oral administration is the most frequent (Voorspoels et al., 2002). The most common and unpleasant side effects related to oral administrations are gastric intolerance e.g. nausea accompanied by headache, anorexia and vomiting. Furthermore, bitter and metallic taste occurs (Voorspoels et al., 2002, Brandt et al., 2008). These troublesome side effects can result in failure of completion of therapy and higher risk of recurrence or even worse lead to the development of antibiotic resistance (Brandt et al., 2008). Intravaginally applied metronidazole is as effective as orally administrated, but displays fewer side effects (Cunningham et al., 1994). The systemic availability of vaginal applied metronidazole was 47 % lower than 500 mg tablet administered orally, which resulted in fewer side effects (Cunningham et al., 1994).

1.3 Vaginal formulations

Traditionally, vagina has been used as a site for local treatment with anti infectiva and systemic delivery of hormones for hormonal replacement therapy and contraception. Gels and creams are frequently used. Creams are usually emulsions while gels are a polymer-solvent system containing a three-dimensional network of stable bonds. They are used for delivery of hormonal replacement therapy and treatment of vaginosis. A disadvantage with these systems is the low residence time due to the self-cleansing action of the vaginal tract (Valenta, 2005). Other drawbacks are discomfort, messy to apply and in some cases embarrassing when they leak into the undergarments (Hussain and Ahsan, 2005). Several attempts have been done to eliminate these drawbacks by using mucoadhesive polymers in gel formulations. It was shown that a polycarbophil gel remained on vaginal tissue for 3-4 days (Baloglu et al., 2009). Delivery of insulin via vagina from chitosan gel formulation has been developed (Degim et

al., 2005). Delivery of a cholera vaccine via gels exhibited better response in vagina than oral administration (Hussain and Ahsan, 2005). A micro-emulsion has been developed for delivery of a derivate of zidovudine which has anti HIV effect (D'Cruz et al., 1999). Gels and creams may not provide an exact dosing which can affect the efficacy of the drug therapy.

In addition, vaginal tablets and vagitories are other delivery system which is widely used. Their action is to provide a sustained release as they gradually dissolve or melt in the vaginal cavity (Hussain and Ahsan, 2005, Brannon-Peppas, 1993). Vagitories are used to administer drugs for cervical ripening prior to child birth, miconazole for vaginal candida and hormonal replacement therapy (Hussain and Ahsan, 2005). Vagitories are slight modifications of suppositories for rectal delivery. Those that contain cocoa butter are readily expelled from the vagina because of their quick melt and provide a low bioavailability because of the short residence time (Brannon-Peppas, 1993). Intravaginal tablets contain disintegrants and binders. If there are present hydrophobic and release-retarding materials, it may decrease drug absorption, but by adding surfactant the absorption can be enhanced (Hussain and Ahsan, 2005). Several intravaginal tablets for treatment of infections and hormonal replacement therapy exist. Incorporating a mucoadhesive polymer in the tablet can increase the residence time. Voorspoels developed a bioadhesive metronidazole tablet consisting of polyacrylic acid and showed a suitable alternative to oral administration of metronidazole (Voorspoels et al., 2002). Starch-based pellets is another promising intravaginal delivery system, which after disintegration showed complete coverage of the vaginal mucosa in women (Poelvoorde et al., 2009).

New systems like vaginal rings have been introduced to the market for delivery of contraceptives. This device is based upon silicone or polymers which allows non-daily, low and continuous dosing with a controlled release (Baloglu et al., 2009, Hussain and Ahsan, 2005). Resulting in enhance patient compliance and reduced fluctuations in the level of estrogen (Baloglu et al., 2009).

1.4 Multiparticulate delivery system

Multiparticulate delivery system (MUDS) comprises of many small unites dispensed in a hard gelatin capsule or compressed to a tablet (Bechgaard and Nielsen, 1978). These small items includes mini – tablets, granules or pellets (Munday, 1994). Dividing the dose into several small units provides higher flexibility than single unit dosage forms, such as conventional tablets (Asghar and Chandran, 2006, Bechgaard and Nielsen, 1978). Upon disintegration of the hard gelatine capsule, either in the GI tract or vaginal cavity, the small units would be distributed over an increased surface area due to their smaller sizes (Poelvoorde et al., 2009, Asghar and Chandran, 2006). Thus, avoiding dose dumping that may cause irritation of the mucous membrane (De et al., 2000). MUDS allow better control of the release profile and can easily facilitate combination of different release profiles within one system (Bechgaard and Nielsen, 1978).

1.4.1 Mini-tablets

Mini-tablets, see figure 1, has during the last years developed into an attractive method to prepare MUDS. Mini-tablets are defined as tablets with a diameter less than 2-3 mm (Lennartz and Mielck, 1998) and are made by tabletting machines using multiple tooling (De et al., 2000, Munday, 1994).

Figure 1: Illustration of mini-tablets (2 mm diameter)

The advantages of mini-tablets are their uniform size, smooth surface, low porosity and high attainable strength (Lennartz and Mielck, 1998, Munday, 1994). The high attainable strength could be explained by the increased ratio between the surface and volume compared to conventional tablets, which leads to wider distribution of relative density over the tablet. This may lead to more binding sites, which enhanced the strength and prevents capping (Lennartz

and Mielck, 1998). Compared to pellets, they are more robust and need lees coating material (Lopes et al., 2006). Several have produced matrix system of mini-tablets (De et al., 2000).

The manufacture of mini-tablets causes problems which normally would not occur in the production of normal tablets. First of all, higher demand on particle size of the powder is required due to smaller matrices and would affect the filling (Lennartz and Mielck, 1998, Flemming and Mielck, 1995). Due to narrow diameter of the die, requires a strict upper limit for the maximum particle size for avoiding blocking of the die. If the particle size is too small, cohesion occurs and causes poor flow. Good flowability is important in the filling for reducing mass variations in the tablets. Another factor is to make sure of proper volumetric filling for reducing variations between the different punch-positions (Flemming and Mielck, 1995). Obtaining volumetric filling when producing mini-tablets can cause problems under up-scaling of production. This can result in variations both in mass content and content of uniformity. Higher requirement is demanded of the equipment, due to higher friction on the matrice wall. High abrasion is reported on punches and matrice if they are not perfectly fitted (Lennartz and Mielck, 1998).

One of the most used methods for controlling drug delivery is to introduce a polymeric matrix in the tablets or as a coating around the tablet (Lopes et al., 2006). Hydrophilic polymers are widely used due to their flexibility to produce desirable drug release profiles, cost – effectiveness and broad regulatory acceptance. Another benefit is their ability to adhere on the mucosa surface (Smart, 2005). Several formulations of mini-tablets have been investigated for use an oral sustained release form and local delivery in the colon (Riis et al., 2007, De et al., 2000). For vaginal delivery there are no mini – tablets used as drug delivery, only normal tablets and pellets are reported (Karasulu et al., 2002, Poelvoorde et al., 2009).

1.5 Mucoadhesion

Mucoadhesion is a more specific example of the general phenomenon of adhesion. This occurs when a material attaches to a biological surface, e.g. mucosa, which is held together by interfacial forces for an extended period of time (Shaikh et al., 2011). Increased interest in this field is due to the retaining effect on mucosal surfaces, such as the buccal mucosa or the vaginal mucosa (Voorspoels et al., 2002, Sandri et al., 2004). Even though there is no mucin secretion in the vaginal mucosa; this is still regarded as a site for mucoadhesion (Valenta, 2005). Hydrophilic polymers are investigated due to their ability to interact with the vagina mucosa, which could increase the retention time of the dosage form and reducing the dose and frequency (Alexander et al., 2004).

1.5.1 Mechanism in mucoadhesion

The mechanism in mucoadhesion is a complex process which is described in different theories. The four main are: the electron, wetting, adsorption and diffusion theory (Shaikh et al., 2011, Smart, 2005, Andrews et al., 2009, Dodou et al., 2005).

In the electron theory it is assumed that an electron transfer occurs between mucus and the mucoadhesive substrate. This creates a double layer of electrical charges in the interface of them and causes electrical attraction inside the double layer and maintaining contact between them (Dodou et al., 2005).

The wetting theory is probably the oldest established theory of adhesion. For the adhesive to swell and spread onto the surface, e.g. mucosa, it must overcome any surface tension effect at the interface. This spreading is essential for adhesion to occur. Afterwards, the mucoadhesive could penetrate into surface layer which ultimately hardens and creates adhesive anchors (Shaikh et al., 2011, Dodou et al., 2005).

According to the adsorption theory, after an initial contact the attraction between the material and the mucosal is achieved by secondary forces like van der Waals, hydrogen bonding and hydrophobic bonding (Shaikh et al., 2011, Dodou et al., 2005).

The diffusion theory describes that polymeric chains from the mucoadhesive penetrates into the mucus layer to a sufficient depth where semi permanent bonding occurs (Dodou et al., 2005, Shaikh et al., 2011, Smart, 2005). The ability for a sufficient depth of penetration is dependent on the contact time, molecular weight and cross-linking. Possibility for a sufficient penetration is reduced upon increased cross-linking density (Shaikh et al., 2011).

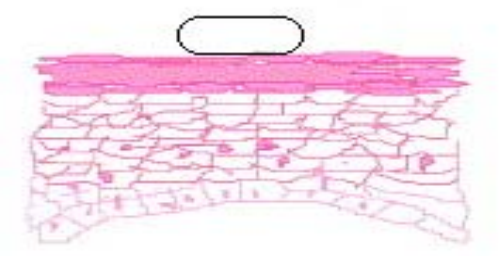


Figure 2: Modified illustration of a tablet attach to the vaginal mucosa where the dosage forms is attached to the surface with thin/discontinous mucus layer. (Smart, 2005)

None of these theories alone is enough to describe how mucoadhesion between mucus and a substrate arises (Figure 2), but a combination of these theories is more likely to occur. First, the polymer is wetted and then swells (the wetting theory). Then, interaction between polymer and mucus arises, which leads to adhesion (Dodou et al., 2005, Smart, 2005).

1.5.2 Material properties of mucoadhesive polymers

1.5.2.1 Molecular weight and branching

Molecular weight of the polymer is important for mucoadhesion (Asane et al., 2008, Hagesaether et al., 2008). The optimum molecular weight for maximum adhesion depends on the type of polymer. It is believed that higher molecular weight is favouring entanglements, while low molecular weight causes higher degree of interpenetration

1.5.2.2 Spatial conformation

According to the adsorption and the electronic theory, the presence of groups which can create covalent (disulfide) and non covalent (ionic, hydrogen and van der Waals) bonds contribute to high mucoadhesion (Smart, 2005). The presence of hydrogen-bonding groups like hydroxyl, carboxyl and amin groups in the polymer is crucial for good mucoadhesive properties (Dodou et al., 2005, Sogias et al., 2008).

1.5.2.3 Flexibility of polymer chains

Chain flexibility of the polymer is crucial for interpenetration and formation of entanglements (Smart, 2005). Cross-linking of hydrophilic polymers reduces the mobility of the chain and the effective length of the chain that can penetrate into the mucus layer decreases. This leads reduced mucoadhesive strength (Shaikh et al., 2011).

1.5.2.4 Swelling and cross-linking

The ability of a polymer to swell is a prerequisite for mucoadhesion to occur . Swelling of a polymer includes wetting, uncoiling and spreading, in which bonds with mucus or cell membranes can arise. The degree of swelling is affected by the properties of the medium, such as pH and ionic strength. Increased ionization leads to repulsive forces between chains and the polymer start to swell. Over hydration results in the formation of a wet and slippery mucilage which will not retain long on the mucosa (Smart, 2005, Asane et al., 2008). Over hydration could be avoided using cross – linking of two polymers by ionic interactions . The higher degree of cross – linking, causes lower flexibility and hydration rate. Another consequence is reduced surface area and mucoadhesion (Shaikh et al., 2011).

1.6 Chitosan

1.6.1 General

Chitosan is a biopolymer derived from chitin by alkaline hydrolysis or deacetylating processes (Illum, 1998). Chitin is one of the most abundant polysaccharides in nature, second to cellulose, and has its origin from crab and shrimp cell wastes (Felt et al., 1998, LeHoux and Dupuis, 2007). Chitosan is considered as a non-toxic, biocompatible and biodegradable polymer, with pharmacological effects in hyper-cholesterolemic and wound-healing, in addition to the bacteriostatic and antiulcer activity (Felt et al., 1998, Sogias et al., 2008). The polymer is used in cosmetics, but has during the last years attracted interest also in the pharmaceutical field (Rossi et al., 2010).

1.6.2 Structure and properties

Chitosan (poly-β-(1,4)-2-amino-2-deoxy-D-glucose) (Figure 3) is a cationic, linear copolymer consisting of glucosamine and N – acetyl glucosamin monomers connected by β - 1.4 – linkages, see figure 1 (Kast et al., 2002). The residues are found randomly distributed and in different quantity throughout the polymer chain (George and Abraham, 2006a). Because of the heterogeneity in the molecule, the classification of chitosan is usually based on molecular weight and deacetylating degree (DD), see table 1 (Hiorth et al., 2006, George and Abraham, 2006a).

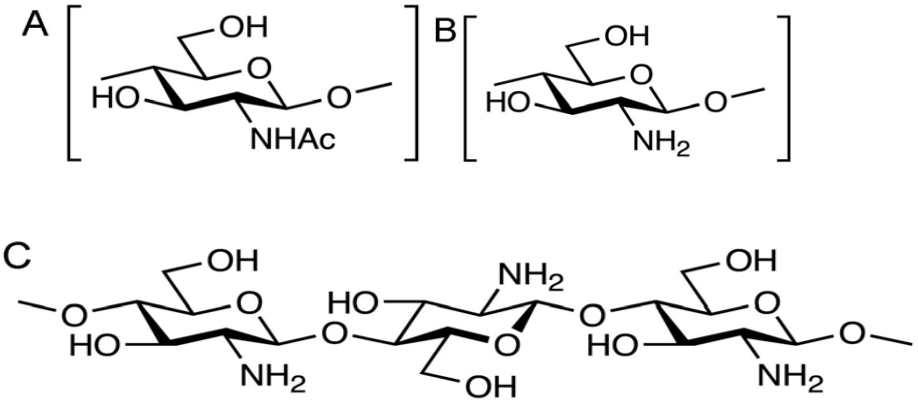


Figure 3: A) chitin monomer B) Chitosan monomer (Poly-β-(1,4)-2-Amino-2-deoxy-D-glucose) C) 100 % deactylated chitosan (Kurita, 2006)

Molecular weight and deacetylating degree are the main parameters which has an influence on the physico – chemical properties of the polymer. Chitosan is a weak base in which the amino groups in D-glukosamin units have a pKa between 6.2 and 7.0. The pKa is influenced by the deacetylation degree. Low DD (<40 %) is soluble in pH unto 9.0, while high DD (>85 %) is insoluble in solution above pH 6.5. The addition of salt could interfere with the solubility, where higher ionic strength causes decreased solubility and a salting – out effects may occur (LeHoux and Dupuis, 2007).

1.6.3 Chitosan in pharmaceutical applications

Chitosan has been widely investigated for pharmaceutical application and has been processed in gels, tablets, beads and films for controlled delivery system, mucoadhesive and rapid release dosage forms (Kristl et al., 1993, Sabnis et al., 1997, Knapczyk, 1993). Chitosan as an excipient in tablets is sporadically investigated (Picker-Freyer and Brink, 2006). Authors report of disintegrant properties,for concentration of chitosan exceeding 50 % (Knapczyk, 1993, Ritthidej et al., 1994). However, chitosan shows high elastic recovery after compression

but produce tablets with acceptable mechanical strength. The mechanical properties were found to be dependent of DD which also affected the flowability (Rege et al., 1999, Picker-Freyer and Brink, 2006). In general, the chitosan showed poor flow(Picker-Freyer and Brink, 2006). It is also suggested that chitosan may be suitable for use as diluents in direct compression processes (Sawayanagi et al., 1982).

1.6.4. Chitosan in mucoadhesion

Chitosan is considered to be mucoadhesive and is one of the most investigated polymers for this purpose (Bonferoni et al., 2009, Hagesaether et al., 2009). The mucoadhesive properties of chitosan are dependent on pH and the degree of ionization. In the acidic environment of the vagina, the amino groups of chitosan are ionized and ionic interactions with sialic acid groups in mucin occur (George and Abraham, 2006b). In more neutral and basic milieu, like in post menopausal women, chitosan is insoluble and interactions with mucosa will depend on hydrogen bond formation only. Increased molecular weight is shown to enhance the mucoadhesive ability (Bonferoni et al., 2009).

1.7. Pectin

1.7.1 General

Pectin is a complex polysaccharide originating from the cell wall of most of plants (Sungthongjeen et al., 2004). The number of sources that may be used for commercial use is very limited, because their ability to form a gel is dependent on the degree of methoxylation (DM) and molecular weight (MW).Commercially acceptable pectin is mainly obtained from apple pomace and citrus peels (Sriamornsak, 2011). Pectin has been widely used in the food industry as a thickening agent and a gelling agent (Thirawong et al., 2007). It is non – toxic and is considered as a safe additive.

1.7.2 Structure and properties

Pectin consists mainly of D – galacturonic acid and its methyl-ester, which are connected by α-1→4 linkages (Figure 4). The linear structure is sporadically interrupted by α -1 → 2 linked L– rhaminose residues, which cause a bend in the backbone due to (1-2) –linkage in the molecule (Sande, 2005). Sugars like arabinose, galactose and xylose pccur in the side chains

(Sriamornsak, 2011). Pectin can be classified into two main categories on the basis of different gelling properties: high-methoxy (HM) pectin (DM>50%) and low-methoxy pectin (DM<50%). Pectins with DM below 10 % is often referred to as pectinic acid (Salbu et al., 2010b) The demethoxylation process is causing reduction in molecular weight (Sande, 2005).

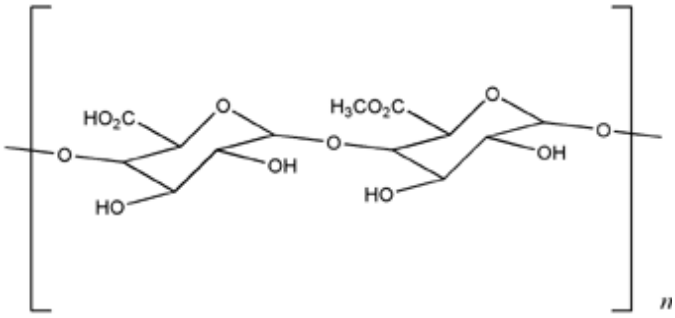


Figure 4: Illustration of pectin showing demethoxylated D-galacturonic acids

The carboxyl groups of the galacturonic acids in pectin are negative charged at neutral pH and exhibits a pKa approximately 3.5 and is affected by the degree of methoxylation (Vandamme et al., 2002). Pectin is considered as water soluble but the metoxylation degree of pectin would have an impact on the solubility, where higher DM causes enhanced solubility. LM and HM pectin creates gels in different manner. The gel formation of LM is caused by cross – linking with calcium ions in solution which occur instantly. Specific amount of calcium ions is required for the creation of gel. Calcium ions interact with the negative charged carboxyl groups. HM pectins formes gel upon hydrogen bonding and hydrophobic interactions within the molecule or with neighbouring chains (Sriamornsak, 2011).

1.7.3. Pectin in pharmaceutical application

Pectins have been used for controlling the drug release due to the ability for a prolonged release based on their ability to swell and form a gel (Salbu et al., 2010b). Other formulations where pectins have been used is coatings, spray-drying, microencapsulation and beads(Sande, 2005). Mura et al have reported of high degree of fragmentation and very small plastic deformation. Kim et al found that pectin is a hard, rigid and poorly compactible material and therefore other excipients was added for improvement of the compactability (KIM ET AL). While Salbu et al showed that pectin form satisfying tablets by direct compression of pectin of low DM (Salbu et al., 2010b).They found that pectins powder with DM < 40 % are potential candidates for direct compression (Salbu et al., 2010a).

1.7.4. Pectin in mucoadhesion

Pectin has previously been listed as a polymer with poor mucoadhesive properties, but studies have shown that pectin exhibit mucoadhesive properties (Hagesaether and Sande, 2007). The mucoadhesive properties are dependent on substitution degree and molecular weight (Sriamornsak, 2011). It is shown that decreased amount of substitution on pectin gives increased interaction between pectin and mucin. Reports have shown that the carboxyl groups are an important contributor to pectins mucoadhesive properties (Hagesaether and Sande, 2007).

1.7.5 Cross-linking of pectin and chitosan

Combinations of pectin and chitosan are often used, in order to overcome the fast release when used alone. In the pH range 3-7, the amino groups in chitosan and the carboxyl groups in pectin becomes ionized and electrostatic interactions occur and a polyelectrolyte complex (PEO) is formed (Hiorth et al., 2006).

2. Aims of the study

The main objective in this study was the development of bioadhesive mini-tablets for improved vaginal therapy of metronidazole.

More specific aims were:

- Formulate a simple composition containing chitosan and/or pectin as matrix with desired mechanical strength
- Investigate and achieve prolonged release characteristics *in vitro* at pH 4.5 and 6.8 simulating pre-and postmenopausal women
- Investigate bioadhesive properties of mini-tablets *ex vivo* on animal tissue

3. Materials and Method

3.1 Materials

Acetic Acid (Merck KGaA, Darmstadt, Germany), lot no K40377663933

Bovine serum albumine (Merck Eurolab, Darmstadt, Germany), lot no K2821518109 6225725

Brilliant blue (Sigma – Aldrich, Steinheim, Germany), lot no MKBD3009 EC 223-339-:WGK3;

Calcium Oxide (Norsk Medisinaldepot, Oslo, Norway) lot no 7F12411

Chitosan(Sigma – Aldrich Chemistry, Milwaukee, USA)

- low molecular, lot no 61496 MJ
- medium molecular, lot no MKBC00060
- high molecular, lot no MKBB0585

Glukose (Norsk Medisinaldepot, Oslo, Norway), lot no 1A102/4

Glycerol (Merck, Darmstadt, Germany) lot no K29746193142

Klucel hydroxypropylcellulose EF (Hercules, Wilmingston, USA), lot no 1882

Lactic acid (Norsk Medisinaldepot, Oslo, Norway), lot no 8EO1812

Metronidazol (Fluka analytical, Steinheim, Switzerland), lot no 1319323 34008265

GA12172, 1319323 34008265

Pectin, (Herbstreith and Fox KG, Neuenbürg/Würth, Germany)

- 10 % DM, lot no 00807DM10
- 25 % DM, lot no 310707DM25
- 40 % DM, lot no 310707DM40

Pottasium hydroxide (Norsk Medisinaldepot, Oslo, Norge), lot no 6D03712

Sodium chloride (Fluka analytical, St Louis, USA), lot no SZB82660

Ureum (Norsk Medisinaldepot, Oslo, Norway), lot no 06B078/2

Table 1: Deacetylation degree of the different chitosans

Chitosan type	**Degree if deacetylation (DD) (%)***	**viscosity (cps)***
Low molecular weight	92	48
medium molecular weright	82	522
high molecular weight	77	1220

*according to certificate of analysis obtained from supplier

Table 2: Degree of methoxylation on the different pectins

Pectin type	Degree of methoxylation (DM) (%)*	Relative information of molecular weight *
Pectin 10%DM	8,0	Low molecular weight
Pectin 25 %DM	26,1	Medium molecular weight **
Pectin 40 % DM	41,7	Medium-high molecular weight**

*according to supplier, ** according to supplier is DM 25% produced by demethoxylation of DM 40 %

3.2 Methods

3.2.1 Powder mixtures

Totally, sixteen different powder mixtures were prepared of metronidazole in combination with one or more of the biopolymers pectin (10, 25 and 40 % DM), chitosan (DD) and hydroxypropylcellulose (HPC-EF), see table 1. 10 % (w/w) metronidazole was used in all mixture. The components were grinded in a mortar, passed through 0.3 mm sieve and blended by volumetric principal. The powder mixtures were stored in a container in dry and dark place. For further information on the experiment on the experimental design, see section

3.2.2 Compaction of tablets

3.2.2.1 Compaction Simulator

Powder was pressed using a costume made compaction simulation consisting of a Schmidt ServoPress 450 (Schmidt Technology GmbH, St. Georgen, Germany) (Figure 5a) equipped with a powder compression device (IBR Reichenbach, Waldkirch, Germany) (Figure 5b). Positioning of press simulator was accomplished by Schmidt Presscontrol 4000 control panel (Schmidt Technology Gmbh, St. Georgen, Germany).

A) B)

Figure 5: A) Schmidt ServoPress 450 A) Compression modul from IBR. Red arrows indicate the position of sensors which measures the distance

3.2.2.2 Data collection

The measure of distance was done by LS 487 sensor (Dr. Johannes Heidenhan, GmbH, Traumreut, Germany). These sensors are placed on the right and left hand side of the dies; see figure 1 b). The measure of force was performed by Kistler piezoelektrical force sensor type 9363 (Kistler Instrumente AG, Winterhur, Switzerland). These sensors measure the force from upper and lower punch and the signals was collected by Kistler charge meter type 5015A1000.

3.2.2.3 Tablet tooling

Multiple mini-tablet tooling was used for the production of mini-tablets (Ritter Pharma-Technik, GmbH, Stapelfeld, Germany). The multiple tooling consists of a punch holder with 15 concave punches and corresponding dies. These are organized in two lines, were each punch has a diameter of 2 mm (Figure 5a). The upper punch was lubricated with 0.5 % magnesium stearate suspension in acetone.

11 mm tablets were compacted using flat faced punches (Ritter Pharma-Technik GmbH, Stapelfeld, Germany), see figure 6B. The upper punch was lubricated with 5 % magnesium stearate suspension in acetone.

A)

B)

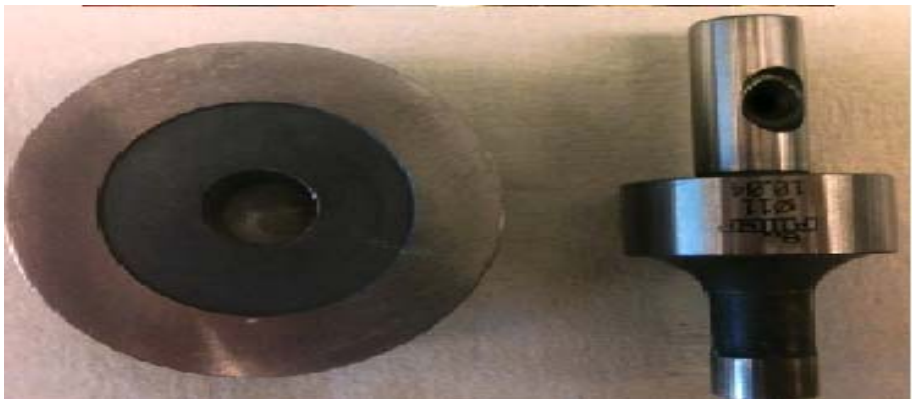


Figure 6: Tablet tooling A) 2 mm, concave multiple mini-tablet tooling. B) 11 mm flat-faced punch.

3.2.2.4 Tabletting conditions

All tablets were compressed using a low velocity of 10 mm/s by the upper punch and a compaction pressure around 241 MPa. The end position of the upper punch decided the force. The pressure was then calculated by formula 1, by using the average force of the upper and lower punch.

Equation 1: $P = F / \pi r^2 n$

P = pressure

F = force

r = radius

n = amount concave punches

3.2.2.5 Temperature and humidity conditions

Relative humidity and temperature in the room during tablet preparation was recorded by an air humidity-and temperature meter (ebro Electronic Gmbh, Ingolstadt, Germany).

3.2.2.6 Manuel Filling of the matrices

The powder was weighed in on a wax paper using an analytical balance (Mettler AE 163, Küsnacht, Switzerland) and the matrices were filled by hand. The mini – tablet dies were filled by volumetric filling. A standard procedure was used to diminish mass variations in the batches. The powder was placed in the back, front and both sides as illustrated in figure 7. The powder was distributed 2 series of forward-back and left-right and the excess powder was removed from the die. After each compression, all tablets were weighed and then dispensed in a container. Average tablet mass and standard deviation was calculated. A relative standard deviation of 2 % was accepted and tablets with deviation above 5 % were eliminated

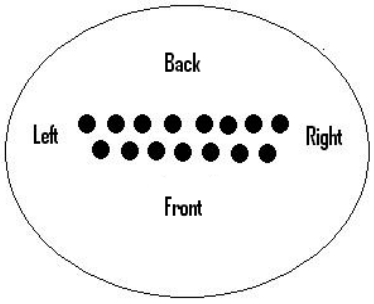


Figure 7: The filling of mini-tablet dies was done by placing the powder back, front, left and right.

3.3 Characterizing of mini-tablets

3.3.1 Friability

The friability of the mini-tablets was determined using a friability tester from Erweka (TAR 20 GmbH, Heusenstamm, Germany). 20 mini-tablets were weighed and placed in the drum. The rotation of the drum was set to 25 rpm for 4 minutes. The mini-tablets was weighed again afterwards and the deviation (%) was calculated. A maximum loss of mass higher than 1 % was considered as not acceptable. In cases of maximum loss higher than 1 %, the test was carried out 3 times and the average was determined according to the European Pharmacopeia.

3.3.2 Crushing strength

The axial crushing strength of the mini-tablets was determined using a texture analyser TA.XT.plus (Stable Micro System, Godalming, UK) in a "return to start" mode, see figure 8. The mini-tablet was placed on radial side and tested in a compression test using a probe of 4 mm in diameter. The velocity of the probe was set to 2 mm/sec until contact with the mini-tablet (trigger force 5 g), and a test speed of 0.3 mm/sec was used. 30 mini-tablets were tested for each composition. A force-distance diagram was recorded and the maximal force was determined for each of the mini-tablets separately. Average and standard deviation was calculated.

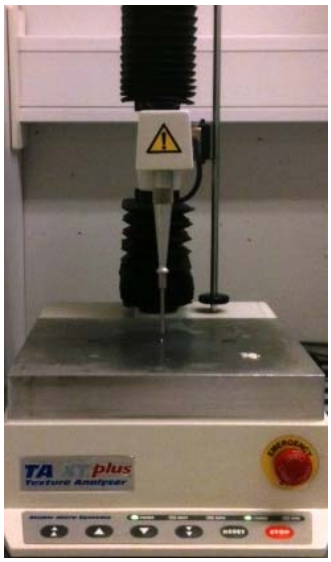


Figure 8: Illustration of crushing strength measurement of mini-tablets by using Texture Analyser**.**

3.3.3 Mini-tablet Height

The height of the mini-tablets was measured by texture analyser TA.XT. plus (Stable Micro Systems Ltd, Godalming, UK) (Figure 9A). The mini-tablets was placed on axial side and tested using a probe of 4 mm. A `return to start mode was used` and in `test configurations`, the term "parameters" was selected. Choose `Use value on setting` in strain height. The velocity of the probe was set at 0.5 mm/sec until contact with the mini-tablet and a test speed of 0.10 mm/sec was used. The trigger force was set at 10 g (in a compression test set-up).

A) **B)**

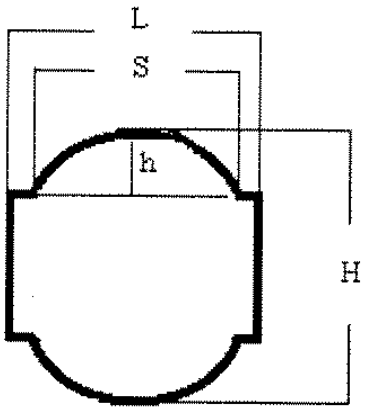
Figure 9: Illustration of A) measurement of height of a mini-tablet. B) Calibration for distance with calibration block**.**

The instrument was calibrated for distance measurements with calibration blocks after DIN 861 (W & Z Computer – Vertrieb GmbH, Dresden, Germany), see Figure 9B. The size of the blocks was 1.000, 1.300, 1.400, 1.500, 1.600, 1.700, 1.800, 1.900, 2.000, 2.100 and 3.000. Three parallels were measured per block and a calibration curve was established (see section 8.4). The height of the measured mini-tablets was corrected using the calibrating curve. The height of mini – tablets was used for calculating the volume and apparent height of mini-tablets as described by S.N.Solum (Solum, 2007), see section 3.3.4 and 3.3.5.

3.3.4 Volume of mini-tablets

Volume of convex mini-tablets was calculated according to equation 2 and figure 10:

Equation 2: $$V = \pi L^2 (H - 2h) + 2 (1/6\ h\ \pi\ (h^2 + 3\ S^2)$$



V = volume

L = diameter cylinder

H = total height tablet (mean of n=)

h = height segment

S = radius segment

Figure 10: Illustration of parameters used to calculate the volume of a convex mini - tablet.

Radius and height of segment was measured by electronic microscopy earlier (Solum, 2007).

Radius and height of segment was kept constant during calculations.

3.3.5 Apparent height of mini – tablets

The volume of a convex mini-tablet was used to calculate the apparent height of a corresponding flat mini-tablet, see equation 2. The apparent height is calculated from the volume of a flat tablet where the apparent height is equal to the flat tablet of the corresponding volume and diameter.

Equation 3: $h = \pi r^2 / v$

h = apparent height of mini-tablets
r = radius
v = volume

3.3.6 Tensile strength

Tensile strength of the convex 2 mm tablets was calculated from equation 4 using the apparent height from equation 3:

Equation 4: $\sigma = 2F / \pi dh$

σ = tensile strength
F = crushing strength
d = diameter
h = apparent height of mini-tablets

3.4 *In vitro* drug release

3.4.1 Cumulative release from mini-tablets

Dissolution rate of the different mini-tablet formulations was investigated by the rotating paddle method using a dissolution apparatus (Sotax, Basel, Switzerland) (Figure 11).

Figure 11: Dissolution apparatus**.**

Samples of approximately 80 mg (13 mini-tablets) where tested in 1000 ml of test medium (sink conditions) at 100 rpm and 37 ˚C (± 0.5) (USP). Ammonium acetate buffer pH 4.5 and vaginal fluid simulant (VFS) were used as test media (for composition, see section 3.4.2). Samples of 5 ml were withdrawn at predetermined time intervals (5, 10, 15, 20, 30, 45, 60, 120 and 240 minute). The withdrawn amount was not replaced by fresh test medium, but corrections were made for changes in volume during calculations. Samples were filtered using 0, 2 µm syringe filter (Acrodisc, Ann Arbor, USA) and quantified photometrically (Aglient Technologies GmbH, Waldbronn, Germany) at λmax = 320 nm against standard solutions (section 3.4.2.3). Cumulative release (%) was calculated for each time point expressed as the fraction of released drug from the mini – tablets. 3 parallels were carried out in each of the test media.

3.4.2 Test media

3.4.2.1 Ammonium acetate buffer pH 4.5

77.1 g/l of ammonium acetate was weighed and dissolved in demineralised water. Seventy ml of acetic acid was added and the volume adjusted to 1000 ml with demineralised water. (Ph.Eur). pH was checked and adjusted if necessary using 1 M HCl.

3.4.2.2 Vaginal fluid simulant (VFS) pH 4.5 and 6.8

VFS pH 4.5 was prepared from 3.51 NaCl, 1.40 g/l KOH, 0.222 g/l $Ca(OH)_2$, 0.018 g/l bovine serum albumin, 2 g/l lactic acid, 1 g/l acetic acid, 0.16 g/l glycerol, 0.4 g/l urea, 5 g/l glucose according to literature (Owen and Katz, 1999). The solution was sonicated until the components were dissolved. pH of the mixture was adjusted to 4.5 using 2 M HCl and 2.5 % acetic acid. This pH was chosen to mimic the pH of normal for healthy vaginal conditions of women in fertile years. For simulating the conditions in post menopausal women (pH 6-7.5), VFS pH 6.8 was prepared from the same method as pH 4.5, with the exception of acetic acid. Acetic acid (glacial and 2.5 %) was only used to obtain the required pH. pH was measured also right before dissolution testing just to assure correct pH-value.

3.4.2.3 Calibration curve

1 mg/ml stock solution was made of 250 mg metronidazole in 250 ml test medium. Standard solutions of 2, 5, 7 and 10 µg/ml was prepared. Separate calibration curves were required for the different test media (see section 8.4).

3.5 Mucoadhesion

The cow vaginal mucosa was chosen as a biological matrix in for the mucoadhesion studies as it is widely used as a model membrane and, is well recognised as suitable for the simulation of human vaginal mucosa properties (das Neves et al., 2008a). As there is limited amount of tissue available from one animal we kept also the rectal tissue from the same animal. The rectal tissue was used in supporting studies (section 3.5.3.), and for the measurement of mucoadhesive strength (3.5.2.).

3.5.1 Preparation of tissue

Samples from newly sacrificed heifer were obtained from the slaughterhouse (Nortura, Bardufoss, Norway). The vaginal and rectal mucosa was carefully removed from the underlying tissue and cleaned with acetate buffer pH 4.5. The tissue was cut into smaller pieces and packed in plastic (cling film) and aluminium foil before freezing -20 ºC. Prior to mucoadhesion testing, the vaginal and rectal tissue was defrosted in VFS pH 4.5 at 37 ± 1ºC for 60 minutes using a magnetic stirrer and heated disc (das Neves et al., 2008b). The tissue was cut into desirable pieces

3.5.2 Detachment force measured using texture analyzer

Investigation of the mucoadhesive strength of tablets to the tissue was done using a texture analyzer TA.XT.plus (Stable Micro Systems, Godalming, UK). Calibration of weight was carried out before the experiments. Vaginal mucosa was placed on a mucoadhesion rig after defrosting of tissue (section 2.6.1). One 11 mm flat-faced tablet was attached to a 10 mm probe on the upper, movable arm of texture analyser using a bilateral tape (figure 5). The mucosa was wetted with 5µl VFS pH 4.5 before testing. The contact time of the tablet with the tissue was set to 30 seconds applying a low force (5.0 g). The detachment test was carried out using a test speed of 0.10 mm/s. Eight parallels were performed for each composition. All tests were performed at room temperature.

Figure 12: Picture of mucoadhesion rig and 11 mm tablet attached to the probe with a bilateral tape.

The force and Displacement of detachment were recorded. Based on the force versus displacement (distance) plot, the peak detachment force (Fmax, N), e.g. the force at the point when the tablet detaches from the tissue, and the work of adhesion (AUC, N*mm) were obtained as described in figure 12. Fmax is the maximum force required to separate the tablet from the tissue. AUC is the total amount of forces involved in the tablet withdrawal from the tissue, which is calculated from the area under force versus distance curve.

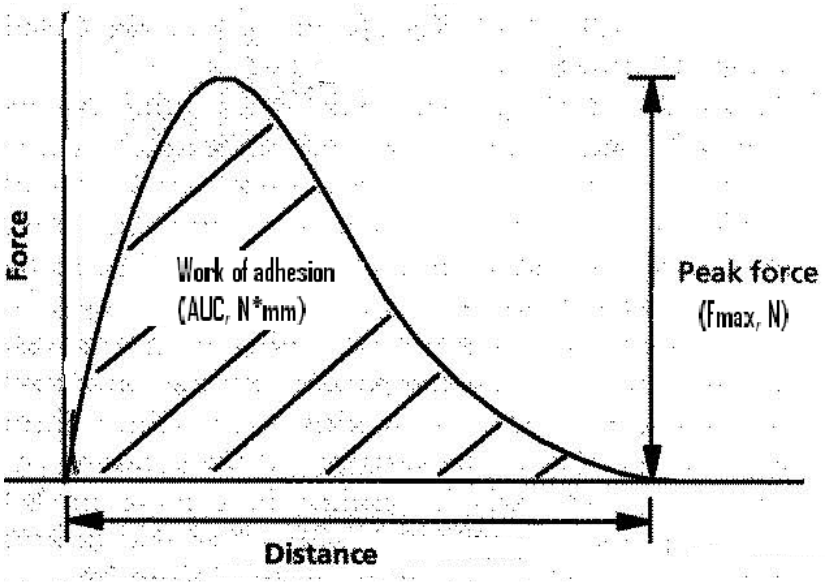


Figure 13: Modified illustration of force-distance curve defining work of adhesion and the peak force (Hagerstrom et al., 2004)

3.5.3 Rotating cylinder method

As the mini-tablets were too small and convex shaped they were not suitable for detachment force test described above. Therefore a supporting test was introduced applying the mini-tablets. For this test the rectal tissue from the same animal was used as a model biological membrane. Investigation of the degree of mucoadhesiveness was tested using a modified version of the rotating cylinder method (Hagesaether et al., 2008). The tissue was fastened onto a cylinder (the basket of a dissolution test apparatus) as shown in figure 13. The cylinder with the tissue was placed in a beaker without any test medium to mimic the *in vivo* situation in vagina. The tissue was hydrated with 300 µl of VFS pH 4.5 prior to the test. Mini – tablets of the same formulation were placed gently onto the mucosa as shown in figure 13, without application of any force. The cylinders were rotated at a rotating speed of 250 rpm for 10 minutes, and the percentage remaining on the tissue after agitation was recorded. 10 mini-tablets of each formulation was tested in 3 replicates.

Figure 14: Experimental set-up of the rotating cylinder method showing how rectal tissue is fixed to the rotatingcylinder and mini - tablets attached on the surface

3.6 Simulated wetting test

The test on simulated wetting time was conducted according to Park et al. (Park et al., 2008). 5 mm Whatman filter paper disk was placed into each of the wells of a 96-well titer plate. Solutions of 0.1 % (w/w) of Brilliant Blue 85E dissolved in VFS pH 4.5 and 6.8 were prepared and 20 µl of the blue colour solutions was added to each well. One mini-tablet was carefully placed on axial side in each of the wells on top of the colour soaked filter paper disc. The time (sec) until the mini-tablet was coloured blue was taken as the swelling time (figure 14), and measured using a stop clock. 5 mini – tablets from each of the formulations were tested at each pH (i.e. 0.1 % (w/w) Brilliant Blue in VFS pH 4.5 and 6.8).

Figure 15: Photo of test set up: 96-titeplate with filter, sensient blue solution and mini-tablets. Fully coloured mini-tablets, confirming the end point of the test, can be seen in the back row**,**

3.7 Experimental design

Firstly, chitosan of different % DD and molecular weight and pectin of different %DM and molecular weight were tested separately as pure matrix for 10 % (w/w) metronidazole mini-tablets. The dissolution rate in acetate buffer pH 4.5 and in VFS pH 4.5 was determined.

Secondly, a 2^2-factorial design with centre point was employed to screen for combinations of chitosan and pectin resulting in high mucoadhesion and a sustained release of metronidazole. The investigated factors and levels are indicated in figure 15. The mini-tablets contained equal amounts of chitosan and pectin (45+45) in addition to 10 % (w/w) metronidazole mini-tablets.

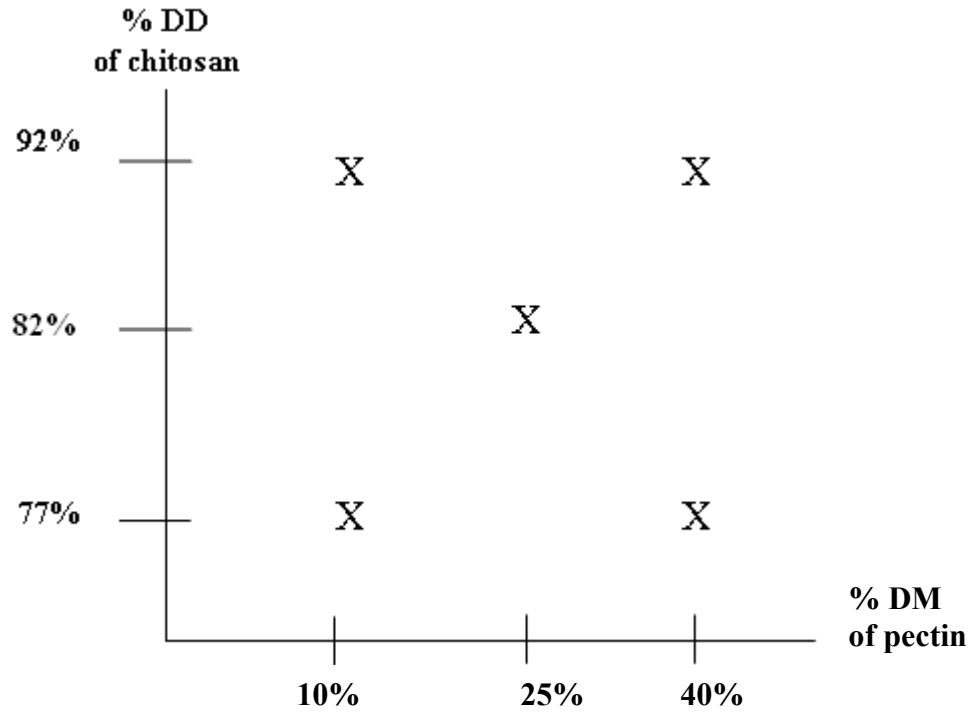


Figure 16: Combinations investigated in the 2^2-factorial design with centre point

Finally, for optimization purposes, different ratios of chitosan:pectin were added to cover a larger experimental area. For pectin DM 10% and Pectin DM40 % both in combination with chitosan DD92%, the series were completed (chitosan+pectin): 0+90, 20+70, 45+45, 70+20 and 90+0. All formulations contained 10% metronidazole to make 100%. The full overview of the different formulations is given in Table 3.

Table 3: Composition of mixtures given in percentage (%, w/w)

Mixtures	MET*	Pectin 10 % DM	Pectin 25 % DM	Pectin 40 % DM	Chitosan DD 92 %	Chitosan DD 82 %	Chitosan DD 77 %	HPC - EF
1	10	70	-	-	20	-	-	-
2	10	-	-	70	20	-	-	-
3	10	90	-	-	-	-	-	-
4	10	20	-	-	70	-	-	-
5	10	-	-	20	70	-	-	-
6	10	-	-	90	-	-	-	-
7	10	45	-	-	45	-	-	-
8	10	-	-	45	45	-	-	-
9	10	-	-	-	90	-	-	-
10	10	-	90	-	-	-	-	-
11	10	-	-	-	-	90	-	-
12	10	-	-	-	-	-	90	-
13	10	-	-	-	-	-	-	90
14	10	45	-	-	-	-	45	-
15	10	-	-	45	-	-	45	-
16	10	-	45	-	-	45	-	-

*metronidazole

4. Results and discussion

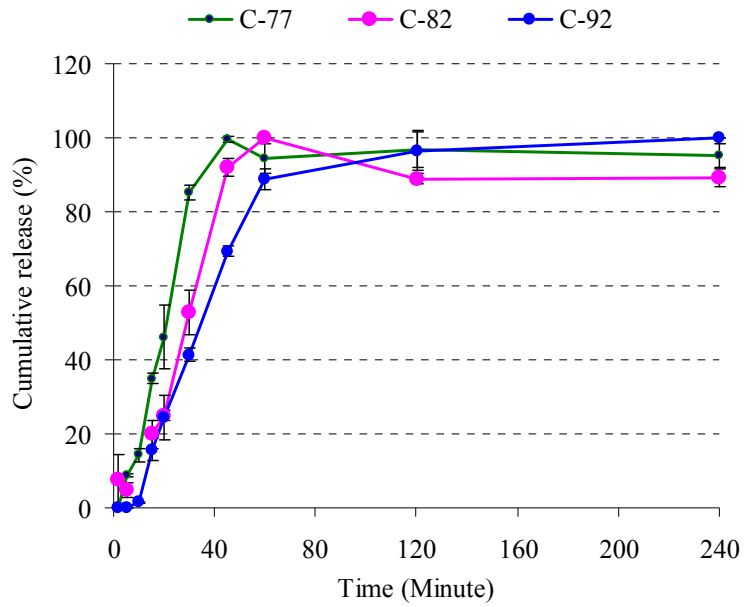
This section is divided into three main parts: The first part is a screening of pure chitosan and pure pectin matrices for sustained release of metronidazole from mini-tablets in media of pH 4.5 and 6.8 mimicking the vaginal pH of pre- and post-menopausal women. The second part consists of a 2^2-factorial design with centre point, screening for combinations of chitosan grade (%DD) and pectin grade (%DM) resulting in high mucoadhesion and sustained release of metronidazole from mini-tablet in vaginal fluid stimulant (VFS) of the same pH as mentioned above. The final part contains a further optimization of the composition by varying the chitosan to pectin ratio in order to obtain the formulation best suited for improved vaginal delivery with prolonged release properties and high mucoadhesive strength.

4.1 Screening of chitosan and pectin as pure matrix for mini-tablets

4.1.1 *In vitro* release of metronidazole from mini-tablets of chitosan

The dissolution rate of metronidazole from pure chitosan mini-tablets was tested in ammonium acetate buffer and VFS (figure 16 A and B, respectively). The effect of different pH (4.5 and 6.8) that simulates the environment in vagina in pre-and post-menopausal women was also tested. The release rate was observed from mini – tablets containing different grades of chitosan, e.g. degree of deacetylation of 77%, 82% and 92%. Figure 17 illustrates the release of metronidazole in ammonium acetate buffer (Figure 17A) and VFS (Figure 17B) in pH 4.5.

A)



B)

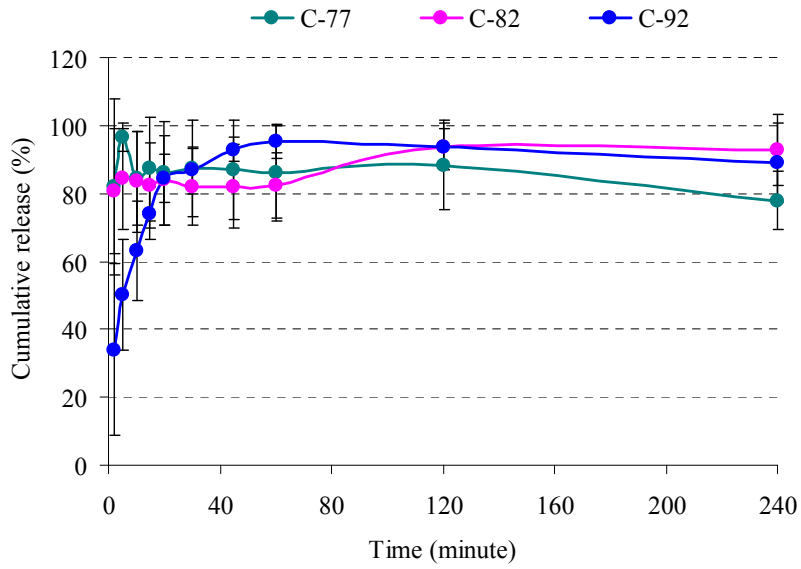


Figure 17: Drug release profile of metronidazole from mini-tablets containing 90% chitosan of various grade (77%, 82% and 92% DD) A) ammonium acetate buffer pH 4.5, B) Vaginal fluid stimulant pH 4.5; (n=3)

The release rate of metronidazole in the two test media was different, where the fastest release was observed in VFS, see figure 17B. The slowest discharge was seen from mini-tablets containing chitosan DD 92% in the ammonium acetate buffer pH 4.5 (Figure 17A), where 80 % of metronidazole is released after 60 minutes in the buffer. The same amount is released within only 20 minutes in VFS of the similar pH. Faster release of the drug is observed from

mini-tablets of C-77 and C-82 in the acetate buffer. However, the mini-tablets were found to fully dissolve in ammonium acetate and not in VFS, despite similar pH and temperature. Clearly, there is a salting-out effect of chitosan caused by the components in VFS. This may be due to high amount of chloride ions in the medium (refer section 3.4.2.2), which could possibly dehydrate chitosan due to competition of the water molecules. Chloride ions ability to precipitate chitosan has also been reported by others (LeHoux and Dupuis, 2007). As a consequence of the participation of the matrix, the metronidazole is quickly released and to full extent. It is interesting to notice that C-92 shows a slower release of metronidazole than the other chitosan grades. This could be due to the higher amount of ionized amino groups in C-92, which could result in a protective gel layer around the precipitate and results in a more prolonged release as described in literature (Kavanagh and Corrigan, 2004).

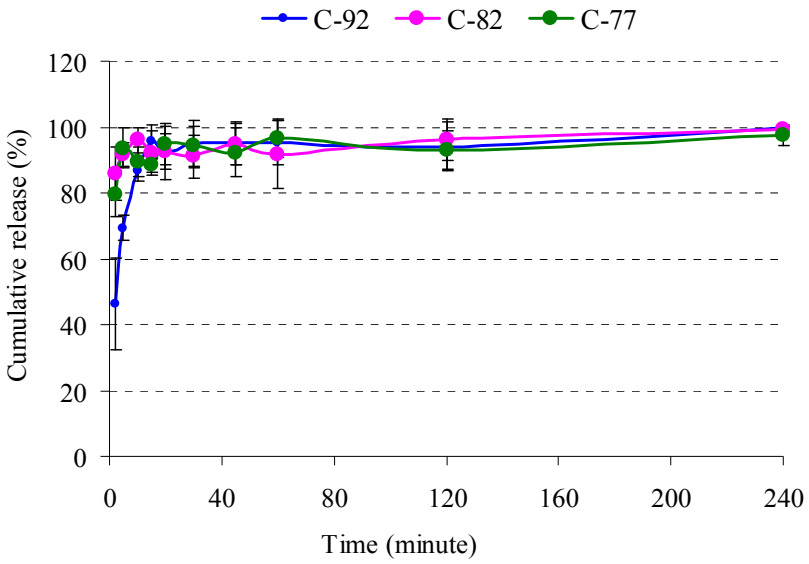


Figure 18: Drug release profile of metronidazole from mini-tablets containing 90 % chitosan of various grade (77%, 82% and 92%); (n=3) in VFS pH 6.8.

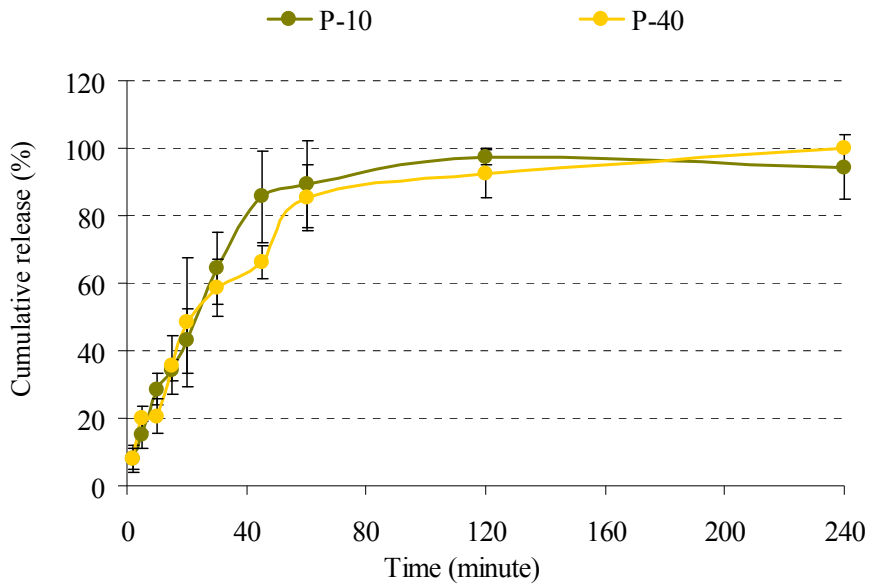
A faster release in pH 6.8 (Figure 18) is observed, which is due to the lower extent of ionization of amino groups and lower ability to swell and form a gel layer around the precipitate. Fast release of drug from chitosan tablets is reported in literature (Mi et al., 1997), and they related this to the lower capacity of chitosan forming a gel at higher pH. Normally, when the pH rises, a sustained release is obtained due to lower solubility of chitosan at neutral and alkaline pH (Sabnis et al., 1997).

Since VFS is a composition of components found in vaginal environment, the VFS was used further experiments for allowing better *in vitro/ in vivo* correlation.

4.1.2 In vitro release of metronidazole from mini-tablets of pectin

Figure 19 shows the release behaviour from mini-tablets of pectins and a different profile was obtained. In general pectins are water soluble and upon contact with water it starts to swell and releases the drug through diffusion and/or erosion of the outer gel-layer (Sungthongjeen et al., 2004). The pH of the dissolution medium affected the drug release of pectin DM 10% and 40%. The profiles in figure 19A demonstrate a similar discharge of metronidazole from mini-tablets the two pectins. While in figure 19B, a tendency of increased pH causes a slower release of metronidazole from P-40 mini-tablets. Sixty % of metronidazole is released after 40 min from the P-40. Whereas mini-tablets containing P-10 is showing a higher up take of fluid which causes a faster drug release, where approximately 95 % is released after 40 minutes. P-10 contains more free carboxyl groups and high charge density causes faster release. The components in VFS could have an impact of the release due to calcium ions in the medium (refer section 3.4.2.2). Low methoxylated pectin with higher content of anionic carboxyl groups are described in literature to interact with calcium ions (Sungthongjeen et al., 2004, Powell et al., 1982). Cross-linking of pectin cause a stronger gel network as observed at higher pH (Figure 19 B), and therefore a slower release is observed.

A)



B)

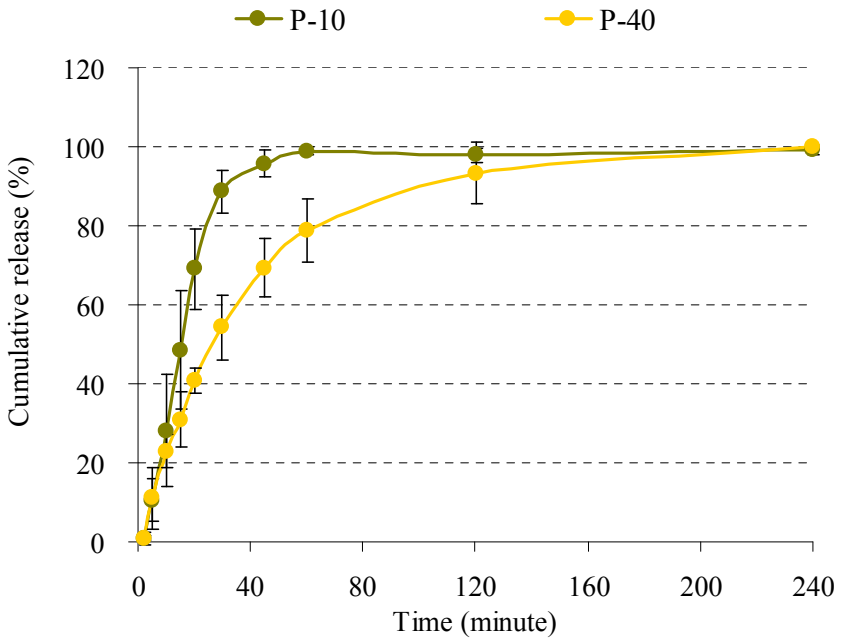


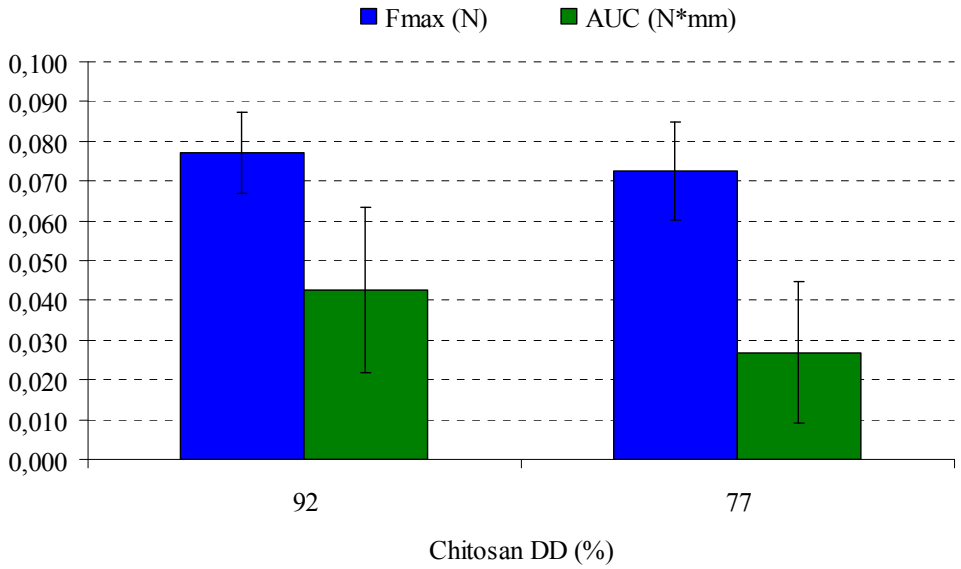
Figure 19: Drug release profile of metronidazole from mini-tablets containing 90 % pectin of various grade (DM 10% and 40 %) A) in VFS pH 4.5 B) in VFS pH 6.8.;(n=3)

4.1.2 Mucoadhesion of metronidazole containing matrix tablets of chitosan versus pectin

Figure 20A presents the results from mucoadhesion tests of 11 mm tablets of the different chitosan grated (C-92, and C-77) to cow vaginal tissue. No significant difference between C-92 and C-77 is found for either Fmax or AUC. There is a tendency where C-92 shows the highest detachment force, also the highest work of adhesion is found for C-92. The tissue was wetted with small amount (20 µl) of VFS pH 4.5 prior to measurements, which may cause ionization of the amino groups in chitosan. Mucin plays an important role in mucoadhesion and bonding occurs as a result of many complex mechanisms. For chitosan it is believed that the good mucoadhesive potential is due to high molecular mass and high charge density (Bonferoni et al., 2009). Also other authors have found the strongest mucoadhesion for the chitosan of high %DD (Qaqish and Amiji, 1999). Strong ionic interactions between positively charged chitosan and negatively charged sialic acids moieties on mucin is expected to occur.

Figure 20B demonstrates mucoadhesion peformed by pure pectin 11 mm tablets. In mixing process of metronidazole and pectin, there were used 1 % metronidazole and not 10 % which is used elsewhere. This was done by a mistake and was corrected for (10%).

A)



B)

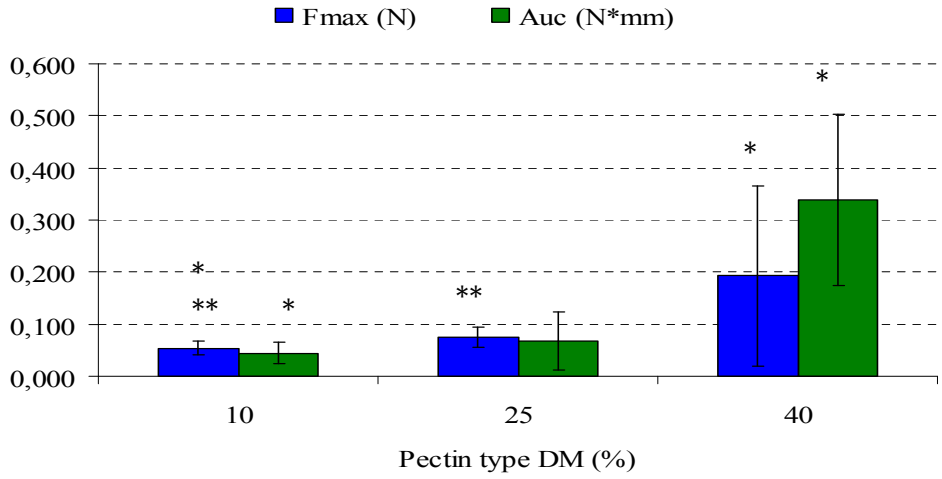


Figure 20: Mucoadhesion determined between 11 mm tablets of matrix tablets and cow vaginal tissue wetted with VFS pH 4.5 A) 90 % chitosan matrix (n=8), B) 99 % Pectin matrix (Amount parallels for the respective pectin type: n10%=7, n25%= 7 and n40%= 8) * significantly different (one and two tailed t-test, 95 % confidence level), ** significantly different (one and two tailed t-test, 95 % confidence level)

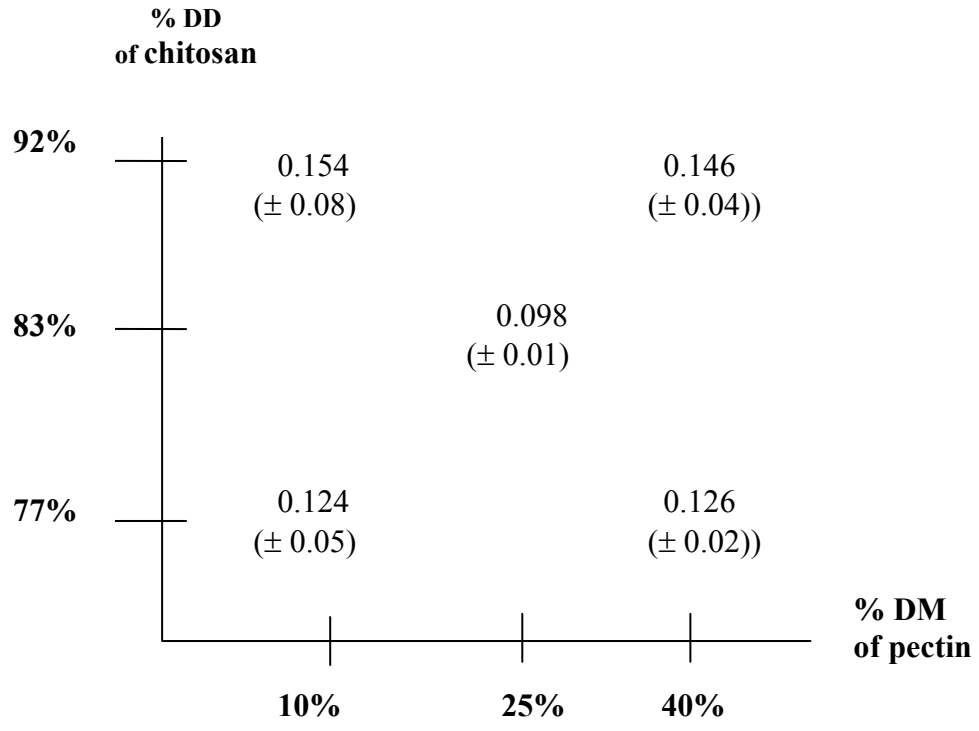
Figure 20 B indicates significant difference in both Fmax and AUC of P-10 and P-40 ($p<0.05$). This indicates that P-40 exhibit higher work of adhesion and maximum detachment force than P-10 and P-25. Significant difference in Fmax is found between P-10 and P-25, where P-25 exhibit higher Fmax. No significant difference in AUC is found ($p<0.05$). Pectins with higher DM have a higher molecular weight which is known to enhance mucoadhesion

due to more pronounced interpenetration with tissue surface (Hagesaether and Sande, 2007). This is also observed in the current study.

4.2 Mucoadhesive screening for suitable combinations of chitosan and pectin using a 2^2-factorial design

Several studies have shown that a cross-linking of pectin and chitosan could be favourable (Hiorth et al., 2006, Hagesaether et al., 2009, Ofori-Kwakye and Fell, 2001). At pH between 3-7 both pectin and chitosan is ionized thus making formation of a polyelectrolyte complex possible (Hiorth et al., 2006). A 2^2-factorial design with centre point was put up to investigate combinations of pectin and chitosan: different type of pectins (DM 10, 25 and 40%) and chitosan (DD 92, 82 and 77%). Figure 21 shows the results from the test on mucoadhesiveness of 11 mm tablets to vaginal tissue. Both % DM of the pectin and % DD of the chitosan seems to have an influence on the maximum detachment force (Fmax) (Figure 20A) as well as on the work of detachment (AUC), (Figure 21B). The measurements of mucoadhesion contain high variation. However, the trends can be recognised, which can be explored in further investigations. Both tested variables are favourable on high level, e.g high %DM and high %DD, and the high degree of deacetylation chitosan is more important for high detachment force and high work of adhesion than a high degree of methoxylation. Therefore, DD 92% was selected as the chitosan type for further optimization studies.

A)



B)

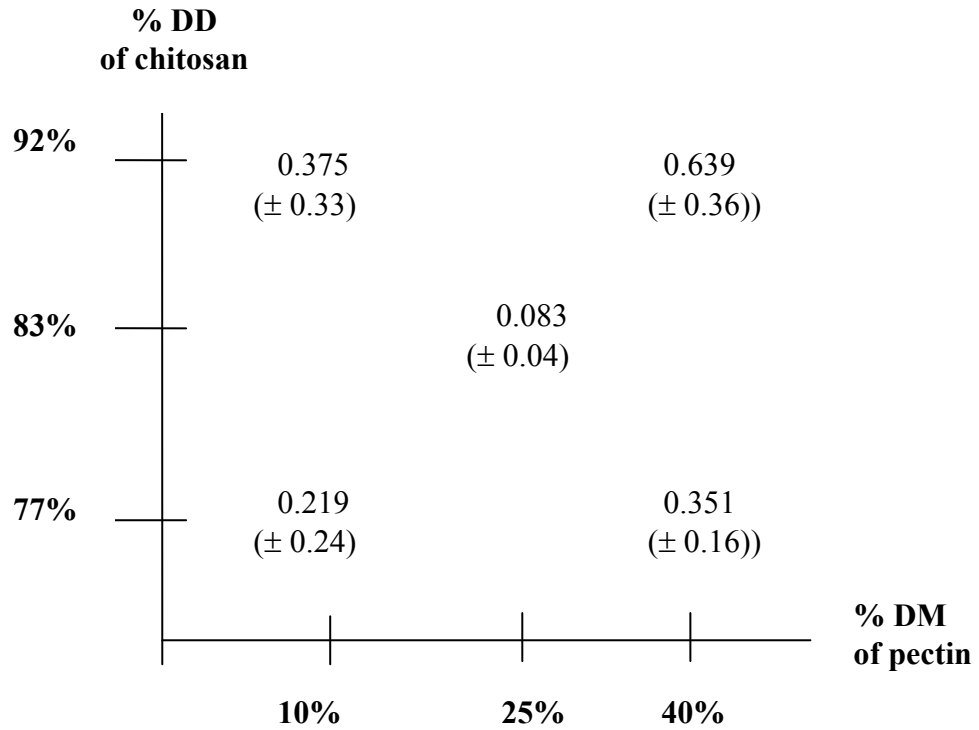


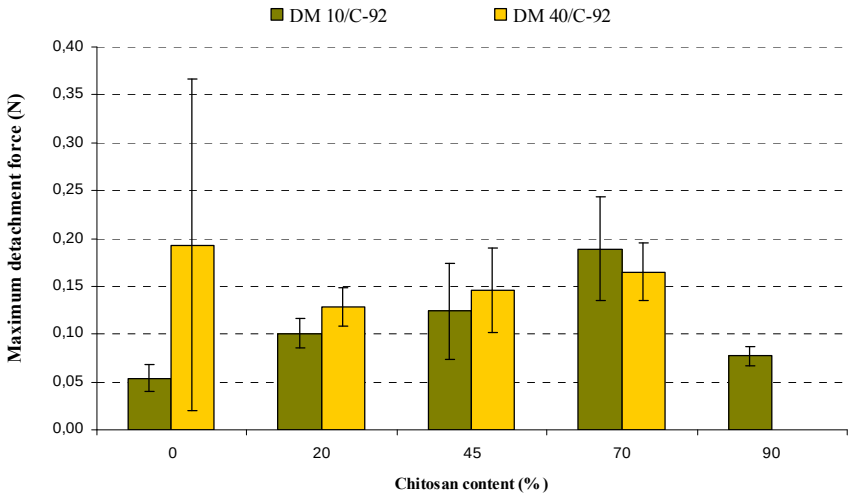
Figure 21: Test on mucadhesivity of a 11 mm tablet of different combinations of chitosan and pectin onto cow vaginal tissue A) maximum detachment force (Fmax), B) work of adhesion (AUC) in the same setup (n=8)

4.3 The effect of chitosan to pectin ratio on formulations properties

4.3.1 Mucoadhesion

Figure 22 is presenting maximum detachment force (Fmax) and work of adhesion (AUC) for all tested ratios of chitosan and pectin

A)



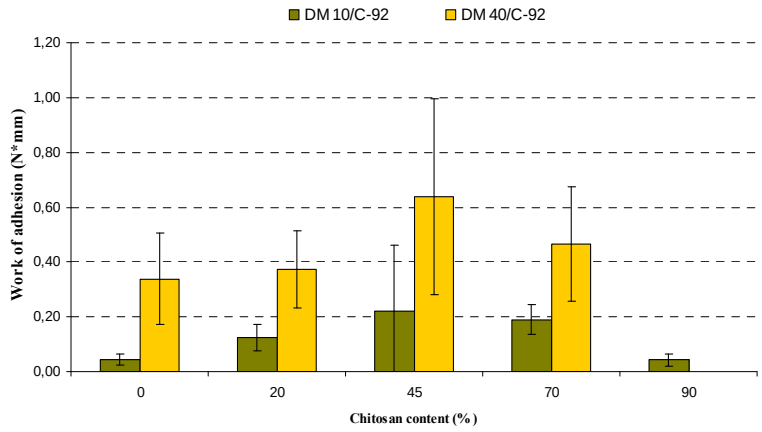


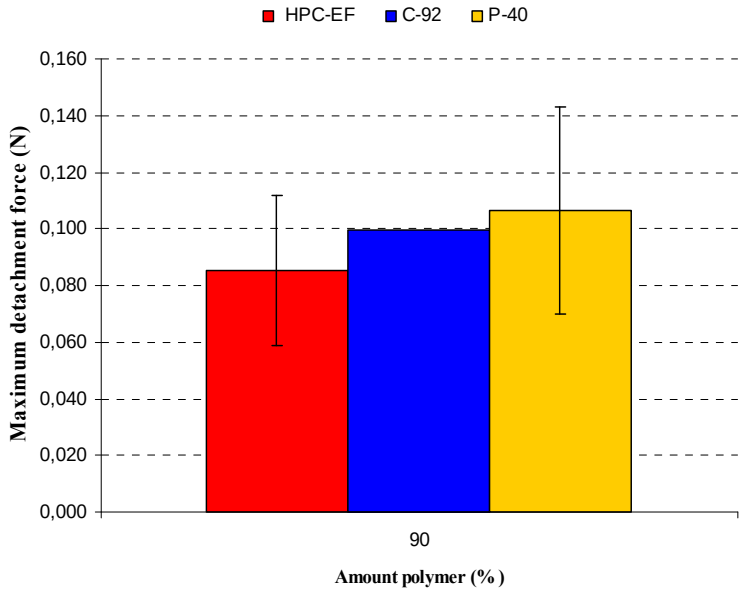
Figure 22: Mucoadhesion of 11 mm tablets to cow vaginal tissue for various combinations of chitosan:pectin ratio A): Maximum detachment force B) Work of adhesion. Data presented as mean ± SD (n=3). (n=10)

There is a wide spread in the results but a tendency can be recognised where increasing chitosan content up to 70% results in a higher maximum detachment force. But chitosan alone (i.e. 90%, 10 % drug) results in a much lower detachments force. Based on visual observation during performance of the mucoadhesivity test, it was clear that tablets containing P-40 had a

higher mucoadhesivity compared to all others. This is clearly described by the results from work of adhesion in the figure 22B. There is a significant difference between P-40 and P-10 at all ratios (except the formulation of equal ratios), where P-40 exhibits the strongest mucoadhesion. When comparing the tablets/formulations where the polymers are used alone, P-40 displays higher AUC while it appears to be small or no differences between P-10 and C-93. The high degree of mucoadhesion observed for P-40 can be caused by its high ability to swell and form gels, even without addition of calcium ions. Also P-40 has the highest molecular weight of the tested pectins and is therefore expected to cause the highest interaction with the mucosal tissue (Bonferoni et al., 2009, Thirawong et al., 2007).

Unfortunately, the results of the combination of equal amounts of chitosan and pectin (seen as 45% chitosan in the plot figure 22 show extremely large variation, therefore it is difficult to conclude whether this combination has a higher mucoadhesion than the others or not. But there might be a similar trend as seen for Fmax where increasing amount of C-92 (up to 70%) causes higher AUC. It is worth keeping in mind that the C-92 is the chitosan of the low molecular weight. The overall conclusion from the mucoadhesion test is that it seems to be beneficial to combine chitosan with pectin in order to increase the mucoadhesive properties of the formulation. The highest mucoadhesion is probably obtained for the formulation of 70 % Chitosan DD93% combined with pectin D40%. Also the same combination of pectin DM10% gives reasonably high mucoadhesion. Pure pectin DM 40% show promising results in the mucoadhesion test, but the variation is high, therefore this formulation is not selected among the best suitable. Due to lack of vaginal tissue from the one animal, a final comparison was set up using rectal tissue from the same animal to include mucoadhesion test of HPC in the study. Formulation of 90 % HPC (10 % is drug) was compared to formulations of 90 % chitosan DD 92% and 90 % pectin DM 40%. Figure 23 shows Fmax and AUC of these experiments. The detachment force showed marginal differences between the polymers (Figure 23A), while figure 23B demonstrates variations between the polymers where P-40 attained the highest work of adhesion. Despite high variations in results, a significant difference was found between HPC and P-40 (one and two-tailed t-test, 95% confidence interval). In addition, significant difference is also found between HPC and C-92 (one-tailed t-test, 95% confidence interval). No significant difference is found between P-40 and C-92 (t-test).

A)



B)

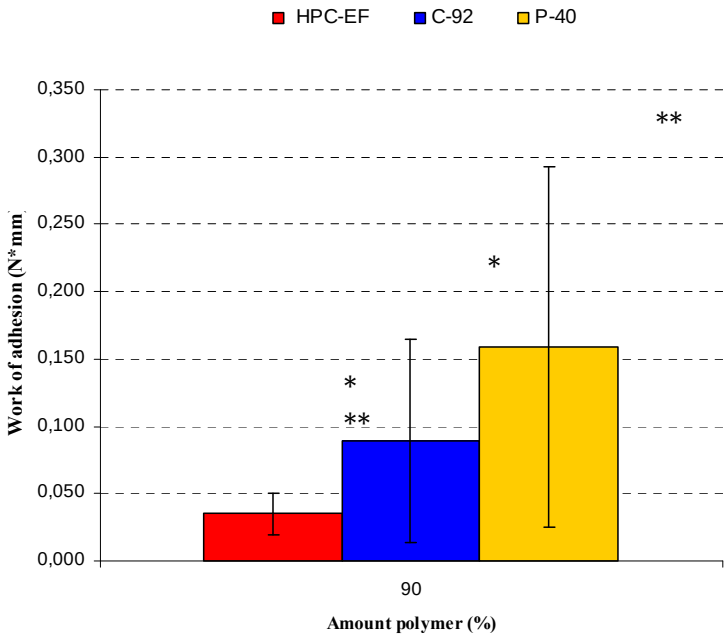


Figure 23: Mucoadhesion of HPC-EF, C-92 and P-40 A) Fmax (N) B) AUC (N/mm^2). Data presented as mean ± SD (n=10). * significantly different (one tailed t-test, 95% confidence level), ** significantly different (one and two tailed t-test, 95% confidence level)

Smart et al lists cellulose derivatives among the polymers possessing good mucoadhesive properties (Smart, 2005). In this study both chitosan and pectin showed the higher work of adhesion (AUC) compared to HPC. Both, chitosan and pectin, consist of groups that are known to be directly involved in mucoadhesion (amino groups and carboxyl groups, respectively), in addition to the hydroxyl groups. The presence of hydrogen-bond forming

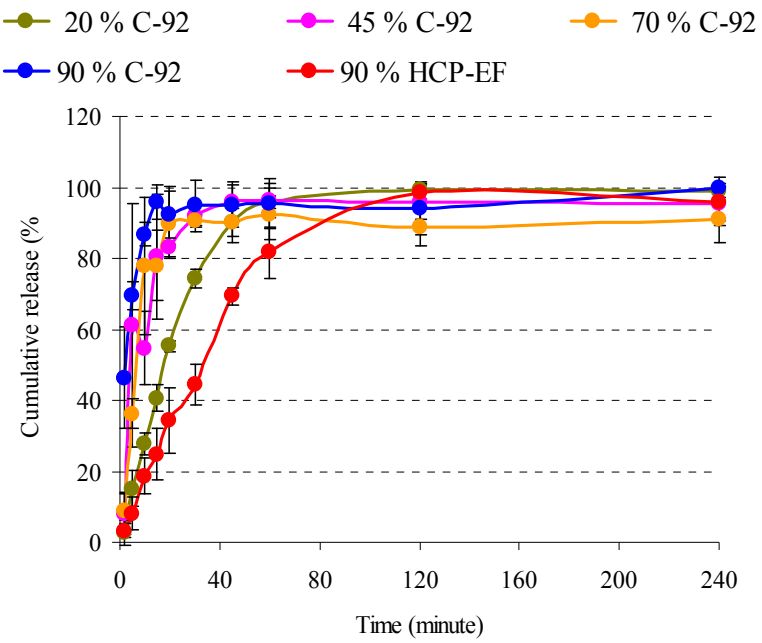
groups is crucial to obtain good mucoadhesivity (Dodou et al., 2005). Since HPC consist mainly of hydroxyl groups, this could be the reason for the lower mucoadhesive strength compared to chitosan and pectin; the overall hydrogen bond forming groups are less. HPC is a non-ionic polymer which is used as a matrix for extended release formulations. This causes lower rate of swelling upon hydration, which in this case was limited amount of fluid, thus resulting in lower flexibility and mobility of the polymer chain which could interact with the tissue.

4.3.2 Dissolution profiles

The drug release from mini-tablets containing pure C-92 was not satisfying as previously seen in figure 17B and 18. An uncontrolled behaviour was observed due to the salting out, and therefore a more controlled release is wanted, despite the fact that disintegrating properties could be desirable in vaginal delivery (Poelvoorde et al., 2009, Bigucci et al., 2008). Several authors reports on cross-linking of chitosan and pectin, especially for specific delivery of drugs to the large intestine (Bigucci et al., 2008). In our study, the polyelectrolyte complex was created at higher pH, which is favourable in bacterial vaginosis and caused by an increased the vaginal pH (Kukner et al., 1996).

The drug release studies of the different ratios of pectin and C-92 (20:70, 45:45, 70:20) (for more information on the formulations see table 3 (3.7) were performed in VFS of pH 4.5 and 6.8 (figure 24 and 25). As in the mucoadhesion studies there are two series of experiments, one for pectin DM10% and one for DM40%. In both series the chitosan grade DD92% is used. Hydroxypropylcellulose (HCP-EF) was included in the study because it is a widely used polymer in matrix tablets and shows good release characteristics, and it is known to possess mucoadhesive properties (Smart et al, 2005).

A)



B)

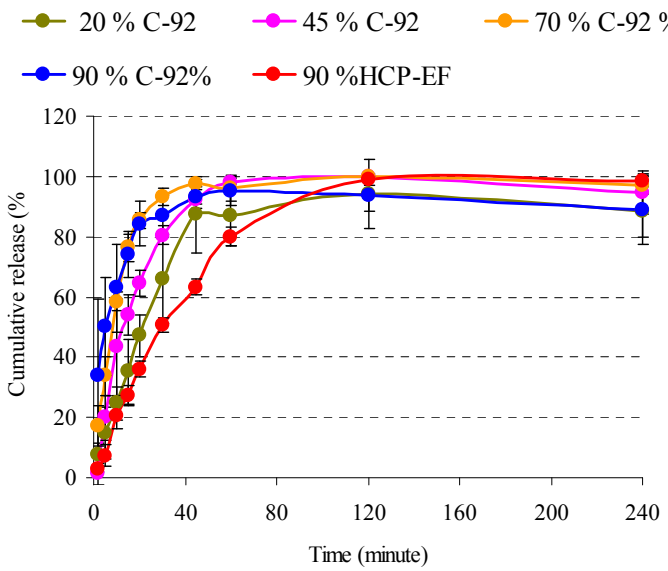
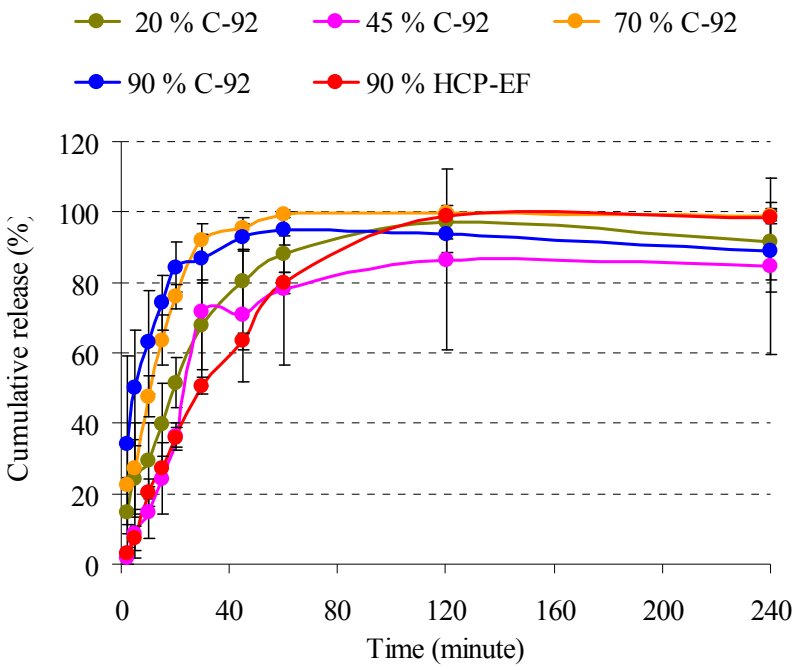


Figure 24: Dissolution profile of metronidazole (10% w/w) from different combinations of chitosan to pectin. Pectin grade used DM 10 %. Hydroxypropylecellulose added as refernce A) VFS pH 4.5 B) VFS pH 6.8. Data presented as mean ± SD (n=3).

A)



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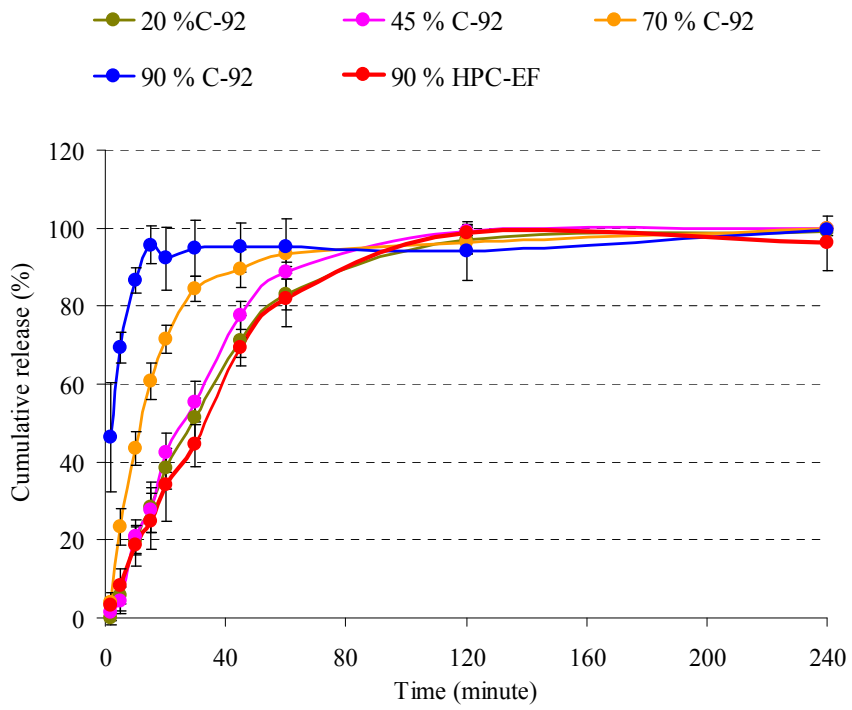


Figure 25: Dissolution profile of metronidazole (10% w/w) from different combinations of chitosan to pectin. Pectin grade used pectin 40 %DM. Hydroxypropylecellulsoe added as reference. A)VFS pH 4.5 B) VFS 6.8. Data presented as mean ± SD (n=3).

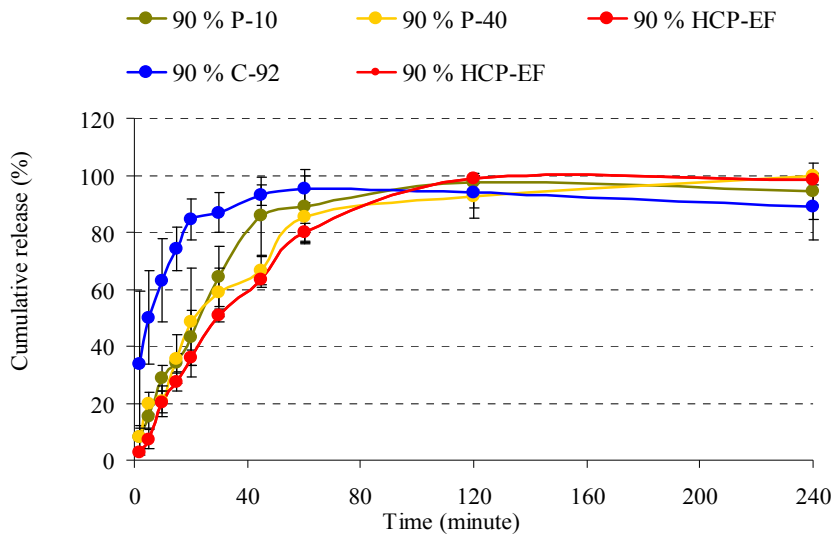
The slowest release in both media was seen for the formulation containing HPC. Mini-tablets containing HPC was found to release 80% of the metronidazole content after 75 min

independently of pH of the media. Since this is a non-ionic polymer, it was also expected not to be influenced by the pH of the media and therefore served as a control for the ionic polymers. The drug release results of the different formulations of chitosan and pectin showed slight pH-dependency. For the series containing pectin DM10% (figure 24A and B) the formulation containing 20% chitosan was able to prolong the release of drug to the highest extent. This formulation released 80 % of the drug load after approximately 40 min at both pH levels. But at increasing chitosan content (corresponding to reducing pectin content) the formulation is becoming more pH dependent. The formulations containing 70% and 90% chitosan (C-92) released 80% of the drug load within 15 minutes in pH 6.8, the corresponding release in pH 4.5 seems to be approximately 30 minutes. The effect of ionization of carboxyl groups is visible. Upon increasing the content of P-10 causes a more desired release profile. This profile also is improved when pH increases. Also for the formulations containing pectin DM 40%, (figure 25A and B) it seems favourable with high pectin contents. Both formulations of 45% and 70 % (i.e. 45% and 20 % chitosan in the plot) show sustained release of the drug. In VFS pH 6.8 the two formulations show release profiles very similar to the release from HPC, whereas the release is slightly faster in VFS pH 4.5.

To summarize, a high content of pectin is important to prolong the release of metronidazole at both pH levels. There slowest release is found when pectin DM40% is applied. This difference observed between formulations containing P-10 and P-40 could be explained by the higher ability of P-40 to create a gel due to higher degree of hydrofobicity than P-10. When this gel network is created, it will make it more difficult for fluid to penetrate in and dissolved drug to penetrate put, which results in prolonged release. Generally, the ability of P-10 for creating a gel network alone is limited. Thus gelling of P-10 is produced by the presence of Ca^{2+} ions in the VFS. Pectin of low DM is known to cross-linked with calcium ions. Since DM 40% per definition also is a low methoxylated pectin (DM<50%), cross-linking can also be contributing to the slow release for seen in DM40%. However, for DM10% cross-linking is the only way of producing a gel network alone. Further, the cross-linking of pectin chains with calcium ions happen readily, whereas the interaction with chitosan is expected to be much slower reaction since both pectin and chitosan must first be hydrated before they can interact (Sungthongjeen et al., 2004). The calcium ions are available in the solution and ready for cross-linking as soon as the outer layer of the pectin-containing tablet is hydrated. The cross-linking effect of C-92 and P-10 is low in those cases where C-92 is in excess (>50 %). Figure 25 show that formulations containing 45% and 70 % of P-40 in

the mini-tablets exhibit a similar release rate as HPC-EF. The effect of cross-linking the two polymers is more prominent at higher pH and for higher degree of esterification in the molecule.

A)



B)

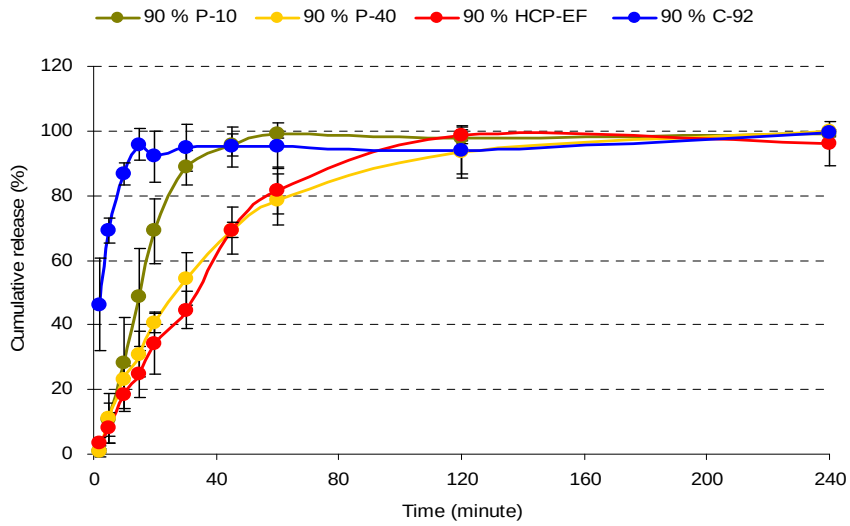


Figure26: Dissolution profile of metronidazole (10% w/w) from mini-tablets of different polymer matrixes. A) VFS pH 4.5, B) VFS pH 6.8 (n=3)

Finally, to point out the major difference between the different polymers of the study, drug release profiles of the mini-tablets containing only drug and polymer is plotted in figure 26. The release from mini-tablets of pectin DM40% is in all respects equal to the release seen

from the pH independent polymer HPC, and it is similar at both pH levels. This suggests that pectin DM40 % could be suitable for improved vaginal therapy. It is a major advantage that the release rate is not changed by changing pH of the vaginal environment. Also pectin DM 10% is well suited for sustaining the release of drug at pH 6.8, but the release is much faster at pH 4.5. However, considering that the vaginal pH often is elevated as a result of infection these formulations should not be excluded for vaginal therapy.

4.3.3 Tensile strength

Figure 27 illustrates the differences in dimension between 11 mm flat-punch tablet and 2mm mini-tablet.The height of the convex mini-tablets was measured and converted to the apparent height of corresponding flat faced tablets before tensile strength was calculated using formula 1. Ideally, the height of the individual 30 mini – tablets used in the crushing test should have been quantified before the crushing force was determined. The mini – tablets were produced using volumetric filling of the powder into the matrices', which resulted in different tablet masses due to density variations of the powder mixtures. Therefore, the average height of 5 mini-tablets was used as an indication of the height of the mini-tablets from that particular formulation and employed in calculations of tensile strength to allow comparison of the mechanical strength between the different blends. Due to the approximation of the high and the transformation from convex tablets to apparent height in flat-faced tablets the tensile strength results are not accurate. However, they should be suitable for a comparison among the different batches that are all treated in the same manner. This was done to evaluate how the different ratios would affect the ability to create strong inter-particulate bonds.

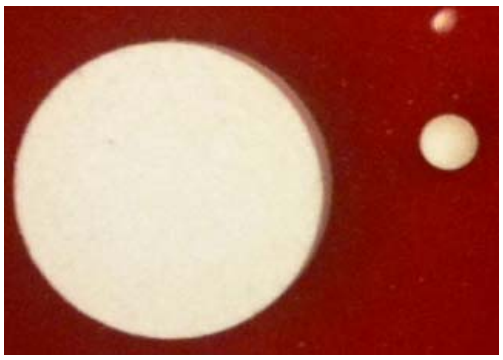


Figure 27: Illustration of dimension difference between 2 mm mini - tablet and 11 flat faced tablet

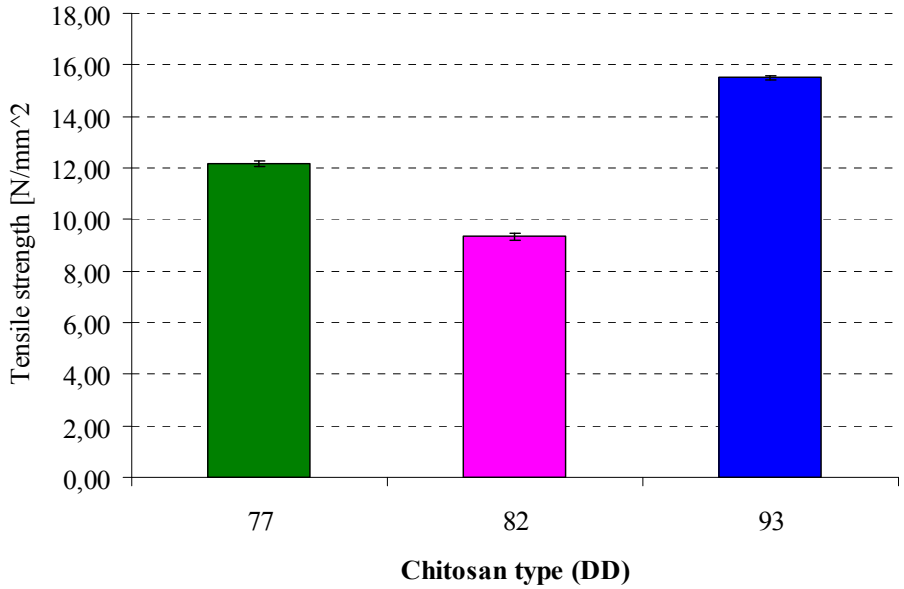


Figure 28: Tensiles strength based on height of 5 mini-tablets containing various chitosan types (DD 77%, 82% and 93%) Data presented as mean ± SD (n=5).

In figure 28, the tensile strength of the different formulations is presented. When comparing the chitosans, the tendency shows a decreased tensile strength in following order C-92 > C-77 > C-82. For tabletting, chitosan have been studied sporadically and the studies showed the ability to form strong mechanical tablets are dependent on deactetylation degree. Rege et al reported that increasing DD of chitosan in tablets produces tablets with higher crushing strength (Rege et al., 1999). They show a tendency of increasing elastic recovery increased with decreasing DD. In this study, C-82 show the weakest mini – tablets. Another factor that could possibly explain the mechanical strength observation is particle size distribution and powder flowability (Picker-Freyer and Brink, 2006), but neither of these parameters were investigated in the current study. However, the observation in the laboratory was that C-82 contained large particles which were retained in the meshes during sieving.

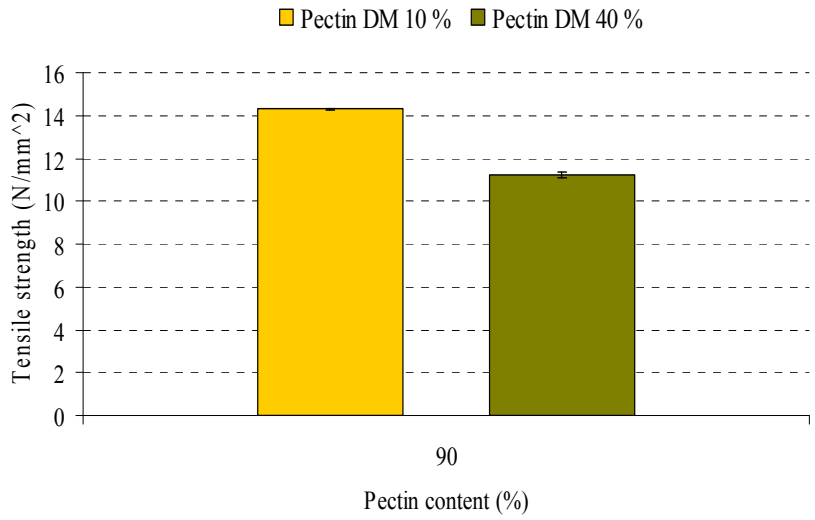


Figure 29: Tensile strength of mini – tablets containing pure pectin type (DM10% and DM 40%) (n= 5)

With respect to the mini – tablets consisting mainly of pectin, P-10 exhibits the highest tensile strength (figure 29). This observation is also according to literature, where higher degree of methoxylating (DM) yields weaker tablets (Salbu et al., 2010a). Salbu et al proposed that higher amount of DM cause an increased hydrophobic particle surface which results in weaker inter –particulate bonding between the particles (Salbu et al., 2010a). Pectin and chitosan are both reported to be rather soft and ductile materials to compress (Salbu et al., 2010a, Mir et al., 2008).

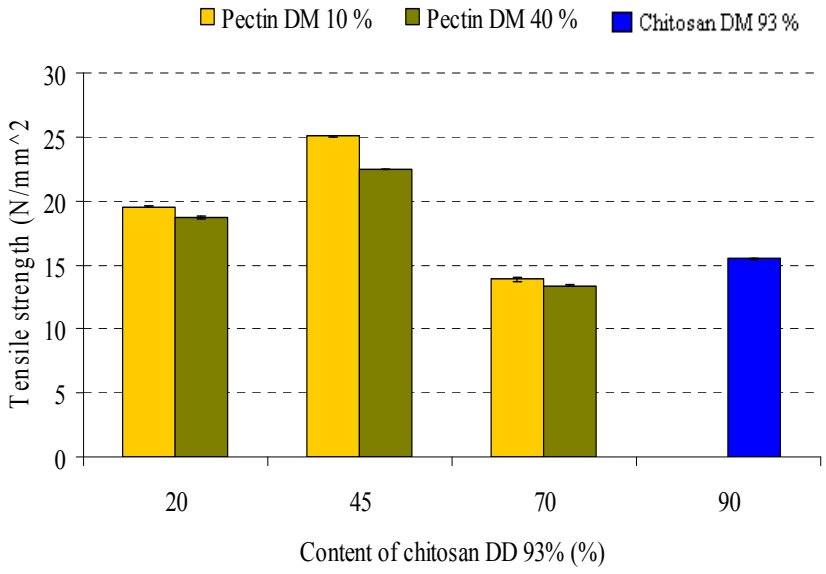


Figure 30: Tensile strength of various pectin type (DM 10% and 40%) and varying degree of C-92

The effect of mixing pectin and chitosan in different ratios had a positive impact on the tensile strength, see figure 30. Mini-tablets containing 20 % of C-92 resulted in an increased mechanical strength for both pectin types and reached maximum at the ratio 45/45 of C-92 to P-10. It appears that when the fraction of chitosan C-92 is high (70%), there is no improvement of mechanical strength compared to the polymers used alone. The tableting capability of P-40 improves when blended with C-92, the tensile strength increases showing improved ability of P-40 to form strong tablets. The effect of mixing C-92 with pectins may result in formation of attractive forces between amino groups in C-92 and carboxyl groups in pectins. These attractions could possibly cause stronger interparticulate bonding between them. It may even be speculated that the opposite charge on particle surfaces could reduce or help to overcome the hydrophobicity described by Salbu et al at the surface of pectin particles (Salbu 2010). This could explain the increased mechanical strength seen in P-40 upon addition of chitosan. Increasing the amount of added C-92 dilutes the pectin and the hydrophobic character of the particles is less pronounced. When excessive amount of C-92 is added, the elastic behaviour of C-92 appears.

The combination of equal amounts of chitosan to pectin results in the strongest mini-tablets. This behaviour has also reported by another group; high tensile strength in 45/45 ratios (Chen et al., 2010), however they tested porous membranes of chitosan and pectin prepared by casting (dissolving the polymers and then drying). They found that molar ratio of carboxyl to amino groups was highest in 45/45 ratio. This could maybe explain the ability for amino and carboxyl groups to create highest tensile strength to more hydrogen - bonding sites in the particles.

4.3.4 Friability

Friability of the formulations was also tested and gives an indication of the tablets ability to withstand handling. From table 5 (see section 8.1) mini-tablets containing 90 % of pectin DM 10 % and 40 % showed a weight loss higher than 1 %, which is not acceptable. This indicates that tablets are not resistance enough against local permanent deformations. A progressive reduction in tablet weight would occur and change its appearance. This was observed with 90% pectin DM 40 % mini – tablets during storing.

To summarize the mechanical strength of chitosan:pectin mini-tablets: During measurements of tensile strength and friability testing demonstrate that both types of pectin has low

capability to produce strong tablets when used alone and are not resistance enough to withstand handling. Adding 20 % C-92 resulted in increased mechanical strength for both pectin types and acceptable weight loss during friability testing. Tensile strength reached maximum with 45:45 C-92 and P-10. Both P-10 and P-40 can be used in mini – tablets with sufficient amount of C-92. The friability of the mini – tablets also showed acceptable weight loss; only pure pectin's not resistance enough against local deformations.

4.3.5 Rotating cylinder test

A modified version of the rotating cylinder method was performed under rather though conditions, where high rotating time was used and swelling was excluded (Hagesaether et al., 2008). Normally, fluid is surrounding the cylinder during the experiment, but since the amount of fluid present at the vaginal surface is relatively low, the test was run in empty beakers for a better in vitro/ in vivo correlation. No fluid was used except for wetting of the tissue before test run. Tablet 4 summarises the percentage of mini - tablets adhering on the tissue after vigorous rotation of the cylinder at 250 rpm for 10 minutes.

Table 4: Percentage of mini – tablets remaining on the tissue after rotation at 250 rpm for 10 min (n=30)

Pectin type (DM)	**Pectin (%)**	**Chitosan type (DD)**	**Chitosan (%)**	**% adhering mini – tablets**	**Standard deviation**
10	90	-	0	96.67	5.77
10	70	92	20	100	0
10	45	92	45	100	0
10	20	92	70	100	0
40	90	-	-	96.67	5.77
40	70	92	20	100	0
40	45	92	45	100	0
40	20	92	70	100	0
-	-	92	90	100	0
-	-	82	90	100	0
-	-	77	90	100	0

All mini-tablets showed high degree of adherence to the tissue, except the formulations containing pure P-10 and P-40, where mini-tablets were detached. The ability of the mini-tablets to swell and remain adhering under such rough conditions can be seen as a proof of the mucoadhesive properties of the formulations and very promising for further investigation.

4.3.6 Simulated wetting test

The simulated wetting test performed in the study, was originally developed for orally disintegrating tablets (Park et al., 2008). This method makes it possible to observe the wetting process of the mini – tablets in an environment with low amount of fluid (20 µl). The results are presented in table 5 shows those mini-tablets, which was soaked by the blue dye solution within reasonable time at pH 4.5 and 6.8, respectively. Only 4 types of mini-tablets turned completely blue during the experiment. The mini – tablets containing pure chitosan had the fastest wetting time, as also seen in the dissolution test, due to high ionic strength of VFS. There might be a trend where C-77 is showing the slowest wetting time, but since the results show large variation one should be careful to draw conclusions.

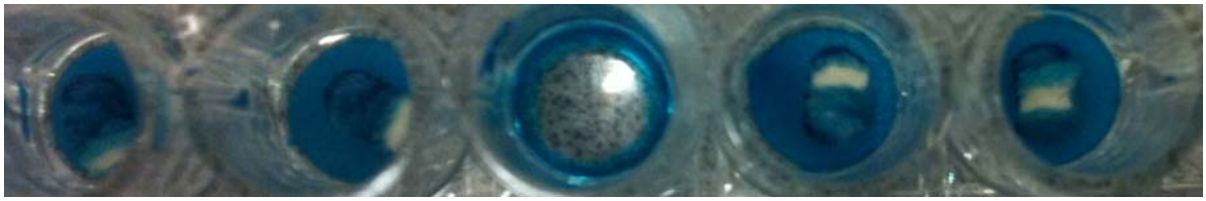
Table 5: wetting time of mini - tablets which turned blue in pH 4.5 and 6.8 in Brilliant blue solution (n=5)

Amount chitosan (%)	Type chitosan (DD)	Amount Pectin (%)	Type pectin (DM)	Simulated wetting time pH 4.5 (s)		Simulated wetting time pH 6.8 (s)	
				Average (s)	SD	Average (s)	SD
90	92	-	-	12.80	3.35	8.00	1.58
90	82	-	-	12.80	4.09	17.00	4.12
90	77	-	-	20.40	7.92	22.00	7.65
70	92	20	40	49.80	32.65	24.60	14.33

At pH 4.5, the amino groups of chitosans are ionized, which increases the solubility and because the amount of fluid is limited the burst release that was seen in the dissolution experiment, is not so striking. Higher charge density on the polymer results in higher uptake of the blue solution and therefore faster wetting time from C-92. Fast wetting time is favourable because swelling causes flexibility and mobility where the polymer chains can interact with each other, with the other polymer and penetrate into the mucus layer (Smart, 2005). The same trend is also observed in pH is 6.8. The mini – tablets containing the mixture of 20:70 (P-40/C-92) was the only formulation of pectin/chitosan blends that gave measureable results in the experimental set-up. They were soaked by the blue solution, but the time until they turned blue was very long compared to the chitosan formulation, one parallel

require 96 s to be fully soaked. Compared to the pure chitosan it is a slower wetting process. When comparing the 70:20 (P-40 and C-92) with the same ratio of P-10 and C-92 (figure 31A and B), the solution did not penetrate fully into the mini – tablets either at pH 4.5 and 6.8. This is probably a limitation of the method. If more fluid was available, the tablets that swell to a higher extent could also have a reasonable chance to be coloured. However, it is a prerequisite that the fluid is fed through the filter paper disc and not floating in the well.

A)



B)

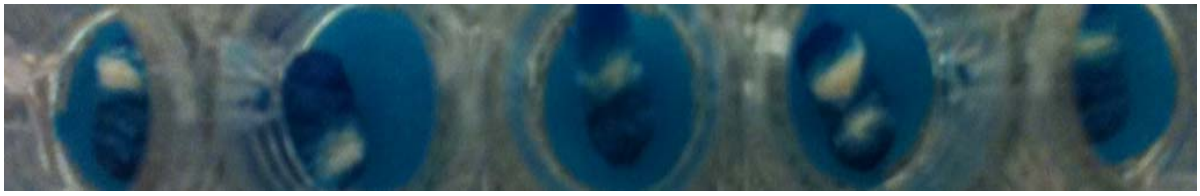
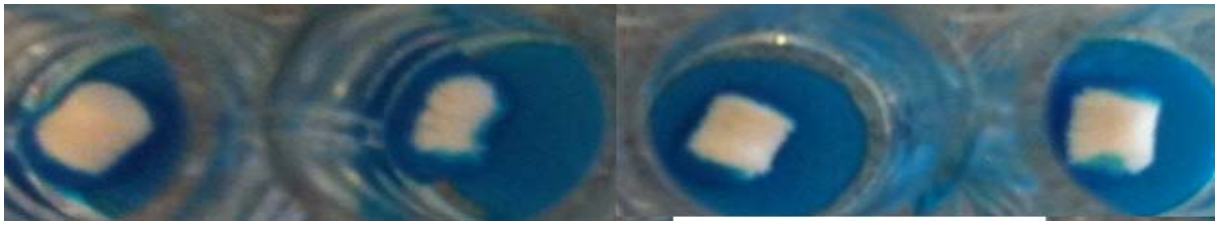


Figure 31: Results of simulated wetting time for mini-tablets of 20 % pectin 10 and 70 % C-93 A) pH 4.5 B) pH 6.8

In general, pectins are soluble in water and the swelling rate is considered to be a function of the hydrophilicity of the polymer. Since P-10 exhibits higher amount of free carboxyl groups, it is expected that P-10 is more hydrophilic and should therefore be wetted much faster than P-40. There are presume that strong hydrogen bonds appear between the polymer chains in P-10, which make it more difficult for dissolving (Khutoryanskiy, 2007). Figure 32 illustrates the fluid uptake of both, pure pectin in pH 6.8 and the difference between them. Clearly, there is a higher degree of swelling in P-40 due to increased fluid uptake than in P-10. Again the limited amount of liquid available is a restricting factor.

A)



B)

Figure 32: Results of simulated wetting time for mini-tablets in pH 6.8 solution A) 90 % pectin 10 % B) 90 pectin 40 % **(n=5)**

By increasing the amount of both pectin types resulted in lower uptake of the fluid and therefore no wetting time was observed at al. When the fluid is wetting the mini – tablets, the water would penetrate into the tablet. Both chitosan and P-40 would get ionized at their respective pH and therefore cross-linking between the polymers could occur. This would make it more difficult for water to penetrate and erode into the mini – tablet due to strong ionic interactions and possible hydrogen bonding. As mentioned earlier, fast wetting time is favourable for avoiding low residence time and increasing bioadhesive bonds.

A)

B)

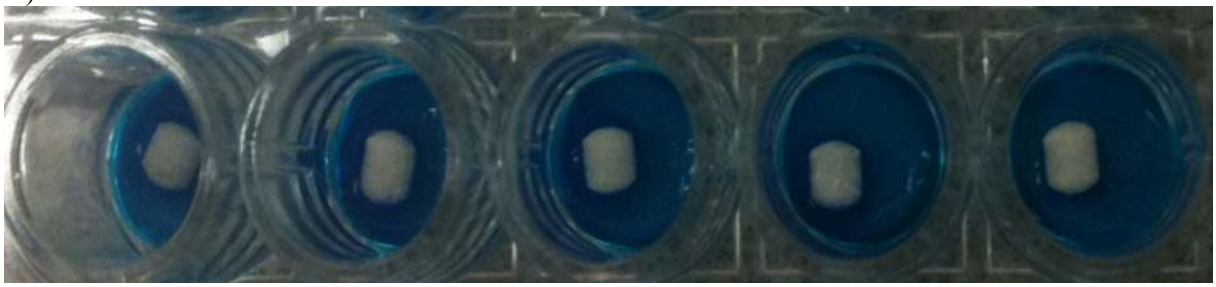


Figure 33: 90 % HPC-EP in A) pH=4.5 B) pH =6.8; (n=5)

Mini-tablets of HPC was also tested as a reference and showed no sign for absorbing the fluid. Because of its non-ionic character causes a slower water uptake and therefore no visual water uptake is observed.

5. Conclusions

Optimized mini-tablets containing metronidazole were developed based on chitosan and pectin mixtures, as both polymers alone failed to prolong the release of metronidazole in simulated vaginal fluid. Chitosan with the highest degree of deactylation (92%) and pectin with a low degree of methoxylation (DM 10% and 40%, respectively) showed suitable properties for vaginal delivery.

Formulations with increasing amount of C-92 showed higher mechanical strength, where mini-tablets containing 45:45 C-92/P-10 achieved maximum tensile strength among all formulations tested. Acceptable strength was also reached for the mixing ratios 45:45 C-92/40 achieved.

Release characteristics of mini-tablets containing C-92 and P-10/40 showed pH-dependent release of metronidazole and required high amount of pectin included in formulation to obtain prolonged release. The most prolonged release was achieved for pectin DM 40%, in both pH 4.5 and 6.8. It was also obvious that cross-linking between chitosan DD 92% and pectins was most prominent in media of higher pH. The mixing ratios which showed the most prolonged release was 45:45 C-92/P-40 and 70:20 C-92/P-40.

Mini-tablets prepared from ratio 70:20 C-92 /P-40 exhibited the highest bioadhesiveness, however the same ratio with P-10 also showed satisfactory bioadhesive properties. The ratio 45:45 C-92 /P-40 showed good mucoadhesion, but the results showed large variations.

It is possible to obtain desired mucoadhesive strength and prolonged release of metronidazole from mini–tablets by combining chitosan of high deacetylating degree with pectin types of low methoxylation degree which has sufficient tensile strength and friability.

6. Future perspectives

Based on literature survey and experience during this study, the next step would be optimization of the powder mixture with focus on powder flowability. It is important to have good powder flow properties in order to reduce mass variations and secure high uniformity of content in mini-tablets during production. Particle size is also an issue in production because of the small size of mini-tablets. Therefore, reducing particle size and obtaining a narrow particle distribution throughout the mixture is desired. Characterisation of chitosan as an excipient for direct compression would also be valuable, due to limited literature.

More *ex vivo* experiments would provide deeper insight on bioadhesive properties of the formulations, such as employing vaginal tissue from animals of various age and different species. The experiments should also be repeated in media containing mucin, to observe the potential influence of mucin on the adhesion as well as on the drug release profile.

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8. Appendix

8.1 Table showing characteristics of mini-tablets and bioadhesive properties of respective formulations (mean ± SD)

8.1 Table showing characteristics of mini-tablets and bioadhesive properties of respective formulations (mean ± SD)

Table 6: Characteristics of mini-tablets and bioadhesive properties of respective formulations (mean ± SD)

Pectin type (DM)	Amount pectin (%)	Chitosan type (DD)	Amount chitosan (%)	Mass tablets (mg)	Compression pressure (MPa)	Crushing strength (N)	Tensile strength (N/mm^2)	Friability (%)	Fmax (N)	AUC (N*mm)	SWT (s)	
											pH 4.5	pH 6.8
10	90	-	0	6.406 ± 0.034	246.94 ± 3.16	8.17 ± 1.86	14.30 ± 0.033	3.23 ± 0.84	0.054 ± 0.014	0.044 ± 0.021	-	-
10	70	92	20	6.11 ± 0.07	227.60 ± 8.85	11.90 ± 2.42	19.55 ± 0.046	0.50	0.101 ± 0.015	0.124 ± 0.047	-	-
10	45	92	45	5.67 ± 0.036	232.44 ± 4.44	16.44 ± 2.76	25.04 ± 0.186	0.44	0.124 ± 0.05	0.219 ± 0.243	-	-
10	20	92	70	5.49 ± 0.05	248.33 ± 11.48	10.37 ± 2.86	13.87 ± 0.023	0.51	0.189 ± 0.054	0.403 ± 0.209	-	-
40	90	-	-	6.90 ± 0.106	226.62 ± 7.95	5.40 ± 1.05	11.21 ± 0.137	8.87 ± 1.10	0.193 ± 0.173	0.339 ± 0.165	-	-
40	70	92	20	6.31 ± 0.04	233.97 ± 4.92	10.41 ± 2.09	18.70 ±0.035	0.94	0.128 ± 0.020	0.374 ± 0.140	-	-
40	45	92	45	6.70 ±0.048	257.62 ± 12.68	13.96 ± 2.39	22.492 ±0.067	0,43	0.146 ± 0.044	0.639 ± 0.358	-	-
40	20	92	70	4.96 ± 0.054	248.20 ± 7.79	9.62 ± 1.92	13.38 ± 0.060	0.49	0.165 ± 0.030	0.465 ± 0.209	49.80 ± 32.65	24.60 ± 14.33
-	-	92	90	4.92 ± 0.09	251.38 ± 14.35	11.71 ± 2.46	15.50 ± 0.098	0.303	0.077 ± 0.010	0.043 ± 0.021	12,80 ± 3.35	8.00 ± 1.58
-	-	82	90	4.78 ± 0.138	247.35 ± 22.68	6.67 ± 1.28	9.34 ± 0.152	0.62	-	-	12.80 ± 4.09	17.00 ± 4.12
-	-	77	90	4.94 ± 0.35	244.37 ± 9.24	8.54 ± 1.93	12.17 ± 0.094	0.00	0.0724	0.027 ± 0.018	20.40 ± 7.92	22.00 ± 7.65

8.2 Overview over experimental conditions (temperature and humidity)

Table 7: Experiment condition during tabletting of 2 mm mini-tablets

Formulation compressed				Mean relative humidity (%)	Mean temperature (℃)
Pectin type (DM)	Pectin amount (%)	Chitosan type (DD)	Chitosan amount (%)		
10	90	-	-	16.6	23.9
40	90	-	-	17.6	21.3
10	70	92	20	18.4	22.8
40	70	92	20	20.3	22.0
10	45	92	45	17.8	23.5
40	45	92	45	18.7	23.6
10	20	92	70	18.5	24.6
40	20	92	70	17.3	24.9
-	-	92	90	17.6	23.8
-	-	82	90	15.2	23.5
-	-	77	90	22.5	15.3

8.3 Calibration curves

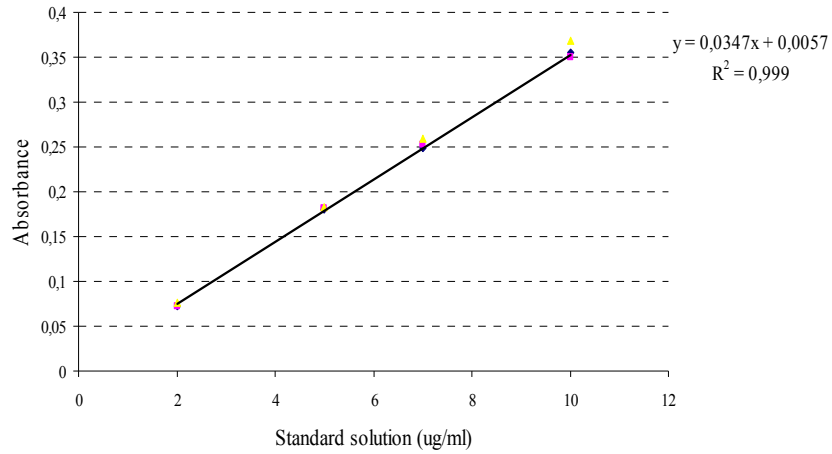

Figure 34: Standard solution of metronidazole in ammonium acetate buffer pH 4.5

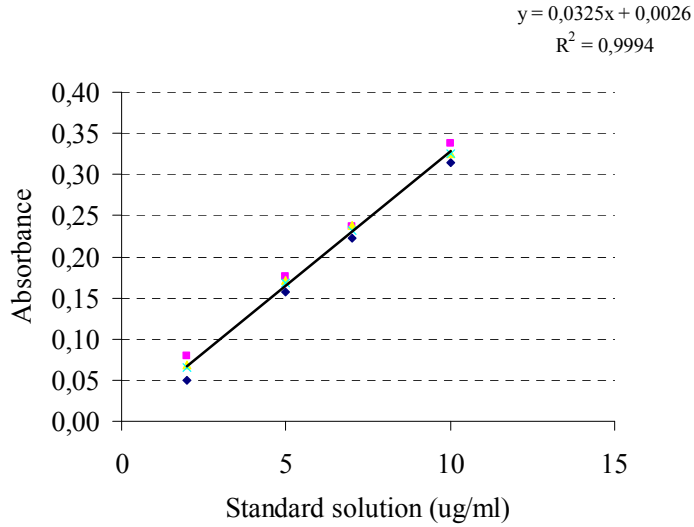

Figure 35: Standard curve of metronidazole in VFS pH 4.5

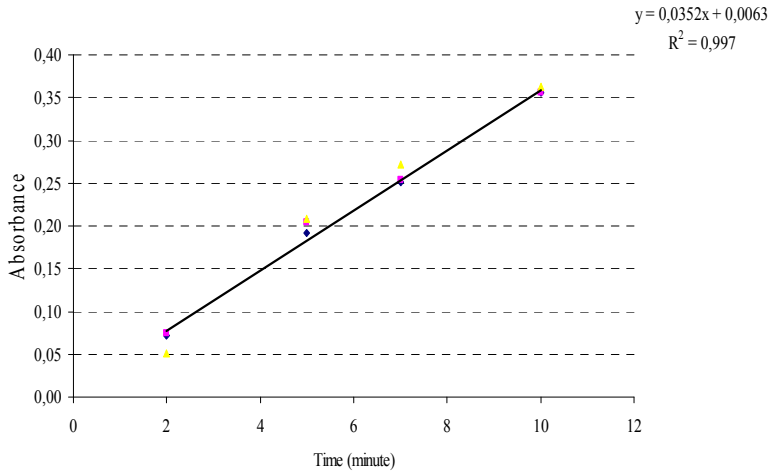

Figure 36: standard curve ofmetronidazole in VFS pH 6.8